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Kinetic Studies on Human Lactate Dehydrogenase Isoenzyme-Catalyzed Lactate-to-Pyruvate Reaction

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In order to evaluate the functional differences that may exist in human lactate dehydrogenase (LDH) isoenzymes widely used for clinical examination the kinetic and thermodynamic properties of the lactate to pyruvate reaction that they catalize were examined. Small but significant differences in the kinetic properties of the three isoenzymes were observed. The difference in the rate constants might affect the activity measurement of the individual isoenzyme as the initial velocity for the L–P reaction catalyzed will not be the same for an equal amount of enzyme. Equilibrium constants for the overall reaction in the presence and absence of pyruvate have been determined. On the basis of transition-state theory, the standard enthalpy and free-energy changes for formation of ternary activated complex were positive, while the standard entropy change was negative. Although the standard free-energy change was the same for activation by the three isoenzymes, the enthalpy and entropy changes for the LDH-3-catalyzed reaction were different from the respective values for others. A large positive value for the free-energy change and a negative value for the entropy change indicated unfavorable production of the activated complex ($K^{\ddagger}_{eq} = 1.89 \times 10^{-16}$). The enzyme appears to stabilize and retain the activated complex until it dissociates into the products.

KEY WORDS: lactate dehydrogenase isoenzymes; lactate-to-pyruvate reaction; kinetics.

INTRODUCTION

Lactate dehydrogenase (LDH, L-lactate-NAD^+ oxidoreductose), EC 1.1.1.27, is a tetrameric protein composed of one or more subunits, muscular (M) or cardiac (H), in different proportions (Holbrook *et al.*, 1975). Although the metabolic role of LDH in the conversion of pyruvate to lactate in biological systems has been known for many decades, the characterization of LDH into multiple forms (isoenzymes) by Market and Moller (1959) did indeed stimulate considerable research interest in the nature, function, clinical use, and control of these isoenzymes. For example, determination of LDH isoenzyme profiles of serum and/or other body fluids from patients suffering from various diseases has been found to be a valuable diagnostic and evaluation tool (Cohen *et al.*, 1966; Vasudevan *et al.*, 1978; Nair *et al.*, 1985). However, the determination of the total LDH and of the individual isoenzyme

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activities is based on the assumption that the maximum velocity v_f for the lactate-to-pyruvate (L–P) or the backward reaction

$$v_f = k_1[E]_0,$$

where k_1 is the rate constant and $[E]_0$ is the initial concentration of the enzyme, is the same for identical amounts of all isoenzymes. In other words, it is assumed that the kinetic parameters for LDH-catalyzed reaction are identical regardless of the nature of the isoenzyme used for activity measurement.

Although the isoenzymes have the same molecular weight (140,000), they differ in amino acid composition (Holbrook *et al.*, 1975), in thermal stability (Menon *et al.*, 1986), in electrophoretic movement (Davis, 1964) and even in immunological properties (Fondy *et al.*, 1964). If there are significant differences in the rate constants for the forward reaction, L–P or the backward reaction, which the isoenzymes catalyze, the currently used measurements of the total LDH and isoenzyme activities will be in error. The isoenzyme ratios derived from the activity measurements being used for the diagnosis of various diseases may also affect the physician's interpretation of the data. The purpose of this investigation was to measure the relative kinetic parameters for the L–P reaction catalyzed by individual human LDH isoenzymes 1, 2, and 3, under identical conditions.

Several investigators (Hakala *et al.*, 1956; Winer and Schwert, 1958; Zewe and Fromm, 1962, 1965; Anderson *et al.*, 1964) studied the kinetics and mechanism of LDH-catalyzed L–P reaction using both lactate and pyruvate as substrates. However, all these workers used nonhuman LDH such as rat liver LDH (Anderson *et al.*, 1964), LDH from rabbit muscle (Zewe and Fromm, 1962, 1965), and LDH from beef heart (Winer and Schwert, 1958). Recently Place and Powers (1984) observed significant differences in the kinetic properties of the allelic isoenzymes of B-type LDH in the fish *Fundulus heteroclitus.* However, no investigation has been conducted to determine whether there are similar differences in the kinetic properties of human LDH isoenzymes. In order to develop radioimmunoassays (RIAs) for LDH-1 and LDH-2 isoenzymes (Menon *et al.*, 1985), these isoenzymes were extracted and purified along with LDH-3 from human myocardial tissue collected from autopsy material, using anion-exchange chromatography. The isolated and purified isoenzymes 1, 2, and 3 were then used for kinetic studies.

EXPERIMENTAL PROCEDURES

Materials and Equipment

Unswollen DEAE-cellulose anion-exchange resin (Sigma) was equilibrated with tris(hydroxymethyl)amino-methanehydrochloride (Tris–HCl) according to manufacturer's instructions. Myocardial extract was prepared from heart muscle separated from autopsy material (Memorial Medical Center, Savannah, Georgia). Sodium lactate (60% syrup), sodium pyruvate, NAD^+ (98% pure), and all other chemicals used in this investigation were purchased from Sigma. Bio-Rad laboratories supplied the protein assay kit. Reagent chemicals were used without repurification. The equipment used in this investigation included Perkin Elmer Model Lambda 3 UV visible spectrophotometer, Beckman LB65 Ultracentrifuge,

Dubnoff metabolic shaking incubator, Corning model 12 research pH meter, Helena's Titan A Gel electrophoretic apparatus, and their model Quick Scann densitometer.

