Inactivation of the endogenous argininosuccinate lyase activity of duck δ -crystallin by modification of an essential histidine residue with diethyl pyrocarbonate

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The argininosuccinate lyase activity of duck δ -crystallin was inactivated by diethyl pyrocarbonate at 0 °C and pH 7.5. The inactivation followed pseudo-first-order kinetics after appropriate correction for the decomposition of the reagent during the modification period. The plot of the observed pseudo-first-order rate constant versus diethyl pyrocarbonate concentration in the range of 0.17–1.7 mM was linear and went through the origin with a second-order rate constant of $1.45 \pm 0.1 \text{ M}^{-1} \cdot \text{s}^{-1}$. The double-logarithmic plot was also linear, with slope of 1.13, which suggested a 1:1 stoichiometry for the reaction between diethyl pyrocarbonate and δ -crystallin. L-Arginine, L-norvaline or L-citrulline protected the argininosuccinate lyase activity of δ -crystallin from diethyl pyrocarbonate inactivation. The dissociation constants for the δ -crystallin–L-arginine and δ -crystallin–L-citrulline binary complexes, determined by the protection experiments, were 4.2 ± 0.2 and $0.12 \pm 0.04 \text{ mM}$ respectively. Fumarate alone had no protective effect. However, fumarate plus L-arginine gave synergistic protection with a ligand binding interacting factor of 0.12 ± 0.02 . The double-protection

data conformed to a random Uni Bi kinetic mechanism. Fluorescence-quenching studies indicated that the modified δ -crystallin had minimum, if any, conformational changes as compared with the native δ -crystallin. Inactivation of the enzyme activity was accompanied by an increasing absorbance at 240 nm of the protein. The absorption near 280 nm did not change. Treatment of the modified protein with hydroxylamine regenerated the enzyme activity to the original level. These results strongly indicated the modification of an essential histidine residue. Calculation from the 240 nm absorption changes indicated that only one histidine residue per subunit was modified by the reagent. This super-active histidine residue has a pK_a value of ~ 6.8 and acts as a general acid–base catalyst in the enzyme reaction mechanism. Our experimental data are compatible with an E1cB mechanism [Raushel (1984) Arch. Biochem. Biophys. 232, 520–525] for the argininosuccinate lyase with the essential histidine residue close to the arginine-binding domain of δ -crystallin. L-Citrulline, after binding to this domain, might form an extra hydrogen bond with the essential histidine residue.

INTRODUCTION

δ -Crystallin is a taxon-specific protein that constitutes the major lens protein in birds [for a review, see Piatigorsky (1984)]. This protein is coded by two tandemly arranged genes that encode two polypeptide chains having 91% identity (Nickerson et al., 1986). The gene product of $\delta 2$ gene possesses argininosuccinate lyase activity, while that from the $\delta 1$ gene does not. Only the $\delta 1$ gene is expressed in chicken, while both $\delta 1$ and $\delta 2$ genes are expressed in duck [cf. Wistow et al. (1990) and references cited therein]. Both the chicken and the duck $\delta 2$ genes were successfully expressed in mouse cells or yeast (Kondoh et al., 1991; Barbosa et al., 1991b). The salient structural features between $\delta 1$ and $\delta 2$ gene products thus may shed some light on the reaction mechanism of the argininosuccinate lyase. Steady-state kinetic and chemical-modification studies suggested that His and a carboxy group might be required in catalysis (Garrard et al., 1985) and that Lys-51 is involved in the substrate binding (Lusty and Ratner, 1987). Sequence analysis between duck and chicken δ -crystallins revealed that His-91 in duck δ -crystallin was replaced by glutamine in chicken δ -crystallin. Barbosa et al. (1991a) produced mutant enzymes in which His-91 (89 in the $\delta 1$ gene

product) was changed to glutamine and Lys-53 (51 in the $\delta 1$ gene product) to asparagine respectively. These mutations resulted in 10-fold and 2-fold decreases in specific activity respectively.

We have purified δ -crystallin from the duck eyeball by a single chromatographic step and deduced a random Uni Bi kinetic mechanism for the endogenous argininosuccinate lyase activity (Lee et al., 1992). In the present study we used chemical modification techniques to explore the essential His residue required for catalysis. From double-protection experimental results, we confirm the proposed kinetic mechanism and the synergistic binding between L-arginine and fumarate. Our experimental data are compatible with the E1cB mechanism proposed by Raushel (1984) for the argininosuccinate lyase-catalysed reaction.

