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Sequence analysis of pigeon δ-crystallin gene and its deduced primary structure

Comparison of avian δ-crystallins with and without endogenous argininosuccinate lyase activity*

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δ-Crystallin is a major lens protein present in the avian and reptilian lenses. To facilitate the cloning of the δ-crystallin gene, cDNA was constructed from the poly(A)$^+$ RNA of pigeon lenses, amplified by the polymerase chain reaction (PCR). The PCR product was then subcloned into pUC19 vector and transformed into *E. coli* strain JM109. Plasmids purified from the positive clones were prepared for nucleotide sequencing by the dideoxynucleotide chain-termination method. Sequencing two clones, containing 1.4 kb DNA inserts coding for δ-crystallin allowed the construction of a complete, full-length reading frame of 1,417 bp covering a deduced protein sequence of 466 amino acids, including the universal translation-initiating methionine. The pigeon δ-crystallin shows 88, 83 and 69% sequence identity to duck δ2, chicken δ1 crystallins and human argininosuccinate lyase respectively. It is also shown that, in contrast to duck δ2 crystallin which has a high argininosuccinate lyase activity, pigeon δ-crystallin appears to contain very low activity of this enzyme, despite the fact that they share a highly homologous structure. A structural comparison of δ-crystallins with or without enzymatic activity suggested several amino acid replacements which may account for the loss of argininosuccinate lyase activity in the lenses of certain avian species.

Pigeon lens; δ-Crystallin; Argininosuccinate lyase; Polymerase chain reaction (PCR); Sequence comparison; Pigeon; *Columba livia*

1. INTRODUCTION

The lens crystallins of vertebrates comprise a complex group of conserved structural proteins with distant evolutionary relationships [1,2]. Crystallins exist as water-soluble proteins and were previously thought to possess only light-focusing structural roles in the cytoplasm of lens fiber cells. More intriguing is the finding that in the lenses of some avian and reptilian species, two taxon-specific crystallins, i.e. ε- and δ-crystallins, were shown to be homologous to the metabolic enzymes, lactate dehydrogenase and argininosuccinate lyase, respectively [3,4]. Recently we have studied avian ε- and δ-crystallins with genuine enzymatic activity from duck, caiman and goose lenses, establishing a close inter-relatedness between these presumably structural proteins and those authentic enzymes isolated from other tissues with regard to catalytic properties and kinetic mechanisms [5–10].

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*The sequence data of the pigeon δ-crystallin gene have been deposited in the EMBL Data Library under accession number: X66404.

In the course of a systematic study on the presence of these enzymatic crystallins in various species of vertebrates, it was deemed essential to establish a cDNA library and solve the primary structures from the disparate species possessing these crystallins in order to allow a defined structure–function comparison and shed some light on the biological significance for the existence of these stable crystallins in certain species of Aves and Reptiles. In this report we have determined the primary structure of pigeon δ-crystallin by the application of a facile PCR technique. A structural comparison of δ-crystallin sequences from pigeon, chicken and duck lenses revealed some salient differences in these avian crystallins. In contrast to the recent claim [11] that His^{89}→Gln mutation in the chicken δ-crystallin could account for the loss of argininosuccinate lyase activity in this avian species, as compared to the high enzymatic activity in the duck crystallin, pigeon δ-crystallin without a mutation at this position and also showed no enzymatic activity.

