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Biochemical comparison of lens crystallins from three reptilian species

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Lens crystallins were isolated from the homogenates of reptilian eye lenses derived from three different species by gel-permeation chromatography and characterized by gel electrophoresis, amino-acid analysis, N-terminal sequence analysis and circular dichroism. Four fractions corresponding to α-, δ/ε/β-, β- and γ-crystallins were obtained for the crystallins from caiman lenses, whereas δ- and γ-crystallin fraction were present in lesser amounts or missing in the turtle and snake lenses, respectively. The native molecular masses for these purified fractions and their polypeptide compositions were determined by gel filtration and SDS-gel electrophoresis, respectively, revealing the typical subunit compositions for each classified crystallin. The spectra of circular dichroism indicate a predominant β-sheet structure in α-, β- and γ-crystallins, and a major contribution of α-helical structure in δ/ε-crystallin fraction, which bears a resemblance to the secondary structure of δ-crystallin from the chicken lenses. Comparison of the amino-acid contents of each orthologous class of reptilian crystallins with those of evolutionary distant species still exhibited similarity in their amino-acid compositions. N-terminal sequence analysis of the crystallin fractions revealed that all fractions except that of γ-crystallin are N-terminally blocked. Extensive sequence similarity between the reptilian γ-crystallin polypeptides and those from other vertebrate species were found, which establish the close relatedness of γ-crystallins amongst the major classes of vertebrates.

Introduction

The lens crystallins of vertebrates comprise a complex group of conserved structural proteins with distant evolutionary relationships [1–3]. We have recently characterized the crystallins from several different species encompassing one class of invertebrates [4] and all five major classes of vertebrates [5–10] with the aim of searching for their evolutionary relatedness and phylogenetic relationships on the basis of their protein and gene structures. The different classes of crystallins not only vary between species, but are also differentially expressed during lens development. In addition, the recent reports of sequence similarity between a minor ε-crystallin of the duck lens and lactate dehydrogenase [11,12] plus the relatedness between ρ-crystallin of the amphibian lens and aldose reductase/aldehyde reductase family [13] have added some interesting aspects of crystallin evolution in relation to the general topics of protein evolution. It is imperative to compare crystallins of various species from different phylogenetic levels in order to shed some light on the mecha-

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nism underlying the process of crystallin diversification and evolution. The molecular approach to this important problem will lie in the extensive characterization of crystallins at both the protein and gene levels from the available species in the animal kingdom. In this report we have furnished some basic information on the physicochemical properties of reptilian crystallins from three different species, which should form a useful basis for the genomic comparison between reptilian and other vertebrate crystallins in the future.

Materials and Methods

Isolation of lens crystallins

Caiman (*Caiman crocodylus apaporiensis*), snake (*Trimeresurus mucrosquamatus*) and river turtle (*Amyda sinensis*) lenses were obtained from local reptile farms. The decapsulated lenses were homogenized in 10–20 ml 0.05 M Tris-sodium bisulfite buffer (pH 7.5) containing 5 mM EDTA as described before [14,15]. The supernatant from centrifugation at 27000 × *g* was adjusted to give a concentration of about 20–30 mg/ml, and the 5.0 ml aliquot was applied to Fractogel TSK HW-55 (Superfine Grade, Merck). It offered good and well-defined resolution, similar to that found in high-performance liquid chromatography (HPLC). Native molecular masses of the eluted fractions were determined on the same column (2.5 × 115 cm) using the following standard proteins: thyroglobulin (670 kDa), catalase (240 kDa), transferrin (80 kDa), ovalbumin (45 kDa) and soybean trypsin inhibitor (20 kDa).

The dialyzed and lyophilized γ-crystallin fraction from the gel-filtration column was further separated into its subfractions on a TSK CM-650 (S) cation-exchange column with a linear gradient of 0.05–0.25 M ammonium acetate (pH 5.9). The separated subfractions and the unfractionated γ-crystallin were taken for amino-acid analysis and circular dichroic studies without detecting substantial differences.

Gel electrophoresis

Isoelectric focusing in 5% 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. The gel was fixed in 12.5% trichloroacetic acid, washed several times with 20% methanol/7% acetic acid and stained with Coomassie blue. SDS-polyacrylamide slab gel (5% stacking/14% resolving gel) was as described [16] with some modifications.

