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Evolution

Genetic variation and relative catalytic efficiencies: Lactate dehydrogenase B allozymes of *Fundulus heteroclitus*

(protein polymorphism/steady-state kinetics/temperature/pH)

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ABSTRACT **In order to evaluate whether functional differences exist between allelic variants of a B type lactate dehydrogenase (LDH; L-lactate:NAD$^+$ oxidoreductase, EC 1.1.1.27) in the teleost fish *Fundulus heteroclitus* (Linnaeus), the kinetic properties of pyruvate reduction were examined. While the pH dependence and the temperature dependence for maximal catalysis were indistinguishable among the allozymes, reaction velocities at low pyruvate concentrations were significantly different. At pH values below 8.00, the LDH-B^{b}B^b allozyme showed a greater reaction rate at lower temperatures (e.g., 10°C) than LDH-B^{a}B^a. The phenomenon was reversed at higher temperatures (e.g., >25°C) for pH values between 6.50 and 7.00. The rates for the heterozygous phenotype, LDH-B^{a}B^b, were not the arithmetic average of the two homotetrameric allozymes. When reaction rates were compared at constant relative alkalinity, that is, a constant [OH$^-$]/[H$^+$] ratio, the findings were similar. The differences in the temperature dependence and the pH dependence for pyruvate reduction found between the LDH-B allozymes may reflect a selective adaptation and help explain the geographical variation in the *Ldh-B* gene frequencies of *F. heteroclitus.***

In recent years no subject in evolution has been more debated than the significance of protein polymorphisms (1, 2). Most of the discussion concerning this phenomenon has centered on two contrasting views: the "selectionist" and the "neutralist." Proponents of the former theory advocate that a form of selection operates to maintain protein polymorphisms, while those of the latter viewpoint argue that the majority of genetic variability at the molecular level is selectively neutral. Although adequate theoretical treatment has been developed for both schools of thought, Stebbins and Lewontin (3) and Lewontin (2) have shown that numerical manipulations alone will *not* resolve the conflict. Clarke (4) has pointed out that present estimates of evolutionary rates, mutation rates, genetic loads, effective population sizes, and numbers of genes are so inexact that, by a suitable choice of values, either case can be favored.

Implicit in the neutralist hypothesis is that most structural differences are, in essence, functionally equivalent (1), or in Darwin's words ". . . of no service or disservice to the species, and which consequently have not been seized on and rendered definite by natural selection . . ." (5). Yet there are examples in which functional nonequivalences of protein variants are known (6–13), the most noted being that of sickle cell hemoglobin (13).

We have been investigating the structural and functional properties of allelic variants in the common killifish, *Fundulus heteroclitus* (Linnaeus). Most of our work has focused on the heart-type or B lactate dehydrogenase (LDH; L-lactate:NAD$^+$ oxidoreductase, EC 1.1.1.27), whose *Ldh* gene is the only one expressed in the liver, heart, and erythrocytes of *F. heteroclitus* (14). There is a dramatic north–south cline in *Ldh-B* gene frequency along the Atlantic coast of the United States (Fig. 1). This same region features a 1°C change in annual mean water temperature per degree change in latitude (15), making it one of the steepest thermal gradients in the world. Moreover, in the southern marshes, summer temperatures in excess of 40°C are commonly recorded (16), while winter temperatures in northern marshes result in extensive ice formation. Because temperature has a profound effect on enzyme structure and function, we asked whether the *Ldh* gene frequency pattern illustrated in Fig. 1 resulted from a selective adaptation to differing thermal regimes. In other words, is there a correlation between the catalytic efficiency of each allozyme and the temperature in which that phenotype is most abundant? This report presents evidence for such a correlation.

MATERIALS AND METHODS

Chemicals. Sodium pyruvate (type II, lot 24C-0390), bovine heart LDH-B (crystalline, lot 63C-0462-9), and bovine serum albumin were purchased from Sigma; NADH (Chromatopure, lots 465001 and 565001) was purchased from P-L Biochemicals. All other chemicals were reagent grade.

