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EFFECT OF TEMPERATURE UPON CATALYTIC PROPERTIES OF LACTATE DEHYDROGENASE ISOENZYMES FROM A POIKILOTHERM

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SUMMARY

Lactate dehydrogenase isoenzymes A_4 and B_4 (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27) have been purified from muscle and heart of the snake *Bothrops neuwiedii*. Catalytic properties at different temperatures and pH values have been studied comparatively with those of the same isoenzymes from beef.

Values of K_m for ophidian isoenzymes were markedly reduced when the temperature decreased. At low substrate concentrations, the increased enzyme–pyruvate affinity compensates for the lower thermal energy and the reaction rate appears to be independent of temperature. Under the same conditions, the activity of beef isoenzymes was closely related to thermal energy.

These findings suggest that lactate dehydrogenases from the poikilotherm are able to function with the same efficiency at all temperatures within the range of habitat and to adjust immediately to thermal changes.

INTRODUCTION

During the last five years there have been important observations made, particularly in the laboratory of Hochachka and Somero, concerning the relationship between temperature and catalytic activity of enzymes from poikilotherms[1–6].

The most general finding is that of the coincidence between temperature at which the enzyme–substrate affinity is maximal and the temperature of the animal's habitat. The same correlation is shown by certain enzyme variants which appear after acclimatization of fish[3,4,7]. It was proposed that compensation of environmental thermal changes is attained by a reduction of K_m as the temperature decreases[8].

In a study of lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27) isoenzymes purified from tissues of the snake *Bothrops neuwiedii*, we found the same type of K_m–temperature relationship. But this behavior is not exclusive to enzymes from poikilotherms. Plagemann *et al.*[9] demonstrated that lactate dehydrogenases from mammals showed lower K_m values at lower temperatures. Thus,

it is not possible to acknowledge a difference of biological significance without a comparative study of enzymes from warm- and cold-blooded animals.

This paper presents the results of such an investigation of the properties of lactate dehydrogenase isoenzymes from beef and snake tissues.

METHODS

Adult specimens of the snake *B. neuwiedii* were obtained at different seasons of the year from the Instituto Nacional de Microbiología Carlos Malbrán (Buenos Aires) and from the Instituto de Animales Venenosos (Santiago del Estero, Argentina). Other specimens were caught in different areas of the province of Córdoba (Argentina).

Animals were killed by chloroform anesthesia, dissected, and the tissues removed and stored at −20 °C until used.

Extraction and purification of lactate dehydrogenase

The enzyme was extracted from skeletal muscle and pooled hearts. All steps were carried out at 4 °C.

50 g of tissue were minced with scissors, suspended in 150 ml of distilled water and homogenized with a Waring blendor. The suspension was left at 4 °C for 1 h and then filtered through cheese cloth, centrifuged at 10 000 × *g* for 20 min and the sediment discarded.

$(NH_4)_2SO_4$ was added to the supernatant up to 50% saturation. The pH was maintained at 7 by adding diluted NH_4OH. After 1 h, the mixture was filtered through paper and the precipitate discarded. $(NH_4)_2SO_4$ was added to the filtrate to obtain a 75% saturation. The precipitate was then collected by filtration on paper, and dissolved in 5 ml of 0.01 M phosphate buffer, pH 7.0. The solution was dialysed against 2 l of the same buffer, with two changes of buffer during 12 h.

Separation of isoenzyme

Dialysed samples were added to a DEAE-cellulose column (20 cm × 2.5 cm) previously equilibrated with 0.01 M phosphate buffer, pH 7.0. Then, a stepwise gradient was established by adding the following solutions: (I) 70 ml of 0.01 M phosphate buffer, pH 7.0; (II) 60 ml of 0.01 M phosphate, 1 mM NaCl, pH 6.8; (III) 60 ml of 0.01 M phosphate, 2 mM NaCl, pH 6.6; (IV) 60 ml of 0.01 M phosphate, 5 mM NaCl, pH 6.4; (V) 80 ml of 0.01 M phosphate, 10 mM NaCl, pH 6.2; (VI) 80 ml of 0.01 M phosphate, 25 mM NaCl, pH 6.0; and (VII) 120 ml of 0.01 M phosphate, 50 mM NaCl, pH 6.0. The eluate was collected in 10-ml fractions.

