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Structural Characterization of Lens Crystallins and the Perspectives on the Evolution and Biosynthetic Applications of Enzymatic Crystallins

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This account describes work in our laboratory on lens crystallins during the past decade. The major classes of crystallins are reviewed. A systematic and detailed analysis of the kinetic properties of two presumably structural proteins with stable enzymatic activities revealed subtle differences in kinetic mechanisms of these enzyme crystallins and their corresponding cellular enzymes. Bioorganic synthesis based on ε- and δ-crystallins isolated from avian and reptilian lenses, two crystallins possessing lactate dehydrogenase and argininosuccinate lyase respectively, provides an efficient and practical tool for enantioselective synthesis of valuable organic intermediates. Understanding the crystallin diversity at the molecular and cellular levels constitutes a major goal of crystallin research, as it provides the framework to unravel the complex process underlying the evolution of lens crystallins in general and may provide insight into their biological significance.

INTRODUCTION

The lens crystallins of vertebrates comprise a complex group of conserved structural proteins with distant evolutionary relationships. More than 90% of the proteins in lenses of various animal species consist of crystallins of several classes, which exist as water-soluble proteins and were previously thought to possess only the structural role of maintaining lens transparency and optical clarity in the lens fiber cells. Many crystallins have been identified, of which some are expressed as complex isoforms in either their size or charge heterogeneities. Crystallins in three major families, i.e. those classified as α-, β-, and γ-crystallins, are found in all vertebrates,[1-3] and other taxon-specific crystallins, e.g. δ-, ε-, λ-, τ-, appear in specific species.[4] In the lenses of the Cephalopod (squid and octopus) of invertebrates, of which the eyes are considered as complex as those of vertebrates, crystallins of only two types, designated Ω- and S-crystallins,[5-8] have been found. The former exists only in octopus, and the latter in both. Characterization of crystallins from the vertebrate and invertebrate lenses may provide a test of two major hypotheses of evolution of proteins, i.e. convergent or divergent evolution. The morphological similarity and immunological non-cross reactivity of lens proteins from these two groups of lenses led to the prevalent view of convergent evolution of crystallins in the animal kingdom.[9] Critical evidence to support divergent or convergent evolution lies in the unequivocal identification of sequence homology at the DNA or protein level between lens crystallins of disparate origins.

We have pursued the isolation and characterization of crystallins from several different species encompassing two classes of invertebrates[5-7] and all five major classes of ver-

Table 1. Classification and Characterization of Eye Lens Crystallins in Vertebrates and Invertebrates

Crystallin Components	Occurrence	Structural Relations	References
α-Crystallin (polymeric, > 600 kDa)	All vertebrates	Related to heat-shock proteins	3, 10-15, 17
β-Crystallin (polymeric, about 60-180 kDa)	All vertebrates	Unknown, similar to γ-crystallin	3, 10-15, 17
γ-Crystallin (monomeric, about 20 kDa)	All vertebrates (low in birds)	Unknown, similar to β-crystallin	3, 10-15, 18
ε-Crystallin (tetrameric, about 150 kDa)	Some birds and reptiles	Homologous to heart-type LDH	10, 20, 21, 23
δ-Crystallin (tetrameric, about 200 kDa)	All birds and reptiles	Homologous to ASL	29, 30-32, 41
ρ-Crystallin (monomeric, about 39 kDa)	Amphibian frog	Homologous to aldehyde reductase	3, 10, 12
S-Crystallin (dimeric, about 50-60 kDa)	Squid and Octopus	Homologous to GST	5, 6, 8, 39, 53
Ω-Crystallin (tetrameric, about 230 kDa)	Octopus	Related to aldehyde dehydrogenase	5, 39, 52

This review is invited to commemorate the sixtieth anniversary of the Chinese Chemical Society.

tebrates[10-15] to discover their evolutionary relations and phylogenetic relationships on the basis of their protein and gene structures. Crystallins not only vary between species but also are differentially expressed during lens development. Table 1 summarizes different classes of crystallins we have studied during the past decade and some salient features of these lens proteins regarding structural properties and biological function so far identified.

