The relative conformational stability of the alcohol dehydrogenase alloenzymes of the fruitfly *Drosophila melanogaster*

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The effect of temperature on four purified alloenzymes of the alcohol dehydrogenase (Adh^s , Adh^f , Adh^D and Adh^{n-s}) of the fruitfly *Drosophila melanogaster* was investigated in detail. Initial-velocity studies showed that the naturally occurring Adh^f and Adh^s enzymes differed only in their temperature optima, and evidence of kinetic adaptation to high and low temperature was not apparent. All four alloenzymes denatured irreversibly on heating purified enzyme solutions at pH 6.0. This technique revealed only small differences in thermostability between Adh^f and Adh^s , although the two mutant enzymes from Adh^D and Adh^{n-s} were considerably more labile. Electrophoresis of the enzymes through a stable transverse temperature gradient proved to be a discriminating and reproducible technique. Enzymes of different net charge were compared on the same polyacrylamide gel. The Adh^f enzyme was shown to be significantly less stable than the Adh^s enzyme. Subunit interchange was observed at temperatures below the point at which the unfolding occurred. At pH 4.0, the Adh^f/Adh^s heterodimer was as stable as the Adh^s homodimeric enzyme, and more stable than the Adh^f homodimer. Adh^{n-s} and Adh^D alloenzymes were relatively thermolabile. The stability of the alloenzymes towards urea denaturation was studied by urea-gradient electrophoresis. Only small differences in stability between the Adh^f and Adh^s enzymes were observed. The Adh^D and Adh^{n-s} mutants were denatured at the same urea concentration, which was much lower than in the case of the wild-type enzymes. Except at pH 4.0, subunit dissociation could not be distinguished from the unfolding of the monomer.

The notion that the proteins of poikilothermic species are adapted to the particular temperature of the ecological niche they inhabit is well supported experimentally (Somero & Low, 1976; Argos *et al.*, 1979). By contrast, the temperature adaptation of enzyme variants segregating within populations of the same species has been little studied. Most of the intra-species molecular variation that we know exists at the protein level has been revealed by the technique of gel electrophoresis, and it is self-evident that this is only a proportion of the total variation existing. However, the amount of electrophoretically 'silent' variation at the Adh (alcohol dehydrogenase EC 1.1.1.1) locus of the fruitfly, *Drosophila melanogaster*, is thought to be low in comparison with other loci (Sampsell, 1977; Retzios & Thatcher, 1979; Kreitman, 1980). Therefore a study of the molecular properties of alcohol dehydrogenases purified from representative isogenic lines of the two major alloenzymes Adh^f and Adh^s may be legitimately

used in the interpretation of naturally occurring patterns of Adh electromorph variation.

Adh^f and Adh^s occur at different frequencies in different natural populations of *Drosophila*. Generally the frequency of the Adh^f electromorph has been found to increase with latitude (Vigue & Johnson, 1973; Malpica & Vassallo, 1980) or with altitude (Grossman *et al.*, 1970; Pipkin *et al.*, 1976) and in some environments with the abundance of ethanol (Briscoe *et al.*, 1975). A number of experiments and observations suggest that these frequency clines are directly related to the temperature adaptation of the Adh^f and Adh^s gene products. For example, partially purified extracts of isogenic Adh^f and Adh^s flies differ in the thermostability of their alcohol dehydrogenases (Gibson & Miklovitch, 1971; Vigue & Johnson, 1973; Day *et al.*, 1974a,b; Sampsell, 1977). On the other hand, these clines could be due to the known genetic linkage of Adh^s to an inversion in chromosome 2, which may contain

