Biochemical characterization and kinetic analysis of duck δ -crystallin with endogenous argininosuccinate lyase activity

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δ -Crystallin, the most abundant crystallin in the avian species, was isolated and purified from duck lenses. It was shown to possess endogenous argininosuccinate lyase activity catalysing the reversible cleavage of argininosuccinate to give fumarate and arginine with an equilibrium constant of 1.8 ± 0.23 mM. In contrast, chicken lens δ -crystallin showed only 0.4–0.8% of the enzyme activity of duck δ -crystallin under identical assay conditions. Biochemical comparison of δ -crystallins from these two species revealed distinct differences in their structural and kinetic properties. An activity-staining method was developed for the easy detection of endogenous enzyme activity of δ -crystallin from crude lens extracts of different avian species. Two-dimensional gel electrophoresis of lens homogenates indicated that, in the chicken lens, δ -crystallin is composed mainly of a subunit with a pI of 5.9 and a subunit mass of 50 kDa, whereas that of duck lens possesses various 50 kDa subunits in a pI range of 5.9–6.8. Activity staining corroborated the fact that all charge isoenzymes of duck δ -crystallin possess enzyme activity whereas that of chicken δ -crystallin is devoid of activity. For duck δ -crystallin, variation of the enzyme activity with argininosuccinate concentration in the forward reaction followed saturation kinetics with an apparent Michaelis constant for the substrate of 17 ± 5 μ M. In the reverse reaction, initial-velocity studies showed intersecting patterns. Inhibitions of the forward reaction by products (fumarate and arginine) were both non-competitive with respect to argininosuccinate. Citrulline, an analogue of arginine, inhibited the enzyme activity in both directions and was competitive with respect to arginine but non-competitive with respect to fumarate or argininosuccinate. Succinate, which inhibited the bovine argininosuccinate lyase, did not affect the δ -crystallin enzyme activity in a concentration range between 1 and 300 mM. These results suggest a random Uni Bi kinetic mechanism for the argininosuccinate lyase activity of duck δ -crystallin with the formation of various abortive δ -crystallin–argininosuccinate–arginine, δ -crystallin–argininosuccinate–fumarate and δ -crystallin–argininosuccinate–citrulline ternary complexes.

INTRODUCTION

The lens crystallins of vertebrates comprise a complex group of conserved structural proteins with distant evolutionary relationships [1–3]. Previously, crystallins were thought to play only a structural role in the maintenance of lens transparency and optical clarity [4–6]. 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 [7,8]. Among them ϵ -crystallin is closely similar to heart-type lactate dehydrogenase [(S)-lactate:NAD⁺ oxidoreductase, EC 1.1.1.27] and δ -crystallin is found to be homologous with argininosuccinate lyase (L-argininosuccinate arginine-lyase, EC 4.3.2.1) [9,10]. These unexpected findings have opened up avenues to the study of the evolution of lens crystallins with enzymic functions and their possible biological significance.

Argininosuccinate lyase, one of the key enzymes in the urea cycle, has been extensively studied in the bovine liver and kidney [11,12] and found in all human tissues examined [13]. The enzyme is found in greater concentrations in the livers of urea-forming animals, where it catalyses the interconversion between argininosuccinate and arginine plus fumarate. In the uricotelic birds and reptiles it is probably involved in the biosynthesis of arginine from ornithine or citrulline. We have developed an ion-exchange method for the single-step purification of the predominant δ -crystallin [14] to near-homogeneity for enzymic characterization. In this report, the charge heterogeneity of duck δ -crystallin subunits was further demonstrated by high-resolution

two-dimensional electrophoresis. Activity staining of the gels under native conditions allowed us to locate all δ -crystallin isoforms with enzyme activity. A detailed kinetic analysis for the argininosuccinate lyase activity of duck δ -crystallin was performed to elucidate the kinetic mechanism of this dual-function crystallin and its possible role in the lens metabolism of avian species.

MATERIALS AND METHODS

Materials

Bovine liver argininosuccinate lyase (specific activity approx. 0.5 unit/mg), fumarase (EC 4.2.1.2) (from pig heart, affinity-purified, 360 units/mg), argininosuccinic acid (barium salt), fumarate, arginine, citrulline, NADP⁺, phenazine methosulphate and Nitro Blue Tetrazolium were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). PhastGels were from Pharmacia-LKB (Uppsala, Sweden). Other chemicals were of reagent grade obtained either from Sigma Chemical Co. or E. Merck (Darmstadt, Germany).