Amino-acid analysis

The amino-acid compositions were determined with the LKB-4150 amino-acid analyzer using a single-column system. The dialyzed and lyophilized protein samples were hydrolyzed at 150 °C on a dry heating block in Pierce re-usable vacuum hydrolysis tubes with constant-boiling 6 M HCl (Pierce Chemicals, U.S.A.) for 1.5 h. We have shown the applicability of high temperature (150 °C) and shortened time (1.5 h) in achieving comparable amino-acid composition data similar to those obtained by the tedious conventional 110 °C/24 h protocol (Ref. 17 and unpublished results).

The half-cystine content was determined separately after performic acid oxidation. Tryptophan was not determined. The procedure of Marchalonis and Weltman [18] was used to analyze and compare the relatedness of amino-acid compositions using the equation:

$$S\Delta Q = \Sigma(X_{ij} - X_{kj})^2$$

where the subscripts i and k identify the particular protein pairs being compared, X_j is the mole content of a given amino acid of type j. The summation is carried out over the 17 types of amino acids typically determined on the 6 M HCl hydrolysates of crystallin samples.

N-terminal sequence analysis

The N-terminal sequences of the four major fractions from the gel-permeation column and the fractionated γ-crystallin subfractions from TSK CM-650 (S) cation-exchange chromatography were carried out by automated Edman degradation with a microsequencing sequenator (Model 477A, Applied Biosystems). The lyophilized crystallin samples each containing about 1–5 nmol of protein were dissolved in 200 μl of 0.1% trifluoroacetic acid or 0.1% SDS/0.1% trifluoroacetic acid (1 : 1, v/v) and 10 μl each for sequence determinations.

Circular dichroism

The circular dichroic spectra for each crystallin were obtained on a Jasco J-41C spectropolarimeter under constant nitrogen flush and the sample was placed in a cell thermostated at 20 °C. The instrument was calibrated with an aqueous solution of (+)-10-camphorsulfonic acid. The crystallins were dissolved in 0.05 M Tris buffer (pH 7.8) at a concentration of 0.5–1.2 mg/ml, depending on the samples. All protein solutions were filtered through an Acrodisc (Gelman) membrane (0.2 μm) before spectra were taken. The ellipticity data were converted to mean-residue-weight ellipticity using a mean residue weight of 115 for all crystallins. Analysis of CD 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. [19]. A nonlinear least-squares curve fitting in the 205–240 nm region at 2 nm intervals of CD-spectra was used to find the best estimate for the percent contribution of each structural element in the studied crystallins.

Protein concentration determinations

The protein concentrations of crystallin solutions were determined by amino-acid analysis. The absorption coefficient (1 mg/ml, at 280 nm) was then determined for each purified crystallin. The protein contents of crystallin samples for the CD study were estimated by their absorbances at 280 nm.

Results and Discussion

There have been some reports on the comparative studies of α-, β- [20–22] and δ-crystallins [23–24] of the submammalian species. However, the systematic characterization and comparison on the crystallin fractions from different species belonging to the reptilian class are still lacking. We have previously detected similar amino-acid compositions for the crystallins of different vertebrate species, which suggested the existence of the sequence similarity in their primary structures [3,5–9]. A more extensive characterization of the crystallins from the species of the the same class of vertebrates may provide some insight into the phenomenon of species diversification and the accompanying molecular origin of crystallin evolution within the vertebrate.