Buffers

Five different Tris–HCl buffers of varying pH and chloride concentrations (*p*H 7.2–8.0; [Cl^-], 0.05–0.25 M) were prepared for chromatographic separation of LDH isoenzymes. Lactate substrate of varying concentrations (10–50 mM) was prepared in sodium phosphate buffers by mixing proportional quantities of disodium hydrogen phosphate and sodium lactate in water and adjusting the *p*H to the desired value. Kinetic studies were carried out at a *p*H of 8.8, the value recommended by Wacker *et al.* (1956) for the measurement of LDH activity.

Isolation of Human LDH-1, LDH-2, and LDH-3 Isoenzymes

LDH isoenzymes were isolated from myocardial extract by anion-exchange chromatography on DEAE cellulose column (85 × 1.5 cm) using a *p*H-coupled salt gradient elution method described by Menon *et al.* (1985). Chromatography of isolated fractions was repeated until the isoenzyme purity was established by electrophoresis. The lower concentration and activity of LDH-3, least stable out of the three, limited the number of experiments conducted with this isoenzyme. Sodium azide, 100 mg, was added to each enzyme preparation to prevent bacterial action.

Measurement of the Concentration and Activity of LDH Isoenzymes

The concentration of the protein (enzyme) was measured using the Bio-Rad protein determination kit and the procedure recommended by Bradford (1976). All activity measurements were made at 37 ± 0.5°C using the method of Wacker *et al.* (1956) involving LDH-catalyzed conversin of L–P in the presence of NAD^+. The final concentrations in millimoles per liter of each reagent in the assay mixture were the following: sodium phosphate buffer (pH 8.8), 50.0, sodium lactate 50.0, and NAD^+ 5.0.

Determination of the Product (NADH) Concentration

The product concentration was determined, whenever necessary, from the absorbance of NADH at 340 nm using the relationship

$$A = \varepsilon b C_M$$

where A is the absorbance, ε is the molar extinction coefficient, 6.22×10^3 M/cm (Place and Powers, 1984) b is the path length (1 cm in this case), and C_M is the molar concentration of NADH.

Kinetic Studies

Except for the studies on activation energy, all rate measurements were carried out at a temperature of 25.5 ± 0.5°C. Previously prepared buffer–lactate solutions (*p*H

8.8) were mixed with appropriate amount of NAD^+ just before the experiment to give the desired final concentrations and then kept in the shaking incubator at 25.5°C for more than 15 min. The substrate-coenzyme mixture (2.8 ml) was pipetted into a 1-cm cell, and the reaction was initiated by the rapid addition of 0.2 ml of the enzyme. The sample was vortexed before being placed into the spectrophotometer to make absorbance measurements at 340 nm. Absorbance was measured in 15-sec intervals for 1–2 min. For the L–P reaction, absorbance increases linearly at least for 2 min (Howell *et al.*, 1979). Initial velocities were computed from the absorbance change for the first 15 or 30 sec.

Determination of K_{eq}

Mixtures of phosphate buffer, lactate, and NAD^+ were allowed to come to equilibrium in the presence of LDH-1 or LDH-2 at a *p*H of 8.8. Absorbance of the product, NADH, was measured at the end of 2 hr and 21 hr to ensure that equilibrium was reached. NADH concentration was measured from the absorbance level. Final concentrations of lactate and NAD^+ were computed from their initial concentrations and that of NADH.

Transition-State Kinetics

In order to measure the first-order rate constant for the slowest step, the decomposition of the substrate-enzyme-NAD^+ complex, on the basis of a transition-state model (Gutfreund, 1971) mixtures containing the steady-state concentrations of lactate and NAD^+ (lactate, 50 mM; NAD^+, 5 mM) in phnosphate buffer (*p*H 8.8) was treated with 0.2 ml of the desired isoenzyme (~500 U/L) at $t = 0$. The mixture was vortexed, and absorbance of the product NADH was measured at 20 sec and every 30 sec thereafter, for 5 min at the desired temperature. The mixtures, at the end of 5 min, were kept at the same temperature for the measurement of equilibrium absorbance A_α after more than 30 min.

RESULTS

Kinetic Constants

It has been suggested by Alberty (1953) that the initial reaction velocities for an enzymatic reaction involving a substrate and a coenzyme follow the general rate law given by

$$-(d[A]/dt)_0 = v_f/\{1+(K_A[A])+(K_B/[B]+(K_{AB}/[A][B])\} \quad (1)$$

where v_f, K_A, K_B, and K_{AB} are various kinetic constants and $[A]$ and $[B]$ are concentrations of A and B. Several workers (Winer and Schwert, 1958; Zewe and Fromm, 1962; Anderson, *et al.*, 1969), have demonstrated that the initial reaction velocity of LDH-catalyzed reaction from both the lactate and pyruvate sides follow a rate law of the form given by Eq. (1). We have therefore used the following

equation to determine the various kinetic parameters for the reaction catalyzed by LDH-1 and LDH-2 isoenzymes:

$$1/v_i = 1/v_f + K_L/[L] + K_N/[N] + K_{LN}/[L][N] \quad (2)$$

where v_i and v_f are the initial and final (maximum) velocities, K_L and K_N are the Michaelis constants for lactate and NAD^+, respectively and K_{LN} is a complex constant dependent on the product of concentrations of the lactate [L] and NAD^+ [N]. At a constant concentration $[N]_0$ of NAD^+, which is of the order of magnitude of K_N, the following equation is valid for the treatment of kinetic data:

$$1/v_i = (1/v_f)(K_L + (K_{LN}/[N_0]))(1/[L]) + (1/v_f)(1 + K_N[N]_0) \quad (3)$$

