CHAPTER 3

THE UBIQUITOUS CRYSTALLINS: STRESS PROTEINS RECRUITED TO LENS

Three classes of crystallins, belonging to two protein superfamilies, are represented in the lenses of all vertebrates. These are the α, β and γ-crystallins. The ubiquity of these crystallins suggests that their recruitment occurred at a very early stage in the evolution of the lens. Furthermore all three classes of ubiquitous crystallins have at least some connection with various stress responses.

α-CRYSTALLINS: MEMBERS OF THE SMALL HEAT SHOCK PROTEIN SUPERFAMILY

α-Crystallins are present in the lenses of all vertebrates; indeed in many mammals they may be the major protein components of the lens.[1-3] Species as divergent as dogfish (*Squalus acanthias*) and mammals express the same two α-crystallin subunits, αA and αB.[4] αA-crystallin is usually the most abundant subunit and indeed may be the single most abundant gene product in the lens, although the αA/αB ratio varies considerably among species and αB seems to predominate in the dogfish lens.[1,2,4] Both subunits are about 20 kDa in size and have considerable sequence similarity; in chicken, αA[5] and αB[6] are 59% identical. This degree of conservation is reflected in the slow rate of sequence change in αA-crystallins[7] which has made them useful tools for molecular phylogenetic studies. αA-crystallin sequences form one of the most comprehensive databases for this purpose.[8-12]

Both α-crystallin gene products undergo a remarkable array of post-translational modifications including peptide bond cleavage,[1,2,13] cAMP dependent phosphorylation,[14-18] autophosphorylation,[19] deamidation,[20,21] transglutamination,[22] fatty acylation,[23] racemization[24] and cytoplasmic glycosylation.[25] The functional and structural consequences of these modifications are not clear although it seems likely that they could

have roles in modifying interactions of α-crystallins which each other and with other lens components.

Tertiary and Quaternary Structure

In spite of the growing interest in this family little is known of the tertiary structure of the protein subunits or their quaternary interactions. CD (circular dichroism) and ORD (optical rotary dispersion) spectroscopy both suggest that α-crystallin subunits have predominantly β-sheet structure with only a small content of α-helix.[26,27] ^{1}H NMR analysis has found that the C-terminal regions of bovine lens α-crystallin subunits are highly flexible and disordered.[28] However an x-ray structure determination is urgently needed.

One of the most notable features of α-crystallins is their propensity for aggregation. Large and variable aggregates form from populations of one subunit or from mixtures of both.[29,30] A number of different models for the quaternary structures for α-crystallins have been proposed in attempts to reconcile data from different experimental systems. Based on a variety of experiments which suggest that α-crystallin subunits form distinct populations with regard to accessibility, three-layer models of α-crystallins have been proposed.[31-33] From a structural point of view these models have the unsatisfactory feature of requiring identical subunits to occupy non-equivalent positions. In a different model the difference in hydrophobicity between the N-terminal and C-terminal regions of α-crystallins inspired the suggestion that α-crystallin subunits form protein micelles.[34]

Other experiments on the aggregation behavior of recombinant α-crystallin domains[35] led to the proposal that α-crystallin subunits assemble as tetramers or pseudo-tetramers on each face of either a rhombic dodecahedron or a cube,[36] a model in which all subunits occupy equivalent positions. Finally another model has been proposed based on the structure of molecular chaperones.[37] It seems very likely that α-crystallins have multiple modes of assembly depending on conditions, with dynamic interchange of subunits and easy transitions between alternative quaternary structures.[36,38,39]

Ontogeny

α-Crystallins are among the earliest crystallins to be expressed during vertebrate lens embryogenesis and continue to be expressed at high levels throughout life.[1,40-42] However the patterns of expression of the two genes appear to vary among species. For example, in human lens development αB-crystallin has been detected in the lens placode (see Fig. 1.2) while αA-crystallin was not observed until the lens vesicle stage.[42] In contrast, in the developing rat lens αA-crystallin appears first in the lens pit at embryonic stage E12,[42] similar to the timing of α-crystallin in mouse,[41] while αB-crystallin was not detected until stage E14 in elongating lens fibers.[42] The same authors also found differ-

ences in the distribution of the two α-crystallins in human and rat lenses such that αB-crystallin was found in both epithelial and fiber cells in human lens but was found only in fiber cells in rat lens. Interestingly, the work on developing mouse lens suggested that there was an association between the onset of crystallin synthesis and cell elongation.[41]

THE SMALL HEAT SHOCK PROTEIN CONNECTION

Although it was appreciated that crystallins must have arisen from molecular ancestors which predated the existence of the vertebrate lens,[2] it was also assumed that α- and other crystallins would be lens-specific structural proteins derived by gene duplication and specialization from those non-lens ancestors. The first indication of a non-lens relationship for crystallins came when the sequences of *Drosophila* small heat-shock proteins (sHSP) revealed surprisingly close similarities with αB-crystallin.[43]

The sHSP superfamily is ubiquitous in eukaryotes.[44-50] Distinct members of this superfamily are also found as egg antigens in *Schistosoma mansoni*[51,52] and muscle-associated proteins in the ascidian *Halocynthia roretzi*.[53] The superfamily is even represented in prokaryotes as surface antigens in *Mycobacterium leprae*[54] and *M.tuberculosis*,[55] as a ribosomal associated protein in *M.bovis*[56] and as stress induced proteins in *Escherichia coli*,[57] *Stigmatella aurantiaca*[58] and *Clostridium acetobutylicum*.[59] Recently a new vertebrate member has been added to this family, p20 a bovine protein with closer sequence similarity to α-crystallins than to any mammalian sHSP.[60] The large scale sequencing project for the nematode *Caenorhabditis elegans* has also uncovered a gene with closer similarity to the α-crystallin family than to other sHSP.[61]

The significance of the similarity between α-crystallins and the sHSP family became clearer when, following the discovery of non-lens expression of taxon-specific enzyme crystallins,[62] αB-crystallin too was detected outside the lens during difference library screening of hamster brains affected with the prion disease scrapie.[63] Indeed, this theme of elevated expression of αB-crystallin associated with neurological disorders has continued with the detection of high concentrations of αB-crystallin in Alexander's disease,[64] Lewy body disease[65] and the human prion associated Creutzfeldt-Jakob disease[66] among others.[3] Lower constitutive levels of αB-crystallin were also found in many adult tissues in both mammals and birds.[3,67-70]

The connection with the sHSP family was strengthened when it was discovered that mouse αB-crystallin was induced by heat-shock in mouse embryonic fibroblasts.[71] Thus in this species αB-crystallin itself is a sHSP. Like other heat shock proteins, αB-crystallin can also be induced by other insults such as osmotic stress,[72,73] ischemia[74] and by expression of some oncogenes.[75] However this stress response may be specific to mammals. In duck embryonic fibroblasts the endogenous

αB-crystallin gene is neither constitutively expressed nor induced by either heat or osmotic stress under conditions which elicit a stress response and HSP70 induction.[73] This correlates with an absence of canonical heat shock response elements in the promoter of the duck αB-crystallin gene.[73] It seems likely that both heat and osmotic stress responses in mammalian αB-crystallin genes are mediated through these elements and that αB-crystallin in the duck has lost this kind of inducibility. If the stress response is an ancestral feature of αB-crystallin predating its role in lens then it appears that duck αB-crystallin (and presumably its homologues in other birds) has become more specialized for lens expression and has given up a large part of the ancestral non-lens role.

There is no evidence for inducibility of αA-crystallin, but very low levels of the protein have been detected in non-lens tissues, particularly in spleen and thymus.[76] In general, however, it seems that αA-crystallin is much more specialized for lens-expression than αB.

Molecular Chaperones of the Cytoskeleton

The functions of heat shock proteins are beginning to become clear. The large heat shock proteins of the HSP70 family are ATPases with structural similarity to actin.[77] These proteins are ubiquitous and highly conserved from bacteria to vertebrates. They and other families of heat shock proteins such as the HSP60 class act as molecular chaperones guiding protein folding pathways and the transport and assembly of multisubunit complexes.[78] They also serve to prevent inappropriate interactions between partially assembled proteins.[78] Recent results suggest that α-crystallins and their sHSP relatives may lack a direct role in protein folding but may share some of the other aspects of molecular chaperones.

In contrast to HSP70, α-crystallins lack an identifiable ATPase domain, although there is some evidence for an association between α-crystallins and ATP.[19,79] However there is clear evidence that α-crystallins can prevent the insolubilization of proteins denatured by heat stress.[80] Unlike the HSP70 family, α-crystallins do not appear to have a direct role in protein folding or refolding since they are unable to protect or restore the enzyme activity of heat stressed enzymes.[3,80] This chaperone-like activity may be a reflection of a physiological role of α-crystallins in what might be described as an auxiliary chaperone role. α-Crystallins might participate in the assembly and disassembly of complex structures without a direct role in protein folding. This could be regarded as catalyzing a process which would otherwise have an unacceptably high activation energy due to the formation of incorrect interactions between constituent subunits of an unassembled structure.

A likely beneficiary of this chaperone effect of sHSP and α-crystallins may be the cytoskeleton. There is considerable evidence linking sHSP

and α-crystallins with management and protection of cytoskeleton in lens and in other cell types. For example, an actin depolymerizing protein of turkey gizzard was found to be a sHSP[81] while αB-crystallin itself can interact with actin,[74,82,83] desmin[82] and vimentin.[84] Chicken αB-crystallin has been associated with extensive cytomorphological remodeling in lens, notochord and myotome during embryogenesis.[85] In lens, α-crystallins participate in the ATP-dependent assembly and disassembly of vimentin and GFAP components of cytoskeleton during lens cell differentiation.[84] This role as a chaperone for cytoskeleton may be the basis for the increased thermotolerance conferred on cultured cells by overexpression of αB-crystallin.[86]

The Recruitment of a sHSP as a Crystallin

It seems likely that a sHSP was recruited as a crystallin in the primitive lens of an ancestor of vertebrates. This sHSP may have been associated with cytoskeleton in the elongating cells of the proto-lens. Shortly after recruitment this gene duplicated to produce the ancestors of the modern αA- and αB-crystallin genes. One gene was able to retain a general stress response role in many tissues in addition to its role as a crystallin. This gene became the αB-crystallin seen today. In contrast, the other gene specialized for the lens environment in both protein sequence and gene expression and substantially lost any non-lens role. This gene became the modern αA-crystallin. Indeed, although αA-crystallin and αB-crystallin are equivalent in many ways[3] there is evidence that αA-crystallin has some specialized properties which may have evolved specifically for its role in lens. In particular, some of the interactions between α-crystallins, lens cytoskeleton and membrane fractions seem to be specific to αA subunits.[87-91] This specialized interaction may be associated with the presence of the unusual "beaded filaments" found only in lens cytoskeleton.[92] It seems that the evolutionary specialization of α-crystallins for lens may be continuing since in birds the non-lens and stress role of αB-crystallin appear to have been lost.[73]

GENE STRUCTURE

Their ubiquity in modern species suggests that the genes for αA- and αB-crystallin must have arisen by duplication in a distant common ancestor of all vertebrates.[3] In man the two genes are found on different chromosomes, αA-crystallin (CRYA1) on chromosome 21[93-95] and αB-crystallin (CRYA2) on chromosome 11,[96] which is perhaps another reflection of the antiquity of their duplication. In spite of this ancient separation, αA- and αB-crystallins show a high degree of sequence similarity and identical gene structures. Genes for αA-crystallin have been cloned and at least partially sequenced from hamster,[97] mole rat,[98] mouse,[99,100] human[101,103] and chicken.[5] Genes for αB-crystallins have been cloned from hamster,[93] mouse,[67,104] rat,[105] human[106] and duck.[73] All these genes have the same general structure of three exons, with

the interesting exception that mammalian αA-crystallin genes also contain an alternatively spliced insert exon or pseudoexon in the first intron[97-99,101] (Fig. 3.1).

