The Adaptation of Enzymes to Temperature: Catalytic Characterization of Glucosephosphate Isomerase Homologues Isolated from *Mytilus edulis* and *Isognomon alatus*, Bivalve Molluscs Inhabiting Different Thermal Environments[1]

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Homologues of glucosephosphate isomerase (GPI, EC 5.3.1.9) were purified to homogeneity and kinetically characterized from *Mytilus edulis* and *Isognomon alatus*, two bivalve molluscs experiencing contrasting thermal environments. The enzyme isolated from *I. alatus* functions at warmer temperatures (25–35 C) than GPI from *M. edulis*, a species that inhabits colder marine littoral habitats (5–20 C). The former exhibits apparent first-order (with respect to substrate) catalytic rate constants (V_{max}/K_M) in vitro that become progressively greater than the mussel enzyme as the assay temperature is raised. Apparent zero-order catalytic rate constants (V_{max}) are relatively less differentiated. Catalytic efficiency, defined as the rate at which a catalytic event occurs in either reaction direction for reference standard states (substrate concentrations), is greater for the enzyme from the tropical species (*I. alatus*) at all realistic combinations of temperature and substrate concentration except for the lowest temperatures and highest substrate concentrations, where the GPI from the boreal/temperate *M. edulis* is more efficient. This pattern of catalytic divergence appears to be due primarily to differentiation in V_{max}/K_M. These results and other published data are reviewed and shown to be inconsistent with claims that adaptation of enzymes to higher cell temperatures requires a loss in catalytic efficiency.

Introduction

Ectothermic organisms inhabit virtually every conceivable thermal environment between the freezing and boiling points of water, and a major preoccupation of comparative biochemists and physiologists has been to understand the interactions among the molecular, metabolic, and physiological layers of organization that allow species to exploit such a wide range of temperatures. Temperature has profound effects on the structure and catalytic functions of enzymes embedded within metabolic pathways. The temperature dependencies of enzymatic properties, in turn, contribute to the thermal sensitivities of metabolic and physiological processes and therefore ultimately determine the range of thermal environments habitable by a species. Variation in thermal sensitivity at the level of the individual enzyme does exist among species. When enzyme homologues have been isolated and kinetically

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characterized from ectotherms experiencing contrasting thermal environments, their kinetic and structural properties are often divergent in ways that correlate remarkably well with the enzymes' respective thermal environments. For example, in their work with homologous fish lactate dehydrogenases (LDH), Somero, his colleagues, and others, have shown that an inverse relationship exists between median body temperature and the Michaelis constants (K_M) for substrate and coenzyme as well as an apparent conservation of K_M values at species' physiological temperatures. There is also a generally negative association between enzyme reaction velocities at saturating substrate concentrations (V_{max} or, normalized for active enzyme concentration, k_{cat}) and both the median cell temperature and the resistance of homologous proteins to thermal denaturation (Borgmann and Moon 1975; Somero and Yancey 1978; Yancey and Somero 1978; Graves and Somero 1982; see Somero 1978 and Graves et al. 1983).

The persistent associations between environmental or cell temperature and enzyme variants' kinetic phenotypes obviously reflect some kind of catalytic differentiation and undoubtedly real catalytic adaptations to different thermal environments in many cases. However, adaptive inferences in much previous work have not been convincing, as they usually have been based on either partial characterizations or the consideration of only one or two kinetic parameters, commonly K_M or k_{cat} (e.g., Borgmann and Moon 1975). One reason for this is that enzymes with lower K_M or higher k_{cat} values have often been presumed to be catalytically more efficient. Such assertions depend on the validity of several often dubious assumptions that relate these parameters to catalytic efficiency (e.g., Somero 1978; McDonald et al. 1980; Watt 1983; Hochachka and Somero 1984). The association of a lower K_M with greater catalytic efficiency implies relationships of K_M to enzyme-substrate affinity and of enzyme-substrate affinity to catalytic efficiency that are not always valid (for discussion of this topic, see Fersht [1977]; Page [1980]). For the second relationship, we may define catalytic efficiency as the rate, v_0, at which an enzyme catalyzes a change in substrate concentration ($v_0 = -d[S]/dt$) for specified conditions of pH, ionic strength, etc. Then associating a higher k_{cat} with greater catalytic efficiency is accurate only when a saturating substrate concentration or standard state is specified. It does not necessarily reflect an enzyme's catalytic effectiveness at physiological substrate concentrations, which tend to be far lower. Strictly speaking, k_{cat} reflects only the catalytic potential of the enzyme-substrate complex(es); it is easily shown that the relative catalytic ability of the *free* enzyme is defined by k_{cat}/K_M—the catalytic parameter that is rate determining at very low substrate concentrations (for reviews, see Fersht [1977]; Hall and Koehn [1983]). For this reason, this parameter is generally a better indicator of catalytic efficiency than k_{cat}.

The purpose of the present work was to isolate the homologues of an enzyme with both a primarily catalytic function and a well-defined substrate specificity from species that conduct their metabolic processes at characteristically different temperatures. The enzymes were then characterized with respect to both *apparent* steady-state rate parameters, k_{cat} and k_{cat}/K_M. A comparison of enzymes' overall catalytic efficiencies that is based on these two parameters and estimates of in vivo substrate concentrations has the advantage of allowing the calculation of efficiency estimates at physiologically relevant temperatures and substrate concentrations that are independent of such assumptions. A further advantage is that it can provide a perspective concerning the relative contribution of these two parameters to any

observed catalytic differences between the enzyme forms (see Albery and Knowles [1976] and Hall and Koehn [1983] for reviews).

Glucosephosphate isomerase (GPI, EC 5.3.1.9) is the model chosen to approach these questions of molecular adaptation. A dimer of two identical subunits, this enzyme catalyzes the interconversion of glucose 6-phosphate (G6P) and fructose 6-phosphate (F6P) (Noltmann 1972). The metabolic role of glucosephosphate isomerase appears to be primarily catalytic: the reaction is freely reversible with an equilibrium constant (K_{eq}) of about 0.3, and there is near universal agreement between the equilibrium constant and observed mass action ratios, for both vertebrates and invertebrates (e.g., Newsholme and Start 1973; Beis and Newsholme 1975; Cameselle et al. 1980). Consequently, GPI is generally thought to provide a coupling function in glycolysis and gluconeogenesis at the glucose 6-phosphate metabolic branch point. The enzyme appears to have no regulatory functions (Noltmann 1972), although several pentose phosphate shunt intermediates, such as 6-phosphogluconate, are known competitive inhibitors, a fact that conceivably could reflect a regulatory effect upon glycolytic flux under some circumstances (Atkinson 1977; Palumbi et al. 1980; Eanes 1984).