A plot of $1/v_i$ versus $1/(L)$ should give a straight line with a slope equal to $(1/v_f)(K_L + K_{LN}/[N]_0$ (Alberty, 1953). If two experiments with two different concentrations ($[N]_0$) of NAD^+ but with varying concentrations of lactate are run, two different slopes and two different intercepts will result, from which v_f, K_L, and K_{LN} can be evaluated. A similar technique can be used to determine K_N and, if necessary, v_f and K_{LN}, by performing experiments with two different concentrations ($[L]_0$) of lactate but varying concentrations of NAD^+. The kinetic equation that will fit the data, in this case, is given by

$$1/v_i = (1/v_f)(K_N + (K_{LN}/[L]_0))1/[N] + (1/v_f)(1 + K_L/[L]_0) \quad (4)$$

Apparent Maximum Velocity and Apparent Michaelis Constants

If only two sets of experiments involving a constant concentration of either the lactate or the coenzyme, NAD^+, with the varying concentration of the other are performed, one can still measure the apparent maximum velocity and apparent Michaelis constants for lactate and NAD^+ using Lineweaver-Burk equations:

$$1/v_i = (1/v_f') + K_L'/(v_f')[L] \quad (5)$$

$$1/v_i = (1/v_f') + K_N'/(v_f')[N] \quad (6)$$

where v_f', the apparent maximum velocity, is $v_f/(1 + K_N/[N]_0)$; K_L', the apparent Michaelis constant for L, is $(K_L[N]_0 + K_{LN})/K_N + [N]_0)$; and K_N', the apparent Michaelis constant for N, is $(K_N[L]_0 + K_{LN})/(K_L + [L]_0)$. Equations (5) and (6) are valid only when concentration of NAD^+ $[N]_0$, and concentration of lactate, $[L]_0$, are held constant during the rate measurement. Figure 1a is a plot of initial velocity versus lactate (substrate) concentration for the LDH-3 catalyzed L–P reaction at 25.5 ± 0.5°C. Figure 1b depicts the Lineweaver–Burk plot for the same system. During the initial stage of this work, rate measurements were made for the LDH-1 and LDH-2 catalyzed P–L reaction to obtain similar Lineweaver–Burk plots. From such plots, the relative values for the apparent maximum velocity, v_f', and for the apparent Michaelis constants for lactate and NAD^+ were computed for the L–P reaction catalyzed by each of the isoenzymes. These results are presented in Table I. Because of insufficient LDH-3 isoenzyme, the rate measurements with constant concentration of lactate and varying concentration of NAD^+ could not be carried out to measure the value for K_N' for the LDH-3 catalyzed reaction.

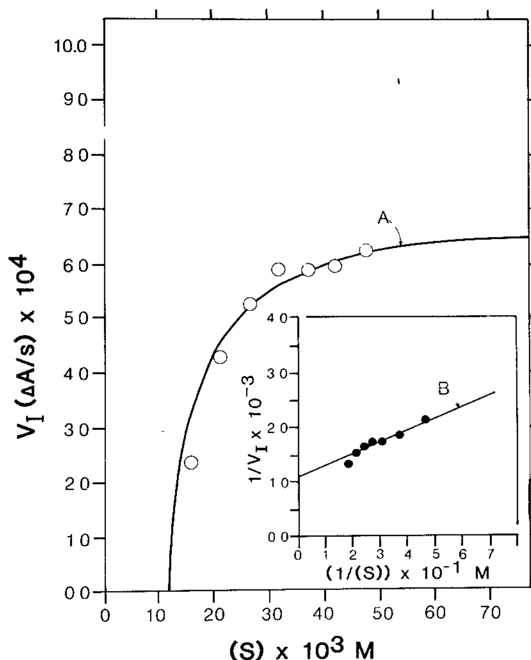


Fig. 1. Typical plots of initial velocity versus lactate concentration (A) reciprocal initial velocity versus reciprocal of lactate concentration (B) for LDH-3 isoenzyme. Concentration of NAD^+ is 5×10^{-3} M.

Kinetic Constants v_f, K_L, K_N, and K_{LN}

From product-inhibition studies, Anderson *et al.* (1964), Hakala *et al.* (1956), Place and Powers (1984), Hammes (1982), and Gutfreund (1971) reported that reactions catalyzed by lactate dehydrogenase follow an ordered sequential mechanism involving one or more catalytically significant ternary complexes. However, Zewe and Fromm (1962) contended that their data are consistent with a compulsory sequence of substrate binding to the enzyme involving kinetically significant binary complexes. Since our main objective in this work was to determine whether there are significant differences in the kinetic constants for the L-P reaction catalyzed by

Table I. Apparent Michaelis Constants and Apparent Maximum Velocities for Human LDH Isoenzyme-Catalyzed Lactate-to-Pyruvate Reaction

LDH isoenzyme	K'_L ($\times 10^3$ M)	K'_N ($\times 10^3$ M)	V'_f ($\times 10^{-2}$)[a]
LDH-1	26.3 ± 0.7	0.62 ± 0.06	1.1 ± 0.1
LDH-2	28.0 ± 0.5	0.21 ± 0.02	2.4 ± 0.2
LDH-3	21.5 ± 1.0	—	0.3 ± 0.03

[a] Moles of product per min per mole of enzyme per liter.