Malic enzyme (EC 1.1.1.40) from pigeon liver was purified to homogeneity according to our previously described procedure [15]. Argininosuccinate from Sigma Chemical Co. was purified and converted into the potassium salt according to the method of Ratner [16]. The concentration of argininosuccinate was checked by the method of Kuchel *et al.* [17]. Argininosuccinate solution stored at 4 °C for 1 month showed only about 2.5% cyclization, which was considered to be within the experimental error of the

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kinetic studies. Correction for the argininosuccinate cyclization was found unnecessary for the freshly prepared argininosuccinate solution.

Ducks (mule ducks, a hybrid between *Cairina moschata* and *Anas platyrhynchos* var. *domestica*) were obtained from Taiwan Livestock Research Institute at Yinan, Taiwan. δ -Crystallin was purified from duck eye-balls by one-step TSK DEAE-650(M) anion-exchange chromatography as described previously [14]. The purities of the crude lens homogenates and all fractions obtained from the above DEAE chromatography were routinely checked by SDS/PAGE before they were used in the kinetic analysis. δ -Crystallin concentration was determined spectrophotometrically at 280 nm by using an absorption coefficient of 5.5 for a 1% (w/v) protein solution.

One- and two-dimensional SDS/PAGE

SDS/PAGE (slab gel; 5% stacking/14% resolving gel) was as described previously [18] with some modifications. Two-dimensional electrophoresis was performed according to a modified procedure of O'Farrell [19], using isoelectric focusing of carrier ampholytes of pH 3.5–10 in the first dimension and SDS/PAGE in the second dimension.

PAGE under native conditions and activity staining of argininosuccinate lyase activity in gels

Electrophoresis under native conditions was performed in duplicate with a Pharmacia PhastSystem in PhastGel homogeneous 12.5% mini-slab gel and PhastGel native buffer strip (containing 0.88 M-L-alanine and 0.25 M-Tris/HCl buffer, pH 8.8). The samples were subjected to electrophoresis at 400 mA and 15 °C for 220 V·h. After electrophoresis, one of the gels was subjected to protein staining with Coomassie Blue and the other to enzyme activity staining. Activity stain solution consisted of 0.1 M-Tris/HCl buffer, pH 7.7, containing 1 mM-argininosuccinate, 7 units of fumarase/ml, 2.8 mM-MnCl₂, 0.38 mM-NADP⁺, 0.1 unit of malic enzyme/ml, 0.4 mg of Nitro Blue Tetrazolium/ml and 0.07 mg of phenazine methosulphate/ml. The stain solution was prepared just before use.

Staining for enzyme activity was performed by immersing the gel in 10 ml of stain solution. The gel was allowed to stay in the solution overnight at room temperature for maximal colour development. The reaction was stopped by replacing the stain solution with 10% (v/v) ethanol/5% (v/v) acetic acid fixing solution. All components in the stain solution were found to be essential. Good correlation between the colour yield and incubation time or amounts of δ -crystallin applied was also demonstrated in our laboratory.

Isoelectric focusing of the native δ -crystallin was also performed in duplicate with PhastGel IEF 4–6.5 plate (coating with polyacrylamide gel and forming a linear pH gradient from 4 to 6.5 with Pharmalyte). Electrophoresis was performed at 25 mA, 2000 V and 15 °C (500 V·h). One plate was subjected to activity staining and another to protein staining. Various marker proteins covering a wide pI range were used as calibrating standards.

Assay of enzyme activity

Argininosuccinate lyase activity of duck δ -crystallin was assayed at 25 °C. The formation of fumarate in the forward direction and disappearance of fumarate in the reverse direction were monitored continuously at 240 nm in a Varian DMS-100 spectrophotometer. In a standard assay for the forward reaction, the reaction mixture contained 50 mM-Tris/HCl buffer, pH 7.5, 0.2 mM-argininosuccinate and an appropriate amount of δ -

Fig. 1. Gel electrophoresis of the fractionated duck crystallins from TSK DEAE-640(M)

Denaturing conditions (SDS/PAGE) in the presence of 5 mM-dithiothreitol. Lane S, standard proteins used as molecular-mass markers (from top to bottom): BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa) and soyabean trypsin inhibitor (20 kDa). Lanes 1–6 correspond to the six separated peak fractions from anion-exchange chromatography. Lane 7 is the crude lens homogenate before purification. Note that the 37 kDa protein band in lane 1 is ϵ -crystallin with lactate dehydrogenase activity whereas lanes 2–4 contain δ -crystallin (50 kDa) with argininosuccinate lyase activity. δ -Crystallin from peak 3 (lane 3) with the highest purity of the three fractions was used for the detailed kinetic analysis. The gels were stained with Coomassie Blue.