MATERIALS AND METHODS

Materials

L-Arginine, argininosuccinate, L-citrulline, diethyl pyrocarbonate (DEP), fumarate, imidazole, α -oxoglutarate, maleic acid, hydroxylamine, L-norvaline, *cis*-oxaloacetate and pyruvate were

Abbreviation used: DEP, diethyl pyrocarbonate.

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purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were obtained from Sigma or E. Merck (Darmstadt, Germany). We used our recently published procedures (Lee et al., 1992) for the purification of argininosuccinate and duck δ -crystallin, determination of protein concentration and assay for the argininosuccinate lyase activity.

DEP was stored in 100% ethanol at 4 °C. The concentration of DEP was determined in each experiment by allowing it to react with 10 mM imidazole in 50 mM sodium acetate buffer, pH 6.5, at 0 °C. The maximum absorbance at 240 nm was measured, and an absorption coefficient of 3600 mM⁻¹·cm⁻¹ for the *N*'-ethoxycarbonylimidazole was used in calculation (Holbrook and Ingram, 1973).

Inactivation experiments

We performed the modification at 0 °C by adding freshly diluted DEP to the δ -crystallin solution in 0.1 M potassium phosphate buffer, pH 7.5. We monitored the progress of enzyme inactivation by assaying the enzyme activity in small aliquots withdrawn at the designated time intervals. The data always followed pseudo-first-order kinetics after appropriate correction for the DEP decomposition during the modification period according to eqn. (1) (Gomi and Fujioka, 1983):

$$\ln(E/E_0) = -(k_1/k')[\text{DEP}](1 - e^{-k't}) \quad (1)$$

where [DEP] is the initial DEP concentration, E is enzyme activity, k_1 is the second-order rate constant for the reaction of δ -crystallin and DEP and k' is the first-order rate constant for the hydrolysis of DEP that was found to be 0.013 min⁻¹ under the experimental conditions. Therefore a plot of $\ln(E/E_0)$ versus $(1 - e^{-k't})/k'$ was linear with slope ($k_{\text{obs.}}$) equal to $k_1[\text{DEP}]$. The second-order rate constant was obtained from the $k_{\text{obs.}}$ -versus-[DEP] replot.

The ethanol introduced into the reaction mixture by the DEP solution never exceeded 5%. In our control experiment, this amount of ethanol did not affect the enzyme activity during the experimental period.

Protection experiments

When the effects of various ligands on the inactivation rate were to be studied, we mixed the δ -crystallin with the ligands and left the mixtures for 3 min. We then added DEP and the enzyme activity was assayed as described above.

Fumarate or structurally related compounds were found not to affect the decomposition rate of DEP under the protection experimental conditions. L-Citrulline or L-norvaline up to 1 mM also did not affect the DEP hydrolysis rate. However, L-arginine was found to increase the DEP decomposition rate. In the presence of 0.5 or 1 mM L-arginine, k' was found to be 0.024 min⁻¹, which increased to 0.034 min⁻¹ at 2 or 3 mM L-arginine. This factor was considered in the protection experiments.

Stoichiometry studies

We performed this experiment with 2.09 μ M δ -crystallin and 0.28 mM DEP in potassium phosphate buffer, pH 7.5, in tandem cuvettes (light path 0.437 cm in each chamber) with the following arrangement: in the control cell, chamber 1 contained DEP in

buffer and chamber 2 contained δ -crystallin in buffer; in the sample cell, chamber 1 contained buffer only and chamber 2 contained δ -crystallin and DEP in buffer. We continuously monitored the difference absorbance at 240 nm and assayed the enzyme activity at appropriate time intervals. We used the absorbance increase at 240 nm to calculate the number of histidine residues modified. These values were then correlated with enzyme activity loss by using an M_r of 200000 for the δ -crystallin tetramer. After completion of the reaction we recorded the difference absorption spectrum between 200 and 300 nm.

Fluorescence-quenching studies

We performed the fluorescence studies with a Perkin-Elmer LS-5 luminescence spectrometer. Quenching of the protein intrinsic fluorescence at 340 nm with acrylamide was performed in a similar manner as for the pigeon liver malic enzyme (Lee et al., 1988).

