Effects of Proteolytic and Thermal Action on the Activity of Lactate Dehydrogenase Isozymes LD-1 and LD-5 (43481)

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Abstract. **L-Lactate dehydrogenase (LD) catalyzes the interconversion of pyruvate and lactate. Using a spectrophotometric assay to determine LD activity, incubation of rabbit, porcine, and bovine LD-1 and LD-5 isozymes with the protease subtilisin (Carlsberg) gave first-order degradation kinetics. Degradation half-lives were significantly lower for the LD-5 isozymes from the three species when incubated with subtilisin at temperatures from 4°C to 25°C. The energy involved in the degradation process, however, was not different. The activation energy for the conversion of pyruvate to lactate by LD-1 at pH 7.4 was significantly higher than that for LD-5 for all three species examined ($P < 0.005$). Thermocalorimetry showed that the LD-1 isozymes have both a higher mean temperature of denaturation and a higher heat uptake during the denaturation process than corresponding LD-5 forms. The results suggest that the LD-5 isozymes in the species studied are more metabolically efficient, whereas the LD-1 forms have greater structural stability.**
[P.S.E.B.M. 1992, Vol 201]

L-lactate dehydrogenase (LD) (E.C. 1.1.1.27) catalyzes the conversion of pyruvate to L-lactate to provide one means of regenerating the NAD^+ needed for continuation of glycolysis:

$$\text{Pyruvate} + \text{NADH} + H^+ \leftrightharpoons \text{L-Lactate} + NAD^+$$

The reverse reaction, under strongly aerobic conditions with elevated levels of NAD^+ normally found in hepatic tissues, allows the lactate formed in skeletal muscle to be reoxidized for gluconeogenesis. LD is a tetramer composed of two dissimilar subunits, A (or M, muscle) and B (or H, heart). These subunits combine to form five different isozymes, numbered from LD-1 (B_4) through LD-5 (A_4). The isozymes differ in their tissue distribution as well as in their physicochemical properties. The predominance of LD-5 in many types of malignancies is well established and is attributed to the A subunit's increased ability to function under anaerobic conditions (1–4). LD-5 has also been shown to predominate in NB-2 lymphoma mitochondria as well as in mitochondria from nontumorous heart, kidney, and liver (5–10).

LD-1 is more stable than LD-5 at both high and low temperatures (3, 11). The isozymes also respond differently to factors that accelerate or prevent heat inactivation, such as oxaloacetate, malate, and changes in pH or ionic strength (12). LD-1 is inhibited to a greater extent than LD-5 by high pyruvate concentrations (13, 14). LD-5 has an affinity (K_m) for both pyruvate and lactate two to 10 times higher than that of LD-1 (15). All of these differences suggest unique roles for the different isozymes in metabolic function. By further studying the thermodynamic stability of the isozymes, their catalytic efficiency, and their ability to withstand degradation, it may be possible to better understand their *in vivo* function. Activation energies, mean temperatures of denaturation, and the heat uptake during degradation were, therefore, determined for bovine, porcine, and rabbit LD-1 and LD-5, along with degradation half-lives and degradation energies obtained by incubating the isozymes with the protease subtilisin.

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Received January 7, 1992. [P.S.E.B.M. 1992, Vol 201]
Accepted April 24, 1992.

0037-9727/92/2011-0066$3.00/0

Materials and Methods

Enzyme Preparations. The following LD isozymes, in an insoluble suspension of $(NH_4)_2SO_4$, were obtained from Sigma Chemical Co. (St. Louis, MO): rabbit heart LD-1, No. L-2889, Type XXXVI; rabbit muscle LD-5, No. L-5123, Type V-S; porcine heart LD-1, No. L-9382, Type VII-S; porcine muscle LD-5, No. L-5762, Type XXXII-S; bovine heart LD-1, No. L-0377, Type IX; and bovine muscle LD-5, No. L-0508, Type XXVI. For analysis, a 50-μl aliquot of the LD enzyme suspension was centrifuged at 2310*g* for 10 min at 5–10°C in a Sorvall RC-5B refrigerated centrifuge. The supernatant fluid was discarded and the enzyme pellet was dissolved in 100 μl of 0.1 *M* potassium phosphate buffer (pH 7.4) containing 0.1% bovine serum albumin (BSA) as a stabilizer. An approximate 1/500 dilution of the resuspended LD isozyme solution was prepared in phosphate-BSA buffer and used to establish initial reaction rates and for protease incubation. To achieve approximately equal baseline activity for all isozymes, the dilution factor was slightly modified in some cases. Enzyme solutions were kept on ice before use.

