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Enzymatic correlates of activity metabolism in anuran amphibians

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BENNETT, ALBERT F. *Enzymatic correlates of activity metabolism in anuran amphibians.* Am. J. Physiol. 226(5): 1149–1151. 1974.— The activities of regulatory glycolytic enzymes (phosphofructokinase and lactate dehydrogenase) were assayed in skeletal muscle of *Rana pipiens* and *Bufo boreas* to determine whether the differential lactate production during maximal activity in these species has an enzymatic basis. The frog is primarily anaerobic during activity and exhausts rapidly; the toad moves slowly, forms little lactate, and remains aerobic. Maximal and half-maximal (apparent K_m) reaction rates for both enzymes were twice as great in the frog as in the toad. When the greater muscle mass of the frog is combined with this differential activity, satisfactory agreement is obtained between the ratios of calculated and observed rates of maximal lactate production: toad capacity is 15–25% of frog. The inhibition and affinity characteristics for each enzyme are identical in both species. It is suggested that differential enzymatic concentration, not alteration in enzymatic composition or structure, is responsible for the interspecific difference.

anaerobiosis; enzymatic inhibition; enzyme-substrate affinity; glycolysis; lactate dehydrogenase; lactic acid; muscle physiology; phosphofructokinase

THERE IS CONSIDERABLE DIVERSITY in the behavioral responses and metabolic sources of energy among amphibians during activity (6, 7, 13). The range of this behavior is exemplified by that of the leopard frog, *Rana pipiens*, and the western toad, *Bufo boreas*. The frog responds to threat or stimulation with rapid escape behavior. Powerful leaps are used to remove the animal from danger rapidly. Stamina is, however, low and exhaustion ensues in 2–5 min (6, 13). Most of the energy utilized during this activity is derived from anaerobic sources: aerobic scope is low and lactic acid production is very great (6, 13). The toad, in contrast, seems incapable of more activity than a moderate walk. It cannot be induced to move rapidly, but it sustains low levels of activity almost indefinitely (6, 13). Predation is avoided by the adoption of an inedible posture or reliance on the possession of poisonous skin secretions rather than by escape behavior (S. Arnold, personal communication). *Bufo* forms very little lactate during activity, has a high aerobic scope, and derives most of its energy aerobically (6, 13). Surveying lactate production and activity patterns in amphibians, Bennett and Licht (6) concluded that rapid activity in this group is achieved only at the cost of extensive anaerobiosis.

An unresolved question concerning this differential activity is whether it has a physiological or behavioral basis. Does, for instance, the toad have capacities for activity and for anaerobiosis which are not revealed in laboratory experiments or is it physiologically incapable of rapid activity? These experiments were performed to examine the activities of the regulatory enzymes associated with lactate formation, phosphofructokinase (PFK), and lactate dehydrogenase (LDH). The former is the rate-regulating enzyme of glycolysis (1, 8) and thus exerts control over the rate at which lactic acid can be formed. In lizards, PFK activity is correlated directly with anaerobic scope and capacity (2–4). Lactate dehydrogenase may exert another type of regulation through inhibition (14). Under certain conditions, LDH is inhibited by high concentrations of pyruvate, limiting the total amount of lactate formed (12, 14). The activities of these enzymes in unpurified homogenates of skeletal muscle were measured at 20°C, a temperature well within the activity range of both animals.

METHODS

Five adult specimens each of *Rana pipiens* (mean weight: 67.0 g) and *Bufo boreas halophilus* (66.8 g) were utilized in these experiments. The animals were maintained at 20–25°C for over 1 wk and were fasted at least 3 days before experimentation. All assays were run in June when the animals are normally active.

Animals were killed rapidly by decapitation. All skeletal muscle on the hindlimbs was immediately weighed and iced. All subsequent manipulations of the tissue except the final activity determination were carried out on ice or at 4°C. The tissue was ground in 0.25 M sucrose in a Sorvall Omnimixer for 10 s to reduce connective tissue elements and was homogenized in a conical glass homogenizer. The samples were centrifuged at 1,000 × *g* for 10 min, and the sediment was resuspended, homogenized, and recentrifuged for 10 min. The sediment was discarded and the soluble fraction was diluted to a final concentration of 20% by weight (4 cm^3 0.25 M sucrose to 1 g tissue).

All assays were run at 20 ± 0.2°C in a thermostatically controlled Beckman DB spectrophotometer set at 340 nm and connected to a Varian strip-chart recorder. Reactions were run in duplicate and mean values were reported as nanomoles of product formed per minute per milligram protein or milligram wet weight of tissue. Protein determinations of homogenates were run by biuret reaction with standards of purified bovine serum albumin. Rates of endogenous metabolism before the addition of substrate were minimal in both species, depleting only 2–3% of the

exogenous NADH. Maximal rates of reaction were used; these developed during the 1st min of LDH analysis but not until 5 min in the PFK assay. The following assays were used; concentrations are expressed in total volume of the final reaction mixture.

