Kinetic analysis of duck ε-crystallin, a lens structural protein with lactate dehydrogenase activity

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Biochemical characterization and kinetic analysis of ε-crystallin from the lenses of common ducks were undertaken to elucidate the enzymic mechanism of this unique crystallin with lactate dehydrogenase (LDH) activity. Despite the structural similarities between ε-crystallin and chicken heart LDH, differences in charge and kinetic properties were revealed by isoenzyme electrophoresis and kinetic studies. Bi-substrate kinetic analysis examined by initial-velocity and product-inhibition studies suggested a compulsory ordered Bi Bi sequential mechanism with NADH as the leading substrate followed by pyruvate. The products were released in the order L-lactate and NAD^+. The catalysed reaction is shown to have a higher rate in the formation of L-lactate and NAD^+. Substrate inhibition was observed at high concentrations of pyruvate and L-lactate for the forward and reverse reactions respectively. The substrate inhibition was presumably due to the formation of ε-crystallin–NAD^+–pyruvate or ε-crystallin–NADH–L-lactate abortive ternary complexes, as suggested by the product-inhibition studies. The significance and the interrelationship of duck ε-crystallin with other well-known LDHs are discussed with special regard to its role as a structural protein with some enzymic function in lens metabolism.

INTRODUCTION

The abundant presence of various common and specific classes of structural proteins in the lenses, i.e. lens crystallins, in different species of vertebrates and invertebrates provides a good model system to unravel the complex process of protein evolution [1,2]. Previously crystallins were thought to play only a structural role for the maintenance of lens transparency and optical clarity [3–5]. The unexpected findings that sequence similarities existed between crystallins and some metabolic enzymes, for example ε-crystallin and lactate dehydrogenase (LDH) [6], ρ-crystallin and aldehyde/aldose reductase [7] plus bovine lung prostaglandin F synthase [8], and δ-crystallin and argininosuccinate lyase [9], have aroused a lot of interest in aspects concerning the evolution of lens crystallins and their possible enzymic functions in general.

ε-Crystallin has been found to be present as a minor crystallin in duck and caiman lenses [10–12]. It appeared to be structurally similar to avian heart-type lactate dehydrogenase [(*S*)-lactate:NAD^+ oxidoreductase, EC 1.1.1.27], as revealed by peptide mapping [6] and sequence comparison [13]. We have corroborated the finding by re-evaluation of the native molecular mass and the inherent subunit number of ε-crystallin with sedimentation, gel-filtration and cross-linking experiments [14] to establish the apparent similarities in the native and subunit structures of these two proteins. However, detailed enzymic properties of ε-crystallin have not been established. Since the kinetic mechanisms of cytosolic LDHs are well documented [15,16], the present investigation was aimed at the elucidation of the kinetic mechanism of the purified ε-crystallin from the duck lens in order to make a defined mechanistic comparison with other well-known LDHs.

MATERIALS AND METHODS

Crystallin isolation and purification

Duck (also called kaiya duck, a cross-bred hybrid between *Cairina moschata* and *Anas platyrhynchos* var. *domestica*) lenses were obtained from Taiwan Livestock Research Institute at Yinan, Taiwan. The batch preparations of crystallins were routinely carried out from the pooled lenses of about 50 duck eyeballs. They were freshly collected from the above Institute without freezing, decapsulated, and homogenized in 20–30 ml of 0.05 M-ammonium bicarbonate buffer, pH 7.7, containing 5 mM-EDTA as described previously [11,12]. Anion-exchange chromatography on a TSK DEAE-650(M) (2.5 cm × 15 cm) (Merck, Darmstadt, Germany) column was employed for the separation of ε-crystallin from δ-, α- and β-crystallins in the crude lens extract. Elution was carried out in three steps: (A) 200 ml of starting buffer, (B) a 450 ml linear gradient of 0.05–0.5 M-ammonium bicarbonate buffer, pH 8.0, and (C) 200 ml of 1.0 M-ammonium bicarbonate buffer, pH 8.3.

Authentic LDHs from pig heart and muscle and chicken heart and muscle were obtained from Sigma Chemical Co. Chemicals for the activity staining of LDH were from Merck. All reagents and chemicals used were of the highest grades commercially available.

SDS/PAGE and isoenzyme electrophoresis

SDS/PAGE was as described by Laemmli [17] with some modifications. For activity staining native gel (8% resolving gel without stacking) electrophoresis was adopted with Nitro Blue Tetrazolium chloride/5-methylphenazonium methosulphate in the coupled oxidoreduction conversion of lactate/NAD^+ and pyruvate/NADH [18] in the Tris/glycine buffer of Davis [19].

LDH activity assay

LDH activity of ε-crystallin was assayed by monitoring the absorbance change at 340 nm as a function of time. In a standard assay for the forward reaction, the reaction mixture contained 0.1 M-sodium phosphate buffer, pH 7.5, 0.1 mM-NADH and 0.3 mM-pyruvate and an appropriate amount of enzyme in a total volume of 3 ml. For the reverse reaction, NADH and pyruvate were replaced by 1.4 mM-NAD^+ and 20 mM-L-lactate respectively. One unit of enzyme activity is defined as the amount

Abbreviations used: LDH, lactate dehydrogenase; LDH-A_4, muscle-type lactate dehydrogenase; LDH-B_4, heart-type lactate dehydrogenase.
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of enzyme to reduce 1.0 μmol of pyruvate to L-lactate/min at pH 7.5 and 25 °C. A molar absorption coefficient of 6.2×10^3 $M^{-1} \cdot cm^{-1}$ for NADH was used for calculation. Protein concentration was determined spectrophotometrically at 280 nm, by using an absorption coefficient of 1.4 for a 0.1% (w/v) protein solution.