The chromatographic profile from heart preparations gave one peak of intense enzymatic activity, comprising fractions 49–52, and a minor peak in fractions 19–21. Electrophoretic control of the eluates corresponding to each peak, showed a single band of lactate dehydrogenase. The isoenzyme in the most prominent peak showed the same mobility as the predominant band in whole heart extract (Fig. 1). Specific activity of crude extract from heart was 4.5 units/mg, and that of the pooled eluate fractions 49–52, 150 units/mg.

The enzyme extracted from muscle gave a profile with three peaks of enzymatic activity. The first and most prominent, from fractions 7 to 10, the second, from

fractions 12–14, and the third, from 19–22. When studied electrophoretically, eluates pertaining to the first peak showed a single band with the mobility of the least negatively charged fraction in muscle extract (Fig. 1). Specific activity of crude extract from muscle was 5.05 units/mg, and that of eluates corresponding to the first peak, 170 units/mg.

Eluates from heart extract containing the anodical fraction, and those from muscle comprising the first peak, were treated with $(NH_4)_2SO_4$ up to 60% saturation, the precipitate was collected and stored at 4 °C until used for studies. These preparations were electrophoretically single bands, not contaminated with other isoenzymes.

Beef lactate dehydrogenase isoenzymes

Lactate dehydrogenase purified from beef heart (B_4) and from beef muscle (A_4) were purchased from Sigma (U.S.A.).

Enzyme assay

A method adapted from that of Wróblewski and LaDue[10] was used. The absorbance at 340 nm was read every minute during 6 min in a spectrophotometer in which the temperature of the cuvette compartment was controlled with a water circulator.

Electrophoresis

Starch gel electrophoresis was performed with a vertical device. The gel was prepared with 12 g of hydrolyzed starch for each 100 ml of the buffer proposed by Markert and Faulhaber[11]. A current of 20 mA was applied for 18 h at 4 °C.

After electrophoresis, the starch block was sliced and stained for lactate dehydrogenase activity with the same method used by Blanco *et al.*[12].

Blood pH determination

Two lots of four adult *B. neuwiedii*, were each allowed to become acclimatised at 5 and 35 °C for seven days. The animals were then slightly anesthesized with chloroform and blood was extracted from the posterior cava vein with a heparinized Becton–Dickinson syringe. The blood was immediately transferred to a microelectrode for pH measurement.

Blood from snakes which had become acclimatise at 5 °C had an average pH of 7.35 ± 0.02, and those at 35 °C, 7.05 ± 0.02.

RESULTS

Electrophoretic patterns

As shown in Fig. 1, aqueous extracts of tissues from the snake *B. neuwiedii* present multiple forms with lactate dehydrogenase activity. Although there are some differences on comparison with lactate dehydrogenase from other species (for example, skeletal muscle shows six isoenzymes), a feature common to most is observed: the predominance of the most anodic isozyme in heart, and of that least negatively charged in muscle and liver. By analogy, we will consider the enzyme predominant

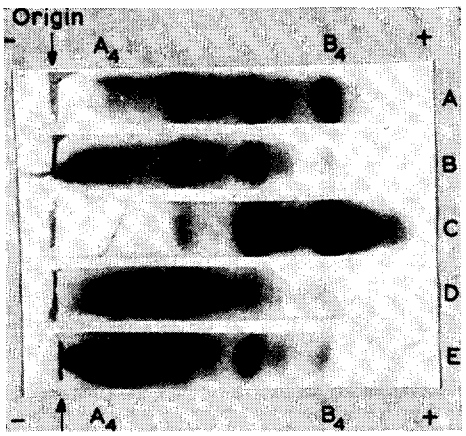

Fig. 1. Starch gel electrophoretic patterns of lactate dehydrogenase isoenzymes from tissues of the snake *B. neuwiedii*. Extracts were prepared with 1 part of tissue and 20 parts of water (1:20, w/v). A, Retina; B, kidney; C, heart; D, liver; E, skeletal muscle. A_4 and B_4 indicate the position of the corresponding isoenzyme.

in heart as equivalent to the homotetramer B_4 (or H_4) present in heart of other species, and the fraction most abundant in muscle, to the tetramer A_4 (or M_4).

There were no significant differences in the relative distribution of isoenzymes from animals caught in different regions or in different seasons of the year. Patterns of tissue extracts from animals which had become acclimatised at 5 and 35 °C were practically identical.

Catalytic properties

Assays were carried out at 10, 20 and 35 °C, temperatures which are in the range of the snake's habitat. All results presented are averages of five determinations on different samples.

