Aldehyde Dehydrogenase-derived Ω-Crystallins of Squid and Octopus

SPECIALIZATION FOR LENS EXPRESSION*

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Ω-Crystallin of the octopus lens is related to aldehyde dehydrogenases (ALDH) of vertebrates (Tomarev, S. L., Zinovieva, R. D., and Piatigorsky, J. (1991) J. Biol. Chem. 266, 24226–24231) and ALDH1/7-crystallin of elephant shrews (Wistow, G., and Kim, H. (1991) J. Mol. Ecol. 32, 262–269). Only very low amounts of Ω-crystallin are present in the squid lens. Here, we have cloned Ω-crystallin cDNAs of the octopus (Octopus dofleni) and squid (Ommastrephes sloani pacificus) lenses. The deduced amino acid sequences of Ω-crystallin from these species are 78% identical to each other, 56–58% identical to cytoplasmic ALDH1 and mitochondrial ALDH2 of vertebrates (which are 66–68% identical to each other), and 40% identical to Escherichia coli and spinach ALDHs. These data are consistent with the idea that the ALDH1/ALDH2 gene duplication in vertebrates occurred after divergence of cephalopods from the line giving rise to vertebrates, before the separation of squid and octopus. Southern blot hybridization indicated that Ω-crystallin is encoded by few genes (possibly just one) in octopus and squid. Northern blot hybridization revealed two bands (2.7 and 9.0 kilobases) of Ω-crystallin RNA in the octopus lens and one band (4.2 kilobases) in the squid lens; Ω-crystallin RNAs were undetectable in numerous non-lens tissues of octopus and squid, suggesting lens-specific expression of this gene(s). Finally, extracts of the octopus lens had no detectable ALDH activity using different substrates, consistent with Ω-crystallin having no enzymatic activity. Taken together, our results suggest that Ω-crystallin evolved by duplication of an ancestral gene encoding ALDH and subsequently specialized for refraction in the transparent lens while losing ALDH activity and expression in other tissues.

The eyes of cephalopods (squid and octopus) and vertebrates show striking similarities, although they have been formed independently and are considered examples of convergent evolution (1). Despite their independent evolution, cephalopods and vertebrates have employed the same strategy of recruiting crystallins from pre-existing enzymes or stress-related proteins. The major crystallins of squid and octopus, Ω-crystallins, are related (but not identical) to glutathione S-transferase (2–6). S-crystallins do not possess glutathione S-transferase activity and demonstrate 42–44% identity to active glutathione S-transferase isolated from squid digestive gland (6).

The octopus lens also has a less abundant crystallin, called Ω-crystallin (7), which is related to aldehyde dehydrogenase (ALDH) (4). Since cytoplasmic ALDH1 is the major crystallin (7-crystallin) in the mammalian elephant shrew (8), Ω- and 7-crystallins represent the first example of a similar enzyme-crystallin in vertebrates and invertebrates. An ALDH-related protein also comprises ~70% of the water-soluble proteins in the lens of the light organ of the squid Euprymna scolopes (9), and the liver-tumor-induced ALDH3 isoenzyme accumulates to high concentrations in the mammalian cornea, where it is suspected of having both structural and enzymatic roles (10–13).

Only limited information is available on the structure of ALDH-related crystallins in cephalopods. The partial structures of five tryptic peptides covering ~15% of the complete sequence of Ω-crystallin in Octopus do fleni (4) and three peptides covering ~9% of the sequence of the light organ lens protein in E. scolopes (9) have been determined. Determination of the complete structure of Ω-crystallin would be very useful for elucidating its structural and functional relationships with ALDHs of vertebrates and with the major protein of the squid light organ lens. In this investigation, we have cloned Ω-crystallin cDNAs of the squid and octopus lenses and showed that the sequences of their encoded proteins are equally similar to cytoplasmic ALDH1 and mitochondrial ALDH2 of vertebrates.

MATERIALS AND METHODS
Obtaining Squid and Octopus—The octopus O. dofleni and the squid O. sloani pacificus were collected on the Russian Pacific coast (Vladivostok). The squid Leigea opalescens was collected at the Hopkins Marine Station (Pacific Grove, CA). The lenses and specified organs were removed and stored in liquid nitrogen.

