Ostrich crystallins

Structural characterization of δ -crystallin with enzymic activity

Shyh-Horng CHIOU,*† Chih-Hung LO,* Chien-Ying CHANG,* Takehito ITOH,† Hiroyuki KAJI† and Tatsuya SAMEJIMA†

*Laboratory of Crystallin Research, Institute of Biochemical Sciences, National Taiwan University

and Institute of Biological Chemistry, Academia Sinica, P.O. Box 23-106, Taipei, Taiwan, Republic of China,

and †Department of Chemistry, College of Science and Engineering, Aoyama Gakuin University, Setagaya-ku, Tokyo, Japan

Lens crystallins from the African ostrich (*Struthio camelus*) were isolated and characterized. Four crystallin fractions corresponding to α -, δ/β - and β -crystallins similar to those of duck crystallins were isolated, but ϵ -crystallin was found to be absent. The native molecular masses and subunit structures of the purified fractions were analysed by gel filtration, SDS/PAGE and isoelectric focusing, revealing various extents of heterogeneity in each orthologous crystallin class. An ion-exchange chromatographic method was used for the large-scale preparation of δ -crystallin suitable for structural and enzymic studies. It was unexpectedly found that the purified native δ -crystallin of ostrich lens possessed high argininosuccinate lyase activity, in contrast with chicken δ -crystallin. The c.d. spectra indicated a predominant β -sheet structure in α - and β -crystallins, and a significant contribution of α -helical structure in the δ -crystallin fraction. The estimate of secondary structures from c.d. spectroscopy for each crystallin class bears a resemblance to that of duck crystallins, except that ostrich δ -crystallin possesses much less helical content than duck δ -crystallin. Comparison of crystallin compositions and structures from aquatic and terrestrial birds revealed distinct differences.

INTRODUCTION

Understanding the evolution of various classes of proteins remains a major aim of current research in protein chemistry and molecular biology. The mechanism governing the presence and distribution of various common and specific classes of structural proteins, e.g. lens crystallins, in evolutionarily distant classes of vertebrates is considered to be an excellent system for studying the complex process of evolution in structurally homologous proteins [1,2]. Recent reports on sequence similarities in class-specific ϵ - and δ -crystallins of avian species and some metabolic enzymes [3,4] have contributed to the study of crystallin evolution with regard to their possible enzymic functions.

The African ostrich (*Struthio camelus*), a terrestrial flightless bird, is often regarded as a vestige or living fossil of primitive birds [5]. It is one of the tallest birds, belonging to the avian subclass Ratitae (running birds), and is a good model for comparative and structural studies of avian crystallins. We have recently characterized ϵ -crystallins with lactate dehydrogenase activity from various avian species [6–8]. In the course of the enzymic study of ϵ -crystallin from the Anatidae Family, it was found that the predominant δ -crystallin, which is present almost universally in the avian and reptilian classes [9,10], can be purified to near homogeneity for structural and enzymic characterization. In the present investigation a general characterization of ostrich crystallins was performed, with emphasis on the study of the δ -crystallin.

MATERIALS AND METHODS

Crystallin isolation and purification

Ostrich (*Struthio camelus*) lenses from birds aged about 12–14 months (sexes unidentified) were provided by Dr. W. Oelofsen of the University of Port Elizabeth, Port Elizabeth, Republic of South Africa. The lenses were collected from the abattoir in ice and kept frozen in the laboratory until being freeze-dried and transported to Taiwan. Duck (a hybrid between *Cairina moschata*

and *Anas platyrhynchos* var. *domestica*) lenses were obtained from Taiwan Livestock Research Institute at Yinan, Taiwan.