The presence of contamination with other isozymes in each of the LD enzyme suspensions was assessed by nondenaturing electrophoresis using a Corning cassette electrophoresis cell with Corning universal agarose gel films (No. 470100). A 2-μl aliquot of each isozyme in 0.1 *M* phosphate buffer (pH 7.4) was applied to the gels. Gels were developed in a Corning barbital buffer (pH 8.6) for 40 min at a constant potential of 90 V. Corning colorimetric LD isozyme substrate kit (No. 470214), an activity stain specific for LD, was used in detection.

Enzyme Assay. Assays were performed on a Gilford 252 modified Beckman DU spectrophotometer equipped with an automatic cuvette positioner and Gilford strip chart recorder. The cuvette chamber was maintained at a constant temperature using a Lauda K-2/R circulating water bath. LD activity was measured using a method similar to that of Kornberg (16). A 40-μl aliquot of the enzyme preparation to be assayed was pipetted into a 1-cm^2 cuvette containing 2.96 ml of a solution of 0.13 m*M* NADH (No. N-6005; Sigma) and 0.32 m*M* pyruvate (No. P-2256; Sigma) in 0.1 *M* phosphate buffer (pH 7.4). Enzyme activity was determined by measuring the decrease in absorbance of NADH at 340 nm as the coenzyme was oxidized to NAD^+ concomitant with the production of lactate. Duplicate samples of each enzyme solution were analyzed at each time period.

Activation Energy Determination. Activation energies were calculated using the Arrhenius equation:

$$\log k = \text{constant} - Ea(2.303\ RT)^{-1}$$

where Ea is the apparent activation energy, k is the reaction velocity, R is the gas constant (1.987 cal mol^{-1} °K^{-1}), and T is the reaction temperature in °K. The activation energy was determined by varying the temperature of the cuvette chamber between 15°C and 30°C. The rate of oxidation of NADH (μmol/sec) in the LD reaction was used as the reaction velocity. An equilibration period of 20 min was allowed after each temperature change before measuring reaction rates. The logarithm of k was plotted against the reciprocal of the reaction temperature in °K (Arrhenius plot). The slope of the resulting line multiplied by $2.303R$ gave the apparent activation energy for the reaction.

Protease Incubation. Previous reports from this laboratory have shown that the protease Type VIII:bacterial (subtilisin Carlsberg) (P-5380; Sigma) was effective in catalyzing the hydrolysis of the LD isozymes from rat tissue at different rates for LD-1 and LD-5 (12). Before use of each new subtilisin preparation, the activity was confirmed by an assay for protein degradation using Folin-Ciocalteu reagent (17). A 100-mg/ml solution of the protease was prepared in phosphate buffer (pH 7.4). For LD isozyme incubation, a volume of the subtilisin solution was added to the LD preparation to give a protease concentration of approximately 10 mg/ml. The concentration was modified slightly in some cases depending upon the determined activity of the protease to maintain equal proteolytic activity in all of the incubation mixtures. To determine the effects of temperature on the rate of isozyme degradation, the protease incubation was performed at different temperatures with the LD assay temperature kept constant at 25°C. Degradation energies were calculated using a modified version of the Arrhenius equation by plotting the logarithm of the degradation rate constant ($0.693 \times [\text{degradation } t_{1/2}]^{-1}$) for a particular isozyme at a given temperature in place of the reaction velocity, as described previously.

Thermocalorimetry. Thermochemical analysis of a 1-mg/ml sample of each isozyme (in 0.1 *M* phosphate buffer [pH 7.4]) was performed with a Microcal MC-2 scanning calorimeter. Temperature scans from 20°C to 100°C were measured at a rate of 60°C/hr. Samples were degassed under vacuum for 20 min before analysis.

Statistical Analysis. Grouped data were compared using a two-tailed Student's t test. Differences were considered to be significant at $P < 0.05$.

