Chemical mechanism of the endogenous argininosuccinate lyase activity of duck lens δ2-crystallin

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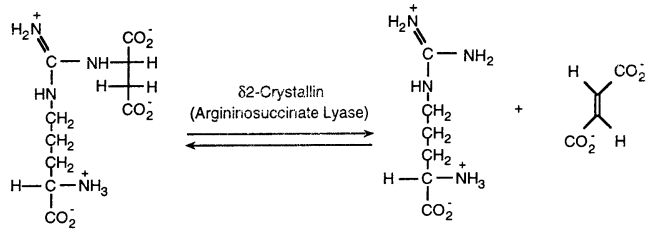
The endogenous argininosuccinate lyase activity of duck δ2-crystallin was specifically inactivated by the histidine-specific reagent, diethyl pyrocarbonate. The protein was protected by L-citrulline or L-arginine from the diethyl pyrocarbonate inactivation. To characterize further the chemical mechanism of the δ2-crystallin-catalysed reaction, deuterium-labelled argininosuccinate was enzymically synthesized from fumarate and L-arginine with δ2-crystallin in ^2H_2O. The argininosuccinate synthesized contained about 19% of the anhydride form; however, the deuterium was clearly demonstrated to be incorporated enantioselectively. Only the *pro*-H_R atom at C-9 of the succinate moiety was labelled in the [^{2}H]argininosuccinate-9-d synthesized, which indicates an *anti*-elimination mechanism for the endogenous argininosuccinate lyase activity of δ2-crystallin. The enzymic activity of duck lens δ2-crystallin in the pH range 5.5–8.5 was investigated using both protium- and deuterium-labelled argininosuccinate as the substrate. From the log k_{cat} versus pH plot, two molecular pK_a values of 6.18 ± 0.02 and 8.75 ± 0.03 were detected in the δ2-crystallin–argininosuccinate binary complex. The former must be dehydronated and the latter hydronated to achieve an optimum reaction rate. The log k_{cat}/K_m versus pH plot suggested two molecular pK_a values of 5.96 ± 0.09 and 8.29 ± 0.10 for the free δ2-crystallin to be involved in the substrate binding. Small kinetic isotope effects of 1.17 ± 0.02 and 1.05 ± 0.09 were found for k_{cat} and k_{cat}/K_m respectively. Combining results from labelling and kinetic analysis indicates that the endogenous argininosuccinate lyase activity of duck δ2-crystallin is compatible with a stepwise E1cB mechanism, the rate-limiting step probably at the C–N bond-cleavage step.

INTRODUCTION

δ-Crystallin is a taxon-specific protein and is the major protein in all avian lenses [1,2]. δ2-Crystallin from the duck eye lens possesses endogenous argininosuccinate lyase activity, which catalyses the reversible breakdown of L-argininosuccinate to L-arginine and fumarate (Scheme 1). This reaction is involved in L-arginine biosynthesis and may be involved in NO detoxification in duck lens [3,4]. We have previously purified δ2-crystallin from the duck eyeball and deduced a random Uni-Bi kinetic mechanism for the endogenous enzymic activity of this crystallin [5]. The results were found to be compatible with the steady-state kinetic [6] and positional isotope exchange [7,8] studies of authentic bovine liver argininosuccinate lyase. Hoberman et al. [9] have suggested that the chemical reaction involves a *trans* elimination of the arginino moiety and the hydrogen atom at C-9 of the argininosuccinate molecule (see Figure 1 for carbon numbering).



Scheme 1 δ2-Crystallin- or argininosuccinate lyase-catalysed cleavage of L-argininosuccinate

Raushel and colleagues proposed a stepwise E1cB mechanism for authentic bovine liver enzyme [10,11]. We postulated the involvement of an essential histidine residue in the catalytic reaction of δ2-crystallin by using chemical modification with diethyl pyrocarbonate [12].

To probe unambiguously the chemical mechanism of the endogenous argininosuccinate lyase activity of δ2-crystallin, we synthesized deuterium-labelled argininosuccinate. The labelling position and the kinetic isotope effect on the enzymic activity were examined. Our experimental data correlate with a carbanion intermediate E1cB mechanism for the δ2-crystallin-catalysed cleavage reaction, with the rate-limiting step probably being at the C–N bond cleavage.

MATERIALS AND METHODS

Materials

L-Arginine, barium argininosuccinate, fumarate, argininosuccinate lyase (bovine liver), and ^2H_2O (99.9 atom% ^{2}H) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). All other chemicals were obtained from Sigma or Merck (Darmstadt, Germany). Conversion of barium argininosuccinate to potassium argininosuccinate was carried out by the procedure of Ratner [13]. Purification of duck δ2-crystallin and determination of protein concentration were as described previously [5,14].

Synthesis of [^{2}H]argininosuccinate

[^{2}H]Argininosuccinate was prepared enzymically by allowing L-arginine to react with fumarate in ^2H_2O in the presence of argininosuccinate lyase or δ2-crystallin. L-Arginine (174 mg, 1 mmol) and fumarate (116 mg, 1 mmol) were dissolved in H_2O

Abbreviations used: $^Hk_{cat}/^{2H}k_{cat}$, kinetic isotope effect on k_{cat}; $^H(k_{cat}/K_m)/^{2H}(k_{cat}/K_m)$, kinetic isotope effect on k_{cat}/K_m.