The pooled freeze-dried lenses, each weighing about 0.8–1.0 g (dry wt.), were homogenized in 10–20 ml of 0.05 M-ammonium bicarbonate buffer, pH 7.5, containing 5 mM-EDTA, as described previously [9,10]. The supernatant fraction from centrifugation at 27000 g was adjusted to give a concentration of about 30–50 mg/ml, and a 3.0 ml portion was applied to a column of Fractogel TSK HW-55 (Merck, Darmstadt, Germany). Anion-exchange chromatography of crude ostrich crystallins was also carried out on a TSK DEAE-650(M) (2.5 cm $\times$ 15 cm) column. Freeze-dried crystallins were dissolved in the starting buffer (0.05 M-ammonium bicarbonate buffer, pH 7.7, containing 5 mM-EDTA and 0.1% 2-mercaptoethanol) and centrifuged before application to the column equilibrated in the same buffer. Elution was carried out in two steps: elution with starting buffer and then elution with a 500 ml linear gradient of 0.05–0.5 M-ammonium bicarbonate buffer, pH 7.5–7.7.

Authentic lactate dehydrogenases and argininosuccinate lyase were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Chemicals for chromatography and activity staining of electrophoresis were from Merck. All reagents and chemicals used were of the highest grades commercially available.

SDS/PAGE, isoelectric focusing and detection of enzyme activity

SDS/PAGE (5% stacking gel, 14% resolving gel) was performed as described by Laemmli [11] with some modifications. Isoelectric focusing in 6.6% polyacrylamide gel containing 2.8% (v/v) carrier ampholytes of pH 3.5–10 was carried out on a slab gel with the incorporation of 0.1% 2-mercaptoethanol and 6 M-urea. For the detection of lactate dehydrogenase activity of crystallin fractions, native PAGE was adopted, with detection with Nitro Blue Tetrazolium chloride/5-methylphenazine methosulphate [8].

† To whom correspondence should be addressed.

Amino acid analysis of ostrich crystallins

Amino acid compositions were determined with the Beckman high-performance amino acid analyser (model 6300) with a dual-channel data system using a single-column ion-exchange chromatography. The procedure for the preparation of protein hydrolysates by microwave irradiation [12] used custom-made Teflon/Pyrex re-usable hydrolysis tubes (4 mm internal diam. $\times$ 150 mm) that can resist high temperature and pressure in the microwave oven ordered from the local glass/plastics shop (The Continuity Enterprise, Taipei, Taiwan). The design of the tubes is based on flushing with inert gas for the removal of O_2 from the tubes and the use of a Teflon cap and plunger to ensure leak-free operations under microwave irradiation [13,14]. Constant-boiling 6 M-HCl and 4 M-methanesulphonic acid containing 0.2% 3-(2-aminoethyl)indole in 1 ml ampoules were obtained from Pierce Chemical Co. (Rockford, IL, U.S.A.). The half-cystine content and tryptophan could be accurately determined by hydrolysis with 4 M-methanesulphonic acid as described previously [14].

The procedure of Marchalonis & Weltman [15] was used to analyse and compare the amino acid compositions of crystallins by using the equation:

$$SAQ = \sum (X_{ij} - X_{kj})^2$$

where the subscripts i and k identify the particular protein pairs being compared, and X_j is the mole content of a given amino acid of type j . The summation is carried out over 18 different amino acids, including tryptophan.

Antibody preparation and immunoblotting

Rabbit antiserum against the various purified crystallins from bovine and avian lenses was prepared by mixing 2 mg of crystallin in 2 ml of phosphate-buffered saline (0.15 M-NaCl/10 mM-sodium phosphate buffer pH 7.4) with 3 ml of complete Freund's adjuvant and injecting it into the backs of several New Zealand White rabbits. Immunoblotting was carried out by electroblotting of protein bands from a SDS/PAGE gel to an Immobilon-P transfer membrane (Millipore Corp., Bedford, MA, U.S.A.) and verifying the presence or absence of the corresponding ϵ - and γ -crystallins with polyclonal antisera against duck ϵ -crystallin and bovine β - and γ -crystallins [16].

Enzymic activity assay

Argininosuccinate lyase activity of various crystallin fractions was assayed by monitoring the absorbance change at 240 nm due to the formation of fumarate as a function of time. In a standard assay for the forward reaction at 25 °C, the reaction mixture contained 2.0 ml of 0.1 M-potassium phosphate buffer, pH 7.5, 0.25 ml of 0.01 M-potassium argininosuccinate and various amounts of crystallin solutions in the same buffer to a final volume of 3.0 ml. The reactions were initiated by the addition of the substrate, and absorbance at 240 nm was read against a blank cell containing all the components of reaction mixture except the substrate. The rate of change was linear with time for about 5 min. Protein content of the assay mixture was estimated by the Coomassie Blue dye-binding method [17].