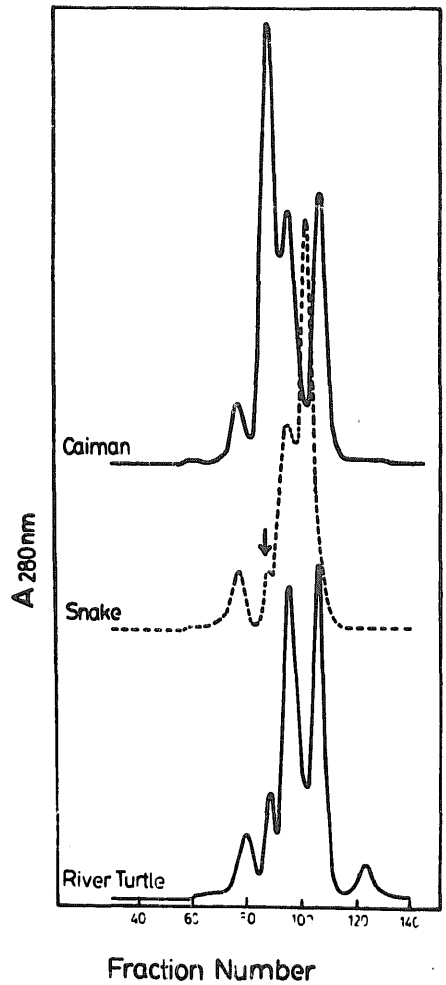

Fig. 1. Gel-permeation chromatography of reptilian crystallins on Fractogel TSK HW-55(S) (2.5×115 cm column). The column eluates (3.5 ml/tube per 4.1 min) were monitored for absorbance at 280 nm. The peak fractions were collected and used for amino-acid analyses (Table I) and SDS-polyacrylamide gel electrophoresis in Fig. 2. The absorbances at 280 nm are relative concentrations in arbitrary units. The small broad peak at the end of elution pattern of river turtle lens is nonprotein components of low molecular masses. The arrow indicates the elution position of δ-crystallin (Sk2) for the snake lens.

Characterization of crystallins of three reptilian species from the gel-permeation chromatography

Fig. 1 shows elution patterns of lens extracts from several reptile species. Four peaks were obtained for all three species in contrast to three for the fish and five for the mammal [3,8]. The identification of each crystallin fraction from the coi-

umn was based on the subunit analysis by SDS-gel electrophoresis of Fig. 2. The clean and characteristic pattern of doublet band for α-crystallin is clearly shown in the first peaks of all three species. In contrast to the case of frog crystallins [9], the reptilian α-crystallin fraction is well separated from β- and other classes of crystallins. The native molecular mass was estimated to be about 600 kDa, which is slightly lower than that of bovine α-crystallin [14].

Re-chromatography of the peaks of gel permeation on the same column to remove some cross-contaminating fractions is sufficient to obtain over 90% pure proteins as indicated in the SDS gels, except that of peak 2 which consistently showed cross-contamination of β- and ε-crystallins in the major fraction of δ-crystallin as revealed by SDS-gel electrophoresis.

It is of interest to note that δ-crystallin seemed to be absent from the river turtle lens [10] when comparing the subunit compositions of the three reptilian species (lanes 2 of Fig. 2A–C). The possibility that it aggregated as a high-molecular species as indicated by the band in the top region of the gel with molecular mass greater than 120 kDa (see peak 2 of Fig. 2C) can be ruled out since the electrophoresis was run in the presence of 5 mM dithiothreitol and 0.1% SDS. However, Williams et al. [25] reported the prominent presence of δ-crystallin in the lenses of the red-eared turtles (*Pseudemys scripta*), with a subunit molecular weight of about 48000. The reason for the discrepancy as to δ-crystallin in the two species of turtles is not clear. However, the river turtle used in this study belongs to the order of Amphichelydia and the sea turtle that of Cryptodira (both were evolved from the order of Anapsida). The absence of δ-crystallin in the river turtle may just reflect the diversification between the two species of turtles. This should constitute an interesting topic for the future phylogenetic comparison of lens crystallins in these two groups of modern turtles from the viewpoint of protein evolution and species diversification.

Another interesting aspect in the comparison of the three species of reptiles lies in the apparent lack of γ-crystallin in the snake as judged from the subunit composition of snake crystallins (Fig. 2B). The amino-acid compositions of these crystal-

