ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 284, No. 2, February 1, pp. 285–291, 1991

Kinetic Mechanism of the Endogenous Lactate Dehydrogenase Activity of Duck ϵ-Crystallin[1]

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Received July 13, 1990, and in revised form September 19, 1990

Initial velocity, product inhibition, and substrate inhibition studies suggest that the endogenous lactate dehydrogenase activity of duck ϵ-crystallin follows an order Bi–Bi sequential mechanism. In the forward reaction (pyruvate reduction), substrate inhibition by pyruvate was uncompetitive with inhibition constant of 6.7 ± 1.7 mM. In the reverse reaction (lactate oxidation), substrate inhibition by L-lactate was uncompetitive with inhibition constant of 158 ± 25 mM. The cause of these inhibitions may be due to ϵ-crystallin–NAD^+-pyruvate and ϵ-crystallin–NADH–L-lactate abortive ternary complex formation as suggested by the multiple inhibition studies. Pyruvate binds to free enzyme very poorly, with a very large dissociation constant. Bromopyruvate, fluoropyruvate, pyruvate methyl ester, and pyruvate ethyl ester are alternative substrates for pyruvate. 3-Acetylpyridine adenine dinucleotide, nicotinamide 1,N^6-ethenoadenine dinucleotide, and nicotinamide hypoxanthine dinucleotide serve as alternative coenzymes for ϵ-crystallin. All the above alternative substrates or coenzymes showed an intersecting initial-velocity pattern conforming to the order Bi–Bi kinetic mechanism. Nicotinic acid adenine dinucleotide, thionicotinamide adenine dinucleotide, and 3-aminopyridine adenine dinucleotide acted as inhibitors for this enzymatic crystallin. The inhibitors were competitive versus NAD^+ and noncompetitive versus L-lactate. α-NAD^+ was a noncompetitive inhibitor with respect to the usual β-NAD^+. D-Lactate, tartronate, and oxamate were strong dead-end inhibitors for the lactate dehydrogenase activity of ϵ-crystallin. Both D-lactate and tartronate were competitive inhibitors versus L-lactate while oxamate was a competitive inhibitor versus pyruvate. We conclude that the structural requirements for the substrate and coenzyme of ϵ-crystallin are similar to those of other dehydrogenases and that the carboxamide carbonyl group of the nicotinamide moiety is important for the coenzyme activity.

Recent developments in the crystallin chemistry of various animal eye lenses have revealed the salient features of sequence similarities between crystallins and some metabolic enzymes, such as ϵ-crystallin and lactate dehydrogenase [(*S*)-lactate:NAD^+ oxidoreductase, EC 1.1.1.27] (LDH)[3] (1), ρ-crystallin and aldehyde/aldose reductase (2), plus bovine lung prostaglandin F synthase (3), δ-crystallin, and argininosuccinate lyase (4). Previously crystallins were thought to play only a structural role in the maintenance of lens transparency and proper light focusing (5–7). These unexpected findings have opened avenues to study the evolution of lens crystallins with enzymatic functions and their possible biological significance.

ϵ-Crystallin was isolated from avian and reptilian eye lenses (8). Later sequence comparison has revealed that it is closely similar to heart-type LDH (9). The recruitment of enzymes as lens structural proteins has been postulated as a result of evolutionary pragmatism, i.e., the selection of existing stable structures for a new structural role through gene sharing strategy (10, 11). Despite the structural similarities between duck ϵ-crystallin and chicken heart LDH, differences in charge and kinetic properties were revealed by isoenzyme electrophoresis and kinetic studies (12). Although the enzyme activity per se may not be required for the role of the protein as crystallin,

[1] This work was supported by the National Science Council, Republic of China. S.-M.H. was a recipient of the College Student Research Award of the National Science Council.