2. MATERIALS AND METHODS

2.1. *Preparation of lens mRNA and construction of a cDNA library*

The lenses of common domestic pigeons (*Columba livia*) of about

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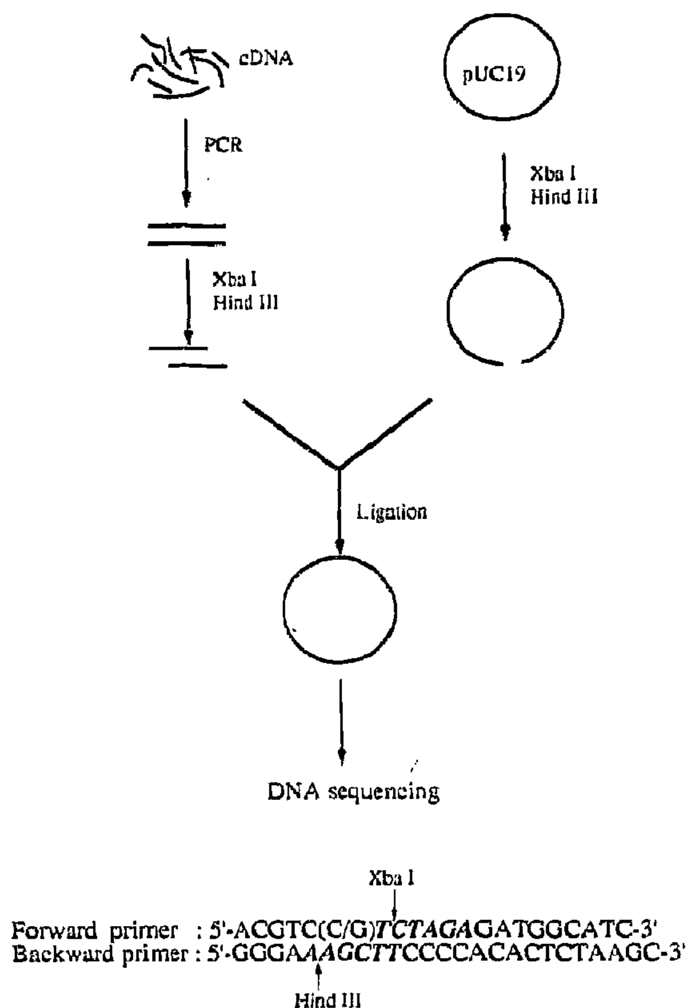
1-month old were obtained from local livestock markets (Taipei, Taiwan). Lenses were removed and stored in liquid nitrogen immediately after the pigeons were sacrificed. For isolation of mRNA, about 2–4 deep-frozen lenses were homogenized and RNA was extracted according to standard procedures [12]. To obtain the full-length δ-crystallin gene of pigeon lens, lens poly(A)$^+$ RNA was purified using the Quick-Prep mRNA preparation kit (Pharmacia, Uppsala, Sweden), and then subjected to the synthesis of a cDNA library using the RiboClone cDNA synthesis system (Promega, Madison, WI, USA).

2.2. *Primer synthesis and PCR amplification*

Two oligonucleotide primers with opposing orientations (covering the 5'- and 3'-coding regions of duck δ1- and δ2-crystallin clones [13]), the forward, 5'-ACG TC(C/G) T|CT AGA GAT GGC ATC-3' and the reverse 5'-GGG AA|A GCT TCC CCA CAC TCT AAGC-3', were first synthesized, each containing *Xba*I and *Hin*dIII restriction sites, respectively, with vertical lines indicating the cleavage sites.

PCR reactions were carried out in a 100 μl volume containing 100 ng of template, 500 ng of each primer, 0,225 mM of each dNTP, 2.5 U of *Taq* polymerase and other buffer components as recommended by Promega Corp. The reactions were subjected to 40 cycles of heat denaturation at 94°C for 1.5 min, annealing the primers to the DNAs at 50°C for 1 min, and DNA chain extension with *Taq* polymerase at 72°C for 2 min, followed by a final extension at 72°C for 10 min. The PCR products were separated on a 1.2% agarose gel, electroeluted, and digested with *Xba*I and *Hin*dIII. The DNA fragments were subcloned into pUC19 previously digested with these two enzymes, and then transformed into *E. coli* strain JM109. Plasmids purified from the positive clones were prepared for nucleotide sequencing the dideoxynucleotide chain-termination method [14].



Scheme 1. General strategy for the amplification of cDNA by polymerase chain reaction (PCR) and cloning of amplified δ-crystallin DNA for nucleotide sequencing. Two oligonucleotide primers with *Xba*I and *Hin*dIII restriction sites covering 5'- and 3'-coding regions of duck δ-crystallin cDNA clones are used for PCR amplification.

