J. Mol. Evol. 16, 37–46 (1980)

Journal of Molecular Evolution

Adaptation of *Drosophila* Enzymes to Temperature. III. Evolutionary Conservatism in Mitochondrial Enzymes

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Summary. The evolutionary behavior of two mitochondrial enzymes (L-glycerol 3-phosphate:cytochrome c oxidoreductase E.C.1.1.1.95, αGPO, and L-malate: NAD^+ oxidoreductase, E.C.1.1.1.37, m-MDH) obtained from several temperate and tropical *Drosophila* species was examined by comparing their catalytic properties, which related to temperature (K_m–E_a–Q_{10}–Thermostability). Mitochondrial αGPO or m-MDH obtained either from temperate or from tropical species was found to exhibit similar catalytic properties while for both cytosolic enzymes, the αGPDH and s-MDH, K_m patterns were similar among species from the same thermal habitat and different between thermal habitats. In combination with other observations reported in the literature these facts support the view that the function, and probably the structure, of mitochondrial enzymes are better conserved in evolution than those of the corresponding enzymes found in the cytosol. It is proposed that the relative invariance of the mitochondrial enzymes structure is probably linked to a necessary relative invariance of molecular interactions inside the mitochondrion.

Key words: Evolution – *Drosophila* – Temperature – Mitochondrial enzymes – Kinetic properties

Introduction

On the basis of the positive and negative thermal modulation model proposed by Hochachka and Somero (1973) for metabolic rate conpensation, we have recently shown that *Drosophila* cytosolic (cytoplasmic) enzymes obtained from temperate species exhibit a pattern of strong positive thermal modulation (Alahiotis, et al. 1977; Alahiotis and Berger, 1978; Alahiotis, 1979a,b). In contrast, enzymes obtained from tropical species were found to exhibit either a pattern of negative thermal modulation or a pattern characterized by a lack of interaction between K_m values and temperature (α-GPDH, Alahiotis, et al., 1977; ACHE and IDH, Alahiotis and Berger, 1978; MDH, ADH, Alahiotis, 1979a,b). On the basis of these previous results we can now classify a

0022-2844/80/0016/0037/$ 02.00

Drosophila enzyme as being 'tropical' or 'temperate'. This association of K_m with temperature in combination with an analogous correlation of other catalytic properties (e.g. Q_{10}) has been considered as strong evidence in favor of the selection model (Alahiotis, et al., 1977; Alahiotis and Berger, 1978; Alahiotis, 1979a,b).

While the above situation is true for several cytosolic enzymes, there is not much information of this sort for *Drosophila* mitochondrial enzymes. We have previously shown (Alahiotis, 1979a), that mitochondrial malate dehydrogenase shows signs of conservative evolutionary changes in the genus *Drosophila* while this is not true for the cytosolic malate dehydrogenase. In the present investigation we provide evidence that the same situation holds for the α-Glycerophosphate dehydrogenases (cytosolic/mitocondrial; αGPDH/αGPO). Furthermore, it is known that there are several enzymic systems which participate in the same biochemical cycle, but act either in the cytosol (cytosolic enzymes) or in a subcellular organelle (mitochondrial enzymes) and represent products of different structural genes. Representative systems include malate dehydrogenase (McReynolds and Kitto, 1970; O'Brien, 1973) and α-Glycerophosphate dehydrogenase (O'Brien and MacIntyre, 1972). The α-Glycerophosphate cycle is composed of two distinct α-Glycerophosphate dehydrogenases: a cytosolic enzyme which requires NAD and a mitochondrial, particle-bound oxidase (a flavin-linked enzyme) which does not require NAD for catalysis (Hansford and Sacktor, 1971; O'Brien and MacIntyre, 1972). The former enzyme catalyzes the reduction of dihydroxyacetone phosphate to α-glycerophosphate in the soluble fraction of the cell, while the latter carries out the reverse reaction in the particulate fraction (O'Brien and MacIntyre, 1972). The existence of such enzymic systems permits us to raise the following question: Do such mitochondrial and cytosolic enzymes respond coordinately to temperature selection?