Glucosephosphate isomerases were isolated from two species of bivalve molluscs. The blue mussel, *Mytilus edulis* Linné, 1758 (Mytilidae), is an abundant marine bivalve that lives in circumboreal/temperate littoral and sublittoral habitats. It occurs as far south as North Carolina along the eastern coast of North America. This species is incapable of acclimating to or surviving at sustained water temperatures above 27 C, which is apparently the major factor that prevents its colonization of otherwise suitable habitats south of Cape Hatteras (Hutchins 1947; Wells and Gray 1960; Read and Cumming 1967). However, physiological studies of individuals from both European and North American populations suggest that adults may experience increasing degrees of metabolic stress as the acclimatization temperature is raised above 20 C (Bayne et al. 1973; Gonzales and Yevich 1976; Widdows 1978; Incze et al. 1980). These physiological studies and the species' latitudinal distribution are indicative of a fairly eurythermal metabolism between the temperatures of 5 and 20 C.

The enzyme was also isolated from *Isognomon alatus* Gmelin, 1791 (Isognomonidae), a species chosen to contrast maximally with the boreal/temperate thermal habitat of *M. edulis* but to be as similar phylogenetically and in life habit to *M. edulis* as possible. *Isognomon alatus* is also an abundant littoral/sublittoral pteriomorphic bivalve, but the geographic ranges and thermal habitats of these species do not overlap: *I. alatus* has an exclusively tropical distribution and extends only as far north as central Florida. It is commonly found among mangrove roots from south Florida through the Caribbean (Andrews 1971; Abbott 1974; Siung 1980).

The physiological range of temperatures for *I. alatus* has not been studied specifically, but experiments concerned with the effects of temperature on heart rate suggest normal physiological function between 25 and 35 C (Trueman and Lowe 1971; cf. Siung 1980). The average monthly range of temperatures for Biscayne Bay, Florida, where individuals of *I. alatus* were collected, is 22–30 C (1961–1966) with an average yearly temperature in 1966 of 26 C (USCGD 1972).

The results of the present work show that conclusions regarding the relative catalytic efficiencies of homologous enzymes that are based on the evaluation of overall catalytic function in many cases can be quite different from those derived from partial kinetic characterizations.

Material and Methods

Materials

Proteins, enzymes, substrates, inhibitors, coenzymes, and buffers used in enzyme assays were obtained from the Sigma Chemical Company. Other inorganic and organic chemicals were reagent grade and obtained from Fisher Chemical Company.

Study Animals

For enzyme preparations several hundred *Mytilus edulis* >50 mm in length were collected from Shinnecock Bay, Hampton Bays, New York. Individuals of the tree oyster, *Isognomon alatus,* were collected from mangrove roots in Matheson Hammock State Park, on Biscayne Bay near Miami, Florida. The animals were frozen at −70 C until used.

Electrophoresis

Individuals to be pooled for enzyme preparations were first screened for allozyme variation by starch gel electrophoresis (Hall 1983). Sodium dodecyl sulfate (SDS)-7.5% polyacrylamide gel electrophoresis was conducted, according to Weber et al. (1972).

Isolation of Glucosephosphate Isomerase from *Mytilus edulis* and *Isognomon alatus*

The extraction and isolation of the bivalve GPIs will not be detailed here, but the scheme involves a sequence of mild fractionation steps, including gel filtration and anion-exchange and hydrophobic interaction chromatography (Hall 1983). The purity was checked with both native and SDS polyacrylamide gel electrophoresis. SDS gels were loaded with 5–15 μg of purified protein. Only a single protein band was detectable after staining with Coomassie blue R-250. Densitometric scanning of SDS gels at 590 nm indicated that the two GPIs were greater than 99% and 97% for the *M. edulis* and *I. alatus* enzymes, respectively, by this criterion.

Enzyme Assays

Initial enzymic reaction velocities were measured using a Gilford Model 2400-2 UV/VIS recording spectrophotometer, equipped with a Gilford ThermoSet thermoelectric temperature control device. Optical-density readings were recorded by a Motorola M6800 microcomputer interfaced with the spectrophotometer.

For pH control, assay cocktails and incubation solutions contained sodium MOPS [3-(N-morpholino) propanesulfonic acid] and were 0.1 in ionic strength (I) at all pH's and temperatures employed in this study. Steady-state kinetic parameters were determined both at pH 7.10 and at pH's that decrease with temperature according to the pK_a of imidazole (Chemical Rubber Company 1974). The latter experimental protocol is more relevant for physiological inference than one in which the same pH is maintained at all temperatures (see Reeves 1977; Somero 1981).

Initial rates of glucosephosphate isomerase were determined in the glycolytic reaction direction at 340 nm (glucose 6-phosphate → fructose 6-phosphate), with a modified version of the multiply coupled enzyme assay (Tilley et al. 1974). Assay mixtures consisted of 0.1 I NaMOPS, 8 mM $MgCl_2$, 0.15 mM NADH, 0.2 mM ATP, 4 mM NH_4Cl, .05% β-mercaptoethanol (βME; vol/vol), 0.02 mM cyclic AMP, various glucose 6-phosphate concentrations (monosodium salt), and enough phos-

phofructokinase, α-glycerophosphate dehydrogenase, triosephosphate isomerase, and trout muscle aldolase not to limit the reaction (Hall 1983). Following thermal equilibration, an enzyme aliquot was added to bring the volume to 0.5 ml and to begin the reaction. The substrate consumed during the assay period was less than 3% at both high and low substrate concentrations, and initial rates were determined using an extinction coefficient of 12.44 mM^{-1} cm^{-1}, by unweighted linear least-squares analysis of the first 15–20 points of the virtually linear progress curve (there was no detectable "lag" phase).