The inner-filter effect due to the absorption of acrylamide at the excitation wavelength (295 nm) was corrected according to Birdsall et al. (1983). Together with the volume dilution correction, the observed fluorescence ($F_{\text{obs.}}$) and the corrected fluorescence ($F_{\text{corr.}}$) were related by eqn. (2):

$$F_{\text{obs.}} = F_{\text{corr.}} (10^{-A}/2.303A) [V_{\text{init.}}/(V_{\text{init.}} + V_{\text{incr.}})] \quad (2)$$

where A was the absorbance at 295 nm of added acrylamide. $V_{\text{init.}}$ denoted the initial volume of the protein solution. $V_{\text{incr.}}$ denoted the increment volume introduced during the titration with acrylamide. At end of the titration the total volume change was 6%.

We first analysed the quenching data according to the classical Stern-Volmer equation (eqn. 3):

$$F_0/F = 1 + K_{\text{sv}}[Q] \quad (3)$$

where F_0 and F are the fluorescence intensities in the absence and presence of acrylamide respectively, $[Q]$ is the acrylamide concentration and K_{sv} is the dynamic quenching constant of the system.

When a downward Stern-Volmer plot was obtained, we plotted the fluorescence quenching data as $F_0/\Delta F$ versus $1/[Q]$ according to a modified Stern-Volmer equation (Lehrer and Leavis, 1978) (eqn. 4):

$$F_0/\Delta F = (1/K_{\text{sv}}f_a)(1/[Q]) + 1/f_a \quad (4)$$

where $\Delta F = F_0 - F$ and f_a is the fractional maximum accessible protein fluorescence.

RESULTS

Inactivation of duck δ -crystallin endogenous argininosuccinate lyase activity by DEP

DEP caused a time-dependent inactivation of the argininosuccinate lyase activity of duck δ -crystallin at 0 °C and pH 7.5. The inactivation followed pseudo-first-order kinetics after appropriate correction for the decomposition of DEP in the inactivation period (Figure 1a). When the observed inactivation rate ($k_{\text{obs.}}$) was plotted versus [DEP], a linear plot going through the origin was obtained (Figure 1b). This result indicated that DEP did not form a Michaelis-type complex with δ -crystallin. The second-order rate constant (k_1) estimated from Figure 1(b) was $1.45 \pm 0.1 \text{ M}^{-1} \cdot \text{s}^{-1}$. A $\log k_{\text{obs.}}$ -versus- $\log [\text{DEP}]$ plot was also linear, with a slope of 1.13 (results not shown), which suggested a 1:1 stoichiometry for the reaction between δ -crystallin and DEP (Levy et al., 1963; Carlson, 1984).

Figure 1 Inactivation of the endogenous argininosuccinate lyase activity of duck δ -crystallin by DEP

The reaction mixture contained $5.45 \mu\text{M}$ δ -crystallin in 0.1 M potassium phosphate buffer, pH 7.5, and various concentrations of DEP. Incubation was at 0°C . (a) Semilogarithmic plot for the inactivation. E_0 , original enzyme activity; E_t , enzyme activity at time indicated. The DEP concentrations were: $\bullet$, 0.17 ; $\circ$, 0.34 ; $\blacktriangle$, 0.68 ; $\triangle$, 1.02 ; $\blacksquare$, 1.36 ; and $\square$, 1.7 mM . For clarity, some of the data from the same experiment are not shown. In the absence of DEP, δ -crystallin was completely stable during the experimental period. (b) Effects of [DEP] on the inactivation rate.

Protection of the argininosuccinate lyase activity of duck δ -crystallin from DEP inactivation

Partial protection of δ -crystallin from DEP inactivation was afforded by L-arginine, L-citrulline or L-norvaline. Among them, L-citrulline gave the most effective protection (Table 1).

We further explored the protection mechanism of L-citrulline (Cit) by performing the inactivation experiments in the presence of various concentrations of L-citrulline. The result is shown in Figure 2(a). Further examining the data by plotting $1/k_{\text{obs}}$ versus [L-citrulline] according to the method of Mildvan and Leigh (1964) resulted in a hyperbolic curve (Figure 2b). The data thus conformed to a partial protection. A general scheme and rate equation for a diagnostic plot for this kind of protection were derived by Renosto et al. (1985) as shown in eqn. (5):

$$1/\Delta k_{\text{obs}} = [K_{\text{Cit}}/k_2'(1-\beta)](1/[Cit]) + [1/k_1'(1-\beta)] \quad (5)$$

where $\Delta k_{\text{obs}} = k_1' - k_{\text{obs}}$ is the difference between the k_{obs} at [citrulline] = 0 and k_{obs} at unsaturated [citrulline]. K_{Cit} is the dissociation constant of the δ -crystallin–L-citrulline complex, which gave a pseudo-first-order inactivation rate constant of k_2' and $k_2'/k_1' = \beta$. Now the plot of $1/\Delta k_{\text{obs}}$ versus $1/[citrulline]$ was linear, with intercepts of $-1/K_{\text{Cit}}$ and $[1/k_1'(1-\beta)]$ at the

