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τ-Crystallin/α-Enolase: One Gene Encodes Both an Enzyme and a Lens Structural Protein

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Abstract. τ-Crystallin has been a major component of the cellular lenses of species throughout vertebrate evolution, from lamprey to birds. Immunofluorescence analysis of the embryonic turtle lens, using antiserum to lamprey τ-crystallin showed that the protein is expressed throughout embryogenesis and is present at high concentrations in all parts of the lens. Partial peptide sequence for the isolated turtle protein and deduced sequences for several lamprey peptides all revealed a close similarity to the glycolytic enzyme enolase (E.C. 4.2.1.11). A full-sized cDNA for putative duck τ-crystallin was obtained and sequenced, confirming the close relationship with α-enolase. Southern blot analysis showed that the duck genome contains a single α-enolase gene, while Northern blot analysis showed that the message for τ-crystallin/α-enolase is present in embryonic duck lens at 25 times the abundance found in liver. τ-Crystallin possesses enolase activity, but the activity is greatly reduced, probably because of age-related posttranslational modification. It thus appears that a highly conserved, important glycolytic enzyme has been used as a structural component of lens since the start of vertebrate evolution. Apparently the enzyme has not been recruited for its catalytic activity but for some distinct structural property. τ-Crystallin/α-enolase is an example of a multifunctional protein playing two very different roles in evolution but encoded by a single gene.

The lenses of vertebrates consist of concentric layers of terminally differentiated fiber cells (8). The major macromolecular components of these cells, comprising most of their refractive structure are highly abundant, soluble proteins, the crystallins. These proteins exist at high concentration, exposed to light and other insults essentially without turnover often for many years while maintaining the optical properties of the lens. It might have been expected that these proteins would be highly specialized, adapted for the unusual lens environment. However, it has recently been discovered that some crystallins appear to be common enzymes expressed at remarkably high levels in the lens (3, 10, 29, 33, 34, 35). Some of these enzyme-crystallins may be recently acquired components of the lens, but one, τ-crystallin, is a major protein of vertebrate lenses in lampreys, some fish, reptiles, and birds (24, 31, 32), suggesting an ancient origin in vertebrate evolution. Previously, limited sequence data for purified turtle τ-crystallin has suggested a close relationship with the glycolytic enzyme enolase (33). Here the nature of this relationship is elucidated, showing that τ-crystallin and α-enolase are the products of the same gene and that a well-characterized enzyme has another distinct function as a lens structural protein.

Materials and Methods

Tissues

Peking duck (Anas platyrhynchos) and chicken embryos were obtained from Truslow Farms (Chesterstown, MD). Turtle (Pseudemys scripta) embryos were obtained from Tangi Turtle Farm (Ponchatula, LA). Sea lampreys (Petromyzon marinus) were caught in River Mass (The Netherlands).

Preparations of τ-Crystallin and Lens Extracts

Turtle and lamprey τ-crystallins were isolated as described previously (24, 31). Lens extracts were the supernatant fractions of homogenates of lenses in enolase reaction buffer (30) after pelleting of insoluble fractions in an Eppendorf microfuge (Brinkman Instruments Co., Westbury, NY).

Sectioning and Staining Turtle Embryos

Embryos were embedded in paraffin and sectioned using an American Optical rotary microtome. Sections were dehydrated by treatment with xylene, followed by ethanol and PBS washes. They were then treated with proteinase K and prehybridized with BSA at room temperature. Rabbit anti-lamprey τ-crystallin antiseraum (diluted 10×) was then applied at 4°C for 20 min. After washing with PBS, sections were treated with fluorescein isothio-
Antiserum

The preparation of rabbit anti-lamprey γ-crystallin serum was described previously (24). Antiserum was purified using CNBr-activated sepharose (Pharmacia Fine Chemicals, Piscataway, NJ). A mixture of bovine and chicken lens extracts was coupled to the column to remove contaminating activities. Fractions were monitored by spectrophotometer for protein content. Peak fractions were pooled and purity was determined by western blotting using a Hoeffer electro-blotting apparatus (Bio-Rad Laboratories, Richmond, CA).

Photography

Microscope magnifications were 10 or 20×. Photographs were taken with an Olympus model BHS fluorescence microscope with blue light excitation filters. The film used was 3M 4000, ASA 1000, and the camera lens was set for 7.5× magnification.