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[3] Abbreviations used: LDH, lactate dehydrogenase; AAD^+, 3-aminopyridine adenine dinucleotide; $APAD^+$, 3-acetylpyridine adenine dinucleotide; ϵ-NAD^+, nicotinamide 1,N^6-ethenoadenine dinucleotide; $NAAD^+$, nicotinic acid adenine dinucleotide; α-NAD^+, α anomer of the usual β-NAD^+; NHD^+, nicotinamide hypoxanthine dinucleotide; $SNAD^+$, thionicotinamide adenine dinucleotide.

0003-9861/91 $3.00

unlike other enzyme-like crystallins ϵ-crystallin seems to maintain high and stable LDH activity under *in vitro* conditions. We have studied the kinetic mechanism for the LDH activity of ϵ-crystallin. Initial-velocity and product-inhibition studies suggested that the endogenous lactate dehydrogenase activity of duck ϵ-crystallin follows an order Bi–Bi sequential mechanism with NADH as the leading substrate, followed by pyruvate. The products are released in the order of L-lactate and NAD^+ (12). Substrate inhibition was observed in both forward and reverse reactions by high concentrations of pyruvate and L-lactate, respectively. In this report, a detailed kinetic mechanism of the endogenous LDH activity of duck ϵ-crystallin is further examined with the aid of various substrate and coenzyme analogs.

MATERIALS AND METHODS

Materials. L-Lactate, D-Lactate, pyruvate, fluoropyruvate, bromopyruvate, pyruvate methyl ester, pyruvate ethyl ester, tartronate, NAD^+ (β-NAD^+), α-NAD^+, $NADP^+$, NADH, NADPH, $SNAD^+$, AAD^+, NHD^+, $NAAD^+$, $APAD^+$, and ϵ-NAD^+ were obtained from Sigma (St. Louis, MO). Other chemicals were of reagent grade obtained either from Sigma or E. Merck (Darmstadt, Germany).

Purification of ϵ-crystallin from duck eyeball was as described previously (12). The purity of the protein was routinely checked by polyacrylamide gel electrophoresis.

Kinetic studies. Lactate dehydrogenase activity of duck ϵ-crystallin was assayed at 25°C. The decrease of NADH in the forward direction and formation of NADH in the reverse direction were monitored continuously at 340 nm in a Varian DMS-100 spectrophotometer. In a standard assay for the reverse reaction, the reaction mixture contained 100 mM sodium phosphate buffer (pH 7.5), 1.4 mM NAD^+, 20 mM L-lactate, and an appropriate amount of ϵ-crystallin in a total volume of 1 ml. For the forward reaction, NAD^+ and L-lactate were replaced by 0.2 mM NADH and 2 mM pyruvate, respectively. One unit of enzyme activity was defined as an initial rate of 1 μmol of NADH transformed per minute under the assay conditions. A molar absorption coefficient of 6.2×10^3 M^{-1} cm^{-1} for NADH was used for calculation. Protein concentration was determined spectrophotometrically at 280 nm, using an extinction coefficient of 1.4 for a 0.1% (w/v) protein solution (12).

Initial-velocity studies at noninhibitory substrate level were performed by varying the concentration of NADH from 10.4 to 44 μM and that of pyruvate from 0.14 to 0.76 mM in the forward reaction, and the concentration of NAD^+ from 50 to 250 μM and of L-lactate from 0.8 to 6 mM in the reverse reaction. Initial-velocity studies at inhibitory substrate levels were performed with 2–7 mM pyruvate for the forward reaction and 20–70 mM L-lactate for the reverse reaction.

Inhibition studies were performed in a similar manner, with NADH or pyruvate as the varied-concentration substrate in the forward reaction and NAD^+ or L-lactate as the varied-concentration substrate in the reverse reaction. The inhibitor was held at several fixed concentrations around its inhibition constant.

In the kinetic mechanism studies variable substrate concentrations used were near the K_m values. The full scale of the recorder was set to 0.01–0.05 absorbance. A chart speed of 3–10 cm/min was used. The amounts of ϵ-crystallin were selected to give significant reaction rates and yet be small enough so that the reaction velocity remained linear for 2 to 3 min. The initial slopes of the recorder tracings were taken as initial velocities.