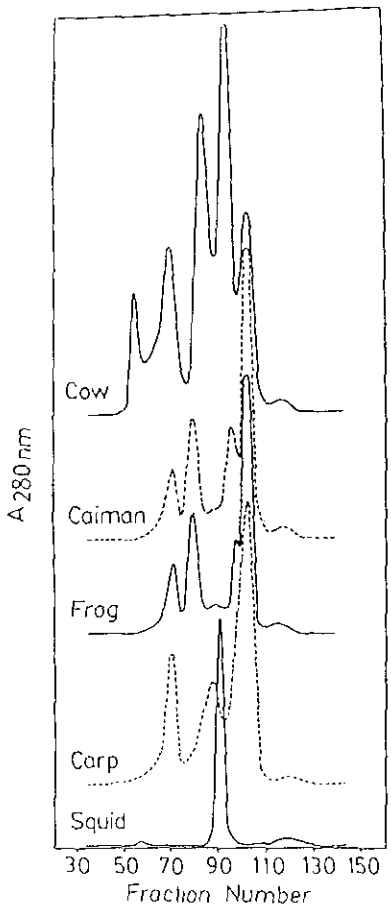

Fig. 1. Comparative gel-permeation chromatography on Fractogel TSK HW-55(S) (2.5 x 115 cm column) of lens extracts from the lenses of four species of vertebrates and one species of invertebrate. The absorbance at 280 nm of column eluates (3.9 mL/tube per 4.6 min) was monitored. The peak fractions eluted about tube no. 103 for the four species of vertebrates represent γ-crystallins and the only major peak in the squid lens is the squid S-crystallin. These fractions were collected, further purified on ion-exchange column and used for amino-acid and sequence analyses. The absorbances at 280 nm denote relative concentrations in arbitrary units. The small broad peaks after the γ-crystallin peak are non-protein components of low molecular masses.

PHYSICOCHEMICAL CHARACTERIZATION OF VERTEBRATE CRYSTALLINS

Isolation and Sequence Characterization of γ-Crystallins

A systematic and general approach has been carried out to provide basic structural information on various lens crystallins. Fig. 1 shows a general elution pattern of lens extracts of several representative species from four major classes of vertebrates and one class of invertebrates. Only one fraction was obtained from the squid lens of invertebrates in contrast to three from the carp and five from the cow. The last peaks of the major eluted fractions of all vertebrates except the bird contain monomeric γ-crystallin of molecular mass 20 kDa according to the subunit analysis by SDS-gel electrophoresis. Charge heterogeneity is detected for γ-crystallins from all species, showing at least four charge-isomeric forms.[16-18] As γ-crystallin of the vertebrate lens is the only crystallin class with a free amino-terminal group amenable to terminal sequence determination by classical Edman degradation, a special effort was directed to isolate and purify various γ-crystallin fractions for *N*-terminal sequence determination by means of the newly acquired gas-phase sequencer in our Institute. Extensive sequence similarity between γ-crystallin polypeptides from various species of major vertebrate classes was thus revealed, which establish these γ-crystallins isolated from these disparate lens origins to be closely related, and establish them as a multi-gene family of lens crystallins (Fig. 2). By the unambiguous determination of partial sequences of

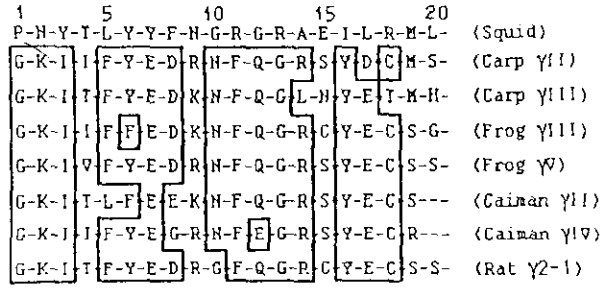

Fig. 2. Comparison of *N*-terminal sequences of squids- and γ-crystallins from various species. The sequences listed were taken from our studies except that of rat γ crystallin.[53] The sequences of the subfractions of γ crystallins from each species were determined on purified fractions upon cation-exchange chromatography of crude γ-crystallins from gel-permeation column. The homology regions are boxed. Amino-acid residues are denoted by one-letter symbols.

γ-crystallin polypeptides from reptilian species,[13,14] we were able to dispute the conventional claim that γ-crystallin is absent from the avian and reptilian species. By gel permeation chromatography and characterization 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 crystallins from caiman lenses, whereas δ- and γ-crystallin fractions were present in lesser proportions or missing from the turtle and snake lenses respectively.[14] 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 the δ/ε-crystallin fraction, which resembles to the secondary structure of δ-crystallin from the chicken lenses. Comparison of the amino-acid contents of reptilian crystallins of each orthologous class with those of evolutionarily distant species 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 cast doubt on the previous claim of the absence of γ-crystallin from the reptilian class, and establishing that γ-crystallins amongst the major classes of vertebrates are closely related.