other temperature-sensitive loci that are under selective pressure (Voelker *et al.*, 1977; Yoshimaru & Mukai, 1979; Malpica & Vassallo, 1980). As there is no reason, *a priori*, why amino acid substitutions that cause variations in net charge should be linked to another molecular property such as thermostability, and as the results obtained by the denaturation of crude enzyme preparations are ambiguous and do not necessarily dissociate the properties of the alloenzymes from their genetic background (Thatcher, 1977; Anderson *et al.*, 1980), it is particularly important to establish whether or not the *Adh^f* and *Adh^s* alloenzymes do differ inherently in stability. With this objective in mind we have investigated the stability of four purified alloenzymes: two from naturally occurring populations, *Adh^f* and *Adh^s*, and two mutant strains derived from *Adh^f*, *Adh^D*, and *Adhⁿ⁻⁵*. The *Adh^f* and *Adh^s* alloenzymes used differed in covalent structure by a single threonine (*Adh^f*)-to-lysine (*Adh^s*) substitution at sequence position 192 (Thatcher, 1980). *Adh^D* is an enzymically active electrophoretic mutant that differs by a glycine-to-glutamic acid substitution (at protein sequence position 232) from the parental *Adh^f* enzyme (Schwartz & Jörnval, 1976; Thatcher, 1980). *Adhⁿ⁻⁵* is a temperature-sensitive mutant (Vigue & Sofer, 1974) of unknown structure, although it is known that the substitution does not involve a change in net charge (Schwartz & Sofer, 1976).

Experimental

Organisms

Drosophila melanogaster (fruitfly) strains were obtained from the collection of Professor B. C. Clarke (University of Nottingham, Nottingham, U.K.). *Adh^s* was originally isolated from the Kaduna (Nigeria) population of Professor A. Robertson University of Edinburgh, Edinburgh, Scotland, U.K.). *Adh^D* was originally produced by ethyl-methanesulphonate mutagenesis of an *Adh^f* strain captured by Professor E. H. Grell in Samarkand. *Adhⁿ⁻⁵* was a mutant of *Adh^D* produced in the laboratory of W. Sofer (University of Baltimore, Baltimore, MD, U.S.A.).

Chemicals

Acrylamide monomer was obtained from Eastman Kodak, Kirby, Liverpool, Merseyside, U.K., and was recrystallized from chloroform before use. *NN'*-Methylenebisacrylamide 5,5'-dithiobis-(2-nitrobenzoic acid), ATP and NAD⁺ were bought from Sigma, Poole, Dorset, U.K. Sepharose 6B was purchased from Pharmacia, Hounslow, Middx., U.K., and all other reagents and biochemicals were obtained from BDH, Poole, Dorset, U.K.

Enzyme preparation

Flies were grown in bulk as described in Thatcher (1980), and alcohol dehydrogenases were purified as detailed for the *Adh^s* alloenzyme in that paper, except that the final gel-filtration step was replaced with an affinity-chromatography step. The ligand ATP-8-(6-aminoethyl)amino-Sepharose was prepared by the method of Lee *et al.* (1977). The enzyme, in 5 mM-sodium phosphate buffer, pH 6.5, was applied to a column (50 cm × 2.5 cm diam.) of the affinity Sepharose and was eluted under the conditions suggested by Lee *et al.* (1979). Each alloenzyme preparation gave a single band on agarose-gel electrophoresis at pH 8.6. The enzyme stock solutions (approx. 10 mg/ml) were stored at 5°C, as repeated freezing and thawing caused rapid precipitation of all enzymes.

Enzyme-activity measurements

The activity of the enzyme was measured under saturating conditions (1 mM-NAD⁺ and 300 mM-propan-2-ol) as described by Thatcher (1977). The temperature was controlled by using a Haake thermostatically controlled circulating-water bath in line with a copper cooling coil immersed in 10 litres of melting ice.

Thermal-aggregation experiments

These experiments were performed in a Pye-Unicam SP.1800 split-beam spectrophotometer fitted with an SP.876 temperature program controller. The enzymes were diluted to a final volume of 1 ml with 0.1 M-sodium phosphate buffer, pH 6.0, in a quartz semi-micro cuvette. Both the temperature increase and the *A*₂₈₀ were monitored simultaneously by using a W+W Tarkan 600 two-channel recorder. The enzymes were denatured by increasing the temperature at a given rate, and the temperature at which denaturation commenced was taken as the aggregation temperature, a technique that has been called 'temperature-scanning kinetics' by Fujita & Imahori (1974). A theoretical analysis of the irreversible denaturation of proteins by this method has been developed by Fujita *et al.* (1979).