Table 1 Protection of the endogenous argininosuccinate lyase activity of duck δ -crystallin against DEP inactivation

The enzyme inactivation was determined at pH 7.5 and 0°C with $4.18 \mu\text{M}$ δ -crystallin and 1.28 mM DEP. All ligands were dissolved in 0.1 M potassium phosphate buffer, pH 7.5, and the pH was re-adjusted to 7.5 if necessary. Protection (%) = $[k_{\text{obs}}(\text{unprotected}) - k_{\text{obs}}(\text{protected})]/k_{\text{obs}}(\text{unprotected}) \times 100\%$.

Protecting ligand	Protection (%)
None	0
L-Citrulline (1 mM)	86
L-Norvaline (1 mM)	39
L-Arginine (1 mM)	37
Fumarate (4 mM)	0
Pyruvate (4 mM)	0
α -Oxoglutarate (4 mM)	0
cis-Oxaloacetate (4 mM)	0
Maleic acid (4 mM)	0
L-Citrulline (1 mM) + fumarate (4 mM)	90
L-Norvaline (1 mM) + fumarate (4 mM)	66
L-Arginine (1 mM) + fumarate (4 mM)	61
L-Arginine (1 mM) + maleic acid (4 mM)	53
L-Arginine (1 mM) + pyruvate (4 mM)	48
L-Arginine (1 mM) + cis-oxaloacetate (4 mM)	45
L-Arginine (1 mM) + α -oxoglutarate (4 mM)	37

horizontal and vertical axes respectively. Figure 2(c) shows the data of Figure 2(b) replotted according to eqn. (5). The replot yields a K_{Cit} of $0.12 \pm 0.04 \text{ mM}$, which was much smaller than the value obtained from a steady-state kinetic analysis ($K_1 = 7.6 \pm 1.8 \text{ mM}$) (Lee et al., 1992). This discrepancy will be discussed below (see the Discussion section).

On the other hand, fumarate, pyruvate, α -oxoglutarate, cis-oxaloacetate and maleic acid were without any protective effect (Table 1). However, the presence of fumarate or related compounds seemed to potentiate the protective effect of L-arginine, L-citrulline or L-norvaline (Table 1). We thus further investigated the synergistic effect between fumarate and L-arginine.

Double protection of the argininosuccinate lyase activity of duck δ -crystallin from DEP inactivation by L-arginine and fumarate (fum.)

We investigated the synergistic effect between L-arginine and fumarate by a double-protection experiment with various concentrations of L-arginine and fumarate as the protectors. The results presented in Figure 3 conform to the mechanism shown in Scheme 1. In this mechanism, δ -crystallin is proposed to bind with L-arginine and fumarate in a random manner, forming δ -crystallin–L-arginine and δ -crystallin–fumarate binary complexes with dissociation constants of K_{Arg} and K_{fum} , respectively. Binding of either ligand favoured the binding of the other, with an interacting factor of α , and an L-arginine– δ -crystallin–fumarate ternary complex was formed.

Renosto et al. (1985) derived a rate equation for Scheme 1 (eqn. 6):

$$k_{\text{obs}} = \frac{k_1'(1 + [\text{fum.}]/K_{\text{fum}})}{1 + [\text{Arg}]/K_{\text{Arg}} + [\text{fum.}]/K_{\text{fum}} + [\text{Arg}][\text{fum.}]/\alpha K_{\text{Arg}}K_{\text{fum}}} \quad (6)$$

Plots of $1/k_{\text{obs}}$ versus [L-arginine] at different fixed concentrations of fumarate were linear with an intersection point on the $1/k_{\text{obs}}$.