Isolation and Analysis of Peptides

Purified turtle γ-crystallin was digested with V8 protease. Peptides were isolated by HPLC (Applied Biosystems, Inc., Foster City, CA) and sequenced by automated Edman degradation (470A; Applied Biosystems, Inc.) with an Applied Biosystems DNA synthesizer. To confirm the sequence of the reverse transcriptase, as described in the Bethesda Research Laboratories cloning manual, using 35S-dATP from Amersham Corp. The preparation of rabbit anti-lamprey γ-crystallin serum was described previously (24). Antiserum was purified using CNBr-activated sepharose (Pharmacia Fine Chemicals, Piscataway, NJ). A mixture of bovine and chicken lens extracts was coupled to the column to remove contaminating activities. Fractions were monitored by spectrophotometer for protein content. Peak fractions were pooled and purity was determined by western blotting using a Hoeffer electro-blotting apparatus (Bio-Rad Laboratories, Richmond, CA).

Enzyme Activity

Enolase activity was measured by both coupled and direct methods (18, 30, 36), giving similar results. One unit of enolase activity converts 1 μg of diphosphoglycerate per minute. Rabbit muscle enolase and other reagents were purchased from Sigma Chemical Company (St. Louis, MO).

Nucleic Acids

RNA was extracted by the guanidinium isothiocyanate method (4). Polyadenylated RNA was prepared using Hybond paper (Amersham Corp., Arlington Heights, IL). Genomic DNA was extracted as described previously (16). Human α-enolase cDNA (7) was a gift from Drs. L. Shawe and D. Andrews at the Harvard University Microchemistry Facility, Cambridge, MA. Purified lamprey γ-crystallin was digested with trypsin. Peptides were separated by two-dimensional electrophoresis and amino acid compositions determined after acid hydrolysis by methods described previously (25).

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Computer Analysis

Sequences were analyzed by a variety of programs including the IDEAS package (M. Kanehisa, National Cancer Institute).

Results

Immunofluorescence of Turtle Embryos

γ-Crystallin was first identified as an abundant lens protein in turtle lenses (31, 32). Sections of embryonic turtles from 3 to 30 d after fertilization were examined by immunofluorescence, using an antibody raised against lamprey γ-crystallin (Fig. 1). Nonimmune serum gave no significant reaction with any tissue at any stage (not shown). At 3 d there was little reaction with the anti-γ-crystallin serum in any tissues (Fig. 1 A) but by 5 d there was a distinct reaction in the innermost (apical) regions of the anterior epithelial cells of the lens (Fig. 1 B). By 6 d the positive staining was strong throughout the lens and much stronger in lens than in any other tissue (Fig. 1 C). Immunofluorescence was apparent in all epithelial and fiber cells throughout lens development up to the last stage taken 30 d after fertilization (Fig. 1 D). Turtles hatch after 60 d of development. While lens clearly gave the strongest immunofluorescence with the anti-γ-crystallin serum from 5 d onwards there was some apparent background in other tissues, particularly in the early brain at 6 d of development (Fig. 2). This is explicable in the light of subsequent findings demonstrating non-lens expression of γ-crystallin/α-enolase (see below).

Peptide Sequencing

Sequence was obtained for several V8 peptides from turtle γ-crystallin (Fig. 3). All gave a close match to the known α-enolase sequences, extending results obtained previously (33). Some peptides (see Fig. 3) were poorly separated and were sequenced as mixtures of two species. These could be interpreted by comparison with the known enolase sequences. Similarly it was possible to deduce the sequences of the peptides from lamprey γ-crystallin by comparison of their amino acid composition with the human α-enolase sequence (Fig. 3). Together the peptides from turtle and lamprey γ-crystallin accounted for about half of the sequence of α-enolase.

Enolase Activity

Enolase activity was measured in lens extracts and preparations of purified γ-crystallin (Table I). Turtle γ-crystallin and 30-d embryonic turtle lens extract had significant activity, but much less than that of a commercial enolase preparation. Enolase activities of extracts of other γ-crystallin containing lenses from lamprey and duck were even lower, although higher than those of lenses in which γ-crystallin has not been identified as an abundant protein. The activity in embryonic duck lenses did not vary significantly between 14, 21, and 28 d of development (data not shown).