It is generally accepted that the mitochondrion is a semiautonomous organelle whose propagation depends in part on its own genetic and enzymatic resources (for review see Tzagoloff, 1977). This organelle seems to have arisen by endosymbiosis (much earlier than 2 billion years ago; Woese, 1977). Given the great age of this organelle and its partial physiological and genetic autonomy we would like to determine whether genes that control specific mitochondrial functions respond differently to evolutionary pressures as compared to those associated with the cytosol functions.

Since the catalytic properties of several cytosolic enzymes (especially patterns of K_m vs. temperature) were found to be diagnostic for tropical and temperate species and to reflect genetic differences (Alahiotis, et al., 1977; Alahiotis and Berger, 1978; Alahiotis, 1979a,b) among homologous enzymes, we utilized this tool as a first approach in order to test the aforementioned hypothesis. Homologous mitochondrial αGPO were obtained from tropical, temperate and cosmopolitan species and a spectrophotometric assessment was made of their kinetic properties at different temperatures.

Materials and Methods

Materials

Stock and Culture Conditions: Two tropical species (*D. willistoni, D. arizonensis*), two temperate species (*D. americana, D. virilis*) and a strain of the cosmopolitan *D. melanogaster (Sw-c)* were examined in this study. Stocks were provided by the Stock Center, University of Texas, Austin (Paterson and Stone, 1952) and were maintained at 25 °C in a dead yeast-sugar-agar food medium at 25 °C (Alahiotis and Pelecanos, 1978).

Abbreviations: The following abbreviations are used in this paper – BSA: bovine serum albumin; αGPDH/αGPO: cytosolic/mitochondrial αGlycerophospate dehydrogenases (L-glycerol 3-phosphate: NAD^+ oxidoreductase, E.C.1.1.1.8/L-glycerol 3-phosphate: cytochrome c oxidoreductase, E.C.1.1.1.95); KP_i: potassium phosphate buffer 0.05M; I.M.: mitochondrial-isolating medium (100ml 0.05M KP_i pH 7.1, 32.7mg EDTA (ethylene-diaminotetraacetic acid, Na_2), 500mg BSA, 13g sucrose plus 1% Triton X-100); INT: P-iodonitrotetrazolium violet; PMS: phenazine methosulphate; HEPES: N-2-Hydroxyethyl piperazine-N'-2-ethanesulfonic acid; ACHE: Acetylcholinesterase (Acetylcholine acetyl-hydrolase, E.C.3.1.1.7); s-MDH/m-MDH: supernatant (cytosolic)/mitochondrial malate dehydrogenases (L-malate: NAD^+ oxidoreductase, E.C. 1.1.1.37); ADH: Alcohol dehydrogenase (Alcohol: NAD oxidoreductase, E.C. 1.1.1.1.); NAD: nicotinamide adenine dinucleotide; IDH: NADP-dependent isocitrate dehydrogenase (L_S-isocitrate: NADP oxidoreductase (decarboxylating)).

Chemicals: The following reagents were purchased from Sigma Chemical Co.: BSA, Na_2 α-glycerophosphate, INT, HEPES, potassium phosphates, PMS, Gelatin. The following reagents were obtained from BDH Chemicals Ltd, Triton X-100, $MgCl_2$, Na_2 EDTA.

Methods

Preparation and Assay of α-GPO: Mitochondrial αGPO was obtained and assayed according to O'Brien and MacIntyre (1972) and O'Brien and Gethman (1973) with some modifications. 300 mg of 4–10 days old adults were homogenized in a motor-driven glass homogenizer (in the ice) in 2 ml of I.M. (without Triton X-100). The crude extract was filtered through an I.M.-saturated cotton-cheesecloth mat and centrifuged at 200 xg for 10 min. The supernatant was then centrifuged at 7.000 xg for 30 min in order to pellet mitochondria, and the supernatant was discarded. Following two I.M. washes the mitochondrial pellets are completely devoid of αGPDH activity. Soluble αGPO was obtained from mitochondria by extraction with the nonionic detergent Triton X-100. Mitochondria preparations were suspended in 1% Triton X-100 in KP_i . pH 7.1 and shaken at 2° C for 2h, followed by centrifugation at 30,000 xg for 30 min. The color was removed from the supernatant by means of dialysis in KP_i pH 7.1 buffer for 20h at 2° C. Small molecular weight ligands were also removed with this procedure. This enzyme preparation was used immediately.