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(20 ml). After the pH had been adjusted to 7.5 with NaOH, the solution was lyophilized and redissolved in ^2H_2O (19 ml). Then 2 mg of δ2-crystallin or bovine liver argininosuccinate lyase (both lyophilized twice in 10 ml of ^2H_2O) was added, and the reaction mixture was allowed to react at 25 °C for 48 h. The pH of the solution was kept at 7.5 during the reaction period. The barium salt of argininosuccinate was obtained by adding $BaCl_2$ (1 mmol) to the solution and the pH adjusted to 9.5 with NaOH. After lyophilization, the solid product was washed with 80% ethanol (3 × 10 ml). The final product was further purified using a TSK-HW 40(S) column to obtain the purified barium argininosuccinate, which was treated with H_2SO_4 to form [^{2}H]-argininosuccinic acid-9-d. The carbon number was assigned as described by Kowalsky and Ratner [15].

The purity and labelling position of the [^{2}H]argininosuccinic acid-9-d synthesized was confirmed by proton-NMR (Bruker AM; 400 MHz) and MS. In the fast-atom-bombardment mass spectrum, a major ion fragment was observed at m/z 292, corresponding to argininosuccinate. Assignments of proton signals were as described by Kim and Raushel [8] and Burns and Iles [16].

The [^{2}H]argininosuccinate synthesized contains a substantial amount (19%) of the cyclized anhydride form as determined by the relative integration area of the respective resonance peaks (see Figure 1B) [17]. This amount was corrected in the calculation of argininosuccinate concentration, because the argininosuccinate anhydride is not active as a substrate for the argininosuccinate lyase activity [15]. The anhydride form is reported not to have an inhibitory effect on the enzyme activity [18].

Substrate specificities

L-Arginine (1 mmol) and fumarate (1 mmol) or their analogues were dissolved in H_2O (19 ml). After the pH had been adjusted to 7.5 with NaOH, δ2-crystallin (2 mg) was added, and the reaction mixture was allowed to react at 25 °C for 48 h. The pH was kept at pH 7.5 during the reaction period and not changed after completion of the reaction. After lyophilization, the relative activities of the analogues were determined with an amino acid analyser (Beckman model 6300) by following the decrease in L-arginine or its analogues.

Enzymic kinetic analysis

The initial velocities of the endogenous argininosuccinate lyase activities of duck δ2-crystallin were assayed at 25 °C in a Perkin–Elmer lambda 3B spectrophotometer by continuously monitoring the formation of fumarate at 240 nm [5]. The reaction mixture contained Tris/HCl (50 mM, pH 7.5), argininosuccinate (1 mM) and δ2-crystallin (20 μg/assay) in a total volume of 1 ml. One unit of enzyme activity was defined as 1 μmol of fumarate released/min using a molar absorption coefficient of $2.44 \times 10^3\ M^{-1} \cdot cm^{-1}$ for fumarate.

pH studies

The endogenous argininosuccinate lyase activity of δ2-crystallin was assayed at various pH values under different argininosuccinate concentrations. KH_2PO_4/K_2HPO_4 buffer was used throughout the pH range studied. The actual pH values were measured before initiation of each assay. No appreciable pH perturbation was found after completion of the assays.

The kinetic data were analysed by a non-linear regression program SigmaPlot V5.00 (Jandel Scientific, San Rafael, CA, U.S.A.). pH profiles were fitted to the following equation to determine K_1 and K_2 values:

$$\log Y = \log \left(\frac{C}{1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}} \right) \quad (1)$$

where Y is the value of the parameter of interest measured at any pH, C the pH-independent value of Y, and K_1, K_2 dissociation constants for the titration groups.

Kinetic isotope effect

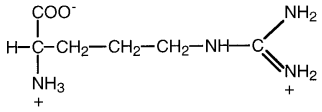
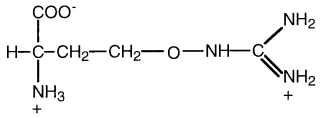
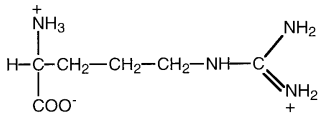
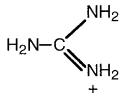
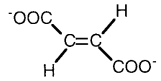
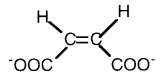
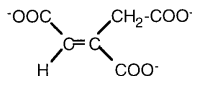
Using [^{2}H]argininosuccinate, we performed pH studies as described above. The kinetic isotope effects on k_{cat} ($^Hk_{cat}/^{2H}k_{cat}$) or k_{cat}/K_m [$^H(k_{cat}/K_m)/^{2H}(k_{cat}/K_m)$] were estimated by comparing the kinetic parameters obtained with [^{1}H]- or [^{2}H]-argininosuccinate.

RESULTS

Substrate specificity

δ2-Crystallin is a stable protein and can be easily purified from duck lens. This protein thus has a bio-catalytic potential for the enzymic synthesis of argininosuccinate [19]. To test the substrate specificities of the endogenous argininosuccinate lyase activity of

Table 1 Substrate specificities for the endogenous argininosuccinate lyase activity of duck δ2-crystallin

Substrate analogues	Chemical structure	Yield (%)
L-Arginine		100
L-Canavanine		78
D-Arginine		0
Guanidine		0
Fumarate		100
Maleic acid		0
trans-Aconitate		0

δ2-crystallin, various substrate analogues were examined for their ability to substitute for the substrate. Of the L-arginine analogues examined, only L-canavanine, which has a -CH_2 in the side chain of the arginino moiety replaced by an oxygen atom, showed 78% overall yield compared with L-arginine (Table 1). D-Arginine and guanidine were inactive. The fumarate analogues, including maleic acid and *trans*-aconitate, were both shown to be completely inactive.