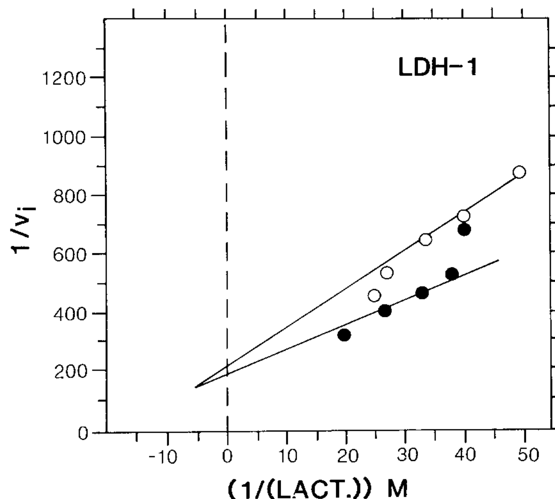


Fig. 2. Plots of reciprocal initial velocity versus reciprocal concentration of lactate for LDH-1 isoenzyme. Concentrations of NAD^+: (○) 6×10^{-4} M; (●) 1×10^{-3} M.

each of the three isoenzymes, we chose to make only two sets of measurements for LDH-1 and LDH-2 isoenzymes holding either lactate or NAD^+ concentration constant. All four kinetic constants—v_f, K_L, K_N, and K_{LN}—can be obtained by solving simultaneous equations using data presented in Figs. 2–5 (Alberty, 1953). It can be seen that the initial reaction velocities follow the rate laws represented by Eqs. (3) and (4). Since the two lines in each plot intersect outside the ordinate, our

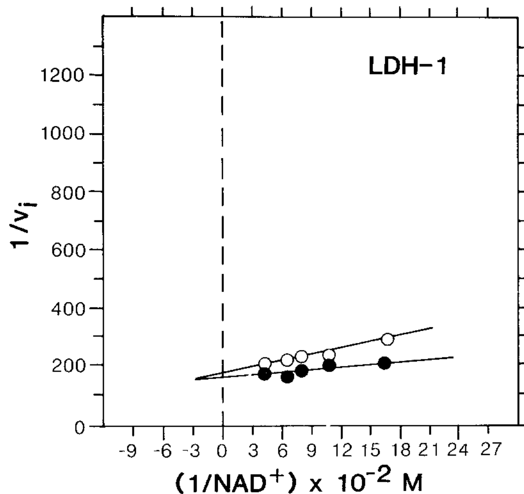


Fig. 3. Plots of reciprocal initial velocity versus reciprocal concentration of NAD^+ for LDH-1 isoenzyme. Concentrations of lactate: (○) 4×10^{-2} M; (●) 5×10^{-2} M.

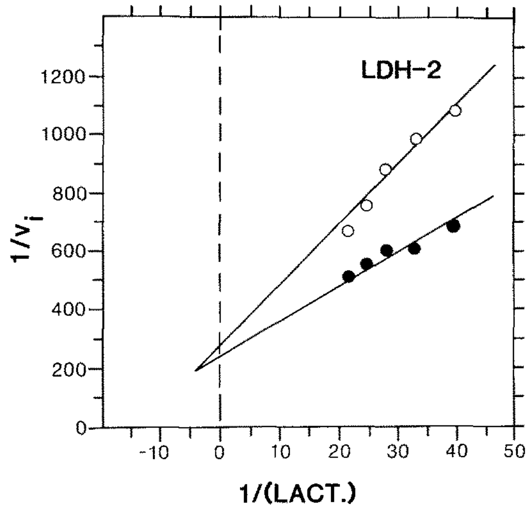


Fig. 4. Plots of reciprocal initial velocity versus reciprocal concentration of lactate for LDH-2 isoenzyme. Concentrations of NAD^+: (○) 6×10^{-4} M; (●) 1×10^{-3} M.

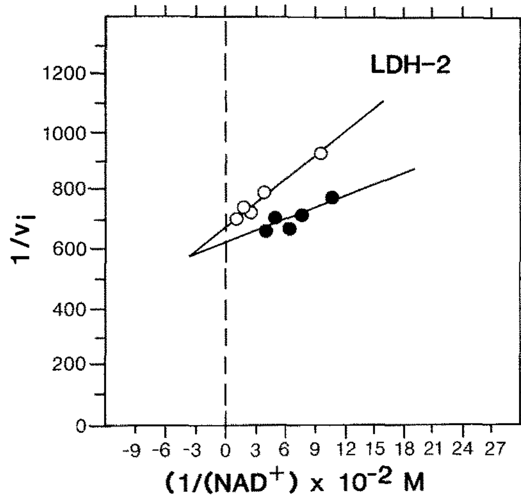


Fig. 5. Plots of reciprocal initial velocity versus reciprocal concentration of NAD^+ for LDH-2 isoenzyme. Concentrations of lactate: (○) 3.5×10^{-2} M; (●) 4×10^{-2} M.

Table II. Relative Kinetic Constants for the Human LDH Isoenzyme-Catalyzed Lactate-to-Pyruvate Reaction

Kinetic constants	Human LDH isoenzymes[a] (pH 8.8, $t=25.5$°C)		Rabbit muscle LDH[b] (pH 6.8, $t=28$)	Rat muscle LDH[c] (pH 8.6, $t=25$)	Beef heart LDH[d] (pH 8.6, $t=28.5$)
	LDH-1	LDH-2			
$v_f \times 10^{-2}$[e]	1.53	1.86	2.09×10^{-7}[f]	426.0	190.0
K_L $M \times 10^3$	7.33	4.84	6.70	24.0	2.8
K_N $M \times 10^4$	4.12	1.72	2.53	1.6	0.66
K_{LN} $M^2 \times 10^5$	5.31	5.07	0.426	1.1	0.11

[a] The present study.
[b] Zewe and Fromm (1962).
[c] Anderson *et al.* (1964).
[d] Winer and Schwert (1958).
[e] Expressed as moles of product per min per mole of enzyme per liter.
[f] Concentration of enzyme was not known, expressed as moles of product per min per liter.

data are in support of a ternary complex mechanism (Gutfreund, 1971). The values for the kinetic constants V_f, K_L, K_N, and K_{LN} for LDH-1 and LDH-2 isoenzymes measured for the forward reaction are listed in Table II. For comparison, kinetic constants reported by other investigators on non-human lactate dehydrogenases are included.