Figs 2 and 3 show curves of enzymatic activity at different substrate concentrations, determined at pH 7.4 at 10 and 35 °C. For both bovine and ophidian enzymes, the activity increased more rapidly and reached a maximum at lower pyruvate concentrations when assayed at 10 °C than at higher temperatures. Although the trend is the same for beef and snake isoenzymes, the changes produced by thermal variations were more marked for the snake isoenzymes.

Table I presents values of K_m for pyruvate of A_4 and B_4 isoenzymes at 10, 20 and 35 °C. The Michaelis constant was calculated from Lineweaver–Burk plots. For all the isoenzymes, the K_m values become lower as the temperature decreases, but the relative reduction is greater for ophidian lactate dehydrogenase.

Curves of activity of snake isoenzymes at 10 and 35 °C show a convergence at low concentrations of pyruvate, while curves of beef enzymes are clearly separated (Figs 2 and 3). The same phenomenon can be expressed by calculating the ratio of activity at a given temperature over the activity at the temperature at which the minor K_m was recorded (10 °C), both determined with a concentration of substrate corresponding to that of the minor K_m. For bovine enzymes the value of the ratio increases with temperature; this means that the increment of affinity does not compensate for the lower thermal energy. Snake isoenzymes show ratios close to

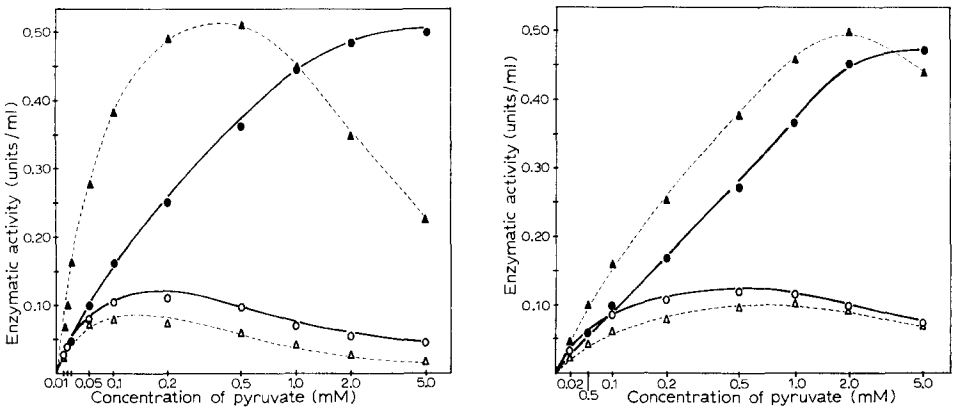

Fig. 2. Effect of substrate concentration and temperature upon activity of lactate dehydrogenase B_4 from beef heart (— — —) and snake heart (———). Reaction mixture contained 0.113 mM NADH and 0.1 M phosphate buffer, pH 7.4. Final concentrations of pyruvate were 0.01, 0.015, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 mM. ● and ▲, determinations at 35 °C; ○ and △: Determinations at 10 °C.

Fig. 3. Effect of substrate concentration and temperature upon activity of lactate dehydrogenase A_4 from beef (— — —) and snake muscle (———). Other details same as in Fig. 2.

TABLE I

EFFECT OF TEMPERATURE UPON K_m FOR BOVINE AND OPHIDIAN PYRUVATE ISOENZYMES LACTATE DEHYDROGENASE A_4 AND B_4

Determinations were carried out at pH 7.4. Concentrations are expressed in M × 10^5.

Temperature of assay (°C)	*Beef enzyme*		*Snake enzyme*	
	A_4	B_4	A_4	B_4
10	11.1	8.6	7.7	4.5
20	15.3	9.8	22.5	12.5
35	23.5	10.5	32.1	19.2

TABLE II

EFFECT OF TEMPERATURE UPON ACTIVITY OF BOVINE AND OPHIDIAN ISOENZYMES LACTATE DEHYDROGENASE A_4 AND B_4

Values represent ratio activity at indicated T/activity at 10 °C.
Determinations were carried out at pH 7.4, concentration of substrate was that of the minor K_m for each isoenzyme.

Temperature (°C)	*Beef enzyme*		*Snake enzyme*	
	A_4	B_4	A_4	B_4
10	1.00	1.00	1.00	1.00
20	1.63	2.53	0.91	0.72
35	2.86	4.66	1.03	1.17

unity at the different temperatures, which indicates that enzymatic activity is practically the same, regardless of the assay temperature (Table II).

