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Drosophila xanthine dehydrogenase variants re-visited

In *Drosophila melanogaster*, the enzyme xanthine dehydrogenase is coded by the much studied *rosy* gene, expression of which affects the eye colour of the flies. More than 200 *rosy* mutant strains are available, of which at least 23 are known to be point mutations leading to known amino acid substitutions at specific sites in the sequence. Characterization at the molecular level of the variant xanthine dehydrogenases from these strains should provide new insights into the structure and functions of this class of enzymes, the molybdenum-containing hydroxylases.

Recently two papers (Hughes et al., 1992a,b) were published from this laboratory on biochemical characterization of such xanthine dehydrogenase variants. Specific claims were made concerning patterns of enzymic activity observed in assays using different reducing and oxidizing substrates, carried out on gel-filtered extracts of various strains of the flies. A particular claim was that the E89K mutation resulted in an enzyme variant lacking iron–sulphur centres as well as lacking, specifically, activity in the xanthine:phenazine methosulphate (PMS)/cytochrome *c* oxidoreductase assay. The main parts of this work have now been repeated by the authors of this Letter, and results are presented here. Additional studies (W. A. Doyle and R. C. Bray, unpublished work) on the properties of selected *rosy* xanthine dehydrogenase variants will be presented elsewhere. Our findings are completely at variance with the published work, and, for the reasons discussed below, we have to conclude that this was unsoundly based. Accordingly, we no longer wish to be associated with the main conclusions of Hughes et al. (1992a,b).

In more detail, Hughes et al. (1992a) developed procedures for the spectrophotometric assay of different catalytic activities of xanthine dehydrogenase in gel-filtered extracts of whole flies. Reproducible and consistent results were reported. In seeking to extend the work, we encountered, however, a lack of reproducibility that we thought might be associated with slight turbidities observed in the assay mixtures prepared according to these workers. We therefore modified these procedures by clarifying the extracts by centrifugation at 100 000 *g* instead of 25 000 *g*. We used a modern double-beam spectrophotometer (Perkin–Elmer Lambda 16) in place of the single-beam instrument originally employed. Also, instead of correcting for ‘blanks’ as described originally, we found it more satisfactory to preincubate the extracts with the oxidizing substrate until endogenous reductants were exhausted, and then to start the assays by addition of the reducing substrate. It was particularly important to do this for xanthine:PMS/cytochrome *c* oxidoreductase activity assays.

With these modifications we obtained reproducible results which, however, differed dramatically and in many ways from those of Hughes et al. (1992a). Thus, for example, those workers reported that the activity of Canton S wild-type flies in the xanthine:NAD⁺ oxidoreductase assay was 0.26 nmol/min per mg of protein, whereas we found a value of 5.3 ± 0.5 nmol/min

per mg of protein, i.e. some 20 times higher. Hughes et al. (1992a) also reported that another wild-type strain, *ry*⁺¹⁰, showed activities indistinguishable in all assays from those of Canton S. This is surprising, since the former strain carries a mutation in the promoter region that is reported (McCarron et al., 1979) to result in a 50 % diminution in expression of immunologically detected xanthine dehydrogenase cross-reacting material. In agreement with this, but in contrast with the original work, we found that xanthine:NAD⁺ oxidoreductase activity in *ry*⁺¹⁰ extracts was 53 ± 5 % of that in those from Canton S, with comparable relative activity levels in several other assays. Finally, and most significantly in relation to our earlier work, we assayed the E89K strain in the xanthine:NAD⁺ and the xanthine:PMS/cytochrome *c* oxidoreductase assays. Activity was clearly present with both assay systems at levels of about 25 % of that in Canton S. This result was confirmed on two separate stocks of the E89K (*ry*⁵²³¹) strain. Results of additional studies (W. A. Doyle and R. C. Bray, unpublished work) will be presented elsewhere. Note, however, that we were unable to perform NADH:PMS/cytochrome *c* and Methyl Viologen:NAD⁺ oxidoreductase assays by the procedures described, because of rapid non-enzymic reactions with the reagent samples that we used.

These findings lead us to believe that the numerical data of Hughes et al. (1992a) are unreliable. They prompted us further-

Figure 1 SDS/PAGE of xanthine dehydrogenase samples

Two samples of the *ry*⁺⁵ wild-type enzyme (lanes a and b) and one of the E89K enzyme (lane c) were run. A_{280}/A_{450} values for the samples were respectively approx. 15, 6.6 and 17.9. Silver staining was used. The left-hand lane shows the positions of *M_r* standards.