Data processing. For the forward reaction, data conforming to a linear sequential initial-velocity pattern, to linear competitive inhibition, and to linear noncompetitive inhibition were fitted to Eqs. [1], [2], and [3], respectively.

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \quad [1]$$

$$v = \frac{VA}{K_a(1 + I/K_{is}) + A} \quad [2]$$

$$v = \frac{VA}{K_a(1 + I/K_{is}) + A(1 + I/K_{ii})}, \quad [3]$$

where K_a, K_b, and K_{ia} are Michaelis constants for A and B and dissociation constant for A, respectively. A and B are reactant concentrations, A for NADH and B for pyruvate in the forward reaction. Similar rate equations were derived for the reverse reaction with A and B replaced by P and Q, where P denotes L-lactate and Q denotes NAD^+. V denotes the maximum velocity in the measured direction. I is the inhibitor concentration. K_{is} and K_{ii} are the apparent inhibition constants associated with the effect of the inhibitor on slope (K_{is}) and intercept (K_{ii}) of the double-reciprocal plots, respectively.

Data conforming to initial-velocity pattern including uncompetitive and noncompetitive substrate inhibition were fitted to Eqs. [4] and [5], respectively.

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB + AB^2/K_{IB}} \quad [4]$$

$$v = \frac{VAB}{K_{ia}K_b(1 + B/K_{IB}) + K_aB(1 + B/K'_{ib}) + K_bA + AB + AB^2/K'_{ib}}, \quad [5]$$

where K_{IB} and K'_{ib} are the inhibition constants for B, K_{IB} for the E–Q–B, and K'_{ib} for the E–B complexes.

Data for linear multiple inhibition by product and inhibitory substrate were fitted to Eq. [6].

$$v = \frac{V}{1 + I/K_i + J/K_j + IJ/\beta K_iK_j}, \quad [6]$$

where K_i and K_j are inhibition constants for I and J, respectively. I and J denote pyruvate and NAD^+ in the forward reaction and L-lactate and NADH in the reverse reaction, respectively. β is the interaction factor between the two inhibitors.

Whenever possible all fitting of experimental data to the respective equation was carried out by the EZ-FIT (13), a curve-fitting microcomputer program using the Melder–Mead Simplex and Marquardt nonlinear regression algorithms sequentially. The data were also analyzed by another general applicable nonlinear-regression program DNRP-53 (14) where any equation may be entered and analyzed. Both programs gave comparable results.

RESULTS

Initial-Velocity Patterns at Inhibitory Substrate Levels

Linear intercepting initial-velocity patterns for the LDH activity of ϵ-crystallin were obtained at noninhibi-