Initial rates were determined in the gluconeogenic direction (fructose 6-phosphate → glucose 6-phosphate) with an assay mixture, containing 0.1 I NaMOPS buffer, 8 mM $MgCl_2$, 1 mM $NADP^+$, various fructose 6-phosphate concentrations, 1–10 units of glucose 6-phosphate dehydrogenase (torula yeast), and a GPI aliquot to a total volume of 0.5 ml. Initial velocities were determined by progress-curve analysis using an extinction coefficient of 6.22 mM^{-1} cm^{-1} (Waley 1981; Hall 1983). Progress curve analysis is applicable both in the case of progressive substrate depletion and when an enzyme-inhibiting reaction product accumulates during the reaction (Cornish-Bowden 1979). This is important since the competitive inhibitor, 6-phosphogluconate, accumulates during the course of this assay. Each temperature, pH assay condition was checked to ensure that the aliquot of GPI used was in the range in which reaction velocity was proportional to enzyme concentration.

Substrate concentrations in assay cocktails were determined enzymatically (Lowry and Passonneau 1972). Glucosephosphate isomerase was diluted for enzyme assays in a solution containing 50 mM Tris, 50 mM NaCl, 1 mM Na_2EDTA, 0.1% βME (vol/vol), and 0.05% bovine serum albumin (wt/vol), pH 8.5, at 5 C. The enzyme was very stable even in very dilute solution in this medium at 0 C for at least a week with no detectable loss in activity.

Experimental design followed recommendations for kinetic parameter estimation (Duggleby 1979; Atkins and Nimmo 1981; Endrenyi and Chan 1981; Currie 1982). In the glycolytic direction this design consisted of 7–11 replicated assays at each of two glucose 6-phosphate concentrations—a concentration approaching saturation (~1.5 mM) and a low substrate level, giving an initial rate approximately one-half that of the higher substrate concentration. The experimental design in the gluconeogenic direction was similar, except that an additional condition was incorporated to estimate the K_i for 6-phosphogluconate. This consisted of another set of replicated assays at the higher fructose 6-phosphate (~0.5 mM) concentration but containing sufficient inhibitor to lower the initial velocities by approximately one-half (Duggleby 1979). Data were analyzed by nonlinear regression (Cornish-Bowden and Endrenyi 1981) and were fitted to a simple Michaelis-Menten model in the glycolytic direction and to a competitive inhibition model in the gluconeogenic direction.

Equilibrium Constants

The thermodynamic equilibrium constant (K_{eq}) for fructose 6-phosphate and glucose 6-phosphate was measured at temperatures from 5 to 35 C (Dyson and Noltmann 1968) to check the internal consistency of the kinetic data via the Haldane relation (fig. 1).

Thermal Denaturation Experiments

Glucosephosphate isomerases were diluted into 0.1 I NaMOPS buffer with 0.05% bovine serum albumin, pH 7.10, at the respective temperatures. Duplicate

$$v_0 = \frac{\frac{V^f_{max}[A]}{K_A} - \frac{V^r_{max}[P]}{K_P}}{1 + \frac{[A]}{K_A} + \frac{[P]}{K_P}} = \frac{\left(\frac{k^f_{cat}[A]}{K_A} - \frac{k^r_{cat}[P]}{K_P}\right)[E_0]}{1 + \frac{[A]}{K_A} + \frac{[P]}{K_P}} \quad (1)$$

$$y = \frac{[E_0]}{v_0}\left(1 - \frac{[P]}{[A]K_{eq}}\right) \quad (2)$$

$$= \frac{K_A}{k^f_{cat}} \cdot \frac{1}{[A]} + \frac{1}{k^f_{cat}} + \left(\frac{[P]}{[A]} \cdot \frac{1}{K_{eq}}\right)\frac{1}{k^r_{cat}} \quad (3)$$

$$\text{HALDANE RELATION}: \quad K_{eq} = \frac{[P]}{[A]} = \frac{k^f_{cat}/K_A}{k^r_{cat}/K_P} \quad (4)$$

FIG. 1.—Rate equations for a reversible unisubstrate enzyme reaction and the derived catalytic efficiency parameter, y, of Albery and Knowles (1976). [A] and [P] denote substrate and product concentrations, respectively; K_A and K_P are the corresponding Michaelis constants; k^f_{cat} and k^r_{cat} are the turnover numbers for the forward and reverse reactions.

aliquots were incubated for increasing intervals at 40–50 C and transferred to an ice bath and assayed for remaining GPI activity. The proportion of enzyme activity remaining was relative to a simultaneously assayed control aliquot remaining in the ice bath. The decrease in enzyme activity with time was treated as an exponential decay process:

$$(E/Eo)_t = \exp(-k_D t),$$

where $(E/Eo)_t$ is the proportion of enzyme remaining at time, t, and k_D is the apparent denaturation rate constant. The slope of the line, calculated by the linear regression of $\ln(E/Eo)$ on time (minutes), is an estimate of the apparent denaturation rate constant, k_D.

Protein Determination

General protein concentrations were measured by means of the Coomassie blue G-250 dye-binding assay of Bradford (1976), with bovine serum albumin (BSA) used as a standard. Bovine serum albumin and yeast GPI exhibited virtually identical dye-binding sensitivities. Consequently, several BSA concentrations (1–12 μg) were used as relative standards.

Catalytic Efficiency

Because GPI catalyzes an equilibrium reaction in vivo, evaluation of its catalytic efficiency requires determination of steady-state kinetic parameters in both the glycolytic and gluconeogenic reaction directions (cf. Albery and Knowles 1976). Expression 1 in figure 1 describes the dependence of reaction velocity, v_0, on the steady-state kinetic parameters and substrate concentrations for a reversible unisubstrate reaction like that catalyzed by GPI. Taking the reciprocal of this expression and rearranging the terms, we obtain in expressions 2 and 3 the catalytic efficiency parameter, y (Albery and Knowles 1976). This expression has the dimensions of time and is a function of not only the kinetic parameters but also substrate and

product concentrations. The reciprocal of this efficiency expression, y^{-1}, has the dimensions of reciprocal time, i.e., that of a first-order rate constant. Therefore, y^{-1} may be interpreted as the rate at which a catalytic event occurs in either reaction direction and is a measure of overall catalytic efficiency at a given standard state (i.e., substrate and product concentrations). It may be calculated from the steady-state kinetic data for one or more chosen standard states.

