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Use of *rosy* mutant strains of *Drosophila melanogaster* to probe the structure and function of xanthine dehydrogenase

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The usefulness in structure/function studies of molybdenum-containing hydroxylases in work with *rosy* mutant strains of *Drosophila melanogaster* has been investigated. At least 23 such strains are available, each corresponding to a single known amino acid change in the xanthine dehydrogenase sequence. Sequence comparisons permit identification, with some certainty, of regions associated with the iron–sulphur centres and the pterin molybdenum cofactor of the enzyme. Procedures have been developed and rigorously tested for the assay in gel-filtered extracts of the flies, of different catalytic activities of xanthine dehydrogenase by the use of various oxidizing and reducing substrates. These methods have been applied to 11 different *rosy* mutant strains that map to different regions of the sequence. All the mutations studied cause characteristic activity changes in the enzyme. In general these are consistent with the accepted assignment of the cofactors to the different domains and with the known reactivities of the molybdenum, flavin and iron–sulphur centres. Most results are interpretable in terms of the mutation affecting electron transfer to or from one redox centre only. The activity data provide evidence that FAD and the NAD^+/NADH binding sites are retained in mutants mapping to the flavin domain. Therefore, despite some indications from sequence comparisons, it is concluded that the structure of this domain of xanthine dehydrogenase cannot be directly related to that of other flavoproteins for which structural data are available. The data also indicate that the artificial electron acceptor phenazine methosulphate acts at the iron–sulphur centres and suggest that these centres may not be essential for electron transfer between molybdenum and flavin. The work emphasizes the importance of combined genetic and biochemical study of *rosy* mutant xanthine dehydrogenase variants in probing the structure and function of enzymes of this class.

INTRODUCTION

Molecular genetic techniques and, in particular, site-directed mutagenesis are currently finding wide application in the study of enzyme mechanisms. The genes for a number of xanthine dehydrogenases which are enzymes belonging to the class of molybdenum-containing hydroxylases (Bray, 1975; Coughlan, 1980; Hille & Massey, 1985; Wootton *et al.*, 1991) have now been cloned and sequenced (Lee *et al.*, 1987; Keith *et al.*, 1987; Houde *et al.*, 1989; Riley, 1989; Amaya *et al.*, 1990). In principle, the way is thus open for such studies of enzymes of this class. However, these are complex enzymes containing molybdenum (as the pterin molybdenum cofactor and bearing a sulphido [Mo=S] ligand) as well as FAD and two types of [2Fe–2S] iron–sulphur centres. It is thus not a simple matter to find a suitable expression system for molecular genetic work that permits correct protein folding and cofactor incorporation to yield the active enzyme. Furthermore, the enzyme is required in sufficient quantity for the physical studies (Bray, 1988; Wootton *et al.*, 1991) that are needed for detailed characterization.

For xanthine dehydrogenase from *Drosophila melanogaster*, an alternative approach is possible. Mutations in the *rosy* locus have a characteristic effect on the eye colour of the flies, with the *rosy* gene (Sang, 1985) that codes for the enzyme being one of the most extensively studied of eukaryotic genes. Of the many *rosy* strains of *D. melanogaster* currently available, at least 23 have been sequenced (Gray *et al.*, 1991) and are point mutations, each corresponding to a known amino acid substitution at a different site in the sequence. Biochemical characterization of these enzyme variants offers exciting possibilities for advancing knowledge of the structure and function of this enzyme. Work of this type may provide information on outstanding problems in molybdenum-containing hydroxylases, concerning electron transfer between and interaction amongst the different cofactors and the domains that bear them and the role of these in the overall catalytic reactions of the enzymes. Results, furthermore, could also have wider relevance to the functioning of other multi-centre redox enzymes.

Molybdenum-containing hydroxylases are a group of widely distributed enzymes of low specificity that are involved in a variety of types of oxidative metabolism. The enzymic reaction (studied most extensively for milk xanthine oxidase but no doubt similar for all enzymes of this class) involves interaction of the reducing substrate with the molybdenum, reducing the metal from the molybdenum(VI) to the molybdenum(IV) state. Electron egress from the reduced enzymes to oxygen or to NAD^+ (the oxidizing substrates for oxidase and dehydrogenase forms of the enzymes respectively) 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 enzyme molecule at some distance from the molybdenum atom. Despite intensive study (e.g. Olson *et al.*, 1974), the precise role of the iron–sulphur centres 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).

Native eukaryotic molybdenum-containing hydroxylases are α_2-dimers with a subunit M_r of about 150000. X-ray crystallographic information is not available but sequence comparisons

Abbreviations used: MV, methyl viologen; MV•, methyl viologen free radical; PMS, phenazine methosulphate; DCPIP, 2,6-dichlorophenol indophenol.

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and affinity labelling have provided evidence (Amaya *et al.*, 1990; Wootton *et al.*, 1991) that the *N*-terminal segment of the enzymes contain the iron–sulphur clusters, the middle segment of the flavin and NAD^+-binding sites, and the *C*-terminal segment the molybdenum cofactor. Boundaries for these three domains, as defined in rat liver xanthine dehydrogenase by the sites of cleavage by trypsin (Amaya *et al.*, 1990), are between residues 184–185 and 539–540 respectively.

In this paper we describe the characterization, from amino acid sequence comparisons and from enzyme activity measurements, of a number of *rosy* mutant xanthine dehydrogenases. We draw conclusions concerning sites of interaction of certain artificial oxidizing substrates with the enzyme and concerning intramolecular electron-transfer reactions in its catalytic cycle. A preliminary note on part of this work has appeared in the proceedings of a symposium (Bray *et al.*, 1991*a*).