The Insert Exon of Mammalian αA-Crystallins

In spite of the close similarity in exon-intron structure of α-crystallin genes in mammals and birds and the high degree of conservation of sequence in α-crystallins, mammalian αA-crystallin genes have an interesting peculiarity. This was first discovered when a minor form of αA-crystallin in rat lens was found to contain an insertion of 23 amino acids in an otherwise perfectly conserved sequence.[107-109] Gene sequencing showed this insertion was due to alternative mRNA splicing of an insert exon in the first intron of rodent αA-crystallin genes.[97-99,110] Perhaps because of a slightly sub-optimal splice site recognition sequence (GC for GT) this alternative or insert exon was found to be spliced into mRNA in only about 10% of mature processed transcripts.[99,110] The function of the insert peptide is still unknown and the αA^{ins} subunit behaves much like αA-crystallin in assembly studies.[30]

The αA^{ins} subunit was originally thought to be a curiosity of the murine rodents, however its distribution has turned out to be more extensive (Fig. 3.2). By western blotting it has been detected in lens extracts of several other rodents, the European hedgehog (*Erinaceus europaeus*), elephant shrew (*Elephantulus rufescens*), both mega- and microchiropteran bats and in a marsupial, the tamar wallaby (*Macropus eugenii*).[102,111,112] It was not detected in the lenses of many other mammals or in any non-mammals. At the level of the genome, the alternative insert exon has also been detected by hybridization in elephant shrews, rodents, bats, marsupials and even the spiny anteater (*Echidna sp.*), a monotreme.[102] Again, there was no hybridization of alternative exon probes with birds, reptiles or amphibians[102] while gene sequencing of chicken αA-crystallin confirms the lack of a recognizable insert exon.[5] αA^{ins} may thus constitute a synapomorphy which distinguishes all mammalian lineages from other vertebrates.

The αA^{ins} subunit is not found in human or other primate lenses. Nevertheless, when the human gene for αA-crystallin was cloned a sequence very similar to the insert exon was found in the first intron.[101] Although closely matching the insert exon of rodent αA-crystallin genes this part of the human gene contains a number of non-silent sequence changes and a deletion which would cause a shift of reading frame if translated. Thus the insert-exon sequences in the human αA-crystallin gene constitute a pseudoexon which, like a pseudogene unprotected by selection, is undergoing accelerated sequence drift. DNA hybridization studies suggest that similar pseudoexons are also likely to be present in chimpanzee, Old World monkey and rabbit which, like humans, do not express the αA^{ins} subunit while in ungulates it appears that any sequences related to an ancestral insert exon have been lost.[102]

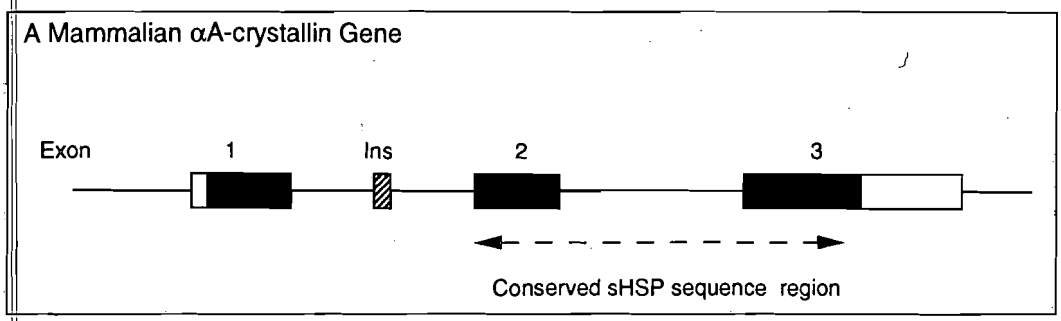

Fig. 3.1. Gene structure in the α-crystallins. The general structure of a mammalian αA-crystallin gene. Exons are shown as boxes, coding sequences are shaded. The alternative insert exon in intron 1 is peculiar to mammalian αA-crystallins. With this exception, αB-crystallin genes have the same general structure.

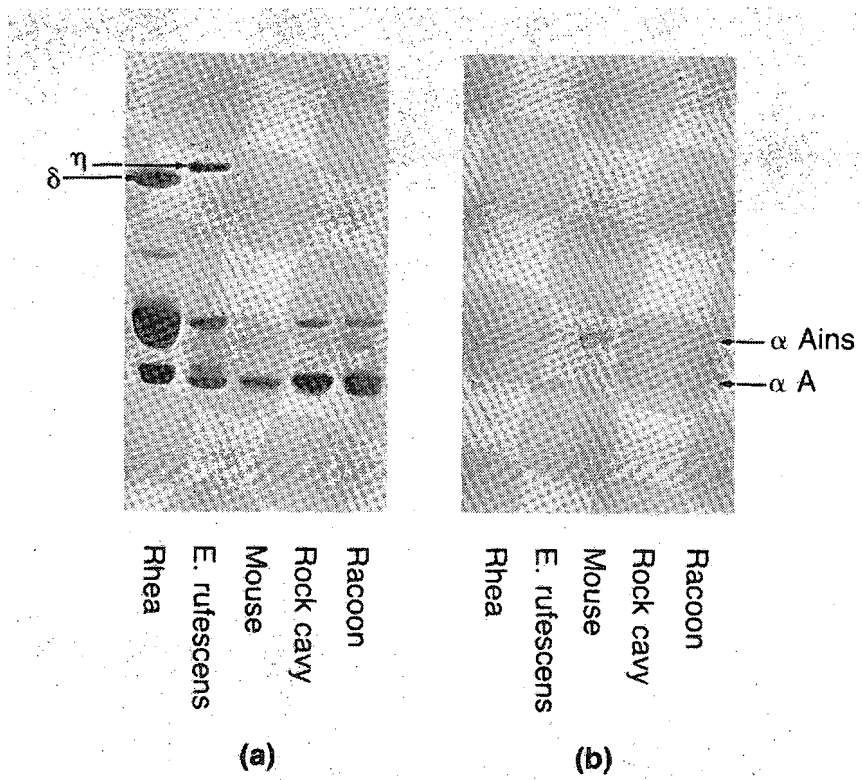

Fig. 3.2. Detection of the αA^{ins} subunit in lenses of some mammals. Proteins were separated by SDS PAGE (left) and subjected to western blotting (right) with antisera to mouse αA-crystallin.[231] Lens extracts are from Rhea (Rhea americana) *a bird; an elephant shrew* (Elephantulus rufescens)*; mouse* (Mus musculus)*; Rock cavy or moco* (Kerodon rupestris)*; Raccoon* (Procyon lotor)*. Migration positions of αA-crystallin and αA^{ins} subunits are marked. Two taxon specific crystallins, δ and η, are also marked.*

It thus appears that far from being a recent evolutionary experiment in murine rodents the insert exon and the encoded peptide have a much more ancient origin in mammalian evolution. Since its sequence is so well conserved it seems reasonable to suppose that αA^{ins} has a useful function when expressed. However, many modern placental species have abandoned its use altogether. It seems that this experiment in protein engineering answered a particular requirement of the lens in a common ancestor of monotreme, marsupial and placental mammals, a requirement which has been superseded by more recent evolutionary developments in many lineages. The story of αA^{ins} is another example of the dynamic molecular evolution of crystallins in the complex history of mammals.

Implications of Gene Structure for Protein Structure

The region of closest similarity in protein sequence between α-crystallins and other members of the sHSP superfamily corresponds to sequences coded by exons 2 and 3 of α-crystallin genes[3,113] (Fig. 3.1). This region of similarity is also that most highly conserved among all members of the superfamily, suggesting that it corresponds to an ancestral functional unit or structural domain. In p40 of *S.mansoni* there are actually two of these structural units in tandem.[4,51] In contrast, N-terminal protein sequences, which correspond to exon 1 of α-crystallins, are much more variable throughout the superfamily. In the α-crystallins themselves there is evidence for a two fold repeat in this region which contains limited patches of similarity with some vertebrate sHSP sequences.[4,113,114]

It has been proposed that the exons of α-crystallins encode discrete structural units.[113] The two fold repeat encoded by exon 1 in the α-crystallins suggests that this exon may have arisen by duplication and fusion of an ancestral motif. Intron 1 neatly separates the more variable N-terminal sequences from those which are universally conserved among members of the sHSP superfamily. Furthermore, when the sequences encoded by exons 2 and 3 are aligned according to intron positions another weak two fold repeat is observed. This repeat is also apparent in *Drosophila* sHSPs which themselves come from intronless genes.[113] This has been interpreted as reflecting the presence of two related structural motifs in the C-terminal domain of α-crystallins which are the result of an ancient duplication of a gene corresponding to one exon.

The observations of possible mapping between exons and protein sequence motifs led to the suggestion that α-crystallins and sHSPs have a bipartite structure consisting of a variable N-terminal domain which may or may not have its own internal symmetry, and a C-terminal domain (or pair of domains) with two fold symmetry.[113] The proposed C-terminal domain contains the most important conserved structural features of the sHSP family. This two-domain model was investigated

by cloning and expressing the putative domains of bovine αA-crystallin.[35] The C-terminal domain behaved as a well-folded discrete structure capable of forming dimers or tetramers while the more hydrophobic N-terminal domain tended to assemble in large aggregates. It was these results which led to the idea that α-crystallins may be able to form tetramers or pseudo tetramers which in turn assemble into larger aggregates through non-specific interactions of the more hydrophobic N-terminal domain.[36]

Like α-crystallins other sHSP are also synthesized as protein subunits of about 20 kDa size and aggregate to form large multimeric complexes very similar to those formed by α-crystallins.[115-117] Typically, for both sHSP and α-crystallins, these aggregates are spherical assemblies of 300-800 kDa, although a variety of complex sizes and shapes can be observed under different conditions.[3] Whatever the methods of subunit assembly, they are promiscuous enough to allow the formation of mixed assemblages of αA- and αB-crystallins with mammalian hsp27[116,118] and with the recently discovered p20 proteins.[60] The ability of these disparate proteins to multimerize is further evidence that assembly is primarily dictated by the evolutionarily conserved C-terminal domains.