2.3. *Assay of enzymatic activity*

Argininosuccinate lyase activities of duck, chicken and pigeon δ-crystallins, plus total lens homogenates from these three species, were assayed at 25°C. The formation of fumarate in the forward direction and its disappearance in the reverse direction were monitored continuously at 240 nm in a Hitachi UV-Visible (Model U-2000) spectrophotometer. In a standard assay for the forward reaction, the reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 0.2 mM argininosuccinate and an appropriate amount of δ-crystallin in a total volume of 1 ml. Authentic argininosuccinate lyase from the bovine liver was obtained from Sigma.

2.4. *Hydropathy profile*

A program analysis of the local hydrophilicity of a protein along its amino acid sequence, based on the Kyte–Doolittle hydropathy scale [15], was carried out on the MacVector sequence analysis software for Macintosh computers (International Biotechnologies, Inc., New Haven, CT), however, the signs of the values have been reversed in order to plot the hydrophilicity instead of hydrophobicity scale. A window of size $n = 7$ was run along the length of the protein; for each window, the hydropathy values of the 7 amino acids were summed and divided by 7 to obtain the average hydrophilicity per residue for the window. Values above the axis denote hydrophilic regions which may be exposed on the outside of the protein molecule, whereas those values below the axis indicate hydrophobic regions which tend to be buried inside the protein.

3. RESULTS AND DISCUSSION

The different classes of crystallins not only vary between dispartate taxons of vertebrates but are also differentially expressed during lens development. The avian lens differs, at least quantitatively, from other

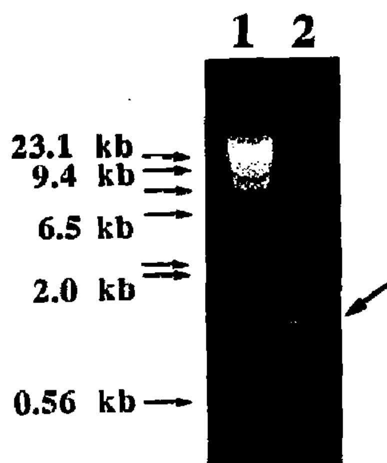


Fig. 1. Identification and size determination of amplified δ-crystallin cDNA. Electrophoresis was carried out in a 1.2% agarose gel. Lane 1, DNA size markers of the *Hin*dIII digestion products of λDNA, ranging from 0.56 to 23.1 kb; lane 2, amplified PCR product of about 1.4 kb (arrow) coding for pigeon δ-crystallin.

species' lenses in several aspects [16]: (i) it has a very low proportion of protein; (ii) it is very soft throughout life, never developing the hard nucleus of many mammalian and piscine lenses. It appears that many of these properties may be correlated with the presence in the bird lens of a unique and predominant component, δ-crystallin (previously called FISC, or first important soluble crystallin) [17], which constitutes about 30–60% of most avian lens proteins. Especially noteworthy is the association of δ-crystallin with genuine argininosuccinate lyase (L-argininosuccinate arginine lyase, EC 4.3.2.1) activity [4,9,10] in some of the avian species, one of the key enzymes in the urea cycle. This has prompted us to solve the primary structure of this relatively large tetrameric protein of 200 kDa by cDNA cloning and sequencing.

3.1. *cDNA amplification by PCR and sequence analysis*

Previous N-terminal sequence analyses of the purified pigeon δ-crystallin isolated from TSK DE-650 anion-exchange columns [5,6] by Edman degradation were carried out in the microsequencing sequenator. None were found to yield N-terminal residues upon repeated trials, indicative of a blocked N-terminus in this crystallin (data not shown).

We have therefore resorted to the analysis of protein sequence by molecular cloning of cDNAs constructed from total mRNA of the fresh pigeon lenses. Subse-