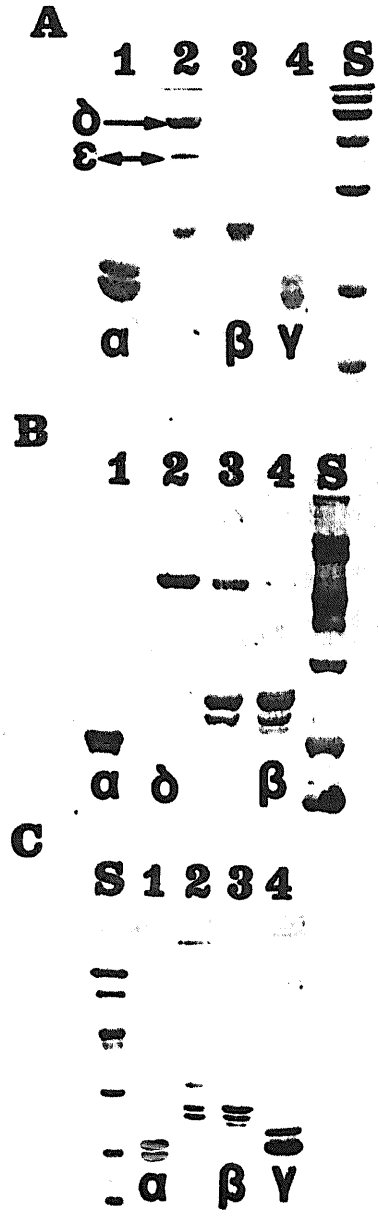

Fig. 2. Gel electrophoresis of the fractionated reptilian crystallins under denaturing conditions (SDS-polyacrylamide gel electrophoresis) in the presence of 5 mM dithiothreitol. Lanes S, standard proteins used as molecular mass markers (in kDa): phosphorylase b (94), bovine serum albumin (66), ovalbumin (45), carbonic anhydrase (30), soybean trypsin inhibitor (20.1) and lysozyme (14.5). Lanes 1–4 correspond to the four peak fractions of of Fig. 1. The gels were stained with Coomassie blue in (A) caiman and (C) river turtle and silver staining for (B) snake crystallins. The unambiguous assignment of fractionated crystallins to each class of crystallins has been indicated below each lane. Arrow and double-head arrow in (A) represent the electrophoretic positions of δ-crystallin and ε-crystallin, respectively.

TABLE I

COMPARISON OF AMINO-ACID COMPOSITIONS OF CRYSTALLINS

Amino acid [a]	Cm1	Sk1	Rt1	Sk2	Cm3	Rt3	Sk4	Cm4	Rt4
Half Cys	0.5	0.5	0.6	0	0.6	0.7	1.4	3.2	2.6
Asx	9.7	10.0	9.4	12.0	9.5	9.9	8.1	9.2	9.9
Thr	4.1	4.2	4.0	7.5	3.2	3.3	2.9	3.0	2.3
Ser	12.2	12.0	10.7	8.1	8.6	7.7	10.6	5.9	6.8
Glx	9.6	10.5	9.9	10.7	15.8	15.3	14.6	14.0	12.7
Pro	7.5	7.6	8.7	3.3	6.6	5.8	7.5	3.6	5.2
Gly	5.3	5.2	5.6	6.5	10.1	10.8	9.4	7.8	7.6
Ala	3.2	3.1	4.1	7.0	3.3	4.5	5.5	2.5	1.9
Val	5.4	5.5	4.7	6.1	6.2	7.4	5.7	3.5	4.5
Met	1.9	1.3	1.2	1.4	1.6	0.8	1.8	2.9	3.0
Ile	6.1	4.7	5.2	6.2	5.8	4.3	4.2	4.7	4.4
Leu	9.6	8.8	8.6	11.7	5.5	5.8	5.8	10.6	9.0
Tyr	3.6	2.6	2.1	3.6	4.7	3.5	3.8	9.3	8.1
Phe	6.0	7.8	7.5	3.5	5.2	5.2	4.6	5.0	5.7
His	4.6	4.7	5.0	1.7	2.7	2.2	2.5	2.1	3.2
Lys	3.9	3.7	5.2	5.8	5.3	7.5	5.2	2.3	2.1
Arg	6.8	8.0	7.8	5.1	5.7	5.8	6.0	10.5	10.8
Trp	n.d. [b]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
$S\Delta Q$ [c]	10	8	115	133	15	28	142	14	

[a] Data of amino-acid analyses are expressed as mol%, and the abbreviations Cm, Sk and Rt are for caiman, snake and river turtle, respectively, with the numbers indicating the peak fractions of gel filtration chromatography in Fig. 1.

