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Evolution

Evidence for neutral and selective processes in the recruitment of enzyme-crystallins in avian lenses

(molecular evolution/Apodiformes/δ-crystallin/ε-crystallin)

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ABSTRACT **In apparent contrast to most other tissues, the ocular lenses in vertebrates show striking differences in protein composition between taxa, most notably in the recruitment of different enzymes as major structural proteins. This variability appears to be the result of at least partially neutral evolutionary processes, although there is also evidence for selective modification in molecular structure. Here we describe a bird, the chimney swift (*Chaetura pelagica*), that lacks δ-crystallin/argininosuccinate lyase, usually the major crystallin of avian lenses. Clearly, δ-crystallin is not specifically required for a functionally effective avian lens. Furthermore the lens composition of the swift is more similar to that of the related hummingbirds than to that of the barn swallow (*Hirundo rustica*), suggesting that phylogeny is more important than environmental selection in the recruitment of crystallins. However differences in ε-crystallin/lactate dehydrogenase-B sequence between swift and hummingbird and other avian and reptilian species suggest that selective pressures may also be working at the molecular level. These differences also confirm the close relationship between swifts and hummingbirds.**

In many vertebrate species recruited enzymes comprise a large fraction of the crystallins, the soluble, structural proteins of the lens (1, 2). This phenomenon, a form of gene-sharing or protein multifunctionality, has wide implications for the processes of protein evolution and the mechanisms of differential gene expression. It also raises questions about the origins and roles of specific crystallins, why particular proteins were recruited as crystallins, and how important they are for correct lens development and function. Here we show that the expression of δ-crystallin/argininosuccinate lyase (ASL) (3) varies widely in avian lenses and appears to be more dependent on phylogeny than on any obvious functional or environmental selection. We have shown previously that two δ-crystallin genes are abundantly expressed in the embryonic duck lens (refs. 4, 5; G.W. and J.P., unpublished data), whereas only one gene contributes significantly to δ-crystallin in the chicken lens (6, 7). Now we describe an avian species, the chimney swift (*Chaetura pelagica*), a fast-flying insectivore, that has no detectable lens expression of δ-crystallin at all, although its lens does contain abundant ε-crystallin/lactate dehydrogenase B (LDH-B) (8). The hummingbirds are thought to be most closely related to the swifts in spite of marked superficial differences, most notably in feeding behavior. We have found that Anna's hummingbird (*Calypte anna*) does express detectable δ-crystallin but at a rather low level and also has extremely abundant ε-crystallin. In contrast, the more distantly related barn swallow (*Hirundo rustica*), another fast-flying insectivore with similar feeding habits to the swift, has a quite different lens crystallin composition, with a more typical level of δ-crystallin and low or absent ε-crystallin. Surprisingly, moreover, the ε-crystallins of swift and hummingbird lack the interesting modifications to LDH-B sequence associated with ε-crystallin/LDH-B in other species (8).

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MATERIALS AND METHODS

Lenses. Peking duck lenses were extracted from 14-day embryos (Truslow Farms, Chestertown, MD). Other bird lenses came from natural adult casualties (resulting from collisions with windows or vehicles) or as postmortem samples from the National Zoo (Washington) and San Diego Zoo. Because of the great difficulty in obtaining such specimens only single examples of each wild or zoo species were examined.

Protein Analysis. Native protein samples were obtained by homogenization of lenses in TE buffer (10 mM Tris·HCl, pH 7/1 mM EDTA) and examined by SDS/polyacrylamide gel electrophoresis (SDS/PAGE) (9). Gels were stained directly with Coomassie blue or transferred to nitrocellulose where they were stained with amido black or Ponceau S as appropriate. Approximate abundances of subunits were estimated by densitometric scanning of blotted and stained gels. Individual bands were isolated by excision from stained blots and were then eluted, digested, and sequenced as described (3, 10). Sequencing was performed as a service by the Harvard University Microchemistry Facility (Cambridge, MA). Western blotting with antisera to isoelectrically focused duck (unpublished) or chicken (11) δ-crystallins or lamprey τ-crystallin (12) was performed by standard methods (13). Enzyme assay for LDH was performed essentially as described by Stolzenbach (14), using reagents from Sigma.

Computer Analysis. Sequences were examined using the IDEAS programs (15) to search the GenBank and National Biomedical Research Foundation databases.