Isotope labelling experiment

Figure 1(A) shows the 400 MHz NMR spectrum of the commercial argininosuccinate. The proton signals c1 and c2 for the prochiral centre C-9 were clearly resonated at 2.71 and 2.87 p.p.m. respectively. Other proton signals are labelled (Figure 1A). Comparison of the spectra with that reported by Burns and Iles [16] showed two major differences. Methanol and ethanol contaminant peaks were absent at 3.38 and 3.68 p.p.m. Instead, signal H(f)′ corresponding to an anhydride form of the argininosuccinate was clearly observed at 4.49 p.p.m. Obviously, methanol and ethanol were removed during the conversion of the barium salt of argininosuccinate into the potassium salt. However, this procedure also caused partial conversion of argininosuccinate (I) into argininosuccinate anhydride (II) (structures shown in Figure 1A). The ionizable hydrons at nitrogen atoms were undetectable under our conditions.

To determine which hydrogen atom was removed from the prochiral centre C-9 during the catalytic reaction, we synthesized argininosuccinate from L-arginine and fumarate by the duck δ2-crystallin-catalysed reaction in ^2H_2O. The [^{2}H]argininosuccinate was then isolated and subjected to NMR analysis (Figure 1B). The peak at 2.87 p.p.m. (corresponding to the c2 proton) disappeared, which clearly indicates that the *pro*-9R hydrogen (H_R), instead of H_S, of the prochiral centre C-9 is labelled in the argininosuccinate molecule. Identical results were obtained with authentic argininosuccinate lyase (Figure 1C).

pH studies and kinetic isotope effects

Purified duck δ2-crystallin is stable between pH 5.5 and 8.5 for at least 20 min under the assay conditions. Detailed pH effects on the enzyme activity were studied in this pH range, using either [^{1}H]argininosuccinate or [^{2}H]argininosuccinate as the substrate. A plot of $\log k_{cat}$ versus pH suggested two pK_a values of 6.18 ± 0.02 and 8.75 ± 0.03 in the δ2-crystallin–argininosuccinate binary complex, which may be involved in the catalytic reaction (Figure 2A). To yield an optimum reaction rate, the former must be dehydronated and the latter must be hydronated. Similarly, the plot of $\log k_{cat}/K_m$ versus pH for the free δ2-crystallin or substrate yielded pK_a values of 5.96 ± 0.09 and 8.29 ± 0.10. These two groups may be involved in substrate binding (Figure 2B). The kinetic parameters are summarized in Table 2 for comparison.

At pH optimum, an isotope effect of 1.17 ± 0.02 for the k_{cat} was obtained. The isotope effect on k_{cat}/K_m was essentially unity (1.05 ± 0.09). The small isotope effects would suggest that hydron transfer may not be the rate-limiting step in the reaction.

DISCUSSION

The assignment of critical amino acid residues presumed to be involved in catalysis or substrate binding requires information on both reaction kinetics and protein tertiary structure. Detailed three-dimensional structures of δ1-crystallin from turkey lens [20] and a histidine to asparagine mutant δ2-crystallin (H91N) from duck eye lens [21] have been elucidated by X-ray-crystallographic analysis to 2.5 Å resolution. The structure of the H91N mutant δ2-crystallin is indistinguishable from that of the wild-type δ2-crystallin, for which only a low-resolution structure (~ 3 Å) is available [21]. More recently, the structure of the counterpart cytosolic enzyme argininosuccinate lyase was also determined to 4 Å resolution [22]. There is extremely high sequence identity (94%) between δ1- and δ2-crystallins [21,23]. The overall structures of δ1- and δ2-crystallins are topologically similar. This structure is also found in argininosuccinate lyase [22] and other metabolic enzymes of this superfamily, which includes aspartase ammonia-lyase [24] and fumarase [25]. Each polypeptide chain of the protein contains three domains, with each domain composed almost completely of helical structure. Domains 1 and 3 have a similar overall topology, with each domain consisting of two helix–turn–helix motifs. The central domain 2 is composed of five long helices and forms a 20-helix bundle at the core of the tetramer. The protein has a double-dimer quaternary structure [21,26]. The structure reveals a putative active-site cleft located at the boundary of the three subunits of the tetramer, thus each of the four active sites is composed of residues from three monomers. The highly conserved His-160 in the B-subunit, and Glu-294 and Lys-287 in the D-subunit are located close to each other in the cleft, making these residues strong candidates for an acid–base catalysis [20–22] (Figure 3). The proximity of Glu-294 and its strong hydrogen bonding to His-160 are consistent with the proposal that a type of charge relay increases the nucleophilicity of His-160, making it a good candidate for the hydron abstractor [21,22,25]. The involvement of histidine, carboxyl and lysine residues in the catalytic mechanism of bovine liver argininosuccinate lyase or duck δ-crystallin has been demonstrated by chemical modification [12,27,28] and site-directed mutagenesis [3,29].