Lactate dehydrogenase (*E.C. 1.1.1.27*). This activity was determined according to the method of Kornberg (9). The cuvette contained Sørensen phosphate buffer, pH 7.4, 33 mM; NADH, 0.067 mM; sodium pyruvate, 0.01–10 mM; and 0.25 mg wet wt of tissue in homogenate. The reaction was initiated by addition of pyruvate.

Phosphofructokinase (*E.C. 2.7.1.11*). This was assayed with a modification of the method of Prichard and Schofield (11). The cuvette contained imidazole buffer, 21 mM; $MgCl_2$, 5.2 mM; KCl, 209 mM; NADH, 0.050 mM; ATP, 1.0 mM; AMP, 2.1 mM; KCN, pH 7.5, 0.31 mM; α-glycerophosphate dehydrogenase, 2.1 U; glucose 6-phosphate, 0.006–33.3 mM; and 20 mg wet wt of tissue in homogenate. The reaction was started by addition of glucose 6-phosphate.

RESULTS

Lactate dehydrogenase. Representative LDH activity of frog and toad skeletal muscle as a function of substrate concentration is shown in Fig. 1. Mean values of maximal velocity (V_{max}) and substrate concentrations at V_{max} and half-maximal velocity (apparent K_m) are given in Table 1. The activity at maximal and half-maximal reaction rates is twice as great in *Rana* as in *Bufo*; the frog muscle homogenate produced twice as much lactate per unit time as that of the toad. Except for this difference in absolute activity,

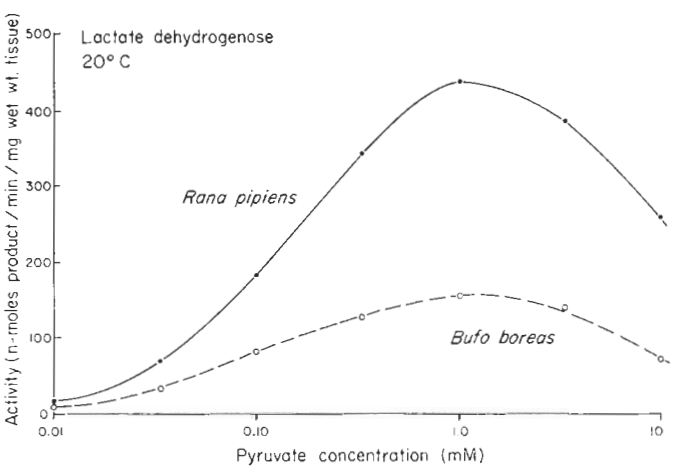

FIG. 1. Activity of lactate dehydrogenase from frog and toad skeletal muscle.

TABLE 1. *Activity and substrate affinity of lactate dehydrogenase from frog and toad skeletal muscle*

	Rana	*Bufo*
Maximal velocity		
nmol/mg wet wt per min	425 ± 63	188 ± 69
nmol/mg protein per min	5,640 ± 1,110	2,910 ± 850
Pyruvate concn at V_{max}, mM	1.0	1.0
Apparent K_m, mM	0.105 ± .020	0.101 ± .023

Mean values and 95% confidence limits are reported (n = 5 for both species).

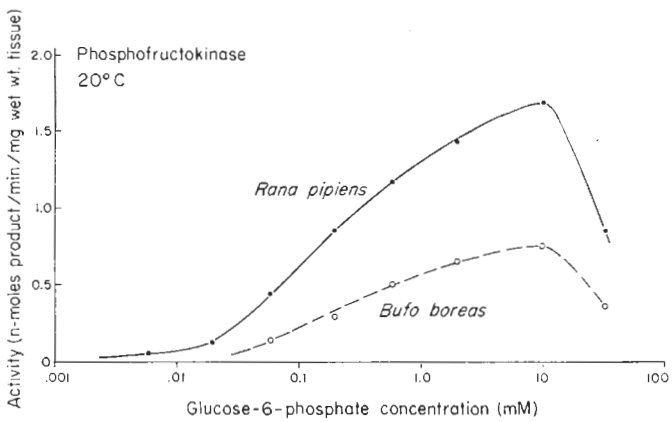

FIG. 2. Activity of phosphofructokinase from frog and toad skeletal muscle.

TABLE 2. *Activity and substrate affinity of phosphofructokinase from frog and toad skeletal muscle*

	Rana	*Bufo*
Maximal velocity		
nmol/mg wet wt per min	1.81 ± 0.51	0.69 ± 0.24
nmol/mg protein per min	24.9 ± 10.9	12.6 ± 9.51
Glucose 6-phosphate concn at V_{max}, mM	10	10
Apparent K_m, mM	0.234 ± .038	0.338 ± .232

Mean values and 95% confidence limits are reported (n = 5 for both species).

the form of the curves for the two species is very similar. Reaction velocity was maximal at 1.0 mM pyruvate in all specimens of both *Rana* and *Bufo*; higher substrate concentrations inhibited the reaction rate. This value is identical to that reported by Salthe (12) for crude extract and purified LDH from *R. pipiens* muscle at 25°C. The apparent K_m values for the two species were determined graphically and are not significantly different ($P > 0.8$).