Thermal-gradient electrophoresis

This technique was described in the preceding paper (Thatcher & Hodson, 1981). The method is analogous to the urea-gradient electrophoresis method of Creighton (1979) (see below), employing a transverse temperature gradient instead of urea as the denaturant. The temperature gradient was formed by circulating water at 0°C through one end of a metal heating jacket and water at 50°C through the other end. The jacket was clamped across the slab-gel plates and left for 1 h to equilibrate. The temperature of the heating jacket was recorded

throughout the electrophoresis run to ensure that the stability of the temperature gradient had been maintained. Electrophoresis was continued for 3 h at 2 mA/cm of gel, and at the end of each run the approximate position of the thermocouple in the heating jacket was marked on the gel plates and the temperature recorded. The gel was then removed, the thermocouple positions marked with Indian ink and stained in 0.1% Coomassie Blue [in methanol/acetic acid/water, 5:1:4 (by vol.)]. The gels were destained in methanol/acetic acid/water (1:1:8, by vol.) and photographed through transmitted light. The buffers used and the polyacrylamide-gel recipes were described in detail by Thatcher & Hodson (1981).

Urea-gradient electrophoresis

The method of Creighton (1979) was adopted. The gel size was 140 mm × 140 mm × 1 mm and a Raven slab-gel apparatus was used. The electrophoresis runs were performed at 5°C in a Colara cold-box, the slab-gel apparatus being immersed in a chromatography tank (22 cm × 22 cm × 40 cm) filled with cold electrophoresis buffer. The pH 4.0 buffer described by Creighton (1979) was employed and the Tris/EDTA/borate buffer described by Ursprung & Carlin (1968) was used at pH 8.6. Gel polymerization was induced by fluorescent-light irradiation of gel solutions containing riboflavin (0.5 ml of a 0.08 mg/ml solution per 15 ml of gel mixture) and *NNN'*-tetramethylethylenediamine (10 µl/15 ml of gel mixture). A 10–8% polyacrylamide-gel gradient was formed in the opposite direction to the urea gradient. The gels were poured by using a normal laboratory gradient maker, 2 ml of liquid paraffin being added to the non-urea solution to equalize the hydrostatic head with that of the 8 M-urea solution. Bromophenol Blue to a final concentration of 0.005% was included in the urea solution so that the linearity of the urea gradient in the polymerized gel could be checked by densitometry. Gels were run at 1.5 mA/cm of gel 14 h at pH 8.6 and for 4 h at pH 4.0. The urea in the gels was leached out in methanol/acetic acid/water (5:1:4, by vol.) before staining. Gels were stained either for activity by the method of Grell *et al.* (1968) or for protein by the method described above for the thermal-gradient gels.

Results and discussion

Effect of temperature on the reaction catalysed

The effect of temperature on the initial velocity of the enzyme under saturating conditions was determined (Fig. 1) in order to establish whether or not the kinetic properties of enzyme variants were adapted to temperature. The *Adh^f* and *Adh^s* alloenzymes, however, had identical Arrhenius activa-

Fig. 1. Effect of temperature on the initial velocity of the *Adh^f* and *Adh^s* alloenzymes of alcohol dehydrogenase. Purified enzyme was assayed at various temperatures. The *Adh^f* (○) enzyme and the *Adh^s* (●) enzyme were preincubated at the assay temperature for 5 min before assay. The temperature was recorded at the beginning and end of each assay by using a thermometer immersed in a blank cuvette in the constant-temperature cell housing of the spectrophotometer.

tion energies and were therefore not adapted. They did, however, differ in their temperature optima, the *Adh^s* alloenzyme being more active at higher temperatures.

Effect on temperature on the stability of the enzyme

Aggregation. Enzyme solutions were subjected to a constant rate of temperature increase, the point at which aggregation occurred being determined spectrophotometrically. This aggregation temperature was found to be independent of protein concentration above 50 µg/ml (Fig. 2) but dependent on the rate of heating (Table 1). The *Adh^s* enzyme was slightly more thermostable at a heating rate of 1°C/min than the *Adh^f* enzyme, although the method lacked sensitivity at a rate of 7°C/min. The *Adhⁿ⁻⁵* enzyme was more thermolabile than the wild-type enzymes, as expected from the temperature-sensitive phenotype of *Adhⁿ⁻⁵* flies. Surprisingly, the parental strain of this mutant, *Adh^D*, also produces a thermolabile alcohol dehydrogenase.