Fig. 2. Activity staining for the argininosuccinate lyase activity of δ -crystallin in polyacrylamide gel

Electrophoresis under native conditions was as described in the Materials and methods section. (a) Gel stained for protein with Coomassie Blue. (b) Gel stained for argininosuccinate lyase activity (cathode is at the top). Lane 1, molecular mass markers (from bottom to top): BSA (66 kDa), lactate dehydrogenase (140 kDa), catalase (232 kDa), ferritin (440 kDa) and thyroglobulin (670 kDa); lane 2, crude duck lens extract (11.2 μ g); lanes 3–5, duck δ -crystallin fractions of Fig. 1 (peak 2, 3.1 μ g, peak 3, 4.2 μ g, and peak 4, 3.8 μ g respectively); lane 6, crude chicken lens extract (13 μ g); lanes 7 and 8, chicken δ -crystallin fractions isolated under identical conditions for duck crystallin purification (peak 2, 6.1 μ g and peak 3, 3.1 μ g, respectively).

crystallin in a total volume of 1 ml. For the reverse reaction, argininosuccinate was replaced by 0.2 mM-fumarate and 1 mM-arginine. One unit of enzyme activity was defined as an initial rate of 1 μ mol of fumarate released or consumed/min under the assay conditions. A molar absorption coefficient of $2.44 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for fumarate was used for calculation [20].

Fig. 3. Two-dimensional gel analysis of the duck crude homogenate and purified δ -crystallin

About 50–100 μ g of crude homogenate (a) and 5 μ g of purified duck δ -crystallin (b) were layered on a 5% polyacrylamide gel for isoelectric focusing (IEF) in the first dimension (basic end is to the right), followed by discontinuous SDS/PAGE in the second dimension (higher molecular mass is at the top). In (a) and (b) a concurrent run of the standard marker proteins (the four proteins from Fig. 1 plus 14 kDa lysozyme) in the second dimension is shown on the left lane. Both samples were run under denaturing conditions in 8 M-urea and 0.1% 2-mercaptoethanol. Arrows indicate the positions of δ -crystallin subunits with approximate pI values between 5.9 and 6.8 determined from a pI calibration kit (range 4.7–10.6; BDH Chemicals). In the chicken lens homogenates, more than 90% of δ -crystallin exists as the acidic form of pI 5.9 (results not shown).

Kinetic studies

Detailed kinetic studies were performed on peak 3 duck δ -crystallin, which showed highest purity (Fig. 1). Initial-velocity studies for the reverse reaction were performed by varying the concentration of arginine from 0.2 to 1 mM and of fumarate from 0.1 to 0.5 mM. The other components were held constant.

Inhibition studies were performed in a similar manner, with one of the substrates as the varied-concentration substrate while the other substrate was held constant. The inhibitor was held at several fixed concentrations around its inhibition constant.

In all kinetic studies the variable substrate concentrations used were near the K_m value. The full scale of the recorder was set at 0.01 absorbance unit. A chart speed of 1–10 cm/min was used. The amounts of δ -crystallin were selected to give significant

Fig. 4. Isoelectric focusing of duck and chicken δ -crystallins under native conditions

Duck and chicken δ -crystallins were subjected to isoelectric focusing under native conditions as described in the Materials and methods section (basic end is at the top). Lanes 1 and 8, calibration standards (pI values 4.15–6.55; Pharmacia-LKB); lanes 2–4, duck δ -crystallin fractions of Fig. 1 (peak 2, 3.1 μ g, peak 3, 4.2 μ g, and peak 4, 3.8 μ g respectively); lanes 5 and 6, chicken δ -crystallin fractions isolated under conditions identical with those used for duck crystallin purification (peak 2, 6.1 μ g, and peak 3, 3.1 μ g respectively); lane 7, bovine liver argininosuccinate lyase. The gels were stained with Coomassie Blue to reveal the extensive charge-isomeric forms of duck δ -crystallin. Note that in lanes 5 and 6 chicken δ -crystallin showed almost no activity-staining bands, in contrast with strong staining intensity for duck δ -crystallins (see Fig. 2b).

reaction rates and yet to be small enough so that the reaction velocity remained linear for 2–3 min. The initial slopes of the recorder tracings were taken as initial velocities.

