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Kinetic Comparison of Caiman ε-Crystallin and Authentic Lactate Dehydrogenases of Vertebrates

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Kinetic comparison of ε-crystallins isolated from the avian and reptilian species and the authentic lactate dehydrogenases (LDHs) was undertaken in order to clarify the identities of these structural lens proteins in relation to their enzymatic activity. Caiman ε-crystallin similar to the previously characterized duck ε-crystallin appeared to possess a genuine and stable LDH activity as detected by nitro blue tetrazolium staining on polyacrylamide gels and conventional kinetic assays. Kinetic parameters for pyruvate, L-lactate, NAD^+, and three structural analogues of the coenzyme in this ε-crystallin catalyzed reaction were also determined and compared. Despite the structural similarities between ε-crystallins and chicken heart LDH, differences in charge and kinetic properties have been revealed by native isozyme electrophoresis and kinetic analysis as examined by initial velocity and substrate inhibition studies. It is found that the kinetic data analyzed for caiman ε-crystallin were more fitted with a compulsory ordered Bi-Bi sequential mechanism similar to those for the authentic LDHs and duck ε-crystallin. Caiman ε-crystallin has for the first time been established as a heart-type LDH based on the kinetic analysis and comparison with the authentic heart- and muscle-type LDHs from pig and chicken.

KEY WORDS: Lens crystallin; ε-crystallin; lactate dehydrogenase; enzyme kinetics; isozyme electrophoresis; eye lens; duck; caiman.

1. INTRODUCTION

Lactate dehydrogenase (LDH; EC 1.1.1.27) is a "house-keeping" enzyme in the glycolysis, the major metabolic pathway that metabolizes glucose to lactate and generates the energy in the form of ATP in the absence of oxygen for most organisms (Metzler, 1977). The presence of the specific distribution of different LDH isozymes 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 (Cahn *et al.*, 1962). Recent crystallin studies have revealed that one of the structural lens proteins (i.e., ε-crystallin isolated from the avian classes) appeared to be structurally and functionally similar to heart-type lactate dehydrogenase as revealed by sequence comparison (Wistow *et al.*, 1987; Hendriks *et al.*, 1988) and kinetic analysis (Chiou and Chang, 1989*a*; Chiou *et al.*, 1990).

ε-Crystallin, similar to another class-specific δ-crystallin, has been found to be present only in the lenses of certain avian and reptilian species (Stapel *et al.*, 1985; Chiou *et al.*, 1987; Chiou *et al.*, 1988*a*). We have carried out a detailed kinetic analysis on the purified duck ε-crystallin to establish the genuine enzymatic activity and a compulsory ordered Bi-Bi sequential mechanism associated with this crystallin (Chiou *et al.*, 1990). The present report was aimed at the isolation and comparison of another ε-crystallin

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isolated from the caiman lenses with duck ε-crystallin and some well-documented avian and mammalian LDHs regarding their kinetic properties. It is of crucial importance to know which type of LDH isozymes the reptilian ε-crystallin belongs to, and the kinetic differences of these dual-function crystallins as compared with other authentic LDHs.

2. MATERIALS AND METHODS

Duck (a cross-bred hybrid between *Cairina Moschata* and *Anas platyrhynchos var. domestica*) lenses were obtained from Taiwan Livestock Research Institute at Yinan, Taiwan. Caiman (*Caiman crocodylus apaporiensis*) lenses were collected from a local reptile farm. The preparation and purification of ε-crystallins from these two species were identical, according to the previous report (Chiou *et al.*, 1989*a*). Anion-exchange chromatography of caiman lens extract was carried out on TSK DEAE-650(M) (2.5 × 15 cm) column. Crude crystallins dissolved in the starting buffer of 0.05 M ammonium bicarbonate with 0.5 mM EDTA, *p*H 7.7, were applied to the column equilibrated in the same buffer. Elution was carried out in two steps: (a) elution with starting buffer, and (b) elution with a 500 ml linear gradient of 0.05–0.5 M ammonium bicarbonate, *p*H 7.9. Authentic lactate dehydrogenases (LDH) from the porcine heart and muscle, and chicken heart and muscle were obtained from Sigma (St. Louis, Missouri). NAD^+ and its three structural analogues were also from Sigma. Chemicals for the activity staining of LDH were from E. Merck (Germany). All reagents used were of the highest grades commercially available.