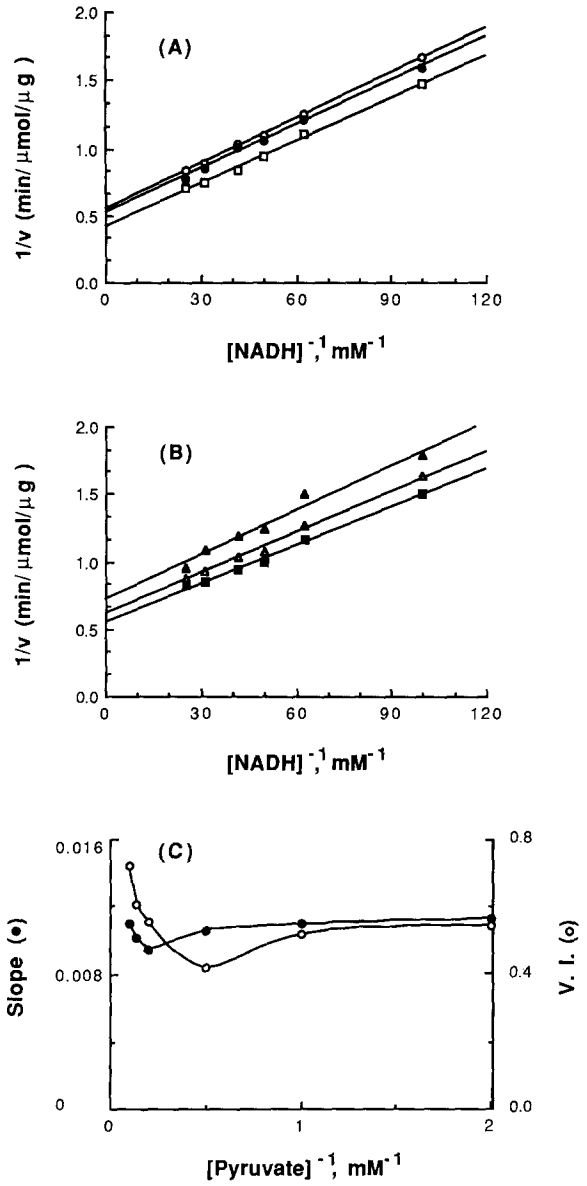

FIG. 1. Uncompetitive substrate inhibition in the forward reaction by pyruvate at several levels of NADH. (A and B) The double-reciprocal plots at subinhibitory and inhibitory pyruvate range, respectively. The pyruvate concentrations were (○), 0.5; (●), 1; (□), 2; (■) 5; (△) 7; and (▲), 10 mM. The slope and intercept versus 1/[pyruvate] replots are shown in C. The ϵ-crystallin used in each assay was 0.167 μg.

tory substrate concentrations (12). However, high concentrations of pyruvate or L-lactate caused substrate inhibition in the forward and reverse reaction, respectively. Figure 1 shows the uncompetitive substrate inhibition by pyruvate with K_{IB} of 6.7 ± 1.7 mM. Both the slopes and intercepts appeared to be changed (Fig. 1C) and the substrate inhibition pattern might be noncompetitive. This result would indicate that pyruvate not only forms an abortive ϵ-crystallin-NAD^+-pyruvate ternary complex but also binds very weakly with free enzyme (15). However, the latter may be not significant since very large dissociation constant (K'_{ib}) was obtained when fitting our data to Eq. [5]. The inhibition pattern thus appears to be uncompetitive, not noncompetitive.

In the reverse reaction (lactate oxidation), uncompetitive substrate inhibition by L-lactate was observed with K_{IB} for L-lactate of 158 ± 25 mM (Fig. 2). This result is consistent with either combination of L-lactate with ϵ-crystallin-NADH or the central ternary complex.