C.d.-spectral studies

The c.d. spectra for δ -crystallin were obtained on a Jasco J-600 automatic recording dichrograph at room temperature (20 °C). The instrument was calibrated with an aqueous solution of (+)-10-camphorsulphonic acid. The crystallins were dissolved in 0.05 M-Tris/HCl buffer, pH 7.8, at a concentration of 0.050–2.25 mg/ml. All protein solutions were centrifuged and the clear supernatant fractions were used for measurement of c.d. spectra. The protein concentrations of δ -crystallin solutions for the c.d.

study were calculated by using an absorption coefficient of 0.55 (1 mg/ml, at 280 nm), determined from the protein dye-binding method [17], with BSA as standard. The ellipticity data were converted into mean-residue-weight ellipticity by using a mean-residue weight of 115 for δ -crystallin. Analysis of c.d. spectra in the far-u.v. region in terms of the fractions of the structural elements, i.e. helix, β -sheet, β -turn and unordered form, was carried out according to the procedure of Chang *et al.* [18]. A non-linear least-squares curve fitting in the 190–250 nm region at 1 nm intervals of the c.d. spectra was used to find the best estimate for the percentage contribution of each structural element in the crystallin studied.

RESULTS AND DISCUSSION

There have been some reports on physicochemical studies of δ -crystallins from avian and reptilian classes of vertebrates [19–22]. All these studies were mainly concerned with the structural aspect of the major avian δ -crystallin, which is present at more than 50% of the total soluble proteins in some of the avian species. The recent finding [4] that δ -crystallin shares sequence similarity with argininosuccinate lyase (L-argininosuccinate arginine-lyase, EC 4.3.2.1), one of the enzymes in the urea cycle and probably involved in the biosynthesis of arginine from ornithine or citrulline in the uricotelic birds and reptiles, prompted us to characterize this crystallin from some other unique species in the Aves.

Isolation and purification of ostrich crystallins

Fig. 1(a) shows the elution pattern of ostrich lens extracts on a gel-permeation column. Five peaks were obtained for this species, with the last peak being shown to be non-protein small molecules. The identification and classification of each crystallin fraction from the column were based on the subunit analysis by SDS/PAGE (Fig. 1b). The characteristic pattern of the doublet bands locating at about 20 kDa clearly belonged to that of α -crystallin subunits (lane 1 in Fig. 1b). In contrast with the fractionation of duck crystallins reported previously by the same gel-filtration procedure [23], the separation of ostrich crystallins seemed to be less well-defined, with some extent of cross-contamination among isolated peak fractions as indicated in SDS/PAGE (Fig. 1b). However, gel electrophoresis under native conditions (Fig. 1c) clearly identified that peak 1 was mainly composed of α -crystallin, peak 2 was δ/β -crystallins and peaks 3 and 4 were β -crystallins. The native molecular masses for each peak fraction were estimated from a concurrent run of standard proteins calibrated on the same column (Table 1).

The absence of γ -crystallin from ostrich lens is not apparent from the SDS/PAGE pattern shown in Fig. 1(b), since β_L -crystallins (low-molecular-mass β -crystallin) also contain subunits with molecular masses close to that of γ -crystallin. However, from the amino acid compositions (Table 2) of the isolated fractions in Fig. 1(a), peak 4 shows a composition similar to that of β -crystallin instead of γ -crystallin. Immunoblotting of the electroblotted bands of Fig. 1(b) with the antiserum against bovine γ -crystallin revealed no discernible cross-reacting protein bands, corroborating the absence of γ -crystallin from ostrich lens (results not shown). This is consistent with most crystallin studies of the avian species, which indicated γ -crystallin is in general lacking in the avian lenses [24]. The anomalous disappearance of γ -crystallin from most species in the avian class of vertebrates during evolution is a noteworthy observation.