Results

Kinetic Characteristics

The most common allelic isozyme from each bivalve species was purified and kinetically characterized. The experimentally determined values of the kinetic parameters at 20 C, pH 7.10, are summarized in table 1. V_{max} and V_{max}/K_M are normalized for enzyme protein concentration and are considered—for the comparative purposes of this paper—to represent relative values for the *apparent* catalytic rate constants, k_{cat} and k_{cat}/K_M, respectively. V_{max} and V_{max}/K_M represent the product of the respective rate constant and active enzyme concentration. Although k_{cat} and k_{cat}/K_M could not be directly measured in this study, several steps were taken to ascertain the levels of purity and to minimize the proportion of inactive enzyme in the preparations. First, the enzyme preparations were virtually homogeneous by the criteria of native and SDS polyacrylamide gel electrophoresis. The enzymes from both sources were quite stable at all stages of the purification and were isolated by an identical sequence of mild fractionation techniques (Hall 1983). This procedure gave identical yields of GPIs that retain their original specific activities for at least 2 mo. The final step of the isolation protocol, a kind of affinity elution technique, was employed specifically as a final step in the protocol to separate "dead" enzyme from active GPI (Hall 1983). That this procedure worked in this way was supported by the observation that a significant increase in specific activity was obtained above that for the immediately preceding step, even though only a single protein band could be detected on overloaded SDS gels (12–26 μg) in both the penultimate and final chromatographic steps. Employing an additional purification step following this last step alters neither specific activities nor kinetic characteristics.

Comparison of the glucosephosphate isomerases from the cold water (*Mytilus edulis*) and warm water (*Isognomon alatus*) bivalves at 20 C, an intermediate temperature, reveals that the *I. alatus* enzyme has significantly lower K_M's, a lower K_i for the competitive inhibitor, 6-phosphogluconate, and higher V_{max}/K_m's in both the glycolytic and gluconeogenic reaction directions (table 1). Values for the equilibrium constant, calculated from the Haldane relation (fig. 1), are in good agreement with the K_{eq}'s determined experimentally at 20 C (table 1) and at other temperatures as well (Hall 1983). The differences in V_{max} between the two species are relatively small compared with the differences in V_{max}/K_M. The *I. alatus* V_{max}'s are generally somewhat lower in the glycolytic reaction direction and higher in the gluconeogenic direction (table 1).

These kinds of differences are also noted at other temperatures. At pH 7.10 the Michaelis constants for both glucose 6-phosphate and fructose 6-phosphate are lower for the *I. alatus* enzyme at all temperatures (fig. 2). The K_i for 6-phosphogluconate is also consistently lower in *I. alatus* (fig. 3). The *I. alatus* enzyme exhibits higher V_{max}/K_M values in both assay directions at all temperatures; however, the temperature sensitivity of the *M. edulis* enzyme is markedly less than the tropical

Table 1
Experimental Values (± SEM) for Glucosephosphate Isomerase Kinetic Parameters for *Mytilus edulis* and *Isognomon alatus* GPI, determined at 20C, pH 7.10, 0.1 Ionic Strength

Kinetic Parameter	Enzyme	
	Mytilus 1.00	*Isognomon* 0.66
Glucose 6-phosphate → Fructose 6-phosphate:		
K_M (μM)	95.6 (±0.6)	55.0 (±1.7)
V_{max} (μmol/min/mg)	251 (±0.3)	230 (±1.6)
V_{max}/K_M	2.63 (±0.02)	4.18 (±0.11)
Fructose 6-phosphate → Glucose 6-phosphate:		
K_M (μM)	25.8 (±0.7)	19.8 (±0.4)
V_{max} (μmol/min/mg)	282 (±0.3)	311 (±1.4)
V_{max}/K_M	10.9 (±0.3)	15.7 (±0.3)
K_i (μM, 6PG)	13.9 (±0.5)	6.7 (±0.2)
Equilibrium constant (experimental)	0.25	0.25
Equilibrium constant (calculated from Haldane relation)	0.24	0.27

I. alatus GPI, which diverges from 20% higher V_{max}/K_M's at 5 C to about 70% greater values at 35 C (fig. 4). Compared with the differences observed for V_{max}/K_m's, the V_{max}'s are relatively similar for the two species, although there are definite temperature-related differences for this parameter also. The glycolytic V_{max} for the mussel enzyme is similar to but somewhat higher than the *I. alatus* enzyme at most temperatures but tends to converge on that of *I. alatus* at higher temperatures (30–35 C; fig. 5). In the gluconeogenic direction, on the other hand, the *I. alatus* V_{max} is greater than that of *M. edulis,* and the two enzymes converge to similar values at low temperatures (5–10 C). The enzymes are most divergent in V_{max} in the gluconeogenic direction at high temperatures (30–35 C), where the *I. alatus* value is approximately 30% higher than the corresponding *M. edulis* V_{max} (fig. 5). The Arrhenius plots of figure 5 are nonlinear, a phenomenon that has also been reported for the rabbit muscle and oyster enzymes (Dyson and Noltmann 1968; Martin 1979). This suggests changes in the rate-determining step(s) with temperature and/or temperature-dependent conformational changes in the enzyme (Dyson and Noltmann 1968; Londesborough 1980). It is also possible that thermal denaturation occurring at the higher temperatures is responsible for the changes in slope in the Arrhenius plots of the *M. edulis* enzyme. However, Selwyn plots (Selwyn 1965) for assays above and below the "break" temperature at both high and low substrate concentrations provide no evidence for significant denaturation at higher assay temperatures. The enthalpies of activation ($\Delta H^{\neq}$) or temperature dependencies of the glycolytic V_{max}'s are apparently linear and nearly identical within the physiological temperature range: 12.5 ± 0.7 kcal/mol (5–25 C) for *M. edulis* and 12.8 ± 0.2 kcal/mol (10–35 C) for *I. alatus.* The apparent breaks at 20–25 C for the *M. edulis* enzyme temperatures coincide with the upper range of normal habitat temperatures for this species.

