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Sequence comparison of γ-crystallins from the reptilian and other vertebrate species

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Lens crystallins were isolated from homogenates of reptilian eye lenses (*Caiman crocodylus apaporiensis*) by gel-permeation chromatography and characterized by gel electrophoresis, and amino acid and N-terminal sequence analyses. Four fractions corresponding to α-, δ/ε/β-, β- and γ-crystallins were identified on the basis of their electrophoretic patterns as revealed by SDS gel electrophoresis. Comparison of the amino acid contents of reptilian crystallins with those of mammals suggests that each orthologous class of crystallins from the evolutionarily distant species still exhibits similarity in their amino acid compositions and probably sequence homology as well. All fractions except that of γ-crystallin were found to be N-terminally blocked. N-terminal sequence analysis of the purified γ-crystallin subfractions showed extensive homology between the reptilian γ-crystallin polypeptides themselves and also those from other vertebrate species, suggesting the existence of a multigene family and their close relatedness to γ-crystallins of other vertebrates.

Crystallin; Subunit structure; Microheterogeneity; Sequence homology; Multigene family; (Reptile)

1. INTRODUCTION

The lens crystallins of vertebrates form a complex group of highly 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 [5–8] with the aim of searching for their evolutionary relatedness and phylogenetic relationships. It has been emphasized repeatedly that the evolution and complexity of different classes of lens crystallins are developmentally regulated [9,10]. However, the mechanism governing the process of crystallin diversification remains largely unknown and speculative. 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. Here, we have furnished the first sequence information on crystallins of the reptilian class, which should form a useful basis for future genomic comparison between reptilian and other vertebrate crystallins.

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2. MATERIALS AND METHODS

Caiman (*Caiman crocodylus apaporiensis*) lenses were obtained from the local reptile farm. The decapsulated lenses were homogenized in 10–20 ml 0.05 M Tris-Na bisulfite buffer, pH 7.5, containing 5 mM EDTA as described [11]. The supernatant from centrifugation at 27 000 × *g* was adjusted to give a concentration of about 20–30 mg/ml and a 5.0 ml aliquot was applied to Fractogel TSK HW-55 (superfine grade, Merck).

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The 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 SDS-polyacrylamide slab gel (5% stacking/14% resolving gel) was as described [12] with some modifications. The amino acid compositions were determined with an LKB-4150 amino acid analyzer using a single-column system. The dialyzed and lyophilized protein samples were hydrolyzed at 110°C in evacuated tubes with constant-boiling 6 N HCl (Pierce, USA) for 24 h. Half-cystine was determined separately after performic acid oxidation. Tryptophan was not determined.

The N-terminal sequences of the 4 major fractions from 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 477, Applied Biosystems). The lyophilized crystallin samples each containing about 1–5 nmol protein were dissolved in 200 μl of 0.1% trifluoroacetic

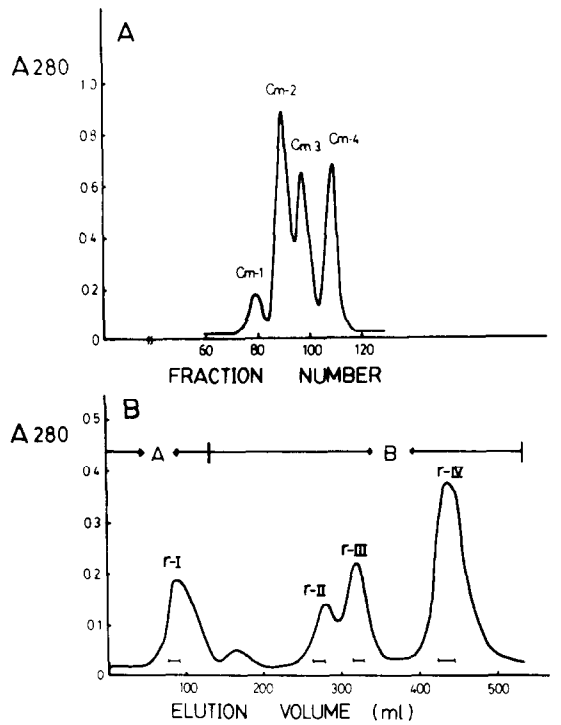

Fig.1. Isolation and purification of caimans lens crystallins. (A) Gel-permeation chromatography on Fractogel TSK HW-55(S) (2.5 × 115 cm column) of lens extract was carried out as described in section 2. The column eluates (3.5 ml/tube per 4.1 min) were monitored for absorbance at 280 nm. The peak fractions (Cm-1–Cm-4) were collected and used for amino acid analyses (table 1) and SDS-PAGE in fig.2. (B) Cation-exchange chromatography on TSK CM-650 (S) (2.5 × 15 cm column) of crude γ-crystallin fraction (Cm-4) from (A). The column eluates (2.4 ml/tube per 4.4 min) were monitored for absorbance at 280 nm. About 30 mg crude crystallin dissolved in the starting buffer (0.05 M ammonium acetate, 0.05% 2-mercaptoethanol, pH 5.9), was applied to the column equilibrated in the same buffer. Elution was carried out in two steps: starting buffer was used in step A and linear gradient of 0.05–0.25 M ammonium acetate in step B. Solid bars under the 4 major subfractions (γ-I–γ-IV) indicate pooled fractions used for sequence analyses.