Purification of δ -crystallin by ion-exchange chromatography

We failed to purify the predominant ostrich δ -crystallin (peak

Fig. 1. Characterization of ostrich lens crystallins

(a) Isolation and fractionation of ostrich lens crystallins by gel-permeation chromatography on Fractogel TSK HW-55(S). The column eluates (3.2 ml/tube per 4.1 min) were monitored for absorbance at 280 nm. Four crystallin fractions (1–4) labelled at the top of each peak correspond to α -, δ/β_H -, β_H - and β_L -crystallins respectively (where β_H - and β_L -crystallins represent high-molecular-mass and low-molecular-mass β -crystallins respectively). The small broad peak (fraction 5) at the end of elution is non-protein components of low molecular masses. The percentage yields for each crystallin class were estimated from the areas under each crystallin peak. (b) Gel electrophoresis of the fractionated ostrich crystallins under denaturing conditions (SDS/PAGE) in the presence of 5 mM-dithiothreitol. Lane S, standard proteins used as molecular-mass markers are indicated by thin bars; transferrin (80 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soyabean trypsin inhibitor (20.1 kDa) and lysozyme (14.5 kDa). Lanes 1–4 correspond to the four peak fractions of (a). The gel was stained with Coomassie Blue. The arrow indicates the electrophoretic position of δ -crystallin. (c) Gel electrophoresis of the fractionated ostrich crystallins under native conditions. Lanes 1–4 correspond to the four peak fractions of (a). The arrow indicates the electrophoretic position of δ -crystallin. Note that the lower mobility of α -crystallin (lane 1) is due to its high molecular mass under native conditions.

1 in Fig. 1a) by repeated gel filtration. The co-eluted and contaminant β -crystallins were always found in variable amounts with the major δ -crystallin, as indicated in the SDS/PAGE gels. Therefore ion-exchange chromatography on a TSK DEAE-650 column was used for the isolation and purification of δ -crystallin from total ostrich lens extract (Fig. 2a). Surprisingly a better

resolution of this crystallin from the β -crystallin is achieved. Over 95% pure δ -crystallin is easily obtained, as shown in SDS/PAGE pattern of Fig. 2(b). δ -Crystallin showed a subunit protein band of about 50 kDa, in contrast with those of β - and α -crystallins with lower molecular masses. It is evident that our protocol can provide a much larger amount of pure δ -crystallin than that obtained with isoelectric focusing [4,19,20,22], which should prove very useful in obtaining enough quantities of protein for the characterization of δ -crystallin from other avian species as well.

Since ϵ - and δ -crystallins were the major taxon-specific crystallins found in duck lens and caiman lens [9,10], the immunoblotting technique with the antiserum against purified duck ϵ -crystallin was also used to check whether ϵ -crystallin exists in ostrich lens. No cross-reacting protein bands were detected on the SDS/PAGE gels of ostrich fractions. LDH activity staining for all ostrich fractions of a native PAGE gel also revealed no activity bands similar to those of duck ϵ -crystallin reported previously [8], corroborating the previous finding [7–9] that ϵ -crystallin is uniquely expressed in some species of birds and reptiles with natural habitats close to aquatic environments.

Table 1. Physicochemical parameters of ostrich crystallins

α -, δ -, β_H - and β_L -crystallins correspond to peaks 1–4 fractions in Fig. 1(a) except that δ -crystallin purified from anion-exchange chromatography (Fig. 2a) was substituted for peak 2 in the estimation of percentage yield and c.d. study. The methods used for determination of these parameters are described in the Materials and methods section. Estimation of percentage yield was based on the absorption-peak areas of four fractions in Fig. 1(a) except that that of peak 2 was corrected for contamination by β -crystallin in this peak by using the purified δ -crystallin from Fig. 2(a) and taking into account different absorption coefficients for different crystallins. The values shown for percentage secondary structure represent the estimates from non-linear least-squares curve fitting of c.d. spectra $\pm$ (estimated uncertainty in the fitting procedure).