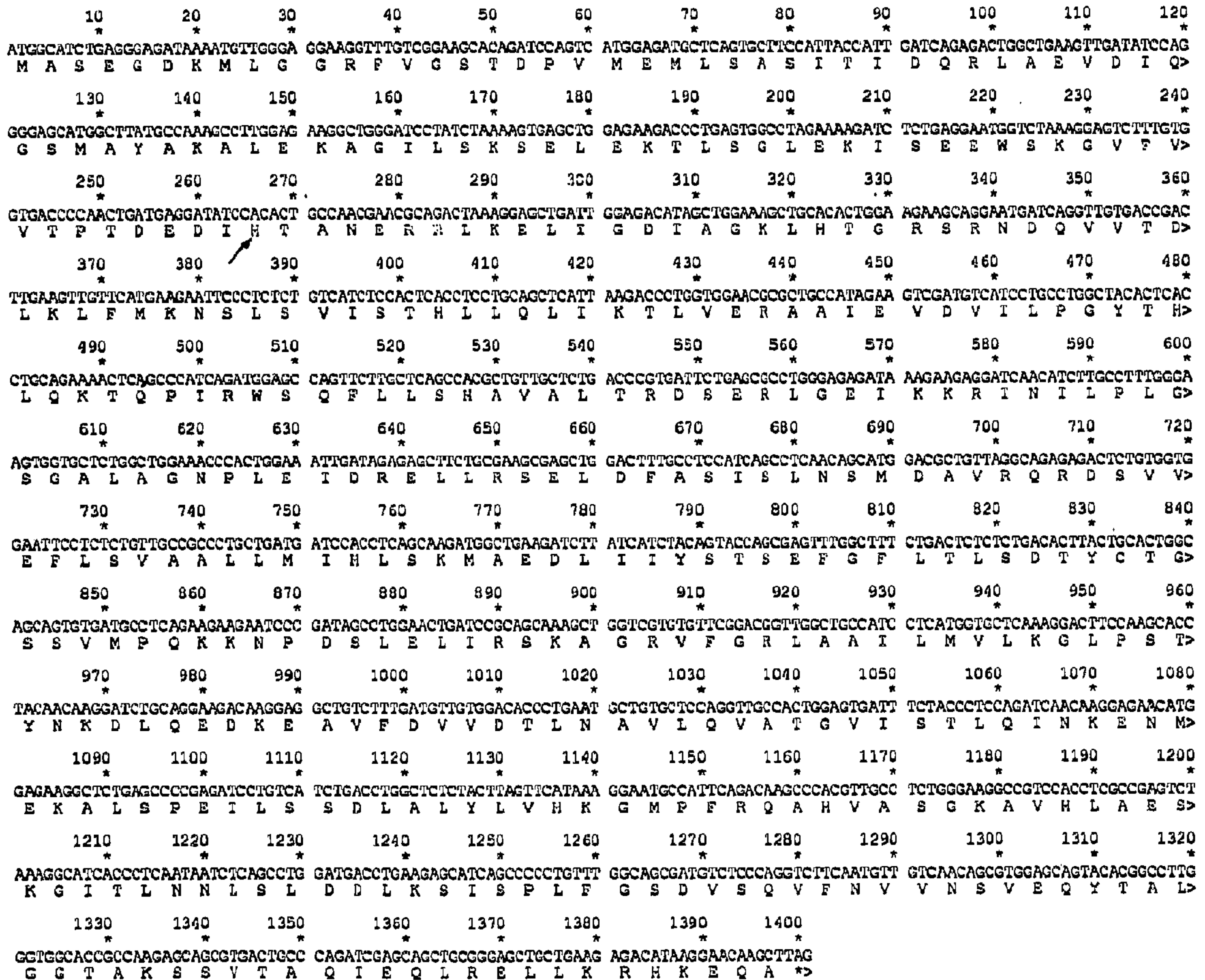


Fig. 2. Nucleotide and deduced protein sequences of pigeon δ-crystallin. The nucleotide sequence of 1,401 bp is shown above the amino acid sequence of 466 residues, which includes the translation initiation methionine as the first amino acid. An asterisk indicates every 10-nucleotide segment for easy tracing of sequence contents. Amino acids are denoted by one-letter symbols. The arrow points to His[89] which is implicated as the major amino acid residue responsible for the high activity in duck δ2 crystallin in contrast to the low activity of chicken δ1 crystallin (His[89]→Gln) [11].

quent PCR amplification of cDNA encoding δ-crystallin achieved the determination of a complete sequence. Scheme 1 summarizes the general strategy in the isolation of mRNA, construction and amplification of cDNAs by PCR and subsequent cloning of PCR-amplified product for DNA sequencing. Fig. 1 shows the size determination of PCR-amplified cDNA coding for pigeon δ-crystallin based on the designed primers covering 5′- and 3′-coding regions of duck δ1- and δ2-crystallin clones [13]. The DNA band was estimated to be about 1.4 kb, in agreement with a protein of about 465 amino acid residues. The actual cDNA sequence determined by sequencing is 1,417 bp in length, which encodes a protein of 466 amino acids, including the initiating methionine. The deduced protein sequence, together with its genetic coding sequence, is shown in Fig. 2.