Results

Control Studies. To determine whether BSA in the phosphate buffer used to redissolve the enzyme pellet affected the rate of proteolytic degradation, rabbit LD-5 was subjected to proteolysis with and without BSA stabilization. No differences in degradation half-lives were found. No change in the absorbance of the reaction mixture was observed in the absence of either NADH or pyruvate, or in reaction mixtures containing

Table I. Reaction Velocities for LD Isozymes LD-1 and LD-5 at Five Reaction Temperatures

Species	Reaction velocity (10^{-10} moles NADH/sec)[a]									
	LD-1					LD-5				
	15°C	18°C	22°C	25°C	30°C	15°C	18°C	22°C	25°C	30°C
Rabbit	2.6 ± 0.5	3.1 ± 0.4	3.9 ± 0.5	4.7 ± 0.5	5.2 ± 0.6	5.7 ± 0.1	6.4 ± 0.3	7.2 ± 0.3	7.7 ± 0.3	8.8 ± 0.5
Porcine	4.4 ± 0.9	5.1 ± 0.9	5.0 ± 0.8	5.7 ± 0.8	6.1 ± 0.7	5.3 ± 1.3	7.1 ± 0.9	7.9 ± 1.4	9.6 ± 1.5	11.6 ± 1.5
Bovine	3.0 ± 0.4	3.5 ± 0.4	4.0 ± 0.6	4.1 ± 0.8	4.9 ± 0.8	4.3 ± 0.5	5.3 ± 0.4	6.4 ± 1.0	8.2 ± 1.3	8.2 ± 1.1

[a] Reaction velocity is 10^{-10} moles of NADH oxidized per second in the LD reaction (pH 7.4) at temperature indicated ± 1°C. Values represent mean ± SD for five analyses.

both NADH and pyruvate to which subtilisin was added in the absence of LD. Electrophoresis of the enzyme preparations showed single bands for all isozymes except bovine and porcine LD-1, in which slight contamination with the LD-2 forms was present (approximately 5%).

Activation Energies for LD Isozymes. Reaction velocities at five different temperatures are given in Table I. Arrhenius plots, showing the rate of change in reaction velocity with temperature, were linear in the temperature range examined (15–30°C), with correlation coefficients of 0.93 ($P < 0.05$) or greater. A representative Arrhenius plot for rabbit LD-1 and LD-5 is shown in Figure 1. Apparent activation energies for the isozymes studied are given in Table II. For each species, the apparent activation energy for the LD-1 isozyme was significantly higher than for the LD-5 form ($P < 0.001$ for rabbit, $P < 0.001$ for porcine, and $P < 0.01$ for bovine) ($n = 5$ for each species). The mean (±SD) combined apparent activation energy for the LD-1 isozymes in the three species studied, 8.6 ± 1.5 kcal/mole ($n = 15$), was significantly higher than the mean of 4.9 ± 0.4 kcal/mole ($n = 15$) for the LD-5 isozymes ($P < 0.005$).

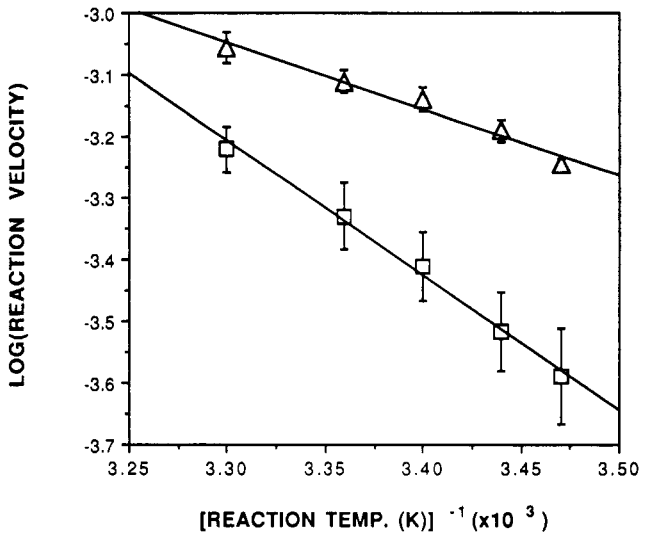

Figure 1. Logarithm of reaction velocity versus the reciprocal of the absolute reaction temperature (Arrhenius plots). Rabbit LD-1 (□) and LD-5 (△) data are shown. Reaction velocity is rate of oxidation of NADH at 340 nm in the reaction at the temperature indicated. Each data point represents mean ± SD for five duplicate analyses at pH 7.4.