MATERIALS AND METHODS

Protein-sequence comparisons

Protein-sequence comparisons were carried out as described previously (Wootton *et al.*, 1991).

Fly stocks and growth

All *Drosophila melanogaster rosy* mutations used were originally isolated in mutagenesis experiments by Chovnick and co-workers. Two wild-type strains were used, Canton S and ry^{+10}; the origin of the latter is described by McCarron *et al.* (1979). The ry^{506} mutation (Coté *et al.*, 1986) is a deletion of approximately two-thirds of the 3′-end of the gene, and this strain was used as a negative control for all enzyme assays, since it does not give rise to a protein that cross-reacts with antisera raised against xanthine dehydrogenase. $Df(3R)ry^{36}$ is a deletion-bearing chromosome that lacks all of the *rosy* gene, as well as several adjacent vital genes responsible for the homozygous lethality of this chromosome (Hilliker *et al.*, 1980). The molecular lesions of all other *rosy* mutations are described by Gray *et al.* (1991). The ry^{5185} mutation is carried on a chromosome bearing a mutation in a vital gene located near *rosy*, and thus cannot be examined in homozygotes. However, $ry^{5185}/Df(3R)ry^{36}$ individuals survive, due to complementation of all vital functions, thereby permitting examination of the mutant protein. For the same reason, the *rosy* mutations ry^{406}, ry^{5184} and ry^{606} were employed not as homozygotes but as heterozygotes with $Df(3R)ry^{36}$.

All genotypes were raised at approximately 25 °C on a standard maize meal/yeast/sugar/agar medium containing the fungicide Nipagin M (4-methoxybenzoic acid methyl ester; Nipa Laboratories Ltd, Pontypridd, Wales, U.K.). Unless otherwise indicated, approximately 3-day-old adult flies were used. Flies were stored at the temperature of liquid nitrogen until required. If large-scale growth was required, techniques based on those of Roberts (1986) were employed, with the use of population cages (approximately 30 cm × 30 cm × 30 cm) in place of glass bottles. Large-scale harvesting was by suction from a domestic vacuum cleaner into muslin bags.

Preparation of gel-filtered extracts of *Drosophila* enzyme assays

All operations were carried out at approx. 4 °C. Frozen flies (4 g) were ground to a smooth paste in a pestle and mortar with acid-washed sand (2 g) and 8 ml of 0.1 M-Tris/HCl buffer, pH 8.0 (at 4 °C), containing the following protease inhibitors (all from Boehringer): leupeptin (1 μM), pepstatin (1 μM), phenylmethanesulphonyl fluoride (200 μM), and the following further additives: EDTA (100 μM), sodium salicylate (1 mM) and dithiothreitol (1 mM). The paste was then diluted to 20 ml with the same buffer and, after centrifugation at 10000 $g_{max.}$ for 20 min, the supernatant was passed through a sintered glass filter (pore size 3) and re-centrifuged at 25000 $g_{max.}$ for 20 min. The supernatant was then purified by gel filtration (Sephadex G-25 column, 1.5 cm × 5.0 cm) by using the same buffer but with no additives except EDTA (1 mM). The protein-containing fraction separated out from coloured, low-M_r material and was retained for enzyme assays. This gel-filtration step was most important, serving to remove unidentified inhibitors (perhaps pteridines) and without it a linear relationship between enzyme concentration and rate was not observed. Attempts to use charcoal (Norite-A) (Seybold, 1974) to remove the inhibitors were unsuccessful in our hands and resulted in the loss of about 50% of the pterin:NAD^+ oxidoreductase activity.

Enzyme assays

Assays were carried out aerobically, unless otherwise indicated, at 23.5±0.2 °C in 0.1 M-Tris/HCl buffer, pH 8.0, containing 1 mM-EDTA. A Phillips PU-8720 spectrophotometer or a Perkin–Elmer LS-3 spectrofluorimeter was used. For the various spectrophotometric assays (1 cm light path), substrate concentrations, final volumes, wavelengths and extinction coefficients used were as follows: xanthine:NAD^+ assay, 100 μM-xanthine (Sigma), 0.33 mM-NAD^+ (β-NAD^+ from Boehringer), 1.5 ml, 340 nm, ϵ 6.2 $mM^{-1}\cdot cm^{-1}$; xanthine:oxygen assay, 100 μM-xanthine, 1.5 ml, 295 nm, $\Delta\epsilon$ 9.6 $mM^{-1}\cdot cm^{-1}$; xanthine:DCPIP assay, 100 μM-xanthine, 12.5 μM-DCPIP (Sigma), 1.5 ml, 600 nm, ϵ 21 $mM^{-1}\cdot cm^{-1}$; xanthine:PMS/cytochrome *c* assay, 100 μM-xanthine, 44 μM-PMS (Sigma), 13 μM-cytochrome *c* (Sigma, Type VI), 3.0 ml, 550 nm, $\Delta\epsilon$ 19.6 $mM^{-1}\cdot cm^{-1}$; NADH:DCPIP assay, 100 μM-NADH (Boehringer), 12.5 μM-DCPIP, 1.5 ml, 600 nm, ϵ 21 $mM^{-1}\cdot cm^{-1}$; NADH:PMS/cytochrome *c* assay, 100 μM-NADPH, 44 μM-PMS, 13 μM-cytochrome-*c*, 3.0 ml, 550 nm, $\Delta\epsilon$ 19.6-$mM^{-1}\cdot cm^{-1}$; $MV^{\cdot}$:NAD^+ assay, 0.3 mM-MV (Sigma), 0.33 mM-NAD^+, 3.0 ml, 600 nm, ϵ 13.4 $mM^{-1}\cdot cm^{-1}$ (Corbin & Watt, 1990) [the procedure was that of Kemp *et al.* (1975) and $MV^{\cdot}$ was generated *in situ* by anaerobic addition of $Na_2S_2O_4$ to give an A_{600}-value of approx. 0.5]. For the fluorimetric assay of pterin:NAD^+ activity, 11 μM-pterin (Sigma) and 0.33 mM-NAD^+ were used, at a volume of 3.0 ml, with excitation at 342 nm and emission measured at 410 nm, and the instrument was standardized by measurements with the product (isoxanthopterin, from Sigma) and the substrate.