RESULTS

The compositions of soluble lens extracts from several avian species were visualized by SDS/PAGE (Fig. 1), revealing some variability. In most species a prominent band or doublet corresponding to δ-crystallin/ASL is apparent at 48–50 kDa in size. Some species exhibit a slightly larger band that remains to be identified. However the most surprising difference is the apparently complete absence of δ-crystallin in the chimney swift. Although this species has a minor band at about 48 kDa, this exhibits no immunoreactivity with anti-chicken δ-crystallin serum, giving instead a clear reaction with antiserum to lamprey τ-crystallin/α-enolase (10, 12) (Fig. 2). The swift lenses examined were clear and after homogenization yielded very little insoluble material, and this material contained no detectable crystallin (not shown). The absence of δ-crystallin is, therefore, unlikely to have

Abbreviations: ASL, argininosuccinate lyase; LDH, lactate dehydrogenase.

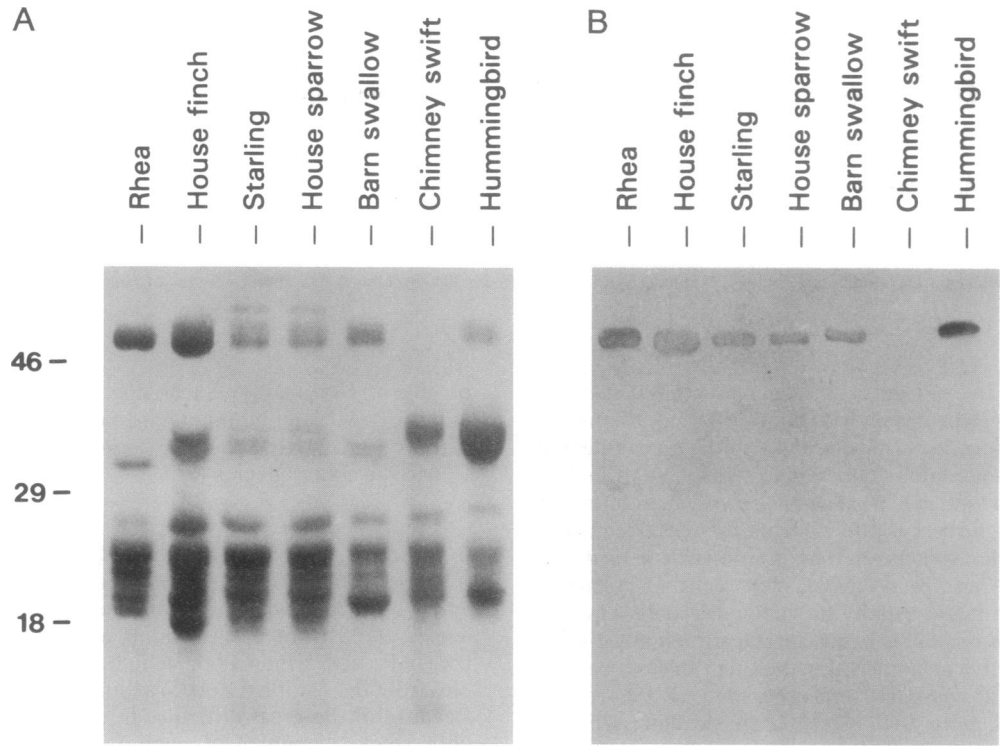

FIG. 1. Soluble lens protein composition of some avian species: SDS/PAGE (9) gels of lens extracts blotted onto nitrocellulose and either stained with amido black (*A*) or used for Western analysis (13) with anti-duck δ-crystallin serum (*B*). Relative subunit sizes are indicated in kDa. Species are as follows: greater rhea (*Rhea americana*), house sparrow (*Passer domesticus*), common starling (*Sturnus vulgaris*), house finch (*Carpodacus mexicanus*), barn swallow (*Hirundo rustica*), chimney swift (*Chaetura pelagica*), and Anna's hummingbird (*Calypte anna*).

been the result of age or disease-related insolubilization. In this swift, δ-crystallin seems to have been substantially replaced by a major species of 35-kDa subunit size that accounts for about 20% of total protein. This protein was isolated and partially sequenced. All major peptides sequenced gave a close match with avian ε-crystallin/LDH-B (8, 16) (Fig. 3). Furthermore the adult swift lens extract had considerable LDH activity, 40 units/mg of total protein, compared with 12 units/mg in the embryonic duck lens (1 unit represents oxidation of 1 μmol of NADH per min). The