In this study, we used kinetic analysis to elucidate the probable chemical mechanism of the endogenous argininosuccinate lyase activity. We examined the variation of kinetic parameters with pH, which may detect the essential amino acid residues involved in catalysis or substrate binding. However, it should be noted that pH–rate profiles cannot be used directly to determine pK_a values for the enzyme when different steps in the overall process depend on different levels of hydrogenation. The assignments of pK_a values must remain tentative because of the many factors that may influence the ionization constant, e.g. the neighbouring group effect, i.e. the ionization of a group changes value depending on the ionic state of other groups. If two groups have pK_a values very close to each other, i.e. the pK_a values differ by less than 0.6, then each titrating group is the sum of the fractions of the molecular pK_a values [30].

The k_{cat}/K_m versus pH profile reveals the titratable groups of free enzyme or substrate. Our experimental results confirm the involvement of two ionizable groups with apparent pK_a values of 5.96 and 8.29 in substrate binding. Three carboxyl groups in the substrate and the conserved Glu-294 of the enzyme molecule may be ionized around pH 6. The guanidino group of the substrate ionizes above pH 12, beyond the pH range employed in this study. The other possible ionizable group of argininosuccinate that can be ionized at pH about 8.29 is the α-amino group of the arginine moiety. However, ionization of Lys-287 of the protein molecule may also be responsible for the pK_a value of 8.29, since this residue is conserved and is located at the active-site cleft of the aspartase–fumarase superfamily [20–22,24,25], which includes δ-crystallin and argininosuccinate lyase [20–22].

