Ca\(^{2+}\)-binding Motif of \(\beta\gamma\)-Crystallins

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\(\beta\gamma\)-Crystallin-type double clamp (N/D)(N/D)XX(S/T)S motif is an established but sparsely investigated motif for Ca\(^{2+}\) binding. A \(\beta\gamma\)-crystallin domain is formed of two Greek key motifs, accommodating two Ca\(^{2+}\)-binding sites. \(\beta\gamma\)-Crystallins make a separate class of Ca\(^{2+}\)-binding proteins (CaBP), apparently a major group of CaBP in bacteria. Paralleling the diversity in \(\beta\gamma\)-crystallin domains, these motifs also show great diversity, both in structure and in function. Although the expression of some of them has been associated with stress, virulence, and adhesion, the functional implications of Ca\(^{2+}\) binding to \(\beta\gamma\)-crystallins in mediating biological processes are yet to be elucidated.

Ca\(^{2+}\)-binding Proteins: One Ligand, Many Motifs

Ca\(^{2+}\) binding, already a very abundant physiological process, has a repertoire of associated Ca\(^{2+}\)-binding proteins (CaBP), including numerous proteins involved in signaling events (a few reviews, see Refs. 1–4). Ca\(^{2+}\)-binding proteins are grouped according to the nature of their binding motifs. The well studied all-\(\alpha\)-helical EF-hand motif and all-\(\beta\)-sheet-containing C2 domains, which possess diverse functions, predominate sensory CaBP (5–8). Many CaBP with extracellular EGF domains, \(\beta\)-propeller-like domains, and cadherins are also known (9–12). These independent structural units perform their Ca\(^{2+}\)-dependent roles either as complete proteins or as modules in multidomain proteins.

The Ca\(^{2+}\)-binding motif of the \(\beta\gamma\)-crystallin type is a recently established motif with a wide prevalence. Here, we lay out our understanding of this motif with respect to the geometry of binding sites, modes of Ca\(^{2+}\) coordination, and prediction of functional, disabled, or degenerate (nonfunctional) motifs. The well characterized founding members of the \(\beta\gamma\)-crystallin superfamily are lens \(\beta\)- and \(\gamma\)-crystallins, which are major constituents of the vertebrate eye lens, rendering it with a high refractive index and transparency (for reviews, see Refs. 13–18). As in the case of some other Ca\(^{2+}\)-binding proteins (C2 domains, EGF domains, and cadherins), \(\beta\gamma\)-crystallins possess an all-\(\beta\) fold, made of strand exchanged Greek key motifs (19, 20).

The \(\beta\gamma\)-crystallin domain is an ancient protein fold, and several proteins across different domains of life are found to have this fold, a majority of them being expressed in bacterial species (15, 17, 21). Many \(\beta\gamma\)-crystallin domains have been studied in the recent past, and insights obtained from ion binding in these \(\beta\gamma\)-crystallins have led to the proposition that proteins of this superfamily possess a universal Ca\(^{2+}\)-binding motif (22, 23).

Notwithstanding that \(\beta\gamma\)-crystallins have been established as a superfamily of Ca\(^{2+}\)-binding proteins, their involvement in the cellular Ca\(^{2+}\) metabolism or signaling is far from being recognized yet. We summarize a critical analysis of the Ca\(^{2+}\)-binding motif present in this superfamily of proteins.