Purification of LDH-B Allozymes from *F. heteroclitus*. The full details of the purification scheme will be presented elsewhere, but the essential step involves affinity chromatography on *N*-(6-aminohexyl)oxamate-Sepharose (17). The purity of each preparation was greater than 95% by several criteria (18). The specific activity of the allozymes measured at 25°C in 0.1 M sodium phosphate buffer (pH 7.50) with 0.167 mM NADH and 0.33 mM pyruvate varied between 450 and 490 μmol/min per mg of protein. Protein was estimated by the microbiuret assay (19) with bovine LDH-B as the primary standard.

Nomenclature. The terminology used in designating each genetic variant of LDH corresponds to its relative electrophoretic mobility. The two homozygous allozymes are designated as LDH-B^{a}B^a and LDH-B^{b}B^b, in which the superscripts *a* and *b* indicate fast and slow relative electrophoretic mobilities, respectively. The heterozygous phenotype is represented by the symbol LDH-B^{a}B^b.

Kinetic Procedure. Assays were performed in 0.1 M sodium phosphate buffers at temperatures of 10°C, 25°C, and 40°C and pH values of 6.50, 7.00, 7.50, and 8.00. All buffers were standardized at 25°C and no corrections were made for ionic strength differences or for changes in temperature.

Experimental determinations of the kinetic constants were conducted with quadruplicate samples at 0.5 mM NADH and each of seven to nine different pyruvate concentrations. Thus, each velocity vs. substrate curve contained a minimum of 28 data points.

All reagents except buffers were prepared daily and kept on ice. Ammonium sulfate suspensions of each enzyme were diluted (>100 fold) into the phosphate buffer to be used con-

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Abbreviations: LDH, lactate dehydrogenase; Prv, pyruvate.

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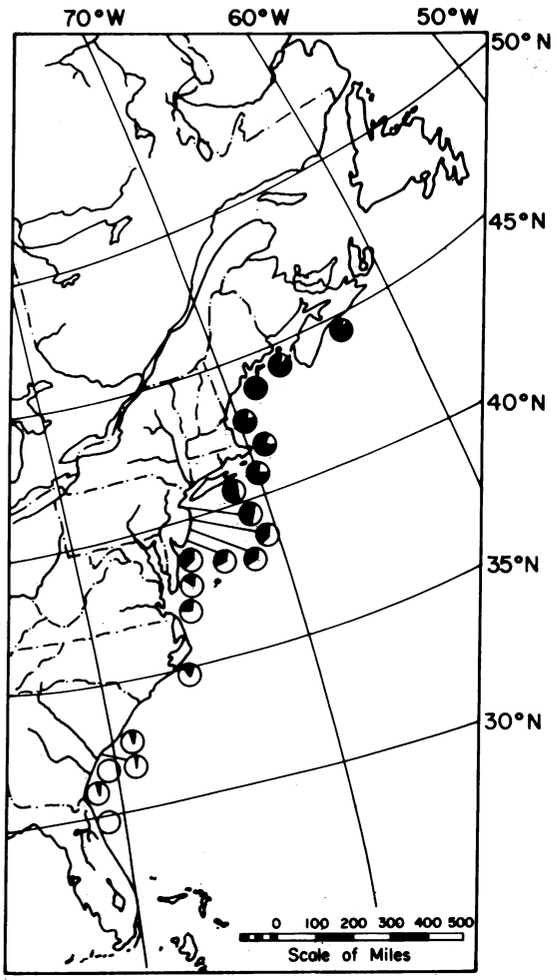


FIG. 1. Geographical variation in the *Ldh-B* gene frequencies of *F. heteroclitus*. Shaded areas of the circles represent the B^b gene frequency while open areas represent the B^a gene frequency. This region of the Atlantic coast of North America features a 1°C change in annual mean water temperature per degree change in latitude (15). (One mile = 1.6 km.)