Property	α	δ	β_H	β_L
Yield (%)	35.1	38.5	20.5	5.9
Native molecular mass (kDa)	440	190	135	85
Subunit molecular mass (kDa)	20, 22	50	26–29	24–28
			34	
Major secondary structure (α -helix/ β -sheet)	β	α	β	β
Secondary structure estimation for ostrich δ -crystallin from c.d. (%)				
α -Helix	β -Sheet	β -Turns	Unordered	
28 $\pm$ 5	18 $\pm$ 6	27 $\pm$ 4	27 $\pm$ 9	

Classification of crystallins by comparison of amino acid compositions

The amino acid compositions of the crystallins (Table 2) also corroborate the correct classification of ostrich crystallins on the basis of their native molecular masses and subunit structures as revealed by gel filtration and SDS/PAGE. In general, the amino acid composition of each orthologous class of crystallins is closely similar and related to that of the corresponding crystallin class from various mammalian species, which reflected the conservative nature of crystallin evolution. Previous statistical comparison of the reliability of phylogenetic information deduced from amino acid sequences and compositions [25,26] has indicated the applicability of amino acid compositions in the comparison of structurally and functionally homologous or

Table 2. Comparison of amino acid compositions of crystallins

Data of amino acid analyses represent means of duplicate determinations. αA_2 , β and δ indicate the amino acid compositions of bovine αA_2 -crystallin, β -crystallin subunits and chicken δ -crystallin based on the cDNA or protein sequences of the respective crystallins. Os-1 and Os-4 indicate ostrich crystallin fractions taken from peaks 1 and 4 of the gel-filtration column (Fig. 1a), and Os-D1 and Os- δ indicate peaks 1 and 3 purified by anion-exchange chromatography (Fig. 2a). $S\Delta Q$ represents the pairwise comparison of amino acid contents between adjacent crystallins as described in the Materials and methods section.

Amino acid	Amino acid composition (mol %)						
	αA_2	Os-1	Os-4	Os-D1	β	Os- δ	δ
CyS	0.6	0.8	1.0	0.9	0.8	0.3	0.0
Asx	9.4	9.1	8.8	7.5	8.6	8.2	7.1
Thr	2.9	3.6	3.1	3.3	2.9	7.1	7.6
Ser	13.3	10.1	7.1	7.7	7.2	9.2	10.1
Glx	9.8	10.7	16.4	16.6	15.9	11.7	11.4
Pro	6.9	7.0	5.3	5.1	6.8	3.1	2.5
Gly	5.9	6.3	9.1	9.5	9.2	5.9	5.6
Ala	3.5	4.6	5.5	5.9	5.2	9.1	8.3
Val	5.7	4.9	5.9	5.3	6.1	6.7	6.9
Met	1.2	2.0	1.7	1.8	1.4	2.0	2.2
Ile	5.2	6.4	4.6	5.4	3.7	7.4	8.1
Leu	8.1	8.5	5.9	5.7	5.7	12.9	14.5
Tyr	3.4	2.5	3.1	3.5	4.8	0.3	1.1
Phe	8.1	6.2	4.5	3.7	4.5	3.3	2.2
His	4.1	3.9	3.3	3.4	3.2	1.4	1.3
Lys	4.1	5.6	5.8	6.5	5.3	6.6	6.5
Arg	7.5	7.4	6.1	5.5	5.7	4.7	4.3
Trp	0.6	0.5	3.2	2.8	3.3	0.3	0.2
$S\Delta Q$		23	77	5	13	182	9

related proteins. We have used a crude index, $S\Delta Q$, proposed by Marchalonis & Weltman [15], i.e. sum of the squares of differences of each amino acid in the composition data (mol %) in any protein pair, to estimate the relatedness of ostrich crystallins to those of well-characterized crystallins. It is noteworthy that the similarity in amino acid content is evident between the pair with $S\Delta Q < 50$, e.g. $S\Delta Q = 23$ between α -crystallins isolated from

bovine and ostrich lenses (Table 2). It is also evident from this comparison that peak 4 of Fig. 1(a) and peak 1 of Fig. 2(a) are almost identical ($S\Delta Q = 5$), with amino acid composition similar to that of bovine β -crystallin. The purified ostrich δ -crystallin was shown to have a composition closely related to that of chicken δ -crystallin except for slightly lower contents of leucine and tyrosine.