Figure 2 Protection of duck δ -crystallin endogenous argininosuccinate lyase activity against DEP modification by L-citrulline

Experimental conditions were the same as in Figure 1, except that different concentrations of L-citrulline were added. The $[\delta\text{-crystallin}]$ and $[\text{DEP}]$ were $4.18 \mu\text{M}$ and 1.12 mM respectively. (a) Semilogarithmic plot for the inactivation in the presence of different L-citrulline concentrations: $\bullet$, 1; $\circ$, 0.3; $\blacktriangle$, 0.2; $\triangle$, 0.1; $\blacksquare$, 0.05; and $\square$, 0 mM. (b) Reciprocal of the inactivation-rate-versus-[L-citrulline] plot. (c) Linear replot of (b). Δk_{obs} was taken as the difference between k_{obs} at $[\text{L-citrulline}] = 0$ and k_{obs} in the presence of L-citrulline.

axis at $1/k_1'$ (Figure 3a). The line where $[\text{fumarate}] = 0$ intercepted at the horizontal axis at $-K_{\text{Arg}}$, which was found to be 4.3 mM . The slope of these lines was $\{(1 + [\text{fum.}]/\alpha K_{\text{fum.}})/k_1' K_{\text{Arg}}(1 + [\text{fum.}]/K_{\text{fum.}})\}$, which gave a hyperbolic slope versus $[\text{fumarate}]$ replot with limits of $(1/k_1' K_{\text{Arg}})$ (at $[\text{fumarate}] = 0$) and $(1/\alpha k_1' K_{\text{Arg}})$ (at $[\text{fumarate}] = \infty$) (Figure 3b). From the slope replot, the ligand-binding interacting factor (α) was estimated to be ~ 0.15 . (An $\alpha < 1$ means that bound fumarate decreases the arginine dissociation constant and vice versa.)

Figure 3 Synergistic protection of the endogenous argininosuccinate lyase activity of duck δ -crystallin against DEP modification by L-arginine and fumarate

The δ -crystallin concentration was $3.9 \mu\text{M}$; that of DEP was 1.7 mM . (a) $1/k_{\text{obs}}$ -versus-[L-arginine] plot. The fumarate concentrations were: $\triangle$, 0.2; $\blacktriangle$, 0.1; $\circ$, 0.05; and $\bullet$, 0 mM. (b) The slope-versus-[fumarate] replot of (a). Different symbols represented data from separate experiments. (c) The $1/\Delta\text{slope}$ versus $1/[\text{fumarate}]$ replot of (b). The Δslope was taken as the difference between slope (in the presence of fumarate) and slope (in the absence of fumarate). (d) $1/k_{\text{obs}}$ -versus-[fumarate] plot. The L-arginine concentrations were: $\blacksquare$, 3; $\triangle$, 2; $\blacktriangle$, 1; $\circ$, 0.5; and $\bullet$, 0 mM (see eqn. 8). (e) Slope-versus-[L-arginine] replot of (d). (f) Vertical-intercept-versus-[L-arginine] replot of (d).

To obtain the $K_{\text{fum.}}$ value, we transformed the hyperbolic slope equation to a linear form by a similar procedure described by Segel (1975). The slope value in the presence of non-saturating amounts of fumarate was subtracted by the minimum slope value (at $[\text{fumarate}] = 0$), which resulted in eqn. (7):

$$\begin{aligned} \Delta\text{slope} &= \frac{\alpha K_{\text{fum.}} + [\text{fum.}]}{\alpha K_{\text{fum.}} k_1' K_{\text{Arg}} (1 + [\text{fum.}]/K_{\text{fum.}})} - (1/k_1' K_{\text{Arg}}) \\ &= \frac{(1 - \alpha)[\text{fum.}]}{(K_{\text{fum.}} + [\text{fum.}]) \alpha k_1' K_{\text{Arg}}} \end{aligned} \quad (7)$$

The $1/\Delta\text{slope}$ versus $1/[\text{fumarate}]$ replot was linear with slope of $[K_{\text{fum.}} \alpha k_1' K_{\text{Arg}} / (1 - \alpha)]$ and vertical intercept of $[\alpha k_1' K_{\text{Arg}} / (1 - \alpha)]$. Figure 3(c) shows such a plot. The $K_{\text{fum.}}$ obtained from this value was 0.83 mM .

On the other hand, according to eqn. (6), plots of $1/k_{\text{obs}}$ versus $[\text{fumarate}]$ at different fixed concentrations of L-arginine were hyperbolic. This was indeed what we observed with a wide range

Scheme 1 Proposed reaction mechanism for the inactivation of duck δ -crystallin endogenous argininosuccinate lyase activity by DEP in the presence of L-arginine and fumarate

Only L-arginine protected the enzyme activity. Fumarate, however, gave a synergistic protection with L-arginine ($\alpha < 1$). Abbreviations etc.: Crys, duck δ -crystallin; Arg, L-arginine; Fum, fumarate; k_1' , pseudo-first-order inactivation rate constant; k_1 , second-order inactivation rate constant.