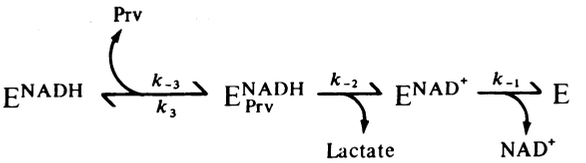
taining 0.1% (wt/vol) bovine serum albumin. When diluted in this manner and maintained at 4°C, the LDH solutions exhibited constant activity over an 11-hr period.

Data Analysis. The estimates for the kinetic parameters were obtained by the nonparametric direct linear method of Eisenthal and Cornish-Bowden (20, 21) and the parametric weighted regression method of Cleland (22). A detailed comparison of the two estimates will be presented elsewhere. In the present study we are concerned with nonparametric estimates because they are more robust than least-squares estimates (21).

The three-dimensional contours of $k_{cat}[E_o]/K_m$ (i.e., V_{max}/K_m), in which $[E_o]$ is total enzyme concentration, in response to changes in pH and temperature were obtained by a cubic spline interpolation (23).

THEORY

The LDH-B allozymes of *F. heteroclitus*, like all vertebrate LDHs studied (24), obey an ordered bisubstrate ternary-complex mechanism. Because the present study concerns itself only with the kinetics of pyruvate reduction in the absence of products and at saturating cofactor concentration (i.e., approximately 25 times the apparent K_m for NADH), the reaction under these conditions can be written as:



(Prv, pyruvate). It is assumed that the two ternary complexes ($E^{NAD^+}_{Lactate}$ and E^{NADH}_{Prv}) are rapidly interconverted and can be treated as a single species.† Thus, the rate of pyruvate reduction at any concentration can be described by:

$$v = \frac{\frac{k_{-1}k_{-2}}{(k_{-1} + k_{-2})}[E_o][Prv]}{\frac{k_{-1}(k_{-2} + k_3)}{k_{-3}(k_{-1} + k_{-2})} + [Prv]}. \quad [1]$$

If we let $(k_{-1}k_{-2})/(k_{-1} + k_{-2})$ equal k_{cat} (an apparent first-order rate constant for maximal catalysis) and let $[k_{-1}(k_{-2} + k_3)]/[k_{-3}(k_{-1} + k_{-2})]$ equal the Michaelis–Menton constant (K_m) for pyruvate, then Eq. 1 reduces to the Michaelis–Menten equation:

$$v = \frac{k_{cat}[E_o][Prv]}{K_m + [Prv]} \quad [2]$$

There are two fundamental rate constants in Eq. 2 that determine the overall kinetics (25): k_{cat} (units, s^{-1}), the limiting velocity observed as the pyruvate molarity tends toward infinity, and the apparent second-order rate constant k_{cat}/K_m (units, $M^{-1}s^{-1}$). As pyruvate concentrations are lowered, the reaction velocity (v) approaches first order relative to the pyruvate molarity, and Eq. 2 becomes:

$$v = \frac{k_{cat}}{K_m}[E_o][Prv] \quad [3]$$

The rate constant k_{cat}/K_m is equal to $(k_{-2}k_{-3})/(k_{-2} + k_3)$.

These two limiting rate constants (k_{cat} and k_{cat}/K_m) vary independently with changes in the concentration of effectors (e.g., inhibitors, activators, etc.) or with changes in the physical environment (e.g., temperature, pH, etc.) (25). By evaluating the pH and temperature dependence of these kinetic constants for the LDH-B allozymes, we can assess whether one allozyme is a more efficient catalyst than the other under similar conditions. Although a comparison could be made employing k_{cat} and K_m, we have chosen not to do this for the following reasons: K_m is *not* a rate constant; k_{cat} and K_m are too highly correlated (26); the expression for k_{cat}/K_m requires one less constant than K_m; and the biochemical significance of k_{cat}/K_m has been well documented (22, 27, 28). Moreover, when our comparisons are confined to k_{cat} and k_{cat}/K_m rather than V_{max} and V_{max}/K_m, the results are applicable at any enzyme concentration.