For all the assay systems under the conditions employed, enzyme activity was linearly proportional to the volume of extract used. In all cases, blank rates, obtained by omitting one component at a time (reducing substrate, oxidizing substrate, or enzyme) were added together and subtracted from the rate obtained with the complete system. The highest blanks obtained were those in the xanthine:NAD^+ assay without reducing substrate. In some cases these were as high as 40–50% of the activity in the complete system. Other blanks were much lower, ranging from 0 to 20% of those for the complete systems.

Protein determination on the *Drosophila* extracts was carried out by the dye-binding method of Bradford (1976) (Protein Assay Kit; Bio-Rad Laboratories). Milk xanthine oxidase, having an A_{280}/A_{450} value of 5.0, purified by steps H1, H2, N1 of Ventom *et al.* (1988), was used as a standard, taking $A^{1\%}_{1cm}$ (280 nm) = 11.7 (Bray, 1975). Enzyme activities are expressed as nmol of substrate $(2e^-)\cdot min^{-1}\cdot(mg\ of\ protein)^{-1}$.

RESULTS AND DISCUSSION

Location of mutations in *rosy* gene

We selected 11 *rosy* mutant strains of *D. melanogaster* for study. Details are given in the Materials and methods section. For three strains (ry^{5231}, E89 → K; ry^{5281}, L127 → F; ry^{606},

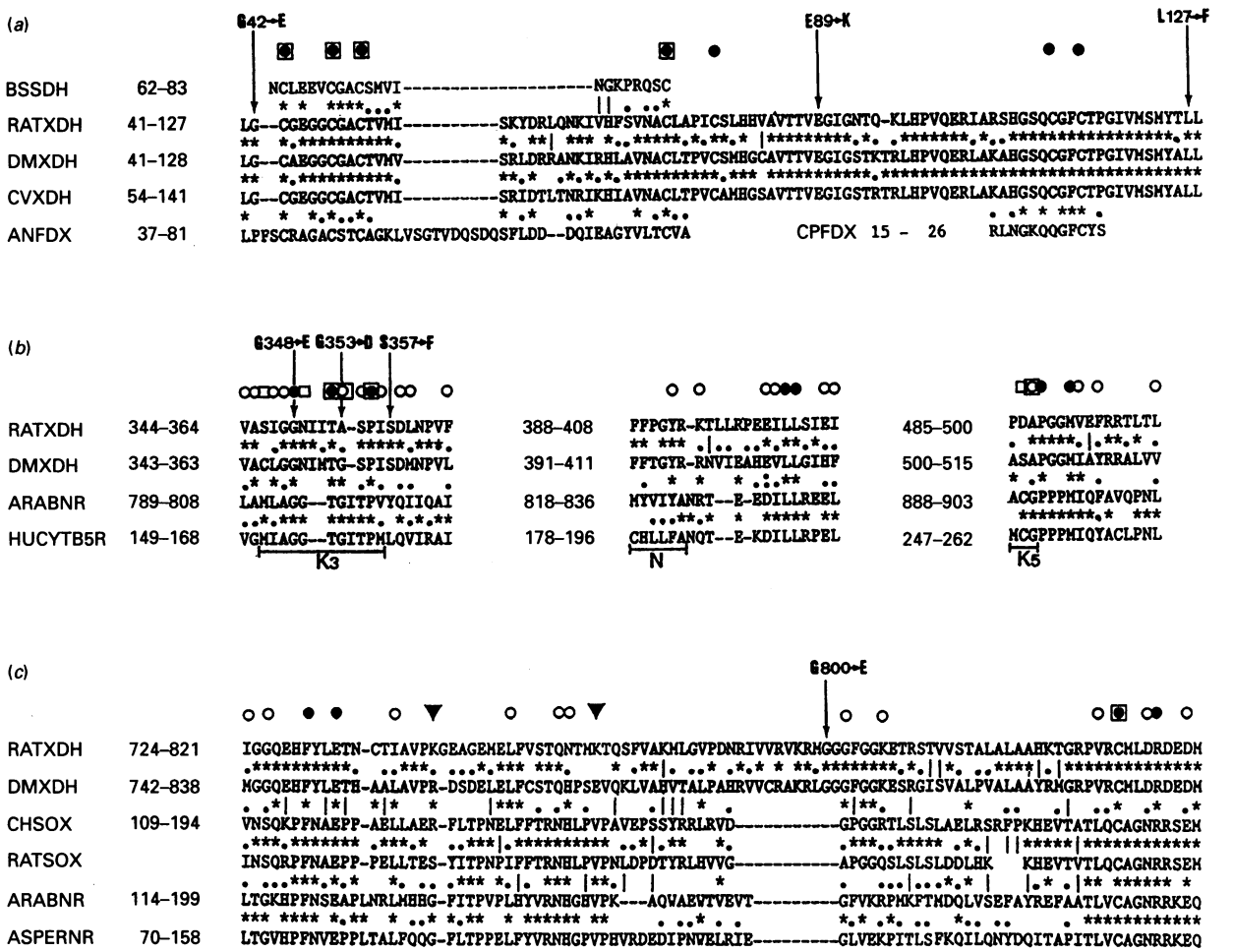

Fig. 1. Alignment of the sequences of xanthine dehydrogenases with those of other proteins in the regions presumed to bind: (*a*) the Fe/S centres, (*b*) the FAD and NAD$^+$/NADH and (*c*) the pterin molybdenum cofactor