\(\beta\gamma\)-Crystallins and Ca\(^{2+}\) Binding: A Chronology

Although the very first non-lens protein belonging to the \(\beta\gamma\)-crystallin superfamily was Protein S from the bacterium Myxococcus xanthus (24), it took almost two decades to classify these proteins as a separate group of CaBP. In the absence of structural information, Protein S was earlier speculated to possess extensively modified EF-hand motifs (25). Weak binding of Ca\(^{2+}\) to lens \(\beta\)-crystallins was reported on the basis of equilibrium dialysis and interaction with the Ca\(^{2+}\) mimic dye Stains-all (26, 27). Another decade passed before Spherulin 3a, a protein from slime mold Physarum polycephalum that was predicted to be a single domain \(\beta\gamma\)-crystallin (28), was finally shown to bind Ca\(^{2+}\), with clues from Ca\(^{2+}\) binding to lens \(\beta\)-crystallin (29–31). Based on Ca\(^{2+}\) binding to a peptide corresponding to a Greek key motif of \(\gamma\)-crystallin (32), a proposition that \(\beta\gamma\)-crystallin-type Greek key could form the motif for Ca\(^{2+}\) binding was formulated. Concurrent structural studies shed light on the coordination pattern of Ca\(^{2+}\) binding to Spherulin 3a and Protein S (22, 33).

The recognition of \(\beta\gamma\)-crystallins as a distinct superfamily of Ca\(^{2+}\)-binding proteins was still in its infancy. The factors responsible for this were: (i) undefined motif of Ca\(^{2+}\) binding, (ii) lack of information about the role of Ca\(^{2+}\) in protein functions, and (iii) no substantial addition of novel members to this superfamily. For a long time, this superfamily was considered a sparsely distributed family with only a few scattered members, although some more proteins (e.g. WmKT, SKLP, and SMP1) were identified based on structural similarity (34–36); these did not belong to the \(\beta\gamma\)-crystallins lineage and may have arisen from convergent evolution (37). With the advent of genomic sequence information, many members from diverse species (bacteria, archaea, and urochordate) were added to the superfamily and also confirmed to be Ca\(^{2+}\)-binding proteins (22, 23, 38–43), leading to the recognition of a common motif for ion binding, and thus prompting the organization of these proteins as a separate set of Ca\(^{2+}\)-binding proteins (23, 44).

Architecture of the \(\beta\gamma\)-Crystallin-type Ca\(^{2+}\)-binding Motif

The domain topology of \(\beta\gamma\)-crystallins is based on a pair of Greek key motifs (19, 28). The Greek key motif, a terminology...
based on the pattern and supersecondary features observed in proteins, is a basic theme of many all-β proteins (45, 46). Greek keys are quite diverse in topology and hence were further classified as (4,0), (3,1), and (2,2) patterns, based on the arrangement and connectivity of strands (46). According to the above classification, proteins of the βγ-crystallin superfamily fall into the (3,1) category (47), where one strand (third strand, c and c’) of the respective Greek key motif out of the four is shared by a partner motif (Fig. 1).

βγ-Crystallin-type Greek key motif has a distinct signature sequence of residues “(F/Y/W)XXX(F/Y)XG” (28) in the β-hairpin loop between the first and the second strands (19) (Fig. 1). Along with this signature sequence, ~24–30 residues downstream, a conserved Ser is located (on the fourth β-strand), which plays a structural role by stabilizing this β-hairpin (19, 20, 48). The third strand of each Greek key is swapped and becomes a part of the partner Greek key motif ((3,1) arrangement of β-strands) (Fig. 1b). The fourth strand folds back to the previous sheet via a connecting loop, which is variable in length and occasionally has a small helical segment. This loop (named loop1 in the first Greek key and loop2 in the second Greek key motif) occupies the top of the βγ-crystallin domain (Fig. 1c).

A βγ domain has two juxtaposed Ca2+ binding sites, which are mainly formed of the loops (loop1 and loop2) with an (N/D)(N/D)XX(T/S)S sequence stretch, running in opposite directions in three-dimensional space, along with the residues from β-hairpins (22, 23, 33, 40) (Fig. 1, c and d). Thus, each Ca2+ binding site is formed by four residues located at three different regions in the primary sequence. The first Ca2+ binding site is formed via one residue from β-hairpin1, two residues from loop1, and one residue from loop2, and in a similar way, the second site is formed by one residue from β-hairpin2, two residues from loop2, and one residue from loop1 (22, 23) (Fig. 1d). This arrangement is common in the βγ domains studied structurally; Protein S (33), Spherulin 3a (22), Ci-βγ-crystallin (40), Clostrillin, Flavollin, M-crystallin (23, 41), and Geodin (42).