Kinetic data processing

Data conforming to saturation kinetics were fitted to eqn. (1):

$$v = \frac{V[A]}{K_a + [A]} \quad (1)$$

where K_a is the Michaelis constant for substrate A. V denotes maximum velocity.

Data from the initial-velocity studies for the reverse reaction were fitted to eqn. (2) assuming an equilibrium condition (see the Discussion section):

$$v = \frac{V[P][Q]}{K_{ip}K'_{iq} + K'_{ip}[Q] + K'_{iq}[P] + [P][Q]} \quad (2)$$

where K_{ip} , K'_{ip} and K'_{iq} are all dissociation constants and are defined in Table 1. P denotes arginine and Q denotes fumarate.

Product and dead-end inhibitions, conforming to linear competitive and linear non-competitive inhibition, were fitted to eqns. (3) and (4) respectively:

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

$$v = \frac{V[A]}{K_a(1 + [I]/K_{is}) + [A](1 + [I]/K_{ii})} \quad (4)$$

Fig. 5. Reversible cleavage of argininosuccinate catalysed by duck lens δ -crystallin

(a) Duck δ -crystallin (peak 3, 42 $\mu\text{g/ml}$) was added to the reaction mixture, which contained 50 mM-Tris/HCl buffer, pH 7.5, and 0.1 mM-argininosuccinate (forward reaction, curve A) or 1 mM-arginine plus 0.2 mM-fumarate (reverse reaction, curve C), and the A_{240} was monitored. For control, A_{240} of the solution containing 50 mM-Tris/HCl and 0.1 mM-argininosuccinate was also monitored (curve B) to demonstrate the stability of the substrate during the assay period. (b) Initial-velocity measurement of duck (traces A, B and C) and chicken (traces D and E) δ -crystallin argininosuccinate lyase activity. Assay conditions were the same as in curve A of (a), except that the δ -crystallins used were: A, duck, peak 2 (3.8 $\mu\text{g/ml}$); B, duck, peak 3 (5.2 $\mu\text{g/ml}$); C, duck, peak 4 (1.5 $\mu\text{g/ml}$); D, chicken, peak 2 (61 $\mu\text{g/ml}$); E, chicken, peak 3 (31 $\mu\text{g/ml}$).

where [I] is the inhibitor concentration. K_{is} and K_{ii} are the inhibition constants associated with the effect of the inhibitor on slope (K_{is}) and intercept (K_{ii}) of the double-reciprocal plots respectively.

Fig. 6. Initial-velocity pattern of duck lens δ -crystallin with argininosuccinate lyase activity in the reverse direction

(a) The varied-concentration substrate was arginine and the fixed-concentration substrate was fumarate. From top to bottom the concentrations of fumarate were 0.1, 0.13, 0.18, 0.3 and 0.5 mM. (b) The same data were plotted with fumarate as the varied-concentration substrate and arginine as the fixed-concentration substrate. From top to bottom the concentrations of arginine were 0.2, 0.25, 0.33, 0.5 and 1 mM. The amount of δ -crystallin used in each assay was 1.4 $\mu\text{g/ml}$.

The equilibrium constant is defined as:

$$K_{eq} = \frac{[\text{fumarate}][\text{arginine}]}{[\text{argininosuccinate}]} \quad (5)$$

All fittings of the experimental data to the respective eqns. (1)–(4) were carried out by a non-linear-regression program DNRP-53 [21]. The points drawn in the Figures showing double-reciprocal plots are the experimentally determined values. The lines drawn through these points are calculated from the fits of these data to the corresponding rate equations.

Table 1. Limiting kinetic parameters of the argininosuccinate lyase activity of duck lens δ -crystallin

Abbreviations: A, argininosuccinate; P, arginine; Q, fumarate; E, δ -crystallin. Values shown are means $\pm$ S.E.M. ($n = 3$).