SDS-polyacrylamide (SDS-PAGE) slab gel (5% stacking/14% resolving gel) was as described by Laemmli (1970), with some modifications. Native gel electrophoresis was carried out on pre-cast gradient gels of 8–25% in a PhastSystem apparatus (Pharmacia-LKB, Sweden). The gels were stained with Coomassie blue for protein bands and detection of LDH activity using nitroblue tetrazolium chloride/5-methylphenazinium methylsulfate (Dietz and Lubrano, 1967).

LDH activity of ε-crystallin was assayed by following 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 (*p*H 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^+, 20 mM L-lactate, respectively.

Initial velocity and substrate inhibition studies were performed essentially as described in Chiou *et al.* (1990). Fitting of experimental data to various kinetic mechanisms was carried out by the EZ-FIT program of Perrella (1988). Calculation of the kinetic parameters was also performed by using the non-linear-regression program (DNRP53) of Duggleby (1984).

The amino acid compositions were determined with the Beckman high-performance amino acid analyzer (Model 6300), using a single-column system based on ion-exchange chromatography. The special procedure for the preparation of protein hydrolysates using microwave irradiation were according to the previous reports (Chen *et al.*, 1987; Chiou and Wang, 1989*b*).

3. RESULTS AND DISCUSSION

Birds and reptiles are phylogenetically more related to each other than other classes of vertebrates (Keeton, 1972). These two classes diverged approximately 200×10^6 years ago and still possess some similar morphological features in the present-day descendant species. ε-Crystallin occurred in crocodiles and alligators (order Crocodylia) and not in snakes (order Squamata) or turtles (order Chelonia) (Stapel *et al.*, 1985; Chiou *et al.*, 1987, 1988*a*). The abundant presence and stability of ε-crystallin with LDH enzymatic activity in the duck lens prompted us to do a kinetic study of the similar ε-crystallin of crocodiles in order to correlate their structural similarity to the corresponding catalytic characteristics and enzymatic mechanism.

Figure 1 shows and compares SDS-PAGE pattern of ε-crystallins isolated from caiman and duck lens and the authentic heart- and muscle-type LDHs of chicken. Over 95% pure ε-crystallin was easily obtained as shown by a single protein band of 37.5 kD in the preparations of this enzymic crystallin from both species using TSK DEAE-650 ion exchanger (Chiou *et al.*, 1989*a*). It differed from the previous gel permeation chromatography of TSK HW-55, and no cross-contamination from the major δ-crystallin was observed. The similarity of duck ε-crystallin to authentic chicken heart LDH regarding some physicochemical properties has been confirmed by estimation of native molecular mass coupled with cross-linking experiments (Chiou *et al.*, 1988*b*). The close

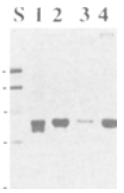

Fig. 1. Gel electrophoresis of purified duck and caiman ε-crystallins under denaturing conditions (SDS-PAGE) in the presence of 5 mM dithiothreitol. Lanes S, standard proteins used as molecular mass markers (in kD): transferrin (80), bovine serum albumin (66), ovalbumin (45), carbonic anhydrase (30), and soybean trypsin inhibitor (20). Lane 1, authentic chicken heart-type LDH (Sigma); lane 2, authentic chicken muscle-type LDH (Sigma); lane 3, duck ε-crystallin; lane 4, caiman ε-crystallin. Ten micrograms of proteins were applied in each lane except duck protein in lane 3 (2 μg). The gel was stained with Coomassie blue. Note that the relatively pure ε-crystallins were obtained by single-step ion-exchange chromatography in contrast to the commercial preparation of avian heart-type LDH which shows two subunit bands (lane 1).

relatedness of the two is also corroborated by similar amino acid compositions (data not shown). Despite some minor difference in aspartic acid, lysine, and arginine, the overall compositions for the rest of amino acids are essentially similar for these four proteins. However, it would be of interest to compare the basic kinetic properties between these proteins in order to provide some insight into the origin of this recently evolved and highly specialized class of protein.