Table II. Activation Energies for Conversion of Pyruvate to Lactate by Lactate Dehydrogenase Isozymes LD-1 and LD-5

Species	Activation energy (kcal/mole)[a]		
	LD-1	LD-5	P^b
Rabbit	10.0 ± 0.9	5.0 ± 0.3	<0.001
Porcine	8.8 ± 1.1	4.4 ± 0.5	<0.001
Bovine	6.9 ± 0.6	5.2 ± 0.9	<0.01

[a] Activation energy = (slope of plot of log [reaction velocity] versus reaction temperature $(K)^{-1}$) × −2.303 × R. Values given as mean ± SD for five duplicate analyses at pH 7.4.
[b] Significance of difference between LD-1 and LD-5 isozymes for given species.

Proteolytic Inactivation of the LD Isozymes and Degradation Energies. Table III lists degradation half-lives (0.693/degradation rate constant at a given temperature) for LD-1 and LD-5 isozymes at varying protease incubation temperatures. The LD assay temperature was held constant at 25°C. Plots showing the loss of activity over time for rabbit LD-1 and LD-5 are shown in Figure 2, A and B, respectively. Plots for porcine and bovine LD-1 and LD-5 were similar. Degradation followed first-order kinetics in all instances. Plots of the logarithm of percentage of initial activity versus incubation period all had correlation coefficients greater than 0.95 ($P < 0.05$) (rabbit data are shown in Figure 3, A and B). Degradation energies are shown in Table IV. A representative plot of the logarithm of the degradation rate constant versus the reciprocal of the protease incubation temperature (°K), from which degradation energies were calculated, is shown for porcine LD-1 and LD-5 in Figure 4. The difference between the degradation energies for the LD-1 and LD-5 isozymes was significant only for rabbit ($P < 0.05$). The mean combined degradation energy for the LD-1 forms from all species was 12.5 ± 2.7 kcal/mole ($n = 9$), whereas that for the LD-5 forms was 10.2 ± 3.1 kcal/

Table III. Degradation Half-Lives for Lactate Dehydrogenase Isozymes LD-1 and LD-5 at Four Protease Incubation Temperatures

Species	Degradation half-lives (min)[a]							
	LD-1				LD-5			
	4°C	10°C	17°C	25°C	4°C	10°C	17°C	25°C
Rabbit	24.8 ± 2.1	13.5 ± 1.6	9.2 ± 1.6	5.6 ± 0.7	9.4 ± 2.2	6.1 ± 0.8	4.3 ± 0.7	3.7 ± 0.2
Porcine	22.8 ± 1.7	12.2 ± 1.3	7.5 ± 0.6	4.2 ± 0.3	15.3 ± 1.2	8.7 ± 0.3	6.0 ± 0.1	3.2 ± 0.6
Bovine	38.4 ± 0.3	20.6 ± 3.2	12.6 ± 2.0	7.8 ± 1.6	13.3 ± 0.8	7.5 ± 0.8	4.9 ± 0.4	3.3 ± 0.8

[a] Degradation with approximately 10 mg/ml of subtilisin (Carlsberg) with incubation at temperature indicated ±1°C. Values given as mean ± SD for three duplicate analyses.