Constant	Description	Value
V_f	Maximum velocity of the forward direction	0.29 ± 0.015 unit/mg
V_r	Maximum velocity of the reverse direction	0.25 ± 0.006 unit/mg
K_a	Michaelis constant for A	0.017 ± 0.005 mM
K_p	Michaelis constant for P	0.098 ± 0.025 mM
K_q	Michaelis constant for Q	0.056 ± 0.02 mM
K_{ip}	Dissociation constant for EP	0.51 ± 0.18 mM
K_{iq}	Dissociation constant for EQ	0.46 ± 0.09 mM
K_{ipq}	Dissociation constant for EPQ (to EQ and P)	0.047 ± 0.02 mM
K_{iqp}	Dissociation constant for EPQ (to EP and Q)	0.042 ± 0.01 mM
K_{eq}	Equilibrium constant	1.8 ± 0.23 mM

RESULTS

Heterogeneity of duck δ -crystallins

Six separated fractions of duck δ -crystallin were eluted from the TSK DEAE-650 column by 0.05–0.5 M-NH₄HCO₃ gradient elution [14]. SDS/PAGE analysis of these fractions is shown in Fig. 1. The 37 kDa protein band in fraction 1 is ϵ -crystallin with lactate dehydrogenase activity which we have characterized previously [14,22]. Fractions 2, 3 and 4 contained δ -crystallin, which characteristically possesses a 50 kDa subunit. Electrophoresis under native conditions revealed heterogeneity of these δ -crystallins (Fig. 2a). Activity staining showed that only the low anodal mobility bands possessed endogenous argininosuccinate lyase activity (Fig. 2b). Chicken δ -crystallin with high anodal mobility was not stained under identical conditions.

The heterogeneity of the δ -crystallin was further analysed by two-dimensional electrophoresis. The chicken lens δ -crystallin is composed mainly of a subunit with a pI of 5.9 and a subunit mass of 50 kDa whereas that of duck lens possesses various 50 kDa subunits in a pI range 5.9–6.8 (Fig. 3). Multiple protein bands with pI values higher than 5.9 were also resolved by isoelectric focusing under native conditions (Fig. 4). Only those protein bands with higher pI values were shown to possess enzyme activity (results not shown). The enzyme activity of δ -crystallin was further corroborated by steady-state kinetics.

Argininosuccinate lyase activity of duck δ -crystallin

Duck δ -crystallin catalyses the cleavage of argininosuccinate to give arginine and fumarate as demonstrated by activity staining

Fig. 7. Product inhibition of the argininosuccinate lyase activity of duck lens δ -crystallin

(a) Inhibition of the forward reaction by fumarate. From bottom to top the concentrations of fumarate were 0, 0.1, 0.2 and 0.3 mM. (b) Inhibition of the forward reaction by arginine. From bottom to top the concentrations of arginine were 0, 3, 5 and 7 mM. The amount of δ -crystallin used in each assay was 1.4 μ g/ml.

Fig. 8. Dead-end inhibition of the argininosuccinate lyase activity of duck lens δ -crystallin by citrulline

(a) Non-competitive inhibition of the forward reaction. (b) Non-competitive inhibition of the reverse reaction with fumarate as the varied-concentration substrate. (c) Competitive inhibition of the reverse reaction with arginine as the varied-concentration substrate. In (a) and (b), from bottom to top the concentrations of citrulline were 0, 25, 50 and 75 mM. In (c), from bottom to top the concentrations of citrulline were 0, 5.7, 17.1 and 37.5 mM. The amount of δ -crystallin used in each assay was 2.8 μ g/ml.

(Fig. 2b). The enzyme reaction was also manifested by the fact that, in the assay period, absorbance at 240 nm was increased due to fumarate formation (Fig. 5a, curve A). Under identical conditions but without δ -crystallin, argininosuccinate was shown to be stable (Fig. 5a, curve B). A commercially available bovine liver argininosuccinate lyase was also assayed under the same conditions and gave results comparable with those for δ -crystallin (results not shown).