[b] n.d., not determined.

[c] $S\Delta Q$ represents the pairwise comparison of amino-acid contents of adjacent crystallins as described in 'Materials and Methods'.

TABLE II

PHYSICOCHEMICAL PARAMETERS OF REPTILIAN CRYSTALLINS

	Caiman (Cm)	Snake (Sk)	River turtle (Rt)
1. classes of crystallins	α, β, δ, ε, γ	α, δ, β	α, β, γ, δ (?)
2. Native molecular mass (kDa)	Cm1 (600)	Sk1 (580)	Rt1 (510)
	Cm2 (190)	Sk2 (190)	Rt2 (160)
	Cm3 (120)	Sk3 (140)	Rt3 (120)
	Cm4 (23)	Sk4 (60)	Rt4 (25)
3. Subunit molecular mass (kDa)	Cm1 (20, 22)	Sk1 (21, 22)	Rt1 (20, 22)
	Cm2 (50, 27, 26)	Sk2 (50)	Rt2 (150, 33, 26, 23)
	Cm3 (26, 24)	Sk3 (50, 26, 23)	Rt3 (26, 23, 22)
	Cm4 (19–23)	Sk4 (23–26)	Rt4 (20–23)
4. Major secondary structure	Cm1 (β)	Sk1 (β)	Rt1 (β)
	Cm2 (α)	Sk2 (α)	Rt2 (β)
(α-helix/β-sheet)	Cm3 (β)	Sk3 (β/α)	Rt3 (β)
	Cm4 (β)	Sk4 (β)	Rt4 (β)

5. % Secondary structure for caiman crystallins

	Cm1	Cm2	Cm3	Cm4
α-helix	9± 5	52±6	15± 4	12± 3
β-sheet	35± 8	17±5	41± 6	38± 6
β-turn	23±10	20±6	24±10	21±12
Unordered form	33± 6	11±8	20± 8	29± 5

The methods used for determination of these parameters are described in 'Materials and Methods'. The values shown for % secondary structure represent the estimates from nonlinear least-squares curve fitting of CD spectra ± (estimated uncertainty in the fitting procedure).

lins (Table 1) also corroborate that none of the fractions of the snake crystallins corresponded to the composition of γ-crystallin, and the peak 4 fraction of snake lens (Sk4) indeed belonged to β-crystallin family. Since the crystallins isolated from the caiman lens [15] comprise both δ- and γ-crystallins which are missing in the river turtle and snake lenses, respectively, the detailed biochemical comparison has been carried out based on the characterization of caiman crystallins.

Physicochemical parameters of reptilian crystallins

Table II summarizes the physicochemical properties of reptilian crystallins characterized in this study. The native molecular weight and the subunit structures of the orthologous classes of α-, β- and γ-crystallins are in fair agreement with other vertebrate species characterized before [26]. Despite the apparent homogeneity of each fraction as shown by the well-defined subunit compositions similar to those reported for mammalian crystallins [26,27], the charge heterogeneity can be detected by isoelectric focusing under denaturing conditions as indicated in Fig. 3. Caiman γ (Cm4, lane 4 of Fig. 3) shows more than four charge-isomeric forms (p*I* 6.8–8.6) similar to carp and bovine γ-crystallin [7,8,29]. α-Crystallin (lane 1 of Fig. 3) shows four distinct bands with low p*I* corresponding to well-characterized α-A and α-B chains [27]. Peak 2 (Cm2, lane 2 of Fig. 3) exhibited the highest heterogeneity among all crystallin fractions. Williams and Piatigorsky [30] have shown five major and nine minor δ-crystallin bands from the mallard lens by isoelectric focusing. Apparently, the subunits of δ-crystallin possess an extensive charge heterogeneity. We have failed to purify caiman δ-crystallin by repeated gel filtration and various ion-exchange chromatographies. The coeluted and contaminated ε- and β-crystal-