Heat-stabilities of ϵ-crystallin and authentic LDHs were determined by measuring the activities of portions of the sample solutions incubated in phosphate buffer, pH 7.5, in a 50 °C water bath for different periods of time.

Kinetic studies

Initial-velocity studies were performed by varying the concentrations of NADH from 10.4 to 44 μM and of pyruvate from 0.14 to 0.76 mM in the forward reaction, and the concentrations of NAD^+ from 50 to 250 μM and of L-lactate from 0.8 to 6 mM in the reverse reaction. Concentrations of the other components were held constant. A Cary 2290 spectrophotometer was used to achieve maximum sensitivity. The full scale of the recorder was set to 0.01–0.05 absorbance units. A chart speed of 3–10 cm/min was used. The amounts of enzyme (2.5 pmol) were selected to give significant reaction rates and yet to be small enough so that the reaction velocity remained linear for 2–3 min. The signal-to-noise ratio was large enough so that it was not necessary to include hydrazine in the reaction mixture to pull the reaction in the reverse reaction (lactate oxidation). The initial slopes of the recorder tracings were taken as initial velocities. The observed rate of reaction was also shown to be proportional to the amount of protein added.

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.

Kinetic-data processing

Double-reciprocal plots of initial-velocity and product-inhibition data were used to examine the pattern. Slope and intercept replots were used to obtain various kinetic parameters. For the forward reaction, data conforming to a sequential initial-velocity pattern, to linear competitive inhibition and to linear non-competitive inhibition were fitted to eqns. (1), (2) and (3) respectively:

$$v = \frac{V_{forward}[NADH][Pyruvate]}{K_s^{NADH}K_m^{Pyr} + K_m^{NADH}[Pyruvate] + K_m^{Pyr}[NADH] + [NADH][Pyruvate]} \quad (1)$$

$$v = \frac{V_{forward}[NADH]}{K_m^{NADH}(1 + [I]/K_i^{Slope}) + [NADH]} \quad (2)$$

$$v = \frac{V_{forward}[NADH]}{K_m^{NADH}(1 + [I]/K_i^{Slope}) + [NADH](1 + [I]/K_i^{Int})} \quad (3)$$

where K_m^{NADH}, K_m^{Pyr} and K_s^{NADH} are Michaelis constants for NADH and pyruvate and dissociation constant for NADH respectively. $V_{forward}$ denotes the maximum velocity of the forward reaction. [I] is the inhibitor concentration. K_i^{Slope} and K_i^{Int} are the apparent inhibition constants associated with the effect of the inhibitor on slope and intercept of the double-reciprocal plots respectively. K_s^{Pyr}, the dissociation constant for pyruvate, could be obtained from the slope replot of the product inhibition by pyruvate in the reverse reaction when L-lactate was the varied-concentration substrate.

Kinetic studies and data treatment for the reverse reaction were performed in a similar manner with NADH and pyruvate replaced by NAD^+ and L-lactate respectively. The maximum velocity for the reverse reaction is $V_{reverse}$. K_m^{Lact}, $K_m^{NAD^+}$, K_s^{Lact} and $K_s^{NAD^+}$ are Michaelis constants for L-lactate and NAD^+ and dissociation constants for L-lactate and NAD^+ respectively. Similar initial-velocity and product-inhibition equations were used to analyse the data. Whenever possible all fitting of experimental data to the respective equation was carried out by using EZ-FIT [20], a curve-fitting microcomputer program using the Melder–Mead Simplex and Marquardt non-linear-regression algorithms sequentially.

RESULTS

Purification and characterization of duck ϵ-crystallin

Preliminary study of duck crystallins separated on gel-permeation chromatography [11] has indicated contamination by δ- and β-crystallins in the fraction containing ϵ-crystallin, and an attempt has hence been made to separate ϵ-crystallin from δ- and β-crystallins by anion-exchange chromatography [21]. Fig. 1(*a*) shows a rapid one-step purification of crude lens extract from duck lenses on a TSK DEAE-650 anion-exchange column. Eight peaks were obtained, with ϵ-crystallin being eluted in the first fraction. It is notable that ϵ-crystallin can be obtained with greater than 95% purity by such a simple step, as shown by the SDS/PAGE pattern of Fig. 1(*b*). Further purification of ϵ-crystallin by rechromatography of this crystallin on a TSK HW-55 gel-permeation column did not increase the specific enzyme activity of this crystallin (S.-H. Chiou, unpublished work). Therefore all enzymic analyses were performed on ϵ-crystallin directly isolated from the TSK DEAE-650 chromatography and stored in 0.05 M-ammonium bicarbonate buffer, pH 7.5, containing 10% (v/v) glycerol at 4 °C without freeze-drying. It is evident that this new protocol can provide a much larger amount of native ϵ-crystallin than that obtained by gel filtration [10], and it should prove very useful for the isolation and characterization of this crystallin from other related avian species. An identical protocol has also been applied to the chicken lens extract. The first peak eluted from the anion-exchange column showed only the presence of β-crystallin, reinforcing the previous observations that ϵ-crystallin is totally absent from the lenses of chicken and some other avian species, in contrast with its abundant existence in the duck species of Anatidae family {[6,10,21] and S.-H. Chiou (unpublished work)}. The relative yield of this crystallin is estimated to be about 14% from determination of protein contents of various peaks in Fig. 1(*a*).