Intron Positions in the sHSP/α-Crystallin Superfamily

The origins and functions of the introns which divide the coding sequences of most vertebrate genes have been the subjects of considerable speculation and controversy.[119-121] In many genes introns map very closely to structural divisions in protein sequences. This has led to the "introns early" hypothesis that introns were present from the earliest time when these genes were assembled and indeed that introns were responsible for the assembly of modern genes.[119,122] For others however the mapping of introns and protein motifs is a statistically insignificant "hopeful illusion." In this view introns were inserted randomly relatively late in the evolution of the eukaryotic genome.[121] An intermediate hypothesis has also been proposed which accepts the special positions of introns in many genes but suggests that they could still have been the result of "introns late" insertion.[123] This scheme envisages that intron insertion could have been directed by RNA structure retained from ancestral RNA "genes."

Although the tertiary structure of α-crystallin subunits is unknown, there are indications that the exons of α-crystallins do in fact correspond to structural motifs. Introns delineate regions of duplicated sequence and in particular they separate the sequences most highly conserved among α-crystallins and small heat shock proteins (sHSP) from more variable regions.[4,113] Thus exons 2 and 3 are hypothesized to correspond to a structural domain conserved in the superfamily[113] and this prediction has received support from recombinant studies expressing the isolated domains.[35]

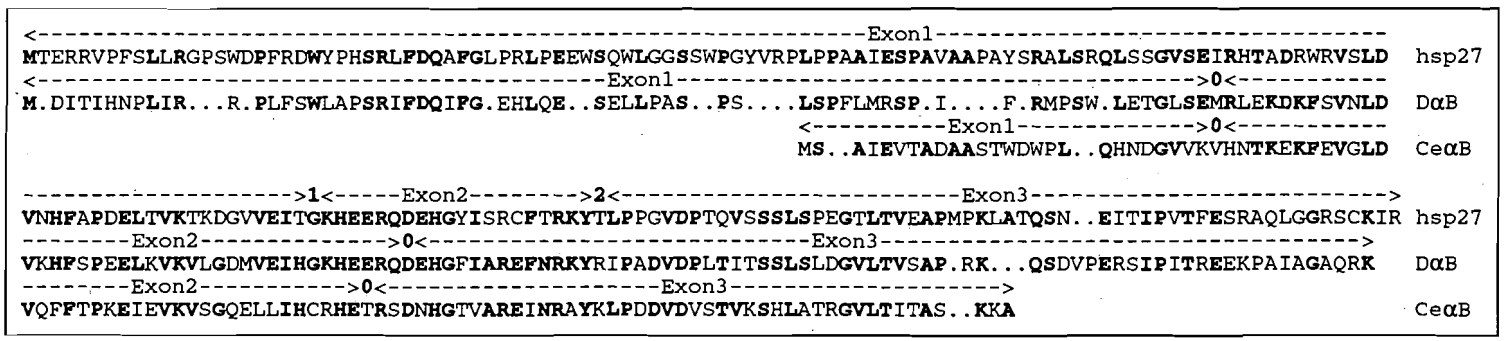

Fig. 3.3. Sequence alignment and relative intron positions of members of the sHSP/α-crystallin superfamily. Protein sequences are aligned and relative positions and phases of introns indicated. CeαB: predicted product of an "αB-like" C.elegans gene;[61] DαB: predicted product of duck αB-crystallin;[73] HSP27: predicted product of human HSP27 gene.[45]

Although the introns in α-crystallin genes seem to correspond to structural divisions they are not conserved in position or phase in the sHSP genes of mammals,[45,49,50] while several sHSP genes in yeast, plants and various vertebrates contain no introns at all.[46-48] It is not clear whether all these intronless genes are functional or may instead be processed pseudogenes.[50] However, some other sHSP family genes do share intron positions with α-crystallins. The relative position and splicing phase of the first intron of α-crystallin genes is exactly conserved in the hsp16 genes of *C.elegans*[44,113,124] and in the muscle-associated 29 kDa protein of *H.roretzi*.[53] Even greater similarity is found in an "αB-like" *C.elegans* gene discovered by large scale sequencing.[61] This gene has two introns both of which are in the same phase (0) as in α-crystallins (Fig. 3.3). The first is identical in position to intron 1 of α-crystallins and hsp16, while the second is displaced relative to α-crystallin intron 2 by only 3 codons.

In contrast the two introns of human hsp27 and mouse hsp25 genes do not closely correspond to the position of either α-crystallin intron and are in different phases (1 and 2 respectively)[45,49,50] (Fig. 3.3). This makes it highly unlikely that they are related to the α-crystallin introns by positional slippage. The 29 kDa protein gene has an intron at a relative position 6 codons downstream of intron 2 in α-crystallins.[53] This intron is in phase 2.

The following scenario for the evolution of gene structure in the sHSP/α-crystallin gene superfamily can be envisaged. The intron positions in α-crystallins may reflect the ancient assembly of a proto-sHSP from small fragments of RNA or DNA genetic material which in turn coded for protein structural motifs. This could have occurred either by intron-mediated assembly[119] or by subsequent intron insertion into RNA genes or transcripts at linker regions between ancestral folded RNA structural domains.[123] In an ancestor of the *C.elegans* hsp16 gene the equivalent of intron 2 was lost. In the gene for the 29 kDa protein such a lost intron may have been replaced by subsequent nearby insertion. In other lineages both introns were lost, perhaps via processed pseudogene intermediates. In some vertebrate sHSP genes there was then a reinsertion of introns which might have occurred directly at the DNA level with no potential for directed insertion. In this hypothesis the genes for α-crystallins and the αB-like sequence of *C.elegans* retained the ancestral structure mapping protein motifs and exons.

β- AND γ-CRYSTALLINS: A SUPERFAMILY IN THE VERTEBRATE LENS

In addition to the α-crystallins there are two other classes of ubiquitous crystallin which are represented in all vertebrate lenses. These are the β- and γ-crystallins. Several physico-chemical criteria led to the separate designation of these two classes.[1,125-127] The β-crystallins, with one interesting exception, were multimeric while the γ-crystallins were

monomers; the β-crystallins had blocked N-termini while those of γ-crystallins were free and the β- and γ-crystallins of mammals had different ranges of pI. Nevertheless, the β- and γ-crystallins have turned out to be more closely related than expected and belong to the same protein superfamily, the βγ-crystallins.[128-130] Furthermore, one original β-crystallin, βs-crystallin, is now redefined as a γ-crystallin.[131,132]

β-Crystallin Polypeptides

The first completely characterized set of β-crystallin polypeptides were those from bovine lens.[133] Several homologues from other species, including clear homologues in chicken, have also been sequenced.[6,129,130,134,135] All the β-crystallin polypeptides have sizes in the range 22-28 kDa. They are subclassified into βA (relatively acidic) and βB (relatively basic) subunits, although all β-crystallins have isoelectric points between 5.7 and 7.0.[1] In the bovine lens there are four βA polypeptides; βA1, A2, A3 and A4, and three βB polypeptides, βB1, B2 and B3.[130,133] The same nomenclature has generally been adopted for all β-crystallins in mammals and birds. Protein and nucleic acid sequencing has shown that βA1 and βA3 are products of the same gene and result from use of alternative initiator methionines.[133,136-138] Remarkably this feature of unknown utility is conserved in mammals, birds and frogs.[137-139]

All β-crystallin form dimers and higher aggregates of mixed composition.[1] The major β-crystallin, βB2, forms stable homodimers.[140] βA3/A1 is also able to form homodimers in vitro[141] but otherwise mixed multimers involving interactions between βA and βB subunits seem to be favored.[140] Dimers, trimers and perhaps tetramers of β-crystallin subunits form a β-crystallin fraction called βL (for low) in a native size range between about 40-100 kDa. In mammalian lenses larger aggregates of up to 200 kDa form the βH (for high) fraction which is dominated by octamers[142,143] containing up to 20% βB1 subunits and up to 35% βB2 subunits.[144]

Taxon-specific Differences

In spite of the conservation of clear homologues among the β-crystallins of mammals and birds there are some interesting differences in their properties which may relate to differences in lens structure and composition. This is illustrated in some taxon-specific variation in properties of βB1-crystallins. The βB1-crystallin of the chicken lens was originally called β35-crystallin for its apparent size in SDS PAGE.[145] This is considerably larger than the βB1-crystallins of mammals and larger than the 27 kDa deduced from its cDNA sequence.[134] Furthermore, the apparent size of this polypeptide even varies among birds, falling into two major mobility classes.[146] This anomalous behavior appears to be due to an essentially quantitative, post-translational modification involving some form of cytoplasmic glycosylation.[146] While there

is no evidence for any similar post-translational modification of β-crystallins in mammals, their βB1-crystallins have their own peculiarity. As discussed in detail below, mammalian βB1-crystallins alone among members of the βγ-crystallin superfamily have a unique sequence change in one of their characteristic structural motifs. Again, this may have altered the surface properties of the protein in a taxon-specific manner.

Other possible taxon-specific differences among β-crystallins of birds and mammals may be found in their phosphorylation patterns. In bovine lenses βB2-crystallin seems to be partially phosphorylated[147] while in chicken lens the only β-crystallin subject to this post-translational modification is βB3-crystallin.[148] Finally there may be taxon-specific differences in the relative abundance of certain β-crystallins. For example it has been suggested that the level of βA4-crystallin is lower in chicken than in bovine lens.[135]

The consequences of these differences is not known. However they may relate to the differences in protein content and "softness" of avian and mammalian lenses. In the bovine lens there is evidence that βB1-crystallin has an important role in the formation of βH.[149] The differences in βB1-crystallin among birds and mammals and perhaps even the differences in phosphorylation behavior of other βB-crystallin subunits may affect this aggregation into octamers such that bird lenses may not achieve these larger, more densely associated aggregates. This in turn may contribute to the greater hydration and softness of the bird lens.

γ-Crystallin Polypeptides

γ-Crystallins were originally characterized as the major components of a low molecular weight fraction in mammalian lenses.[1,150] In contrast to β-crystallins, they are strictly monomeric under in vitro conditions. Six γ-crystallins genes and their products were identified in the rat.[151,152] They were named γA-F and these systematic names have generally been applied to the γ-crystallins of other mammals, although not without certain problems (see below). This group of six γ-crystallins, constitute the originally defined γ-crystallin fraction of lens soluble proteins and they meet the original criterion of unblocked N-termini.

Although the best defined γ-crystallins are those found in mammals, multiple γ-crystallins have also been identified in the amphibians *Rana*[153] and *Xenopus*,[154] in the crocodilian *Caiman*[155] and in fish.[156,157] In all cases the multiple γ-crystallins are more similar within than among species, suggesting independent radiations or else highly constrained modifications to suit the requirements of each species. In the carp (*Cyprinus carpio*) γ-crystallins have remarkably high contents of methionine residues and this may be associated with the extremely high protein density of fish lenses.