PCR of Octopus Ω-Crystallin cDNA—Degenerate oligonucleotide 5’-ATC/TGGT/AAGC/T(C/G)G(G/A)G/GTG/AAGC/TGGA/T(G/A)C/GG/TA(A/G)/TCTTTC/CGTG(GA/AT/TA-3') corresponding to the tryptic peptides NT66 and NT104, respectively, of octopus Ω-crystallin (4) were made on an automated DNA synthesizer (Model 380B, Applied Biosystems, Inc.) and used as primers for PCR using an octopus lens cDNA library (4) as template. Samples underwent 30 amplification cycles of denaturation at 94°C for 2 min, followed by annealing at 50°C for 2 min and extension at 72°C for 3 min in an automated thermocycler (Perkin-Elmer Cetus Instruments). A fragment with the expected length of 200 bp was excised from an agarose gel, purified using Geneclean.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank accession number(s) L06902 and L06903.

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The abbreviations used are: ALDH, aldehyde dehydrogenase; PCR, polymerase chain reaction; bp, base pair(s); kbp, kilobase pair(s).
RESULTS

Isolation and Characterization of cDNA Clones Encoding Squid and Octopus \(\Omega\)-Crystallins—One positive plaque (pOAL1) was identified among \(10^9\) plaques of an octopus lens cDNA library after screening with an \(\Omega\)-crystallin-specific PCR probe obtained as described under “Materials and Methods.” The combined nucleotide sequence of the two longest overlapping cDNAs (pSAL) is shown in Fig. 1. It is much longer (3831 bp) than the longest octopus \(\Omega\)-crystallin cDNA (1724 bp), but encodes a protein that is only 1 amino acid shorter (494 residues) and clearly homologous to the octopus \(\Omega\)-crystallin (see below). The 5′- and 3′-untranslated regions are both longer in the squid \(\Omega\)-crystallin cDNA (167 nucleotides and 2176 nucleotides, respectively) than in the octopus cDNA. The squid polyadenylation signal is located 16 nucleotides upstream of the poly(A) tract. The octopus and squid \(\Omega\)-crystallin mRNAs are 74% identical in the coding region; there are no significant regions of similarity in the untranslated regions.

The deduced amino acid sequences of pOAL1 and pSAL are 78% identical to each other (Fig. 2). Each shows 56–58% identity to cytoplasmic ALDH1 (19) and mitochondrial ALDH2 (20) of vertebrates (see Fig. 2 where rat sequences are shown). The identity of the deduced cephalopod \(\Omega\)-crystallins to tumor-induced ALDH3 (21) or microsomal ALDH4 (22) is only 25–27%. Squid and octopus \(\Omega\)-crystallins as well as vertebrate ALDH1 and ALDH2 show ~40% identity to betaine ALDH of Escherichia coli (23) and spinach (24).

ASSAYS FOR ALDH ACTIVITY—In view of the structural relationship of \(\omega\)-crystallin with ALDH of vertebrates, extracts of the octopus lens were assayed for ALDH activity; extracts from the octopus digestive gland were also examined for comparison. Three concentrations were examined for each substrate tested (see “Materials and Methods”). The digestive gland extracts gave activity with all substrates tested. The maximum activity was observed with 1 and 10 mM acetaldehyde and propionaldehyde or with 200 \(\mu\)M hexanal and octanal. The digestive gland ALDH activity ranged from 0.008 to 0.019 units/mg of protein with different substrates and in different experiments. The lens extracts had virtually no detectable ALDH activity with any of the substrates tested.

Since \(\omega\)-crystallin is clearly more abundant in the lens than are proteins of similar molecular mass in the digestive gland (Fig. 3), these results indicate that \(\omega\)-crystallin possesses no ALDH activity with these commonly used substrates. As in these tests on ALDH activity in the eye lens, the light organ lens of *E. scolopes*, which also has an ALDH-like protein as its major crystallin, did not show significant ALDH activity (9). The same was true for \(\gamma\)-crystallin of the elephant shrew, which also had no ALDH activity (8).

Lens-specific Expression of Squid and Octopus \(\Omega\)-Crystallins—We next examined the tissue specificity of squid and octopus \(\Omega\)-crystallin gene expression by Northern blot hybridization...
oration. The squid tides in length were observed (Fig. 1404-1674) were used in these experiments and gave identical results. They hybridized exclusively with lens RNA; and in (see below), these results may reflect the usage of two poly-

* Asterisks mark the beginning and the end of the pOAL1 sequence. **Underlined** mark the gaps that were introduced underlined. **Genes-Southern blot hy-