LDH activity stain and thermostability of ϵ-crystallin

LDH activity stains of ϵ-crystallin and some authentic LDHs after PAGE under native conditions were carried out (Fig. 2). The oxidoreduction reactions involved in the activity stain routinely revealed the high LDH activity from duck lens, in contrast with low activities in the chicken and mammalian lenses [21]. We have included the purified LDH enzymes from pig and

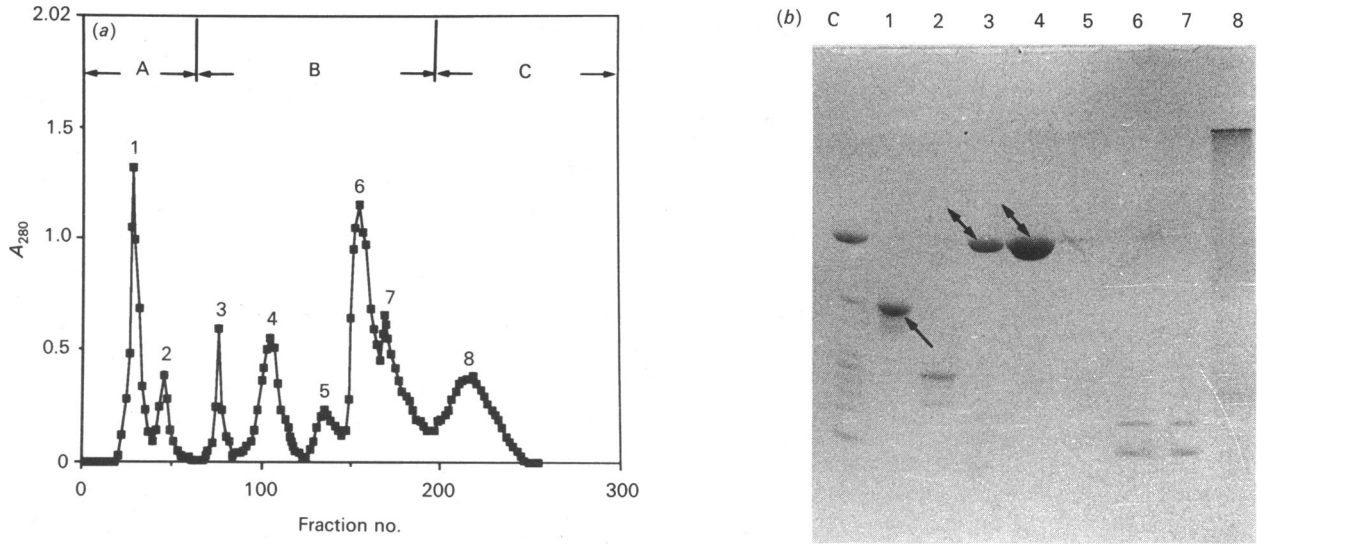


Fig. 1. (*a*) Anion-exchange chromatography of duck lens extract on TSK DEAE-650(M) and (*b*) SDS/PAGE of the resulting fractionated duck crystallins in the presence of dithiothreitol

(*a*) Anion-exchange chromatography of duck lens extract on TSK DEAE-650(M) (2.5 cm × 15 cm column). The column eluate fractions (2.6 ml/tube per 2.8 min) were monitored for absorbance at 280 nm. About 110 mg of crude crystallins dissolved in the starting buffer (0.05 M-ammonium bicarbonate buffer, pH 7.7, containing 0.5 mM-EDTA) was applied to the column equilibrated in the same buffer. Elution was carried out in three steps: A, elution with 200 ml of starting buffer; B, elution with a 450 ml linear gradient of 0.05–0.5 M-ammonium bicarbonate buffer, pH 8.0; C, elution with 200 ml of 1.0 M-ammonium bicarbonate buffer, pH 8.3. Peaks labelled with numbers indicate the eight fractions taken for SDS/PAGE in (*b*). (*b*) SDS/PAGE of the isolated fractionated duck crystallins from (*a*) in the presence of 5 mM-dithiothreitol. Lane C, total crude extract before purification. Lanes 1–8 are the eight peak fractions of (*a*). The gel was stained with Coomassie Blue. Note the relatively pure ε-crystallin (arrow) and δ-crystallin (double arrows) in lanes 1, 3 and 4 by single-step ion-exchange chromatography. The purity of ε-crystallin was shown to be greater than 95%.

chicken tissues for comparison of their electrophoretic mobilities. It is to be noted that LDH of chicken heart tissue from the commercial source (Sigma Chemical Co.) contained two isoenzymes with the heart-type LDH (LDH-B_4) moving further toward the anodal site and another slower species lying slightly above it (Fig. 2, lane 3), presumably the muscle-type LDH (LDH-A_4). In contrast, LDH isoenzymes from duck heart, liver and muscle all moved closely together [14] with similar mobilities to that of purified duck ε-crystallin (Fig. 2, lane 7). In accordance with the pioneering study of chicken LDHs by Pesce *et al.* [22], the faster anodal species was designated as LDH-B_4. Therefore duck ε-crystallin seemed to be close to the mobility of LDH-A_4 despite the convincing sequence similarity of ε-crystallin to that