Equilibrium Constant K_{eq} in the Presence and Absence of Added Pyruvate

The equilibrium constant for the reaction

$$\text{lactate} + \text{NAD} \rightleftharpoons \text{pyruvate} + \text{NADH} + \text{H}^+ \quad (7)$$

was experimentally determined by computing the concentrations of the reactants and products from the initial composition of the mixture and the final concentration of NADH. Absorbance of NADH was measured at 2 hr and 21 hr after initiation of the reaction to ensure that equilibrium had been reached. There was a 5% increase in the absorbances at 21 hr over that at 2 hr when LDH-2 was used and a 10% increase when LDH-1 was used in the absence of added pyruvate, but in the presence of pyruvate as much as 16% increase was observed in each case. Thus, an absorbance of NADH measured after 21 hr was used to measure the concentration in each case. The results of the experiments performed in the absence and presence of added pyruvate are presented in Table III. Although equilibrium constants were measured using two different concentrations of each isoenzyme, K_{eq} was found to be independent of the enzyme concentration, as expected.

pH Dependence of Equilibrium Constant, K_{eq}

Reaction mixtures with different amounts of lactate, NAD^+, and pyruvate in phosphate buffer at different pH values were allowed to come to equilibrium in the presence of LDH-1 or LDH-2 isoenzyme. The equilibrium concentrations of all the

Table III. Equilibrium Constant at 25.5°C (pH 8.8) for the Reaction Lactate + NAD^+ $\rightleftharpoons$ Pyruvate + NADH + H^+

Isoenzyme	Activity U/L	A_{20} Hrh$_r$	C^f_{NADH} (M × 10^4)	C^f_L (M × 10^2)	C^f_P (M × 10^4)	$C^f_{NAD^+}$ (M × 10^3)	K_{eq} (× 10^{14})
			Without any added pyruvate				
LDH-2	6550	0.718	1.15	4.99	1.15	4.89	8.6
	6550	0.727	1.16	4.99	1.16	4.88	8.8
	1210	0.705	1.12	4.99	1.13	4.89	8.3
	1210	0.690	1.10	4.99	1.10	4.89	7.9
LDH-1	10,200	0.700	1.13	4.99	1.13	4.89	8.3
	10,200	0.693	1.11	4.99	1.11	4.89	8.0
	1020	0.715	1.15	4.99	1.15	4.89	8.6
	1020	0.715	1.15	4.99	1.15	4.89	8.6
							Average = 8.39
			With added pyruvate (2.0 × 10^{-2} M)				
LDH-2	6550	0.708	1.14	4.99	201	4.89	1490
	6550	0.720	1.16	4.99	201	4.88	1520
	1210	0.750	1.21	4.99	201	4.88	1590
	1210	0.675	1.09	4.99	201	4.89	1430
LDH-1	10,200	0.687	1.10	4.99	201	4.79	1440
	10,200	0.727	1.17	4.99	201	4.88	1540
	1020	0.675	1.09	4.99	201	4.89	1430
	1020	0.685	1.10	4.99	201	4.89	1440
							Average = 1490

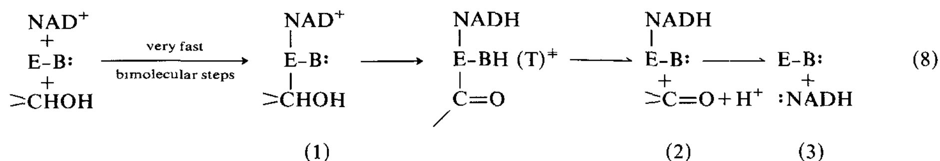
Table IV. pH Dependence of the Equilibrium Constant K_{eq} at 25°C for the Reaction Lactate + NAD^+ $\rightleftharpoons$ Pyruvate + NAD + H^+

*p*H	$(L)_i$ (× 10^2 M)	$(P)_i$ (× 10^4 M)	$(NAD^+)_i$ (× 10^3 M)	$A_{20\,hr}$ (0.275)	$(NADH)_f$ (× 10^5 M)	$(L)_f$ (× 10^2)	$(P)_f$ (× 10^4 M)	$(NAD^+)_f$ (× 10^3)	K_{eq} (× 10^{12})
				LDH-2 (Activity = 565 U/L)					
8.0	8.0	4.0	1.0	0.275	4.42	7.99	4.44	0.96	2.56
8.1	7.0	3.0	2.0	0.351	5.64	6.99	3.56	1.95	1.18
8.3	6.5	2.0	1.5	0.259	4.16	6.50	2.42	1.46	0.53
8.5	5.5	1.5	2.0	0.289	4.65	5.50	1.97	1.95	0.27
8.7	7.5	1.0	1.0	0.236	3.79	7.50	1.38	0.96	0.14
8.9	3.0	2.0	1.5	0.170	2.73	3.00	2.27	1.47	0.18
9.1	6.0	4.0	2.0	0.241	3.87	6.00	4.39	1.96	0.11
				LDH-1 (Activity = 1020 U/L)					
8.0	8.0	4.0	1.0	0.281	4.51	8.00	4.45	0.96	2.61
8.1	7.0	3.0	2.0	0.361	5.80	6.99	3.58	1.95	1.21
8.3	6.5	2.0	1.5	0.221	3.55	6.50	2.36	1.46	0.44
8.5	5.5	1.5	2.0	0.243	3.91	5.50	1.89	1.96	0.22
8.7	7.5	1.0	1.0	0.243	3.91	7.50	1.39	0.96	0.15
8.9	3.0	2.0	1.5	0.165	2.65	3.00	2.27	1.47	0.17
9.1	6.0	4.0	2.0	0.253	4.07	5.99	4.41	1.96	0.12

species were computed from the final concentration of NADH and the initial composition of the mixture. The results of these experiments are summarized in Table IV.