In addition to the "classical" embryonic γ-crystallins there is another monomeric crystallin which is more widely distributed and better conserved in vertebrates and which was only recently redefined into this family. This is βs-crystallin, the protein which was formerly described as a monomeric β-crystallin.[1] By other criteria, such as the presence of a blocked N-terminus, this polypeptide seemed to be closer to the β-crystallins than to the γ-crystallins. However, protein, cDNA and gene sequencing has now shown that βs-, renamed γs-crystallin, is actually a more distantly related member of the γ-crystallins.[131,132] γs-Crystallin is expressed in fish, mammals and birds and is well conserved in sequence.[131,158-160] Indeed, while there are no clearly homologous relationships between any other fish or amphibian γ-crystallins and those of mammals, γs-crystallin is 70% identical between carp and bovine lens.[131,158] γ-Crystallins were thought to be absent from birds, however the reclassification of βs-crystallin, which is expressed in birds has altered this perception and allowed γ-crystallins the claim of ubiquity in vertebrates.[161,162] Even so, the absence of the classical γ-crystallins from bird lenses is striking and probably highly significant for the properties of the soft avian lens.

Expression Patterns

The expression patterns of β- and γ-crystallins in the developing vertebrate lens reveal something of their roles in building and maintaining the optical properties of the tissue. Both classes are expressed predominantly in the fiber cells. The γ-crystallins and certain β-crystallins such as βB1-crystallin are particularly strict in their preference for these terminally differentiated cells.[163-169] Although β-crystallins are expressed from early developmental stages in the lens, their expression continues and rises after birth so that the highest concentrations are usually found in the lens cortex. However, the pattern of expression varies among the individual β-crystallins. In contrast, the main group of mammalian γ-crystallins are expressed predominantly during embryogenesis, although some continue to be expressed for a time after birth, and there is species variability such that γD is the last to be active in human lens while in rat the last is γB.[152,165,166,170]

The result is that the highest concentrations of γA-F-crystallins are found in the central regions of the lens, the so-called lens nucleus which represents the embryonic lens. As the expression of the lens nuclear γ-crystallins declines γs-crystallin, the outlying member of the family, replaces them.[167] This led to its early identification as "cattle γ" a γ-crystallin peculiar to the mature, as opposed to the embryonic, bovine lens.[171] Thus γs-crystallin is found in the younger, more hydrated cortical fiber cells while the main group of mammalian γ-crystallins is associated specifically with the densest, highest refractive regions of the lens.

In the distribution of γ-crystallins, ontogeny seems to match phylogeny. Among species, the highest abundance of γ-crystallins is found in the hard lenses of fish as well as in nocturnal, burrowing rodents such as rats and mice. Only the cortical γs-crystallin is found in birds[162] and in human lenses only two out six γ-crystallin genes produce significant amounts of protein.[170,172,173] Embryonic γ-crystallins appear to have a specialized role in maintaining the stability of a low-water, high-protein concentration environment. In contrast, γs-crystallin and some of the β-crystallins help to maintain an environment of lower protein concentration. The result is a lens with a refractive index which declines from center to periphery. This increases the apparent convexity of the lens and can also eliminate the spherical and chromatic aberration which afflicts lenses of uniform substance.[174]

This specialization of γ-crystallins seems to be borne out by the lack of evidence for any functional role for them outside the lens. Although sensitive RNA protection methods have detected non-lens expression of γ-crystallin family genes in *Xenopus* larvae, there has so far been no detection of γ-crystallin protein outside the lens.[154] In contrast, there is recent evidence that some β-crystallin proteins are expressed

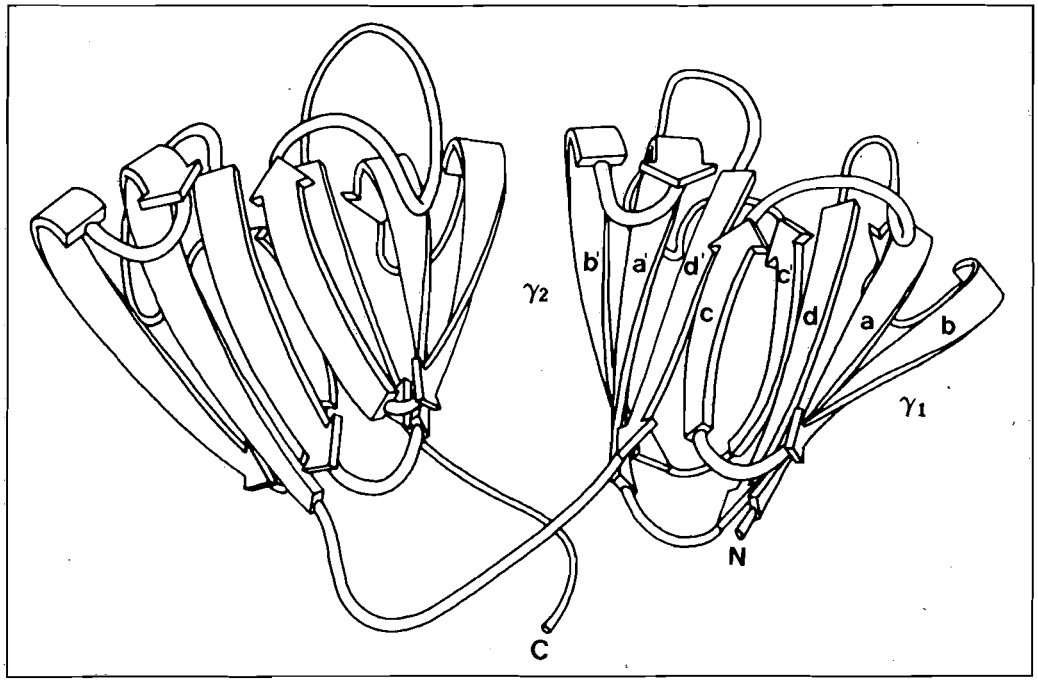

Fig. 3.4. The structure of bovine γB-crystallin[176-179] illustrated as a ribbon tracing of the polypeptide chain. (A modification of a drawing by Jane Richardson.)[206] The first two motifs of the N-terminal domain are shown as γ1 and γ2 with their β-strands lettered a-d and a'-d' respectively.

in non-lens tissues of newly hatched chickens, especially in retina, brain and kidney.[175] Their function in these tissues is not yet known.

PROTEIN STRUCTURE

Although little is known about the non-lens or ancestral function of the βγ-crystallin superfamily, a great deal is known about the three dimensional structure of the proteins and the gene organization of its members. This knowledge suggests a rich and complex evolutionary history. Since structure is so important in an understanding of this superfamily it will be described in some detail.

X-ray crystallography has determined the tertiary structures of several β- and γ-crystallins in detail. The first example solved, which has become the archetype of the class, was bovine γII-crystallin[176-179] (which now has the systematic name γB-crystallin) (Fig. 3.4). Very similar structures have also been determined for bovine γIIIb (γD)[180,181] and γIVa (equivalent to rat γE or mouse γF) crystallins.[182] Bovine γB-crystallin has a remarkably symmetrical structure of two domains, each containing a further two fold repeat of a characteristic structural motif. This γ-crystallin motif fits the structural category of a modified "greek-key." This same pattern is seen in βB2-crystallins for which an x-ray structure analysis is now also available.[183,184] Each motif bears a distinctive sequence signature which is required for correct folding of the polypeptide chain.

The structural motif of the βγ-crystallin superfamily consists of four antiparallel β-strands, *a-d*[177] (Fig. 3.4). The βγ motif fold is achieved as follows. Using the numbering scheme for motif 1 of bovine γB-crystallin as reference (Fig. 3.5), the first pair of strands, *a-b*, form a β-hairpin with an unusual turn between the strands which folds back onto the hairpin, burying some side chains. This is the principal distinguishing feature of this superfamily and requires certain key amino-acid residues. At position 6, on strand *a*, is an aromatic, usually tyrosine, which is buried by the folded back loop. At position 11 on the bend itself is another aromatic whose side chain also contributes to the interaction with the hairpin. At position 13 is an absolutely conserved glycine residue. As the polypeptide backbone negotiates the bend it adopts several positions of unusual Φ/ψ angles, this puts residue 13 in such a position that there is no room to accommodate a side chain, hence the requirement for glycine. After the *a-b* hairpin, strand *c* bends away from the plane occupied by the other three strands, crossing over to the other side of the protein domain. This strand is usually short and leads into the most variable part of the motif, a long *c-d* loop which crosses back over the "top" of the motif to the β-sheet in which the *a,b* and *d* strands of the motif lie.

As it bends back into the *d* strand which is hydrogen bonded to strand *a* in a β-sheet, the polypeptide backbone again encounters the unusual structure formed between strands *a* and *b* which actually bur-

ies the first residue of strand *d*, at position 34. There is only limited space for the side chain of this residue. Furthermore the side chain is in a position to hydrogen bond to the peptide backbone amide of residue 11 which would otherwise be unbonded and shielded from water, an energetically unfavorable situation. Only a serine residue can fit both requirements for size and hydrogen bonding and indeed serine is almost always found at this position. However, glycine and alanine could also fit into the pocket although they would be unable to form hydrogen bonds. It turns out that evolution has already performed this structural experiment in the fourth motif of mammalian βB1-crystallins.[133,185] These polypeptides, unlike their homologue in chicken[134] have alanine rather than serine at position 34 (Fig. 3.5). The structural consequences of this substitution are not clear. It seems probable that it would lead to looser packing of the folded hairpin. Since this change has been conserved among mammals it may modify the surface properties and intermolecular interactions of βB1-crystallin to fit the supramolecular organization of mammalian lenses.

The complete folding pathway results in a supersecondary structural motif with a three stranded β-sheet, *b-a-d*, and a lone β-strand *c*. This structure leaves exposed most of the hydrophobic side chains and it seems unlikely that it could exist in isolation. A complete globular domain is formed by assembly of two motifs around a pseudo-twofold axis such that the lone strand *c* of one motif interacts with strand *d* of the other. This forms a single globular domain, a wedged shaped structure of two β-sheets with strand patterns *b-a-d-c'* with a compact hydrophobic core between the two β-sheets (Fig. 3.4). Each β- and γ-crystallin monomer contains two of these domains.