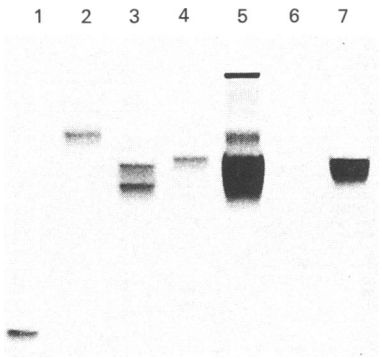


Fig. 2. PAGE under native conditions for the identification of LDHs by activity stain

Conditions were as described in the Materials and methods section. Lane 1, pig heart LDH, 2.0 μg; lane 2, pig muscle LDH, 2.0 μg; lane 3, chicken heart LDH, 3.0 μg; lane 4, chicken muscle LDH, 2 μg; lane 5, duck lens extract, 10 μg; lane 6, purified duck δ-crystallin, 5 μg; lane 7, purified duck ε-crystallin, 1.0 μg. The anode (+) is at the bottom. Note two extra LDH-staining bands in lane 5: one is due to the precipitated enzymes at the top of gel and the other is probably due to the inherent muscle-type LDH in total lens extract.

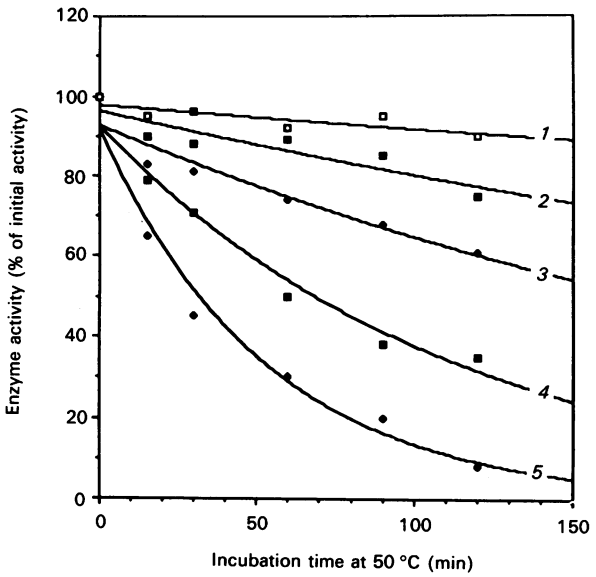


Fig. 3. Comparison of thermostability of LDHs and ε-crystallin

Heat-stabilities of ε-crystallin and authentic LDHs were determined by measuring the activities of portions of the sample solutions incubated in phosphate buffer, pH 7.5, in a 50 °C water bath for different periods of time. The activity measured was the forward reaction of the conversion of pyruvate into L-lactate. Curve *1*, duck ε-crystallin; curve *2*, chicken heart LDH; curve *3*, pig heart LDH; curve *4*, chicken muscle LDH; curve *5*, pig muscle LDH.

Table 1. Comparison of kinetic parameters of LDHs and duck ε-crystallin

The methods used for determination of these parameters are described in the Materials and methods section. LDHs of chicken heart and muscle and of pig heart and muscle were from Sigma Chemical Co. Specific activity was measured in 0.1 M-sodium phosphate buffer, pH 7.5, at 25 °C for the direction of the conversion of pyruvate into L-lactate. K_m values are the Michaelis constants of bi-substrate kinetics for the reaction of the conversion of pyruvate into L-lactate and are derived from the secondary plots of kinetic data.

	Duck ε-crystallin	Chicken LDH-A_4	Chicken LDH-B_4	Pig LDH-A_4	Pig LDH-B_4
Specific LDH activity (units/mg of protein)	510	480	540	620	550
K_m^{NADH} (μM)	14.0	9.2	22.5	11.5	15.6
K_m^{Pyr} (μM)	95.2	185	125	640	180

of chicken heart LDH [6,13]. However, kinetic behaviour in the substrate-inhibition study clearly distinguished ε-crystallin from the chicken muscle LDH, which showed a less pronounced effect of pyruvate inhibition than that of chicken heart-type LDH (see below).

Fig. 3 compares the heat-stabilities of ε-crystallin and various LDHs from pig and chicken tissues. It is noteworthy that ε-crystallin was more stable towards thermal denaturation than LDH of chicken heart, which in turn was more stable than that of chicken muscle. The heat-stability study also corroborated the previous result that avian LDHs were in general more stable than mammalian LDHs, with the avian heart-type LDH most heat-resistant among all vertebrate species studied [23]. It is of interest that duck ε-crystallin is even more stable than chicken LDH-B_4. This may have some bearing for the evolution of ε-crystallin with the kinetic properties of heart-type avian LDH in certain aquatic birds from the viewpoint of structural stability.

Comparison of the enzymic activity of duck ε-crystallin with other LDHs

We have used traditional assays of LDH activity by monitoring the absorbance change of NADH, the essential coenzyme for LDH. The comparisons of this enzymic crystallin and authentic LDHs with regard to some basic kinetic parameters are tabulated in Table 1. ε-Crystallin displays lower Michaelis constants for pyruvate and NADH than those of authentic chicken heart-type enzyme, and moderate substrate inhibition at high pyruvate concentrations. Even after a long period of storage duck ε-crystallin still possessed specific activity comparable with those of purified LDHs.