αGPO activity was assayed according to O'Brien and Gethman (1973) by monitoring the oxidation of α-glycerophosphate and subsequent reduction of the electron receptor molecules PMS-INT, at 490 nm in a Gilford recording spectrophotometer. Assay temperatures were maintained in the cuvette Chamber by a Haake circulating water bath. The 1ml reaction mix contained 20 μl enzyme preparation, 20 μl PMS (from a stock solution 7.66 mg PMS/ml KP_i pH 7.1), 100 μl INT (from a stock solution 10 mg INT/2ml KP_i pH 7.1), varying levels of DL-α-glycerophosphate (22.5-225 mM or 225 mM in the standard assay) and assay mix (the assay mix contained: 100 ml 0.05 M KP_i pH 7.1 (or pH 6.2), 100mg BSA, 203mg $MgCl_2$, 238mg HEPES and 40 mg Gelatin). The assay mix was incubated with enzyme for 4 min after which substrate was added to start the reaction. INT formation production is linear for about 20 min. The reaction velocity was measured 3 min after the substrate was added.

It must be noted that *Drosophila* αGPO exhibits two distinct isozymes (αGPO-1, αGPO-2) revealed by means of isoelectric focusing (O'Brien and MacIntyre, 1972). They

were found to differ in their pH optima. The ubiquitous mitochondrial oxidase αGPO-2 is present in each adult segment and developmental stage and has a pH optimum near 7.1 in the oxidation of α-glycerophosphate (O'Brien and MacIntyre, 1972). Moreover, αGPO-1, the major adult mitochondrial species, is concentrated in the thorax and has a pH optimum near 6.2 (O'Brien and MacIntyre, 1972). Enzyme assay on the same isozyme mixture at both pH 6.2 and 7.1 revealed a constant ratio of enzyme activity (αGPO-2/αGPO-1:*D.virilis* = 1,24; *D.arizonensis* = 1.23; *D.willistoni* = 1.15; *D.anericana* = 1.15; *D.melanogaster (Sw-c)* = 1.26). This indicates that there is no significant variability among the species in the relative levels of the two mitochondrial isozymes. Furthermore, a spectrophotometric assessment was made of αGPO kinetic properties at both pH 6.2 and 7.1, to see to what extent the pH influences the catalytic behavior of the enzyme. While it is quite possible that assays at pH 6.2 or 7.1 probably represent kinetic data for the corresponding isozymes, it is preferable to refer to αGPO instead to αGPO-1 or αGPO-2 isozymes, as the catalytic properties of this enzyme are indistinguishable among tropical and temperate species at either pH (see results).

K_m Determination: for estimating kinetic parameters, five substrate concentrations were assayed with two to four replicates in at least two separate experiments at each of four temperatures. K_m values were determined from 1/S vs. 1/V plots.

Heat Inactivation: Enzyme preparations were incubated at 49° C in a water bath. Samples were removed from the water bath, cooled on ice, and assayed for activity (standard assay at 25° C). The results were plotted as percent of original activity vs. time of incubation.

Results

Experiments were carried out in order to determine the K_m values, as a function of temperature for αGPO obtained from tropical (*D.arizonensis, D.willistoni*) temperate (*D.virilis, D.americana*) and cosmopolitan species (*D.melanogaster; Sw-c* strain). Figure 1 compares the K_m values of αGPO at pH 7.1 at different temperatures for all five species and those at pH 6.2 for *D.virilis* and *D.arizonensis.* αGPO obtained either from tropical or from temperate species exhibits a similar pattern in terms of K_m values vs. temperature. At temperatures below 15° C all species examined at pH 7.1 exhibit a (modest) negative thermal modulation, while above 15° C no essential interaction of K_m values with the temperature was observed. As regards the K_m values of αGPO at pH 6.2 it also appears from Fig. 1 that there is no essential interaction of K_m values with the temperature.