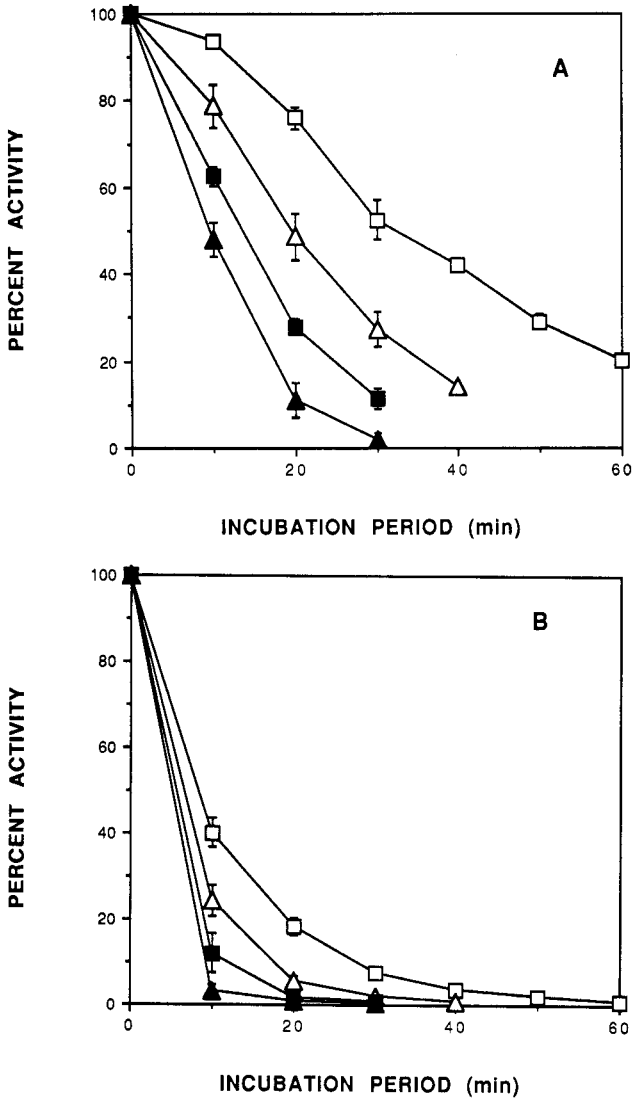

Figure 2. Change in percentage of activity of (A) rabbit LD-1 and (B) rabbit LD-5 with time following incubation with subtilisin at 4°C (□), 10°C (△), 18°C (■), and 25°C (▲). Initial activity was designated 100%. Data points represent mean ± SD of three duplicate analyses.

mole ($n = 9$). The difference in the two means was not significant.

Thermal Stability of the LD Isozymes. The heat uptake and mean temperature of denaturation (Tm) for the isozymes, as determined by plots of heat capacities at constant pressure versus temperature from calorimetry scans, are given in Table V. The mean temperature for the LD-1 isozymes as a combined group, 67.1 ± 1.1°C ($n = 3$), differed significantly from the Tm of 58.6 ± 1.8°C ($n = 3$) for the LD-5 forms ($P < 0.005$). Individually, the LD-1 isozymes all had higher heat uptakes during the denaturation process than their corresponding LD-5 forms. Mean heat uptakes for the LD-1 isozymes as a group were not significantly different from those of the LD-5 isozymes due to the wide variations between species.

Discussion

Plagemann *et al.* (18) reported in 1961 that the activation energy for the conversion of lactate to pyruvate in an unbuffered solution of ammonium sulfate was 8.3 ± 0.4 kcal/mole for rabbit LD-1 and 13.2 ± 0.074 kcal/mole for rabbit LD-5. Based on these activation energies, it was estimated that the LD-1 isozymes should have turnover numbers 3300 times those for LD-5 (3). LD-5 isozymes, however, have consistently been shown to have turnover numbers twice those of LD-1 (5, 19). In addition, an extra hydrogen bond must be broken in the LD-1-catalyzed reaction (20). These observations suggest that the LD-5 isozymes should have lower activation energies than the LD-1 forms, as we are reporting here. A possible explanation for the apparent discrepant results of Plagemann *et al.* (18) is their use of an unbuffered ammonium phosphate solution at pH 9.0. It has been demonstrated that the LD-5 isozymes are more labile at higher pH (12, 21). Since the primary function of an enzyme is to lower the activation energy of the reaction it catalyzes, it is interesting to speculate that the lower activation energies for LD-5-catalyzed reactions reported in this study indicate that the A_4 forms are more efficient in the species examined.