Substrate inhibition

The effect of substrate concentration on the LDH activity of duck ε-crystallin is shown in Fig. 4. Substrate inhibition was observed with high concentrations of pyruvate and L-lactate in the forward and the reverse reactions respectively. The inhibition patterns for ε-crystallin (Fig. 4*a*) and chicken heart LDH (Fig. 4*c*) are quite similar, in contrast with that of chicken muscle LDH, which shows low inhibition only at high substrate concentrations for both directions (Fig. 4*b*). Care has been taken in the use of the substrate concentrations lying only in the linear part of inhibition curves for the subsequent kinetic experiments. It is also found that the reaction rate for the forward reaction (pyruvate reduction) was much higher than that for the reverse (lactate oxidation).

Initial-velocity studies

The initial-velocity patterns for the forward and the reverse reactions are shown in Fig. 5. When NADH was plotted as the varied-concentration substrate, with different concentrations of pyruvate as the fixed-concentration substrate, an intersecting pattern was obtained (Fig. 5*a*). Similar intersecting patterns were obtained when L-lactate and NAD^+ were used as the substrates in the reverse reaction (Fig. 5*b*). This is one criterion that must be satisfied by all mechanisms of the Bi Bi sequential type. Table 2 lists the various kinetic parameters that were obtained by fitting the data to eqn. (1) for the forward reaction and a similar equation for the reverse reaction. K_s^{NADH}/K_m^{NADH}, K_s^{Pyr}/K_m^{Pyr}, K_s^{Lact}/K_m^{Lact} and $K_s^{NAD^+}/K_m^{NAD^+}$ were all greater than 1. K_s^{NADH} and K_s^{Pyr} were in the same range as K_m^{NADH} and K_m^{Pyr} respectively, whereas K_s^{Lact} and $K_s^{NAD^+}$ were at least an order of magnitude

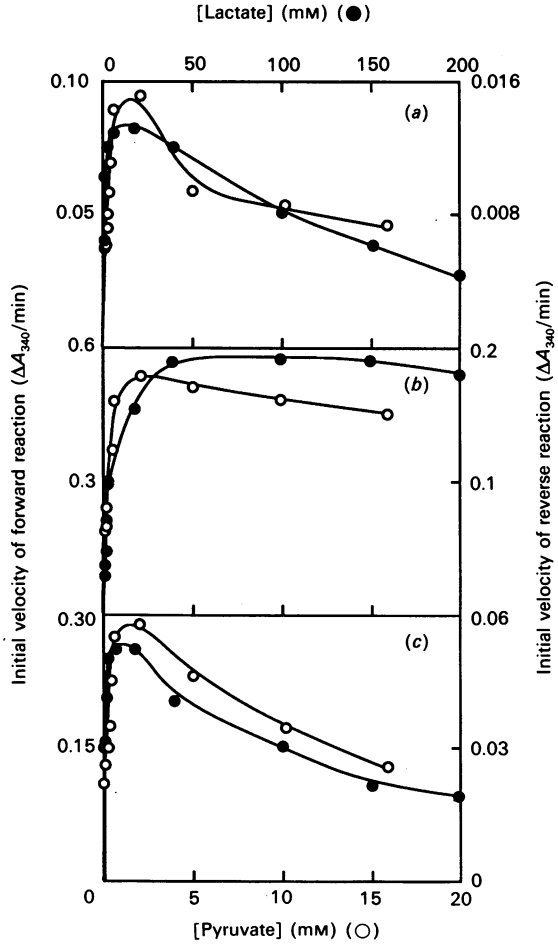


Fig. 4. Substrate inhibition of ε-crystallin and other LDHs

Inhibition of the forward reaction (pyruvate reduction) by pyruvate (○) and the reverse reaction (lactate oxidation) by L-lactate (●) were determined under standard assay conditions. (*a*) Duck ε-crystallin; (*b*) chicken muscle LDH; (*c*) chicken heart LDH.

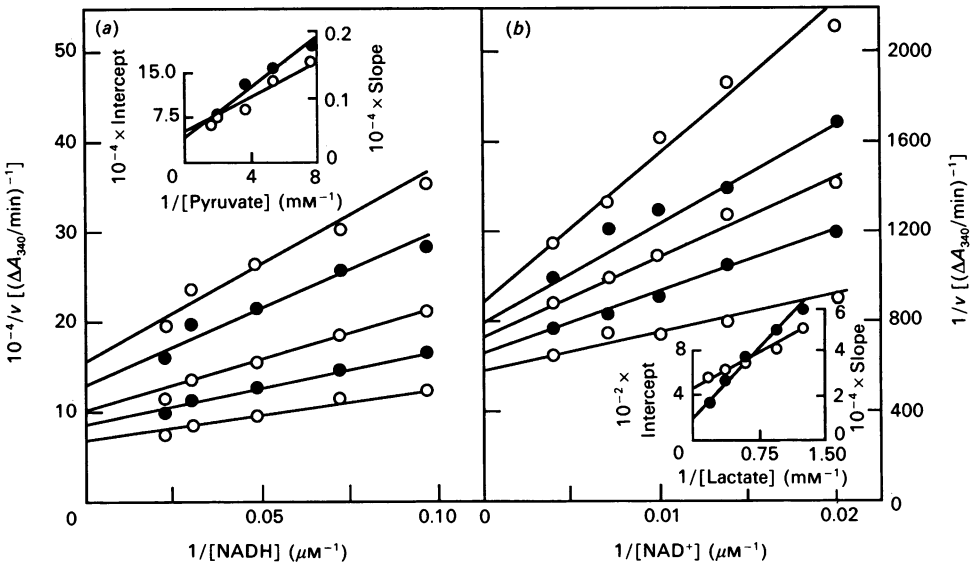


Fig. 5. Initial-velocity patterns of duck ε-crystallin with LDH activity in the forward and reverse directions

(*a*) Forward reaction. The varied-concentration substrate was NADH and the fixed-concentration substrate was pyruvate. From bottom to top the concentrations of pyruvate were 0.76, 0.41, 0.31, 0.18 and 0.14 mM respectively. (*b*) Reverse reaction. The varied-concentration substrate was NAD^+ and the fixed-concentration substrate was L-lactate. From bottom to top the concentrations of L-lactate were 6, 2.8, 1.6, 1.08 and 0.8 mM respectively. The inset Figures were the slope (●) and vertical-intercept (○) replots.