Abbreviations used are as follows: BSSDH, *Bacillus subtilis* succinate dehydrogenase (Phillips *et al.*, 1987); RATXDH, rat xanthine dehydrogenase (Amaya *et al.*, 1990); DMXDH, *D. melanogaster* xanthine dehydrogenase (Keith *et al.*, 1987; Lee *et al.*, 1987); CVXDH, *Caliphora vicina* xanthine dehydrogenase (Houde *et al.*, 1989); ANFDX, *Anabaena* 7120 ferredoxin (Rypniewski *et al.*, 1991); CPFDX, *Clostridium pasteurianum* ferredoxin (Meyer *et al.*, 1986); ARABNR, *Arabidopsis thaliana* nitrate reductase, *nia* 2 gene product (Crawford *et al.*, 1988); HUCYTB5R, human cytochrome-b_5 reductase (Yubisui *et al.*, 1984); CHSOX, chicken sulphite oxidase (Neame & Barber, 1989); RATSOX, rat sulphite oxidase (Barber & Neame, 1990; residue numbers are not known and are omitted); ASPERNR, *Aspergillus nidulans* nitrate reductase (Kinghorn & Campbell, 1989). Symbols between the sequences are as follows: *, identical amino acids in adjacent rows; ·, similar amino acids that are commonly found as alternatives in homologous proteins in adjacent rows; |, identical or similar amino acids in non-adjacent rows. The sites of various mutations used in the present work, in the *D. melanogaster* xanthine dehydrogenase sequence, e.g. G42 → E, are indicated by arrows above the sequences. Other symbols have respectively the following meanings: for (*a*) ● indicates cysteine residues conserved in all three xanthine dehydrogenases, and □, cysteine ligands for the iron–sulphur cluster of ANFDX. In (*b*), the bars marked K3 and K5 correspond to the motifs, identified in ARABNR and HUCYTB5R and in six other flavoproteins by Karplus *et al.* (1991); □, residues conserved in all the eight sequences aligned by these workers; ● and ○, indicate, respectively, residues identical or similar in all four sequences here aligned. The bar marked N corresponds to the sequence identified by affinity labelling in chicken xanthine dehydrogenase by Nishino & Nishino (1989). In (*c*), ● and ○ indicate, respectively, residues identical or similar in all six sequences; □ indicates a cysteine conserved in all six sequences; and ▼ above the alignment indicates the lysine residues susceptible to chemical modification in chicken xanthine dehydrogenase (T. Nishino, Y. Amaya & T. Nishino, personal communication, and data presented at 10th International Symp. on Flavins and Flavoproteins, Como, Italy, 1990).

G42 → E) the mutation is located in the iron–sulphur domain of the xanthine dehydrogenase molecule. In another three (ry^{602}, S357 → F; ry^{5185}, G353 → D; ry^{406}, G348 → E) the mutation is located in the flavin domain, and for the remaining five *rosy* strains (ry^{5264}, G800 → E; ry^{544}, G1011 → E; ry^{573}, G1164 → R; ry^{5205}, G1266 → D; ry^{5184}, S1275 → F) it is the pterin molybdenum cofactor domain. These mutations all map to regions of the sequence that are well conserved between the rat and the *D. melanogaster* enzymes (see Amaya *et al.*, 1990).

Iron–sulphur domain

Fig. 1(*a*) shows a segment of the sequence of the *N*-terminal domain of three xanthine dehydrogenases aligned with part of that of the [2Fe–2S] centre of the ferredoxin from *Anabaena* 7120, the structure of which has recently been established (Rypniewski *et al.*, 1991) by X-ray crystallography. These new data strengthen the conclusion of Wootton *et al.* (1991) that cysteine residues 43, 48, 51 and 73 of *D. melanogaster* xanthine dehydrogenase provide the ligands of one of the [2Fe–2S] centres of the enzyme. This region also shows similarities to sequences associated (e.g. Werth *et al.*, 1990) with the [2Fe–2S] centre in succinate dehydrogenase and fumarate reductase, the similarity being particularly marked for succinate dehydrogenase from *Bacillus subtilis* (Fig. 1*a*). That the iron–sulphur centre involved is the one designated Fe/SI in the molybdenum-containing hydroxylases was shown by e.p.r. data. This

Table 1. Activity of xanthine dehydrogenase in gel-filtered extracts from wild-type and mutant flies in different assay systems

Activities in different assays, as nmol of substrate ($2e^-$)/min per mg of protein, are expressed relative to that for the Canton S wild-type flies in the xanthine:NAD^+ assay, taken as 100. Data refer to extracts, purified by gel filtration on Sephadex G-25, as described in the Materials and methods section, except for the sample marked 'purified enzyme.' Assays were carried out at pH 8.0 and 23.5 °C. Except where otherwise indicated, errors correspond to deviations for duplicate assays on the same extract; in cases (particularly for xanthine:NAD^+ and pterin:NAD^+ activity) where duplicate extracts were assayed, deviations similar to those given were observed. (Absolute activity of extracts from Canton S wild-type flies, grown in bottles, in the xanthine:NAD^+ assay was routinely found to be approx. 0.26 nmol/min per mg of protein. For flies grown in cages somewhat more variable results were observed.) Numbers in parentheses are column numbers referred to in the text.

