ADAPTATION OF DROSOPHILA ENZYMES TO TEMPERATURE—II. SUPERNATANT AND MITOCHONDRIAL MALATE DEHYDROGENASE

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(Received 12 July 1978; revised 3 October 1978)

Abstract—The observed temperature-dependent $K_m$ patterns were similar among species (Drosophila) from the same habitat and different among species from different habitats. The $Q_{10}$ values in general reflected temperature dependent changes in $K_m$. The observed convergence in terms of $K_m$ and $Q_{10}$ values and assay temperature strongly suggests that natural selection is operating at the malate dehydrogenase locus in the genus Drosophila with the temperature being one of the mediating agents. Our dual, based on biochemical analysis, show that mitochondrial malate dehydrogenase seems to be a 'conservative' enzyme from the evolutionary point of view. This enzyme isolated either from temperate or tropical species exhibits "symptoms" of tropical enzyme in terms of $K_m$ vs. temperature. The fact that mitochondrial malate dehydrogenase and soluble malate dehydrogenase do not react coordinately, bears on the significance of the existence of two forms of the enzyme.

Key Word Index: Drosophila, supernatant and mitochondrial malate dehydrogenase, homologous enzymes, temperature compensation, selection

INTRODUCTION

Since temperature is known to affect the higher structures of proteins (3° and 4°) and the interactions of proteins with low molecular weight ligands, many studies have focused on the effects of temperature on the formation of enzyme–substrate complexes and on the conversion of this complex into an 'activated complex' (Low et al., 1973; Low and Somero, 1976). From these studies significance was placed on the mechanism of positive thermal modulation, for metabolic rate compensation (Hochachka and Somero, 1973). According to this mechanism decreases in temperature within the physiological temperature range of an organism, which would normally reduce enzyme reaction rates, are accompanied by an immediate increase of the enzyme substrate affinity. This leads to reaction rate constancy possibly buffering the individual against changes which could take place during daily or seasonal temperature fluctuations.

The present work deals with the temperature dependent kinetic parameters of the double enzymic system of the mitochondrial-soluble malate dehydrogenase (m-Mdh, s-Mdh) by comparing several temperate and tropical species of Drosophila. Investigation, utilizing such enzymic systems, is of considerable interest for studies in biochemical evolution. The aim of this study was to determine the extent to which the temperature-compensatory system mentioned above, is taking place in terrestrial poikilotherms (in the genus of Drosophila). The specific questions whether this is a common scheme and if so, whether there exists a convergence in enzymatic function among terrestrial species inhabiting similar habitats (tropical versus temperate species) were examined.

MATERIALS AND METHODS

Stocks and culture conditions

We examined three tropical (D. willistoni, D. arizonensis, D. equinoxialis) and two temperate species (D. virilis, D. americana). Stocks were provided by the Stock Center, University of Texas, Austin (Patterson and Stone, 1952) and were maintained at 25°C in a dead-yeast-sugar-agar food medium (Alahiotis and Pelécanos, 1978).

Chemicals

DDT (dithiothreitol), EDTA (ethylenediaminetetraacetic acid), NAD$^+$ (oxidized form of $\beta$-nicotinamide adenine dinucleotide), NADH (reduced form of $\beta$-nicotinamide adenine dinucleotide), PMS (phenazine methosulfate), NBT (nitroblue tetrazolium), OAA (oxaloacetate), citric acid and Tris (Tris(hydroxymethyl) amino methane) were obtained from Sigma Chemical Co.

Enzyme separation and assay techniques

For the separation of the cytoplasmic from the mitochondrial malate dehydrogenase a simple method was applied based on the observation that the soluble enzyme migrates to the anode while the mitochondrial enzyme migrates to the cathode in starch electrophoresis (McReynolds and Kitto, 1970). This technique and the gel electrophoresis are described in detail elsewhere (Alahiotis, in press). In short, crude homogenates [300 mg flies/ml 0.1 M potassium phosphate buffer pH 7.5 with 1 mM EDTA (Na$_2$), 1 mM DTT] of 4-10-day-old adults were made in a glass homogenizer. Following two centrifugations (at 20,000 g for 20 min at 4°C) both the soluble and mitochondrial Mdh were present in the supernatant. To collect cytoplasmic or mitochondrial enzyme, electrophoresis was carried out (tris-citrate buffer at pH 7.0), (Alahiotis, in press), for 2 hr on starch plates and a small longitudinal strip of gel removed and stained for malate dehydrogenase (the staining solution was 25 mg NAD, 15 mg NBT, 1 mg PMS, 50 mg l-malate 10 ml 0.1 M glycine-NaOH pH 10.3, 40 ml H$_2$O2 activity appeared within 5-10 min). Just anodal to the position in
which staining took place a section of starch was removed from across the entire gel and substituted with a buffer saturated strip of sponge. Current was turned on again and the enzyme was electrophorized into the sponge during the next 50 min. A strip of dialysis membrane was inserted anodal to the sponge to prevent enzyme from migrating out of the sponge. The sponge was then removed and squeezed to recover the enzyme. To remove starch and sponge particles the solution was centrifuged at 5000 g for 10 min. We tested the purity of the soluble or mitochondrial enzyme by a second electrophoresis (Fig. 1). The partially purified enzyme was used immediately. Malate dehydrogenase activity was assayed by monitoring the oxidation of NADH at 340 nm in a Gilford recording spectrophotometer. Assay temperatures were maintained in the cuvette chamber by a Haake circulating water bath. The reaction was initiated by the addition of 20 µl partially purified enzyme preparation in a circulating water bath, cooled on ice, and assayed for activity. The results were plotted as percent of original activity vs time of incubation.