Figure 2 presents the results of LDH isozyme staining on polyacrylamide gel under native conditions. It clearly indicated that our preparations of ε-crystallins indeed possessed genuine and similar

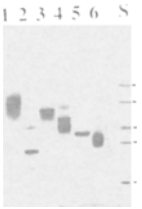

Fig. 2. Isozyme electrophoresis for the detection of lactate dehydrogenase on precast gradient gel of 8–25% under native conditions. Staining conditions are described in Materials and Methods. Lane 1, porcine muscle LDH; lane 2, porcine heart LDH; lane 3, chicken muscle LDH; lane 4, chicken heart LDH; lane 5, caiman ε; lane 6 duck ε; lanes S, standard native proteins used as molecular mass markers (in kD): thyroglobulin (670), ferritin (440), catalase (232), lactate dehydrogenase (140), bovine serum albumin (66).Note that the molecular masses of duck and caiman crystallins correspond closely to marker of lactate dehydrogenase with 140 kD, whereas the authentic LDHs (lanes 1–4) show anomalous mobilities on native gradient gel.

LDH activity when compared with porcine and chicken heart- and muscle-type LDHs. In our previous isozyme electrophoretic experiments using uniform polyacrylamide gel of constant percentage (Chiou *et al.*, 1988*b*, 1989), the mobility pattern of duck ε-crystallin was indistinguishable from those of the authentic chicken heart- and muscle-type LDHs. Using the gradient gel system of native conditions (Fig. 2), caiman and duck ε-crystallins exhibited more mobility differences from those of heart-type LDHs of pig and chicken. This would indicate that avian and reptilian ε-crystallins are distinguishable from the chicken and porcine heart-type LDHs on the basis of charge properties, despite their overall structural similarity. It is intriguing that purified commercial LDH preparations of various types of LDH isozymes still possess a great extent of heterogeneity as revealed by sensitive activity staining on gradient gel in contrast to the result of regular uniform gel. It is difficult to classify ε-crystallin of caiman lens as heart-type LDH as that of duck lens based on the structural data alone. Therefore, a detailed kinetic analysis was carried out to provide some insight on the catalytic behaviors among different classes of LDH isozymes.

We have provided assays of LDH activity by following the absorbance change (340 nm) of NADH, a coenzyme essential for LDH. Even after long periods of storage (more than 6 months), caiman and duck ε-crystallins still possessed comparable specific activity to those of purified LDHs. The comparisons of ε-crystallins and authentic LDHs regarding Michaelis constants of pyruvate and lactate and their ratios (K_m^{lact}/K_m^{pyr}), an index used for distinguishing different types of LDH isozymes, are tabulated in Table I. Caiman ε-crystallin with a ratio of 11.5 was closer to that of 6.4 and 13.7 obtained for chicken and porcine heart-type LDH, and much smaller than that of 38.5 for chicken muscle-type LDH. The apparent maximum velocities were not used for further analysis, since kinetic experiments were performed on different days and did not give comparable maximum velocities because of the instability of the LDH activity of ε-crystallin after high dilution.

The effect of substrate concentration on the LDH activity of duck ε-crystallin is shown in Fig. 3. Substrate inhibition was observed with high concentrations of pyruvate and L-lactate in the forward and reverse reactions, respectively. The inhibition patterns for duck and caiman ε-crystallin (Fig. 3A andB) and chicken and porcine heart LDHs (Fig. 3C and E) are quite similar in contrast to those of chicken and porcine muscle LDHs (Fig. 3D and F), which show