Recently we have also shown that duck δ -crystallin catalyses the synthesis of argininosuccinate from arginine and fumarate very efficiently [23]. The reaction can be easily followed by measuring the decrease in absorbance at 240 nm (Fig. 5a, curve C). Duck δ -crystallin was thus demonstrated to catalyse the reversible cleavage of argininosuccinate. The forward and

Table 2. Inhibition patterns for the product and dead-end inhibitors of the argininosuccinate lyase activity of duck lens δ -crystallinAbbreviations: C., competitive; N.C., non-competitive. Values shown are means $\pm$ S.E.M. ($n = 3$).

Type of inhibition	Varied-concentration substrate	Fixed-concentration substrate	Inhibitor	Inhibition pattern	Inhibition constant (mM)	
					K_{is}	K_{ii}
Product inhibition	Argininosuccinate	–	Fumarate	N.C.	0.13 ± 0.08	0.46 ± 0.2
		–	Arginine	N.C.	4.9 ± 1.8	3.8 ± 0.6
Dead-end inhibition	Argininosuccinate	–	Citrulline	N.C.	37.3 ± 10.8	125 ± 20
	Fumarate	Arginine	Citrulline	N.C.	93 ± 85	65.6 ± 15.3
	Arginine	Fumarate	Citrulline	C.	7.6 ± 1.8	

reverse reactions have comparable maximum velocities (Table 1). The equilibrium constant measured from both directions was 1.8 ± 0.23 mM (Table 1).

The two δ -crystallin fractions isolated from chicken lenses by the same TSK DEAE-650 (M) chromatographic method contained only 0.4% and 0.8% of the argininosuccinate lyase activity of duck δ -crystallin under identical assay conditions (Fig. 5b).

Initial-velocity studies

The initial-velocity patterns for the reverse reaction are shown in Fig. 6. When arginine was plotted as the varied-concentration substrate, with different concentrations of fumarate as the fixed-concentration substrate, an intersecting pattern was obtained (Fig. 6a). When fumarate was plotted as the varied-concentration substrate, with different concentrations of arginine as the fixed-concentration substrate, an intersecting pattern was also obtained (Fig. 6b). Fitting the data to eqn. (2) yielded various kinetic constants that are given in Table 1 together with other constants.

Inhibition studies

Product and dead-end inhibition patterns were analysed to examine the detailed kinetic mechanism of duck δ -crystallin with argininosuccinate lyase activity. The product inhibition for the forward reaction is shown in Fig. 7. Inhibitions by fumarate and arginine were both non-competitive with respect to argininosuccinate. In both cases the double-reciprocal plot showed lines intersecting at a point above the x -axis.

Citrulline was found to inhibit both directions of the enzyme reaction. Citrulline was a non-competitive inhibitor with respect to argininosuccinate in the forward reaction (Fig. 8a). In the reverse reaction citrulline was a non-competitive inhibitor with respect to fumarate (Fig. 8b) but competitive with respect to arginine (Fig. 8c). The inhibition constants obtained by fitting the inhibition data to eqns. (3) and (4) are summarized in Table 2. Succinate, which inhibited bovine argininosuccinate lyase, did not affect δ -crystallin enzyme activity in a concentration range between 1 and 300 mM.

DISCUSSION

We have demonstrated the endogenous argininosuccinate lyase activity of the purified duck δ -crystallin by kinetic assays and direct staining of the enzyme activity in gels. In contrast, we have found that chicken δ -crystallin contained very little enzyme activity. It is also demonstrated that the duck δ -crystallins possess inherent heterogeneities associated with their native forms and subunits. Since protein bands, as exemplified in Figs. 2 and 4, were run under native conditions, these multiple protein

bands could not be due to the artifacts discussed in the previous review [24].

It has been stated that in the embryonic duck lens ten δ -crystallin cDNAs were derived from one δ -crystallin gene and two from another [9]. The avian δ -crystallin locus consists of two tandemly arranged highly homologous genes δ_1 and δ_2 [25,26]. The δ_2 -crystallin is the best candidate for the role of active argininosuccinate lyase. In chicken, the expression of the δ_2 gene is repressed in lens cells [27–29]. The chicken δ_1 -crystallin thus represents a natural mutant of argininosuccinate lyase devoid of enzyme activity. Comparison of the purified δ -crystallin from duck and chicken on two-dimensional gels revealed that the chicken δ -crystallin with low enzyme activity showed one major subunit spot of pI 5.9 in contrast with the multiple subunit chains of different isoelectric points spreading around pI 5.9–6.8 in the duck δ -crystallin (Fig. 3). 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 a gene-sharing strategy [9,30]. However, it still does not explain the expression of δ -crystallin with enzyme activity in the duck and ostrich [31] but not in the chicken. The possibility that the multiple duck δ -crystallin subunits are post-translational products cannot be ruled out since there are only two gene loci [26] to account for multiple isomeric forms in the duck species.