Thermal-gradient electrophoresis. The method is capable of providing data on the effect of tem-

perature on the conformation and quaternary structure of proteins (Thatcher & Hodson, 1981). Because the alleloenzymes studied differ in electrophoretic mobility, their denaturation profiles may be examined simultaneously in the same electrophoresis run.

Fig. 2. Effect of increasing enzyme concentration on the aggregation of alcohol dehydrogenase

Aggregation, produced by exposing enzyme to a linear temperature gradient, was quantified as described in the text. The experiments were performed at pH 6.0 in 0.1 M-sodium phosphate buffer and the temperature was increased at a rate of 1°C/min. The aggregation temperature was taken as the temperature at which the A_{280} began to increase. The concentrations of the Adh^f (○) and the Adh^s (●) enzyme were determined by amino acid analysis as described in Thatcher (1977).

The pattern of denaturation observed was consistent with a process of unfolding and irreversible aggregation (Figs. 3 and 4). No fine structure was apparent between the native enzyme band, which came to an abrupt end at a particular temperature, and the denatured aggregate, which did not enter the gel. This 'melting' temperature was estimated from the temperature of the heating jacket of the apparatus (Table 2) and was found to vary slightly with the concentration of protein applied to the gel and with the duration of electrophoresis. Under standard conditions of 100 μg of enzyme/gel and 200 min of electrophoresis, the Adh^s enzyme denatured at a temperature 5°C higher than did the

Table 1. Aggregation of alcohol dehydrogenase alleloenzymes

All measurements were made on solutions containing 0.5 mg of enzyme/ml in 0.1 M-sodium phosphate buffer, pH 6.0.

Alleloenzyme genotype	Heating rate (°C/min)	'Melting' temperature (°C)
Adh^f	7	48.0
Adh^s	7	48.0
Adh^D	7	46.0
Adh^{n-5}	7	40.0
Adh^f	1	42.5
Adh^s	1	45.0
Adh^D	1	35.0
Adh^{n-5}	1	33.0

Fig. 3. Thermal-gradient electrophoresis of alcohol dehydrogenase alleloenzymes at pH 8.6

The gels were loaded with equal quantities of each alleloenzyme type, 100 μg each for gels (a) and (c) and 50 μg for gel (b), dissolved in the pH 8.6 buffer, diluted with an equal volume of 50% sucrose. Samples were loaded with a 100 μl Eppendorf pipette. The 'melting' temperatures recorded on the outer heating jacket were as follows: gel (a), Adh^f = 30°C, Adh^s = 38°C; gel (b), Adh^f = 33°C, Adh^D = 27.5°C; and gel (c), Adh^f = 32.5°C, Adh^{n-5} = 20°C.

Fig. 4. Thermal-gradient electrophoresis of alcohol dehydrogenase at pH 4.0

The Adh^f and Adh^s enzymes were electrophoresed into a shallow temperature gradient (increasing by $0.5^\circ\text{C}/\text{cm}$ across the gel left to right). The upper (Adh^f homodimer) band disappeared abruptly at a temperature of 23°C , whereas the Adh^f/Adh^s heterodimer and the Adh^s homodimer 'melted' at 33°C .

Table 2. Heat-denaturation temperatures measured by thermal-gradient electrophoresis at pH 8.6

Results are means $\pm$ S.D. for the numbers of experiments given in parentheses.

Alleloenzyme genotype	'Melting' temperature ($^\circ\text{C}$)
Adh^f	32 ± 0.9 (9)
Adh^s	38 ± 1.0 (5)
Adh^D	27.5 ± 1.0 (5)
Adh^{n-5}	21.6 (3)

Adh^f enzyme. Both the Adh^D and Adh^{n-5} enzymes denatured at lower temperatures under the same conditions.