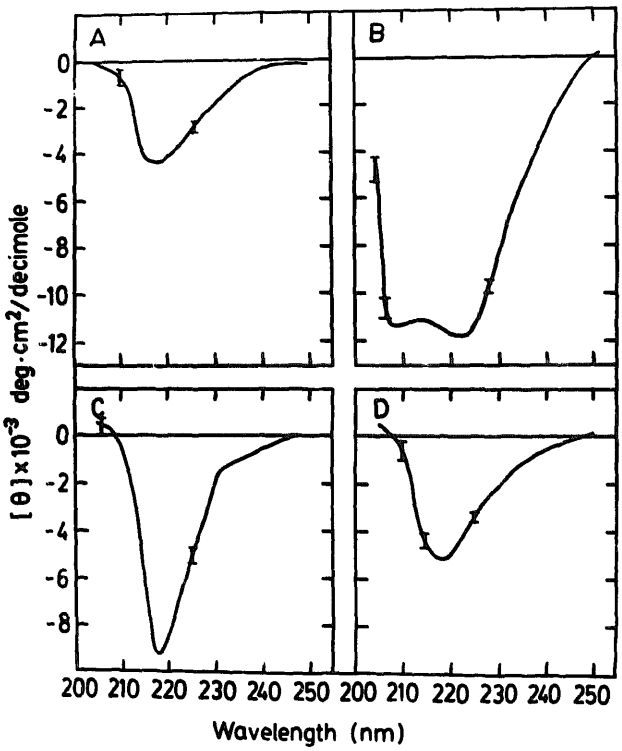

Fig. 4. CD spectra of caiman crystallins in the far-ultraviolet region. Conditions are as described in 'Materials and Methods'. [θ] is the mean residue ellipticity in degrees·cm² per decimole based on a mean residue weight of 115 for all crystallins. (A) Cm1 (B) Cm2 (C) Cm3 and (D) Cm4 correspond to the four re-chromatographed peaks of Fig. 1. The error bars represent the noise levels of the instrument.

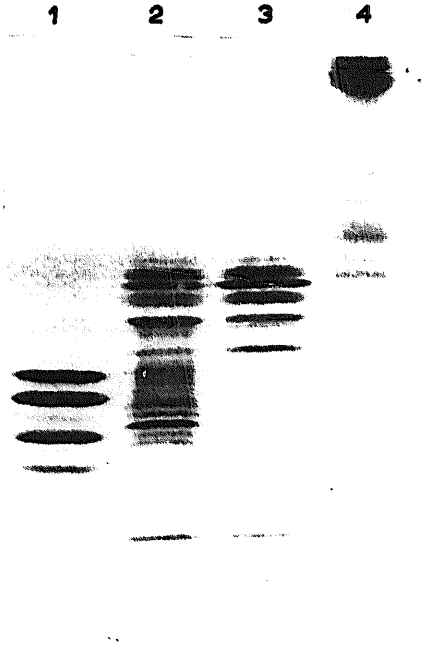

Fig. 3. Isoelectric focusing of caiman crystallins under denaturing conditions. Lanes 1–4 correspond to Cm1-Cm4 of Fig. 1. The slab gel contained 6 M urea and 0.1% 2-mercaptoethanol and the electrophoresis was run at an initial voltage of 200 V for 6 h until a final current of less than 0.5 mA was reached. The approximate isoelectric points of various crystallin polypeptides were estimated from a p*I* calibration kit (range 4.7–10.6, BDH Chemicals). The p*I* values of 4.8–5.9, 5.0–7.1, 6.2–7.1 and 6.8–8.6 were obtained for Cm1 to Cm4, respectively.

Fig. 5. Comparison of N-terminal sequences of γ-crystallins from various species. The sequences listed for caiman, carp and frog crystallins were taken from this study and those for other species from Ref. 15 and the references therein. The homology region is boxed. Amino-acid residues are denoted by one-letter symbols.

lins were always found in varied amounts with the major δ-crystallin in the SDS gels. Therefore, the amino-acid analysis was carried out on a purer δ-crystallin from the snake lens despite a much lower content of this crystallin in the snake species (Fig. 1 and Table I).