RESULTS AND DISCUSSION

Starch-gel electrophoresis; heat inactivation test

Crude extract from each of the five species was subjected to starch-gel electrophoresis at pH 7.0. The mobility of the s-Mdh shows very high interspecific variation (Fig. 1). The same is true when the heat inactivation profiles of s-Mdh are compared (Fig. 2). It seems that enzymes extracted from tropical species are not more stable than those from temperate. The same is true for the acetylcholinesterase (Ache) and NADP-dependent isocitrate dehydrogenase (Idh-NADP) (ALAHOTIS and BERGER, 1978) although in the case of z-glycerophosphate dehydrogenase (z-Gpdh) (ALAHOTIS et al., 1977) a correlation was observed between inactivation rate and natural habitat temperature. The heat inactivation patterns of s-Mdh were found to be species specific which indicate that the primary sequence of s-Mdh for each of the five species was different.

Michaelis constants

Table 3 shows that the enzyme-substrate affinity of s-Mdh is temperature-dependent, indicating that it is sensitive to environmental temperature. All species had a pattern of positive thermal modulation the extent of which differed between tropical and temperate species. In comparison with those of tropical species, K_m values of the temperate species, although indistinguishable at low temperatures are higher at high temperatures. The observation suggests that the K_m-temperature plots, again are habitat temperature specific. It must be noted that the K_m values obtained here for partially purified enzyme are higher than those reported earlier (McREYNOLDS and KITTO, 1970; HAY and ARMSTRONG, 1976) for D. virilis and D. melanogaster. Moreover, enzymes other than Mdh which are present in crude extracts, or partially purified enzyme preparation may also use oxaloacetate as substrate. Thus the discrepancy in K_m values may be due in fact to variation in the degree of purification (and assay conditions); alternatively, it could be due to the interference of low molecular weight compounds that are removed during purification. A parallel case has been reported for the alcohol dehydrogenase of D. melanogaster (McDONALD et al., 1977).

Q_10 and E_a

From the kinetic data temperature coefficients (Q_10) at three 10°C temperature intervals were determined at two substrate concentrations. As one can see from Table I the Q_10 values are lower at below substrate saturation conditions (0.2 mM OAA) than those at saturating concentrations of substrate (3 mM OAA), where K_m is unimportant in determining reaction velocity (SOMERO, 1969). Furthermore, on the basis of positive thermal modulation model, and below saturation concentrations of the substrate (0.2 mM; Table I) the Q_10 values of tropical species are higher than those of temperate as predicted (HOCHACHKA and SOMERO, 1973). Activation energies (E_a) were calculated (ROBERT and GRAY, 1972) from Arrhenius plots of log V_max vs 1/T. All species had similar values (10.24 ± 0.23 kcal/mole).

pH optima

As in the case of D. melanogaster (ALAHOTIS, in press) all Drosophila species examined here exhibited activity peaks at two different pH values (the main peak at pH 9.45 and a shoulder at pH 7.95) although it has not been noticed before (McREYNOLDS and KITTO, 1970). It is quite likely that the observed bimodality in pH optima indicates the presence of two isozymic types (ALAHOTIS, in press) of s-Mdh (it has been suggested by O'Brien (1973), that s-Mdh is a dimeric enzyme with epigenetic modification).

Table 1. Temperature coefficients (Q_10) of s-Mdh as a function of substrate and temperature range. The values represent an average of three separate experiments, and were determined at two concentrations of substrate (1) 3 mM OAA, (2) 0.2 mM OAA. The results are the means ± S.E.M.

| Species          | Temperature range | Q_10 (1) | Q_10 (2) |
|------------------|-------------------|----------|----------|
| D. arizonensis   | 5-15°C            | 2.0 ± 0.25 | 2.0 ± 0.05 |
|                  | 15-25°C           | 1.40 ± 0.15 | 1.36 ± 0.07 |
|                  | 25-35°C           | 1.51 ± 0.05 | 1.33 ± 0.09 |
| D. equinoxialis  | (1)               | 1.72 ± 0.59 | 1.44 ± 0.10 |
|                  | (2)               |           |          |
| D. willistoni    | (1)               | 1.45 ± 0.07 | 1.62 ± 0.21 |
|                  | (2)               | 1.09 ± 0.04 | 1.33 ± 0.18 |
| D. virilis       | (1)               | 1.67 ± 0.35 | 1.07 ± 0.04 |
|                  | (2)               |           |          |
| D. americana     | (1)               | 1.70 ± 0.10 | 1.45 ± 0.08 |
|                  | (2)               | 1.12 ± 0.11 | 1.12 ± 0.07 |