**Coordination Geometry of the Ca2+ binding Site**

In βγ-crystallins, the Ca2+ coordination number varies from five to eight, four being satisfied by protein ligands, the rest being satisfied by water molecules (22, 23, 33, 40, 42). The +x position of the coordination sphere is provided by the main chain carbonyl of the first X residue of (F/Y)XXX(Y/F)XG stretch, which is next to the first conserved aromatic residue of β-hairpin1 (Fig. 2). The second coordination (+y position) is provided by the main chain carbonyl oxygen of the third residue and the third coordination (+z position) by the side chain oxygen of the fifth residue of the (N/D)(N/D)XX(T/S)S stretch of the same Greek key motif (22, 23, 40) (Fig. 2). The involvement of the Ser or Thr hydroxyl group to coordinate Ca2+ is distinctive to this motif as most Ca2+ binding sites do not involve these residues in Ca2+ ion coordination (49). The fourth coordination (−x position) is provided by the side chain oxygen of the second residue (mostly Asp or sometime Asn) of
Thus, providing variations in the microenvironment around the oxygen and is generally a polar residue, but is variable in nature, demonstrated that the amino acid residues forming the motif get its name as a “double clamp” motif (23) (Figs. 1 and 2). The third residue (N/D)XX(T/S) stretch from the partner Greek key motif. The −y and −z positions are satisfied by water molecules.

Both sites of a domain exhibit similar or slightly altered coordination geometry. The coordination number is seven, with pentagonal bipyramidal geometry, but octahedral geometry (coordination number: 6) and square anti-prismatic (coordination number: 8) are also seen. A coordination number of five has also been observed in the first Ca$^{2+}$ binding site of Protein $S$ (33). The average coordination radius at each of these sites varies from 2.4 to 2.8 Å.

Preferred Residues in the Binding Motif and Their Significance

An analysis of >100 sequences of canonical motifs available demonstrated that the amino acid residues forming the (N/D)XX(T/S) fingerprint vary in various proteins of the superfamily (44). The first residue of the fingerprint is involved in stabilizing the pocket through hydrogen bonding with the hydroxyl side chain of Ca$^{2+}$ coordinating Ser/Thr and in some cases supports a water molecule present at the −y position. The second residue, also a polar amino acid (mostly Asp), directly coordinates Ca$^{2+}$ by providing monodentate ligation at the −x position. These first two residues act in trans, i.e. the first two residues of loop 1 of the first Greek key, become part of the second Ca$^{2+}$-binding site and vice versa; thus, the motif gets its name as a “double clamp” motif (23) (Figs. 1 and 2). The third residue (X1) coordinates via main chain carbonyl oxygen and is generally a polar residue, but is variable in nature, thus providing variations in the microenvironment around the Ca$^{2+}$-binding site. The nonligating fourth residue (X2) is generally hydrophobic. It forms a part of the hydrophobic core and may be an important player in relaying signals to the core; local conformational changes in the loop upon Ca$^{2+}$ binding can thus affect the stability of the protein (23, 44). The fifth residue is usually Ser (or sometimes Thr), which coordinates via the hydroxyl oxygen and is a determinant for domain properties. The sixth nonligating residue is highly conserved structural Ser, which stabilizes the β-hairpin loop (48). The crystal structures of βγ-crystallin domains available in the Ca$^{2+}$-bound form display a common theme of Ca$^{2+}$ coordination that led to the proposition of a distinct motif for Ca$^{2+}$ binding in the βγ-crystallin superfamily (Table 1), although some of these structures carry minor variations in this theme, which are discussed below.