Figure 4 Reversible inactivation of the endogenous argininosuccinate lyase activity of duck δ -crystallin

The δ -crystallin concentration was 10.4 μ M; that of DEP was 1.6 mM. After 10 min incubation at 0 $^{\circ}$ C, the residual activity remaining was 30%. The modified δ -crystallin was then treated with 0.63 M hydroxylamine in 0.1 M potassium phosphate buffer, pH 7.5 (pH re-adjusted) (●) or 0.1 M potassium phosphate buffer (pH 7.5) only (○).

of fumarate concentrations (results not shown). However, at $[\text{fumarate}] < K_{\text{fum}}$, eqn. (6) degenerates to:

$$1/k_{\text{obs.}} = \frac{(1 + [\text{Arg}]/\alpha K_{\text{Arg}})[\text{fum.}]}{k_1' K_{\text{fum.}}} + \frac{(1 + [\text{Arg}]/K_{\text{Arg}})}{k_1'} \quad (8)$$

Now the plots of $1/k_{\text{obs.}}$ versus $[\text{fumarate}]$ at different fixed concentrations of L-arginine were linear (Figure 3d). The slope and vertical intercept versus $[\text{L-arginine}]$ replots were also linear (Figs. 3e and f). From these replots, α , K_{Arg} and $K_{\text{fum.}}$ were estimated to be 0.1, 4.9 mM and 0.9 mM respectively.

The above results are completely consistent with our previous kinetic data (Lee et al., 1992). We thus have, by a different

Figure 5 pH-dependence of the inactivation of duck δ -crystallin endogenous argininosuccinate lyase activity by DEP

δ -Crystallin (4.7 μ M) was allowed to react with 1.12 mM DEP in 0.1 M potassium phosphate buffer at various pH values. To avoid other factors that might complicate the interpretation, phosphate buffer was used at all pH values.

approach, confirmed the random Uni Bi kinetic mechanism for the argininosuccinate lyase activity of duck δ -crystallin. The synergistic protection between L-arginine and fumarate with an interaction factor $\alpha \sim 0.12$ was also consistent with our kinetic analysis where binding affinity of fumarate or L-arginine in the L-arginine- δ -crystallin-fumarate ternary complex was an order of magnitude stronger when compared with the affinity in the binary complexes (Lee et al., 1992).

Reversibility of the argininosuccinate lyase activity of DEP-inactivated duck δ -crystallin by hydroxylamine

Reaction of 10.4 μ M δ -crystallin with 1.6 mM DEP at 0 $^{\circ}$ C and pH 7.5 caused a 70% activity loss in 20 min. Addition of hydroxylamine completely restored the enzyme activity within 75 min (Figure 4). Addition of the same amount of buffer alone did not re-activate the enzyme activity. This result excluded a lysine residue as the possible modification site, but strongly suggested modification of a histidine residue (Miles, 1977).

pH-dependence of the inactivation of the argininosuccinate lyase activity of duck δ -crystallin by DEP

The pH-dependent inactivation is shown in Figure 5. Plot of $k_{\text{obs.}}$ versus pH gave a typical titration curve which suggested that an amino acid with a side chain $\text{p}K_{\text{a}}$ value of ~ 6.8 was involved in the inactivation. This $\text{p}K_{\text{a}}$ value was compatible with that for an imidazole group.

Stoichiometry for the inactivation of argininosuccinate lyase activity of duck δ -crystallin and the number of histidine residues modified

Further evidence for the histidine modification came from the difference-absorption-spectrum analysis. The inactivated δ -crystallin showed a u.v. absorption peak with a maximum at 240 nm. The absorption at 280 nm did not change (results not shown). This result ruled out tyrosine modification, which would cause a decreased absorption at 280 nm, but directly indicated the formation of *N*'-ethoxycarbonylhistidine, which absorbs u.v. light at 230–250 nm (Miles, 1977). We observed a gradual increase in A_{240} during the inactivation period (Figure 6a). There was

Figure 6 Stoichiometry between the inactivation of duck δ -crystallin endogenous argininosuccinate lyase activity and the number of His residues modified

Modification experiment was performed in tandem cuvettes. (a) The difference absorption at 240 nm (●) and the enzyme activity (○) were monitored. (b) The residual enzyme activity was plotted against the number of His residues modified per subunit. Different symbols in (b) represent data from separate experiments.