Detection of Taxon-specific Crystallins

During the course of study of lens crystallins isolated from the homogenates of avian and reptilian eye lenses from several different species,[10,13,14] we have found unique crystallin components which are absent from mammalian classes of most species. Salient differences in the orthologous crystallin classes between different species belonging to the same class have also been found, which may be important in the comparison and tracing of evolutionary origin amongst these related species. There was a concurrent report[19] of the uncommon sequence similarity between a minor ε-crystallin of the duck lens and avian heart-type lactate dehydrogenase (LDH; EC 1.1.1.27) by peptide mapping and sequence comparison. Subsequently we have corroborated their report by re-evaluation of the native molecular mass and their inherent subunit number of ε-crystallin with sedimentation, gel filtration and cross-linking experiments[20] to establish the identities between these two proteins. The abundant presence and stability of ε-crystallin with enzymatic activity in the duck lens prompted us to search for lens crystallins with LDH activity from vertebrate and invertebrate of other classes in order to elucidate the enzymatic role of this crystallin in the lens.

Screening of lens homogenates to identify lactate dehydrogenases was undertaken for the representative species from vertebrates of five major classes and the cephalopod of invertebrates.[21] The duck and caiman lenses appeared to contain the greatest enzymatic activity of this glycolytic enzyme among all species examined. Biochemical isolation and characterization of ε-crystallins from the duck and caiman lenses established the apparent similarity of the physicochemical and kinetic properties of this structural crystallin to the enzyme. The duck lens provides an abundant source for the isolation and characterization of this important lens structural protein as an enzyme. Another unique and predominant crystallin present only in avian and reptilian lenses, the so called δ-crystallin, was found to be homologous to argininosuccinate lyase [ASL; EC 4.3.2.1], a key enzyme in the urea cycle.[22] In collaboration with Prof. Gu-Gang Chang of the Department of Biochemistry, National Defense Medical Center, we have sought to elucidate the kinetic mechanisms of these two crystallins with endogenous enzymatic activities to reveal subtle differences in the conventional LDH and ASL and these dual-function crystallins.

KINETIC STUDIES ON ε- AND δ-CRYSTALLINS WITH ENDOGENOUS ENZYMATIC ACTIVITIES

We have carried out a detailed kinetic analysis on the purified duck ε-crystallin to establish the genuine enzymatic activity and a compulsory sequential mechanism associated with this crystallin.[23,24] Despite the structural similarities between ε-crystallin and chicken heart LDH, distinct kinetic properties were revealed from steady-state kinetics. Initial-velocity and product-inhibition studies indicated a compulsory ordered Bi-Bi sequential mechanism with NADH as the leading substrate followed by pyruvate. The product were released in the order L-lactate and NAD^+. Substrate inhibition was also observed at high concentrations of pyruvate and L-lactate for the forward and reverse reactions respectively. The substrate inhibition was presumably due to the formation of ε-crystallin-NAD^+-pyruvate or ε-crystallin-NADH-L-lactate abortive ternary complexes, indicated by the product-inhibition studies. We concluded that the distinct mechanistic kinetic properties found between ε-crystallin and other well documented LDHs might indicate that these proteins may not be identical. In contrast with previous results[25] that duck ε-crystallin and heart-type LDH are identical products originating from the same gene locus, the possibility that ε-crystallin is a natural variant or analogue of this special type

of LDH from post-translational modifications cannot be eliminated. Further investigation into the molecular basis of the non-random existence and distribution of ε-crystallin in avian and reptilian lenses may elucidate the molecular origin of this novel aspect of crystallin evolution. We carried out kinetic comparison of another novel ε-crystallin isolated from the caiman lenses with duck ε-crystallin and some well documented avian and mammalian LDHs.[26] Caiman ε-crystallin similar to previously characterized duck ε-crystallin possessed a genuine and stable LDH activity as detected by nitro-blue-tetrazolium staining on polyacrylamide gels and conventional kinetic assays. The kinetic data of caiman ε-crystallin were fitted better with a compulsory ordered Bi-Bi sequential mechanism, similar to those of authentic LDHs and duck ε-crystallin. Caiman ε-crystallin has thus been established to be a heart-type LDH based on the kinetic analysis and comparison with authentic heart- and muscle-type LDHs. It is important to know to which type of LDH isoenzymes the reptilian ε-crystallin belongs and the kinetic differences of these dual-function crystallins compared with other authentic LDHs. The detailed characterization of ε-crystallins from bird and reptilian lenses and comparison with various lactate dehydrogenases has provided insight into the evolution of crystallins and their enzymatic roles inside the lens. The avian and reptilian species that possess this crystallin all seem to have natural habitats close to aquatic environments, for example most aquatic birds[27,28] and the caiman and the alligator.