Deviations within the Common Theme of the Binding Sites

The second Ca$^{2+}$-binding site of Ci-βγ-crystallin (Protein Data Bank (PDB) ID: 2BV2) from Ciona intestinalis is a minor exception to the theme described above (40). In this site, the protein molecule provides five coordinations, where the third residue (Glu-76) of the (N/D)XX(T/S) fingerprint coordinates through the side chain oxygen at the −z position, in addition to the usual coordination by the main chain carboxyl oxygen at +y (Fig. 3a). Similarly, in the W39D mutant of Clostrilllin, Asp–39 coordinates via the main chain carboxyl oxygen in addition to the side chain carboxyl oxygen, whereas in the wild type, only the main chain carboxyl of Trp–39 coordinates at the +y position (44).
TABLE 1
A glance at \( \beta \gamma \)-crystallins with Ca\(^{2+} \) perspective

| Protein/ PDB ID /Resolution (Å) | Protein S 1NPS/1.80 | Flavolin 3HZB/1.74 | Clostrillin 3J9H/2.00 | Spherulin3a 1HDF/2.35 | Ciona-crystallin 2BVZ/1.55 | M-crystallin 3HZZ/1.86 | Geodin 4IAU/0.99 |
|--------------------------------|----------------------|---------------------|----------------------|------------------------|---------------------------|------------------------|------------------|
| Crystal structures of \( \beta \gamma \)-crystallins |
| Hydrodynamic status | Monomer | Dimer | Dimer | Dimer | Monomer | Monomer | Monomer |
| Ca\(^{2+} \)-binding motifs |
| \( K_d \) (nM) for Ca\(^{2+} \) | 27, 76 | 33 | 4 | 9, 200 | - | 80 | - |
| Chemical Stabity \( c_{12} \) [M] | Apo form | 4.4±0.10 | 1.0±0.08 | 1.9±0.10 | 1.2 | - | - | 1.2 |
| Ca\(^{2+} \)-bound form | 5.0±0.10 | 1.3±0.11 | 2.5±0.08 | 2.8* | - | - | - |
| Thermal Stability \( T_m \) (°C) | Apo form | 52 NPS 68 | 47 | 45 | 65 | - | 55 | 60.5 |
| Ca\(^{2+} \)-bound form | 64 NPS 70 | 48 | 63 | 85 | - | 71 | 65.0 |
| References | 24, 33, 79 | 23, 52 | 23, 52 | 30, 31 | 40 | 23, 41 | 42, 88 |

* Equilibration required about 8 weeks.

FIGURE 3. \( a \), the Ca\(^{2+} \)-binding site of Ci-\( \beta \gamma \)-crystallin where Glu, the third residue of the (N/D)(N/D)XX(T/S) fingerprint, coordinates with the side chain carboxyl oxygen at the –z position along with the main chain carbonyl oxygen at the +y position. \( b \), the Ca\(^{2+} \)-binding site of Geodin showing a glycerol molecule occupying the positions (+y) corresponding to the water molecule in the Ca\(^{2+} \) coordination sphere. \( c \), the first Ca\(^{2+} \)-binding site of Ci-\( \beta \gamma \)-crystallin where Glu-7 from a symmetry-related molecule occupies the +z position corresponding to the water molecule. \( d \), a schematic diagram showing the pattern coordination and mode of Ca\(^{2+} \) ligation along with the primary sequence of the binding site. As in Fig. 1, the broken and continuous lines represent coordination via the main chain and side chain, respectively.
MINIREVIEW: Ca\(^{2+}\)-binding Motif of βγ-Crystallins