One of the pK_a values observed in the log k_{cat} versus pH plot

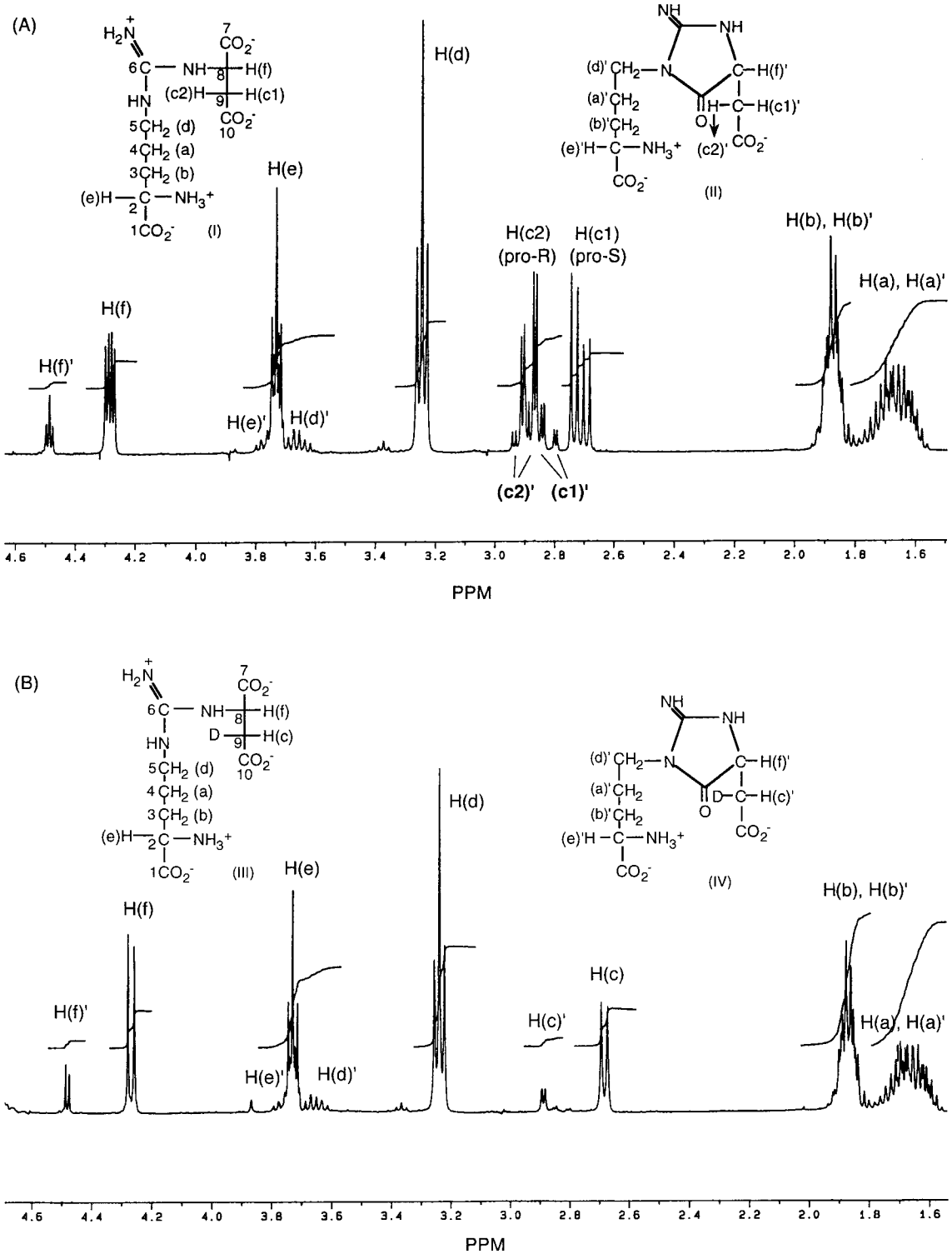


Figure 1 For Legend see facing page

should be from the histidine imidazole group. The pK_a value of 6.18 is comparable with the pK_a value ($\sim$ 6.8) for the histidine residue determined by chemical modification of δ2-crystallin [12]. The ionizable group responsible for the other pK_a value is not clear. It could be due to the ionization of the carboxyl group of a glutamate residue. However, a pK_a value of 8.75 is generally considered to be too high for a carboxyl group, although it is not unusual to find the pK_a value of a carboxyl group to increase to above 6 in the hydrophobic environment of an enzyme active centre [31]. Alternatively, this pK_a value could be the result of

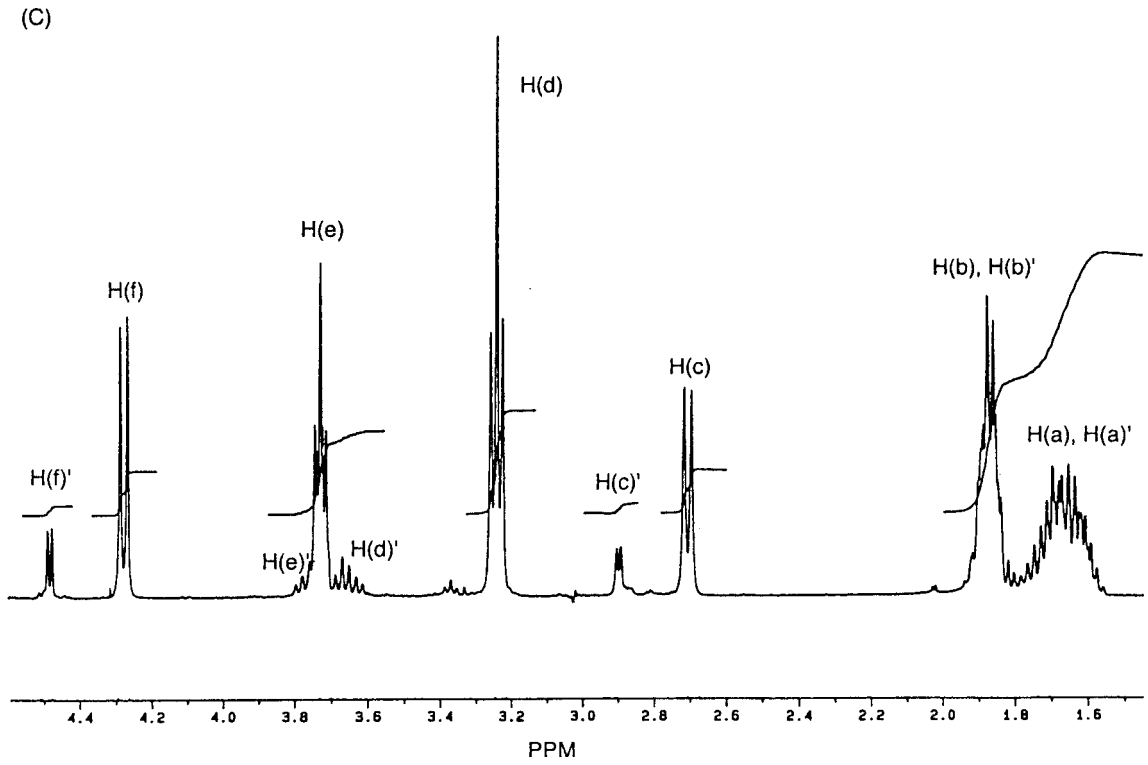


Figure 1 NMR spectrum of synthesized argininosuccinate

400 MHz ^{1}H-NMR spectrum of unlabelled [^{1}H]argininosuccinate (**A**) and [^{2}H]argininosuccinate (**B** and **C**) synthesized from L-arginine and fumarate, in ^2H_2O, by duck lens δ2-crystallin (**B**) or bovine liver argininosuccinate lyase (**C**) are shown with appropriate peak labelling. The peak assignments were as described by Kim and Raushel [8] and Burns and Iles [16]. The carbon atoms were numbered as described by Kowalsky and Ratner [15]. (I) and (III) correspond to the structure of [^{1}H]- and [^{2}H]-argininosuccinate respectively. (II) and (IV) correspond to those of the anhydride forms of argininosuccinate.

ionization of Lys-287, which has been proposed to be involved in neutralization of the negative charges of the carbanion intermediate [20–22]. The differences between pK_1 and pK_2 for both K_m and k_{cat}/K_m parameters (ΔpK_a in Table 2) are in the range 2.33–2.57, which is large enough, indicating that the titration constant effectively becomes equal to the molecular pK_a values [30,32]. However, since only the structure of the apoprotein of δ-crystallin is available, the above essential group assignments must be considered tentative. With these caveats in mind, the possible chemical mechanism of δ2-crystallin-catalysed argininosuccinate cleavage can be analysed.