ζ-Crystallin/Aldehyde Dehydrogenase

Fig. 1. Nucleotide sequences of cDNA inserts of pOAL1 (upper line) and combined nucleotide sequences of pSAL1 and pSAL2 (lower line) clones. The sequence of the pOAL1 insert is shown in full; for pSAL cDNAs, only differing positions are shown. Asterisks mark the beginning and the end of the pOAL1 sequence. Dashes mark the gaps that were introduced to maximize similarity. Initiator ATG and terminator codons are boxed. Possible polyadenylation signals are underlined.
FIG. 2. Comparison of amino acid sequences of octopus (OC) and squid (SQ) ω-crystallins with amino acid sequences of rat ALDH1 (19) and ALDH2 (20) and betaine ALDEHIDE OF E. coli (ALDHB) (23). Sequenced peptides of ALDH-like crystallin from the E. scolopes light organ lens (SQ LO) (9) are also included for comparison. The sequence of the octopus ω-crystallin is shown in full; for other sequences, only differing amino acid residues are shown. Asterisks mark Cys-302 and Glu-268, which may be essential for the catalytic activity of vertebrate ALDEHIDE (see text). Dashes mark the gaps that were introduced to maximize similarity. Plus signs mark the beginning and the end of E. scolopes sequenced peptides. Tryptic peptides obtained from sequencing octopus E. scolopes ω-crystallin (4) are boxed.

FIG. 3. Coomassie Blue-stained SDS-14% polyacrylamide gel of water-soluble proteins of lenses and digestive gland (D.G.). Approximately 5 μg (lens) and 30 μg (digestive gland) of proteins were used. Positions of the marker proteins are shown on the left.

294–2646 of the octopus and squid cDNAs, respectively, were used as probes. With the octopus, two ω-crystallin hybridization bands of −1.2 and 4.45 kbp were present in the EcoRI-digested DNA, and two bands of −1.9 and 4.6 kbp were present in the HindIII-digested DNA (Fig. 5A). The hybridization patterns of the squids enzyme varied with the restriction enzyme. Five bands (1.0, 1.45, 1.8, 3.9, and 6.2 kbp) were obtained after digestion with EcoRI; two bands (2.2 and 11 kbp) were evident after digestion with HindIII; three bands (13, 15, and 20 kbp) were present after digestion with BamHI; and only a single band (10.5 kbp) was seen after digestion with Sall (Fig. 5B). Multiplicity of hybridization bands in some restriction digests can be partially explained by polymorphism, which appears common in natural squid populations (5, 6). Although further experiments are necessary, our data are consistent with the existence of few genes (and perhaps only one) for ω-crystallin in these species.

DISCUSSION

The ALDH-related ω-crystallin of cephalopods (4) and ALDH1/η-crystallin of elephant shrews (8) are the only known examples of similar lens crystallins in invertebrates and vertebrates. They show that independent choices during the convergent evolution of lenses in cephalopods and mammals have resulted in a similar protein for a crystallin. Our earlier protein gel data (4) as well as recent Western blot experiments (9) indicate that the octopus lens has much more ω-crystallin compared with the squid lens. The hybridization data presented here establish that ω-crystallin is present in the squid lens, although at a concentration generally considered too low to qualify as a crystallin. Surveys among vertebrates lenses have also shown that taxon-specific crystallins that exist in abundance in one species may be present at low concentrations in other related species (8). In the squid E. scolopes, ALDH-like crystals are the major proteins of the light organ lens (9). In preliminary experiments during the course of this study, we partially sequenced a PCR-generated 650-bp cDNA fragment of ω-crystallin of the eye lens of E. scolopes (data not shown). The sequences of two peptides (V28 and T44) of the ALDH-like protein from the light organ lens are included (24).
ocular \( \Omega \)-crystallin of \( E. \text{scolopes} \) (see Fig. 2). Indeed, the available sequences of ocular \( \Omega \)-crystallin and light organ L-crystallin suggest that both proteins are identical.\(^2\) Since the cephalopod eye lens is derived from two ectodermal layers formed on the dorsal surface of the head lobe (33) and the light organ lens is derived from muscle of the hindgut (34), we conclude that the ALDH-related \( \Omega \)-crystallin has been independently recruited to serve similar structural functions even in different tissues of the same species.