3.2. *Structural comparison of avian δ-crystallins*

In the comparison of this sequence with those published δ-crystallin sequences in the data banks using software package (DNASTAR Inc., Madison, WI, USA), the pigeon δ-crystallin shows 88, 83 and 69% sequence identity to duck δ2 [13], chicken δ1 [18] crystallins and human argininosuccinate lyase [19], respectively.

In our assays of argininosuccinate lyase activity by monitoring the absorbance change at 240 nm due to the formation of fumarate, the lens extract and purified δ-crystallin of pigeon exhibited similar low activity to that of chicken lens extract, which has been shown to be almost devoid of enzymatic activity compared to duck and goose lenses [9,10]. Therefore we have compared and analyzed the primary sequences of the active

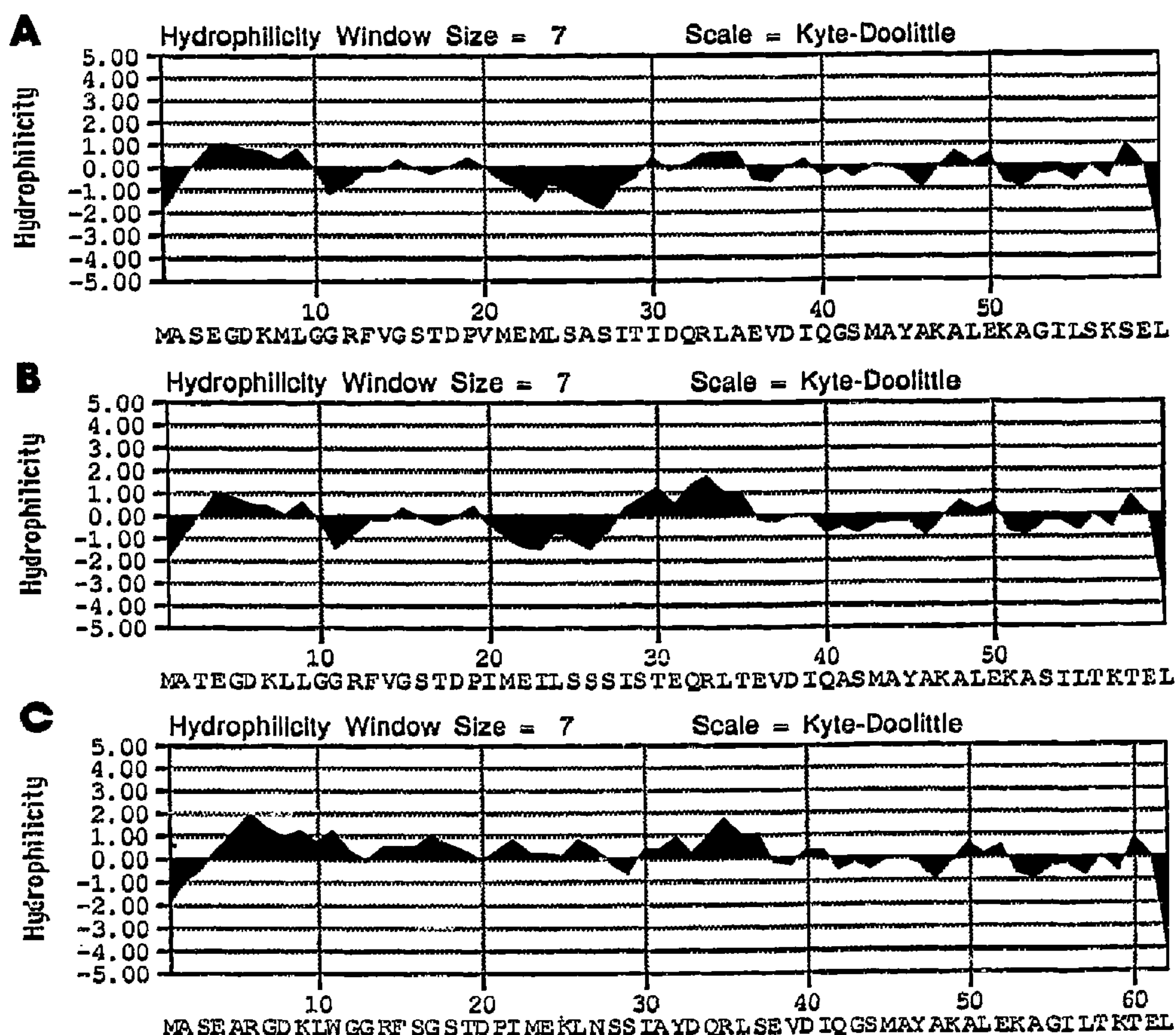


Fig. 3. Hydropathy profiles for pigeon (A), chicken (B) and duck (C) δ-crystallins. The method for the analysis of the local hydrophilicity of crystallin sequences is based on the Kyte-Doolittle scale [15], as described in section 2.4. The sequences of the N-terminal 60–62 amino acid segments for three crystallins are listed below the graphs. Note that salient profile differences are present in the first 30 amino acid segments, which may be due to an insertion of two amino acids (Ala and Arg) at positions 5 and 6 in the duck crystallin.

(duck) and inactive (chicken and pigeon) δ-crystallin in order to locate the active amino acid residues responsible for the enzymatic activity.

A program analysis of the local hydrophilicity of these sequences based on the Kyte–Doolittle hydropathy scale [15] has been employed to reveal the general distribution of surface-charge groups in these functionally distinct crystallins. It is quite noteworthy that the overall profiles share a great similarity for the distribution of hydrophilic amino acids among these three polypeptide chains, with the exception that the hydropathy profile for the N-terminal region covering the first 30 amino acids, which appears to be very different between active duck crystallin and that of the inactive pigeon or chicken. Fig. 3 shows the hydropathy profiles for these three homologous crystallins in the