At pH 4.0, the presence of a band of intermediate electrophoretic mobility in $Adh^f + Adh^s$ enzyme gels indicated that subunit dissociation and re-association must have occurred during the initial stages of electrophoresis. When a shallow gradient of temperature was applied across the gel (Fig. 4), the pattern observed was consistent, with the Adh^f/Adh^s heterodimer being more stable than the Adh^f homodimer and as stable as the Adh^s homodimer. These findings may provide an explanation of the results of Van Delden *et al.* (1978) on the effect of temperature on caged populations of *Drosophila melanogaster*. At higher temperatures the Adh^f frequency in the cages fell to a lower equilibrium value, which would be expected if the Adh^f homozygote (thermolabile gene product) was at a relative disadvantage to the heterozygote and Adh^s homozygote. The formation of the intermediate heterodimer band was most pronounced in the $Adh^f +$

Adh^{n-5} gels, occurring at all temperatures below the denaturation temperature. As this pattern was not observed in $Adh^f + Adh^D$ gels, the Adh^{n-5} mutation must be affecting subunit interaction.

Effect of urea on the stability of the enzyme

Experiments were performed to see if the observed differences in thermostability of the alleloenzymes were reflected in a differential susceptibility towards urea denaturation. The transverse-urea-gradient gel-electrophoretic method described by Creighton (1979) was adopted. The undenatured or native enzymes migrated as discrete bands at low urea concentrations and showed rapid transitions to the denatured state at pH 8.6 (Fig. 5). Most of the enzyme unfolded into a state having an electrophoretic mobility of less than half that of the native enzyme. The presence of distinct transition zones between the native and fully denatured state showed that the unfolding process was both rapid and reversible. At pH 4.0 (Fig. 6) the folding/refolding transition band did not merge with the native band but migrated with a slightly higher mobility at the point at which denaturation begins. This pattern is interpreted as evidence for the dissociation of the alcohol dehydrogenase dimer into subunits before unfolding occurs. Gels loaded with two alleloenzymes never showed bands of intermediate electrophoretic mobility when denatured, although heterodimer native bands were sometimes observed. Staining the pH 8.6 gels for active enzyme showed that the enzyme did not refold to its original structure after urea denaturation, since enzyme could only be detected in the native bands and never in the transition zone or where the enzyme was fully denatured.

Fig. 5. Transverse-urea-gradient electrophoresis of alcohol dehydrogenase at pH 8.6

The interpretation of the photograph (a) is shown diagrammatically in (b). The urea gradient was always 0–8 M (right-hand side). The arrows denote the positions of aggregated enzyme. In expt. A, 100 µg each of the *Adh^s* and *Adh^f* alleloenzymes were loaded on top of the gel. In expt. B, 100 µg each of the *Adh^D* and *Adh^f* alleloenzymes were loaded on to the gel. In expt. C, 100 µg each of the *Adhⁿ⁻⁵* and *Adh^f* alleloenzymes were loaded on to the gel. Experimental details are described in the text.

Fig. 6. Transverse-urea-gradient electrophoresis of alcohol dehydrogenase at pH 4.0

In gel A the *Adh^s* alleloenzyme alone was loaded on to the gel. Gel B was loaded with 100 µg each of the *Adh^f* and the *Adh^s* enzymes.

The *Adh^s* enzyme was only slightly more resistant to urea denaturation than was the *Adh^f* enzyme. Both the *Adh^D* and *Adhⁿ⁻⁵* mutant enzymes

were equally sensitive to urea, unfolding at less than 1 M-urea, and were consequently much more labile than the wild-type enzymes.

We have shown that the alleloenzymes from the *Adh^f* and *Adh^s* electromorphs of *D. melanogaster* do differ inherently in their stability. As at the sequence level these enzymes differed in a single threonine (*Adh^f*)-to-lysine (*Adh^s*) amino acid substitution (Thatcher, 1980), thermostability does in this case correlate with a change in net charge. This substitution also changes a number of kinetic properties (D. R. Thatcher & A. Retzios, unpublished work; Clarke, 1975). It is unlikely that these differences in the molecular properties of the *Adh^f* and *Adh^s* gene products do not affect the physiology of *D. melanogaster* and its ability to compete in alcohol-rich environments.

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