However, the absolute K_m values are somewhat higher than those obtained at pH 7.1. This fact indicates that the differences found may reflect different K_m values for the respective αGPO isozymes. Nevertheless, it is important that the K_m values are indistinguishable among tropical and temperate species at either pH. When we consider the pattern as a whole we see that only half-fold differences in absolute αGPO (at pH 7.1) K_m values, appear among tropical and temperate species at low temperatures. These values range only between 25-40 mM at high temperatures. These findings are in clear contrast to the situation observed as regards the other enzyme, the cytosolic αGPDH, which participates in the α-glycerophosphate cycle, where K_m-temperature relationships were si-

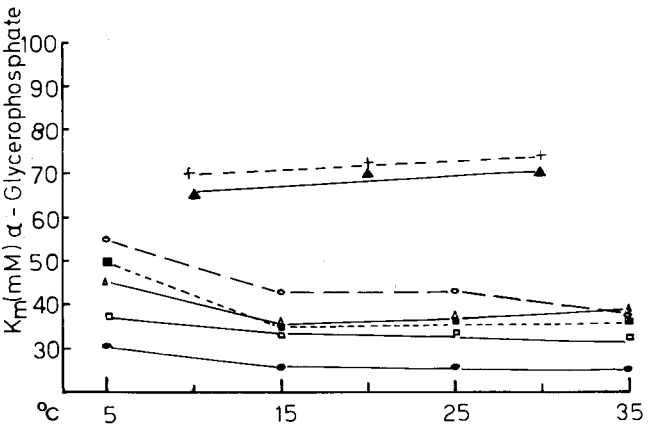

Fig. 1. Apparent K_m for DL αglycerophosphate-α-GPO as a function of assay temperature at pH 7.1 for (□) *D. virilis*, (△) *D.americana*, (■) *D.arizonensis*, (○) *D.willistoni*, (●) *D.melanogaster (Sw-C)* and at pH 6.2 for (▲) *D.virilis* and (+) *D.arizonensis*. Since one of the isomers of DLα-glycerophosphate is used by the αGPO, the K_m values detected have been divided by a factor of 2

Table 1. Temperature coefficients (Q_{10}) of αGPO (at pH 7.1) as a function of substrate concentration and temperature range. The values were determined at four concentrations of substrate (1) 225 mM, (2) 112,5 mM, (3) 46,5 mM and (4) 22,5 mM. The results are the means ± SEM

	Temperature range		
Species	5–15°C	15–25°C	25-35° C
D.virilis	(1) 2.83 ± 0.29	2.83 ± 0.30	2.00 ± 0.25
	(2) 2.77 ± 0.15	2.73 ± 0.29	–
	(3) 2.64 ± 0.30	2.41 ± 0.15	2.08 ± 0.20
	(4) 2.43 ± 0.11	–	1.99 ± 0.07
D.americana	(1) –	1.74 ± 0.20	2.42 ± 0.25
	(2) 2.43 ± 0.09	–	1.92 ± 0.20
	(3) 2.18 ± 0.15	1.99 ± 0.19	–
	(4) 2.17 ± 0.08	2.00 ± 0.18	1.76 ± 0.15
D.arizonensis	(1) 2.46 ± 0.20	–	2.32 ± 0.15
	(2) 2.35 ± 0.15	2.00 ± 0.09	2.32 ± 0.25
	(3) 1.82 ± 0.13	2.98 ± 0.19	–
	(4) –	2.33 ± 0.14	1.95 ± 0.09
D.willistoni	(1) –	2.83 ± 0.30	–
	(2) –	2.50 ± 0.15	2.88 ± 0.26
	(3) –	2.94 ± 0.14	2.02 ± 0.22
	(4) –	2.68 ± 0.12	2.02 ± 0.19
D.melanogasyer	(1) 2.57 ± 0.24	2.56 ± 0.13	2.43 ± 0.25
(Sw-c)	(2) 2.25 ± 0.21	–	2.17 ± 0.15
	(3) 2.47 ± 0.14	2.43 ± 0.17	2.33 ± 0.11
	(4) 2.25 ± 0.12	2.19 ± 0.19	–

milar among species from the same thermal habitat and different for species from different thermal habitats (Alahiotis, et al., 1977). The same relationship between the mitochondrial and cytosolic isozymes has been found for another enzyme system, m-MDH (Alahiotis, 1979a). In this case it is again clear that mitochondrial MDHs isolated either from temperate or from tropical species have relatively more similar kinetic properties (with respect to temperature) than do the cytosolic MDHs obtained from these species.