RESULTS

Comparison of the First-Order Rate Constants k_{cat}. Inspection of the k_{cat} estimates in Table 1 indicates no apparent pH dependence in the maximal rate of catalysis. A strong positive temperature dependence exists, but there are no significant differences between phenotypes. In fact, the thermodynamic activation parameters are indistinguishable (Table 2).

† Although this is an admitted oversimplification, data from porcine LDH-B have shown that the interconversion of the two ternary complexes is indeed more rapid than any of the other unimolecular rate constants (24).

Table 1. Nonparametric estimates of the kinetic constants for the LDH-B allozymes of *F. heteroclitus*

Temp., °C	pH	LDH-B^bB^b K_m, mM	LDH-B^bB^b k_{cat}, s^{-1}	LDH-B^bB^b $10^{-3} \times k_{cat}/K_m$, $M^{-1}s^{-1}$	LDH-B^aB^b K_m, mM	LDH-B^aB^b k_{cat}, s^{-1}	LDH-B^aB^b $10^{-3} \times k_{cat}/K_m$, $M^{-1}s^{-1}$	LDH-B^aB^a K_m, mM	LDH-B^aB^a k_{cat}, s^{-1}	LDH-B^aB^a $10^{-3} \times k_{cat}/K_m$, $M^{-1}s^{-1}$
10	6.50	0.029	159	5460	0.039	188	4860	0.074	187	2540
	7.00	0.038	173	4580	0.046	191	4140	0.083	192	2330
	7.50	0.056	179	3200	0.084	174	2070	0.100	184	1840
	8.00	0.120	169	1420	0.172	187	1100	0.136	176	1290
25	6.50	0.061	482	7930	0.076	480	6300	0.077	509	6580
	7.00	0.080	500	6230	0.078	471	6030	0.084	490	5840
	7.50	0.136	499	3690	0.149	498	3340	0.159	512	3220
	8.00	0.331	513	1550	0.291	498	1720	0.251	497	1980
40	6.50	0.160	1190	7420	0.124	1120	9020	0.133	1170	8790
	7.00	0.227	1140	5020	0.171	1100	6460	0.189	1240	6530
	7.50	0.414	1100	2640	0.409	1073	2630	0.425	1210	2830
	8.00	0.778	1042	1350	0.779	1020	1310	0.743	1140	1540

The nonparametric direct linear method of Eisenthal and Cornish-Bowden (20, 21) was used to obtain the kinetic constants. The estimates of k_{cat} and k_{cat}/K_m are based on a holoenzyme tetramer molecular weight of 140,000.

Temperature and pH Dependence of the Second-Order Rate Constant k_{cat}/K_m. Table 1 indicates that, unlike k_{cat}, the apparent second-order rate constant k_{cat}/K_m differs significantly between allozymes. The most dramatic differences occur at low temperatures and low pH. For example, at 10°C and pH 6.50, k_{cat}/K_m for LDH-B^bB^b is more than twice that of LDH-B^aB^a. These differences become less pronounced with increasing pH. The LDH-B^aB^b phenotype does not reflect a simple average of the two homozygous types. Instead, it more closely resembles the LDH-B^bB^b allozyme for pH values between 6.50 and 7.00.

At 25°C, the differences between the enzymes are less pronounced. The k_{cat}/K_m estimate for LDH-B^bB^b at pH 6.50 is approximately 20% larger than that for either of the other two phenotypes, while at pH 8.00 it is nearly 10% smaller. At 40°C, the LDH-B^bB^b enzyme has a smaller rate constant than LDH-B^aB^a at all pH values examined, thus reversing the order found at 10°C.

By using spline interpolation (see ref. 23) of the data in Table 1, three-dimensional contours for the pseudo-first-order rate constant ($k_{cat}[E_o]/K_m$) in relation to pH and temperature were drawn by computer for each allozyme. The drawings are presented in Fig. 2. The large catalytic differences between LDH-B^bB^b and LDH-B^aB^a are readily apparent.