After the completion of the kinetic analysis and mechanisms on ε-crystallins isolated from the duck and caiman lenses we examined another more abundant δ-crystallin present in only the avian and reptilian classes. δ-Crystallin was previously called FISC (first important soluble crystallin) and only recently found to be homologous to argininosuccinate lyase,[22] a key enzyme in the urea cycle. The enzyme is found in greater concentrations in the livers and kidneys of urea-forming animals, in which it catalyzes interconversion between argininosuccinate and arginine plus fumarate. In the uricotelic birds and reptiles it is probably involved in the biosynthesis of arginine from omithine or citrulline. The unexpected linking of two unrelated functions into one prominent crystallin has provided some impetus to re-examine and compare the structural and enzymatic properties of δ-crystallins from varied vertebrate species in order to ascertain the metabolic or developmental significance of the existence of this dual-function crystallin in certain species of Aves and Reptilia. Screening of lens homogenates from the representative species of vertebrates of five major classes was undertaken to search for δ-crystallin with argininosuccinate lyase activity.[29,30] Purification and biochemical characterization of δ-crystallins from the avian and reptilian species revealed distinct electrophoretic and kinetic properties despite their similar tetrameric structure about 200 kDa in the native forms. Chicken δ-crystallin, in contrast to those obtained from duck, ostrich,[29] goose[30] and caiman, is almost devoid of enzymatic activity. Two-dimensional gel electrophoresis of lens homogenates indicated that in the chicken lens δ-crystallin is composed of a subunit with an isoelectric point 5.9 and a subunit mass 50 kDa whereas that of goose lenses possesses heterogeneous subunits with isoelectric points spread in the range 5.9-6.8. Immunological comparison of inactive and active δ-crystallins from the chicken, duck and caiman lenses established the apparent structural similarity of some common surface epitopes of all δ-crystallins to those of the authentic enzyme, but they are not completely identical. Kinetic constants of two active δ-crystallins, i.e. those from the duck and goose of the Anatidae family, were determined; their catalyzed reaction was shown to conform to a random Uni-Bi kinetic mechanism similar to that of argininosuccinate lyase from bovine liver.

An activity-staining method[31] was developed to detect easily endogenous enzymatic activity of δ-crystallin from crude lens extracts of various avian species. Activity staining corroborated that all charge isozymes of duck δ-crystallin possessed enzymatic activity whereas that of chicken δ-crystallin was devoid of activity. The detailed kinetic mechanism was derived for duck δ-crystallin with endogenous argininosuccinate lyase activity catalyzing the reversible interconversion of argininosuccinate and fumarate plus arginine.[32] For duck δ-crystallin, variation of the enzymatic activity with argininosuccinate concentration in the forward reaction followed saturation kinetics with an apparent Michaelis constant $17 \pm 5\ \mu M$ for the substrate. In the reverse reaction, initial-velocity studies showed intercepting patterns. Inhibition of the forward reaction by products (fumarate and arginine) were both noncompetitive with respect to argininosuccinate. Citrulline, an analogue of arginine, inhibited enzymatic activity in both directions and was competitive with respect to arginine but noncompetitive with respect to fumarate or argininosuccinate. Succinate up to 300 mM that strongly inhibited the bovine argininosuccinate lyase, did not affect the δ-crystallin enzymatic activity. These results indicated a random Uni-Bi kinetic mechanism of the argininosuccinate lyase activity of duck δ-crystallin with formation of various abortive ternary complexes: δ-crystallin-argininosuccinate-arginine, δ-crystallin-argininosuccinate-fumarate, and δ-crystallin-argininosuccinate-citrulline.