Monomers and Dimers

The two domains of γB-crystallin are highly stable structures which are able to fold independently.[186] In the monomeric γ-crystallins, the two of these two-motif domains in the same molecule associate about another pseudo-twofold axis, joined by a bent connecting peptide so that symmetry related motifs 2 and 4 interact[176,177] (Fig. 3.6). In spite of their independent folding pathways, interaction between the two domains seems to be necessary for maximal stability.[187] Most of the residues of a γ-crystallin polypeptide are contained in the globular domains. Apart from the residues of the connecting peptide, the only other residues outside the globular domains are in the short, exposed extension at the C-terminus which is available for intermolecular interactions, perhaps acting as a "spacer" as suggested for β-crystallin extensions.[188] At the N-terminus in contrast there is no extension. The sequence begins with the first residue of strand *a*, a conserved glycine, and its amino terminus does not protrude from the surface of the N-terminal domain. This presumably explains the lack of N-terminal acetylation in γ-crystallins since their N-termini provide no target for aminopeptidase activity.

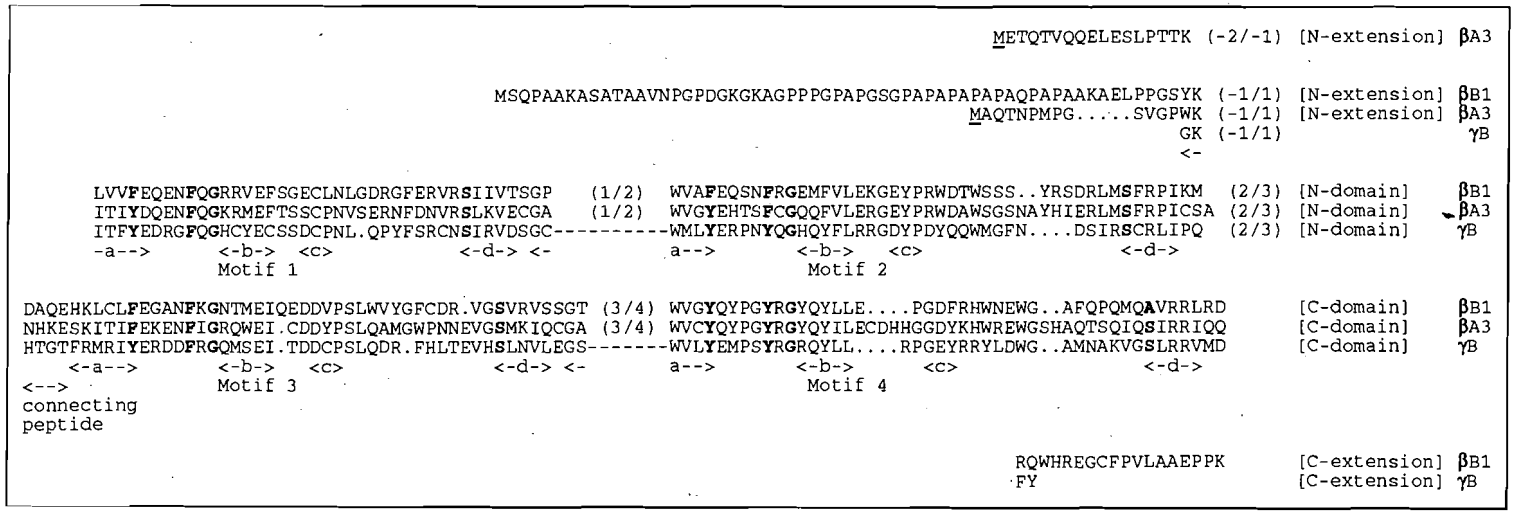

Fig. 3.5. Sequence alignment of β- and γ-crystallins. Three bovine protein sequences taken from the databases, βB1-crystallin, βA3/A1-crystallin and γB-crystallin. Underlines show the two alternative translation starts for βA3- and βA1-crystallins. Sequences are divided to illustrate both exonic and protein structures. Intron positions and phases are indicated, taken from several gene sequences in GenBank (see also ref. 129). Introns labeled according to the scheme in Figure 3.9. Key residues in the motif signature are shown in bold type. The extent of β-strands is shown with arrows.

β-crystallins are different. They can form homo- and heterodimers, and they do this by a unique mechanism revealed by x-ray analysis of bovine βB2-crystallin.[183,184] The basis of the intermolecular interaction is essentially the same as the interdomain interaction in a γ-crystallin monomer except that this time the two domains come from different molecules. Each subunit has a very similar domain structure to γ-crystallins. However, instead of a bent connecting peptide and contact between two domains in the same molecule as in γ-crystallin, the connecting peptide of βB2-crystallin adopts an extended conformation (Fig. 3.6) which allows both domains to interact with another subunit through motif 2/motif 4' contacts across a two fold axis (Fig. 3.7).

β-Crystallins also differ from γ-crystallins in having long, relatively unstructured extensions. The βB family have extensions at both the N- and C-termini (Fig. 3.5). In contrast βA subunits have only an N-terminal extension while their C-termini are actually shorter than those in γ-crystallins. It has been hypothesized that either the terminal extensions or the connecting peptides provide the basis for the different conformations of β- and γ-crystallins.[183,189] This question has been

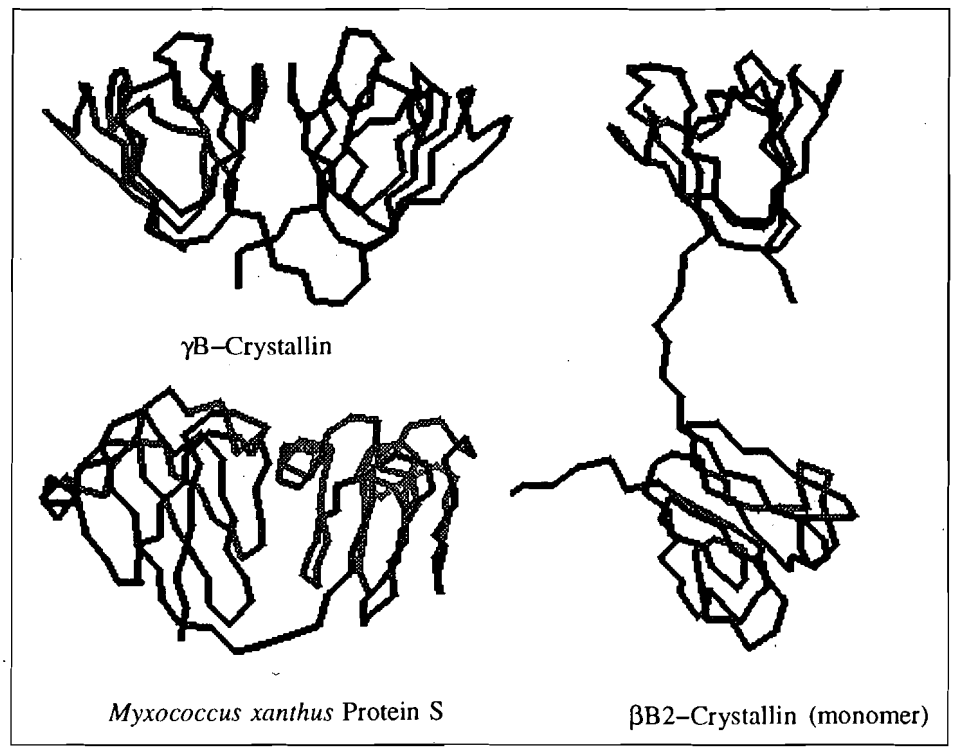

Fig. 3.6. Chain traces of three polypeptides of the βγ-crystallin superfamily. Coordinates were taken from the Protein Data Bank and displayed using the program Quanta on a Silicon Graphics workstation. (See text for references.)

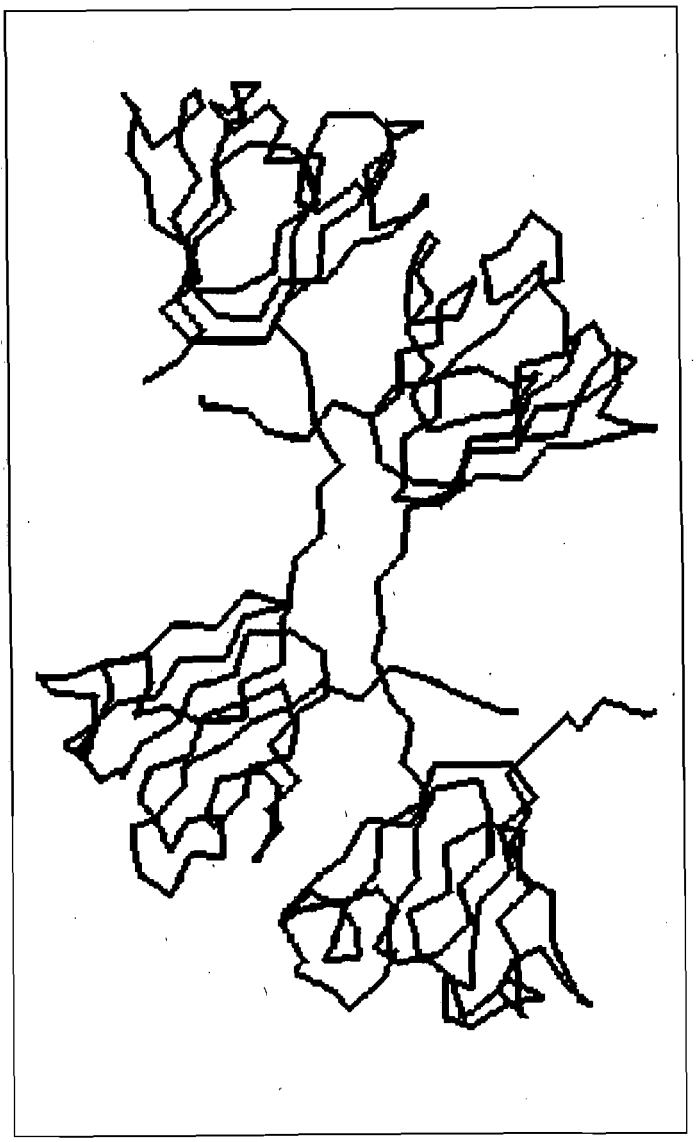

Fig. 3.7. Chain tracing of a bovine βB2-crystallin dimer.

addressed by numerous studies involving proteolytic modification of β-crystallins,[190] x-ray analysis,[183,184,188] ^{1}H NMR spectroscopy[144,191] and synthesis of chimeric recombinant crystallins.[141,187,192-194]

Early model-building[189] and proteolysis studies[190] had suggested that the N-terminal arm of βB2-crystallin was important for dimerization. However more recent studies have contradicted this idea. NMR and x-ray structure analyses[144,183,191] find that the extensions of βB2-crystallin are highly flexible and unstructured. Recombinant protein experiments

also show that dimers of βB2 or βA3/A1 can form in the absence of either or both extensions.[192,194] However x-ray structure analysis does suggest that at least part of the N-terminal extension may be involved in formation of βB2 tetramers.[188] There is less consensus about the role of the connecting peptide. Transposition of the βB2 connecting peptide into γB yields a monomeric protein[187] while similar transposition of the γB connecting peptide into mouse βA3 does not affect the ability of the recombinant protein to dimerize.[193] These results suggest that monomer or dimer formation is independent of the connecting peptide. However when the γB connecting peptide was transposed into βB2, the recombinant protein was found to be monomeric.[196] These apparently contradictory results await resolution.