			Relative activity							
Strain	Mutation	Domain	Xanthine: NAD^+ (1)	Xanthine: oxygen (2)	Pterin: NAD^+ (3)	Xanthine: DCPIP (4)	Xanthine: PMS/cyt. *c* (5)	NADH: DCPIP (6)	NADH: PMS/cyt. *c* (7)	MV: NAD^+ (8)
Canton S	(Wild-type)	–	100±0	4±1	76±1	63±1	37±3	11±1	12±3	57±3
ry^{+10}	(Wild-type)	–	97±1	4±2	72±3	68±1	36±5	17±4	12±3	58±4
Canton S (purified enzyme*)	(Wild-type)	–	100±0	61±9	90±2	54±3	32±3	15±2	10±2	46±3
ry^{506}	(Deletion)	–	2±1	2±0	1±1	3±1	2±1	1±0	1±1	5±1
ry^{606}	G42 → E	Fe/S	1±1	1±0	1±0	2±1	1±0	1±0	2±0	62±5
ry^{5231}	E89 → K	Fe/S	89±2	2±0	72±2	2±1	1±1	10±2	2±1	60±2
ry^{5281}	L127 → F	Fe/S	91±2	3±3	68±2	3±1	2±0	12±1	2±1	57±3
ry^{406}	G348 → E	Flavin	2±1	1±1	2±0	2±0	9±1	1±0	8±3	55±5
ry^{5185}	G353 → D	Flavin	2±1†	3±1†	4±1†	3±1†	7±1†	1±1	7±1	52±5†
ry^{602}	S357 → F	Flavin	2±0	2±0	1±1	1±1	9±1	2±1	6±0	52±0
ry^{5264}‡	G800 → E	Mo	59±1	1±0	42±1	30±2	24±2	5±1	10±0	50±2
ry^{544}	G1011 → E	Mo	2±1	2±1	2±0	2±1	2±1	1±0	12±2	52±0
ry^{573}	G1164 → R	Mo	3±0	1±0	1±1	1±1	2±1	1±0	13±2	53±4
ry^{5205}	G1266 → D	Mo	2±1	2±0	2±1	2±0	2±0	1±0	12±0	61±3
ry^{5184}	S1275 → F	Mo	2±1	1±1	2±1	2±0	2±0	1±0	12±1	55±4

* Calculated from the data of Hughes *et al.* (1992); activity in the xanthine:NAD^+ assay is taken as 100.

† The effect of the age of the flies was investigated for this strain, but no clear activity trends with age were observed. Results given are the overall means±S.D., from duplicated assays on each of the five sets of flies, aged 2–10 days.

‡ Very similar results were obtained with an analogous heterozygote strain.

centre has g_1 2.021±0.004, g_2 1.933±0.005, g_3 1.907±0.010, $g_{av.}$ 1.954±0.005 in a variety of eukaryotic molybdenum-containing hydroxylases (Bray *et al.*, 1991*b*; Hughes *et al.*, 1992), values that are quite different from those of Fe/SII but which are indistinguishable statistically from, e.g., those of *E. coli* fumarate reductase (Werth *et al.*, 1990) or bovine succinate dehydrogenase (Salerno *et al.*, 1979).

The locating of the centre of Fe/SII in the xanthine dehydrogenase sequences is at present on a more speculative basis. Not all the four cysteine residues that Wootton *et al.* (1991) suggested might be involved are conserved in all the xanthine dehydrogenases for which data are now available. It is unclear which of the eight conserved cysteines within residues 78–176 are associated with this centre. Indeed, it is not a foregone conclusion that all the ligands are sulphur atoms (cf. Werth *et al.*, 1990). Of possible significance is the similarity indicated in Fig. 1(*a*) between a short stretch of the xanthine dehydrogenase sequence and a segment of that of the [2Fe–2S] ferredoxin from *Clostridium pasteuranium* (Meyer *et al.*, 1986). However, the cluster ligands have not been identified in the latter protein, so definitive conclusions cannot be drawn.

Thus, as indicated in Fig. 1(*a*), of the three mutations in the iron–sulphur domain of *D. melanogaster* xanthine dehydrogenase listed in Table 1, G42 → E is immediately before the first cysteine ligand of Fe/SI, E89 → K is most likely between the two clusters, and L127 → F is either between them or, more likely, within the Fe/SII binding sequence.