DETERMINATION OF CRYSTALLIN PRIMARY STRUCTURES BY MOLECULAR CLONING AND GENE CHARACTERIZATION

Recent progress in recombinant DNA and molecular cloning has facilitated elucidation of gene structures of crystallins in several lens research laboratories. After several years of extensive effort to characterize the primary structures of crystallin polypeptides by conventional protein-sequencing methods, I realized that the extensive and inherent heterogeneity associated with various crystallins might hinder greatly progress to determine crystallin structures by protein sequencing. Therefore in collaboration with Prof. Wen-Chang Chang of our Institute we embarked on the molecular biological approach on crystallin genes in 1985 after I returned from the Linus Pauling Institute of Science and Medicine in U.S.A. We started from the teleostean carp of the piscine class as it belongs to the lowest class in vertebrates and may provide important clues to the general mechanism related to crystallin evolution. Starting from the isolation of total lens mRNA[33] the collaborative efforts resulted in determination of three carp γ-crystallin cDNA sequences[34,35] and their corresponding genes.[36] Despite the great dissimilarity in amino acid compositions between mammalian and piscine γ-crystallins, notably for the presence of high methionine content (> 12 %) in the latter species, γ-crystallins showed distinct homology in primary sequences between the two and also in overall polypeptide folding in their tertiary structures. Lens crystallins were isolated and characterized from the sharks of cartilaginous fish.[37,38] Four crystallin fractions corresponding to α-, β_H-, β_L- and γ-crystallins similar to those of mammalian crystallins were obtained. Amino-acid analysis and *N*-terminal sequence analysis revealed that the amino-acid composition and *N*-terminal sequence of shark γ-crystallin are more closely related to those of bovine than to carp γ-crystallin. Circular dichroism measurements indicated that significant α-helical structure is associated with γ-crystallin of shark lens in contrast to the predominant β-sheet secondary structure present in the carp. This finding may bear on the divergence and specification of γ-crystallins between the phylogenetic lines of mammals and fish. γ-Crystallin from the primitive shark seems to be more in line with the main evolutionary phylogeny leading to the modern mammalian γ-crystallin. Lens crystallins from the cartilaginous fish such as shark are of special interest from the evolutionary point of view because they constituted the early forms of fish and are thought to have been ancestral to land vertebrates. The isolation of mRNA and establishment of cDNA library of the shark lens crystallins pose an interesting topic for future investigation.

We characterized crystallin genes from the Cephalopod (squid and octopus) after preliminary success in the characterization of the major crystallin S-crystallin in the squids[8] and Ω-crystallin in the octopus.[5] S-Crystallin is a major lens protein present in the octopus and squid of Cephalopods. To facilitate the cloning of S-crystallin genes, we constructed cDNA from the poly(A)$^+$RNA of octopus lenses, and amplification by polymerase chain reaction (PCR) was carried out with two primers designed according to the 5′- and 3′- coding regions of S-crystailin gene.[39] Sequencing two of 15 positive clones obtained shows 37-44% similarity in nucleotide and 23-30% similarity in amino-acid sequences compared with mammalian glutathione S-transferases (GST); hence S-crystallins exist as a multigene family probably derived from GST by gene duplication and subsequent mutational base replacements. Both octopus OctS1 and OctS2 crystallins (214-215 amino acids and 24.6 kDa) determined and those S-crystallins of squid (204-221 amino acids)[40] show 25-30% similarity to that of rat GST Yb subunits (218 amino acids) in pair-wise comparison of their protein sequences. The extent of sequence similarity is greater between these two octopus crystallins (66%) than that between octopus and squid ones (41-56%). Although the percentage of homology between these cephalopod S-crystallins and those GST is low (25-29%), some structural features of critical residues in the GST family are well conserved in these GST-like crystallins.

PCR facilitated our cloning of pigeon δ-crystallin gene.[41,42] Sequencing two clones containing 1.4-kb DNA insert coding for δ-crystallin constructed a complete full-length reading frame of 1417 base pairs 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 $\delta 2$, chicken $\delta 1$ crystallins and human argininosuccinate lyase respectively. In contrast to duck $\delta 2$ crystallin with high argininosuccinate lyase activity, pigeon δ-crystallin contains very low enzyme activity despite the highly homologous common structure. Structural comparison of δ-crystallins with or without enzymatic activity (Fig. 3) indicated that in contrast to the recent claim[43] that $His^{89} \rightarrow Gln$ mutation in the chicken δ-crystallin accounts for loss of argininosuccinate lyase activity in this avian species relative to high enzymatic activity in duck crystallin, pigeon δ-crystallin without mutation at this position showed no enzymatic activity. As shown in Fig. 3 the inactive pigeon δ-crystallin possesses a His residue at this position similar to that in ac-

tive duck protein; hence histidine-89 may not play a major role in the enzymatic activity of δ-crystallin. The inserted sequence Ala-Arg in the duck δ_2-crystallin between positions 4 and 5 of the chick and pigeon sequences is actually the major structural difference between active duck and inactive pigeon δ-crystallins. A site-specific mutagenesis to construct this mutant with the inserted sequence on the pigeon cDNA clone is currently in progress to assess the role of this mutation on enzyme activity.