Each three-dimensional surface in Fig. 2 is defined by twelve points—i.e., $k_{cat}[E_0]/K_m$ values at three temperatures and each of four pH values. In order to evaluate whether data from three temperatures were representative of the actual surface at a given pH, data from eight temperatures were obtained for each of the homotetramers at pH 7.5 (Fig. 3). The symbols represent the experimental values, while the curves are the spline interpolation based on three temperatures: 10°C, 25°C, and 40°C. No significant departures from the interpolated curves are evident. Therefore, the spline-interpolated contours of Fig. 2 are good first-order approximations.

The extracellular and intracellular pH values of cold-blooded animals vary inversely with body temperature (30–32). The consequence of this phenomenon is interpreted as the maintenance of a constant relative alkalinity as temperature changes (33). Whereas the relative alkalinity in the extracellular fluids (e.g., blood) of cold-blooded animals is generally in the range of 15 to 40, measurements on frog skeletal muscle (34, 35) indicate that the intracellular $[OH^-]/[H^+]$ ratio is approximately 4. By using our spline-interpolated estimates, the k_{cat}/K_m rate constants at constant relative alkalinities were examined as a function of temperature. Fig. 4 presents the results for a relative alkalinity of 1.[§] The LDH-B^bB^b allozyme had a greater rate constant at 10°C than the other types, while at high temperatures (above 25°C) the relationship was reversed. The k_{cat}/K_m values of the LDH-B^aB^b allozyme more closely resemble values intermediate between those of the two homotetramers at this relative alkalinity.

It is interesting to note that, had we limited our investigation to a single temperature and pH (e.g., 25°C and pH 7.50), the differences observed between LDH-B allozymes could have gone undetected.

DISCUSSION

Substrate concentrations *in vivo* are normally too low to saturate enzymes (37); most often they are at or below an enzyme's K_m. For example, intracellular pyruvate concentrations have been

[§] The same general results are obtained at higher relative alkalinities (e.g., $[OH^-]/[H^+] = 16$).

Table 2. Thermodynamic activation parameters for the LDH-B allozymes of *F. heteroclitus* (25°C)

Allozyme	E_a, cal/mol	$\Delta H^‡$, cal/mol	$\Delta S^‡$, cal/mol·°C	$\Delta G^‡$, cal/mol
LDH-B^aB^a	10,598 ± 327	10,000 ± 327	−10.5 ± 1.06	13,165 ± 448
LDH-B^aB^b	10,393 ± 330	9,800 ± 330	−11.3 ± 1.07	13,176 ± 390
LDH-B^bB^b	10,341 ± 339	9,749 ± 339	−11.5 ± 1.09	13,186 ± 581

These results in each case represent the mean ± SEM of values determined at pH 6.50, 7.00, 7.50, and 8.00. An analysis of covariance (29) did not allow us to reject the null hypothesis ($P < 0.10$) for colinearity of the group means; i.e., the Arrhenius plots were identical for all three allozymes and independent of changes in pH. The correlation coefficient for the pooled data was −0.995. One calorie = 4.19 J.