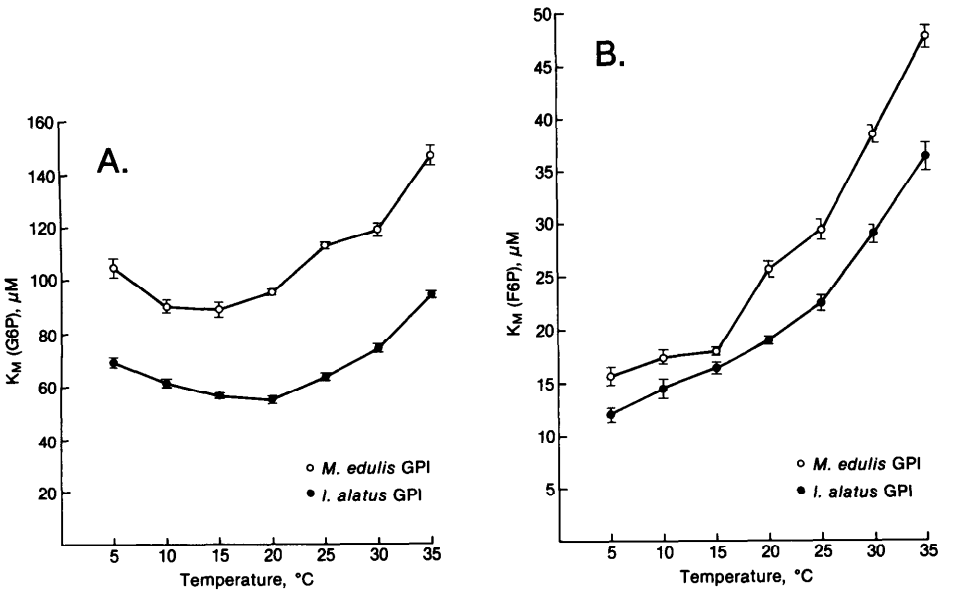

FIG. 2.—Temperature dependence of the Michaelis constants determined at pH 7.10, 0.1 ionic strength, in the glycolytic (*A*) and gluconeogenic (*B*) reaction directions for *Mytilus edulis* (open circles) and *Isognomon alatus* (closed circles) GPI. Bars denote ±1 SEM.

Generally similar patterns of differentiation are also apparent for the physiologically more relevant conditions of an assay pH that varies with temperature as described above. For example, the glycolytic V_{max} data obtained for the two species under these conditions are similar to the data collected at constant pH (fig. 6): the

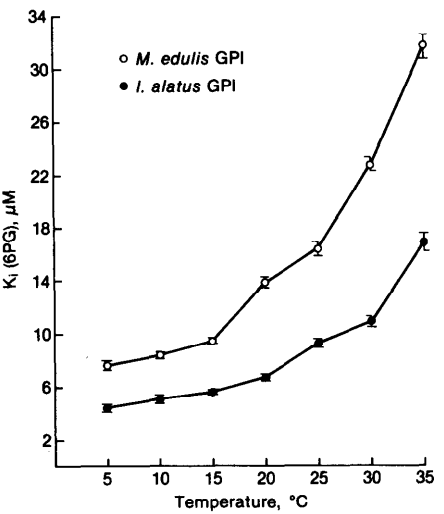

FIG. 3.—Temperature dependence of the inhibition constant (K_i) for 6-phosphogluconate at pH 7.10, 0.1 ionic strength, for *Mytilus edulis* (open circles) and *Isognomon alatus* (closed circles) GPI. Bars denote ±1 SEM.

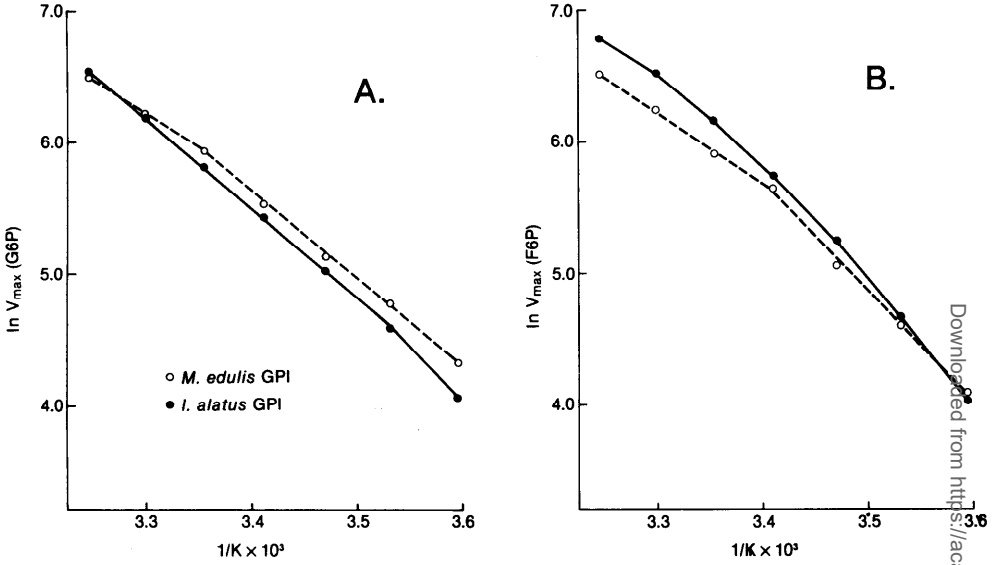

FIG. 4.—Arrhenius plots of glycolytic (*A*) and gluconeogenic (*B*) V_{max} determined at pH 7.10, 0.1 ionic strength, for *Mytilus edulis* (open circles) and *Isognomon alatus* (closed circles) GPI between 5 and 35 C. K refers to temperature in degrees Kelvin.

V_{max} of *M. edulis* GPI is slightly greater at most temperatures but converges on the *I. alatus* enzyme at 30–35 C. The V_{max}/K_M's show the same pattern and degree of differential temperature sensitivity when a variable pH assay is used as when assays are conducted at a constant pH (cf. figs. 5 and 7).