Table I. Kinetic Michaelis Constants for Various ε-Crystallins and LDHs

Protein or enzyme	Substrate	K_m (μM)	K_m^{lact}/K_m^{pyr}
Caiman ε-crystallin	Pyruvate	140	11.5
	Lactate	1610	
Duck ε-crystallin	Pyruvate	82	11.3
	Lactate	930	
Chicken heart LDH	Pyruvate	110	6.4
	Lactate	700	
Chicken muscle LDH	Pyruvate	130	38.5
	Lactate	5000	
Porcine heart LDH	Pyruvate	60	13.7
	Lactate	820	
Porcine muscle LDH	Pyruvate	260	21.5
	Lactate	5600	

small inhibition only at very high substrate concentrations for both directions. Care has been taken in the use of the substrate concentrations lying only in the linear part for the kinetic constants estimation. It is also found that the reaction rate for the forward reaction (pyruvate reduction) was much faster than the reverse reaction (lactate oxidation). Initial velocity pattern for the reverse reaction of LDH activity of caiman ε-crystallin was analyzed to examine the kinetic mechanism (Fig. 4). The data were more fitted with an ordered Bi-Bi mechanism (Perrella, 1988), similar to those for the authentic LDHs and duck ε-crystallin (Chiou *et al.*, 1990). The K_m^{lact}, K_s^{NAD}, and K_m^{NAD} were found to be 1.61 ± 0.3, 0.42 ± 0.1, and 0.16 ± 0.07 mM, respectively.

Several NAD^+ analogues were investigated for their ability to serve as coenzymes in the lactate dehydrogenase activity of caiman ε-crystallin. Nicotinamide-1,N^6-ethenoadenine dinucleotide, nicotinamide hypoxanthine dinucleotide, and 3-acetylpyridine adenine dinucleotide were found to be good alternative coenzymes for the caiman ε-crystallin catalyzed oxidation of L-lactate. Similar results were obtained for duck ε-crystallin, chicken heart or muscle LDH (unpublished observations), and other

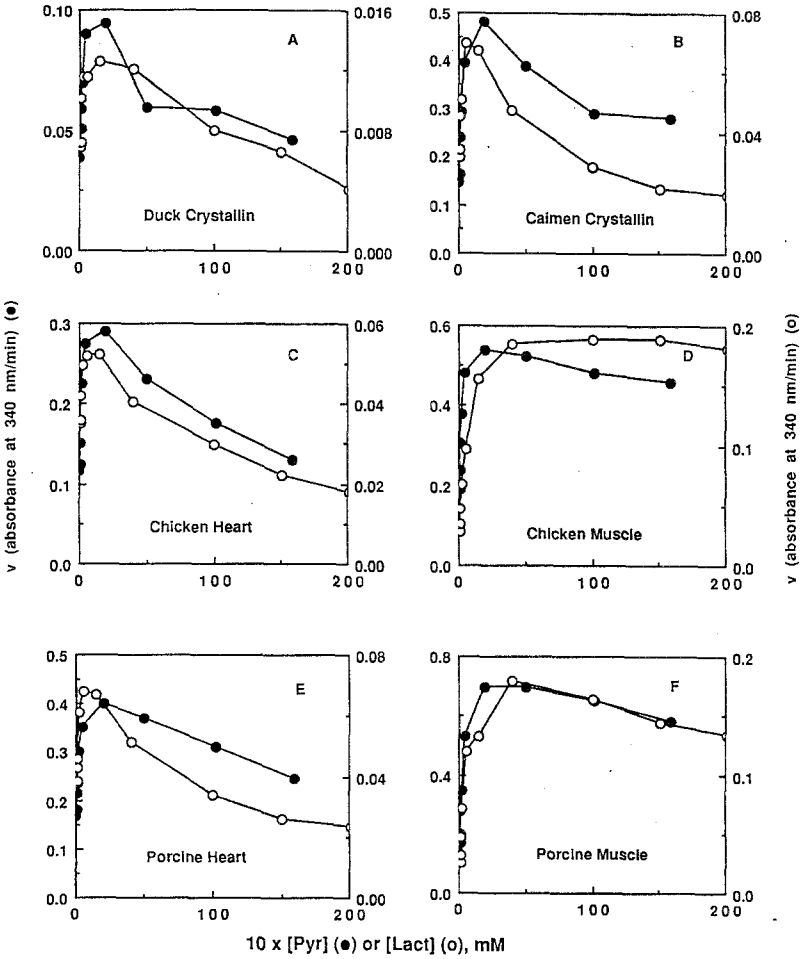

Fig. 3. Substrate inhibition of ε-crystallins and other lactate dehydrogenases. Inhibition of the forward reaction (pyruvate reduction) by pyruvate (●) and the reverse reaction (lactate oxidation) by L-lactate (○) were determined under standard assay conditions. (A) duck ε-crystallin, (B) caiman ε-crystallin, (C) chicken heart LDH, (D) chicken muscle LDH, (E) porcine heart LDH, and (F) porcine muscle LDH.