Values are means ± S.E.M.
Fig. 1. Mdh comparative allozyme pattern of adults *Drosophila* species. Channels 1 and 2 show the purified cytoplasmic enzyme of the *D. arizonensis* and *D. virilis*. Channels 3, 4, 5, 6 and 7 contain crude extracts from the species *D. equinoxialis*, *D. arizonensis*, *D. willistoni*, *D. virilis* and *D. americana*, respectively. Mitochondrial enzyme migrates into the cathodal half of the gel, while cytoplasmic enzyme is localized in the anodal half. O denotes the 'origin'. The photograph does not show the minor band of s-Mdh activity which appears only faintly on the gel.
Adaptation of Drosophila enzymes

**s-Mdh isozymes**

s-Mdh is one of the *D. melanogaster* enzymes which shows no developmental isozymes when single flies, pupae, larvae and embryonic cells are subjected to starch electrophoresis (ALAHiotis and Berger, 1977). Moreover, homogenates of fly parts (head, thorax, abdomen from either females or males) exhibited Mdh activity without showing isozymic differences on starch gel electrophoresis. Hence, the present study was based on enzyme isolated from the whole organism and not from a particular organ.

**Mitochondrial Mdh (m-Mdh)**

Particularly interesting in this study is the observed $K_m$ vs. temperature patterns of the m-Mdh. Figure 4 shows clearly that m-Mdh, isolated either from tropical or temperate species, has 'symptoms' of tropical enzyme, in terms of $K_m$-temperature dependence. This finding, in combination with the absence of interspecific variation in electromorphs (Fig. 1) and with the fact that the m-Mdh of *D. melanogaster* was more thermostable than the s-Mdh (McReynolds and Kitto, 1970) increase, considerably, the possibility that m-Mdh is conservative in evolutionary changes in the genus of Drosophila. Such a 'conservative' enzyme like Drosophila α-glycerophosphate dehydrogenase (Lakovara et al., 1977) has much taxonomic value and is apparently useful as a key in classification. Our results are strengthened by those of McReynolds and Kitto (1970), where mitochondrial malate dehydrogenase from two different species of Drosophila (*D. melanogaster* and *D. virilis*) is catalytically more similar to each other than to the supernatant enzyme from the same species and vice versa. Conservation, but in the opposite direction in electrophoretic mobility of s-Mdh and m-Mdh, was also observed in birds (Kitto and Wilson, 1966). The fact that s-Mdh and m-Mdh do not react coordinately in terms of $K_m$ vs temperature also supports the existence of two forms of the enzyme. While both s-Mdh and m-Mdh are under nuclear control (O'Brien, 1973) it appears that they have different evolutionary capabilities.

**CONCLUSION**

It is widely accepted that the $K_m$ and $Q_{10}$ for any given enzyme is not a change characteristic, but one of major importance in metabolic control and regulation of reaction rates. Hence, it must be under selective pressure (Hochachka and Lewis, 1971). In conclusion, the observed temperature-dependent $K_m$ profiles described in the present paper, as predicted, seem to be based on the species distribution rather than phylogeny. The data strongly suggest that natural selection is operating on malate dehydrogenase in the genus Drosophila, temperature being one of the controlling agents; although it cannot be said with any certainty how these kinetic differences are translated into physiological differences in vivo. This is also true for Ache, Idh-NADP (Alahiotis and Berger, 1978), α-Gpdh (Alahiotis et al., 1978) of Drosophila as well as for s-Mdh of reptiles (Hoskins and Aleksiuk, 1973).

Acknowledgements—I am indebted to Professor M. Pelecanos for critically reading the manuscript as well as for

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Fig. 2. Thermal inactivation of s-Mdh at 52°C. Plots designate *D. virilis* (O), *D. americana* (●), *D. equinoxialis* (+), *D. arizonensis* (△) and *D. willistoni* (□). Bars about representative points (means) indicate S.E.M. values of at least six determinations. The same is true for Figs. 3 and 4.

Fig. 3. Apparent $K_m$ for oxaloacetate-supernatant Mdh as a function of assay temperature for *D. americana* (●), *D. virilis* (O), *D. equinoxialis* (+), *D. arizonensis* (△) and *D. willistoni* (□).

Fig. 4. Apparent $K_m$ for oxaloacetate for *D. equinoxialis* (○), *D. americana* (+) and *D. virilis* (●).
providing the Laboratory facilities; thanks are also due to Dr. E. BERGER (Dartmouth College, N.H., U.S.A.) for supplying the stocks.

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