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From microbial to lens $\beta\gamma$-crystallins: Presence of a universal calcium-binding motif in $\beta\gamma$-crystallin superfamily

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Crystallins which form the major protein components of the eye lens are responsible for its transparency. $\beta\gamma$-crystallin superfamily has a well-characterized protein fold which is present in several members in prokaryotes and eukaryotes. Apart from two members from eukaryote, Spherulin 3a and Ciona crystallin, a majority of them possess the two or more $\beta\gamma$-crystallin domains. The $\beta\gamma$ domain consists of two Greek key motifs arranged as four antiparallel β-strands. Since a very long time, calcium connection with lens crystallins has been a debatable issue. Here in this article, we summarize the recent advances about how this domain evolved from bacteria to chordates to perform specialized functions. Emphasis is put on summarizing the calcium-binding properties of the members from prokaryotes and eukaryotes, and the definition of a universal motif of calcium-binding in the proteins of this superfamily.

1. Introduction

Crystallins are abundant proteins, present in the vertebrate eye lens, which provides transparency and high refractive index to an eye lens. Mörner [1] classified these proteins as α- β-, and γ-crystallins by decreasing molecular weight elution of native proteins from a gel exclusion column. Precipitation, aggregation or crosslinking of crystallins cause lens opacification that leads to light scattering and visual impairment. The structural features of crystallins, their arrangement and interaction is critical to the understanding of the molecular basis for cataract formation. Cataractous lenses were found to have excess of free Ca^{2+} (concentration as high as 1 mM) [2]. The Ca^{2+} ion concentration and its connection to eye lens proteins and transparency is a long drawn and unproven hypothesis. It would be of utmost importance to prevent or slow down the process of cataract formation, at least the age-related loss of lens transparency.

The α-crystallin forms protein complexes of high molecular weight with around 800 kDa. α-Crystallin is known to perform numerous functions. It acts as a small heat shock proteins and has the ability to protect other lens crystallins from stress, mainly oxidative stress [3]. It prevents irreversible aggregation of proteins, thus acting as molecular chaperone [4]. It interacts with the cellular cytoskeleton, to perform various functions such as degradation of denatured proteins and copper binding [5]. α-Crystallin has been shown to possess the protective ability against apoptosis [6].

β- and γ-crystallins are ubiquitous in vertebrate lenses and these were thought largely as structural proteins and were related to the stress response proteins [7]. Both β- and γ-crystallins show the structural similarity and together they are placed in as $\beta\gamma$-crystallins [8]. β- and γ-crystallins share a common structural motif named as Greek key motif, because of its similarity to the paintings in the ancient Greek vases. The $\beta\gamma$-crystallin domain

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consists of two Greek key motifs arranged as four antiparallel β-strands, that share their third β-strand with the opposite motif and together form a domain [8,9]. γ-Crystallins are monomers while β-crystallin forms dimer to octamer in solution because of the presence of a large linker between two domains [10]. Widespread genome sequencing revealed the presence of $\beta\gamma$ domain in the different bacterial species and lower eukaryotes other than the lens. All $\beta\gamma$-crystallins have a common feature in folding pattern, besides that they also belong to the most long lived globular proteins known today [11]. It is thought that the crystallin fold evolved for proteins in such a way to provide an extraordinary long-lasting stability [12].

2. Organization of the $\beta\gamma$-crystallin domain

γ-Crystallin was the first crystallin whose three-dimensional structure was solved [8]. A key feature of the $\beta\gamma$-crystallin fold was the presence of a β hairpin motif, which forms a loop between the first two strands of each motif [8,13]. This conserved aromatic residue interacts with another conserved residue, particularly the serine present at the beginning of fourth strand in each Greek key motif to form a folded hairpin, a characteristic of $\beta\gamma$-crystallin type topology [14]. Both Greek key motifs share some similarities as well as dissimilarities and they have been classified as A-type or B-type Greek key motifs. In the lens $\beta\gamma$-crystallins, the arrangement of these motifs is of AB type. In protein S (a microbial homologue), the organization of domain is BA type as compared with the lens crystallin having AB type organization. The observation of an inverse arrangement of motifs led to the hypothesis that the microbial and the vertebrate $\beta\gamma$-crystallin domains have diverged from a single motif ancestor and followed independent paths of gene duplication and fusion [15]. Tyrosine corner (with a sequence of –GXY-), a type of local motif is usually present in the B type motif [16]. The presence of Tyr corners is also a notable feature in many Greek key β-barrel structures where tyrosine is generally located at the corner between a Greek key connection and the subsequent β-strand. It probably helps in stabilizing the connection relative to a hairpin as well as might be needed in such folding.