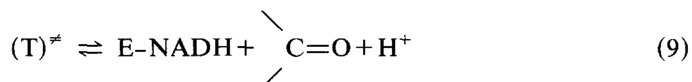
Kinetic and Thermodynamic Constants Based on Transition-State Theory

Early transient kinetic studies of pig H_4 and M_4 LDH reported by Gutfreund (1971) indicate that there are three phases in the reaction path when LDH is rapidly saturated with NAD^+ for the subsequent formation of NADH, on the basis of ternary complex theory. These phases include (1) an instantaneous (<1-msec) formation of the NADH complex, (2) a first-order slow dissociation process, and (3) steady-state production of NADH as given by the following reaction path:



Reaction of lactate dehydrogenase with lactate and NAD^+ at pH 6 was monitored by nucleotide absorbance, nucleotide fluorescence, and protein fluorescence.

A simple-minded approach to the LDH enzyme kinetics involves fast equilibration steps to form the activated ternary complex or transition state, labeled $(T)^+$, among enzyme, NADH, and pyruvate, with the subsequent decomposition of the activated complex into pyruvate and NADH by a slow rate-determining step and the final attainment of the equilibrium or steady state. The species identified as $(T)^+$ may decompose according to the following equation:



$$(T)^{\neq} \rightleftharpoons \text{E-NADH} + \;\rangle C{=}O + H^+ \tag{9}$$

The first-order rate law for this reaction may be written as

$$(dx/dt)_{net} = k_1(a-x) - k_{-1}x^2 \tag{10}$$

where a is the initial concentration of $(T)^+$ and x that of E–NADH and pyruvate at any time t at constant pH. At equilibrium

$$(dx/dt)_{net} = 0 = k_1(a-x_e) - k_{-1}x_e^2 \quad \text{or} \quad k_{-1} = k_1(a-x_e)/x_e^2 \tag{11}$$

Since at equilibrium all of the activated complex is converted to NADH, its concentration will then equal a_e. Since $a_e = K \cdot A_e$ and $x_e = K \cdot A_e$, where x_e and A_e are the equilibrium concentration and absorbance of NADH, respectively, and K is the proportionality constant, the value of k_{-1}, in this special case, becomes zero. Equation (10) can therefore be rearranged as

$$dx/(a-x) = k_1\, dt \tag{12}$$

which on integration and substitution of absorbance A_α for a_e and A_t for x yields

$$\log(A_\alpha - A_t) = -(k_1 \cdot t/2.303) + \log A_\alpha \tag{13}$$

A plot of $\log(A_\alpha - A_t)$ versus t should result in a straight line, the slope of which will yield the value of k_1, the rate constant for the forward reaction. Representative plots for the LDH-1-, LDH-2-, and LDH-3-catalyzed L-P reaction are depicted in Fig. 6. The rate constants for the reaction catalyzed by each of the isoenzymes at various temperatures were measured from the slopes of similar plots. The Arrhenius plots for the temperature dependence of the rate constants for L-P reaction catalyzed by each of the isoenzymes are shown in Fig. 7. Since the slopes of these plots are somewhat different, activation energy for the formation of activated complex seems to be different for the catalytic action of the isoenzymes.

According to the transition-state theory, which is also applicable to enzyme-catalyzed reaction (Page, 1980), the rate constant is related to the enthalpy change for activation by the expression

$$k_1 = (k \cdot T/\mathrm{h}) \exp(\Delta S^{\circ\ddagger}/R) \exp[-(\Delta H^{\circ\ddagger}/RT)] = A \cdot \exp[-(E^*/RT)] \quad (14)$$

where k is the Boltzmann constant, $\Delta H^{\circ\ddagger} = E^*$ for solution reactions, and A is the pre-exponential factor of the Arrhenius equation. The activation energy E^* was calculated from the kinetic data by a curvilinear regression method using the Biostatistics Apple IIe computer program. Knowing the values of $\Delta H^{\circ\ddagger}$ and k_1 at 25 °C, $\Delta S^{\circ\ddagger}$, the standard entropy change, at 25°C can be computed. The standard free-energy change $\Delta G^{\circ\ddagger}$ and the equilibrium constant for the formation of activated complex $K^{\ddagger}_{eq}$, can be calculated from standard thermodynamic equations. The values

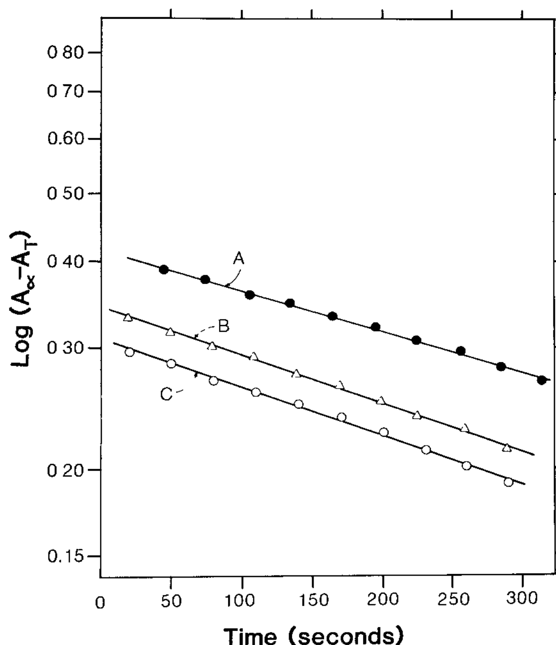


Fig. 6. Typical plots of $\log(A_\alpha - A_t)$ versus time for the measurement of first-order rate constants. (A) LDH-2; (B) LDH-1; (C) LDH-3.