BIOORGANIC SYNTHESIS BASED ON STABLE ε- AND δ-CRYSTALLINS WITH ENDOGENOUS ENZYMATIC ACTIVITIES

Bioorganic synthesis based on enzymatic catalysis provides an increasingly important tool in enantioselective synthesis of valuable organic intermediates (i.e. chiral synthons).[44] Despite many practical applications of enzymes to generate chiral compounds, not many enzymes simultaneously possess broad substrate specificity and high enantioselectivity. Prof. Kung-Tsung Wang of our Institute suggested to me that ε- and δ-crystallins may be employed for enzymatic bioorganic synthesis due to ready availability and high stability. These two crystallins are suitable for application in large-scale synthesis of important chiral synthons. In collaboration with Prof. Wang's group with enthusiastic assistance of Dr. Shui-Tein Chen and Mr. Chi-Yue Wu, we successfully applied ε-crystallin with LDH activity and δ-crystallin with ASL activity for the enantioselective synthesis of important compounds.

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                5        10        15        20        25        30        35        40        45        50
Chick-δ1   MATEG--DKLLGGRFVGSTDPIMEILSSSISTEQRLTEVDIQASMAYAKALE
Pigeon δ   MASEG--DKMLGGRFVGSTDPVMEMLSASITIDQRLAEVDIQGSMAYAKALE
Duck-δ2    MASEARGDKLWGGRFSGSTDPIMEKLNSSIAYDQRLSEVDIQGSMAYAKALE

                55        60        65        70        75        80        85        90        95       100
Chick-δ1   KASILTKTELEKILSGLEKISEESSKGVLVMTQSDEDIQTAIERRLKELI→
Pigeon δ   KAGILSKSELEKTLSGLEKISEEWSKGVFVVTPTDEDIETANERRLKELI→
Duck-δ2    KAGILTKTELEKILSGLEKISEEWSKGVFVVKQSDEDIETANERRLKELI→
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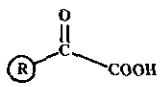
Fig. 3. Comparison of *N*-terminal sequences of chick, pigeon and duck δ-crystallins. The sequence for pigeon δ-crystallin was obtained by PCR-amplification of the cDNA clone constructed from mRNA of fresh 1-month-old pigeon lenses. Those sequences listed for chick and duck crystallins were taken from the literature.[41,42] His residue was identified at the 89th amino acid of pigeon sequence, same as duck δ-crystallin with enzymatic activity. However the pigeon sequence similar to that of inactive chick δ-crystallin lacks inserted Ala-Arg between residues 4 and 5. Amino-acid residues are denoted by one-letter symbols.

δ-Crystallin comprised about 50 % of total protein in the duck lens and was isolated easily by single-step purification. δ-Crystallin with endogenous ASL activity was found to be stable ($t_{1/2}$ at pH 7.5 and room temperature is about 10 days) and maintains its activity in organic cosolvent.[45] Argininosuccinate is synthesized easily and economically using δ-crystallin isolated from the duck lens. Argininosuccinate is commonly used clinically to measure enzymatic activities of argininosuccinate lyase (ASL, EC 4.3.2.1) and argininosuccinate synthetase (EC 6.3.4.5) to diagnose the diseases of argininosuccinic aciduria and argininaemia respectively.[46,47] Synthesis by this enzymatic route supersedes the organic synthesis using N^{α}-benzyloxycarbonyl-argininosuccinic acid trimethyl ester prepared from N^{α}-benzyloxycarbonyl-L-citrulline and dimethyl aspartate as the latter method gave only a total yield of 33% and as a further deprotecting step was needed to generate argininosuccinate.[48] In essence this enzymatic method involved use of an abundant natural crystallin to achieve C-N bond formation and C=C bond addition between arginine and fumarate. The immobilization of the crystallin further recycles the enzyme crystallin and facilitates the isolation of product.

Duck ε-crystallin, an abundant natural mutant of LDH with high stability, is especially abundant in the lenses of birds belonging to the family Anatidae which includes ducks, swans and geese.[27] It comprised about 10-20% of total protein in the duck lens and was isolated easily by ion-exchange chromatography. Whitesides' group at Harvard first demonstrated the preparative efficacy of the LDH group of enzymes in synthesis of chiral synthons.[49] We made a systematic analysis of kinetic properties of duck lens ε-crystallin by employing 19 α-keto acids (Table 2) as substrates for this NADH-dependent LDH-catalyzed reaction.[50] We explored the range of unnatural substrates accepted by ε-crystallin and stereochemical restraints involved in binding of substrates to the active site of this enzyme crystallin. The steady-state Michaelis and catalytic constants (K_m, k_{cat}) were determined for these substrates. From the consideration of different molecular sizes of side chains and the relative affinities, as reflected in varying Km for 19 compounds, we deduced that ε-crystallin possesses stereochemical restraints for substrate binding and affinity to the enzymatic active site (Fig. 4). According to comparison of Km's of these compounds in a broad range, the side chains of α-keto acids with good binding contain one

