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FROG LACTATE DEHYDROGENASE: KINETICS AT PHYSIOLOGICAL ENZYME LEVELS

DENISE EBY*,**, STANLEY SALTHE AND AARON LUKTON

St. Joseph College, Department of Chemistry, Emmitsburg, Md. 21727 (U.S.A.) and Brooklyn College, CUNY, Brooklyn, N.Y. 11210 (U.S.A.)

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SUMMARY

Pyruvate inhibition of frog muscle and heart lactate dehydrogenases (L-lactate: NAD^+ oxidoreductase, EC 1.1.1.27) has been determined at physiological enzyme concentrations. This inhibition is more pronounced with heart lactate dehydrogenase than it is with the skeletal muscle enzyme when the reduced coenzyme (NADH) concentration is near the *in vivo* level. Inhibition differences are independent of temperature, except in the case of muscle lactate dehydrogenase which shows a slight increase in sensitivity to pyruvate when the temperature is lowered to 10 °C.

INTRODUCTION

A difference in substrate inhibition has been suggested as the basis for distinct physiological roles for lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27) isoenzymes in most vertebrates[1,2]. Excess pyruvate inhibits heart lactate dehydrogenase in the reduction of the substrate pyruvate to lactate; whereas muscle lactate dehydrogenase is considerably less inhibited under the same conditions. Inhibition of the heart enzyme channels pyruvate into the Krebs cycle in this highly aerobic tissue; while in anaerobic muscle the muscle lactate dehydrogenase isoenzyme effects swift conversion of pyruvate to lactate with regeneration of the needed oxidized coenzyme, NAD^+.

The mechanism proposed for pyruvate inhibition of lactate dehydrogenase is the formation of an abortive ternary complex, *viz.* lactate dehydrogenase–NAD^+–pyruvate, resulting from the interaction of NAD^+ with pyruvate and the enzyme[3,4]. The oxidation of NADH to NAD^+ during the lactate dehydrogenase-catalyzed reduction of pyruvate to lactate results in the ternary complex formation in a time-dependent reaction. This complex competetively inhibits the reduction of pyruvate by NADH.

* To whom correspondence should be addressed.
** Present address c/o Dr M. Keeney Department of Chemistry, University of Maryland, College Park, Md. 20742 (U.S.A.).

The dissociation constant for the heart lactate dehydrogenase ternary complex is much lower than that for muscle lactate dehydrogenase, with the result that heart lactate dehydrogenase is considerably inhibited at physiological conditions while the muscle form has significant activity. This view is supported by Everse *et al.*[5,6] who show physiological levels of the pure lactate dehydrogenase isoenzyme to be substrate inhibited when the enzyme and pyruvate are pre-incubated with NAD^+, the inhibition being more pronounced with heart lactate dehydrogenase than it is with muscle lactate dehydrogenase.

A report of Wuntch *et al.*[7] claims evidence against the theory that substrate inhibition differences account for the functional differences in the lactate dehydrogenase isoenzymes. Using physiological concentrations of mammalian lactate dehydrogenases they found no pyruvate inhibition and suggest that this occurs *in vitro* only after appreciable dilution of the enzyme as is required for conventional enzyme assays.

The physiological significance of difference in sensitivity to pyruvate by lactate dehydrogenase isoenzymes has also been questioned by those who find that the differences, as observed at 25 °C, are minimized at 37–40 °C, the physiological range for mammals and birds[8,9].

The purpose of the work reported in this paper was to study substrate inhibition of skeletal muscle and heart lactate dehydrogenases isolated from the frog, *Rana pipiens*, at enzyme and substrate levels approaching *in vivo*; and at temperatures near the environmental range for the species. The results were compared with similar studies of other vertebrate species.