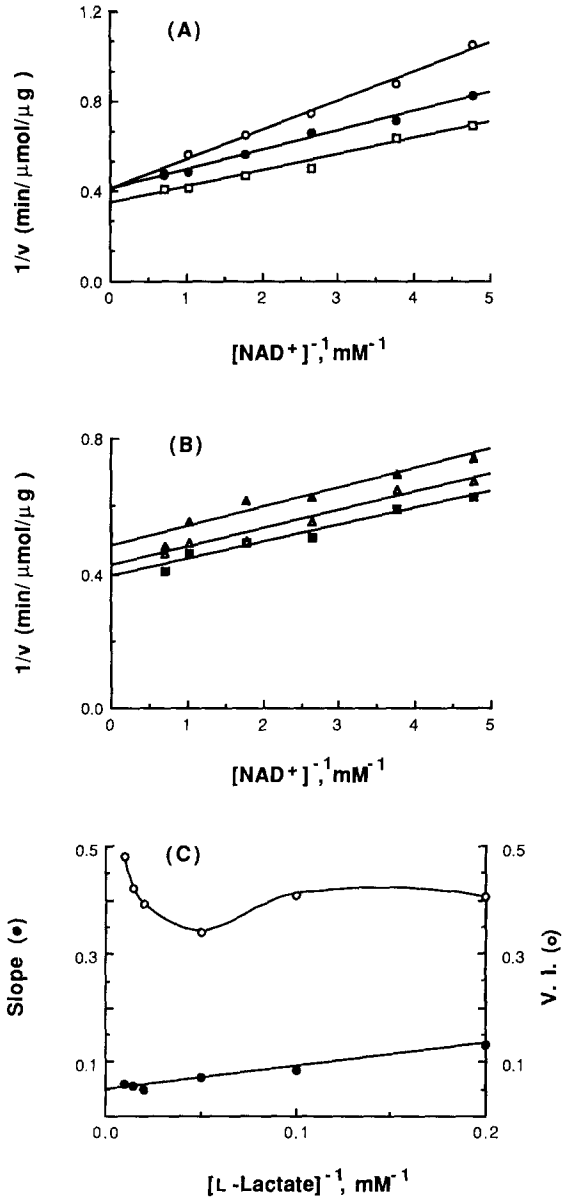

FIG. 2. Uncompetitive substrate inhibition in the reverse reaction by L-lactate at several levels of NAD^+. (A and B) The double-reciprocal plots at subinhibitory and inhibitory L-lactate range, respectively. The concentrations of L-lactate were (○), 5; (●), 10; (□), 20; (■), 50; (△), 70; and (▲), 100 mM. The slope and intercept replots are shown in C. The ϵ-crystallin used in each assay was 0.5 μg.

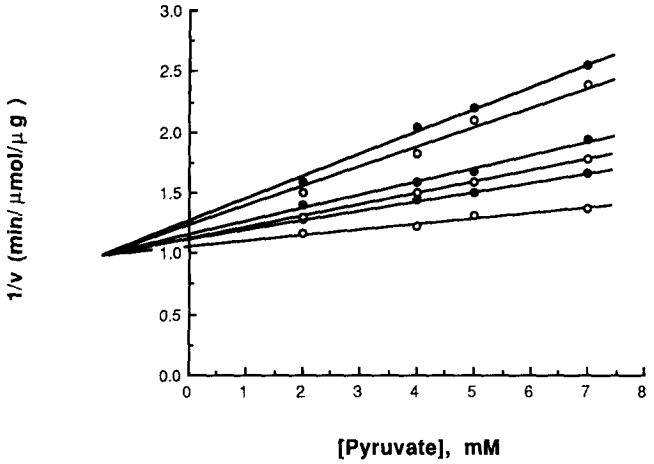

FIG. 3. Multiple inhibition by pyruvate and NAD^+. From top to bottom, the concentrations of NAD^+ were 300, 240, 180, 120, 60, and 0 μM. The ϵ-crystallin used in each assay was 0.2 μg.

Multiple Inhibition Studies

The above results are consistent with an ordered sequential mechanism with substrate inhibition resulting from abortive ϵ-crystallin–NADH–L-lactate ternary complex formation or combination of the inhibitory substrate with the central ternary complexes. Such combination may be tested by multiple inhibition with the second substrate and the final product (16, 17). When product inhibition by NAD^+ was examined at inhibitory levels of pyruvate in the forward reaction (pyruvate reduction), an intersecting pattern was obtained (Fig. 3). The β value estimated from the intersecting point was 0.025.

The substrate inhibition by L-lactate in the reverse reaction was examined in a similar manner. An intersecting pattern was also obtained (Fig. 4), which ruled out the possibility of combination of L-lactate with central complexes. The β value was 0.95.

Interaction of ϵ-Crystallin LDH Activity with Substrate Analogs

Bromopyruvate, fluoropyruvate, pyruvate methyl ester, and pyruvate ethyl ester were found to be alternative substrates in the forward reaction. At noninhibitory substrate levels, all the above compounds gave intercepting initial-velocity patterns. The kinetic parameters obtained by fitting data to Eq. [1] are summarized in Table I. Oxamate (amino oxoacetic acid) was found to be a strong competitive inhibitor versus pyruvate with K_{is} value as low as 27 ± 2 μM.

In the reverse reaction, D-lactate could not replace L-lactate as the substrate, but was a competitive inhibitor with respect to L-lactate. Tartronate (2-hydroxymalonate) was also found to be a competitive inhibitor with respect to L-lactate.