Table 2. Comparison of Relative Reactivities of Various Oxo Acids in ε-Crystallin Catalyzed Reductions

	R group:	Reactivity	Kcat[a]
(A) 2-Oxo Acids			
(1)	H	Very Good	> 100 %
	$HOCH_2$		
	CH_3CH_2		
	$HOOCCH_2$		
(2)	CH_3	Good	> 10 %
	FCH_2		
	$HSCH_2$		
	$CH_3CH_2CH_2$		
	$CH_3CH_2CH_2CH_2$		
	HOOC		
(3)	$C_6H_5CH_2$	Fair	1-10 %
	CH_3COCH_2		
	$CH_3SCH_2CH_2$		
	Imidazolyl-CH_2		
(4)	$(CH_3)_2CH$	Poor	< 1 %
	$(CH_3)_2CHCH_2$		
	$CH_3CH_2(CH_3)CH$		
	$HOOCCH_2CH_2$		
	$HOOCCH_2CH_2CH_2$		
(B) 3-Oxo and 4-Oxo Acids			
	CH_3COCH_2COOH		
	$CH_3COCH2CH_2COOH$	No Reaction	
	$HOOCCH_2COCH_2COOH$		
	$HOOCCH_2CH_2COCH_2COOH$		

[a] K_{cat} values for different substrates are relative to that of pyruvate as 100%.

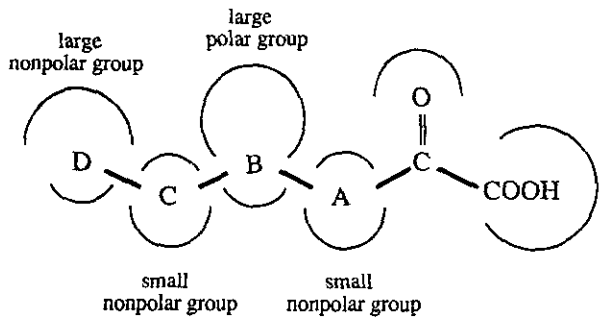


Fig. 4. The substrate binding site of ε-crystallin in relation to the stereochemical properties of side-chain groups on various α-keto acids compounds. The deduced large polar binding site for carbon-B side chain (γ-carbon position for α-keto acids) may explain the possession of malate dehydrogenase activity in duck lens ε-crystallin.

large hydrophilic or charge group at γ-carbon which may account for binding of oxaloacetate to ε-crystallin. However the β-, δ- and ε-carbon containing side-chains are in general more favorable for hydrophobic groups. In addition to the enzymatic activity of lactate dehydrogenase, duck ε-crystallin also possesses the enzymatic activity of malate dehydrogenase.

Application of ε-crystallin to synthesis of (*S*)-2-hydroxy acids

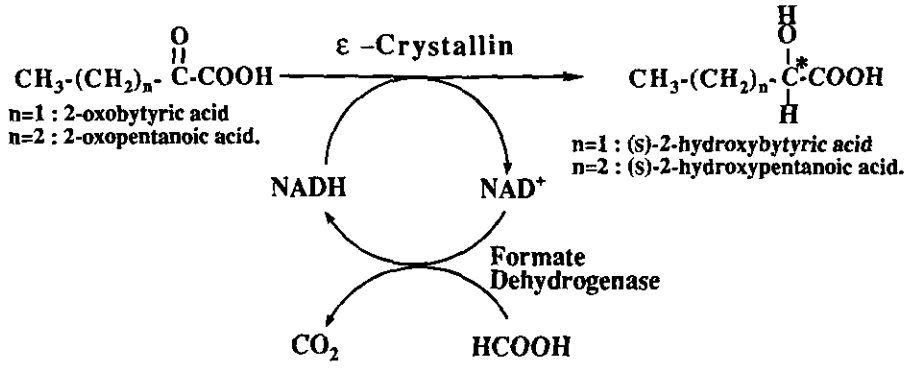
Because ε-crystallin adopts substrates in a broad range and maintains its activity in pure water and at room temperature for a long duration, we reported the application of duck ε-crystallin to synthesis of two chiral 2-hydroxy acids, (*S*)-2-hydroxybutanoic acid and (*S*)-2-hydroxypentanoic acid.[51] By utilizing the inexpensive and stable ε-crystallin with endogenous LDH activity, coupled with an in situ NADH regeneration system of formate/formate dehydrogenase (Scheme I), We synthesized these pharmaceutically useful chiral synthons with high yields and high optical purity, thus circumventing the use of relatively expensive conventional LDH's or other organic syntheses. This work should prove a great potential in the asymmetric synthesis of some other biosynthetic intermediates.