with coordination number 7 is more prevalent in the βγ-crystallin superfamily, where three positions corresponding to −y1, −y2, and −z positions of the coordination sphere are occupied by water molecules (Fig. 2). However, the water molecules coordinating with Ca\(^{2+}\) vary in number, leading to different coordination geometries as well. In Geodin (from a marine sponge), only one Ca\(^{2+}\) bound to a C-terminal domain is observed as only one site is canonical (42), attesting to the prediction made by Clout et al. (22) applying fingerprint comparison. In selenomethionine derivatized crystal, the water molecules correspond to −y1 and −y2 positions, as seen in undervativized crystals, have been replaced by hydroxyl oxygens of glycerol molecule without affecting the overall coordination geometry (Fig. 3b). Cit-βγ-crystallin is another example, where a water molecule at the first Ca\(^{2+}\)-binding site (−z position) is replaced by the side chain carboxyl oxygen of Glu-7 from a symmetry-related molecule as an effect of crystal packing (Fig. 3c) (40). These examples suggest that the water molecules participating in Ca\(^{2+}\) coordination are not very strongly bound in βγ-crystallins and can be replaced by other suitable ligands, if conditions are favorable for their binding.

Signature Sequence and Identification of New Ca\(^{2+}\)-binding βγ-Crystallins

With the growing number of protein sequences in databases, the (N/D)(N/D)XX(S/T)S fingerprint can be used to predict Ca\(^{2+}\)-binding βγ-crystallins as with the consensus sequence of the EF-hand motif. The (N/D)(N/D)XX(S/T)S fingerprint occurs twice in a domain due to the pairing of the two Greek key motifs. As two Greek key motifs participate in constructing a site, both should be taken into consideration for prediction (Fig. 3d). Only two residues use their side chains in coordinating Ca\(^{2+}\), and any change at these positions leads to either complete loss or reduction in Ca\(^{2+}\) binding ability. The first critical residue is Ser/Thr at the fifth position, and the second is Asp or Asn from the juxtaposed motif at the second position in the (N/D)(N/D)XX(S/T)S fingerprint (Fig. 3d). As an example, in Clostrillin, at the first Ca\(^{2+}\)-binding site, Thr from the motif NDWMTS ligates via the side chain hydroxyl oxygen at the +z position and Asp residue from the second motif NDKMTS coordinates via the side chain carboxyl oxygen at the −z position (Table 1). Similarly, for the second site, Thr from the motif NDKMTS would provide its side chain for coordination at the −z position, and the site would be completed by the side chain of the Asp residue coming from NDWMTS (23).

Thus, both sites are well matched for binding Ca\(^{2+}\) (Table 1).

In the case of the first site of the C-terminal domain of Geodin, Lys from the first β-hairpin loop and Gly from the motif IGGVSS would coordinate via main chain, besides Ser at the fifth position in the motif (Table 1) (42). This site would bind Ca\(^{2+}\) only if the second residue of the second motif NDALKS is also favorable for ligation. It is Asp, and hence the first site in this protein is suitable for Ca\(^{2+}\) binding. At the second site, Lys occupies the fifth position in the motif NDALKS, which is not compatible. Also, the second residue of the partner motif IGGVSS is Gly, which, again, cannot provide its side chain for completing the site, and thus the second site of this domain would not bind Ca\(^{2+}\). This consensus sequence has aided into the identification of new members of this family.

Degeneracy of Motif: Constraints and Gains

Not all βγ-crystallins possess the canonical (N/D)(N/D)XX(S/T)S fingerprint of ion binding. Most proteins of this family with functional Ca\(^{2+}\)-binding motifs are from bacterial sources or lower eukaryotes (Table 1). The vertebrate homologues, such as lens βγ-crystallins, AIM1, Crybg3, and a few more diverged domains do not have the canonical sequence, and their binding sites are degenerated to an extent that Ca\(^{2+}\) binding is either very poor or lost (23, 43, 50–54). It is proposed that although Ca\(^{2+}\) binding might have been a vital need for ancestors of lens crystallins, most binding sites in lens βγ-crystallins were evolutionarily disabled to gain extra stability (52, 54) because high domain stability is an indispensable requirement of lenticular proteins (15, 55). It is, however, speculated that these βγ-crystallins may be involved in Ca\(^{2+}\)-dependent functions in non-lenticular tissues as shown in brain and testes (56, 57).