On the basis of the kinetic data we were able to determine Q_{10} values over three 10° C intervals at different substrate concentrations. An examination of these data (Table 1) reveals that there is a decrease in Q_{10} values for αGPO (at pH 7.1) as substrate concentrations are reduced (see also Hochachka and Somero, 1973). However, these values show very little, if any, difference between tropical and temperate species.
A characteristic finding worthwile mentioning concerning the Q_{10} values is that these values for αGPO are higher than those reported for other *Drosophila* enzymes (Q_{10}: αGPO: 1.8–3.0; other enzymes approximately 1–2; Alahiotis and Berger, 1978; Alahiotis, $1979_{a,b}$). This is also true for the absolute K_m values.

Activation energies (E_a) were also calculated using the Arrhenius plots according to the formula followed by Robert and Gray (1972), for all species at both pH. Figure 2

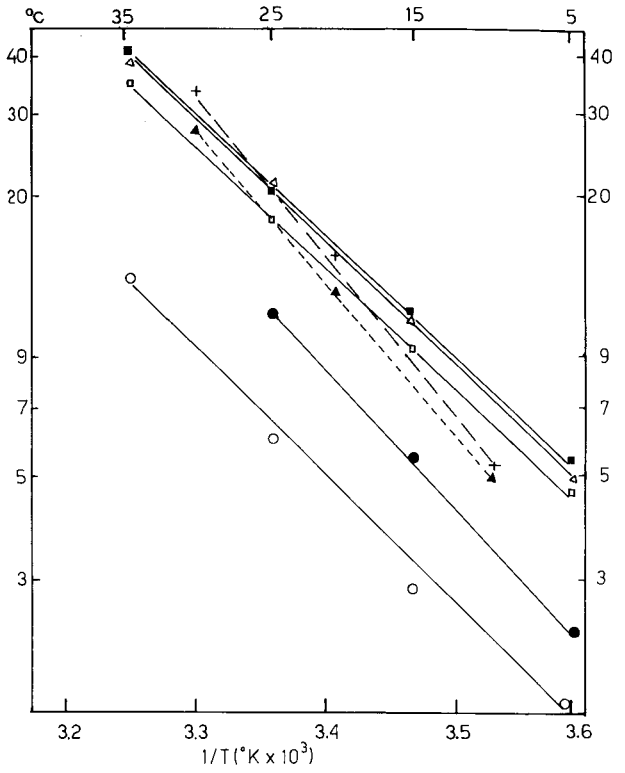

Fig. 2. Arrhenious plots of log V_{max} vs. $^1/T$ of αGPO. The assay was carried out in the presence of saturating substrate concentrations at pH 7.1 for (□) *D.virilis*, (△) *D.americana*, (■) *D.arizonensis*, (○) *D.willistoni*, (●) *D.melanogaster (Sw-c)* and at pH 6.2 for (▲) *D.virilis and (+)* D.arizonensis. E_a was determined from the slope of the line

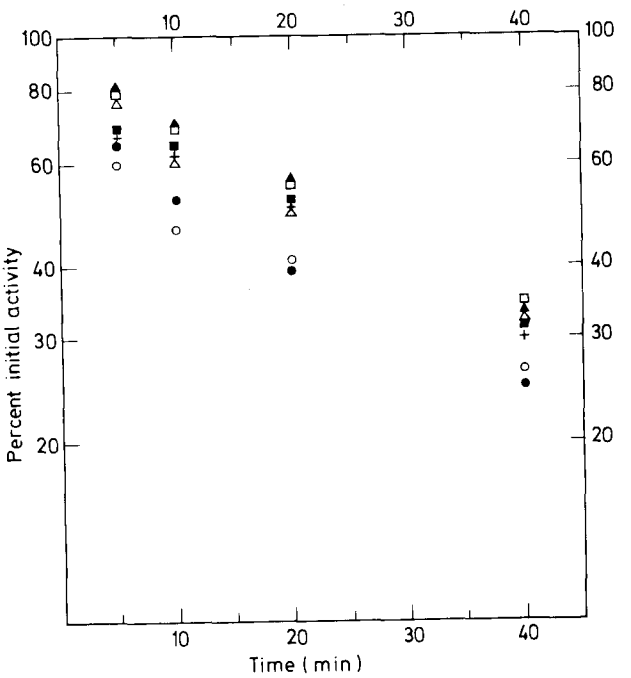

Fig. 3. Thermal inactivation of αGPO at 49° C. Plots designated: at pH 7.1 (□) *D.virilis*, (△) *D.americana*, (■) *D.arizonensis*, (○) *D.willistoni*, (●) *D.melanogaster (Sw-c)* at pH 6.2, (▲) *D.virilis* and (+) *D.arizonensis*