Figure 2 Absorption spectra of xanthine dehydrogenase samples: comparison with milk xanthine oxidase

Curve a (dashed line) shows the E89K *Drosophila* enzyme variant (A_{280}/A_{450} 11.5; note that features in the region of 410 nm suggest the presence of a haem contaminant), and curve b (dotted line) shows the γ^{+5} wild-type enzyme (A_{280}/A_{450} 8.1). For comparison, curve c (continuous line) shows the spectrum of milk xanthine oxidase prepared by the method of Hart et al. (1970). Spectra were recorded in 20 mM Tris/HCl buffer, pH 7.4, containing 1 mM EDTA. Note that curves (a) and (b) have been displaced upwards by 0.002 and 0.001 A unit respectively.

more to repeat the purification, originally reported by Hughes et al. (1992b), of the xanthine dehydrogenases from the wild-type and E89K strains. Purification is difficult because of the relatively low levels at which this enzyme is present in *Drosophila*, and because of the problems of growing the organism on a large scale. We found, however, that the original procedures could with advantage be simplified, and our final procedure consisted of extraction and $(\text{NH}_4)_2\text{SO}_4$ fractionation, essentially as described by Hughes et al. (1992b), followed by chromatography (Pharmacia FPLC system) on Mono-Q, Superose-6 and Phenyl-Superose columns. Our final products were not totally pure ($A_{280}/A_{450} > 5.0$) and were obtained in yields that were inadequate for e.p.r. spectroscopic analysis. The probable reason for the low yields in comparison with the earlier work is considered below. Results were nevertheless clear-cut.

The wild-type enzyme was clearly non-proteolysed, showing (Figure 1, lane b) a main band in SDS/PAGE at about M_r 150 000 and a u.v./visible spectrum (Figure 2, curve b) similar to those of other molybdenum-containing hydroxylases (cf. milk xanthine oxidase, shown in Figure 2, curve c). Furthermore, it showed an exceptionally high specific activity, corresponding to a catalytic centre activity of about 23 s^{-1} in the xanthine:NAD⁺ oxidoreductase assay, at pH 8.0 and 23.5 °C (enzyme concentration calculated from A_{450} , by assuming ϵ $36 \text{ mM}^{-1} \cdot \text{cm}^{-1}$). Finally, it had only a very low activity in the xanthine:oxygen oxidoreductase assay, corresponding to $2 \pm 1\%$ of that in the xanthine:NAD⁺ assay, with these relative activities not changing significantly on 11 days' storage at 4 °C and pH 8.0. All these properties are in marked contrast with those reported by Hughes et al. (1992b), but are in no way inconsistent with what is expected for the *Drosophila* enzyme on the basis of the earlier literature. What is in our view the most likely explanation of the discrepancies is put forward with considerable embarrassment to the senior author (R.C.B.). Accidental contamination of the *Drosophila* enzyme (prepared in quantities of the order of 100 μg) with milk xanthine oxidase is a real possibility in a laboratory that routinely handles quantities of the order of several grams of

the latter. We suggest that such contamination must have occurred, specifically with pancreatin-treated milk xanthine oxidase, largely in the desulpho form. This would explain why the preparations of Hughes et al. (1992b) were extensively proteolysed and showed high oxidase activity. On the basis of earlier work (Edwards et al., 1977; Wahl et al., 1982), the *Drosophila* enzyme is expected, as we now find it, not to be proteolysed and to be resistant to conversion into an oxidase form. In further support of the contamination hypothesis are calculations of the amount of xanthine dehydrogenase in whole flies. Provided that the catalytic centre activity of the enzyme does not change during purification and that activities can be accurately estimated in crude extracts, this quantity may be calculated by dividing the yield of purified enzyme by the overall fractional recovery in the purification process. For the wild-type enzyme, our purification gave an 8% yield of material corresponding to 36 μg of the pure enzyme (assumed to have A_{280}/A_{450} 5.0). The extrapolated xanthine dehydrogenase content of the flies is therefore 450 $\mu\text{g}/40 \text{ g}$ of tissue. On the basis of such calculations, the xanthine dehydrogenase levels of Hughes et al. (1992b) are 4-fold higher, with the corresponding value for the E89K enzyme (see below) being 16-fold. These data thus imply that the samples of these workers were largely milk xanthine oxidase.

Properties of partially purified E89K xanthine dehydrogenase are shown in Figure 1 (lane c) and in Figure 2 (curve a). Though the sample examined was far from homogeneous, the 150 000- M_r band is clearly visible, as is the contribution of iron-sulphur centres to the visible absorption spectrum in the region above 500 nm. Within the limits of our measurements, we were not able to distinguish the properties of the E89K from those of the wild-type enzyme. The two enzymes showed similar catalytic centre activities in the xanthine:NAD⁺ assay. Furthermore, the E89K variant retained full activity in the xanthine:PMS/cytochrome *c* assay (1.4 times the activity in the xanthine:NAD⁺ assay, in comparison with a value of 1.3 times obtained for the wild-type enzyme). We conclude that the claim of Hughes et al. (1992b) that the E89K enzyme lacks iron-sulphur centres is quite insecurely based. We can only point out that discrepancies would be resolved if, in addition to the apparent contamination of the enzyme, denatured samples had inadvertently been used for some of the spectroscopic work and assays reported by these workers.

The senior author accepts full responsibility for the shortcomings of the earlier publications. Our geneticist colleagues, Professor A. Chovnick, Dr. J. R. S. Whittle and Dr. J. F. Burke, who we thank for their continuing help, accept the current findings and were in no way involved in the biochemical parts of the earlier work that we now find to be in error. Similar considerations apply to Mr. B. Bennett, who recorded and interpreted e.p.r. spectra. Also, neither of the present authors was significantly involved in practical aspects of the work. They were, however, responsible for the amino acid sequence comparisons of Hughes et al. (1992a), which remain fully valid.

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