region of the first 60 amino acids. In the duck δ-crystallin the amino-acid segment of residues 3–30 exhibited a highly hydrophilic profile, indicative of its being exposed on the protein surface. In contrast, the profiles for pigeon and chicken are quite similar in this region, with most of the amino acids (residues 10–30) probably buried inside the protein. The only major sequence variation in the N-terminal part among the three sequences lies in the insertion of two extra amino acids, i.e. alanine and arginine, at positions 5 and 6 in the duck δ2-crystallin.

3.3. *Conclusion and perspective*

We have established the complete amino acid sequence of pigeon δ-crystallin by cloning and sequencing of cDNA clones by virtue of the PCR DNA amplification technique to circumvent the inherent difficulty of protein sequencing due to the presence of a blocked N-terminal group in this crystallin. The presence of a histidine residue at position 89 of inactive pigeon δ-crystallin certainly ruled out the claim [11] that the $His^{89} \rightarrow Gln$ mutation in chicken δ-crystallin may mainly account for the loss of argininosuccinate lyase activity in this avian species, when compared to the high enzymatic activity in duck crystallin. In contrast to the conclusion of Wistow and Piatigorsky [13], who suggested that the inserted sequence of Ala–Arg in the duck δ_2-crystallin is not of critical importance for enzyme function, we believe that this inserted sequence is actually the major structural difference between active duck and inactive pigeon δ-crystallins. A site-specific mutagenesis for the construction of a mutant with the inserted sequence in pigeon cDNA clone is currently in progress to assess the role of the insertion on the enzyme activity.

Further studies on the expression of pigeon δ-crystallin in some cell culture systems, coupled with site-specific mutagenesis of the amplified clones, may be conducive to unraveling the intriguing evolutionary process leading to the loss of this crystallin in certain lineages of vertebrates, and the predominant recruitment of this dual-function crystallin in some species of Avian and Reptilian classes.

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