3. Topology of $\beta\gamma$-domains in various members of $\beta\gamma$-crystallin superfamily

Only a few structural homologs of β- and γ-crystallins, found in some lower eukaryote and prokaryote species have been studied. Protein S from the spore coat of the bacterium *Myxococcus xanthus*, and spherulin 3a from a slime mould *Physarum polysephalum* have a remarkable similarity with the two-domain vertebrate lens $\beta\gamma$-crystallins [12,15,17]. They help the organism tide over periods of stress in the environment in a Ca^{2+}-dependent manner [18,19]. The domain organization follows the typical topology of two Greek-key motifs which share their third strand with the opposite domain. They also retain the typical β-hairpin architecture and sequence signatures [13]. They were the first $\beta\gamma$-crystallins in which bound Ca^{2+} was observed in crystal structures [20]. During the process of cyst formation, monomeric protein S forms oligomer in the presence of Ca^{2+}. This oligomerization is necessary to form an extremely stable spore coat to provide viability to myxospores in the adverse condition of bacterium lifecycle.

Spherulin 3a, an encystment-specific protein, is a well-known example of a single domain protein [15]. Recently, some more single domain proteins, such as nitrollin and Ciona crystallin, have been identified [21,22]. These proteins are structurally related to vertebrate lens proteins $\beta\gamma$-crystallins. NMR solution structure confirms that spherulin 3a forms the single domain like γ-crystallin with an additional short β-strand [15,19]. The role of the N-terminal short β-strand was initially presumed to be involved in the dimerization of spherulin 3a through three-dimensional domain swapping thereby replicating an ancestral mode of dimer formation [23]. However, it was later found in the crystal structure that spherulin 3a dimerizes through a different interface and the N-terminal extensions have no role in dimerization. Close to a decade after this hypothesis was made, nitrollin, a single domain protein from a soil bacterium *Nitrosospira multiformis* was found to undergo dimerization through 3D-domain swap using its N-terminal extension [21]. Spherulin 3a was considered an evolutionary off-shoot from conventional $\beta\gamma$-crystallins due to the high level of sequence variation and absence of the typical A and B motifs in its domain organization [15]. The evolutionary relationship of lens $\beta\gamma$-crystallins to spherulin 3a suggests that the lens proteins were derived from an ancestor with a role in stress-response.

Crystal structure of Ciona crystallin from a urochordate *Ciona intestinalis* (sea squirt) illustrates the single domain with $\beta\gamma$ fold having two Greek key motifs. The sequence similarity of Ciona $\beta\gamma$ crystallin with vertebrate crystallins domains are relatively low and contains two Ca^{2+} ions in the crystal structure that are similar to microbial crystallin domains [22]. It could be inferred that

vertebrate $\beta\gamma$-crystallins have evolved from a single ancestral gene which forms a single domain lens protein in common chordate ancestor.

4. Sequence diversity and topology

Signature sequence of $\beta\gamma$-crystallin domains illustrates the flexibility to preserve the $\beta\gamma$ fold, which is required for novel and specific functions. Members of this superfamily are expanding as numerous proteins are being observed which contain typical $\beta\gamma$-crystallin domains. This is made possible due to an explosion of genomic information of different organisms available and with novel structural information added in the recent past. Some examples of monomeric proteins having a fold similar to $\beta\gamma$-crystallins are yeast killer toxins such as *Streptomyces* killer toxin like protein and *Williopsis mrakii* yeast killer toxin (WmKT) protein [23,24]. However, these proteins are thought to be a result of divergent evolution [25]. One of the $\beta\gamma$-crystallin domains (AIM1g1) of A1M1 (absent in melanoma) lacks some conserved residues such as Gly at six and eight position, and an insertion of about 10 residues in region between glycine-8 and serine at 34 positions in one of the domains, thus making it a variant of $\beta\gamma$-crystallins [26]. Despite these variations in the primary sequence, it is shown to retain the typical prototype $\beta\gamma$ fold, thus suggesting a relationship in sequence diversity and prototype fold [27]. As mentioned above, a one-domain protein nitrollin demonstrates the presence of three-dimensional domain swapping [21]. Although the sequence of this protein is highly variant with $\sim$20–24% sequence identity with known $\beta\gamma$-crystallins, this domain retains a typical $\beta\gamma$ fold and the swap provides the stability to the protein [21].