In the light of the above discussion, our results are consistent with the following chemical mechanism for the endogenous argininosuccinate lyase activity of δ2-crystallin. An essential histidine residue (His-160) may act as a base catalyst by providing an electron pair, which abstracts a hydron from C-9 of the substrate. During the cleavage of argininosuccinate, the leaving of the strong basic arginino moiety may require acid catalysis [33]. In addition, the carboxyl group of Glu-294 is proposed to donate a hydron to the guanidino nitrogen of the arginino moiety resulting in the formation of L-arginine and fumarate (Scheme 2).

The above reaction could proceed by a concerted β-elimination mechanism if both the hydron and the arginino moiety leave simultaneously. A requirement for the concerted β-elimination is the presence in the same plane of all the five atoms, including the base and H-C9-C8-N. Two kinds of arrangement can fulfil this requirement: *trans*, between H and the leaving group, resulting in *anti*-elimination; or *cis* orientation leading to *syn*-elimination. The former has a staggered conformation in the transition state and is found in most elimination reactions [34,35]. However, steric, conformational, ion-pairing and other factors cause *syn*-elimination to intervene [34,36,37].

Our results clearly exclude a *syn*-elimination mechanism, which predicts H_S, instead of H_R, to be labelled at the [^{2}H]argininosuccinate-9-d. Our results thus point to an *anti*-elimination mechanism. The experimental results are compatible with a concerted *anti*-elimination or a stepwise carbanion elimination. If the endogenous argininosuccinate lyase activity of δ2-crystallin proceeds via a concerted *anti*-elimination, it would be difficult to conceive the experimental results for the kinetic isotope effect (Figure 2); a large isotope effect is expected for a concerted mechanism [35,38]. However, other factors may mask the intrinsic isotope effect. Thus the data supporting a stepwise carbanion mechanism would also support a concerted elimination mechanism.

The kinetic isotope effect is a powerful technique for determining the structure of an enzyme transition state [39,40]. An isotope effect of 3–8 is expected for E2 elimination if protium is replaced by deuterium and the removal of a hydron is rate-limiting [35,38]; however, we observed only a very small isotope effect (Table 2). This result is consistent with other enzymes of the fumarase–aspartase family, which operate via a carbanion mechanism [20–22,41–44]. However, the chemical mechanism of

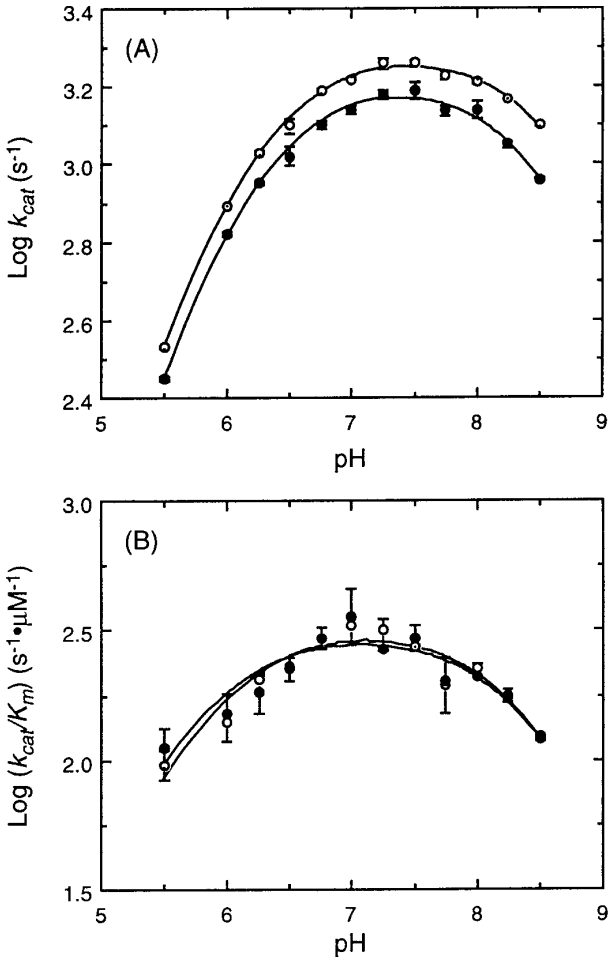
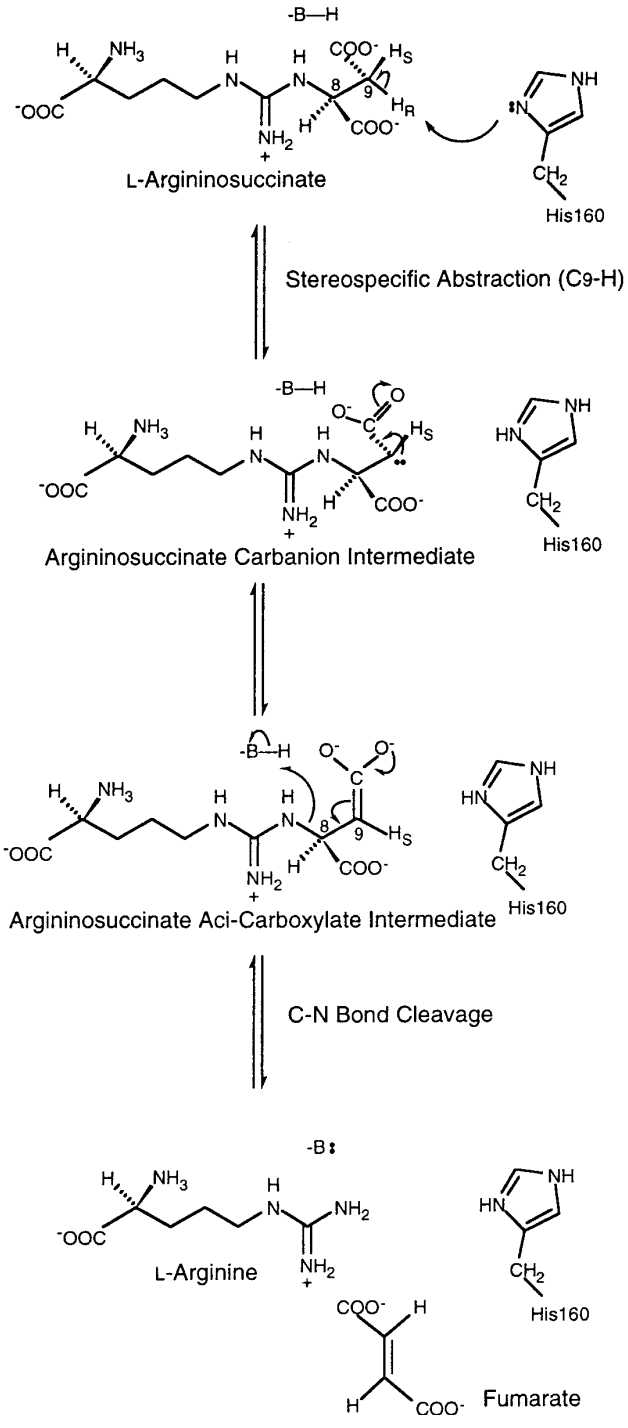