Form for Function: The Role in Lens

Many of the structural features of β- and γ-crystallins can be interpreted in terms of the functional requirements of their role as crystallins.

γ-Crystallins need to be highly stable proteins capable of existing in the highest protein concentration regions of the lens throughout life. Aiding them in this role, they have compact globular domains with few protruding loops exposed for proteolysis. Bovine γB-crystallin is extremely symmetrical, yet the domains achieve an extra degree of close packing between the two four-stranded β-sheets of each domain through adoption of a slight asymmetry.[177] This allows rows of hydrophobic residues from opposing β-sheets to interdigitate, something which a perfectly symmetrical structure could not do.

Another somewhat unusual feature of γ-crystallins is their aminoacid composition. The core residues of γ-crystallins are rich in aromatic and sulfur-containing residues. The high content of cysteine residues seems paradoxical for a lens protein since oxidation is regarded as a major threat and potential cause of cataract.[1] This suggests that the sulfur atoms of γ-crystallins are present for important structural and functional reasons. One possibility which has been suggested[177] is that the d-orbitals of these atoms together with π-orbitals of aromatic residues are involved in formation of molecular bonds, shared networks of electrons binding the hydrophobic core. Some of the cysteine residues are actually exposed on the surface where they might be involved in similar interactions with other molecules in the densely packed regions of the lens. Interestingly, in fish γ-crystallins, which achieve even higher concentrations than those of mammals, the sulfur content, particularly of methionines, is even higher.[156,157] This may also reflect a use of polarizable electrons for non-hydrophobic contacts in low water concentrations.

X-ray analysis of bovine γB-crystallin under somewhat oxidizing conditions also revealed another possible useful function of some cysteine residues. Under these conditions, the protein forms a disulfide

bond between a cysteine in strand *c* and on in the *c-d* loop.[177] This tightens up a bend, but otherwise does not disrupt the structure. Thus this protein has the ability to absorb oxidizing potential harmlessly without forming any intermolecular bonds. This could provide an additional protection for the lens from oxidizing insult.

A final feature of amino acid composition is the very low lysine content of γ-crystallins (2 residues in bovine γB) compared to a considerably higher level of the other basic residue, arginine (20 in γB). Lysine is usually one of the most common residues in animal proteins. Its diminished content in γ-crystallins may help to reduce the potential for various kinds of post-translational modification which have been postulated to lead to cataract, such as carbamylation.[1]

The surface properties of γ-crystallins are key to its interactions with water and with other proteins. Bovine γB-crystallin has about half of its surface polar and charged side chains involved in intramolecular ion pairs or hydrogen bonds,[176,177] accounting for one third of the total molecular surface.[195] This is an unusually high fraction. It reduces the potential for binding shells of surrounding water molecules and may contribute to the usefulness of γ-crystallins in a relatively dehydrated environment. Because of their surface features, γ-crystallins may be less susceptible to certain kinds of phase separation at the high concentrations found in lens. Since they do not bind as much water as other proteins, their presence allows the maintenance of a low-water concentration and hence a high refractive index.

The surface properties of γ-crystallins may explain some unusual aspects of their biophysical behavior. Although they maintain their solubility at high concentrations, three of the mammalian γ-crystallins, γD, γE and γF whose genes form a consecutive group in the gene cluster, have a temperature sensitive phase separation.[152,173,196] This so-called "cold cataract" occurs when a solution of crystallins is cooled and is reversible. It suggests that the affinity of these proteins for a more hydrated environment is not strong and that energetically they are close to favoring a phase with more protein-protein interactions. In fact there is apparently an overall attraction among γ-crystallins while in contrast α- and β-crystallins have repulsive interactions.[197] Indeed, recent NMR studies suggest that at the concentrations of the lens nucleus, γ-crystallins may not be truly monomeric but may enter a state of "macromolecular crowding."[198] This is presumably essential for achieving the high protein concentrations and hence high refractive index of the lens nucleus without precipitation.

Chemical modification studies suggest that exposed cysteine residues may be involved in the phase separation which occurs in cold cataract and hence in attractive interactions among γ-crystallins.[199] Interestingly, in humans two of the four "cold cataract" crystallins, γE and γF, are pseudogenes.[170,200] Their inactivation probably contributes to the softening of the human lens relative to those of rodents in which all six γ-crystallins are expressed.

γ-Crystallins also have another odd behavior. On simple column separations they tend to elute as if they were smaller than half their actual molecular size.[201] The smaller than expected apparent size of the molecule in solution might be due to the presence of a more loosely held shell of water molecules than would be typical for other soluble proteins. Another possibility is that the two domains move rather independently in solution. This is something which could be answered by NMR solution structure analysis of the kind which have been performed for Protein S of *Myxococcus xanthus*.[202,203]

β-Crystallins have many similarities with γ-crystallins in domain structure.[183] However they have fewer surface ion-pairs and hydrogen bonds consistent with a role less specialized for a low-water environment. They are able to form various sizes of aggregates by forming dimers and higher multimers with subunits arranged in antiparallel "bunches."[188] This permits flexibility in supramolecular structure according to the requirements of different regions of the lens.

What is the function of the β-crystallin extensions? They may be involved in higher quaternary structures through direct protein interactions.[188] However it has also been suggested that these exposed polypeptides may act as "spacers" in the supramolecular structure of the lens.[188] Another possibility is that they have a principally entropic role in maintaining the solubility of large β-crystallin aggregates. The free extensions seem to be highly mobile in solution. This freedom would be lost in a precipitated phase. Thus the extensions, spinning like propellers on the outside of β-crystallin multimers, may contribute to an entropic component of the free energy of solution, opposing superaggregation, phase changes and opacity. Indeed, proteolytic cleavage of the N-termini of β-crystallins reduces their solubility.[204] In the rat lens this cleavage occurs as part of a normal maturation process which seems to contribute to the hardening of the lens nucleus and to its high refractive index.[204]

A WIDER SUPERFAMILY

The characteristic structural signature in the protein sequence of βγ-crystallin superfamily members (Fig. 3.5) has allowed the detection of non-lens members whose sequences are in the databases. Two of these proteins are expressed during spore or cyst formation in response to stress in certain micro-organisms.

The sequence of Protein S of the bacterium *Myxococcus xanthus* suggested the presence of a four motif, two domain structure similar to those of β- and γ-crystallins.[205] This was recently confirmed by a solution NMR analysis which revealed a structure remarkably similar to that of γ-crystallin[202,203] (Fig. 3.6). There were two notable differences. First, the long *c-d* loops of motifs 1 and 3 in Protein S form regular α-helices, reminiscent of but much more ordered than the irregular helices of motifs 2 and 4 in γ-crystallins. Second, the interdomain contact in the bacterial protein resembles neither that of a γ- or

β-crystallin. It is unsymmetrical, involving contact between motifs 2 and 3 while that in γ-crystallin is highly symmetrical and involves motifs 2 and 4. This difference may be due to the calcium binding properties of Protein S.

Spherulin 3a is expressed in the eukaryotic slime mold, *Physarum polycephalum*.[206] It too bears the signature required for folding of the βγ-crystallin superfamily motif. However, uniquely for known members of this superfamily, instead of four motifs and two domains spherulin 3a has only two motifs and one domain. It thus illustrates an earlier stage in molecular evolution than related proteins, predating the second internal duplication of sequence. Intriguingly, although it is only a one-domain structure, spherulin 3a has a fairly long N-terminal extension suggestive of those found in β-crystallins. By analogy, this might be expected to participate in higher order quaternary interactions.

The functions of these two proteins are not known. However, unlike more closely related species, prokaryotic *M.xanthus* and eukaryotic *P.polycephalum* share an unusual stress response. Both of them sporulate in response to osmotic stress induced by polyols, sugar alcohols. Since both organisms also share expression of members of the βγ-crystallin superfamily when undergoing spore or cyst formation it is possible that these two phenomena are related and that the ancestral role of the βγ-crystallin superfamily may have been to act as osmotic stress proteins.[62,206]

Until recently, no non-crystallin members of this superfamily were known in vertebrates. However, a candidate for this class has now been proposed.[207] An epidermis differentiation-specific protein (EDSP) in embryos of *Cynops pyrrhogaster*, an amphibian, has been cloned.[208] The N-terminal half of EDSP contains four, somewhat divergent, βγ-like motifs which suggest a two-domain, four motif structure like that of a γ-crystallin.[207] At the protein sequence level, EDSP is more similar to β- and γ-crystallins (28-30% identical) than to Protein S (25%).

Evolution of the Superfamily

The remarkable internal symmetry of each member of the βγ-crystallin superfamily suggests that they are derived from an extremely distant ancestor corresponding to a single structural motif. Such an isolated structure would probably be unable to satisfy hydrogen bonding requirements or to exclude water from its hydrophobic residues. It could achieve a stable conformation only by forming a homodimer. However the x-ray analysis of γB-crystallin shows that a heterodimeric association of two similar but different motifs, as in a γ-crystallin domain, produces a closer packing of core residues than would be possible in a perfectly symmetrical homodimer.[177] The added stability of such a heterodimeric structure could have been attained by duplication of the ancestral "motif gene" followed by sequence divergence to yield two isolated motifs, A and B (Fig. 3.8). These would have been required to fold together. The efficiency of the folding of each motif

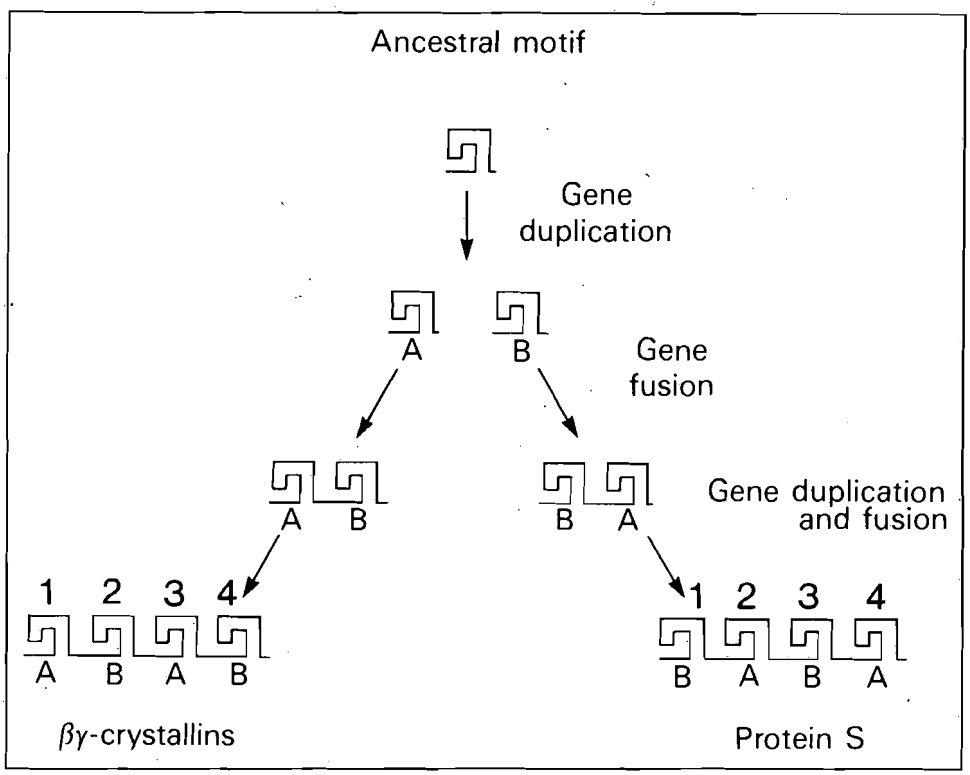

Fig. 3.8. Internal duplications in the evolution of the βγ-crystallin superfamily.

and their dimerization into a stable globular structure would have clearly been enhanced by fusion of the two proto-genes. The resultant fused gene would have coded for a one-domain protein with motif pattern AB, similar to one domain of a γ-crystallin or to spherulin 3a. At this stage there would have been no obvious mechanism to determine the order of fusion of the two motifs, so that AB and BA would have been equally likely.