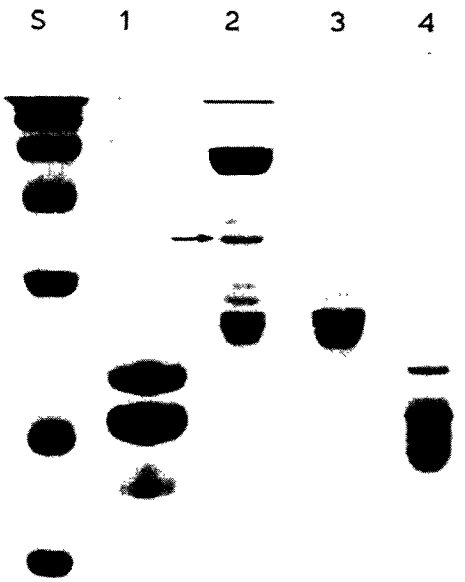

Fig.2. Gel electrophoresis of the fractionated caiman crystallins under denaturing conditions (SDS-PAGE) in the presence of 5 mM dithiothreitol. Lane S, standard proteins used as molecular mass markers (in kDa): transferrein (80), bovine serum albumin (66), ovalbumin (45), carbonic anhydrase (30), soybean trypsin inhibitor (20.1) and lysozyme (14.5). Lanes 1–4 correspond to the 4 fractions (Cm-1–Cm-4) of fig.1A. The gel was stained with Coomassie blue. The arrow in lane 2 indicates the minor ϵ-crystallin subunit of 37.5 kDa.

acid (TFA) or 0.1% SDS/0.1% TFA (1:1, v/v) and 10 μl each for sequence determinations.

3. RESULTS AND DISCUSSION

There have been some reports on comparative studies of δ-crystallins [13,14] of the submammalian species. However, sequence characterization of the crystallin fractions from the reptilian class is still lacking. We have previously detected similar amino acid compositions for crystallins of different vertebrate lenses, which suggested the existence of sequence homology in their primary structures [3,5,15]. A mode defined sequence comparison of the crystallins from different classes of vertebrates may provide some insight into the phenomenon of species diversification and the accompanying molecular origin of crystallin evolution within the vertebrates.

Fig.1 shows a typical elution pattern of lens extracts from the caiman lens of the reptilian class. Four peaks were obtained in contrast to three for carp and five for pig [3]. It is of interest to find that the first peak contains α-crystallin as judged from subunit analysis by SDS gel electrophoresis (fig.2), which showed the characteristic doublet subunit bands similar to that of mammalian α-crystallins [16]. Peak 2 contained α- and β-crystallins plus a minor ϵ-crystallin which was not been shown in the reptilian lens of tegu [14]. This crystallin deserves special attention since it has been shown to be identical to the glycolytic enzyme, lactate dehydrogenase [17]. Peaks 3 and 4 are as expected and were identified as β- and γ-crystallins, respectively. Rechromatography of the peak fractions of gel permeation on the same column is usually sufficient to obtain over 90% pure crystallins for amino acid analyses except the peak 2 fraction which consistently contained about 30% β- and ϵ-crystallins as shown in fig.2. Amino acid analyses (table 1) of the four fractions gave further support for the close relatedness of each orthologous class of crystallin between the reptilian and mammalian classes with the exception of δ/ϵ (Cm-2) which is absent in the mammalian lenses.

It is noteworthy that all crystallins except γ-crystallin are all amino-terminally blocked. Hence, we have concentrated on sequence analyses of γ-

Table 1

Comparison of amino acid compositions of crystallins

Amino acids (mol%)	Cm-1	αB_2	Cm-2	δ	Cm-3	βBp	Cm-4	Cow γ
1/2 Cys	0.5	0	0.2	0	0.6	1.0	3.2	3.6
Asx	9.7	7.4	9.7	7.1	9.5	8.8	9.3	10.9
Thr	4.1	4.0	5.2	7.6	3.2	3.4	3.0	3.0
Ser	12.2	9.7	8.9	10.1	8.6	8.8	5.9	7.3
Glx	9.6	9.7	12.8	11.4	15.8	15.7	14.0	10.9
Pro	7.5	9.7	4.1	2.5	6.6	6.9	3.6	4.9
Gly	5.3	4.6	6.9	5.6	10.1	9.3	7.8	7.3
Ala	3.2	5.1	7.1	8.3	3.3	3.9	2.5	1.2
Val	5.4	5.7	6.6	6.9	6.2	6.9	3.5	3.6
Met	1.9	1.1	2.9	2.2	1.6	1.0	2.9	4.2
Ile	6.1	5.7	5.3	8.1	5.8	3.4	4.7	3.6
Leu	9.6	8.6	11.2	14.5	5.5	4.9	10.6	7.9
Tyr	3.6	1.1	1.1	1.1	4.7	4.4	9.3	9.1
Phe	6.0	7.4	4.5	2.2	5.2	3.9	5.0	4.9
His	4.6	5.1	2.8	1.3	2.7	3.9	2.1	3.0
Lys	3.9	5.7	5.4	6.5	5.3	6.4	2.3	1.2
Arg	6.8	8.0	5.8	4.3	5.7	4.9	10.5	11.5
Trp	n.d.	1.1	n.d.	0.2	n.d.	2.5	n.d.	1.8
$S\Delta Q$	37		52		20		34	