Table 2. Kinetic parameters for the reaction catalysed by duck ε-crystallin with LDH activity

The forward reaction is the conversion of pyruvate and NADH into L-lactate and NAD^+, and the reverse reaction is the conversion of L-lactate and NAD^+ into pyruvate and NADH. Results are given as weighted averages ± S.E.M. calculated from values obtained in two or three experiments.

Forward direction	$V_{forward}$ (units/mg)	K_s^{NADH} (μM)	K_m^{NADH} (μM)	K_s^{Pyr} (μM)	K_m^{Pyr} (μM)
	510	18.7±1.1	14.0±1.7	104±1	95.2±6.8
Reverse direction	$V_{reverse}$ (units/mg)	$K_s^{NAD^+}$ (μM)	$K_m^{NAD^+}$ (μM)	K_s^{Lact} (μM)	K_m^{Lact} (μM)
	9.7	140±0.5	14.5±0.5	33000±1000	590±30

higher than K_m^{Lact} and $K_m^{NAD^+}$ respectively. The higher reaction rate for the forward reaction could be a consequence of its higher $V_{forward}$ or because ε-crystallin had higher affinity for pyruvate and NADH, as reflected in the lower K_s^{NADH} and K_s^{Pyr} than $K_s^{NAD^+}$ and K_s^{Lact} respectively.

Product-inhibition studies

Product-inhibition patterns were analysed to examine the detailed kinetic mechanism of duck ε-crystallin with LDH activity. The inhibition patterns for the reverse reaction are shown in Figs. 6 and 7. With pyruvate as the inhibitor and NAD^+ or L-lactate as the varied-concentration substrate, linear non-competitive inhibition patterns were obtained (Fig. 6). With NADH as the inhibitor and NAD^+ as the varied-concentration substrate, a linear competitive inhibition was obtained (Fig. 7*a*). However, when L-lactate was used as the varied-concentration substrate, the double-reciprocal plot was non-linear (Fig. 7*b*), suggesting the formation of ε-crystallin–NADH–lactate abortive ternary complex.

Similar results were obtained for the forward reaction (results not shown). Inhibition by NAD^+ at low concentration (approx. 2 μM) was non-linear when pyruvate was used as the varied-concentration substrate. At high NAD^+ concentration (180 μM), inhibition of the forward reaction was abolished. The inhibition constants for slope (K_i^{Slope}) and vertical intercept (K_i^{Int}) from various reciprocal plots are listed in Table 3.

DISCUSSION

The abundant presence and stability of ε-crystallin with LDH enzymic activity in the duck lens [14,21] prompted us to make a kinetic study of this crystallin in order to correlate its structural similarity to avian LDH with the corresponding catalytic characteristics. Among the recently characterized crystallins with sequence similarities to those of known functional enzymes [6–9,24], duck ε-crystallin is unique in that it has been definitely shown to possess genuine and comparable enzymic activity *in vitro*, in contrast with some other enzyme-like crystallins devoid of any detectable activities [24].

Kinetic mechanism of duck ε-crystallin with LDH activity

The intersecting initial-velocity results (Fig. 5) ruled out a Ping Pong mechanism, which predicts patterns of parallel lines in the bi-substrate kinetic plots. The LDH activity of duck ε-crystallin

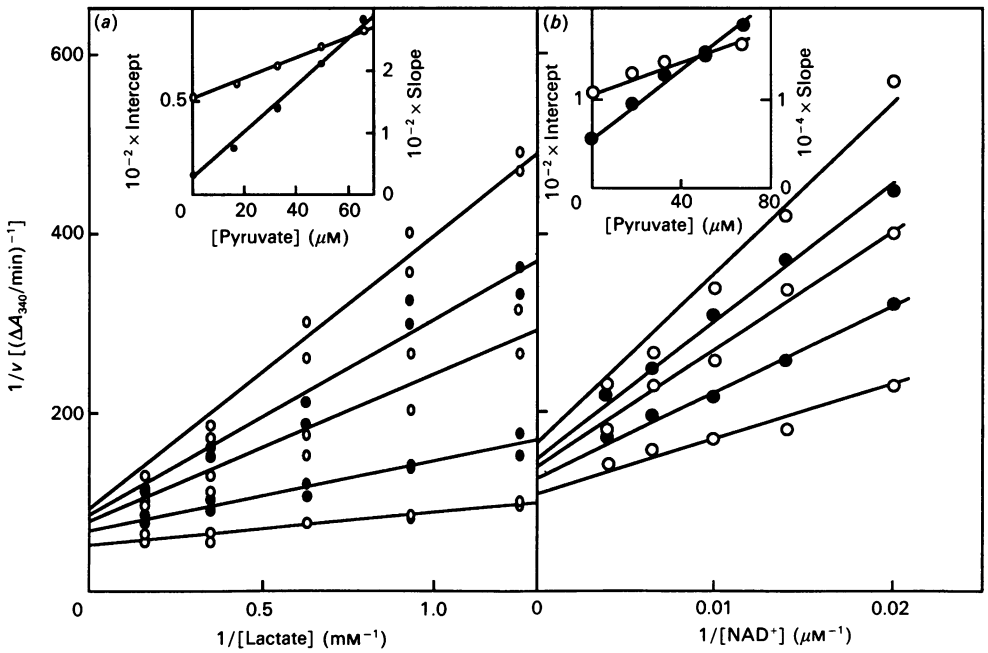


Fig. 6. Inhibition by pyruvate of the reverse reaction of ε-crystallin with LDH activity