Further duplication would have created the ABAB pattern seen in β- and γ-crystallins in which motifs 1 and 3 are both A-type while motifs 2 and 4 are B-type. In the crystallins, the B-type motif generally has a slightly longer *c-d* connecting loop which tends to adopt an irregular α-helical conformation. *Cynops* EDSP seems to lie on the same evolutionary pathway as the crystallins and also has the ABAB motif pattern.[207] In *M.xanthus* Protein S, one pair of motifs also exhibits slightly longer *c-d* loops which in this protein adopt the form of regular α-helices. However, these are motifs 1 and 3, not 2 and 4. This gives Protein S a BABA pattern of motifs,[202,205] suggesting an independent history of duplication and fusion starting from a very early stage.

INTRONS AND INTERNAL DUPLICATIONS IN β- AND γ-CRYSTALLIN GENE EVOLUTION

Further support for the idea that there were multiple independent gene duplication and fusion events in the evolution of this superfamily comes from the gene structure of the β- and γ-crystallins themselves.[129]

In vertebrates, the genes for the related families of β- and γ-crystallins exhibit striking examples of mapping between repeated protein structural motifs and exons. However this mapping is coupled with a clear history of internal duplication and it is by no means easy to discern the path taken in the assembly of each gene family. As we have seen, the members of the protein superfamily have a repeated structure of conserved motifs. Two motifs assemble into one domain and in most cases two domains are linked by a connecting peptide. N- and C-terminal peptide extensions may also be present. In β-crystallins, each of the four conserved structural motifs is encoded by a separate exon while one or two additional exons encode the N-terminal extension (Fig. 3.9). In γ-crystallin genes, in contrast, instead of each motif being encoded in separate exons the motifs are encoded in pairs so that one exon corresponds to a complete domain of two motifs.

For comparative purposes the exons and introns of β- and γ-crystallin genes can be labeled according to the motif structure of the proteins encoded so that homologous exons have similar names (Fig. 3.9). In this scheme the numbering of exons reflects the encoded motifs. Thus the third exon of a β-crystallin codes for motif 1 and can be designated exon 1. In γ-crystallins the second exon codes for motifs 1 and

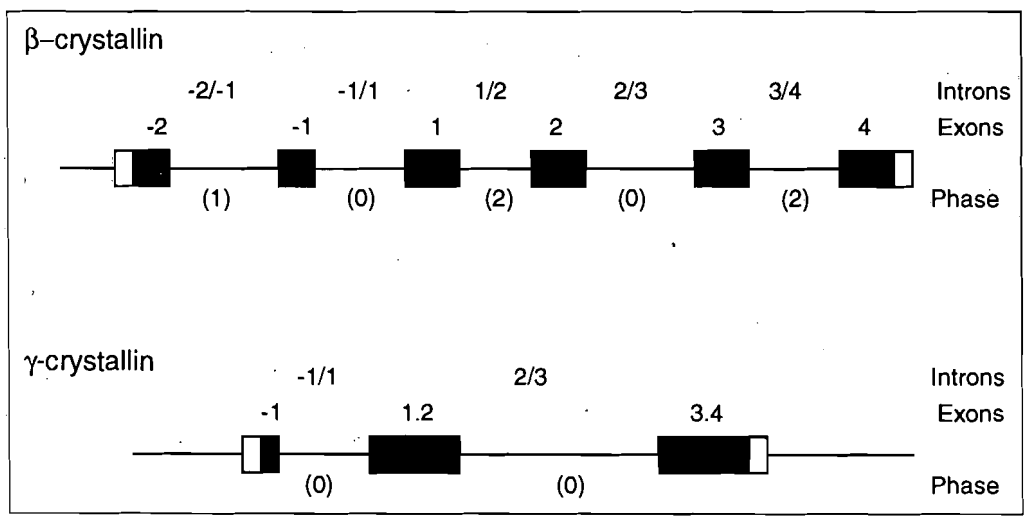

Fig. 3.9. Schematic gene structures for typical β- and γ-crystallins. Exons are shown as boxes. Coding sequences are shaded. In some β-crystallins the first exon is non-coding. Introns and exons are numbered to show similar mapping relative to protein motifs. Intron phases are shown in parentheses.

2 and can be designated exon 1.2. Introns can then be designated according to the flanking exons so that the interdomain intron of both β- and γ-crystallins is intron 2/3. Upstream exons have negative numbers. The first three codons of γ-crystallin motif 1 are contained in exon -1 which has some similarity to both exons -2 and -1, which encode the N-terminal extensions of β-crystallin genes.[129]

In β-crystallins the introns which delineate motifs form two pairs (Figs. 3.5, 3.9). Introns -1/1 and 2/3 are in phase 0. Intron -1/1 divides the N-terminal extension sequences from those of motif 1 while 2/3 divides motif 2 sequences from the connecting peptide. In spite of these differences there are intriguing similarities in the positions of these introns relative to the repeated motif structure (Fig. 3.5). The introns forming the other pair, 1/2 and 3/4, fall between motifs in each domain. Their positions relative to the motif structure are close to that of the other pair but not identical (Fig. 3.5) and they have phase 2. Introns -1/1 and 2/3 in γ-crystallins correspond precisely to those in β-crystallins. The γ-crystallin genes lack introns 1/2 and 3/4.

These two families of genes clearly share common ancestry. The "introns early" model would predict that the original common ancestor gene would have arisen by the intron-mediated assembly of two "motif genes." Duplication of this gene gave rise to the β-crystallins such that introns 1/2 and 3/4 of β-crystallins represent the original intron. γ-Crystallins would have arisen by independent duplication of a copy of the two-motif gene in which the ancestral intron was lost.[129] The general similarity of introns -1/1 and 2/3 could reflect shared ancestry such that one represents a duplication of the other resulting from an unequal crossover between two copies of the same gene. However this occurred, it would have been necessary to generate the extra sequence of the connecting peptide at the N-terminal end of motif 3.

All two-domain members of the superfamily, including β- and γ-crystallins, Protein S of *M.xanthus* and EDSP of *Cynops* have connecting peptides of 4 or 5 residues between domains. From a structural view it seems unlikely that a protein of this family could have two domains and no connecting peptide. Thus when the last round of duplication occurred the connecting peptide must have been present immediately. It could have arisen from an N- or C-terminal peptide of a one-domain precursor, but again it is not simple to reconcile the precise conservation of intron 2/3 in both β- and γ-crystallin families with a model of independent duplication. In fact the simplest explanation for the identical positioning of this intron is that it was already present in a common ancestor of both families. Otherwise this intron could be the result of directed insertion at a special position perhaps delineated by conserved RNA structure.[123]

Spherulin 3a of the eukaryote *P.polycephalum* is a one-domain member of the βγ-crystallin superfamily resembling an ancestral stage before the last internal duplication which gave rise to the crystallins.

The gene for spherulin 3a lacks introns. If introns are ancestral in this family, they were lost in spherulin 3a and in Protein S, partially lost in γ-crystallins and retained in β-crystallins. Alternatively, introns were not present ancestrally but were progressively added to vertebrate crystallin genes. As more members of this superfamily are uncovered, it will be interesting to see what patterns of introns their genes possess. Possibly the ancestral genes of β- and γ-crystallins were intronless even at the four motif stage. Introns may have been gained by directed insertion and then became duplicated within a gene through gene conversion mechanisms. The two families may have diverged prior to the last round of intron insertion and propagation in the ancestral β-crystallin gene.

The additional 5' exon -2 and intron -2/-1 of β-crystallins could have arisen by a variety of mechanisms. Some similarity has been noted between first and second exons of β-crystallins and between these sequences and the first exons of γ-crystallin genes.[129] This has led to the suggestion that there was a duplication of the first exon in β-crystallins.[129] In βA3/A1 both duplicated initiator methionines were retained while in βB1-crystallin the first initiator was lost causing the first exon to become non-coding.[129] However intron -2/-1 is in phase 1 so it seems unlikely that it represents a duplication of intron -1/1.

Gene Multiplication in the Evolution of β- and γ-Crystallins

Distinct families of β- and γ-crystallin are found throughout the vertebrates, from fish and (probably) lampreys to amphibians, reptiles, birds and mammals. This suggests that the molecular lineages of the two families were well established at a very early stage in lens evolution. Possibly both families pre-date the vertebrate lens and were recruited together. However a more parsimonious hypothesis is that an ancestral β-crystallin was recruited as one of the original crystallins. Subsequent gene duplications and divergence give rise to multiple β-crystallins and to the more lens-specialized γ-crystallins.

Homologous βA- and βB-crystallin genes are found in both birds and mammals. In the human genome the genes for βB2, βB3, βA4 and a pseudogene for βB2-crystallin genes are closely linked on chromosome 22.[209-212] βB2, βB3 and βBA4 are linked on mouse chromosome 5[213] while βB2 and βB3 are known to be linked in rat.[209] In chicken βB1 and βA4 are arranged head to head only 2 kb apart.[135] However human βA3/A1-crystallin is located on chromosome 17.[214,215] This suggests that there might at one time have been a single β-crystallin cluster which is now beginning to disperse.

βA3/A1- and βA4-crystallins have been observed in amphibians[39,139] and hybridization studies suggest the existence of βA- and βB-crystallin related sequences in all classes of vertebrates.[216] Thus the βA- and βB-crystallin families also seem to have had a very early origin. In the course of surveying fish for possible taxon-specific crystallins, we have

obtained several tryptic peptides of β-crystallin subunits from the lens of a teleost fish, the surf perch (unpublished). All the peptides clearly belong to the βB family. However it is less easy to assign these peptides to specific family members since individual peptides share some sequence hallmarks of different mammalian/avian βB-crystallins. This preliminary analysis confirms the presence of βB-crystallins in fish but raises the possibility that this family underwent independent radiation in fish and in terrestrial vertebrates.