Arrhenius plots

As previously indicated, for snake isoenzymes the reduction of K_m with temperature can compensate the lower thermal energy when working at low substrate concentrations. This is also evident from Arrhenius plots (Fig. 4). Representation of log v against $1/T$ gave straight lines with comparable slopes for bovine and ophidian enzymes when saturating concentrations of pyruvate were used. If the activity at low concentrations of substrate is plotted, the difference becomes evident. The activation energy was significantly lower for ophidian lactate dehydrogenases.

Values of temperature coefficient (Q_{10}) between 20 and 10 °C at low substrate concentrations are clearly lower for snake isoenzymes (Table III).

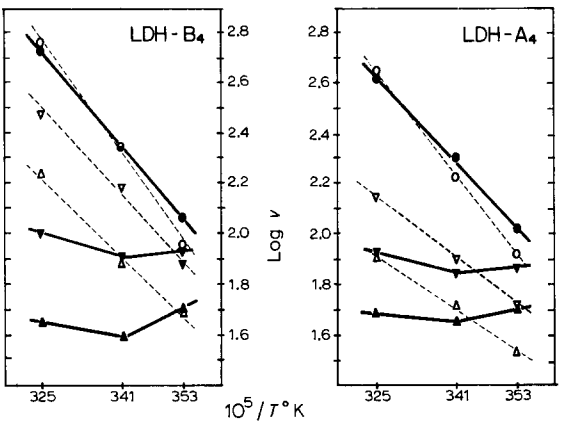

Fig. 4. Arrhenius plots for lactate dehydrogenase isoenzyme B_4 (left) and A_4 (right) from snake (———, black circles and triangles) and beef (— — —, open circles and triangles). ● and ○, activity determined at saturating concentrations of pyruvate; ▲ and △, activity with 0.1 mM pyruvate. Reaction mixture contained 0.113 mM NADH and 0.1 M phosphate buffer, pH 7.4.

TABLE III

TEMPERATURE COEFFICIENT (Q_{10}) BETWEEN 20 AND 10 °C FOR BOVINE AND OPHIDIAN ISOENZYMES LACTATE DEHYDROGENASE A_4 AND B_4

Pyruvate concentration was 0.1 mM for lactate dehydrogenase A_4 and 0.05 mM for lactate dehydrogenase B_4.

pH	*Beef enzyme*		*Snake enzyme*	
	A_4	B_4	A_4	B_4
6.0	1,75	1.26	1.03	0.89
7.4	1.60	1.51	0.90	0.75
8.0	1.42	1.81	0.80	0.50

Effect of pH

It has been demonstrated that reduction of environmental temperature produces an increase in blood and intracellular pH in poikilotherms[13,14]. Our determinations in *B. neuwiedii* agree with those observations. Blood pH increased 0.01 unit for each degree of reduction in temperature.

On the other hand, it is known that pH, as well as temperature, modifies the K_m values for pyruvate of lactate dehydrogenase isoenzymes from different species[15]. It is of interest then, to study the interacting effects of pH and temperature upon catalytic activity.

Values of K_m were determined at pH 6.0, 7.4 and 8.0. Fig. 5 represents the results at different temperatures. At any given temperature, there was an increase of K_m with increasing pH. Ophidian enzymes were much more affected by pH than bovine lactate dehydrogenases. At a constant temperature, the increase of K_m for snake isoenzymes was greater between 7.4 and 8.0 than between 6.0 and 7.4.

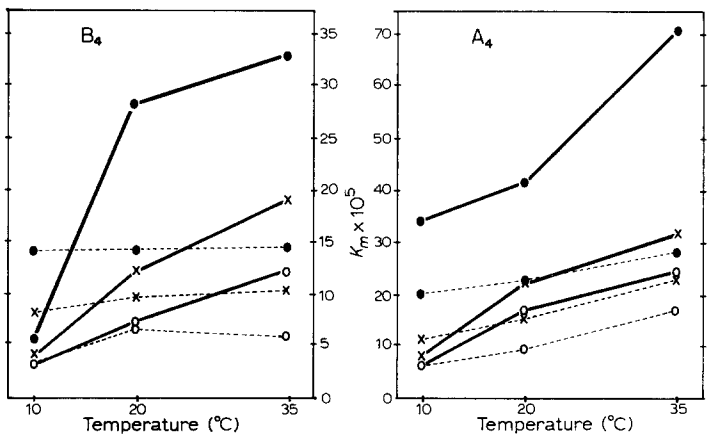

Fig. 5. Effect of pH and temperature upon K_m for pyruvate of lactate dehydrogenase isoenzymes B_4 (left) and A_4 (right) from snake (———) and beef (– – –). ●, pH 8.0; ×, pH 7.4; ○, pH 6.0. Conditions of assay as indicated for Fig. 2 except for pH.