Circular dichroic study

CD spectra in the far-ultraviolet region for the caiman crystallin fractions are shown in Fig. 4. All crystallin fractions except Cm2 corresponding to δ-crystallin showed the predominance of β-sheet structure. The estimations of the fractions of four basic structural elements (Table II) indicated about 35–45% of β-sheet contribution for α-, β- and γ-crystallins, whereas about $52 \pm 6\%$ α-helical structure was found in Cm2. This is consistent with the previous conformation study of chicken δ- and duck ε-crystallins [31,32], further corroborating the existence of similar conformation of the peptide backbone in the orthologous crystallins of the vertebrate lens. The CD spectra of the fractions of the turtle and snake crystallins were also compared with the corresponding fractions of caiman crystallins (data not shown); essentially no qualitative differences could be found in the peptide region, except that there were small quantitative differences in the magnitude of ellipticities. It is noteworthy that α-, β- and γ-crystallins demonstrated essentially similar secondary structures even from the evolutionarily distant species despite their differences in primary sequences and immunological properties (unpublished results). This would indicate some conformational relatedness among the major crystallin classes characterized here.

N-terminal sequence analysis

A more defined sequence comparison of these crystallins is needed for establishing the phylogenetic relationships amongst different classes of crystallins. It is surprising to find that all crystallins except γ-crystallins contain blocked N-terminal residues (Table III). We have also found a blocked N-terminus for the peak 4 fraction of the snake, strengthening our conclusion that the snake lens lacks γ-crystallin fraction which is present in turtle and caiman species of reptiles. Hence, the preliminary sequence analyses were carried out on caiman and turtle γ-crystallin fractions. N-terminal sequence analyses of the crude (turtle) and purified γII-crystallin subfractions (caiman) from cation-exchange column indicated that they were closely related to each other with almost identical N-terminal sequences (Ref. 15 and Table III). Some differences and heterogeneity were detected along the sequences by microsequencing. There are several positions at which two amino acids have been identified, which is indicative of more than four γ-crystallin polypeptides present in the

TABLE III

THE AMINO-TERMINAL SEQUENCES OF REPTILIAN CRYSTALLINS

The residues with more than one amino acid denote those positions where more than one phenylthiohydantoin derivative was detected by automatic sequencing. Dashes indicate the unidentified amino acids. Cm1–3, Sk1–4 and Rt1–3 of Table II were found to be N-terminally blocked.

Amino acid	Rt4	Cm4	Cm γII
1	Gly	Gly	Gly
2	Lys	Lys	Lys
3	Ile/Val	Ile	Ile
4	Thr/Ile	Thr/Ile	Thr
5	Phe	Leu/Phe	Leu
6	Tyr	Tyr/Phe	Phe
7	Glu	Glu	Glu
8	Glu	Glu/Gly	Glu
9	Lys	Lys/Arg	Lys
10	Asn	Asn	Asn
11	Phe	Phe	Phe
12	Gln/Glu	Glu/Gln	Gln
13	Gly	Gly	Gly
14	Leu/Arg	Arg	Arg
15	Ser/Cys	Ser/Cys	Ser
16	Tyr	Tyr/Phe	Tyr
17	Glu	Glu	Glu
18	Cys	Cys	Cys
19	Ser	Arg/Ser	Ser
20	Ser	-	Ser

reptilian γ-crystallin family. Comparison of the amino-terminal sequences of γ-crystallin polypeptides from the six different species encompassing four major classes of vertebrates, and wide evolutionary history showed a high degree of sequence similarity (Fig. 5). γ-Crystallin was found in either decreased amounts or was missing in the lenses of the bird class [5,26] and therefore not included for comparison. The percentage of sequence identity for the first 20 residues of all species is about 70%. The protein sequence study associated with the present report has indicated a great deal of difficulty encountered in the purification of the tryptic and cyanogen bromide fragments of γ-crystallin polypeptides due to their inherent microheterogeneity, which makes the sequence determination of crystallin polypeptides more feasible at the gene than at the protein level. The partial sequences reported here could also provide the crucial information in making several oligonucleotide probes for the future molecular cloning of reptilian crystallin genes.

Conclusions

A systematic and general approach has been carried out to provide some basic information on the protein chemistry of reptilian crystallins. Some salient differences between different species belonging to the same class have been found, which may be important in the comparison and tracing of the evolutionary origin amongst these related species.

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