good correlation between the enzyme inactivation and the number of His residues modified (Figure 6b). Extrapolating the data points to zero activity suggested that only one mol of histidine/mol of subunit was ethoxycarbonylated upon total inactivation. Addition of freshly prepared DEP to the reaction mixture at the 18 min time point (Figure 6a) did not cause further inactivation or an increase in A_{240} . Thus only one His residue per subunit of δ -crystallin was modifiable by DEP. In the presence of 2 mM L-citrulline, only 0.2 His was modified per subunit, with 86% enzyme activity remaining after prolonged incubation of δ -crystallin with DEP under the same experimental conditions.

Fluorescence quenching of the native and DEP-modified δ -crystallin by acrylamide

Fluorescence-quenching parameters are good indicators for protein conformational changes (Chang and Lee, 1984). We quenched the fluorescence of duck δ -crystallin with acrylamide to determine the accessibility of tryptophan (Trp) toward the quencher. A downward Stern–Volmer plot was obtained (Figure 7a). This result was anticipated for a heterogeneous system [for a review, see Eftink and Ghiron (1981)]. Duck δ -crystallin contains a total of three Trp residues/subunit. The downward Stern–Volmer plot suggested that these Trp residues were in different micro-environments. We then analysed the quenching data by the Lehrer plot of $F_0/\Delta F$ versus $1/[\text{acrylamide}]$ according to eqn. (4) (Figure 7b). The f_a and K_{sv} values estimated from Figure 7(b) were 0.22 and 0.155 M^{-1} respectively, which suggested

Figure 7 Fluorescence quenching of duck δ -crystallin with acrylamide

(a) Stern–Volmer plot: ○, 0.25 μM native δ -crystallin; □, 0.25 μM DEP-modified δ -crystallin; ●, 0.24 μM denatured δ -crystallin; ■, 0.24 μM DEP-modified denatured δ -crystallin. (b) Lehrer plot; the same symbols as in (a) were used.

that only one Trp residue was partially accessible to acrylamide. The other two Trp residues were buried deeply in the interior of the protein molecule.

When DEP-modified δ -crystallin was analysed by the same method, a downward Stern–Volmer plot was obtained again (Figure 7a). The Lehrer plot was linear and yielded f_a and K_{sv} values of 0.2 and 0.262 M^{-1} respectively (Figure 7b). Thus modification of a single His residue by DEP did not change the accessibility of Trp toward the quencher. The protein did not seem to have gross conformational changes after modification.

On the other hand, when δ -crystallin was treated with 0.4% SDS and then boiled at 100°C for 5 min, complete denaturation of the protein molecule was observed, and f_a and K_{sv} values increased to 1.1 and 1.7 M^{-1} respectively. This result indicated that other Trp residues were now accessible to the quenching agents, as anticipated. The dynamic quenching constant (K_{sv}) increased by ~ 10 -fold after protein denaturation. The DEP-treated denatured δ -crystallin yielded f_a and K_{sv} values of 1.13 and 2.32 M^{-1} respectively.

DISCUSSION

The experimental results presented here indicate that modification of an essential His residue of δ -crystallin by DEP caused enzyme-activity inactivation and that (a) the inactivation was

completely reversible, (b) the pK_a value of 6.8 involved in the inactivation was consistent with that for the imidazole side chain of a His residue and (c) the difference absorption spectrum with maximum at 240 nm indicated the formation of N' -ethoxycarbonylhistidine. Only limited information about the DEP modification of ox liver argininosuccinate lyase is available (Garrard et al., 1985). There are two major differences between duck δ -crystallin and the liver enzyme in DEP modification: (a) fumarate alone protected the liver enzyme, but not the δ -crystallin, and (b) a total of about four His residues were modified in the liver enzyme but only one in δ -crystallin. δ -Crystallin has a total of eight His residues per subunit. This superactive His residue seems to be His-91, as site-directed mutagenesis of this His to glutamine decreased the specific activity by 10-fold as compared with the wild-type enzyme (Barbosa et al., 1991a). The hydrophobicity profile predicted by amino acid sequence revealed that the micro-environment for the N-terminal region was quite different between duck and chicken δ -crystallin (Lin and Chiou, 1992). However, the presence of a His residue at the position 89 of pigeon δ -crystallin, which has very little argininosuccinate lyase activity, put the above suggestion into jeopardy (Lin and Chiou, 1992). Furthermore, if the putative critical histidine residue acts as a general acid-base, it is hard to explain that mutation of it to Gln (Barbosa et al., 1991a) results in only a 10-fold reduction in activity and not a 100% loss. This suggests that either His-91 is not the His modified by DEP or that Gln can act as a weak general acid-base catalyst. Further studies on the expression of pigeon δ -crystallin in some cell-culture system, coupled with site-directed mutagenesis, are necessary to solve this problem.