Nucleotide Specificity of ϵ-Crystallin LDH Activity

The LDH activity of ϵ-crystallin required NADH as the coenzyme. NADPH is a poor coenzyme and will decrease the reaction rate of NADH. NADPH, at 0.2 and 2 mM, gave only 2.6 and 16.2% activity, respectively, as compared to NADH, but inhibited 16 and 96%, respectively, of the NADH oxidation rate. In the reverse reaction, $NADP^+$ is also a poor coenzyme and inhibited the NAD^+ reduction. No activity was detectable for 0.2 mM $NADP^+$ and only 5% of the NAD^+ activity was detected for 10 mM $NADP^+$. These concentrations of $NADP^+$ inhibited the NAD^+ reduction by 14 and 52%, respectively.

Several NAD^+ analogs were investigated for their ability to serve as coenzyme for the LDH activity of ϵ-crystallin. ϵ-NAD^+, NHD^+, and $APAD^+$ were found to be alternative coenzymes for ϵ-crystallin catalyzed oxidation

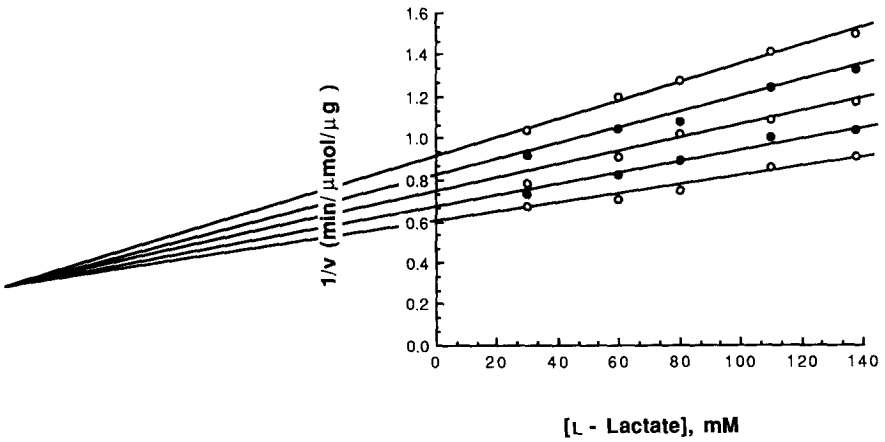

FIG. 4. Multiple inhibition by L-lactate and NADH. From top to bottom, the concentrations of NADH were 80, 40, 24, 8, and 0 μM. The ϵ-crystallin used in each assay was 0.4 μg.

TABLE I

Kinetic Parameters for the Reaction Catalyzed by Duck ε-Crystallin Lactate Dehydrogenase Activity Using Pyruvate Analogs as the Substrate

Pyruvate or analog	k_{cat} (s^{-1})	K_{ia} (μM)	K_a (μM)	K_b (μM)	k_{cat}/K_b ($10^3\ M^{-1}\ s^{-1}$)
Pyruvate	204 ± 32	8.7 ± 1.1	14.0 ± 1.7	95 ± 6.8	2143 ± 492
Fluoropyruvate	98 ± 71	3.3 ± 9.7	11.6 ± 21.6	170 ± 199	577 ± 357
Bromopyruvate	269 ± 195	8.8 ± 4.1	23.8 ± 31.3	620 ± 551	434 ± 354
Pyruvate methyl ester	227 ± 27	3.5 ± 2.9	31.4 ± 6.0	104 ± 24	2183 ± 1125
Pyruvate ethyl ester	176 ± 35	27.9 ± 19	16.8 ± 7.4	62 ± 35	2839 ± 1000

Note. Values shown are averages ± SE.

of L-lactate. Similar results were obtained for chicken heart or muscle LDH (Table II). On the basis of k_{cat}/K_a values, these analogs were less effective than NAD^+ as coenzyme. The initial velocity patterns for the reverse reaction of two NAD^+ analogs, ε-NAD^+ and NHD^+ were examined. When NAD^+ analog was plotted as the varied-concentration substrate, with different concentrations of L-lactate as the fixed-concentration substrate, an intersecting pattern was obtained. When L-lactate was plotted as the varied-concentration substrate, with different concentrations of NAD^+ analog as the fixed-concentration substrate, an intersecting pattern was obtained again. In both cases, the different lines converged to a single point. The K_{ia} for ε-NAD^+ and NHD^+ were found to be 1.14 ± 0.7 and 1.71 ± 0.9 mM, respectively. The K_b for L-lactate was found to be 3.52 ± 0.35 mM.