Figure 2 pH and kinetic isotope effects of the endogenous argininosuccinate lyase activity of duck lens δ2-crystallin

Log k_{cat} (**A**) or k_{cat}/K_m (**B**) of [^{1}H]argininosuccinate (○) or [^{2}H]argininosuccinate (●) was plotted against pH. Curves are computer-fitted results according to eqn. (1). In (**B**), for the data at each pH value, the upward standard error bars were those for the [^{1}H]argininosuccinate and the downward standard error bars were for the [^{2}H]argininosuccinate.

3-methylaspartase, the archetypal enzyme of the fumarase–aspartase family, depends on the substrate used. A primary deuterium isotope effect of 1.7 was observed for 3-methylaspartic acid at 1.6 mM K^+ [45]. From the results of product-inhibition experiments and $^2H/^1H$–$^{15}N/^{14}N$-double isotope fractionation experiments [46], Gani and colleagues [45–47] concluded that a concerted mechanism operates for the 3-methylaspartase when 3-methylaspartic acid or 3-ethylaspartic acid was used as the substrate, but the E1cB mechanism prevails for aspartic acid. The change in reaction mechanism is presumably due to the loss of the hydrophobic interaction between the C_3-alkyl substituent of the substrate and the complementary active site of the enzyme [47,48]. The problem associated with the kinetic isotope effect technique is that the chemical step of an enzymic reaction is sometimes not totally rate-limiting, making the observed isotope effect deviate from the intrinsic isotope effect. Appreciation of this method depends on the relative rates of the turnover number compared with those of reactant association and product release. In our case, since the k_{cat} of the reaction is only $\sim 10^3\ s^{-1}$, which is not large, and the substrate concentration is well above



Scheme 2 Proposed E1cB chemical mechanism for the endogenous argininosuccinate lyase activity of duck lens δ2-crystallin

The E1cB mechanism proceeds via the following steps. First, a histidine residue (His-160) in its conjugated form abstracts the H_R hydron in C-9 of the argininosuccinate, forming a carbanion intermediate. Secondly, redistribution of negative charge into the carboxyl group generates the aci-carboxylate intermediate. The aci-acid or carbanion intermediates provide the driving force for the expulsion of the fumarate group. These intermediates are stabilized by the positive charge of the guanidino group, which provides an electron-withdrawing centre. A general acid-BH (possibly Glu-294) helps the final C-N bond cleavage step by donating a hydron to the guanidino-NH group and generating the olefinic bond of fumarate. Lys-287 (not shown in the Figure) may be involved in stabilizing the argininosuccinate carbanion or the aci-carboxylate intermediate.

Table 2 Kinetic parameters and kinetic isotope effects for the endogenous argininosuccinate lyase activity of duck δ2-crystallin

Values shown are means ± S.E.M.

Molecular forms	Substrate		pK_1	pK_2	ΔpK_a
δ2-Crystallin–argininosuccinate		k_{cat} (s^{-1})			
complex	[^{1}H]Argininosuccinate	1963 ± 27	6.18 ± 0.02	8.75 ± 0.03	2.57
	[^{2}H]Argininosuccinate	1669 ± 36	6.20 ± 0.02	8.59 ± 0.04	2.39
		$^{H}k_{cat}/^{T}k_{cat} = 1.17 \pm 0.02$			
Free δ2-crystallin		k_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$)			
	[^{1}H]Argininosuccinate	326 ± 26	5.96 ± 0.09	8.29 ± 0.10	2.33
	[^{2}H]Argininosuccinate	310 ± 27	5.86 ± 0.12	8.31 ± 0.12	2.45
		$^{H}(k_{cat}/K_m)/^{2}H(k_{cat}/K_m) = 1.05 \pm 0.09$			