Fig. 2. Characterization of duck lens crystallins

(a) Anion-exchange chromatography of duck lens extract on TSK DEAE-650(M). The column eluents (2.6 ml/tube per 2.8 min) were monitored for absorbance at 280 nm. Crude crystallins dissolved in the starting buffer (0.05 M-ammonium bicarbonate buffer, pH 7.7, containing 5 mM-EDTA) were applied to the column equilibrated in the same buffer. Elution was carried out in two steps: elution with 100 ml of starting buffer and then elution with a 500 ml linear gradient of 0.05–0.5 M-ammonium bicarbonate buffer, pH 7.5. Peaks labelled with numbers indicate five fractions used for SDS/PAGE of (b). (b) Gel electrophoresis of fractionated duck crystallins isolated from (a) under denaturing conditions (SDS/PAGE) in the presence of 5 mM-dithiothreitol. Lane S, standard proteins used as molecular-mass markers indicated by thin bars as in Fig. 1b. Lane T, total crude extract before purification. Lanes 1–5 are five peak fractions of (a). The gel was stained with Coomassie Blue. Arrows point to the starting position of the resolving gel. Note the relatively pure δ -crystallin in lane 3, which is used for c.d. spectroscopy.

Fig. 3. Isoelectric focusing of ostrich lens crystallins under denaturing conditions

Lanes 1–5 correspond to the five peaks of Fig. 2(a). The slab gel contained 6 M-urea and 0.1%-mercaptoethanol, and the electrophoresis was run at an initial voltage of 200 V for 6 h until final current of less than 0.5 mA was reached. The approximate isoelectric points of various crystallin polypeptides were estimated from a pI calibration kit (range 4.7–10.6; BDH Chemicals, Poole, Dorset, U.K.) Note the charge heterogeneity of δ -crystallin subunits in lane 3 under denaturing conditions.

Subunit heterogeneity as revealed by isoelectric focusing in 6 M-urea

Despite the apparent homogeneity of most fractions in Fig. 2(b) as shown by the well-defined subunit compositions similar to those reported for mammalian crystallins [27,28], the charge heterogeneity can be detected by isoelectric focusing under denaturing conditions in 6 M-urea, as indicated in Fig. 3. Ostrich α -crystallin (lanes 4 and 5 in Fig. 3) shows more than ten charge-isomeric forms (pI 4.1–7.7), similar to bovine α -crystallin subunits. They are more complex than those α -crystallins shown for the reptilian lenses [29], probably reflective of the genetic polymorphism or some post-translational modifications of this crystallin with aging. β -Crystallin in lanes 1 and 2 in Fig. 3 seemed to exhibit less heterogeneity than the other crystallin fractions. It is of note that ostrich δ -crystallin isolated from the

ion-exchange column shows a relatively high purity on SDS/PAGE (Fig. 2b) and a single band on the native gel (arrow in lane 2 in Fig. 1c). Nevertheless it showed multiple subunit bands in isoelectric focusing under denaturing conditions (lane 3 in Fig. 3). Williams & Piatigorsky [30] have also shown five major and nine minor δ -crystallin bands from the mallard lens by isoelectric focusing under non-denaturing conditions in a sucrose density gradient. Apparently the subunits of most avian δ -crystallin possess an extensive charge heterogeneity.

Conformation study by c.d. spectroscopy

C.d. spectra in the far-u.v. region of the ostrich crystallin fractions were studied to obtain information about the conformation of the peptide backbone. All crystallin fractions except δ -crystallin showed a predominance of β -sheet structure, similar to previous studies on mammalian [31] and reptilian crystallins [29]. The estimations of the fractions of four basic structural elements (Table 1) from c.d. spectroscopy of purified ostrich δ -crystallin (Fig. 4a) indicated about 28% α -helical structure, which is much less than those values (> 50% reported in the previous conformation study of δ -crystallins from chicken and duck [20]. The c.d. spectrum of ostrich δ -crystallin in the near-u.v. region (Fig. 4b), which reflects the intrinsic micro-environments of aromatic amino acid residues, also showed distinct difference from those of δ -crystallins isolated from quail, turkey, chicken, duck and gecko lenses [20]. In these spectra c.d. absorption peaks were all shown to be negative, whereas that of ostrich δ -crystallin possesses several positive ellipticity bands in this region. The difference in helical content is noteworthy in the light of the close amino acid composition index shown in Table 2. Distinct differences in the helical content and the tertiary structures among these closely related crystallins from different species probably indicate subtle differences in their primary structures.