The nicotinamide-modified analogs, α-NAD^+, AAD^+, $NAAD^+$, and $SNAD^+$ were found to be unable to substitute for NAD^+ as coenzyme in the LDH activity of ε-crystallin. They were strong inhibitors for the enzymatic activity. With NAD^+ as the varied-concentration substrate, linear competitive inhibition was obtained. With L-lactate as the varied-concentration substrate, linear noncompetitive inhibition was obtained. The inhibition constants for slope (K_{is}) and vertical intercept (K_{ii}) from the various reciprocal plots are listed in Table III.

DISCUSSION

The cause of substrate inhibition of the ε-crystallin LDH activity was studied in this report. Initial-velocity patterns at inhibitory substrate levels and the multiple inhibition studies suggested the formation of ε-crystallin–NAD^+–pyruvate and ε-crystallin–NADH–L-lactate abortive ternary complexes as the origin of substrate inhibition. The multiple inhibition experiments also ruled out the possibility of combining pyruvate or L-lactate with the "productive central complexes" since in that case a parallel pattern should be observed (17).

Different β values were obtained for the forward and reverse reactions. In an ordered mechanism, the value of β may indicate the degree to which the release of the final

TABLE II

Nucleotide Specificity for the Duck ε-Crystallin, Chicken Heart and Muscle Lactate Dehydrogenases

Species	Nucleotide analog	k_{cat} (s^{-1})	K_a (mM)	k_{cat}/K_a ($10^3\ M^{-1}\ s^{-1}$)
Duck ε-crystallin	NAD^+	6.9 ± 0.19	0.49 ± 0.04	14.2 ± 4.7
	$APAD^+$	1.4 ± 0.05	0.49 ± 0.03	2.8 ± 0.4
	NHD^+	5.5 ± 0.01	1.48 ± 0.03	3.7 ± 0.3
	ε-NAD^+	5.3 ± 0.05	1.17 ± 0.13	4.5 ± 0.4
Chicken heart LDH	NAD^+	12.1 ± 0.02	0.25 ± 0.02	48.2 ± 0.8
	$APAD^+$	1.6 ± 0.01	0.05 ± 0.01	30.3 ± 0.7
	NHD^+	18.8 ± 0.11	2.83 ± 0.5	6.7 ± 0.2
	ε-NAD^+	6.2 ± 0.04	0.57 ± 0.08	10.1 ± 0.6
Chicken muscle LDH	NAD^+	3.3 ± 0.05	0.7 ± 0.09	4.7 ± 0.5
	$APAD^+$	1.6 ± 0.02	0.5 ± 0.06	2.7 ± 0.4
	NHD^+	1.1 ± 0.08	2.75 ± 0.5	0.4 ± 0.2
	ε-NAD^+	0.7 ± 0.12	2.4 ± 0.47	0.3 ± 0.2

Note. Values shown are averages ± SE.

TABLE III

Inhibition Patterns for the Dead-End Inhibitor of Duck ϵ-Crystallin Lactate Dehydrogenase Activity

Varied-concentration substrate	Fixed-concentration substrate	Inhibitor	Inhibition pattern	Apparent inhibition constant (mM) K_{is}	K_{ii}
		Reverse direction			
NAD^+	L-Lactate	$NAAD^+$	C	0.22 ± 0.03	
L-Lactate	NAD^+	$NAAD^+$	NC	0.43 ± 0.08	3.13 ± 3.13
NAD^+	L-Lactate	$SNAD^+$	C	0.14 ± 0.02	
L-Lactate	NAD^+	$SNAD^+$	NC	2.53 ± 1.28	1.2 ± 0.39
NAD^+	L-Lactate	AAD^+	C	0.29 ± 0.06	
L-Lactate	NAD^+	AAD^+	NC	1.42 ± 0.71	1.84 ± 0.75
NAD^+	L-Lactate	α-NAD^+	NC	3.35 ± 0.75	4.28 ± 0.78
L-Lactate	NAD^+	D-Lactate	C	7.93 ± 0.61	
L-Lactate	NAD^+	Tartronate	C	0.12 ± 0.01	
		Forward direction			
Pyruvate	NADH	Oxamate	C	0.027 ± 0.002	