Temperature coefficients at low substrate concentration are presented in Table III. Particularly for snake lactate dehydrogenase B_4, there was a striking reduction of Q_{10} between 20 and 10 °C with increasing pH.

DISCUSSION

Data presented here confirm previous observations pointing out that changes of temperature produce modifications on the enzyme–substrate affinity of lactate dehydrogenase. The response of the enzymes from a homeothermic and a poikilothermic organism was of the same type: a reduction in temperature results in a decrease of K_m values.

However, there was a clear difference between the behavior of bovine and ophidian enzymes. For beef lactate dehydrogenases, the activity rate was closely related to thermal energy, and the modification of K_m with temperature was insufficient to offset that relation. For snake isoenzymes, the activity was independent of temperature when pyruvate concentrations were low.

The same conclusions can be drawn from Arrhenius plots and Q_{10} values. As a result, lactate dehydrogenase isoenzymes from *B. neuwiedii* are able to work with the same efficiency at 10, 20 or 35 °C when pyruvate is at physiological levels (con-

centrations of pyruvate in tissues of other poikilotherms have been reported as ranging from 0.01 to 0.1 mM (refs 5, 16, 17)).

At substrate concentrations below saturating levels, snake lactate dehydrogenase A_4 and B_4 did not exhibit differential properties other than the higher affinity for pyruvate of lactate dehydrogenase B_4 at all temperatures tested. Thermal sensitivity of K_m for both isoenzymes was similar, increasing by a factor of 4 between 10 and 35 °C.

In tissues of the king crab, Somero and Hochachka[5] found two types of lactate dehydrogenase with different K_m values which were distinctly affected by temperature. Hochachka and Lewis[6] observed differences in the relationship K_m–temperature of lactate dehydrogenases from trouts acclimatised at distinct thermal regimes. Whether this phenomenon reflects the preferential synthesis of a particular isoenzyme in different conditions of habitat, is unknown.

We have not found significant differences in the relative distribution of isoenzymes in extracts of tissues from *B. neuwiedii* collected in different areas or at different seasons of the year, or in animals acclimatised for a week at distinct temperatures. From the properties revealed by pure lactate dehydrogenases in this study, we would not expect changes of the relative distribution of isoenzymes in snake tissues as an expression of thermal adaptation.

Hochachka and Somero[1] were the first to recognize the biological importance of the relationship K_m–temperature in poikilotherms, and to emphasize the fact that at physiological concentrations of substrate, the enzyme–substrate affinity is more important than thermodynamic effects to regulate the reaction velocity. The increase of K_m with temperature results in a stabilization of the reaction rate. Thus, decreasing temperature could be regarded as a positive modulator and a very important factor in the regulation of poikilothermic enzymes.

Another factor to be considered is the effect of the hydrogen ion concentration. On account of the modifications of K_m produced by pH changes and, on the other hand, of the variations in pH of organic fluids related to environmental thermal changes, the question was raised as to whether the pH–temperature interaction could cancel the compensation of enzyme velocity obtained from the relation K_m–temperature. The findings presented here indicate that the expected pH changes within the habitat's temperatures do not modify substantially the behavior of lactate dehydrogenase isoenzymes.

Evidence so far accumulated suggests that enzymes from poikilotherms are adapted to work efficiently at the temperature of the animal's habitat. Acclimatization of trouts to different temperatures induced the appearance of distinct variants of citrate synthase[3] and acetyl cholinesterase[4] which showed a minimum K_m at the temperature of acclimatization. Genetic variants of esterases in fish from regions with different thermal regimes, showed a good correlation between temperature of minimum K_m of the variant enzyme and temperature of the habitat where that variant was prevalent[7].

These examples of biological adaptation in poikilotherms involve the production of enzymes functioning most aptly within a defined and limited range of temperatures. Of course, this would have a selective advantage for organisms living in environments in which thermal variations are mild. As a matter of fact, all species previously studied from this point of view were aquatic organisms.

The habitat of *B. neuwiedii*, a terrestrial animal, comprises a region of South America where large and sudden thermal changes are common. Survival of organisms requires an instantaneous adjustment to temperature variations which frequently amount to 20 °C or more. The ability of snake lactate dehydrogenase isoenzymes to work with equal effectiveness at different temperatures within the range of habitat, indicate that they are suited for an immediate adaptation to thermal changes.

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