Involvement of His and carboxy groups in the catalytic mechanism of ox liver argininosuccinate lyase has been suggested by Garrard et al. (1985). The His was proposed to act as an acid-base catalyst by providing an electron pair which abstracts a proton from C-3 of the substrate. The carboxy group donates a proton to the guanidino nitrogen, resulting in the formation of fumarate. The above reaction could proceed by a β -elimination mechanism if both the proton and the arginino moiety left simultaneously. One of the requirements for the β -elimination is that the five atoms, including the base and H-C3-C2-N, be in one plane (cf. Solomons, 1992). Two kinds of arrangement can fulfil this requirement: *trans* between H and the leaving group (dihedral angle = 180°), resulting in *anti*-elimination; or *cis* (dihedral angle = 0°), which leads to *syn*-elimination. Between these two possibilities, the former has been found in most elimination reactions, because in *anti*-elimination the leaving group is in a *staggered* conformation while *syn*-elimination requires an *eclipsed* conformation in the transition state (cf. Carey and Sundberg, 1990).

If the argininosuccinate lyase activity of δ -crystallin proceeds via an *anti*-elimination, it would be very difficult to conceive of the protective effect of L-arginine or L-citrulline. Our protection experimental results suggested that the essential His residue is located at or near the L-arginine-binding domain of the protein. Our protection results may be explained by a *syn*-elimination mechanism. However, this postulation is contrary to the generally accepted *anti*-elimination for the enzyme (Hoberman et al., 1964; Rawn, 1983). Furthermore, our preliminary n.m.r. spectroscopy results indicate that the pro-3S hydrogen atom (H_3), instead of H_{R_3} of the prochiral centre C-3 is labelled in the argininosuccinate molecule synthesized from L-arginine and fumarate by the duck δ -crystallin-catalysed reaction in 2H_2O . This result clearly rules out a *syn*-elimination mechanism (S.-H. Chiou and G.-G. Chang, unpublished work).

Raushel (1984) has prepared a nitro analogue of arginino-

succinate, N^3 -(1-1-carboxy-2-nitroethyl)-L-arginine. This analogue was found to be a strong competitive inhibitor with respect to argininosuccinate with a K_i value of 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 suggests that the inhibitor is an intermediate analogue (Raushel, 1984). The lyase reaction was thus proposed to proceed via a two-step E1cB mechanism. The reaction is initiated by the abstraction of a proton from C-3 of argininosuccinate and generation of a carbanion intermediate. Our experimental results are compatible with this reaction mechanism. The E1cB mechanism is favoured with stronger bases and higher base concentrations (Carey and Sundberg, 1990). Binding of δ -crystallin with argininosuccinate increased the local His concentration near C-3 of the substrate by the proximity and orientation factors. Furthermore, the positive charge in the guanidino group might have contributed another factor in favour of the E1cB mechanism, since positively charged leaving groups shift the mechanism toward the E1cB end of the E1-E2-E1cB spectrum (Abeles, et al., 1992; Carey and Sundberg, 1990).

The E1cB mechanism can also explain the protective effect of L-citrulline. In examining the results presented in Table 1, the protective effect was in the order of L-citrulline > L-norvaline $\geq$ L-arginine. Removing the positively charged guanidino moiety (L-norvaline) did not have appreciable effect on protection. Replacing the positively charged $=NH_2^+$ group with a carbonyl oxygen, on the other hand, greatly increased the protective effect. Furthermore, the K_{cit} value obtained from the protection experiment was at least an order of magnitude smaller than the kinetic determined K_i values (Lee et al., 1992). These results suggest that the carbonyl group might form an extra hydrogen bond with the imidazole group, which greatly decreases the reactivity of His toward DEP and results in the much better protective effect (Table 1).

The protection experiment results also suggest that the succinate-binding domain can accommodate a substrate with maximum of four carbon atoms. α -Oxoglutarate has no synergistic effect. The other structural requirements for this binding domain seem to be less rigorous (Table 1).

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