Thermal Denaturation Experiments

The denaturation rates of the purified GPIs at elevated temperatures (40–50 C) were measured to check their consistency with the generally observed positive

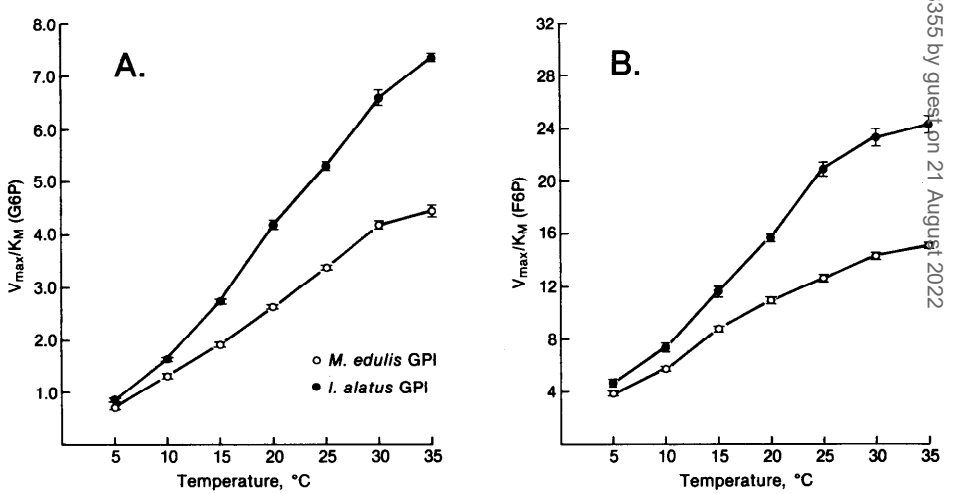

FIG. 5.—Temperature dependence of V_{max}/K_M, determined at pH 7.10, 0.1 ionic strength, in the glycolytic (*A*) and gluconeogenic (*B*) reaction directions for *Mytilus edulis* (open circles) and *Isognomon alatus* (closed circles) GPI. Bars denote ±1 SEM.

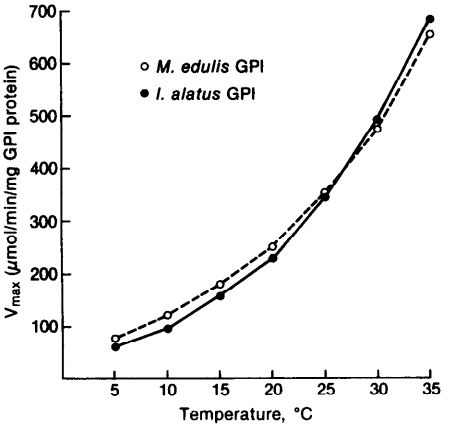

FIG. 6.—Temperature dependence of V_{max} determined at variable pH, 0.1 ionic strength, in the glycolytic reaction direction for *Mytilus edulis* (open circles) and *Isognomon alatus* (closed circles) GPI. Using untransformed plots avoids the distortion inherent in Arrhenius plots when relative rates at low and high temperature are visually compared.

correlation between thermal stability and cell temperature for protein homologues (Somero 1978). A comparison of the two enzymes at 1×10^{-9} M in the presence of .05% BSA indicates good accord with these previous observations (fig. 8). The *Isognomon alatus* enzyme is substantially more thermostabile than the *Mytilus edulis* enzyme, a conclusion that is supported by the results of individual *t*-tests between 42.5 and 50 C ($P < 0.01$ in each case).

Analysis and Discussion

A meaningful evaluation of an enzyme's relative efficiency as a catalyst begins with the specification of a standard state (see Albery and Knowles 1976; Page 1980; Hall and Koehn 1983). For purposes of adaptive inference, this means estimating in vivo substrate levels. Because of this, comparison of the relative catalytic efficiencies for the two GPI homologues requires a caveat. The two enzymes have

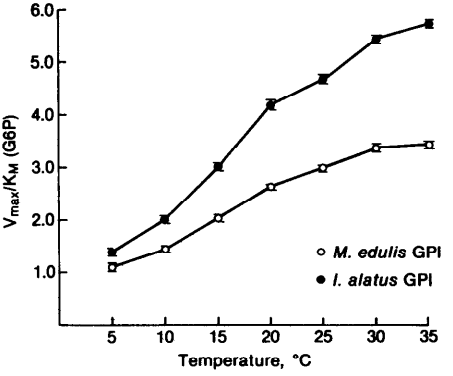

FIG. 7.—Temperature dependence of V_{max}/K_M, determined at variable pH, 0.1 ionic strength, in the glycolytic reaction direction for *Mytilus edulis* (open circles) and *Isognomon alatus* (closed circles) GPI. Bars denote ±1 SEM.

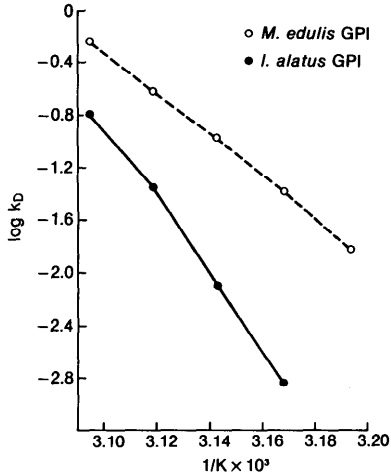

FIG. 8.—Arrhenius plots of thermal denaturation rates (k_D) for *Mytlius edulis* (open circles) and *Isognomon alatus* (closed circles) GPI. Temperatures are from 40 C to 50 C in 2.5-degree intervals. K refers to the temperature in degrees Kelvin. Conditions as described in text.

undoubtedly evolved in different metabolic contexts, one facet of this metabolic context being intracellular substrate concentration. The Michaelis constants of many enzymes in intermediary metabolism, including *Mytilus edulis* and mammalian GPIs, are similar to or somewhat greater than theirs in vivo substrate levels (Bris and Newsholme 1975; see Fersht 1977; Cameselle et al. 1980; Ebberink and de Zwaan 1980). Thus, the fact that the K_M's of *Isognomon alatus* GPI are consistently and significantly lower than those of the *M. edulis* enzyme suggests that the two enzymes' catalytic functions have been evolutionarily "set" for different standard states. Consequently, the comparison of their relative catalytic efficiencies for a single substrate concentration or standard state could be misleading.