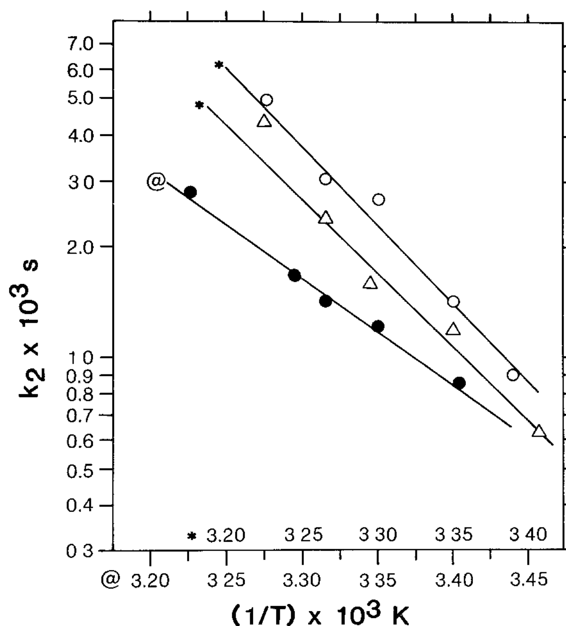


Fig. 7. Arrhenius plots for LDH-1 (●), LDH-2 (△), and LDH-3 (○) isoenzymes. Curvilinear regression analysis gave the following equations: $k_1 = \exp((1/T) \times -6187.37 + 20.93)$ with $r = -0.997$ for LDH-1, $k_1 = \exp((1/T) \times -10235.23 + 34.37)$ with $r = 0.983$ for LDH-2 and $k_1 = \exp((1/T) \times -10296.74 + 34.83)$ with $r = -0.994$ for LDH-3.

for these kinetic and thermodynamic constants for the L–P reaction catalyzed by each of these isoenzymes are presented in Table V.

DISCUSSION

Since the objective of this investigation was only to determine whether there are differences in the kinetic properties of L–P reaction catalyzed by each of the three LDH isoenzymes, no product-inhibition studies were undertaken to distinguish among three possible mechanisms suggested by Alberty (1953). However, the product-inhibition studies conducted by Anderson *et al.* (1964) with rat liver LDH suggest

Table V. Kinetic and Thermodynamic Constants for the Activated Complex, at 298 K

Isoenzymes	k_1 $\times 10^3$ sec)	$K^{\ddagger}_{eq}$ ($\times 10^{16}$)	$\Delta H^{\circ\ddagger}$ (kJ mole)	$\Delta G^{\circ\ddagger}$ (kJ mole)	$\Delta S^{\circ\ddagger}$ (J mole/K)
LDH-1	1.15	1.85	51.442	89.765	−125.6
LDH-2	1.05	1.69	85.096	89.989	−16.42
LDH-3	1.32	2.13	85.607	89.421	−12.80

an ordered sequential mechanism with catalytically significant ternary complexes. The ternary complex mechanism has been supported by the work of many other investigators (Holbrook *et al.*, 1975; Place and Powers, 1984; Holbrook and Gutfreund, 1973).

The results presented in Table I show that there are significant differences in the apparent maximum velocities for the L-P reaction catalyzed by human LDH isoenzymes 1, 2, and 3. Although there are no appreciable differences in the apparent Michaelis constant K'_L for lactate, the similar constant K'_N for NAD^+ is three times higher with LDH-1 than with LDH-2 when used for catalysis. The apparent K_m for pyruvate for the P-L reaction catalyzed by pelagic fish LDH is reported to be influenced by temperature and *p*H (Valkirs, 1978). There appears to be no correlation between kinetic constants and stability of isoenzymes.

Comparison of our results on LDH isoenzymes with those reported for rabbit muscle, rat muscle, and beef heart LDH presented in Table II shows significant differences in the respective values for each of the kinetic parameters. Although there is not much difference in our v_f values for LDH-1- and LDH-2-catalyzed reactions, our values are lower by more than an order of 2 than the values reported for rat muscle (Anderson *et al.*, 1964) and for beef heart (Winer and Schwert, 1958) LDH. This great difference between our values and theirs may be attributed to the difference in the reaction conditions and the computational methods that the authors have employed. For instance, Anderson *et al.* (1964) used a value of 126,000 M_r and Winer and Schwert (1958) a value of 135,000 M_r for LDH instead of 140,000 M_r (Holbrook *et al.*, 1975), the current estimate used to compute the molar concentration of the enzyme. It is not quite clear how they measured the concentration of the product, NADH. The differences in temperature, *p*H, the buffer solution, and purity of the chemicals used by the investigators will also affect the results. The fact that there are smaller differences in the values of other constants indicates that an error in the measurement of the enzyme concentration may be responsible for the considerable difference in v_f values, as the other constants are not dependent on enzyme concentration. It is also possible that v_f for human LDH-catalyzed L-P reaction is significantly smaller than that for nonhuman LDH values. No data on human LDH isoenzymes appear to have been reported in the literature for comparison. The equilibrium constants for the reaction represented by Eq. (8)

$$K_{eq} = (\text{pyruvate})(\text{NADH})(H^+)/(\text{NAD}^+)(\text{lactate})$$

were computed from the data presented in Table III, in the absence and in the presence of pyruvate. The last column of Table III demonstrates that our results are very precise and are independent of the nature of isoenzyme and its activity, as expected. However, our average value of 1.49×10^{-11} for K_{eq}, in the presence of added pyruvate, is different from 2.7×10^{-12} reported by Anderson *et al.* (1964) for rat liver LDH. This is attributed to the fact that the concentration we have used for pyruvate is higher than that used for rat liver LDH by at least a factor of 10. Another factor that will affect the value of K_{eq} is the time of measurement of the absorbance of NADH after initiation of the reaction, to determine the product concentration. A maximum of 20% increase in absorbance was observed when the equilibration time was 21 hr rather than 2 hr. The values computed by Zewe and Fromm (1962)

for the apparent K_{eq} from the kinetic constants for the reaction is 4.85×10^{-5} and 1.10×10^{-5} based on two different mechanisms. However, it appears that they did not include the concentration of H^+ in their calculation.