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cDNA Cloning and Sequencing

To obtain the full sequence of α-crystallin by cDNA cloning a source of lenses more convenient than turtles or lampreys was chosen. Previous results have shown significant levels of α-crystallin, reactive with the antiserum to lamprey α-crystallin, in lenses of Peking ducks (24). In view of the embryonic expression of α-crystallin in turtle, a cDNA library made from 14-d embryonic duck lenses was examined. The same library yielded cDNA clones for duck β-crystallins and αB-crystallin (to be described elsewhere). The library was cloned in the sequencing vector M13 using a recombination deficient host (JM101 r-). Since protein sequence data clearly suggested that α-crystallin was very closely related to α-enolase, a cDNA for human α-enolase was used as a probe. An initial sample of 3,000 plaques proved to be sufficient to isolate the required clones. Two positive clones were sequenced. One of 1,700 bp contained the full coding sequence of putative duck α-crystallin (Fig. 4), while another was identical, lacking only 70 bp of 5' coding sequence.

The identity of this clone then had to be determined. The cDNA could have corresponded simply to an enolase distinct from α-crystallin. It could have encoded α-crystallin while being the product of a duplicated gene similar to α-enolase. Finally, and perhaps most likely in view of the sequence similarity, the cDNA could have encoded both α-crystallin and α-enolase. When the deduced protein sequence for duck α-crystallin and the partial protein sequence of turtle and lamprey α-crystallin were compared with known enolase se-
quence (5, 7, 11, 20, 21, 22), the closest relationships were with α-enolases (not shown).

**Southern Blotting and RNA Sequencing**

It was possible that τ-crystallin was the product of a gene similar to but different from that for α-enolase, the result perhaps of gene duplication or gene conversion. Therefore, the multiplicity of α-enolase-like genes in the duck genome was examined by Southern blotting. Genomic DNA from ducks, chickens, and humans was digested with Eco RI, Bam HI, and Bgl I. Duck and chicken DNA gave single size fragments hybridizing with the putative τ-crystallin cDNA. Fig. 5 shows the results of Bam HI digestion of duck and chicken DNA, followed by hybridization with both the duck lens cDNA and the human α-enolase cDNA. Both probes hybridized to the same single bands suggesting that the avian genomes contained only a single α-enolase gene and that the duck lens cDNA corresponds to duck α-enolase. In contrast to the results using duck and chicken DNA, restriction of human DNA yielded more fragment sizes with all enzymes (data not shown). This suggests the presence of multiple α-enolase-like genes or pseudo-genes in humans.

Others have shown that α-enolase is the predominant enolase gene expressed in liver and embryonic tissues of birds (26). To test further if the duck lens cDNA was encoded by the same gene as α-enolase, the 5' untranslated regions of α-enolase-like mRNA was sequenced from total RNA from 14-d embryonic duck lens and liver using a primer complementary to the first seven codons of the τ-crystallin cDNA. Readable sequence was obtained for both tissues with both labeled and unlabeled sequencing primers. The extended cDNAs from both tissues appeared to be identical in sequence and extent, giving a 5' noncoding region of almost 200 bp. However, as single-stranded sequencing this should not be regarded as definitive until the gene sequence is complete (Lietman, T., G. J. Wistow, and J. Piatigorsky, unpublished data).

**Northern Blotting**

Finally, to ensure that the putative τ-crystallin cDNA actually codes for an abundant lens message, τ-crystallin/α-enolase mRNA was examined in lens and liver. Northern blots of total RNA from 14-d embryonic duck lens and liver were hybridized with the τ-crystallin cDNA probe. Equal quan-
Figure 3. Peptide sequences of turtle (T) and lamprey (L) ρ-crystallin compared with the deduced sequence of human α-enolase (H) (7). Sequences in lower case were deduced from peptide compositions (lamprey) or from sequenced HPLC fractions which contained mixtures of two peptides. In the latter case it was usually easy to distinguish major and minor components. Single letter code is used with X marking positions that could not be determined. An ambiguity exists at residue 433 of the turtle sequence where two possibilities are indicated. Two V8 peptides from turtle ρ-crystallin seem to have arisen by unusual cleavage. These are found at sequence position 309 and 425. The deduced sequence of duck ρ-crystallin/α-enolase (D) from Fig. 4 is included for comparison.

**Table 1. Enolase Activity**

| Extract       | Purified protein |
|---------------|-----------------|
|               | U/mg            | U/mg            |
| Turtle lens   | 3.3             | 6.9             |
| Duck lens     | 0.18            | -               |
| Lamprey lens  | 0.28            | -               |
| Bovine lens   | 0.045           | -               |
| Rabbit muscle | -               | 35              |

Enolase activity of ρ-crystallin and lens extracts compared with that of commercial purified rabbit muscle enolase. The stated activity of the commercial enzyme was confirmed experimentally, however the activity of freshly purified rabbit muscle enolase is expected to be ~90 U/mg (30), suggesting that the commercial preparation itself is not completely active.