The consistently shorter degradation half-life for

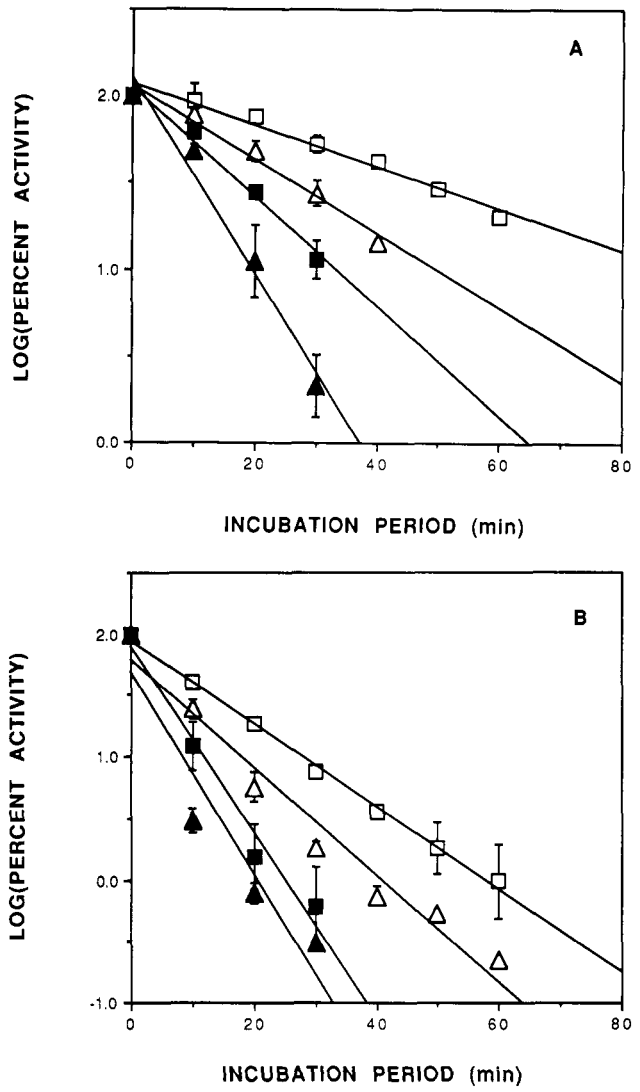

Figure 3. Logarithm of change in percentage of activity of (A) rabbit LD-1 and (B) rabbit LD-5 with time following incubation with subtilisin at 4°C (□), 10°C (△), 18°C (■), and 25°C (▲) demonstrating first-order degradation. Data points represent mean ± SD of three duplicate analyses.

Table IV. Degradation Energies for Lactate Dehydrogenase Isozymes LD-1 and LD-5

Species	Degradation energy[a] (kcal/mole)		P[b]
	LD-1	LD-5	
Rabbit	12.1 ± 1.9	7.1 ± 1.3	<0.05
Porcine	12.9 ± 1.1	12.3 ± 1.9	NS
Bovine	12.6 ± 1.5	11.2 ± 2.1	NS

[a] Degradation energy = (slope of plot of log [degradation rate constant] versus subtilisin incubation temperature $(K)^{-1}$) × −2.303 × R. Degradation with 10 mg/ml subtilisin (Carlsberg). Values given as mean ± SD for three duplicate analyses.
[b] Significance difference between LD-1 and LD-5 isozymes for given species. NS, not significant.

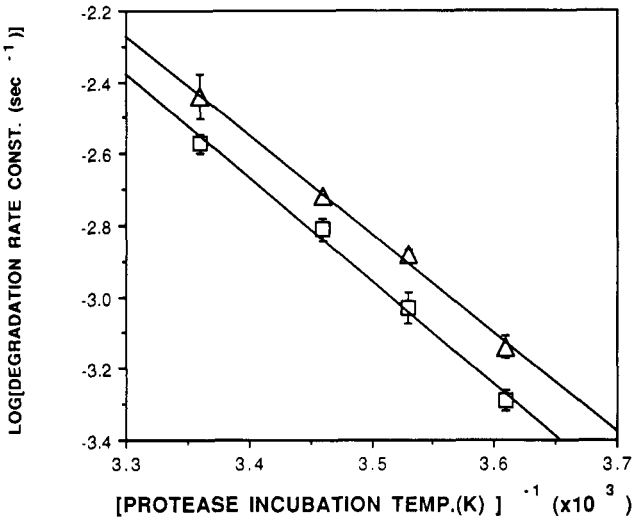

Figure 4. Logarithm of degradation rate constants versus absolute protease incubation temperature (modified Arrhenius plots used for determination of degradation energies). Porcine LD-1 (□) and LD-5 (△) data are shown. Degradation rate constant = (0.693 × degradation $t_{1/2}$)$^{-1}$ at temperature indicated. Each data point represents mean ± SD of three duplicate analyses.