shows that these values are of the same order of magnitude [E_a (kcal/mole) (at pH 7.1): *D.virilis*=12.1; *D.americana*=12.36; *D.arizonensis*=12.24; *D.willistoni*=12.57; *D.melanogaster (Sw-c)*=13.67; at pH 6.2: *D.virilis*=15.5; *D.arizonensis*=16.00)]. However, one notices that the E_a for the αGPO at pH 6.2 are somewhat higher than those of the αGPO at pH 7.1, as in the case of K_m values. Moreover, all these values are higher than those for αGPDH ($\bar{E}_a$=7.7 ± 0.08; Alahiotis, et al., 1977). Nevertheless, the E_a and K_m values for αGPO at both pH are indistinguishable among tropical and temperate species.

Another test performed to reveal differences among the homologous αGPO molecules in the genus *Drosophila* was that of heat denaturation rate. Here again, as it appears from Fig. 3, no distinct differences were detected among tropical and temperate species, when the enzyme assay is performed at either pH 7.1 or 6.2.
Nevertheless, cytosolic αGPDH obtained from tropical species was more thermostable as compared to that obtained from temperate species (Alahiotis, et al., 1977). Moreover, in relation to other *Drosophila* enzymes studied for thermostability (see introduction), we found that the heat inactivation patterns were species specific which is consistent with the hypothesis that the primary sequence of homologous enzymes for each species was different. However, αGPO shows a great conservatism concerning differences in thermostability among tropical and temperate species.

Discussion

Our findings indicate that the substrate-binding abilities (as measured by apparent K_m values) of the mitochondrial αGPO are conserved among the species examined. This

conservation of the apparent K_m values is found in an enzyme that must function over a wide temperature range in *Drosophila*. Thus, one could say that αGPO acts like a eurythermal enzyme, in contrast to cytosolic αGPDH for which the K_m values change relatively markedly as the temperature varies (Alahiotis, et al., 1977). It must be noted here that the absolute K_m values detected in this investigation are higher than those reported from other sources (Scislowski, 1977). Our K_m values probably do not reflect (in absolute numbers) those existing in vivo. For example several activators lower K_m for α-glycerophosphate (e.g. Ca^{2+}; Sacktor, 1976). Thus, since the distribution and concentration of cations is thought to be adjusted during thermal acclimatization (Moon and Hochachka, 1971) it is possible that appropriate adjustment in bivalent cation (e.g. Ca^{2+}) concentrations in the cells during thermal acclimatization could lead to some compensation of αGPO catalytic properties. Moreover, the possibility might be considered that the catalytic properties of the mitochondrial enzymes are affected by their putative seguestration in membranes and that such a process might give rise to compensatory changes in enzyme activity during thermal acclimatization. There would be an analogy here with cold-sensitive assembly processes (vs. catalytic ones) that are known from protein work in other organisms (e.g. Olmsted and Borisy, 1973; Nomura, et al., 1977). An analogous situation seems to hold for temperature sensitive (heat and cold) mutants of the membrane-bound ACHE in *D.melanogaster* (Hall et al.. 1980).

As is known, the local environment of an enzyme plays a major role in shaping the functional properties of the enzyme. Positive and negative organic modulators, hydrogen ions, inorganic ions and the molecules to which the enzyme protein is bound if it possesses a quintary level of structure may all affect the catalytic and regulatory properties of the enzyme (Hochachka and Somero, 1973). A good example of this type of effect (the modulation strategy) is the case of succinic dehydrogenase (SDH). This enzyme is a lipoprotein, and it has been reported by Hazel (1972) that at least a major fraction of the rate compensation characteristic of SDH activity (obtained from 5° C and 25° C acclimated goldfish) is due to differences in the lipid 'environments' of cold and warm-acclimated enzymes (while electrophoretic analysis of the protein moieties of the 5° C and 25° C enzymes revealed that the fish contained the same protein variant of both temperature). As is known, the mitochondrial αGPO (from *Drosophila*) exhibits behavior also typical of a lipoprotein (O'Brien and MacIntyre, 1972). Thus, one cannot rule out the possibility that a similar compensatory situation is due to αGPO in the genus of *Drosophila*.