5. Evolution and ancestral relationship

Crystallins evolved from ancestors with pre-existing functions. It is believed that crystallins evolved from a single domain. The single domain underwent duplication events resulted in two or multiple domains containing proteins in prokaryotes and eukaryotes [28,29]. EP37 (Epidermal differentiation specific proteins) from amphibian *Cynopus phyrrogastor*, expressed in integumental tissues instead of lens. EP37 was the first example of crystallin identified with non-lens functions in vertebrates. Since the lens originates from the ectoderm, it could possibly retain the ancestral features [30,31]. A1M1 (absent in melanoma) protein which is associated with the tumorigenicity in human malignant melanoma, comprises six $\beta\gamma$-crystallin domains. Other studied members of the $\beta\gamma$ superfamily from various other species are geodin from *Geodia cynodium,* cargo proteins from *Tetrahymena*, yersinia crystallin from pathogenetic bacteria *Yersinia pestis* and caulollins from *Caulobactor cresecentus* [32–34]. However, none of these proteins have been structurally characterized.

Ciona crystallin, a single domain protein, is structurally similar to lens $\beta\gamma$-crystallins [22]. Therefore, it is suggested that there is an ancestral relationship of this urochordate crystallin and lens crystallins. Recently, a protein from archaea, M-crystallin, was found to have strikingly similar topology to that of lens $\beta\gamma$-crystallins, thus suggesting the presence of this fold in all three kingdoms of life [21,35]. Based on the structure, it appears that these domains evolved from a common ancestor [29].

6. Calcium-binding and $\beta\gamma$-crystallins

Understanding the functions of the member $\beta\gamma$-crystallins is a matter of debate till date. It was proposed that the lens γ- and β-crystallins show interdomain-intramolecular and intermolecular interactions. Thus, the crystallins in the lens would be well packed to minimize the light scattering without compromising the transparency of lens. It was proposed that lens $\beta\gamma$-crystallins bind calcium though there was no known motif for calcium-binding in these proteins [36–38]. It can be proposed that calcium level could be maintained by these crystallins, though it has not been experimentally verified. Several factors, such as lens lipid, might also play a role in controlling calcium concentration in the lens [39]. Two proteins of the superfamily, protein S and spherulin 3a which are spores and cyst forming $\beta\gamma$-crystallins, also known as calcium-binding proteins [40,41], where they help the organism tide over periods of stress in the environment in a Ca^{2+}-dependent manner [18,19]. Recently, several members have been shown to bind calcium, such as AIM1g1, yersinia crystallin, caulollins from *Caulobacter cresentus*, and geodin from a sponge [33,34,42,43]. In some cases, such as yersinia crystallin and caulollins, the proteins are intrinsically unstructured in apo form and gain β-sheet conformation upon binding calcium [33,34]. In case of some proteins such as AIM1g1, lens crystallins, and protein S, there is no significant change in protein conformation upon binding calcium [37,38,40], whereas in case of other homologues (spherulin 3a), calcium is able to induce minor conformational changes [41], thus suggesting the diversity in their properties.

7. Presence of a universal motif for calcium-binding

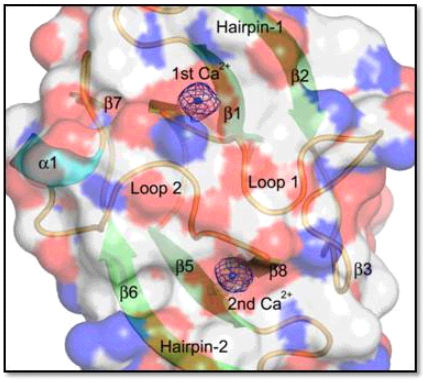
Though several proteins of the superfamily have been shown to bind calcium, the presence of a common, universal motif has not been put forth. Three-dimensional structure of only three proteins, namely, protein S, spherulin 3a and ciona crystallin was known in Ca^{2+}-bound form till recently [15,18,22,40]. The residues involved in Ca^{2+} ligation in these proteins are located at homologous position though it has been thought that the -N/D-N/D-X-X-S-S- sequence which is involved in calcium ligation in protein S and spherullin 3a is not conserved in the fish and vertebrate crystallins [22]. It was therefore not possible to define the universality of Ca^{2+}-binding in this superfamily. In order to define the motif and its universal presence, the presence of the motif of calcium-binding in the proteins of the superfamily has been identified based on the crystal structure of a number of proteins [21]. Since there is similarity in the binding site in all these proteins, it would be prudent to classify this superfamily as a family of calcium-binding proteins [29].