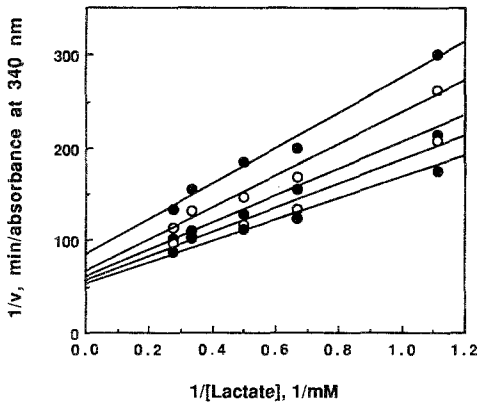

Fig. 4. Initial velocity pattern of the lactate dehydrogenase activity of caiman ε-crystallin in the reverse direction (lactate oxidation). Initial velocity studies were performed with L-lactate as the varied-concentration substrate and NAD^+ as the fixed-concentration substrate. From top to bottom, the [NAD^+] was 0.35, 0.63, 0.98, 1.40, and 2.52 mM, respectively. Concentrations of the other components were held constant. Caiman ε-crystallin used in each assay was 80 ng.

dehydrogenases (Grau, 1982; Denicola-Seoane, 1990). The K_m and k_{cat} values obtained in reactions for caiman ε-crystallin with these analogues are listed in Table II. On the basis of k_{cat} values, the purine-modified analogues were as effective or, in the case of nicotinamide-1,N^6-ethenoadenine dinucleotide, more active than NAD^+ as coenzymes. However, these analogues had higher K_m values, suggesting a weaker binding between the analogues and the ε-crystallin protein. The k_{cat}/K_m values for these analogues were at least an order of magnitude lower than the nature coenzyme NAD^+.

The great variability with which different species and tissues select the specific type of LDH isozymes is at present difficult to understand at the molecular level. The recent finding 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 especially puzzling that in most lens tissues of which the anaerobic glycolysis seem to be the major pathway for glucose metabolism (Kinoshita, 1965), heart-type instead of muscle-type LDH is selected. Substrate inhibition studies shown in this report clearly identify that caiman ε-crystallin, similar to avian ε-crystallin, belongs to heart-type LDH. The genetic basis for the presence of ε-crystallin as a heart-type LDH remains a great challenge for future investigation.

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Table II. Coenzyme Specificity of Lactate Dehydrogenase Activity of Caiman ε-Crystallin

Nucleotide analogues	k_{cat} (sec^{-1})	K_m (mM)	k_{cat}/K_m (10^3 M^{-1} sec^{-1})
Nicotinamide adenine dinucleotide	30.3 ± 0.1[a]	0.19 ± 0.02	163
	(30.7 ± 0.7)[b]	(0.20 ± 0.02)	(154)
Nicotinamide-1,N^6-etheno-adenine dinucleotide	45.8 ± 4.7	3.97 ± 0.86	11.5
	(48.8 ± 8.1)	(4.30 ± 0.64)	(11.3)
Nicotinamide hypoxanthine dinucleotide	19.1 ± 1.0	2.11 ± 0.39	9.1
	(21.0 ± 2.7)	(2.47 ± 0.03)	(8.5)
3-Aminopyridine adenine dinucleotide	3.2 ± 0.3	0.36 ± 0.03	8.8
	(3.2 ± 0.5)	(0.36 ± 0.04)	(8.9)

[a] Calculated by DNRP 53 program of Duggleby (1984).
[b] Values in parentheses were calculated by EZ-FIT program of Perrella (1988).

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