Enzymic activity of ostrich δ -crystallin

We used traditional assays of argininosuccinate lyase activity (Fig. 5) by monitoring the absorbance change at 240 nm due to the formation of fumarate [32]. A comparison of the enzymic activities was performed on chicken, duck and ostrich lens extracts and purified δ -crystallins. Unexpectedly, it was found that ostrich crude extract and δ -crystallin possessed high enzymic

Fig. 4. C.d. spectra of ostrich lens δ -crystallins

(a) C.d. spectrum of ostrich δ -crystallins in the far-u.v. region. Conditions are as described in the Materials and methods section. The ellipticity in degrees $\cdot$ cm² $\cdot$ dmol⁻¹ is calculated on the basis of a mean residue weight of 115. The protein concentration was 0.0545 mg/ml, and ellipticity values were taken from the average of ten scans at a time constant of 2 s. (b) C.d. spectrum of ostrich δ -crystallins in the near-u.v. region. Conditions are as described in the Materials and methods section. The ellipticity in degrees $\cdot$ cm² $\cdot$ dmol⁻¹ is calculated on the basis of a mean residue weight of 115. The protein concentration was 2.250 mg/ml, and ellipticity values were taken from the average of ten scans at a time constant of 2 s.

Fig. 5. Comparison of argininosuccinate lyase activity on avian crystallins

The activity was measured at 25 °C for the forward reaction of the conversion of L-argininosuccinate into L-arginine and fumarate on the basis of 1 mg/ml (based on Coomassie Blue dye-binding method) for each sample. Curve 1, chicken lens extract; curve 2, duck lens extract; curve 3, ostrich lens extract; curve 4, duck δ -crystallin; curve 5, ostrich δ -crystallin. The specific activity of ostrich δ -crystallin was estimated to be 9.7 munits/mg. The specific activity of commercial argininosuccinate lyase from bovine liver (freeze-dried powder from Sigma Chemical Co.) under identical conditions at 25 °C was found to be 9.5 munits/mg.

activity despite its long storage (> 6 months) in freeze-dried form. On the basis of the same amount of proteins analysed, the purified ostrich δ -crystallin exhibited about 1.7-fold higher activity than that of duck lens, which in turn was 24-fold higher than that of chicken lens extract. This implies that the stability and enzymic activity of ostrich δ -crystallin are much higher than that present in duck and chicken lenses. Especially noteworthy is the report by Piatigorsky *et al.* [4], who also found very little enzymic activity in the embryonic chicken lens as compared with duck lens and that the activity seemed to decrease with development. It was suggested that δ -crystallin is encoded by two genes in chicken and duck, with only the $\delta 2$ gene product being responsible for the enzymic activity and the predominant chicken $\delta 1$ crystallin thus being devoid of the enzymic activity. In our purification of δ -crystallins from duck or ostrich lenses by anion-exchange chromatography (Fig. 2a) we consistently obtained two δ -crystallin fractions, with the more acidic form (i.e. peak 3 in Fig. 2a) showing higher activity than the other (peak 2 in Fig. 2a). It would be of interest to examine the structural differences between these two δ -crystallin isoforms in order to shed some light on the structure-function relationship of this crystallin.

Conclusions

A systematic and general approach has been carried out to provide some understanding of the chemistry of the crystallins of the African ostrich. We have shown some salient differences in the structure and function of ostrich and other avian crystallins, and demonstrated that the major ostrich δ -crystallin possesses a high argininosuccinate lyase activity, which may be important in

the comparison and tracing of the evolutionary origin of this enzymic crystallin in the avian class of vertebrates.

This work was supported in part by Academia Sinica and the National Science Council, Taipei, Taiwan, Republic of China. We thank Professor W. Oelofsen of the University of Port Elizabeth for his generous assistance in providing the ostrich lenses for this study.