However, it is possible to analyze the two enzymes' relative catalytic abilities by evaluating their relative y^{-1} values for ranges of temperature and glucose 6-phosphate concentration likely to occur in vivo and assuming that the hexose monophosphates are at equilibrium. Figure 9*A* and 9*B* are plots of several y^{-1} ratios of *I. alatus* to *M. edulis* GPI. For example, the 1.25 contour represents the combinations of temperature and substrate concentration where the *I. alatus* enzyme is 1.25 times more catalytically efficient than the mussel enzyme. Similarly, the 1.00 contour represents the combinations where the two enzymes are catalytically equivalent. This exercise demonstrates that, for assays conducted both at pH 7.0 and at a pH varying in accord with the pK_a of imidazole, the *I. alatus* GPI is apparently more efficient at virtually all realistic combinations of temperature and substrate concentration until below 10 C, where the *M. edulis* enzyme is more efficient at higher substrate levels. However, the catalytic superiority of the *M. edulis* enzyme is marginal (about 5%) at substrate concentrations around K_M (table 2).

The relative catalytic efficiencies of these two bivalve enzymes at different temperatures are consistent with the species' respective thermal environments. The GPI isolated from the tropical bivalve, *Isognomon alatus,* is catalytically more efficient than the *Mytilus edulis* enzyme at higher physiological temperatures, while at the lowest temperatures the two enzymes are catalytically more similar, with the

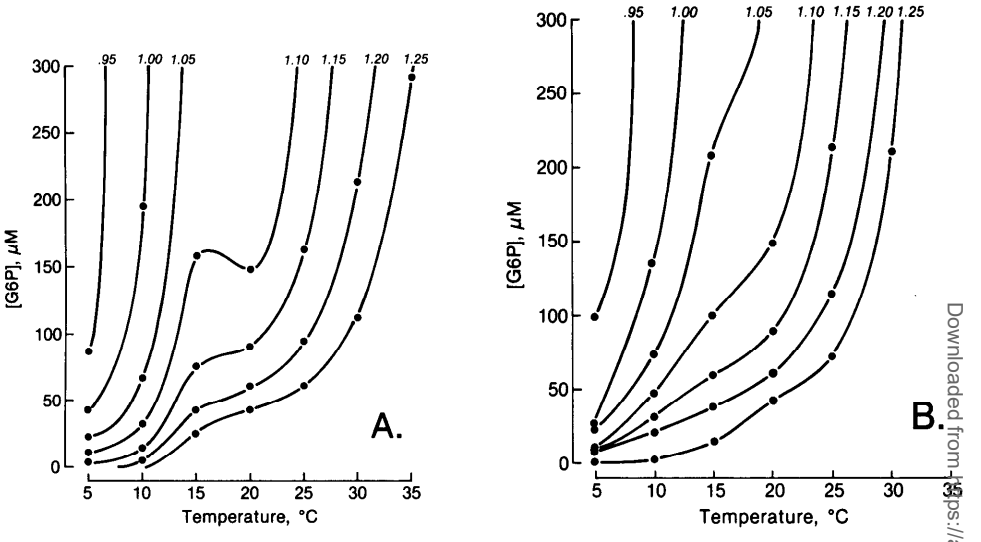

FIG. 9.—Contours calculated for the ratio of *Isognomon alatus* GPI catalytic efficiency (y^{-1}) to that of *Mytilus edulis* as a function of temperature and glucose 6-phosphate concentration at (*A*) a constant pH = 7.10 and (*B*) a pH varying in accordance with the pK_a of imidazole. The mass-action ratio is assumed to track the temperature dependence of K_{eq}. See text for details.

mussel enzyme being slightly more efficient, especially at higher substrate concentrations. The lower temperature sensitivity of the mussel enzyme appears to coincide with the generally observed temperature independence of the species' physiological processes between 5 and 20 C (Bayne et al. 1973) and the greater range of temperatures it experiences, compared with the relatively stenothermal *I. alatus*. These data suggest that environmental temperature has played a selective role in

Table 2
Relative Catalytic Efficiencies (y^{-1}) of *Mytilus edulis* and *Isognomon alatus* Glucosephosphate Isomerases at Different Temperatures and [G6P]'s, Assuming [F6P]/[G6P] = K_{eq}

Assay Condition	[G6P] (μM)	y^{-1} (μmol/min/mg) *Mytilus*	y^{-1} (μmol/min/mg) *Isognomon*	(*Isognomon* y^{-1})/(*Mytilus* y^{-1})
5 C, pH 7.10	10	5.9	6.5	1.10
	20	10.7	10.1	1.06
	40	15.5	15.5	1.00
	50	17.4	17.1	.98
	100	22.9	21.4	.93
	500	30.6	26.8	.88
20°C, pH 7.10	10	22.0	31.8	1.45
	20	37.7	51.2	1.36
	40	49.5	64.4	1.30
	50	66.1	81.0	1.23
	100	88.2	100.4	1.13
	500	121.0	124.0	1.02

shaping these enzymes' catalytic properties. This pattern of catalytic differentiation appears to be largely attributable to the greater temperature sensitivity of the V_{max}/K_M's of the *I. alatus* enzyme. The differences in gluconeogenic V_{max} also contribute to this pattern at higher substrate levels, while the higher glycolytic V_{max} of the *M. edulis* GPI is responsible for its apparent catalytic superiority at low temperatures and high substrate levels.

Comparison of the two GPIs with respect to several of the individual kinetic parameters shows several parallels with similar comparisons involving other homologous enzymes, mostly LDHs, that have been isolated from species with different body temperatures. Inverse correlations between median body temperatures and Michaelis constants and the conservation of apparent K_M values at species' respective physiological temperatures have been reported for fish LDH homologues (Borgmann and Moon 1975; Somero 1978; Somero and Yancey 1978; Yancey and Somero 1978; Graves and Somero 1982; Graves et al. 1983). Similarly, the GPI K_M's for fructose 6-phosphate and glucose 6-phosphate are lower at all temperatures for the warm water *I. alatus* enzyme. The K_M's also appear to be conserved at the two bivalves' median environmental temperatures: the values for glucose 6-phosphate and fructose 6-phosphate at 10 C for the *Mytilus edulis* enzyme are 85 μM and 18 μM, respectively, while the corresponding K_M's for *Isognomon alatus* GPI at 25 C are 75 μM and 24 μM.