The results from measured K_{eq} as a function of pH for the L–P reaction catalyzed by LDH-1 and LDH-2 isoenzymes (Table IV) show that it idecreases with increase in pH. However, its value changes only slightly within the pH range of 8.5–9.1. All our experiments for the kinetic studies were carried out at a pH of 8.8. It is interesting to note that K_{eq} values for L–P reaction catalyzed by LDH-1 and LDH-2 are almost identical at the same pH values.

Application of the transition-state theory to the complicated enzyme-catalyzed reaction may be a new approach, but it deserves consideration. Transition-state theory has been very helpful in providing a theoretical interpretation of A and E^* of the Arrhenius equation without detailed molecular data. In addition, one can also calculate the equilibrium constant $K^{\ddagger}_{eq}$, standard free energy, enthalpy, and entropy changes for the formation of activated complex (transition state). The assumption that thermodynamic equilibrium is attained between the initial and transition states in one or more fast steps has led to the widespread use of free energies, enthalpies, and entropies of activation. The reaction path [Eq. (8)] suggested by Gutfreund (1971) for LDH catalysis of the L–P reaction indeed includes very fast bimolecular and ternary complex interconversion steps leading to an activated complex, $(T)^{\ddagger}$). The activated complex dissociates into pyruvate, H^+ and :B–E–NADH [Eq. (8)] by a first-order slow process culminating in a steady-state production of NADH. Thus, first-order rate measurement, in this case, is based on the measurement of absorbance of :B–E–NADH at 340 nm. The small but significant differences that can be observed in the rate constants for LDH-1, LDH-2, and LDH-3 catalysis from Table V indicate that equal amounts of these isoenzymes do not catalyze the L–P reaction with the same velocity, $v_i = k_1 \cdot [E]_0$. From our work (Menon *et al.*, 1986) as well as the work of Hunter *et al.* (1983), it is known that there is considerable difference in the heat stability of LDH isoenzymes. They are also different in their electrophoretic behavior, probably due to differences in the effective net charge carried by the protein. This may explain the slight difference in kinetic behavior.

The standard enthalpy change $\Delta H^{\circ\ddagger}$ for the formation of the activated complex at 25°C, which is the same as the Arrhenius activation energy E^* for reactions in solution, is also different for the LDH isoenzymes for catalysis. The value, 51.4 kJ mol obtained for LDH-1 isoenzyme is in good agreement with 13+2 kcal/mol (54.4+8.6 kJ/mol) reported by Anderson *et al.* (1964) for rat liver LDH. The standard free energy, $\Delta G^{\circ\ddagger}$ for the formation of the transition state is surprisingly almost identical for the three isoenzymes. This would indicate that there is no difference in the mechanism of formation of the highly unfavorable ternary complex (activated complex), no matter which isoenzyme is used. Although there is some difference in the entropy changes $\Delta S^{\circ\ddagger}$ for the formation of activated complex with the three isoenzymes, it is not significant enough to affect the formation of activated complex. The formation of an activated complex that carries charges will usually lead to a negative change in entropy. Positive values for the standard enthalpy and free-energy changes coupled with a negative entropy change and a very small equilibrium

constant $K^{\ddagger}_{eq}$ make formation of the transition state very unfavorable. As suggested by Holbrook *et al.* (1975), at least one role of the enzyme is to stabilize the ternary-activated complex. The other role may be to keep the activated complex at a high-energy level without rolling downhill before dissociation into pyruvate and NADH takes place.

SUMMARY

Measurement of the apparent kinetic parameters for the lactate-to-pyruvate reaction catalyzed by human lactate dehydrogenase isoenzymes 1, 2, and 3 demonstrates small but significant differences. The apparent maximum velocity for LDH-3 catalysis calculated from Lineweaver–Burk plots is different from the other two by a factor of 4–8. By contrast, the maximum velocities calculated with the use of Alberty's equation for LDH-1 and LDH-2 catalysis are almost identical. The values for the other kinetic constants, such as K_L, K_N, and K_{LN} are in general agreement with the values reported for nonhuman LDH.

The equilibrium constant for the overall reaction was measured in the absence and in the presence of added pyruvate (2×10^{-2} M) at pH 8.8, using the equilibrium composition of mixtures catalyzed by different amounts of each isoenzyme. The K_{eq} was found to have the same value, regardless of the nature and quantity of the isoenzyme used. Nevertheless, the K_{eq} was found to be dependent on the pH value of the mixtures, though to a lesser degree in the pH range of 8.5–9.1.

Application of transition-state theory to the enzyme catalysis enabled us to calculate enthalpy, free energy, and entropy changes for the formation of the activated complex (transition state) during the reaction catalyzed by each of the three isoenzymes. Although the enthalpy and entropy changes for the activation were found to be different for the LDH-1-, LDH-2-, and LDH-3-catalyzed reactions, the free-energy change was almost identical indicating that the mechanism for the formation of activated ternary complex is the same irrespective of the nature of isoenzyme.

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