Flavin/NAD^+/NADH domain

As already noted, the central domain of xanthine dehydrogenase is presumed to bear the FAD- and NAD^+/NADH-binding sites, a conclusion based primarily on results of affinity labelling work with an NAD^+ analogue (Nishino & Nishino, 1989; see the segment marked N in Fig. 1*b*). Wootton *et al.* (1991) aligned the central part of the xanthine dehydrogenase sequence with the flavin- and NADH-binding regions of assimilatory nitrate reductases and cytochrome b_5 reductases, noting that the sequence similarities were not strong. More data have now become available both on xanthine dehydrogenases (Amaya *et al.*, 1990) and on flavoprotein structures and sequence motifs (Karplus *et al.*, 1991). The left-hand portion of Fig. 1(*b*) shows our alignment of *D. melanogaster* xanthine dehydrogenase in the region of the third of the conserved flavoprotein motifs identified by Karplus *et al.* (1991) (marked K3 in Fig. 1*b*). This motif occurs in the $NADP^+$ domain of ferredoxin:$NADP^+$ oxidoreductase, being associated with the binding site for this cofactor. Thus, if our alignment is in any way meaningful, this part of the xanthine dehydrogenase sequence should be related to the NAD^+-binding site. This conclusion is of particular interest since the three mutations in the flavin domain that we have studied (Table 1) are all located in this segment of the chain (Fig. 1*b*). Some weak sequence similarities were also found in the region of the fifth motif of Karplus *et al.* (1991) (marked K5 in Fig. 1*b*). None of the regions of similarity found in the present work corresponds to the regions of the flavin domain provisionally identified from less extensive comparisons by Wootton *et al.* (1991).

Pterin molybdenum cofactor domain

The pterin molybdenum cofactor binding domain is considerably larger than the other two domains. Wootton *et al.* (1991) aligned parts of the *Drosophila* sequence with relevant parts of those of five assimilatory nitrate reductases and one

sulphite oxidase. [Note that these are all enzymes expected (cf. Rajagopalan, 1991) to bear the simple pterin molybdenum cofactor and not one of its dinucleotide variants.] A limited region of significant sequence similarity between approximately residues 740 and 911 of the *Drosophila* enzyme was found. The inclusion of new data (Amaya *et al.*, 1990; Barber & Neame, 1990) further strengthens the earlier evidence that the molybdenum cofactor binding site is associated with this region of the polypeptide chain. Fig. 1(*c*) shows part of the region where the similarities are considered significant. [Similarities between residues 1148 and 1165 of the *Drosophila* enzyme noted by Wootton *et al.* (1991) no longer appear significant.] Amaya *et al.* (1990) drew attention to the motif GGGFGG (residues 783–788 in rat xanthine dehydrogenase), suggesting that it might be associated with the binding of the pyrophosphate moiety of a coenzyme. Our work provides no support for such a role for this part of the sequence. On the other hand, further important evidence concerning the relevance of this region of the chain to the molybdenum site comes from the identification by Nishino and co-workers of lysine residues, the chemical modification of which interferes (Nishino *et al.*, 1982) with interaction of the enzyme with reducing substrates. Two such residues have now been identified (T. Nishino, Y. Amaya & T. Nishino, personal communication and data presented at 10th International Symposium on Flavins and Flavoproteins, Como, Italy, 1990) as K742 and K759 in the rat enzyme (see Fig. 1*c*).

Of the five mutations in the putative molybdenum domain that we have studied (Table 1), only one [G800 → E (Fig. 1*c*)] falls within that part of the polypeptide chain showing similarities to the other molybdenum-containing enzymes.

Enzyme assays employed for comparisons of the enzyme variants

Sequence alignments as discussed above are of great value in delineating sub-domains of complex enzymes and in defining probable regions of interaction of cofactors with the polypeptide chain. In the case of the molybdenum-containing hydroxylases, study of the multiple catalytic activities of the enzymes offers the possibility not only of checking information from sequence comparisons but also of probing the enzymic mechanism in greater depth. To make comparisons of the mutant xanthine dehydrogenases with one another and with the wild-type enzyme we employed eight different enzyme activity assay systems, as listed in Table 1 and detailed in the Materials and methods section. These assays are based on different combinations of one of four reducing substrates with one of four oxidizing substrates. They were selected to include substrates believed to interact with different centres in the enzyme. Before presenting results we summarize prior information relating to the assay systems.

All four redox centres in molybdenum-containing hydroxylases are in rapid redox equilibrium with one another (Olson *et al.*, 1974). Furthermore, the measured rates (Anderson *et al.*, 1986) of the intramolecular electron-transfer reactions involved in these processes are much greater than, and therefore not limiting in, the turnover rate for the enzyme. In such a system, rigorous identification of the sites of interaction of substrates with the enzyme is not a simple task. Problems of interpretation are further compounded by the enzymes existing (Bray, 1975), in normal preparations, with their molybdenum centres as mixtures of functional and non-functional (desulpho, demolybdo) forms. Further complications arise from modification (-SH oxidation and proteolysis) occurring in the flavin domain, with resultant interconversions between dehydrogenase and oxidase forms of the enzymes.

Despite these problems, it is generally accepted (Bray, 1975; Coughlan, 1980), based on well-documented evidence, that in all molybdenum-containing hydroxylases, xanthine, pterin and related substrates act at the molybdenum centre and that NAD^+, NADH and O_2 act at the flavin (see Fig. 2, below). The remaining substrates we used are artificial ones that would not necessarily be expected to react at a single site only. However, for 2,6-dichlorophenol indophenol (DCPIP), there is evidence that it reacts, predominantly if not exclusively (Coughlan, 1980), at molybdenum. Phenazine methosulphate (PMS), sometimes in combination with a secondary electron acceptor, has been widely used (Singer, 1966) in assays of the iron–sulphur flavoprotein enzyme succinate dehydrogenase (Hatefi & Stiggall, 1976) and occasionally used for molybdenum-containing hydroxylases. On one-electron reduction, it yields a free radical of adequate stability and redox potential to reduce, e.g., cytochrome *c*, non-enzymically. Bray (1975) concluded that PMS acts at molybdenum, but Coughlan (1980) suggested that it might act at the iron–sulphur centres (as is presumably the case in succinate dehydrogenase). The methyl viologen free radical, $MV^{\bullet}$, is a reducing substrate widely used in assays of nitrate reductases, though little used in conventional assays of molybdenum-containing hydroxylases. For the former enzymes, it seems accepted (e.g., Solomonson & Barber, 1990), that $MV^{\bullet}$ reacts at molybdenum. For xanthine oxidase, fast kinetic studies employing pulse radiolysis (Anderson *et al.*, 1986) demonstrated that $MV^{\bullet}$ and other viologen radicals are effective reducing agents for all the redox centres of the enzyme, as well as for a specific disulphide bond. Whether the radicals react directly and rapidly both at the molybdenum and at the flavin sites is as yet unclear, though they appear not to do so, at rates that are catalytically significant, at the iron–sulphur centres.