Note. Abbreviations used: C, competitive; NC, noncompetitive. Values shown are averages ± SE.

product is rate-limiting (17). In the reverse reaction, the β value (0.95) approached 1, suggesting that release of NADH was the rate-limiting step. This result was consistent with the fact that the reduced coenzyme bound more strongly to LDH than the oxidized coenzyme. On the other hand, a β value (0.025) approaching zero was obtained in the forward reaction, suggesting that an earlier step (catalysis or release of L-lactate) was rate-limiting.

The initial-velocity data in this report indicate that various pyruvate and NAD^+ analogs act as alternative substrates or coenzymes for ϵ-crystallin with LDH activity and conform to the same sequential kinetic mechanism. The results obtained with the alternative substrates showed that the structure of pyruvate could be varied considerably without losing its activity as a substrate. The carboxyl group could be esterified and the methyl group could be halogenized. However, if the methyl group was replaced with an amino group, the oxamate (amino oxoacetic acid) could no longer serve as a substrate.

Modification of the nicotinamide moiety of NAD^+ could affect the oxidation–reduction potential of the pyridinium ring or the proper orientation of the coenzyme in binding to the dehydrogenase. The finding that ϵ-NAD^+ and NHD^+ could serve as an alternative coenzyme indicated that an intact adenine structure was not essential for activity. On the contrary, structural changes at the nicotinamide part drastically changed the coenzyme activity. ϵ-Crystallin was unable to utilize α-NAD^+, AAD^+, $SNAD^+$, or $NAAD^+$. These analogs were strong inhibitors for the LDH activity of ϵ-crystallin. The binding ability of these analogs with ϵ-crystallin thus seemed not to be impaired. Pyridine nucleotide analogs substituted with highly electron-withdrawing groups in the 3-position of the pyridinium ring exhibited a more negative oxidation–reduction potential than that of NAD^+, and resulted in an active coenzyme analog. The potential of the $APAD^+$–APADH system is −248 mV which is much more positive than that of the NAD^+–NADH system (−320 mV) and indeed $APAD^+$ was found to be more active than NAD^+ in many dehydrogenase systems (18). However, $APAD^+$ was less active than NAD^+ as a coenzyme for the ϵ-crystallin LDH activity. Although topological similarity between the domains involved in NAD^+ binding in LDH and various dehydrogenases were detected and most dehydrogenases exhibited almost identical protein arrangements in both their nucleotide binding and catalytic domains (18), some differences between various dehydrogenases in their activities associated with different analogs were detected.

In contrast to beef heart or rabbit muscle lactate dehydrogenases which utilized $SNAD^+$ as coenzyme with 41 and 3% activity, respectively, compared to NAD^+ (19), ϵ-crystallin could not use $SNAD^+$ as a coenzyme. The $SNAD^+$–SNADH system has a more positive oxidation–reduction potential (−285 mV) than the NAD^+–NADH system. The thionicotinamide moiety of $SNAD^+$ thus could not exhibit specific interactions with ϵ-crystallin, but rather extended out into solution as suggested for dihydrofolate reductase (20). The kinetic differences found between ϵ-crystallin and other well-documented lactate dehydrogenases, although small, might hint that these proteins may not be the identical products generated from the same gene. It seems more likely that they may originate from the same gene loci but with different post-

translational modifications resulting in different kinetic behaviors.

ACKNOWLEDGMENTS

We thank Professor Foo Pan for critically reading this manuscript. We are also indebted to Dr. Perrella for the EZ-FIT program and Dr. Duggleby for the DNRP-53 program.

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