Enolase activity of RNA, estimated spectrophotometrically and with identical intensities of rRNA bands on polyacrylamide gels (visualized by ethidium bromide), were compared (Fig. 6). After hybridization identical sized bands were seen in RNA from both tissues, corresponding to a full mRNA size of ~1,900 bp. Hybridization was much more intense to lens RNA than to that from liver. Liver RNA required a 25× higher loading to give an equivalent hybridization intensity, suggesting that ρ-crystallin/α-enolase mRNA is ~25 times more abundant in lens than in liver.

**Discussion**

ρ-Crystallin is a major component (10% or more of total pro-
tein) of the lenses of various species throughout vertebrate evolution (24, 31, 32), making it as ancient as any of the more familiar α- or β-crystallins. As shown here however, γ-crystallin like some more recently recruited crystallins with more restricted distribution is the product of the same gene as a common enzyme (see reference 34), in this case the glycolytic enzyme γ-enolase.

γ-Crystallin is prominent in the embryonic turtle lens from at least 5 d after fertilization and accumulates throughout early embryonic development. Its distribution in the lens is therefore similar to that observed in other species for β- (9, 15, 37), ε- (1), and δ- (2) crystallins.

Protein sequencing of turtle and lamprey γ-crystallins and cDNA sequencing of putative duck γ-crystallin revealed a striking match with γ-enolases. Indeed, this similarity underlines the remarkable degree of conservation in this protein family during vertebrate evolution, suggestive of strict selective constraints on all parts of the protein structure.

The results of southern blotting, RNA sequencing, and northern blotting clearly suggest that the cDNA does encode duck lens γ-crystallin which is the product of the same gene as α-enolase. Thus by increased expression in lens (by either transcriptional or posttranscriptional mechanisms) the duck α-enolase gene encodes an additional function, that of lens structural protein, an example of the kind of gene-sharing which occurs also in δ- (17) and ε- (10) crystallins. This result was strongly implied by the protein sequence similarity itself. As has been discussed previously (34, 35), it is very unlikely that a crystallin as the product of a duplicated gene would maintain such striking identity of sequence with a related enzyme in the absence of selective pressure to maintain enzymatic activity.

The lack of selective pressure for retention of enzymatic activity in the lens is emphasized by the low enolase activity of purified γ-crystallin and lens extracts. Data for both turtle and lamprey γ-crystallins have suggested that both proteins are present predominantly as 48-kD monomers in lens (24,
have been the root cause of the selection (34, 35). However, enzymes are frequently found at fairly high concentrations do ct-, 13-, and y-crystallins (see reference 34). For example, the recruited enzyme crystallins in mammals may in some way be the result of the long nocturnal history of early mammals (28). During this period there may have been advantages in using y-crystallins as lens components. If y-crystallins are indeed being abandoned in diurnal species they would be expected eventually to be eliminated in many modern mammals. Interestingly at least two y-crystallin genes in humans have been shown to be pseudogenes (13), while in the nocturnal, myopic rat all six genes are actively expressed (27).

Surprisingly, modern mammals with their abundant, multiple y-crystallins and lack of p-, 3-, and e-crystallins more closely resemble amphibians than reptiles in the molecular structure of their lenses (34). This mirrors at the molecular level the remarkable similarity in anatomical structure between the eyes of mammals and amphibians noted many years ago by Franz (see reference 28), who on the basis of this observation was led to propose that mammals had developed directly from amphibians, rather than from an ancestor common to modern reptiles and birds.

Since a-enolase has acquired a distinctly different function as a structural lens crystallin its sequence has presumably been under pressure to adapt to the unusual lens environment. In the case of one of the most recently recruited enzyme crystallins, e-crystallin, identical to LDH-B, one or two amino acids highly conserved in LDH subunits have changed in those species expressing the enzyme also as a crystallin (10, 25, 35). The same thing could have occurred in y-crystallin, but because of its antiquity these effects will be hard to identify. y-Crystallin/a-enolase is an example of a multifunctional protein, perhaps reflecting an unexpected economy in vertebrate genomes. An enolase also acts as a heat-shock protein in yeast (12), suggesting that multifunctionality of this gene product may extend even further.

In a wider context, the possession by well-characterized proteins of unexpected extra functions may become very important when those proteins or their genes become the target of specific medical therapies. In fact, this may explain some instances of unanticipated drug side effects.

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