Data are taken from this study or recalculated from [3,16] and references therein. $S\Delta Q$ represents the pairwise comparison of amino acid contents of the adjacent crystallins and is used as an index of their relatedness [3]. n.d., not determined

crystallin. The crude γ-crystallin (Cm-4, fig.1A) was further separated on a cation-exchange column and 4 major subfractions were obtained as shown in fig.1B. They may represent allelic variants of genetic polymorphism from the pooled lenses of different specimens or nonallelic gene products as exemplified in mammalian γ-crystallin genes [18]. The similar amino acid contents of these four subfractions (not shown) and the N-terminal sequence comparison reported here seem to be in favor of the latter supposition. It is also of interest to find that γ-I-like α-, β-, δ- and ε-crystallins contain a blocked N-terminal residue. It corresponds to the slowest moving band in lane 4 of fig.2 and probably belongs to the class of β$_s$-crystallin as reported by Quax-Jeuken et al. [19].

It is clear that γ-II, γ-III and γ-IV are closely related to each other with almost identical N-terminal sequences except for some differences and minor heterogeneity being detected along the sequences (table 2). There are several positions at which two amino acids have been identified by microsequencing, which is indicative of more than 4 γ-crystallin polypeptides present in this caiman γ-crystallin family. Comparison of the amino-terminal sequences of γ-crystallin polypeptides from the 5 different species encompassing 4 major classes of vertebrates and wide evolutionary history showed a high degree of sequence homology (fig.3). γ-Crystallin was found in lesser amounts or not at all in the lenses of bird class [5,20] and therefore was not included for comparison. The sequence variations of different species at some of the residues are conservative in nature, i.e. Phe/Tyr at residue 6, Arg/Lys at residue 9 and Gln/Glu at residue 12. However, it is not clear why there is a substitution of Cys in the mammal for Ser in the fish and reptile at residue 15. The percentage of sequence identity for the first 20 residues for all species is about 70%. Detailed comparison regarding the protein stability and conformation [21–23] of γ-crystallins from the different species of vertebrates needs to await determination of the complete sequences of these crystallins.

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 protein level. Nonetheless, the partial sequences reported here did provide the crucial in-

Table 2

The amino-terminal sequences of caiman γ-crystallin polypeptides

Amino acid	Crude γ	γ-II	γ-III	γ-IV
1	Gly	Gly	Gly	Gly
2	Lys	Lys	Lys	Lys
3	Ile	Ile	Ile	Ile
4	Thr/Ile	Thr	Thr/Ile	Ile
5	Leu/Phe	Leu	Leu/Phe	Phe
6	Tyr/Phe	Phe	Phe	Tyr
7	Glu	Glu	Glu	Glu
8	Gly/Glu	Glu	Glu/Gly	Gly
9	Arg/Lys	Lys	Lys	Arg
10	Asn	Asn	Asn	Asn
11	Phe	Phe	Phe	Phe
12	Glu/Gln	Gln	-	Glu
13	Gly	Gly	Gly	Gly
14	Arg	Arg	-	Arg
15	Cys/Ser	Ser	Cys/Ser	Ser
16	Tyr/Phe	Phe/Tyr	Phe	Tyr
17	Glu	Glu	Glu	Glu
18	Cys	Cys	Cys	Cys
19	Arg/Ser	Arg/Ser	-	Arg
20	-	-	-	-

Residues with more than one amino acid denote those positions where more than one phenylthiohydantoin derivative was detected by automatic sequencing. Dashes indicate unidentified amino acids. Peaks 1–3 of fig.1A and γ-I were found to be N-terminally blocked

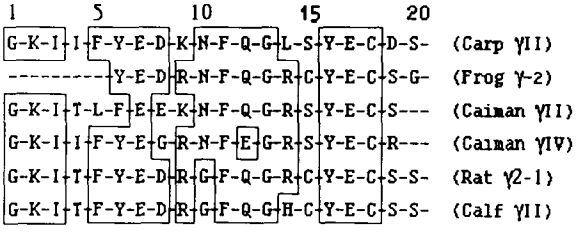

Fig.3. Comparison of N-terminal sequences of γ-crystallins from various species. The sequences listed for caiman crystallins were taken from this study (table 2) and those for other species from [7,18,22,23]. The reported sequence of frog γ-2 lacks the first 5 N-terminal residues. The homology region is boxed. Amino acid residues are denoted by one-letter symbols.

formation in making several oligonucleotide probes for molecular cloning of crystallin genes.

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