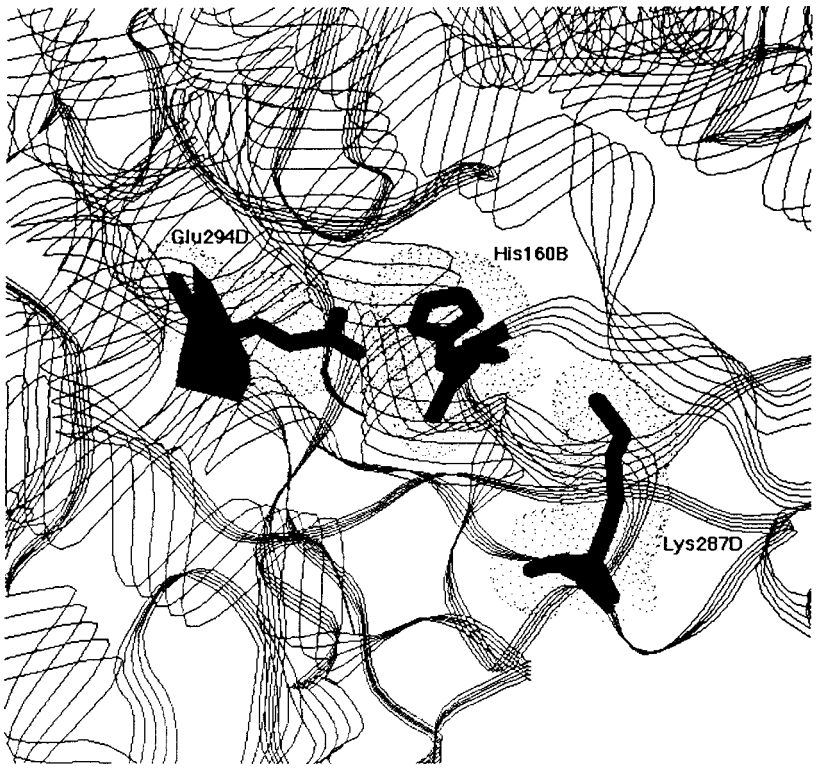


Figure 3 Putative active-site region of δ-crystallin

The model is based on the X-ray-crystallographic structure of turkey δ-crystallin [20]. The possible amino acid residues, His-160 from subunit B and Glu-294 and Lys-287 from subunit D, involved in the catalytic reaction are labelled. The Figure was generated using the RasMol (V2.6) program.

physiological, the substrate is assumed to be non-sticky, and neither substrate association nor product release is considered to be rate-limiting. However, unequivocal discrimination between these possibilities requires determination of the enzyme catalytic rate in the presence of viscogens, e.g. sucrose, glycerol, poly(ethylene glycol) or Ficoll [35].

Raushel [10] observed that the nitro analogue of argininosuccinate, N^3-(L-1-carboxy-2-nitroethyl)-L-arginine, is a strong competitive inhibitor with respect to argininosuccinate of the bovine liver argininosuccinate lyase. The K_i value of this inhibitor is only 2.7 μM, which is 20 times smaller than the K_m value for argininosuccinate. The tighter binding of the inhibitor relative to substrate led Raushel to suggest that the inhibitor is a transition-state analogue [10]. The lyase reaction was thus proposed to proceed via a stepwise E1cB mechanism like aspartate ammonia-lyase [41] or histidine ammonia-lyase [42,43]. The reaction is initiated by the abstraction of a hydron from C-9 of argininosuccinate by His-160 and generation of a carbanion intermediate (Scheme 2). Subsequent redistribution of negative charge generates the aci-carboxylate intermediate similar to that proposed by Blanchard and Cleland for fumarase [44]. A second enzymic site (-BH, possibly Glu-294) is involved to aid arginino group removal from C-8, leading to fumarate formation. Our experimental results agree with this mechanism, with a rate-limiting step being assigned at the last C–N bond-cleavage step, which explains the small kinetic isotope effect detected (Figure 2). Alternatively, the reaction may follow a mechanism proposed by Gerlt and Gassman [33] which involves a concerted general

acid–general base catalysis (the Glu-294–His-160 pair in δ2-crystallin) and the formation of an enolate intermediate, but avoids the energetically unfavourable carbanion intermediate.

However, the E1cB mechanism is supported by the theoretical derivation of Warshel and colleagues [49,50], who predicted that ionic interaction is the major factor contributing to enzyme catalysis. The role of an enzyme molecule in the catalysis is to provide pre-oriented dipoles, which are polarized to stabilize the transition-state charge distribution. In this sense, the positive charge of the ϵ-amino group of a lysine residue (Lys-287) in the protein molecule may be an important contributing factor in favour of the E1cB mechanism [20–22,51] by stabilizing the argininosuccinate carbanion intermediate or the negatively charged carboxyl group of the argininosuccinate aci-carboxylate intermediate or by neutralizing one of the carboxylate groups of the nascent fumarate [20–22]. Lys-287 has been implicated in the reaction mechanism because of its strict conservation throughout the superfamily [21,22]. Indeed, mutation of the corresponding residue (Lys-326) to arginine in aspartase eliminates catalytic activity by abolishing the binding of substrate to the enzyme [52], which strongly suggests that Lys-287 is involved in stabilizing the enzyme–substrate complex.

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