The motif for calcium-binding is formed from the sequence of Y/F-X1-----N/D1-N/D2-X2-X-S1-S2, whereas calcium binding takes place at residues X1, N/D2, X2 and S1. Actually, the binding site is formed between two Greek motifs as shown in figure 1. The ligation for the first Ca^{2+}-binding site takes place between the three residues from first Greek key motif and one from another Greek key motif. Similarly, for the second binding site, one residue from first Greek key motif, and three from second Greek key motifs are involved. This ligation pattern is different from EF-hand proteins where a site is formed with a continuous stretch of a sequence of a loop.

In several proteins of the superfamily, there is the presence of unfavourable amino acids, particularly arginine in place of serine/threonine (where side chain ligation takes place). This makes the protein non-binder as seen in case of nitrollin [21]. It has been demonstrated in protein S that when serine is mutated by arginine, protein S loses the Ca^{2+}-binding ability [18]. The gain of function of the binding site has been shown in nitrollin when arginine was mutated as serine in nitrollin [44]. This suggests the presence of disabled Ca^{2+}-binding site in some proteins of the superfamily, which might be disabled during evolution probably due to selection pressure.

8. Functions of lens crystallins

To understand the function of lens $\beta\gamma$-crystallins and their homologues, only a few studies have been performed. Earlier, it was thought that these proteins exclusively express in the lens, however, later on the expression of lens $\beta\gamma$-crystallins has been observed in several non-lens tissues, including testis and brain. βB2-crystallin carrying a phil mutation is shown to be related with fertility [45]. Addition of $\beta\gamma$-crystallins in the tissue culture medium on intra vitreal injections strongly enhanced axon regeneration in retinal explants and they induce the ciliary neurotrophic factor (CTNF) to activate the major downstream signaling pathways [46]. This suggests the multifunctional facets



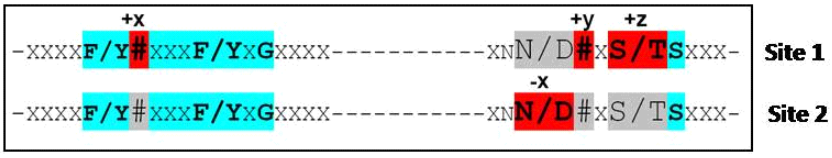


Figure 1. Surface diagram of a $\beta\gamma$-crystallin with Ca^{2+} ions embedded in the loop regions of the domain roof. Below is the $\beta\gamma$-crystallin Ca^{2+} binding motif depicted through primary sequence.

of $\beta\gamma$-crystallins. However, their role with respect to calcium-binding in the lens is not yet demonstrated.

One of the microbial homolog, yersinia crystallin domain shows unique characteristics. Yersinia crystallin domain is a departure from conventional $\beta\gamma$-crystallin domains as the latter are structurally rigid domains with high stabilities. The domain from *Yersinia pestis* is intrinsically unstructured in apo form and gain β-sheet conformation upon binding calcium [33]. It is suggested that these proteins might be associated with the virulency of *Yersinia* as the pathogen is not virulent in the low concentration of calcium, called as low calcium response. Another protein with related characteristics is caulollin from *Caulobactor crescentus*. Caulollins are partially unstructured in apo form and gain β-sheet conformation upon calcium-binding [34]. Therefore, the functions of these homologue proteins could be extrapolated to the pathophysiology and possibly to virulency. In case of protein S and spherulin 3a, it has already been shown that Ca^{2+} protects the cyst under adverse conditions by providing spore coat. Diversity of the protein sequence would thus be related with the specific functions of the protein in the respective organism, and need to be explored further.

9. Conclusions

$\beta\gamma$-Crystallin superfamily is emerging as a widespread superfamily with its presence in all three kingdoms of life. The topology of many domains is similar despite diversity in their sequence or the presence of usual diversity of a sequence and domain topology. The interesting aspect has been calcium-binding, and the presence of a universal motif with a sequence of Y/F-#----N/D-N/D-X-X-S/T-S in the superfamily. Members with this motif are likely to bind calcium. This calcium-binding motif is disabled or distorted in many proteins due to replacement of unfavorable amino acids at ligation sites. Despite similarity in topology, it would be interesting to understand how this domain is recruited for varied functions related to calcium binding in the physiology and evolution. Depending upon the functions in the normal or stressed conditions, the domains exhibit different conformations and Ca^{2+} binding stoichiometry, transitions from unstructured to structured and low to high calcium affinity.

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