Phosphofructokinase. Representative curves and a summary of PFK activity functions are given in Fig. 2 and Table 2. The maximal and half-maximal activity rates are again twice as great in the frog as in the toad. The forms of the activity substrate curves are also similar. Maximal velocity occurred at 10 mM glucose 6-phosphate in all specimens of both species; apparent K_m values are not significantly different ($P = 0.69$).

Weight of tissue. The total hindlimb musculature weighed an average of 16.3 g per animal in *Rana* and represented 24% of the total body weight. The hindlimb muscles of *Bufo* weighed an average of 4.7 g/animal or 7% of the total body weight.

DISCUSSION

The greater anaerobic capacities of the frog are clearly reflected in the regulatory enzymes of glycolysis: *Rana* muscle is able to catabolize twice as much substrate anaerobically at all substrate concentrations as is *Bufo* muscle. In addition, a greater proportion of the frog's body consists of limb musculature, so that a greater total amount of lactate and ATP can be produced during activity. It is instructive to compare these ratios with the actual anaerobic

scopes (maximal rates of lactate production) of which these animals are capable during activity. We must neglect for the moment that *Bufo* also uses its forelimbs during activity and that not all hindlimb muscles function equally during forward movement. We can then calculate that the activity of glycolytic enzymes per gram of muscle in the toad is one-half that in the frog and that the hindlimbs have only 30% of the muscle of the frog's. These values predict that *Bufo* is capable of an anaerobic energy production of only 15% that of *Rana*. The actual anaerobic scopes at 20°C are 0.24 and 1.01 mg lactate formed per gram of body weight per minute for *Bufo* and *Rana*, respectively (6). *Bufo* can form lactate only 23% as rapidly as *Rana*. In view of the assumptions made, the predicted and actual ratios are surprisingly similar. It must be concluded that the differences in activity patterns of *Rana* and *Bufo* are not purely behavioral but are a reflection of the physiological differences within the muscles of these animals.

In view of the more extensive utilization of and dependence on anaerobic metabolism by *Rana*, it is somewhat surprising that the substrate affinity and inhibition points of its enzymes are identical to those of *Bufo*. A priori, it might have been expected that the frog enzymes would remain unaffected by concentrations of substrate which inhibit those of the toad, since the former animal forms and tolerates much greater levels of lactic acid. Likewise, a different affinity of the enzymes for their substrates might have been expected, perhaps an increment in the sigmoid character of the activity-substrate curve, giving maximal activities over a broader range of substrate concentrations. In fact, the catalytic properties of the enzyme molecules have not been altered during the evolutionary development of these species. It is possible that the observed differences in enzymatic activity are the result of differential concentrations of similar enzyme molecules, e.g., *Rana* might have a greater amount of LDH per gram of muscle than does *Bufo* rather than a greater turnover number for each enzyme molecule. Such a conclusion would be of interest in regard to the considerable variability observed among the anaerobic scopes of amphibians, including those of closely related species (6). The differential demands for activity in this group may be met solely by differential enzymatic concentrations and not alterations of enzymatic composition or structure.

The selective value of the inhibition of enzymatic activity by high substrate concentration in a major metabolic pathway is unclear. Inhibition of LDH by high pyruvate levels has been reported previously in both fish (14) and amphibians (12). Such inhibition is inversely temperature dependent: the substrate concentrations at which the enzyme is inhibited increase with increasing temperature. This thermal dependence has been interpreted as a mechanism for shunting substrates through aerobic pathways at low temperatures and through anaerobic pathways at high temperatures (12, 14). No explanation has been given, however, for the advantage of inhibition per se. It is possible that inhibition is a self-regulating mechanism that maintains lactic acid production at manageable levels. The latter is a physiologically noxious product, disrupting buffering systems, blood pH, and oxygen transport by hemoglobin (3) and limiting further activity on the part of an organism (7). The rate of lactate elimination is directly temperature dependent (5, 10); thus, lactate formed at low body temperatures will take a longer time to be removed and will place severe physiological constraints upon activity. However, most lower vertebrates utilize anaerobic metabolism extensively during maximal activity and derive most of their ATP production from this pathway (4–7). Inhibition of LDH closes a pathway of significant energetic importance. A failure to mobilize the requisite energy in a life-or-death escape attempt may be fatal. Maintenance of physiological homeostasis is beside the point in such a situation; survival is the only factor of importance. These arguments of regulation versus maximal energetic mobilization must be deferred, however, in the absence of measurements of intracellular pyruvate: inhibitory concentrations may never in fact be reached even during activity. The apparent inhibition may be a pharmacological property of in vitro analysis and only a reflection of temperature-dependent enzyme substrate affinity.

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