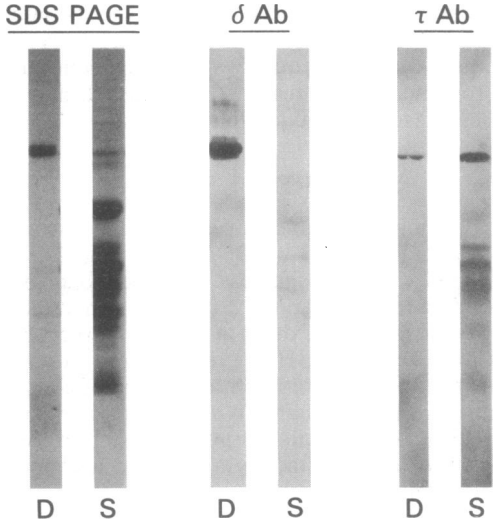

FIG. 2. The minor 48-kDa band in the swift lens extract is τ-crystallin/α-enolase (10), not δ-crystallin/ASL (3). D, lens extract from 14-day embryonic duck; S, lens extract from adult chimney swift. (*Left*) SDS/PAGE stained with amido black. (*Center*) Western blot with anti-chicken δ-crystallin. (*Right*) Western blot with anti-lamprey τ-crystallin.

presence of ε-crystallin in an Old World swift (*Apus apus*) has been noted before (8) and, in retrospect, lens protein analysis for this species was also consistent with low expression or absence of δ-crystallin (see ref. 8, figure 1, lane 8).

For comparison, the lens proteins of the barn swallow and Anna's hummingbird were then examined. In the swallow lens the prominent 35-kDa band yielded peptides only from βB_1-crystallin (19) (to be described elsewhere), not ε-crystallin. The swallow lens also had a high content of δ-crystallin, about 20% of total protein. In contrast, the lens of the hummingbird contained only about 5% δ-crystallin, apparently making a smaller contribution than normal to total lens protein. Furthermore, the hummingbird, like the swift, had a very prominent 35-kDa subunit that was identified as ε-crystallin by microsequencing. Indeed, ε-crystallin may be extremely abundant in the hummingbird lens. The 35-kDa band in the hummingbird extract accounts for about 40% of total protein and all of the peptides sequenced from this band corresponded to ε-crystallin/LDH. However no significant LDH activity was detected in the extract from this sample, which may have suffered postmortem inactivation.

DISCUSSION

δ-Crystallin/ASL Is Not Absolutely Required for Avian Lens Function. Until now, δ-crystallin/ASL has been found in the lenses of all birds and reptiles (6), suggesting that it was recruited as a lens structural protein in a common ancestor of all reptiles and birds after divergence from the line leading to mammals. In birds it seems that δ-crystallin/ASL has replaced the γ-crystallins prominent in the embryonic lens in amphibians and mammals. An unanswered question is whether the recruitment of ASL and of other enzymes in various taxa (1, 2) was the result of neutral evolution or selection for specific catalytic or structural properties.

	60	80	114 118	250	310
hb	XEMMXLQHGSLFL	IVADKDYAVTAN	NVNVFKEIIPQIMK		
sw		IVADKDYAVT	NLVQXNVNVFKEIIPQXMK	GYTNXAXGL	LKDDEVXQLKK
de	KGEMMDLQHGSLFLQ	KIVADKDYAVTANSK	RLNLVQRNVGVFKGIIPQIVK	KGYTNWAIGL	LKDDEVAQLKK
cb	KGEMMDLQHGSLFLQ	KIVADKDYAVTANSK	RLNLVQRNVNVFKFIIPQIVK	KGYTNWAIGL	LKDDEVAQLKK
pb	KGEMMDLQHGSLFLQ	KIVANKDYSVTANSK	RLNLVQRNVNVFKFIIPQIVK	KGYTNWAIGL	LKDDEVAQLKN
ca	KGEMMDLQHGSLFLK	KITSGKDYSVTAHSK	RLNLVQRNVNIFKFIIPNVVK	KGYTSWAIGL	LKPDEEEKIKK

FIG. 3. Sequences of tryptic peptides from the 35-kDa bands isolated from swift and hummingbird lenses. hb, Hummingbird ε-crystallin; sw, swift ε-crystallin; de, duck ε-crystallin (8, 16); cb, chicken LDH-B (17); pb, pig LDH-B (18); ca, chicken LDH-A (17). Circled residues indicate differences between ε-crystallin in swift or hummingbird and duck. Residue numbers for full-length LDH-B are indicated.

Enzyme crystallins do not always retain activity in lenses. For example, the embryonic duck lens, which expresses two δ-crystallin genes at high levels (refs. 4, 5; G.W. and J.P., unpublished data), has >1500 times the ASL activity of the embryonic chicken lens (20), which instead has a great preponderance of δ1-crystallin, a protein that may have specialized for lens, losing ASL activity. As shown here, the chimney swift lacks detectable δ-crystallin/ASL altogether. Thus it seems that very high levels of ASL catalytic activity are not required for the function of avian lenses. Furthermore, the absence of δ-crystallin/ASL from the chimney swift lens suggests that this protein is not even required in bird lenses for any particular structural properties. It is not needed to maintain transparency and can be adequately replaced by other crystallins, including a different enzyme-crystallin.