the LD-5 isozymes suggests greater accessibility to the sites of hydrolysis in the LD-5 forms. This greater susceptibility to proteolysis may be indicative of looser folding and/or faster unfolding in areas critical to LD's function and stability. Although the amino acid sequence has yet to be elucidated for the bovine B chain and the sequences for the rabbit A and B chains are incomplete, comparison shows remarkable sequence identity between both different isozymes in the same species and the same isozyme in different species. The sequence similarity is especially great within a given isozyme form across species lines, with porcine B to rabbit B, porcine A to bovine A, and porcine A to rabbit A each having greater than 98% identity (22–24). As

Table V. Mean Temperature and Heat Uptake of Denaturation for Lactate Dehydrogenase Isozymes LD-1 and LD-5[a]

Species	Mean temperature for denaturation (°C)		Heat uptake of denaturation (cal/g)	
	LD-1	LD-5	LD-1	LD-5
Rabbit	67.4	56.8	3.77	1.51
Porcine	68.0	60.1	6.67	4.99
Bovine	65.9	59.0	5.58	0.49

[a] n = 1 for all values.

Eventoff *et al.* (20) have previously noted, the differences which do exist between the isozyme forms impart subtle but important changes in their resulting structures. For example, it is more difficult for NAD^+ to reach its binding site in the LD-1 forms, but there is tighter binding once it reaches the site due to formation of an extra hydrogen bond (20). It is likely that a similar occurrence accounts for the differing stabilities of the isozymes when subjected to proteolysis. Though the exact site(s) of attack by the protease has not yet been determined, once the protease reaches the site of hydrolysis, the energy involved in the breakage of bonds is apparently equal in magnitude for both isozymes, because degradation energies were found to be equivalent in all but the rabbit isozymes. It is unclear why rabbit LD appears unique in this instance.

Comparing degradation half-lives and using degradation rate constants to further calculate the energy involved in the degradation process is contingent on the proteolytic process occurring in the same manner for all isozymes, i.e., that the same kinetic steps are involved and that the protease or degradation products do not interfere with the LD assay. One justification for this assumption can be found by comparing the preexponential factors (the *y*-intercepts of the degradation energy plots [Fig. 4]). The values differ by less than 10% for the degradation energies of both isozymes, which suggests that the inherent degradation reactions are very similar.

It is generally felt that the loss of enzymatic activity of the LD isozymes with heating or cooling is due to changes in isozyme conformation (11, 12, 19). These changes apparently take place at a lower temperature and with a lower heat uptake for the LD-5 isozymes when compared with their corresponding LD-1 forms. Although the heat uptake in the unfolding process does not represent the enthalpy of denaturation, since the process of unfolding for LD is an irreversible, nonequilibrium process, it can be used to compare the stability of these similar isozymes. The findings suggest that the quaternary structures of LD-5 forms are less stable than those of the LD-1 isozymes, and correlate with and help to explain work by others who have found that LD-1 retains activity at temperatures that result in the inactivation of LD-5 (15, 19).

A final speculative suggestion based on our findings of the increased efficiency of the LD-5 isozymes and other reports from this laboratory that LD-5 is associated with more rapidly proliferating tissue (6) is that this isozyme is associated with the classical Warburg effect, which is the production of lactate by many different tumors even under aerobic conditions.

This research was supported by the Tapan Hazra Memorial Cancer Research Fund and the Blanton Fund and was partially undertaken for fulfillment of the medical student research requirement at The Medical College of Virginia. It is dedicated to the late Professor E. S. Kline, Department of Biochemistry and Molecular Biophysics, Medical College of Virginia.

The authors would like to thank Dr. Mark Brandt, Department of Physiology and Biophysics, University of California, Irvine, and Dr. Bruno Maggio, Department of Biochemistry and Biophysics, Medical College of Virginia, for their help in manuscript preparation. The assistance of Dr. Douglas Lee, Department of Biochemistry and Molecular Biophysics, Medical College of Virginia, in carrying out calorimetry work is also acknowledged.

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