MATERIALS AND METHODS

Sodium pyruvate and NADH were obtained from Sigma Chemical Co. (St. Louis, Mo.). Heart and muscle lactate dehydrogenase were isolated from *Rana pipiens* tissues purchased, frozen, from Pel-freez Biologicals Inc. (Rogers, Ark.), by the method of Pesce *et al.*[10], with some modification. The activities of the purified enzymes were: 1500 enzyme units/mg protein for muscle lactate dehydrogenase and 2000 units for heart lactate dehydrogenase, where one enzyme unit is the mg of enzyme giving an initial rate of 1.00 $A_{340\ nm}$/min per ml at 25 °C. Standard assays were in 0.1 M phosphate buffer, pH 7.2, with a final concentration of 1.0 mM pyruvate and 1.0 mM NADH. The protein concentration of lactate dehydrogenase was determined using an absorbance of 1.45 for 1.0 mg of lactate dehydrogenase per ml at 280 nm[4].

Tissue distribution of the purified lactate dehydrogenases was analyzed by polyacrylamide gel electrophoresis according to the standard procedure published by Canalco Industrial Co. Gels were stained for protein with 2% Coomassie blue, and for enzyme activity with Gelman Instrument Company's lactate dehydrogenase working stain. Both muscle and heart lactate dehydrogenase migrated as predominantly single bands. Bands visualized by protein staining coincided with those having enzyme activity.

For pyruvate inhibition studies and for measuring rates at high (physiological) enzyme concentrations an Aminco–Morrow stopped-flow system with a thermoregulated cell block was employed. The system was attached to a Gilford spectrophotometer. All reagents were in 0.1 M phosphate buffer, pH 7.2. Final NADH concentrations were 0.15 mM or, alternatively, 0.56 mM. NADH was stored desiccated in the

dark at room temperature prior to use, and both NADH and pyruvate solutions were prepared fresh each day to reduce the possibility of inhibitor formation. Enzyme and pyruvate were introduced from one syringe of the stopped-flow system into the cuvette; NADH was introduced from the second syringe. Reaction rates were measured as decrease in absorbance at 340 nm and the reactions were recorded on a Tektronix 564-B storage oscilloscope and photographed. The slope of the initial linear reaction was taken as the reaction rate. Enzyme concentrations ranged from $3.0 \cdot 10^{-8}$ M to $3.0 \cdot 10^{-6}$ M. Measurements were made at 10, 25 and 40 °C.

The K_m (pyruvate) values were obtained at the three temperatures used for the inhibition studies, and at high lactate dehydrogenase concentration, *viz.* $3.0 \cdot 10^{-6}$ M. These values were determined from Lineweaver–Burk plots of the experimental data.

RESULTS AND DISCUSSION

In the pyruvate inhibition experiments maximum rates, expressed as $\Delta A_{340\ nm}$/min, ranged from 9.0 for $3.0 \cdot 10^{-8}$ M lactate dehydrogenase at 10 °C to $1.1 \cdot 10^{3}$ for $3.0 \cdot 10^{-6}$ M lactate dehydrogenase at 40 °C. Data from the experiments at 25 °C are presented in Table I. At all concentrations of heart lactate dehydrogenase maximum activities were reached around 0.33 mM pyruvate; whereas muscle lactate dehydrogenase attained maximum activities about 1.0 mM pyruvate (Table I). Since *in vivo* pyruvate concentrations are usually lower than 1.0 mM[7,11,12] these data suggest that, at least *in vitro*, frog heart lactate dehydrogenase is much more sensitive to pyruvate inhibition than is muscle lactate dehydrogenase at physiological pyruvate levels.

The experimental data (Table I) for both heart and muscle lactate dehydrogenase show that the degree of pyruvate inhibition increases as the enzyme concentration is diluted below probable physiological levels. When 0.15 mM NADH is employed in the assay a marked difference in pyruvate inhibition of the heart and muscle lactate dehydrogenases is noted even at high lactate dehydrogenase concentration (Fig. 1). Assuming the tissue concentrations of lactate dehydrogenase in frog to be similar to the approximations reported for several mammalian tissues[7,13], $3.0 \cdot 10^{-6}$ M lactate dehydrogenase can be considered physiological.