The unexpected lack of δ-crystallin in swifts could be the result of peculiar, stringent selective pressures on the accommodative mechanisms of the eye resulting from the exacting requirements of catching insects on the wing at high speed. If this is true, species that experience similar pressures might be expected to share some characteristics of the swift lens, including reduced expression of δ-crystallin/ASL and elevated ε-crystallin/LDH-B.

To the nonspecialist observer the birds most similar to swifts in terms of general appearance and habit are, perhaps, the swallows (Hirundinidae). In fact, the two groups are not closely related (21) and their similarities as insectivores are presumably the result of convergent evolution. Instead, the closest relative of the swifts are the hummingbirds (both are of the order Apodiformes; see ref. 21). Swifts and hummingbirds are highly specialized but for very different habits. The lens protein composition of the swift does not closely resemble that of a swallow but is instead more similar to that of a hummingbird. The implication of this is that, at least in some cases, enzyme-crystallin expression is more dependent on phylogenetic relationships, the chance effects of ancestry, than on any specific functional requirements. Expression of δ-crystallin may have begun to decline in the common ancestor of hummingbirds and swifts, with replacement by ε-crystallin, a process that seems to have gone to completion in the swift and that has evidently not impaired the vision of these fast-flying insectivores.

Sequence Changes in ε-Crystallin. ε-Crystallin/LDH-B itself has some interesting features. It is found only in the lenses of many avian and crocodilian species (8, 22), suggesting that it was recruited more recently than δ-crystallin/ASL, in a common ancestor of the archosaurs. The fact that ε-crystallin (as such) is not expressed in the lenses of all birds implies that its expression was lost again in some lines of descent. Furthermore, in the amino acid sequences of duck lens ε-crystallin and LDH-B extracted from duck heart, the products of the same gene (16), two residues, Asn-114 and Phe-118, that are otherwise well conserved in LDH-A and -B subunits in vertebrates are replaced by glycine residues (8). In fact, the Phe/Gly-118 change is present in all of the other ε-crystallin sequences previously examined (22). It has been hypothesized that these changes, presumably not beneficial to LDH enzymatic function, were selected for by the requirements of lens, producing flat patches on the surface of the LDH-B4 tetramer, perhaps involved in intermolecular interactions (5, 16). It was surprising therefore to see that in swift and hummingbird ε-crystallin both of these amino acids are the normal, conserved LDH choices.

In the case of the hummingbird the extraordinary metabolic requirements of this bird may have created an extra degree of selective pressure against any lens-driven sequence modification of the LDH-B expressed in heart and other tissues, the product of the same gene as ε-crystallin. However, there is also a possible correlation between conservation of LDH sequence at residues 114 and 118 of ε-crystallin and reduced δ-crystallin expression in the lens suggesting that pressure for change at these positions in ε-crystallin/LDH-B might also arise from some unfavorable interactions between abundant δ- and ε-crystallins in the lens. Although Asn-114 and Phe-118 are conserved in swift and hummingbird ε-crystallin, there are also nonconserved positions, notably the replacement of Val-124 by methionine (Fig. 3). This underscores the relatedness of these birds and may also reflect a lens-driven selective change in LDH-B sequence different from that experienced in other species.

Recruitment of Enzymes as Crystallins. Although there may have been general selective benefits in adding to the repertoire of lens structural proteins, perhaps helping to dilute the effects of lens-hardening γ-crystallins (ref. 23; G.W. and H. Kim, unpublished data), the recruitment of specific enzymes as crystallins does not seem to have been the direct result of positive selection for their particular structure or catalytic function. In different lines of descent different choices of enzyme were made and it is apparently possible for particular enzyme-crystallins to be replaced. Even though the enzymes selected must have been able to satisfy the requirements of lens for stability and appropriate packing (see ref. 1), their selection seems to have been at least partially neutral, drawing from a pool of several equally acceptable choices.

However, selective pressures may have come into play subsequently. For example, it might indeed be the case that very high concentrations of ε-crystallin/LDH-B and δ-crystallin/ASL are not advantageous for the lens in some species. This problem could be solved (*i*) by reduction of ε-crystallin/LDH-B expression back to "normal" enzymatic levels, something that must have occurred in the swallow and other birds species (8); (*ii*) by modification of LDH sequence, perhaps as in duck and other lenses (8, 22); or (*iii*) by modification or, as in the chimney swift, loss of δ-crystallin. Finally, given the pragmatism of the evolutionary process, it would not be surprising if some enzyme-crystallins acquired additional useful functions in lens, as has been suggested previously (8).

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