The C-terminal domain of Geodin with one naturally disabled Ca\(^{2+}\)-binding site (42) is unusual as most βγ domains have two juxtaposed Ca\(^{2+}\)-binding sites. Nitrollin, a β-crystallin from the bacterium Nitrosospira multiformis, which is peculiar for its mode of domain swapping, does not bind Ca\(^{2+}\) due to the presence of Arg in place of conserved Ser along with other unfavorable residues (43). Thus, even bacterial homologues can have disabled sites. Substitution of Ser/Thr with Arg leads to the loss of Ca\(^{2+}\) binding in many homologous proteins, such as Protein S, Flavollin, and Clostrillin (24, 44). This leads to enhanced stability of the protein, which, in some cases, is comparable with stability gained upon Ca\(^{2+}\) binding (52).

Comparisons with Other Ca\(^{2+}\)-binding Motifs

Multiple modules, EF-hands, C2 domains, and EGF domains, have purposes such as sensing and sequestration of Ca\(^{2+}\) (5, 7, 58, 59), and Ca\(^{2+}\) binding takes place at a specific motif forming continuous or discontinuous Ca\(^{2+}\)-binding sites (60, 61). The EF-hand motif, an example of a continuous site, is the predominant class in CaBP. The EF-hand loop between the E and the F helices coordinates Ca\(^{2+}\) ion, usually with coordination numbers of 7 or 8 (6, 62–64). Non-EF-hand proteins utilize fewer protein ligands for coordinating Ca\(^{2+}\) (65). Unlike βγ-crystallins in which the ion is exposed to the solvent with 1–4 water molecules in direct coordination with Ca\(^{2+}\), the EF-hand bound Ca\(^{2+}\) is exposed to a single water molecule (Table 2). Similarly, in βγ-crystallins, fewer protein ligands (4) and more water (1–4) molecules are seen. The affinities of Ca\(^{2+}\) toward EF-hand proteins are moderate to very high with $K_d$ values in the nanomolar range (6), whereas βγ-crystallins display affinities in the micromolar range (Table 1).

Unlike the EF-hand motifs, C2 domains are all-β conforma-
tional Ca\(^{2+}\) sensors and share the β-sandwich arrangement not akin to βγ-crystallins (Table 2) (7). C2 domain-containing synaptotagmins are major players in neurotransmitter exocytosis and are involved in Ca\(^{2+}\)-dependent phospholipid binding (66). The domains are incorporated in multiple proteins (such as phospholipases) for their Ca\(^{2+}\) sensing roles (67). The Ca\(^{2+}\)-
binding site is discontinuous and formed by mutually distant regions in the primary sequence of this domain (68) as is the case with βγ-crystallins. In the C2 domains, the Ca\(^{2+}\) ions are coordinated by a network of aspartate side chains in mono- or bidentate fashion with one aspartate involved in coordination with two Ca\(^{2+}\) ions (58, 69–71).

### Minutiae of Ca\(^{2+}\) Binding and Origin of Domain Diversity

The affinity of Ca\(^{2+}\) for Ca\(^{2+}\)-binding proteins ranges from intracellular nM to extracellular mM, depending on their spatio-temporal localization, which in turn reflects their functions. With the limited information available, it appears that most βγ-crystallins are extracellular or secretory proteins (72–78). βγ-Crystallins bind Ca\(^{2+}\) with affinities in the lower micromolar range (M-crystallin, 32 μM; Clostrillin, 4 μM; Flavollin, 30 μM; Protein S, 27 and 76 μM) (Table 1) (23, 24, 79), Ca\(^{2+}\)-binding affinities of Ci-βγ-crystallin from *C. intestinalis*, DdCad-1, and Geodin have not been reported. Comparatively weak binding affinity is reported for βγ-crystallins from eukaryotic species (9 and 200 μM for Spherulin 3a, and 260 μM for amphibian EP37) (23, 30, 31). Although the affinity of these proteins ranges in μM, a clear-cut variation has been seen not only in different βγ-crystallin domains but also between two juxtaposed sites of the same domain.