CONCLUSION AND FUTURE PERSPECTIVES

The discovery of enzyme crystallins in various taxons of disparate species is part of the allure of crystallin research. Of vital importance and no less interesting in a structural analysis of unique crystallins with some conventional or exotic enzymatic functions is knowledge of their primary structures achieved by the modern molecular cloning or PCR techniques. Only after such information is obtained would it be possible to assign functional roles for the individual crystallin. About my favored invertebrate crystallins, i.e. S- and Ω-crystallins which are similar to glutathione S-transferase and aldehyde dehydrogenase[52] respectively, we are currently examining structure-function relationships by the application of site-specific mutagenesis coupled with protein engineering techniques on the PCR-amplified crystallin products. Understanding the evolutionary conundrum of enzyme crystallins of various classes remains a major focus of current eye research.[3,53] The biological significance of these dual-function crystallins may elucidate the evolution of lens crystallins and its possible metabolic functions in general.

The holistic approach that we adopted is based on the

Scheme I



hypothesis that all abundant lens crystallins present in the animal kingdom may represent variations on a common structural basis at the molecular and genetic levels. Despite the conventional view of the structural purpose for the existence of these lens crystallins, recent work in the crystallin chemistry and molecular biology of various animal eye lenses indicates that these structural proteins may have descended from some enzymes of major metabolic or detoxification pathways. Therefore attention should be directed to common and specific structural features of these crystallins relative to general protein folding in other proteins.

We made a systematic structural comparison of several carp γ-crystallins with the salient feature of high methionine content by the secondary-structure predictions together with computer model-building based on the established X-ray structure of calf γ-II crystallin.[54] The overall profile of surface hydrophilicity and the distribution of helices, β-sheets and β-turns along the polypeptide chains were similar among these carp γ-crystallins. The general polypeptide packing of these carp crystallins[55] is similar to the characteristic two-domain/four-motif Greek key three-dimensional conformation depicted in calf γ-II crystallin. Most hydrophobic methionine residues are located on the protein surface with only a few buried inside the protein or at the interface between two motifs of each domain (Fig. 5). Important insight about the location and distribution of methionine residues in carp γ-crystallins has been revealed through examination of these graphic models. The exposed hydrophobic and polarizable methionine cluster on the protein surface may bear on the crystallin stability and dense packing in the piscine species, and probably also serves to provide a malleable nonpolar surface for the interaction with other crystallin components to maintain a clear and transparent lens. As high-resolution X-ray structures of crystallins are few at present, the approach of adopting computer-graphics model building should prove fruitful to gain important insight into the structural features responsible for crystallin stability based on the known coordinates of available three-dimensional structures of crystallins. Our approach has differed from that of others in that we

Fig. 5. Computer graphics of the ribbon drawing of carp γ-m2 crystallin. All side-chain atoms of methionine residues are shown by van der Waals ball representation, with those residues exposed on the surface shown in green and those buried inside the proteins or in the interfacial region of two domains shown in red. Carp γ-m2 crystallin comprises 24 methionine residues (14%) with 11 buried inside (Met-28, 42, 43, 67, 89, 117, 132, 142, 156, 162 and 164) and 13 exposed on the surface (Met-18, 50, 65, 70, 79, 82, 98, 101, 104, 112, 126, 159, and 168). Carp γ-m1 crystallin with 22 methionine residues (12.4%) showed an essentially similar pattern of distribution of methionine residues on the protein surface.[55] The three-dimensional organization of γ-crystallin molecules consists of *N*-terminal (right side) and *C*-terminal (left side) domains; each is composed of two Greek-key motifs.

have made structural correlation based upon analysis of crystallins in a broad spectrum rather than focused on one or a few major crystallins from one single species.

With the recent advance in DNA recombination and biotechnology, we are developing a programme of bioorganic synthesis and protein engineering with the aim to apply enzymatic crystallins in chiral synthesis and to improve enzyme and protein stability through site-directed mutagenesis. We plan to establish a coordinated and mission-oriented programme by integrating several successful projects done by several colleagues in Taiwan in order to broaden and to expand the mutual interaction under the common theme of protein engineering and enzyme technology. The eventual goal of the project is to provide insight into the mechanism governing the evolutionary process to generate these taxon-specific enzymatic crystallins in lenses and their potential applications to biotechnology.

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Key Words

Lens crystallins; Lactate dehydrogenase; Argininosuccinate lyase; Steady-state enzyme kinetics; Chiral synthesis; 2-Hydroxybutanoic acid; 2-Hydroxypentanoic acid; Molecular cloning; Polymerase chain reaction (PCR); Protein evolution.

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