(*a*) Non-competitive inhibition with respect to L-lactate. (*b*) Non-competitive inhibition with respect to NAD^+. From top to bottom the concentrations of pyruvate were 67, 50, 33, 16.7 and 0 μM respectively. The inset Figures are the slope (●) and vertical-intercept (○) replots.

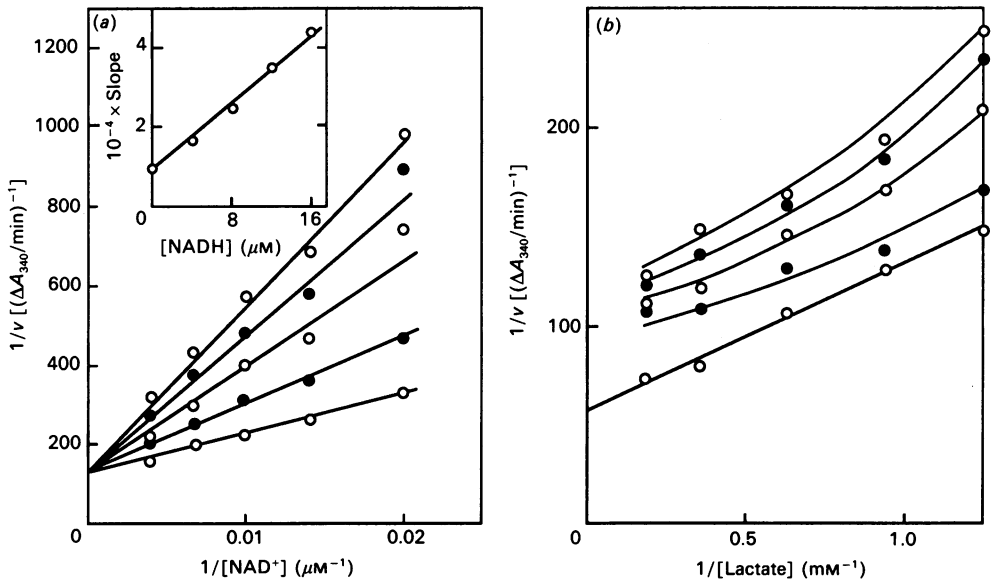


Fig. 7. Inhibition by NADH of the reverse reaction of ε-crystallin with LDH activity

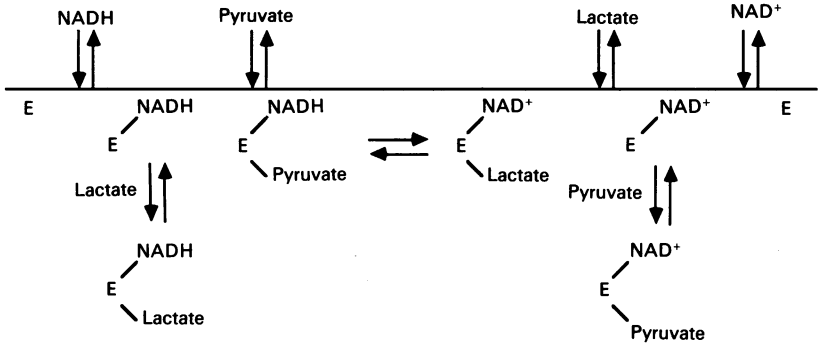
(*a*) Competitive inhibition with respect to NAD^+. From top to bottom the concentrations of NADH were 16, 12, 8, 4 and 0 μM respectively. The inset Figure is the slope replot. (*b*) Non-linear inhibition with respect to L-lactate. From top to bottom the concentrations of NADH were 20, 15, 10, 5 and 0 μM respectively.

thus conformed to the same sequential mechanism as other well-known LDHs [15,16]. The observation of non-competitive inhibition (Fig. 6) by L-lactate with pyruvate as the varied-concentration substrate in the forward reaction and by pyruvate with L-lactate as the varied-concentration substrate in the reverse reaction showed clearly that two or more central ternary complexes are present, and thus ruled out a mechanism of the Theorell–Chance type [15,25]. Random-order mechanisms are also inconsistent with the observed product-inhibition patterns. Furthermore, the points of convergence of the double-reciprocal plots for forward and reverse directions were both above the abscissa, which is allowed for random Bi Bi or ordered Bi Bi, but is forbidden for the Theorell–Chance mechanism [25]. The simplest kinetic mechanism that fits the initial-velocity and product-inhibition data reported in the present study is thus the sequential ordered Bi Bi mechanism. This compulsory-order mechanism requires that both substrates be added before the first product is released, and that the addition of substrates and

Table 3. Inhibition patterns for the product inhibition of duck ε-crystallin with LDH activity

Results are weighted averages ± S.E.M. calculated from two or three experiments.