Another important component of temperature adaptation is the partial compensation of membrane fluidity for environmental temperature that occurs over evolutionary time as well as during seasonal acclimation (Cossins and Prosser, 1978). Membrane fluidity is principally influenced by the fatty acid composition of its phosphoglycerides in various species and related to their respective cellular temperatures (Cossins and Prosser, 1978). Since αGPO is a particle-bound enzyme and because structure and fluidity can change markedly with temperature, one could hypothesize that perhaps such a conformational shift in mitochondrial membranes would alter the conformations and activity of the enzyme in a way that would cause a K_m shift with temperature, a shift that would not be detected in vitro. However, it is quite possible that this mechanism requires a highly precise location of the enzyme in the membrane and it also depends on the degree of membrane association with the enzyme. On the other

hand the direct effect of temperature (in vitro) possibly is stronger, in terms of conformational changes of the enzyme, than that caused by changes in the membrane fluidity. Until these ideas can be tested the in vitro experiments at least give information on the protein structure. From this point of view our data are useful for making comparisons and for drawing information about mechanisms of molecular evolution.

Our present and previous data obtained from in vitro experiments (Alahoitis, et al., 1977; Alahiotis and Berger, 1978; Alahiotis, 1979a,b) show that cytosolic and mitochondrial enzymes do not react coordinately in terms of their catalytic properties vs. temperature. Both mitochondrial enzymes examined so far, the MDH and αGPO, display conservatism in evolutionary changes as revealed by their K_m, E_a and Q_{10} values, heat denaturation rate and electrophoretic behavior (see also Alahiotis, 1979a), in contrast to the behavior of the cytosolic enzymes. A possible explanation of these findings could be that the crucial mitochondrial function, energy production, probably requires rigorous structural adaptations. As is known, m-MDH participates in the citric acid cycle and participates in concert with the α-Glycerophosphate cycle in NAD generation (Sacktor, 1965; O'Brien and MacIntyre, 1972). On the other hand the α-Glycerophosphate cycle participates in the generation of oxidized NAD in the cytoplasm for the continuation of glycolysis (Sacktor and Dick, 1962; Zebe and McShan, 1957) and in the production of energy for flight by the donation of electrons to the respiratory chain of the sarcosomes, the huge mitochondria of insect flight muscle (Sacktor and Dick, 1962).

Both the αGPO and m-MDH loci are nuclear (O'Brien and MacIntyre, 1972; O'Brien and Gethman, 1973). Moreover, experiments have shown that less than 20 proteins are encoded (5–10% of the total protein weight) in the mitochondrial genome (Groot, et al., 1972; Weislogel and Butow, 1971; Tzagoloff, 1977), the remaining mitochondrial proteins being encoded in the nucleus. Thus, while the cause of the difference found in the rates of evolution among mitochondrial and cytosolic enzymes remains open to conjecture, one could speculate that new mutations of these genes probably disrupt the well established balanced mitochondrial operation and that they are not fixed easily by selection in populations. The relative invariance of the enzyme structure is probably linked to a necessary relative invariance of molecular interaction inside the mitochondrion.

It is also important to point out that the mitochondrial form of aspartate aminotransferase (mAAT) of vertebrates is evolving at a slower rate than the cytosolic isozyme as has been reported by Sonderegger, et al., (1977) and Sonderegger and Christen (1978), based on immunological distances. According to the same authors the plots of the immunological distances against the times of divergence of the phylogenetic lineages showed no differences in the rate of change for mAAT between homeothermic and poikilothermic vertebrates, while during the development of mammals, the evolution of the cytosolic AAT proceeded more than twice as fast. These data indicate that the evolution of the AAT is independent of thermal selection at least in the vertebrate classes examined. However, it seems from my data, that this is not true for at least several *Drosophila* enzymes. The above situation is not clearly understood. Nevertheless we would hypothesize that since the mechanisms of changing enzyme function in higher eucaryotes (as compared with insects) are somewhat more complicated and much less understood (Hochachka and Somero, 1973), the evolutionary conservatism in mito-

chondrial enzymes observed in both insects and vertebrates cannot be attributed solely to the action of one and the same evolutionary parameter (e.g. temperate climate).

Acknowledgement: I am indebted to M. Pelecanos for critically reading the manuscript.

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Received March 31, 1979; Revised February, 20, 1980