Validity of assays on gel-filtered extracts of the mutant flies

In Table 1, activities in the various assays of gel-filtered extracts from each of 14 strains of *D. melanogaster* are compared. Conditions for preparation of the extracts and of carrying out the assays and correcting for blanks were rigorously standardized, as summarized in the Materials and methods section, and results were highly reproducible, as indicated in Table 1. Owing to potential problems in drawing detailed conclusions from enzyme assays performed on extracts not purified to homogeneity, we took considerable care to establish the validity of our procedures. We tested (Table 1) two wild-type strains (Canton S and ry^{+10}) and found good agreement between them. Finally, when this became available (Hughes *et al.*, 1992), we compared the activity of the purified wild-type enzyme with that in the gel-filtered extracts. The close agreement of the results (Table 1) in all the assays, except that for xanthine:oxygen oxidoreductase activity (column 2), clearly shows the validity of our procedures. The increase in this oxidase activity in the purified sample in comparison with the extract arises because (Hughes *et al.*, 1992) of dehydrogenase-to-oxidase conversion, a phenomenon well known in molybdenum-containing hydroxylases but not previously noted for the *Drosophila* enzyme. The validity of our measurements was further confirmed by the very low activities found in all assays on extracts from the deletion mutant, ry^{506} (see the Materials and methods section). A potential problem would have been interference in our assays by *Drosophila* aldehyde oxidase. Only limited work on the specificity of this enzyme has been reported (Bray, 1975). On the assumption that *Drosophila* aldehyde oxidase is devoid of activity towards xanthine and pterin, then only in assays with NADH or NAD^+ (Table 1) would it be likely to interfere. That it does not interfere significantly is shown by the low activity in these assays for the *rosy* deletion mutant, as well as by there being only a small diminution in activity in the $MV^{\bullet}$:NAD^+ oxidoreductase assay

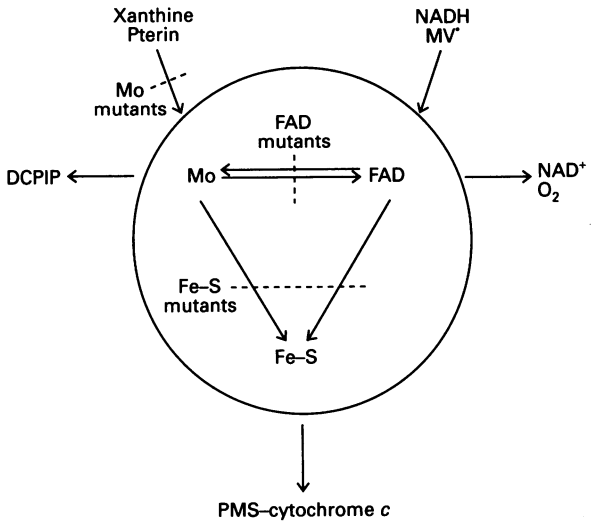

Fig. 2. Minimum scheme for electron-transfer pathways to, from and within xanthine dehydrogenase in wild-type and mutant enzyme variants

Arrows indicate electron transfer; individual steps presumed to be blocked in the three types of mutants are indicated by broken lines. See the text for exceptions. (Note that no attempt has been made to distinguish between the two iron–sulphur clusters of the enzyme.)

(column 8) for the purified wild-type enzyme in comparison with the gel-filtered extract.

Enzymic activities of the xanthine dehydrogenase variants: effects of the mutations

All the mutations produce diminutions in activity in some of the assays in comparison with the wild-type flies (Table 1) and clear-cut patterns emerge with, however, two exceptions (G42 → E and G800 → E). Apart from these two, any mutation within a given domain gives rise to a characteristic pattern of activities in the different assays that is clearly different from the pattern resulting from a mutation in either of the other domains but which is indistinguishable from that from a different mutation in the same domain.

Activity changes (Table 1) may be summarized as follows, ignoring initially the G42 → E and G800 → E mutants. For all the mutants, xanthine:oxygen activity (column 2) is vanishingly small, and for all of them the wild-type activity level is fully retained in the $MV^{\bullet}$:NAD^+ oxidoreductase assay (column 8). Activity in the xanthine:NAD^+ and pterin:NAD^+ assays (columns 1 and 3) is retained in the iron–sulphur mutants as is that in the NADH:DCPIP assay (column 6), but all other activities are lost. For the flavin mutants, activity is retained only in the assays with PMS/cytochrome *c* as acceptor when using xanthine (column 5) or NADH (column 7) as reducing substrate, activity levels being diminished, however, to 20–25% and to about 60%, respectively, of the wild-type levels. For the molybdenum mutants, activity only in the NADH:PMS/cytochrome *c* assay (column 7) is retained. Of the two mutant strains having anomalous activity patterns, G42 → E is unique in showing no activity at all in any but the $MV^{\bullet}$:NAD^+ assay (column 8). Conversely, G800 → E is anomalous in showing activity in all the assays (except xanthine:oxygen; column 2) though at diminished levels ranging from 40 to 90% of those in the wild-type strains.