The molecular determinants of affinity are not well understood in this domain. Either of the sites in a domain is interdependent as abrogating binding in one site attenuates the affinity of the juxtaposed site (44, 80). Nevertheless, it is not clear whether filling of Ca\(^{2+}\) at the two sites is simultaneous or sequential, cooperative or noncooperative. The microenvironment around a Ca\(^{2+}\)-binding site affects its affinity toward Ca\(^{2+}\). Even a homologous replacement of the fifth residue of the motif between structurally very similar domains changes its affinity as well as domain properties (44, 52). Consequently, this motif acts as a tuning knob for such a high diversity in domain properties (52). All the proteins studied so far show little or no binding toward divalent Mg\(^{2+}\), confirming the Ca\(^{2+}\) specificity of these binding sites. Similar observations for ion selectivity have been made in C2 domains (81), whereas Mg\(^{2+}\) is known to bind functionally, at least in the resting state, to Ca\(^{2+}\)/Mg\(^{2+}\)-binding EF-hands (6). Mg\(^{2+}\) may bind even the Ca\(^{2+}\)-specific (regulatory) EF-hands, although with very weak affinity, without affecting the overall conformation of the protein (6).

### Prevalence of the βγ-Crystallin-type Ca\(^{2+}\)-binding Protein Family

The advent of more genomic information has revealed a widespread occurrence of this superfamily and recruitment of βγ-crystallin domains in conjunction with domains of different functions. An analysis demonstrates that it exists in several hundred species (source: Pfam database, accession number PF00030). Three out of four protein sequences of βγ-crystallins from an invertebrate species *Branchiostoma floridae* (amphioxus) also possess Ca\(^{2+}\) binding fingerprints probably similar to that seen in Ci-βγ-crystallin from *Ciona intestinalis* (21). In a limited analysis of sequences, it was observed that the domain is recruited in serine proteases (Sorangium cellulosum), aspartate metalloproteases (Saccharophagus degradans), carbohydrate-binding glycosyl hydrolases (*Flavobacterium johnsoniae* and other bacterial species), and cell adhesion molecules (*Dictyostelium discoideum*). Isolated βγ-crystallins are also found as part of proteins from *Vibrio cholerae*, *Maricaulis maris*, *Oceaniculis alexandri*, and *M. xanthus* and in the archaeal species *Methanosarcina acetivorans*. Such extensive recruitment as modules in proteins of diverse functions clearly indicates some significant and widespread roles of these domains in protein function.

### Ca\(^{2+}\) Binding and Domain Stabilization

CaBP, mostly those of the EF-hand family, undergo large conformational changes upon binding Ca\(^{2+}\). Contrary to this, βγ-crystallins generally do not undergo a drastic change in conformation upon binding Ca\(^{2+}\), suggesting that apo forms are conformationally not very flexible (e.g. Protein S, Spherulin 3a) (30, 82). This is attributed to thermodynamically robust domain architecture (reviewed in Ref. 17). Some individual domains of larger proteins from the pathogenic bacterium *Yersinia pestis* and the extremophilic *Caulobacter crescentus* and *Hahella chejuensis* are intrinsically unstructured (or partly unstructured) in the apo form and gain significant structure upon binding Ca\(^{2+}\) (38, 39, 83).

Although βγ domains do not undergo major structural change upon binding Ca\(^{2+}\), they assume a reduced hydrodynamic size and thermodynamically drift to a state of higher structural stabilization. Therefore, in some βγ-crystallins, Ca\(^{2+}\) plays the role of an extrinsic stabilizer. Within the superfamily, there exists a stability gradient across the domains and differences in the extent of gain in stability upon Ca\(^{2+}\) binding from very low (in Vibriillin) to very high (in Centillin) (Table 1) (52, 84). As noted in *Y. pestis*, βγ domain-containing proteins are differentially expressed in Ca\(^{2+}\)-depleted avirulent strains (85, 86) and are unstructured in apo form (38), raising the prospective of their role in virulence via low calcium response.