Furthermore, the *I. alatus* enzyme is more thermostable (fig. 8), while its glycolytic V_{max} is generally slightly lower than *M. edulis* GPI. These data are also consistent with reports of negative correlations between thermal stability and k_{cat}'s for homologues of fish myofibrillar ATPases (Johnston et al. 1973; Johnston and Goldspink 1975; Johnston and Walesby 1977; Johnston et al. 1977), pyruvate kinases (Hoffman 1976; Low and Somero 1976), and lactate dehydrogenases (Borgmann and Moon 1975).

The phenomenon that enzymes that function at higher temperatures generally have greater thermal stabilities but lower k_{cat}'s than their cooler temperature homologues has been interpreted by several workers to indicate that the adaptation of homeotherms and other organisms to higher cell temperatures has been accompanied by a loss in their enzymes' catalytic efficiencies (i.e., as reflected in lower k_{cat} values; Low et al. [1973]; Somero [1978]; Hochachka and Somero [1984]). To account for the apparent paradox of a loss in enzyme catalytic efficiencies in the otherwise metabolically advanced birds and mammals, it has been proposed that proper enzyme conformations for binding substrate cannot be attained at higher temperatures without the formation of additional weak bonds, such as hydrogen bonds, electrostatic interactions, etc., that stabilize these structures. Such changes are presumed to be reflected as an increased enzyme thermal stability. According to the argument, the formation of additional weak bonds leads to a more conformationally rigid enzyme that is a less effective catalyst. Consequently, present-day enzymes of homeotherms and other "warm-adapted" species must be the result of "evolutionary compromises" between competing functions—that of maintaining adequate catalytic capabilities, on the one hand, and preserving proper conformations for binding substrate, on the other (Borgmann and Moon 1975; Somero 1975, 1978; Johnston et al. 1977; Somero and Yancey 1978; Hochachka and Somero 1984).

If k_{cat} is assumed to be a reliable indicator of catalytic efficiency, then both the GPI and LDH data mentioned above appear to be consistent with this notion.

However, equating k_{cat} with catalytic efficiency implies the choice of a saturating substrate concentration as the standard state. This is not physiologically appropriate—at least for enzymes involved in intermediary metabolism—because such enzymes have evolved to catalyze their reactions, not at saturating substrate concentrations, but at the low levels ($\leq K_M$) that predominate within the cell. Furthermore, it also artificially constrains the evaluation of the "evolutionary compromise" hypothesis to only the enzyme-substrate complex(es) and not the free enzyme. For these reasons it is clear that the use of k_{cat}/K_M and a definition of catalytic efficiency that specifies an in vivo standard state (fig. 1) are more realistic indicators than the former. When catalytic efficiency is defined in this way, the GPI and LDH data do not support the evolutionary compromise hypothesis. *Isognomon alatus* GPI, which functions in a warmer intracellular environment than the mussel enzyme, has (in addition to consistently higher y^{-1} values above 10 C) a higher gluconeogenic V_{max} at virtually all temperatures, a higher glycolytic V_{max} at the highest temperatures, and higher V_{max}/K_M's in both reaction directions at all temperatures. Comparison of the LDH k_{cat}/K_M's is also at odds with the compromise hypothesis. In their comparison of mammalian and fish LDHs, Borgmann and co-workers found the k_{cat}/K_M's for enzyme-coenzyme complexes to be higher for the mammalian enzyme, while the k_{cat}'s were lower at intermediate assay temperatures (Borgmann et al. 1975; Borgmann and Moon 1975). This relationship is apparent in both reaction directions. A similar pattern of differentiation is also evident in the data on the LDHs isolated from species of Pacific barracuda (g. *Sphyraena*): k_{cat}/K_M increases with median body temperature, while k_{cat} decreases, when the enzymes are assayed at 25 C (Graves and Somero 1982; Hall and Koehn 1983). The k_{cat}/K_M is the rate-determining kinetic parameter at low substrate concentrations (cf. Place and Powers 1979; Hall and Koehn 1983). Therefore, these higher k_{cat}/K_M values, obtained for enzymes isolated from species with warmer body temperatures, indicate that it is not valid to conclude that their enzymes are less efficient than those of species with cooler body temperatures at substrate concentrations much less than K_M—and perhaps not at physiological substrate levels (around K_M) either.

Several aspects of these LDH and GPI data suggest that, from the perspective of a single substrate level, the maintenance of catalytic efficiency at different thermal set points involves specific adjustments. First, the general observation of lower K_M's for homologues isolated from warmer cellular environments reflects a *net* tighter binding of substrates *and* intermediates (Borgmann and Moon 1975; Fersht 1977; cf. Hall and Koehn 1983). Second, some of this binding energy apparently is used to stabilize one or more of the transition states in the reaction sequence (higher k_{cat}/K_M's) and thereby facilitate catalysis (Jencks 1975). The lower K_i's of *I. alatus* GPI for 6-phosphogluconate (fig. 3), a competitive inhibitor and transition state analogue, are consistent with this interpretation (Chirgwin and Noltmann 1975). Tighter binding of substrates, intermediates, and transition states, as well as the higher thermal stabilities of such enzyme homologues, may reflect a requirement to conserve particular conformational equilibria for proper function at higher intracellular kinetic energy levels (Somero 1978).

Implicit in this discussion of these data is the conclusion that the relative catalytic efficiencies of two enzymes can be completely reversed, as different individual kinetic parameters are selected in turn as indicators of catalytic efficiency. While this dilemma is only an apparent one, it serves to illustrate the hazards of drawing conclusions about the relative efficiencies of enzyme homologues from

comparisons of individual kinetic parameters without reference to probable in vivo substrate levels. When catalytic efficiencies, as defined earlier, are determined for specified standard states for enzymes that function in different thermal environments, one must conclude that metabolic adaptation to higher cell temperatures does not necessarily compromise enzymic catalytic efficiency. Instead, selection appears to alter the kinetic properties of enzymes so as to tend to optimize catalytic function for different body temperatures and in vivo substrate levels.

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