A diagram of the relationships between \( \Omega \)-crystallins and different ALDHs is shown in Fig. 6. In view of our finding that \( \Omega \)-crystallins of squid and octopus are 78% identical to each other and 56–68% identical to cytoplasmic ALDH1 and mitochondrial ALDH2 of mammals, one may speculate that the gene duplication giving rise to ALDH1 and ALDH2 (which are 66–68% identical) occurred after the divergence of cephalopods from the line leading to mammals (estimated at 550 million years (35)), but before the separation of squid and octopus (estimated at 200–300 million years (35)). This speculation does not consider, of course, possible differences in the rates of evolution of \( \Omega \)-crystallins in the lens and enzymatically active ALDHs. It is interesting to note, however, that \( \Omega \)-crystallins and ALDH1/ALDH2 are each \(~40\)% identical to \( E. \text{coli} \) (23) and spinach (24) ALDHs (see Fig. 6), consistent with the possibility that \( \Omega \)-crystallins and enzymatic ALDHs have evolved at similar rates. In contrast to the relatively high conservation between \( \Omega \)-crystallins of cephalopods and ALDHs of other species, the glutathione S-transferase-related S-crystallins of squid and octopus lenses show only \(~25\)% identity to glutathione S-transferases of vertebrates (3–5) and as little as 42–44% identity to glutathione S-transferase of squid (6). This suggests that glutathione
S-transferases and S-crystallins have evolved faster than ALDHs and Q-crystallins.

One of the characteristic features of enzyme-crystallins of vertebrates is that the identical gene encodes the enzyme and the crystallin (36). Thus, vertebrate enzyme-crystallins are generally expressed at low concentrations in non-lens tissues when performing their metabolic functions and at high concentrations in the lens when fulfilling structural roles. In some cases, the enzyme-crystallin gene has duplicated, such as with α-crystallin, and one of the duplicated genes has specialized for lens expression. Even when this happens, however, both genes are still expressed in non-lens tissues (37, 38), and the gene encoding the enzyme may still be expressed abundantly in the lens, where it probably serves a refractive function (39). In contrast to the situation among vertebrate enzyme-crystallins, our recent studies on S-crystallins of squid showed that the glutathione S-transferase gene from which the S-crystallin family was derived is barely detectable in the lens and that the S-crystallins are found only in the refractive lens and cornea (6, 13). The present study suggests that a similar separation of function has occurred with ALDH/β-crystallin in cephalopods and that α-crystallin and ALDH are encoded in different genes. This is consistent with the fact that ALDH activity is much higher in the digestive gland than in the lens, where activity was not detectable, although we cannot eliminate the possibilities that α-crystallin possesses enzymatic activity with substrates not tested in this study, that an ALDH inhibitor exists in the lens extracts, or that ALDH activity is suppressed by post-translational modification(s) of β-crystallin in the lens. Replacement of Cys-302, which is located at the active site of ALDHs (26, 27, 29, 30), by Arg in both squid and octopus β-crystallins also argues that β-crystallins do not possess ALDH activity. The Northern blot experiments showed that β-crystallin mRNA is abundant in the lens and undetectable in other tissues, including the digestive glands of octopus and squid, which also supports the idea that genes with different expression patterns encode Ω-crystallin and ALDH. In contrast to the ~10 genes encoding the S-crystallins (5), Ω-crystallin appears to be a much smaller family, possibly consisting of a single member. Further experiments are necessary to establish the number of Ω-crystallin genes and to characterize the ALDH genes of cephalopods. We anticipate that site-directed mutagenesis of Ω-crystallin cDNA and determination of the structure of active ALDHs from squid and octopus will help clarify the role of different residues in enzymatic activity.

Finally, the independent recruitment of ALDH-like proteins to be crystallins in the eye lenses of cephalopods (4) and elephant shrews (8) and in the light organ lens of squid (9) is consistent with the generality that crystallins are identical or related to proteins involved in stress responses (40, 41). Protection against oxidative stress and protein denaturation have been considered as factors in the recruitment of crystallins, especially in view of the deleterious effects for transparency of agents leading to protein denaturation and aggregation (42). Indeed, in vertebrates, α-crystallins protect the lens by functioning as a molecular chaperone (43), and many of the taxon-specific crystallins are related or identical to detoxification enzymes (36, 41). Protection against oxidative stress may be especially important for cephalopod lenses since seawater contains high concentrations of photochemically generated oxygen radicals and redox-active transition metal ions (see Ref. 44). Moreover, the squid light organ contains a high concentration of peroxidases that may generate many potentially dangerous oxidants, such as superoxide anion, hydroxyl free radicals, and hypochlorous acid (45, 46), threatening the lens. Thus, the recruitment of Ω-crystallins may have initially involved the induction of enzyme activity for stress protection and subsequent gene duplication and specialization for refraction in the eye and light organ.

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