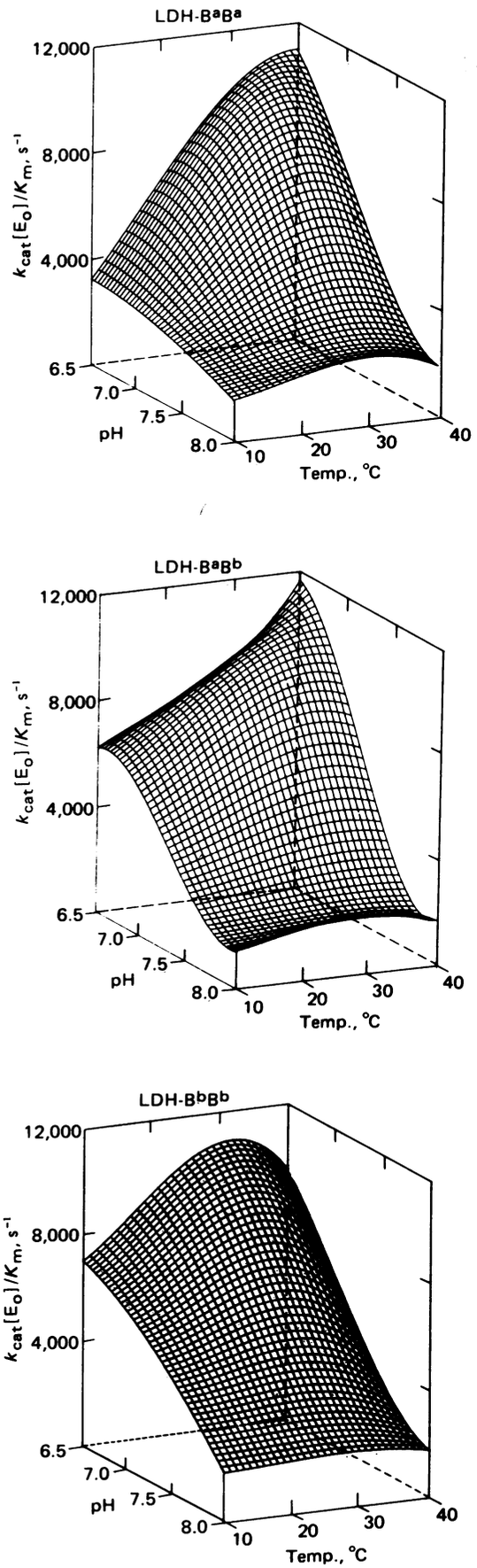


FIG. 2. Three-dimensional contours of $k_{cat}[E_o]/K_m$ as a function of pH and temperature for the three LDH-B phenotypes of *F. heteroclitus*. Cubic spline interpolation (23) was used to generate the surfaces. The enzyme concentration in each case was 1.286 mM.

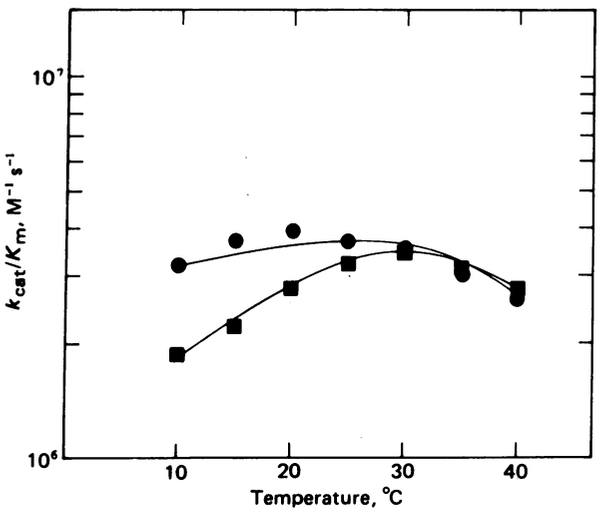


FIG. 3. Comparison of spline-interpolated and measured estimates for k_{cat}/K_m at pH 7.50. The curves are the spline interpolation based on data at 10°C, 25°C, and 40°C for the two homotetramers. The symbols represent the measured estimates over the same temperature range. ■, LDH-B^{a}B^a; ●, LDH-B^{b}B^b.

found to be slightly less than the K_m for pyruvate (38, 39). Therefore, reaction rates at low substrate concentrations ([Prv] $\leq K_m$) are more significant biologically than rates measured at high substrate concentrations. While rates at high substrate concentrations are governed by k_{cat}, velocities at physiological concentrations are dominated by both k_{cat} and $k_{cat}K_m$. When two enzymes have the same k_{cat} but different k_{cat}/K_m values, at identical substrate concentrations their reaction rates will vary solely as a function of k_{cat}/K_m.