### Table 2

A comparative look at some major Ca\(^{2+}\)-binding motifs

| Motif Features | EF-Hand | C2-Domain | βγ-Crystallin |
|----------------|---------|-----------|---------------|
| Representative figures and the respective PDB IDs | ![Image](3CLN) | ![Image](TDO) | ![Image](H2Z2) |
| Nature of motif based on primary sequence | Continuous | Discontinuous | Discontinuous |
| Category (Buffer/sensors) | Sensors | Sensors | not clear |
| Affinity for Ca\(^{2+}\) | μM – mM range | μM range | μM/mM range |
| Conformational change upon Ca\(^{2+}\)-binding | Large | Little | Varies from domain to domain (no change to very large) |
| Coordination numbers observed | 6-8 | 7 | 5-8 |
| Coordination provided by protein ligands | 4-7 | 3-6 | 4-5 |
| Number of water molecules involved in coordination | 1-2 | 1-4 | 1-4 |
| Mg\(^{2+}\)-binding affinity at physiological concentration | may bind (nM–mM) | not shown | not shown |
| References | 5, 6 | 7, 58, 71, 81 | 22, 23, 40, 44 |
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Ca\(^{2+}\)-dependent Functions: An Underexplored Arena

It is clear that the βγ-crystallin domain is widely spread. However, it has been difficult to assign functions in many βγ-crystallins studied so far (22, 23, 38, 41, 40, 83, 87, 88). Protein S of *M. xanthus* is expressed as a soluble protein and self-assembles as a multilayer spore coat in a Ca\(^{2+}\)-dependent manner. Based on the observations that adjacent spores were fused to each other via a common Protein S layer, it was proposed that Protein S is involved in spore-spore interaction in the fruiting body (76). Spherulin 3a is another Ca\(^{2+}\)-binding βγ-crystallin induced during stressful situations, but just like Protein S, its Ca\(^{2+}\)-dependent functional implications in the physiological context have not yet emerged.

Ci-βγ-crystallin from *C. intestinalis* is localized in the otolith and thus might be involved in a primordial sensory system (40). It binds Ca\(^{2+}\) at both binding sites, but the Ca\(^{2+}\)-dependent role of this single domain protein in the urochordate remains an enigma.

βγ-CAT from the skin of the frog *Bombina maxima* is implicated in several in vivo toxic effects on mammals (72, 89, 90). The βγ-Crystallin domain of this protein binds Ca\(^{2+}\) (its affinity has not been reported), and when red blood cells were treated with this protein, increased Ca\(^{2+}\) flux was observed that eventually resulted in hemolysis (73).

The cell adhesion molecule DdCad-1 from *D. discoideum* is involved in cell-cell adhesion in a Ca\(^{2+}\)-dependent manner via dimer formation through two βγ-crystallin domains (91). During its transport, the protein is internalized in vacuoles in a Ca\(^{2+}\)- and conformation-dependent manner (92, 93). The available functional information, although limited, suggests important roles played by this domain that remain yet to be explored.

Perspective

Although βγ-crystallins are distinct in terms of their Ca\(^{2+}\) binding properties with a well defined Ca\(^{2+}\)-binding motif, the functions of many proteins in the protein sequence/structure databases are either uncertain or unknown. The loss of Ca\(^{2+}\) binding ability in recent homologues of *β*-crystallins (as in *M. xanthus*-crystallin II) is yet to be deciphered (95, 96).

In summary, the Ca\(^{2+}\) binding at the (N/D)(N/D)XX(S/T)S motif may serve as a domain stabilizer, and evolutionary imperatives may have replaced this stabilizing function of Ca\(^{2+}\) with an intrinsic stability of the domain. Although the motif has established its identity, the functional analysis of its members still remains to be addressed, which is a challenging yet imminent task considering the appearance of numerous new members.

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