At 40 °C the reaction rates for both muscle and heart lactate dehydrogenase

TABLE I

EFFECT OF PYRUVATE CONCENTRATION ON THE ACTIVITY OF *R. pipiens* LACTATE DEHYDROGENASE AT 25 °C AND 0.15 mM NADH

H-LDH, heart lactate dehydrogenase; M-LDH, muscle lactate dehydrogenase.

Pyruvate concn (mM)	*Activity ($\Delta A_{340\ nm} \cdot 10^2$); concn of LDH*					
	$3.0 \cdot 10^{-8}$ M		*$3.0 \cdot 10^{-7}$ M*		*$3.0 \cdot 10^{-6}$ M*	
	H-LDH	*M-LDH*	*H-LDH*	*M-LDH*	*H-LDH*	*M-LDH*
0.10	1.99		4.68		7.79	
0.33	2.20	1.16	5.03	2.79	8.20	5.52
1.00	1.65	1.26	3.92	3.10	6.56	6.00
3.33	1.17	0.91	3.07	2.57	5.74	5.76
10.00	0.59	0.78	2.16	2.42	5.41	5.64
20.00	0.26	0.63	1.81	2.23	4.51	5.64

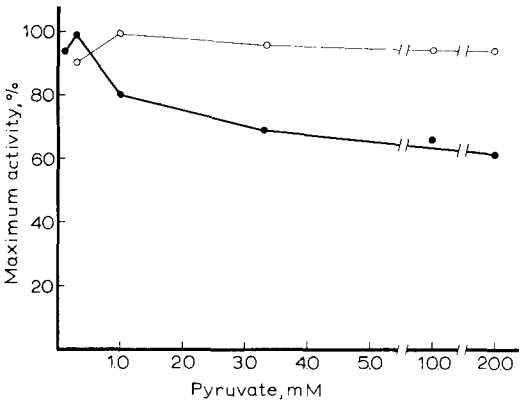

Fig. 1. Pyruvate inhibition of $3.0 \cdot 10^{-6}$ M muscle lactate dehydrogenase (○—○) and $3.0 \cdot 10^{-6}$ M heart lactate dehydrogenase (●—●) from *R. pipiens*, at 25 °C and 0.15 mM NADH. The % maximum activities correspond to the values in Table I.

were greatly increased but the pyruvate inhibition effects were not notably different from those at 25 °C. At 10 °C, however, inhibition of muscle lactate dehydrogenase by pyruvate was seen at somewhat lower pyruvate concentrations (Fig. 2). This is probably due to the fact that the K_m (pyruvate) for frog muscle lactate dehydrogenase at 10 °C is lower than the K_m values at 25 and 40 °C (Table II). An effect similar to this, though much more marked, was observed by Somero[14] in his studies of muscle lactate dehydrogenase from the skeletal muscle of the fish, *Gillichthys mirabilis*. He found that the K_m values (pyruvate) for this isoenzyme became lower as the assay temperature was lowered below 25 °C. The lowered K_m values were accompanied by an increase in the extent of pyruvate inhibition. He interpreted this effect as the poikilotherm's adaptation to conditions where oxygen is relatively plentiful and the activity of the organism is low. Under these conditions, according to Somero, the muscle lactate dehydrogenase of *G. mirabilis* becomes more "heart-like", and muscle metabolism is shifted toward a more aerobic pattern. To make a similar interpretation for the slightly

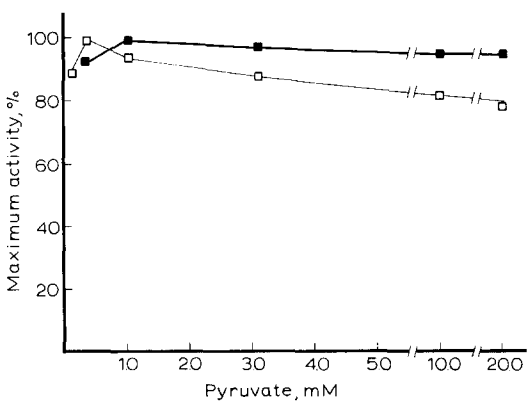

Fig. 2. Pyruvate inhibition of $3.0 \cdot 10^{-6}$ M muscle lactate dehydrogenase from *R. pipiens* at 10 °C (□—□) and at 25 °C (■—■).