Limited but important conclusions may be drawn from the above data. In general, results are in accord with expectations, based on earlier work concerning the centres at which the different substrates react. Similarly, they are consistent with, and thus tend to confirm, the accepted assignment of the cofactors to the different domains and with the domain boundaries that we have assumed. Moreover, results are generally consistent with the assumption that the effect of a particular mutation is localized within the relevant domain, where it has a controlling influence on electron transfer to or from the redox centre in question. Fig. 2 illustrates a minimum scheme in terms of which most of the data may be interpreted, for interaction of the different substrates and for intramolecular electron-transfer pathways. The simplifying assumption has been made that all substrates act at one centre only. (Note that there are certain anomalies in relation to the action of DCPIP which are considered below.) The scheme differs relatively little from those of earlier workers (Bray, 1974; Coughlan, 1980). It does, however, present some novel features, notably the interpretation that PMS reacts at the iron–sulphur centres rather than at molybdenum. Retention of a significant part of the xanthine:PMS/cytochrome *c* activity (column 5) in the flavin mutants, together with complete loss of this activity as well as of NADH:PMS/cytochrome *c* activity (column 7) in the iron–sulphur mutants, indicates that electron transfer from molybdenum to flavin proceeds directly, and not via the iron–sulphur centres. This conclusion from the above work on gel-filtered extracts (Table 1) has already been published in preliminary form (Bray *et al.*, 1991*a*,*b*) and subsequently confirmed directly in work (Hughes *et al.*, 1991; Hughes *et al.*, 1992) on the purified E89 → K mutant enzyme variant.

For the molybdenum and iron–sulphur mutants the data presented are not adequate to allow us to decide whether these redox centres are deleted as a result of the mutations, or whether they remain present but with electron transfer blocked as shown in Fig. 2. It is of course well known that intramolecular electron-transfer reactions between redox centres in proteins are to varying degrees sensitive to the nature of the intervening amino acid residues (Liang *et al.*, 1987; McLendon, 1988; Rees & Farrelly, 1990). For the iron–sulphur mutants, the sequence data (Fig. 1*a*) make it abundantly clear, however, that the sites of the mutations are intimately related to the iron–sulphur binding sites. In the case of the molybdenum mutants, loss of DCPIP activity (column 6; see also below) as well as of activity with xanthine and pterin (columns 1, 3 and 4) could argue for loss of the metal. Conversely, in relation to the flavin site, strong evidence that FAD is present in all the flavin mutants is provided by their retention (Table 1) of $MV^{\bullet}$:NAD^+ activity (column 8), as well as of partial activity in the NADH:PMS/cytochrome *c* assay (column 7). Thus the flavin-associated NAD^+/NADH binding site must be retained in these mutants, notwithstanding the indications from the sequence comparisons as discussed above. We conclude therefore that the sequence of the flavin domain bears no true relation to that of the flavoproteins compared by Karplus *et al.* (1991) and that the alignments of Fig. 1(*b*) have little meaning.

Data with G800 → E show that this mutant is of particular interest both because of its location in the sequence (Fig. 1*c*) and because of the pattern of activities (Table 1) that is unique among the mutants studied. For this mutant, but not for the others in the molybdenum domain, there is clear information from our sequence comparison with other molybdenum enzymes (Fig. 1*c*) linking the position of the mutation to the cofactor-binding site. Retention of some activity in all the assays for this mutant indicates that the structural change is a subtle one meriting further study.

For the results so far discussed, effects of the mutations are interpretable in terms of changes within a single domain. This simple view is not adequate to account for the data in relation to the G42 → E mutant, however, and in relation to the loss of xanthine:DCPIP activity (column 4) in the flavin and iron–sulphur mutants. In relation to both these results some type of

inter-domain interaction needs to be postulated. The G42 → E mutant has the characteristics, in terms of loss of activity in comparison with wild-type strains, both of iron–sulphur and of molybdenum mutants. It seems that in this case mutation in the iron–sulphur domain has a profound influence also on the molybdenum domain. Loss of activity to DCPIP in the flavin and iron–sulphur mutants is not expected in terms of Fig. 2, and we must conclude that our interpretations are an oversimplification. Possible explanations include the reaction of substrates at more than one site. Alternatively, the loss of activity may relate to the numbers of electron pairs that can be accommodated in the mutant enzymes on reduction and the distribution in reduced enzyme forms of these electrons among the different redox centres (cf. Olson *et al.*, 1974).

Conclusions

In addition to the conclusions discussed above, our work provides an essential basis for further studies. Full characterization at the molecular level of a number of *rosy* mutant xanthine dehydrogenases will be of very considerable interest. Our carefully validated results in different enzyme assay systems in conjunction with sequence comparisons of the iron–sulphur and molybdenum domains, but not of the flavin domain, will provide a firm basis for the selection of mutations for such further work. Furthermore our assay procedures will obviously be of value for screening of further *rosy* mutants, whether produced by classical procedures or by site-directed mutagenesis. Finally, the complexity of the enzymes suggests that future approaches to understanding them should include the cloning and expression of specific domains. Our data help further to define these domains and will thus aid further study.

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