REFERENCES

- Chiou, S.-H. (1986) FEBS Lett. **201**, 69–73
- de Jong, W. W. & Hendricks, W. (1986) J. Mol. Evol. **24**, 121–129
- Wistow, G. J., Mulders, J. W. M. & de Jong, W. W. (1987) Nature (London) **326**, 622–624
- Piatigorsky, J., O'Brien, W. E., Norman, B. L., Kalumuck, K., Wistow, G. J., Borras, T., Nickerson, J. M. & Wawrousek, E. F. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 3479–3483
- Oelofsen, W. (1989) in International Symposium on Biologically Active Proteins and Peptides (Chiou, S.-H., Wang, K.-T. & Wu, S.-H., eds.), pp. 165–175, Academia Sinica, Taipei
- Chiou, S.-H., Chang, W.-P. & Lin, H.-K. (1988) Biochim. Biophys. Acta **957**, 313–317
- Chiou, S.-H., Chang, W.-P. & Lai, T.-A. (1989) Curr. Eye Res. **8**, 1055–1061
- Chiou, S.-H., Lee, H.-J. & Chang, G.-G. (1990) Biochem. J. **267**, 51–58
- Chiou, S.-H., Chang, W.-P. & Chen, C.-C. (1989) Biochem. Int. **18**, 1093–1100
- Chiou, S.-H. & Chang, W.-P. (1989) in International Symposium on Biologically Active Proteins and Peptides (Chiou, S.-H., Wang, K.-T. & Wu, S.-H., eds.), pp. 81–89, Academia Sinica, Taipei
- Laemmli, U. K. (1970) Nature (London) **227**, 680–685
- Chen, S.-T., Chiou, S.-H., Chu, Y.-H. & Wang, K.-T. (1987) Int. J. Pept. Protein Res. **30**, 572–576
- Chiou, S.-H. & Wang, K.-T. (1988) J. Chromatogr. **448**, 404–410
- Chiou, S.-H. & Wang, K.-T. (1989) J. Chromatogr. **491**, 424–431
- Marchalonis, J. J. & Weltman, J. K. (1971) Comp. Biochem. Physiol. B. **38**, 609–625
- Burnette, W. N. (1981) Anal. Biochem. **112**, 195–203
- Bradford, M. M. (1976) Anal. Biochem. **72**, 248–253
- Chang, C. T., Wu, C.-S. C. & Yang, J. T. (1978) Anal. Biochem. **91**, 13–31
- Williams, L. A. & Piatigorsky, J. (1979) Eur. J. Biochem. **100**, 349–357
- Horwitz, J. & Piatigorsky, J. (1980) Biochim. Biophys. Acta **624**, 21–29
- De Jong, W. W., Stapel, S. O. & Zweers, A. (1981) Comp. Biochem. Physiol. B **69**, 593–598
- Williams, L. A., Piatigorsky, J. & Horwitz, J. (1982) Biochim. Biophys. Acta **708**, 49–56
- Chiou, S.-H., Chang, W.-C., Kuo, J., Pan, F.-M. & Lo, T.-B. (1986) FEBS Lett. **196**, 219–222
- de Jong, W. W. (1981) in Molecular and Cellular Biology of the Eye Lens (Bloemendal, H., ed.), pp. 221–278, John Wiley and Sons, New York
- Cornish-Bowden, A. (1977) J. Theor. Biol. **65**, 735–742
- Cornish-Bowden, A. (1980) Biochem. J. **191**, 349–354
- Bindels, J. G., Bessems, G. J. J., de Man, B. M. & Hoenders, H. J. (1983) Comp. Biochem. Physiol. B **76**, 47–55
- Bloemendal, H. (1977) Science **197**, 127–138
- Chiou, S.-H., Chang, W.-P. & Lo, C.-H. (1988) Biochim. Biophys. Acta **955**, 1–9
- Williams, L. A. & Piatigorsky, J. (1979) Biochemistry **18**, 1438–1442
- Harding, J. J. & Crabbe, M. J. C. (1984) in The Eye (Davson, H., ed.), vol. 1B, pp. 207–492, Academic Press, London
- Ratner, S. (1970) Method Enzymol. **17A**, 304–309