Before the detailed three-dimensional structures and the salient structural differences between these two crystallins are known, it would be intuitive to deduce the reaction mechanism of this enzymic crystallin on the basis of classical enzyme kinetics. Argininosuccinate lyase is a key enzyme in the urea cycle. Steady-state kinetics of this enzyme have been studied by using the bovine liver enzyme [17,32,33]. Kuchel *et al.* [17] first proposed that it follows an ordered sequential mechanism with arginine being released before fumarate. Raushel and his colleagues [32,34] later deduced a random Uni Bi mechanism from a steady-state kinetic analysis and positional isotope exchange study. Duck δ -crystallin represents an abundant and alternative enzyme source for the study of the mechanistic aspects of this enzyme.

Our initial-velocity and product-inhibition experiments also suggest a random Uni Bi kinetic mechanism for the argininosuccinate lyase activity of duck δ -crystallin. The inhibition patterns of citrulline definitely ruled out a sequential ordered mechanism. Citrulline was found to be a dead-end inhibitor of δ -crystallin and was competitive with respect to arginine and non-competitive with respect to fumarate. The expected inhibition pattern for an ordered mechanism would be uncompetitive with respect to fumarate, the last released product. Furthermore, since inhibition patterns were all non-competitive for arginine, fumarate and citrulline with respect to argininosuccinate, dead-end ternary complexes δ -crystallin–argininosuccinate–arginine,

Scheme 1. Proposed kinetic mechanism for the argininosuccinate lyase activity of duck lens δ -crystallin

Key: E, δ -crystallin; Arg-Suc, argininosuccinate; Arg, arginine; Fum, fumarate; Cit, citrulline.

δ -crystallin-argininosuccinate-fumarate and δ -crystallin-argininosuccinate-citrulline were assumed to be formed. The proposed kinetic mechanism of the reversible cleavage of the argininosuccinate reaction catalysed by duck δ -crystallin is summarized in Scheme 1.

As predicted by the random Uni Bi system, in the absence of added salt, the forward reaction is described by the usual Michaelis-Menten equation (Fig. 5). The random Uni Bi mechanism predicts non-linear reciprocal plots for the reverse reaction. However, departure from linearity was not detected for δ -crystallin, suggesting that the two routes to EPQ are about equally favourable. Therefore similar values were obtained for K_{ip} and K_{iq} or K'_{ip} and K'_{iq} (Table 1) [35]. The equation describing these linear portions of the curve is similar to those derived by the equilibrium treatment of this mechanism and thus may be treated in the same way as the equilibrium case. The K_{ip} , K'_{ip} and K'_{iq} in eqn. (2) are therefore all simply dissociation constants and K_{iq} can be obtained from the simple relationship $K_{ip}K'_{iq} = K'_{ip}K_{iq}$. Although in a random mechanism the protein could bind to either ligand, the binding to the first ligand may induce a protein conformational change which tightens the binding to the second ligand, as suggested by the finding that K'_{ip} and K'_{iq} are an order of magnitude smaller than K_{ip} and K_{iq} . The much lower inhibition constants for the fumarate (Table 2) suggest that it has a higher affinity for the protein and the addition or release of arginine and fumarate is not completely random.

One of the Haldane relationships for a random Uni Bi mechanism is derived as:

$$K_{eq} = V_f K_{ip} K'_{iq} / V_r K_{ia} \quad (6)$$

where K_{ia} is the dissociation constant of the δ -crystallin-argininosuccinate binary complex. We were not able to estimate this value because the substrate was spontaneously cleaved in the reaction mixture. Since we have already used the equilibrium assumption to derive eqn. (2), the Michaelis constant for

argininosuccinate was tentatively used to calculate the predicted equilibrium constant from kinetic constants. The calculated value of 1.46 mM is very close to the experimentally determined value of 1.8 mM (Table 1).

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