Fersht (27) and Crowley (40) have reported elegant studies that suggest that evolutionary pressures increase both k_{cat} and K_m such that k_{cat} is as large as possible and K_m is large compared to physiological substrate levels, the result being a maximal k_{cat}/K_m (27). While Cornish-Bowden (41) has argued that K_m should be within an order of magnitude of the substrate level, all investigators agree on the biological significance of a large k_{cat}/K_m. However, its value is limited by the thermody-

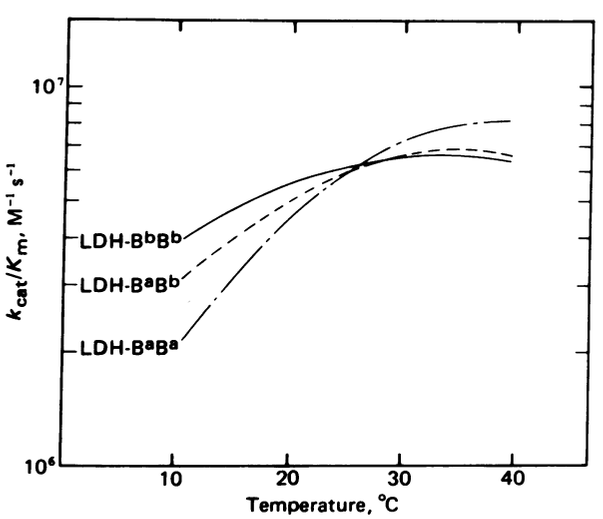


FIG. 4. The k_{cat}/K_m parameter as a function of temperature at the constant ratio $[OH^-]/[H^+] = 1$. The values used were those supplied by the spline approximation used in generating the three-dimensional contours. The neutral pH at each temperature was calculated as $\frac{1}{2}pK_w$, in which pK_w is $-\log_{10}$ of the ionization constant of water at that temperature (36).

namic restraints imposed by the Haldane relation (25). Moreover, because k_{cat}/K_m cannot be larger than any of the second-order rate constants of the appropriate reaction (42), its value sets a lower limit on the rate of enzyme–substrate association.

Borgman and Moon (43), in an interspecific comparison of LDH-A enzymes from cow and flounder, found that the enzyme from the cold-blooded animal had a maximum for k_{cat}/K_m at a lower temperature (≈5°C) than the orthologous protein from the warm-blooded species (≈20°C). More importantly, the absolute value for k_{cat}/K_m above 10°C was greater than that observed for the enzyme from the cold-blooded species. Although such differences between a cold-blooded fish and a warm-blooded mammal are not surprising, they are reassuring to evolutionists who promote natural selection at the molecular level. However, the dichotomy between the neutralist and selectionist hypotheses lies not upon enzymatic differences between divergent species but also upon the existence of a functional nonequivalence for genetic variants within a species. In our intraspecific comparison of *F. heteroclitus*, the allozyme common to the colder northern waters, LDH-B^bB^b, has a maximum k_{cat}/K_m around 20°C, while the phenotype most common in the warmer southern latitudes had a maximal k_{cat}/K_m of around 30°C. If a large k_{cat}/K_m is advantageous, as suggested by Fersht and others (25, 27), then at constant relative alkalinity LDH-B^bB^b would be a more efficient catalyst at low temperatures than either LDH-B^aB^b or LDH-B^aB^a. At higher temperatures, LDH-B^aB^a should be the most efficient. The heterozygous phenotype, LDH-B^aB^b would be intermediate. Viewed in this light, in an annually fluctuating thermal environment such as that experienced by *F. heteroclitus* (15), the heterozygous phenotype would have an advantage with a net heterozygote superiority. These catalytic differences between allozymes, the correlation of LDH-B phenotype with hemoglobin function (44), and the changes in *Ldh-B* gene frequency along the animal's natural distribution (Fig. 1) are consistent with the selectionist hypothesis.

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