TABLE II

APPARENT K_m VALUES FOR *R. pipiens* LACTATE DEHYDROGENASE AT SEVERAL ASSAY TEMPERATURES
H-LDH, heart lactate dehydrogenase; M-LDH, muscle lactate dehydrogenase.

Assay temperature (°C)	*Apparent K_m (mM pyruvate)*	
	H-LDH	*M-LDH*
40	0.13	0.33
25	0.15	0.31
10	0.12	0.24

increased pyruvate inhibition of muscle lactate dehydrogenase from *R. pipiens* would require further and more exhaustive studies at several additional temperatures using frogs acclimated to these temperatures prior to the isolation of heart and muscle lactate dehydrogenase. However, the results reported here suggest the possibility that *R. pipiens*, a poikilotherm, may shift skeletal muscle metabolism toward a more aerobic pattern at low temperatures when activity begins to fall off.

The data obtained from pyruvate inhibition experiments in which the NADH concentration was 0.56 mM (Table III), show that there is considerably less inhibition of heart lactate dehydrogenase at physiological conditions than was noted in the experiments using 0.15 mM NADH. These results are in agreement with those of Wuntch *et al.*[7] who concluded that, at physiological enzyme levels little or no pyruvate inhibition of either isoenzyme occurs *in vitro*. Their conclusions, however, were based on experiments employing 0.56 mM NADH, a concentration much higher than that estimated to exist in the cytoplasm of cells. To be physiologically meaningful experiments should employ *in vivo* levels of the coenzyme. The concentration of NAD^+ in cells is about 1.0 mM (ref. 3). According to Kaplan's[15] analysis of pyridine nucleotide distribution in cell fractions, the concentration of NAD^+ in the soluble cell fraction is about ten times higher than that of NADH; which makes the NADH level around 0.1–0.2 mM. Our data (Table III) show that an increase in NADH from 0.15 mM to 0.56 mM results in minimized inhibition of $3.0 \cdot 10^{-6}$ M heart lactate dehydrogenase. The inhibition by 1.0 mM pyruvate is 20% at 0.15 mM NADH, and 4% when NADH is increased to 0.56 mM. The difference in inhibition becomes greater at higher concentrations of pyruvate. A similar effect was seen by Kaplan *et al.*[4] for chicken heart lactate dehydrogenase. Such an effect is to be expected if NADH is in competi-

TABLE III

THE EFFECT OF NADH CONCENTRATION ON PYRUVATE INHIBITION OF $3.0 \cdot 10^{-6}$ M HEART LACTATE DEHYDROGENASE FROM *R. pipiens*

Pyruvate concn (mM)	*% maximum activity*	
	0.15 mM NADH	*0.56 mM NADH*
0.10	95	97
0.33	100	100
1.00	80	96
3.33	70	90
10.00	66	85
20.00	55	83

tion with the lactate dehydrogenase–NAD^+–pyruvate abortive ternary complex in the reduction of pyruvate to lactate.

Although kinetic differences as seen *in vitro* cannot be applied directly to indicate the real *in vivo* situation, the fact that significant inhibition differences are found for heart and muscle lactate dehydrogenase using physiological levels of enzyme, pyruvate and NADH, is in agreement with the theory that differences in sensitivity to pyruvate is a basis for distinct physiological roles for the two isoenzymes. The results of the experiments with *R. pipiens* lactate dehydrogenases closely parallel those seen in many other vertebrate species.

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