Varied-concentration substrate	Fixed-concentration substrate	Inhibitor	Inhibition pattern	Apparent inhibition constant (mM) K_i^{slope}	Apparent inhibition constant (mM) K_i^{int}
Forward direction					
NADH	Pyruvate	NAD^+	Competitive	0.067 ± 0.003	
		L-Lactate	Non-competitive	104 ± 11	22 ± 6
Pyruvate	NADH	NAD^+	Non-linear		
		L-Lactate	Non-competitive	26 ± 7	33 ± 2
Reverse direction					
NAD^+	L-Lactate	NADH	Competitive	0.004	
		Pyruvate	Non-competitive	0.025 ± 0.005	0.111 ± 0.003
L-Lactate	NAD^+	NADH	Non-linear		
		Pyruvate	Non-competitive	0.007 ± 0.001	0.105 ± 0.001



Scheme 1. Proposed kinetic mechanism for duck ε-crystallin with LDH activity

release of products follow an obligatory order as depicted in Scheme 1.

ε-Crystallin and rat liver LDH showed some differences in detailed kinetic mechanism. Evidence was provided for the formation of ε-crystallin–NADH–L-lactate and ε-crystallin–NAD^+–pyruvate abortive ternary complexes (Fig. 7*b* and Table 3), whereas no significant amount of abortive LDH–NADH–L-lactate complex was detected for rat liver LDH [16]. Rabbit muscle LDH conforms to a Theorell–Chance mechanism, i.e. no detectable formation of central ternary complexes. The kinetic importance of the productive complexes in the ε-crystallin system was evaluated by the method of Janson & Cleland [26]. The ratio of 'central ternary complexes' to all other enzyme forms (see Scheme 1) present was 5.7. Furthermore, from the data presented in Table 1, we conclude that the kinetics of ε-crystallin activity is closer to those of the heart-type LDH than the muscle-type LDH.

The most salient differences between these two major types of LDHs [27] are (*a*) the turnover number of LDH-A_4 (muscle type) is generally about twice that for LDH-B_4 (heart type), and (*b*) the substrate inhibition by lactate/pyruvate is greater for LDH-B_4 than for LDH-A_4. Fig. 4 clearly justified in the classification of ε-crystallin as heart-type LDH. However, the differences in the mechanistic aspect and kinetic parameters found between ε-crystallin and these well-documented LDHs, although small, might give a hint that these proteins may not be structurally identical.

Biological significance and functional adaptation of ε-crystallin

Lactate dehydrogenase is a 'house-keeping' enzyme in glycolysis, the major metabolic pathway that metabolizes glucose to lactate and generates energy in the form of ATP in the absence of O_2 for most organisms [28]. The fact that ε-crystallin with high LDH activity is found in the lenses of certain avian and reptilian species suggests that glycolysis may have some bearing in the maintenance of optical clarity during lens development. It is of crucial importance to know why this crystallin is identified as being more closely related to heart-type LDH than to muscle-type LDH.

In the classical study of the relationship of LDH isoenzymes and lens differentiation in the bovine system [29], LDH-A_4 was found to be more active in the epithelial cells of calf lenses with a transition to LDH-B_4 in the adults. LDH-B_4 was also found to be more active in the fibre cells than in the epithelial cells; in other words, a shift of synthesis to isoenzyme LDH-B_4 is favoured with the progression of differentiation and aging. It is especially puzzling that in most lens tissues, in which anaerobic glycolysis seems to be the major pathway for glucose metabolism [30], LDH-B_4 instead of LDH-A_4 is selected. It is well known that LDH-A_4 of vertebrates is the major isoenzyme adapted to the anoxic environment of skeletal-muscle cells and that LDH-B_4 is the predominant form in the aerobic cardiac tissues. Kinetic analysis of substrate-inhibition and product-inhibition studies indicates the strong effect of pyruvate on the reaction rate of

pyruvate/lactate conversion, which may have some bearing on the presence of ε-crystallin with LDH-B_4 activity. In order to prevent excess pyruvate from being converted into lactate, which is harmful to the lens and may lead to cataract formation in the acidic microenvironment of lens cells, it may be essential to have LDH-B_4, which is strongly inhibited by high concentrations of pyruvate.

The presence of the specific distribution of different LDH isoenzymes in various tissues does not seem to be a random occurrence, but is of functional importance in tissue metabolism and has developed from natural selection [31,32]. The great variability with which different species and tissues select a specific type of LDH isoenzyme is at present difficult to understand at the molecular level. Hendriks *et al.* [13] proposed that selection of ε-crystallin with LDH-B_4 structure may be based on the structural stability, which is corroborated by our thermostability comparison of these enzymes (Fig. 3). However, stability alone still does not explain the high expression of ε-crystallin in some avian species and not others such as chicken and pigeon. It is of interest that the avian and reptilian species that possess this crystallin all seem to have their natural habitats close to aquatic environments, for example most aquatic birds and the caiman and the alligator.

In conclusion, the distinct differences regarding some mechanistic kinetic properties found between ε-crystallin and other well-documented LDHs, although small, might give an indication that these proteins may not be identical. In contrast with a previous study [13], which suggested that duck ε-crystallin and LDH-B_4 are identical products originating from the same gene locus, the possibility that ε-crystallin is a natural variant or analogue of LDH-B_4 from post-translational modifications cannot be completely ruled out. We believe that further investigation into the molecular basis of the non-random existence and distribution of ε-crystallin in avian and reptilian lenses may be important to shed some light on the molecular origin of this novel aspect of crystallin evolution.

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