ANCESTRAL β-CRYSTALLINS?

It is possible that one of the existing β-crystallins is directly homologous to the ancestral β-crystallin which gave rise to the whole β-crystallin family and perhaps even to the γ-crystallins. Several features of βB2-crystallin suggest that it has a special structural and evolutionary significance. βB2-crystallin is the most highly conserved of the βB-crystallin subunits.[39] In those vertebrate lenses which have been examined it is the major β-crystallin subunit[1,2] and it plays a role in organizing other β-crystallin subunits.[140] Unlike other β-crystallins, βB2 shows a strong propensity for formation of highly stable dimers[140,183] and can therefore exist in a stable form in the absence of other β-crystallin subunits. Indeed, βB2-crystallin has the highest thermal stability of any β-crystallin.[217,218] Furthermore, βB2 appears to be the β-crystallin subunit with the highest non-lens expression and is the first crystallin to be detected at elevated level in transdifferentiation of chicken neural retina.[175] In most of these "special" categories βB2 is closely followed by βA3 which also happens to be even more highly conserved in amino acid sequence.[39,219] Could one of these proteins be the modern homologue of the pre-lens ancestor of this family?

γ-CRYSTALLINS

γ-Crystallins present a more complicated picture. The single γ-crystallin of the adult mammalian lens, γs-crystallin is well conserved among mammals and fish.[131,158] In contrast the six embryonic γ-crystallins, γA-F, are highly conserved in mammals,[129] absent from birds[162,220] and apparently non-homologous to γ-crystallins in amphibians and fish.[153,156] The six embryonic genes of mammals form a tight cluster on a single chromosome, 2 in man,[22-223] 1 in mouse[224] and 9 in rat.[151,225] Their products are highly similar to each other, ranging from about 75% to 98% identical. Analysis of the rat γ-crystallin gene cluster has yielded evidence of gene conversion.[226] Nevertheless, clearly homologous genes are present in different mammals. The distantly related but evolutionarily conserved γs-crystallin is not linked to the γ-crystallin cluster and in the human genome is located on chromosome 3.[132]

It seems likely that all modern placental mammals have six homologous embryonic γ-crystallin genes although since only in rat, mouse and human have all six genes been characterized,[129,227] this view is subject

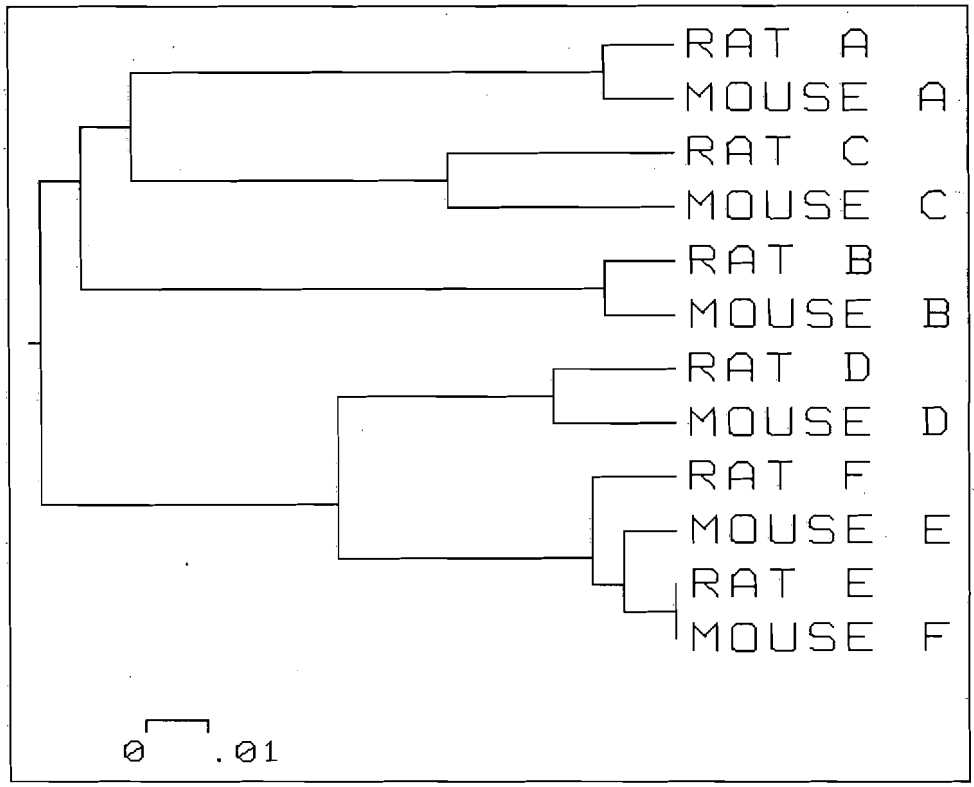

Fig. 3.10. Cladogram comparing protein sequences for mouse and rat γ-crystallins. Sequences taken from Swissprot database. Tree was constructed using the UPGMA p-distance option in the program MEGA.[232] Scale represents 1% difference. (Figure kindly provided by Dr. Cynthia Jaworski). Note that the high-phase separation temperature cryoproteins γD,E and F (152) are on the same branch and that rat γE and mouse γF are identical.

to revision. In rat and mouse four γ-crystallins, γA-D, are clearly homologous as judged by both gene and protein sequence[151,227] (Fig. 3.10). This suggests that these four genes were established prior to the separation of murine rodents 10-20 million years ago. γE- and γF-crystallins are a different story. These two proteins are the most similar pair of γ-crystallins, 98% identical in rat and 96% identical in mouse. However, designating homologues for this pair of crystallins between rat and mouse is not straightforward. When application of a rationalized nomenclature for γ-crystallins was attempted it seemed clear that the protein coded by the cDNA originally named mouse γ2-crystallin was identical to that coded by rat γE-crystallin.[128] However, when gene sequences and relative positions in the γ-crystallin gene cluster were compared it appeared that the mouse γ2-crystallin gene was actually more equivalent to the gene for rat γF-crystallin.[129] Consequently, since it was felt that genomic organization should have precedence over protein

sequence, the genes now known as rat γE and mouse γF-crystallin produce an identical protein (Fig. 3.10).

There are two possible reasons for this paradox. The first is that gene conversion has shuffled the identities of the γ-crystallins. The second explanation is that a prototype γE/F crystallin was present in an ancestor of rats and mice. Just before these species diverged the gene duplicated and untranslated sequences diverged rapidly establishing γE and γF genes with essentially identical protein products. After divergence of the species, the protein products of the two daughter genes diverged in sequence more slowly in response to particular selective pressures. These pressures led to certain coordinated sequence changes in one protein, equivalent to the sequence of rat γE-crystallin. However, in mouse these changes were produced in the γF-crystallin gene, giving the remarkable result of an identical protein produced by a different gene in a closely related species.

The human genome also contains six complete genes for γ-crystallins.[170] In protein sequence, the products of γA-D genes again seem to be homologues of the equivalent rodent genes, suggesting that these proteins and their genes predate the radiation of modern placental mammals. Humans also have two pseudogenes, superficially equivalent to the γE and γF genes of rodents.[170,200] Thus there may have been six γ-crystallin genes in the common ancestor of primates and of rodents. The human genome also contains a fragmentary γ-crystallin gene sequence and it has been suggested that this represents a lost copy of one of the γE/F genes, possibly the true homologue of rat γF, which has been deleted in primates.[170] The remaining gene may have then duplicated again to restore the complement of genes to six. However the history of these genes is by no means unambiguous. Insertion of repetitive elements, gene conversion, sequence drift in pseudogenes and perhaps varying pressure on expressed genes in species whose lenses are as different as humans and rats have all had their effect.[129]

γ-CRYSTALLINS AND THE EVOLUTION OF THE LENS

The γ-crystallins of mammals are much more similar to each other than are the multiple γ-crystallins of a frog, which generally show the same degree of conservation and therefore the same apparent age as β-crystallins.[129] Thus the mammalian γ-crystallins appear to be a much younger family than either β-crystallins or the γ-crystallins of an amphibian. Part of this similarity may be due to gene conversion resulting from the tight clustering of γ-crystallin genes in mammals. However, both the clustering and the conservation of sequence could also be the result of a relatively recent re-invention of γ-crystallins, perhaps a series of duplications of a single gene, the most recent of which gave rise to the γE/γF pair.

In this hypothesis, γ-crystallin gene expression in the distant reptilian ancestors of mammals would have declined just as it did in the

ancestors of birds and for the same reasons. Later in evolution however, the ancestors of modern placental mammals seem to have abandoned the diurnal habit which evidently favors soft lenses and instead adopted a nocturnal, burrowing habit similar to that of mice and rats today. This idea is supported by certain features of the mammalian visual system. In particular, most mammals are essentially color blind, lacking cone cell photoreceptors required for color discrimination and instead relying on the rod cells which are specialized for dim-light vision.[228] Indeed, all placental mammals lack the colored oil-drop bodies which are associated with cone cells in the retinas of fish, reptiles and birds and even in non-placental mammals such as marsupials.[228,229]

Due to the low photosensitivity of cones, color vision requires bright light. It therefore has no value to animals which are active in dim light and in such species there is no selective pressure for its retention. In the same way, animals living as mice do now have no need of a soft, accommodating lens. As rats and mice demonstrate, a harder, spherical lens is more useful, perhaps because its short focal length permits detailed examination of objects at close quarters.

Nocturnal, burrowing ancestors of placental mammals may have lost both their color vision and their soft, diurnal lenses. They may have essentially re-invented the hard lenses of their distant aquatic ancestors by increasing the expression of γ-crystallins. If γ-crystallins were already on the path to elimination, their revival could have easily been achieved by multiplication of a surviving gene to regenerate a family of γ-crystallins forming a tightly linked cluster of recently duplicated genes. Later, when descendent species became diurnal or partially diurnal once more, this dynamic evolutionary process would have reversed yet again, recapitulating the softening of the lens. Thus, while all six γ-crystallin genes are expressed at high levels in rat, only two out of six γ-crystallin genes, γC and γD, are expressed at significant levels in human lens. Two other human γ-crystallin genes, γE and γF, are pseudogenes while γA and γB are expressed at very low levels.[170,172,173] As a result the soft human lens has no more than one third the γ-crystallin content of the hard rodent lens.[152,230]

Modulation of γ-crystallin content seems to be one of the key mechanisms for modifying the properties of the vertebrate lens. This strategy for molecular engineering of the optical properties of the lens has also been hypothesized to be the underlying reason for the recruitment of taxon-specific enzyme crystallins.[62] The introduction of these new proteins into the lens may serve to dilute or replace the γ-crystallins and hence contributes to a lower protein concentration in the lens of terrestrial vertebrates.

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