Calcium Binding Properties of γ-Crystallin

CALCIUM ION BINDS AT THE GREEK KEY βγ-CRYSTALLIN FOLD

Bheemreddy Rajini, Preetha Shridas, C. Sivakama Sundari, Dasari Muralidhar, Sushil Chandani, Fairwell Thomas, and Yogendra Sharma

From the Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad-500007, India and NHLBI, National Institute of Health, Bethesda, Maryland 20892

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The β- and γ-crystallins are closely related lens proteins that are members of the βγ-crystallin superfamily, which also include many non-lens members. Although β-crystallin is known to be a calcium-binding protein, this property has not been reported in γ-crystallin. We have studied the calcium binding properties of γ-crystallin, and we show that it binds 4 mol eq of calcium with a dissociation constant of 90 μM. It also binds the calcium-mimic spectral probes, terbium and Stains-all. Calcium binding does not significantly influence protein secondary and tertiary structures. We present evidence that the Greek key crystallin fold is the site for calcium ion binding in γ-crystallin. Peptides corresponding to Greek key motif of γ-crystallin (42 residues) and their mutants were synthesized and studied for calcium binding. These peptides adopt β-sheet conformation and form aggregates producing β-sheet sandwich. Our results with peptides show that, in Greek key motif, the amino acid adjacent to the conserved aromatic corner in the “a” strand and three amino acids of the “d” strand participate in calcium binding. We suggest that the βγ superfamily represents a novel class of calcium-binding proteins with the Greek key βγ-crystallin fold as potential calcium-binding sites. These results are of significance in understanding the mechanism of calcium homeostasis in the lens.

Calcium homeostasis plays an important role in lens transparency, opacification, and cataractogenesis. Cataracts can occlude both under hypocalcemic and hypercalcemic conditions, so the actual amount of available calcium in the lens is an important parameter for the health of the lens (1, 2). The normal mammalian lens has around 0.2 mM total calcium, of which the amount of free Ca$^{2+}$ is only of the order of a few micromolars. Thus there must exist calcium regulation systems in the lens, and it is of interest to identify what they are and how they change in health and in disease. Vrensen et al. (3) have done an ultrastructural analysis of calcium distribution in the rat lens and have found calcium precipitates in the intermediate cortex fiber membranes, cytoplasm, and the nuclear envelope and very low levels of calcium in gap junctions, epithelial cells, and superficial fibers (3–5). The question of what the calcium-binding and -storing agents are in the lens is open; phospholipids and crystallins have been thought of as candidates. The major components of the lens are cytosolic proteins, crystallins, which account for about 40% of the wet weight of the lens. It is worth investigating whether any of the crystallins could act as a calcium sponge or storage depot in the tissue, particularly since the ultrastructural analysis shows calcium distribution in the cytoplasm. We have earlier shown that the β- and avian core protein δ-crystallins show significant calcium-binding ability (6, 7). Thus, the possibility of crystallins acting as lenticular calcium-sequestering and -storing systems exists. However, the calcium binding properties of γ-crystallin have not yet been reported.

γ-Crystallin is a well studied protein and was the first crystallin whose structure was solved (8). The Greek key crystallin fold was first described in this protein (8). It was later found in another lens protein, β-crystallin, and in several other non-lens proteins, which were together classified as the βγ-crystallin superfamily (9, 10). The crystallin fold, also called the βγ motif, is a super-secondary structure formed from the symmetrical association of the two Greek key motifs that are organized as two four-stranded anti-parallel β-sheets (8, 9). The crystallin fold is a protein domain in which aromatic residues Tyr/Tyr/Trp at position 1 and Gly at position 8 constitute the conserved sequence (Y/F/W)XXG, followed by a Ser at positions 28–34 from the first Y/F/W residue, and this sequence is repeated within 40 residues. Gly-8 is irreplaceable and is needed for forming a dihedral angle, which is not possible with any other amino acid. These residues are required for the stabilization of the folded hairpin of the βγ motif (11). Between Gly-8 and Ser-34 lie two charge clusters of alternate signs (12). More members have been added to the diverse βγ-crystallin superfamily. Protein S, a development-specific protein from Myxococcus xanthus (13–15), spherulin 3a from Physarum polycephalum (16, 17), AIM1 (absent in melanoma) which is associated with the tumorigenicity in human malignant melanoma (18), epidermis differentiation-specific protein family (EDSP or EP37) from the amphibian Cynops pyrrhogaster (19–21), a yeast killer toxin (WmKT) from Williopsis mrakii (22), Streptomyces metalloproteinase inhibitor (SMPI) (23), and the calmodulin-binding membrane protein family (PCM) from Paramecium tetraurelia (24) are the non-lens members of the βγ-crystallin superfamily. βγ-Crystallins are thought to have originated from a single domain ancestor by gene duplication and gene fusion (25). The βγ motif is seen in single domain (spherulin 3a and WmKT), two domain (β- and γ-crystallins, protein S, EP37, SMPI) as well as multidomain proteins (AIM1). Evolutionarily, these proteins are among the most long-lived globular proteins known, generally expressed under stressed, adverse conditions or in differentiating tissues.

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§ To whom correspondence should be addressed. Tel.: 91-40-717-2241; Fax: 91-40-717-1195; E-mail: yogendra@ccmb.ernet.in.
It is believed that the crystallin domain evolved in proteins of extraordinary stability. Members of this superfamily have, therefore, been studied for their stability and architecture. An interesting feature of some of these proteins is their calcium-binding ability, e.g. β-crystallin (6, 7, 26), protein S (14, 27), and spherulin 3a (17). Putative calcium-binding sites have been shown in the EP37 proteins (20). However, these proteins do not have any of the well characterized motifs for calcium binding, such as the EF-hand, lipocortin-, or the annexin-like domains. They thus seem to contain an “orphan” motif, which needs to be identified. Surprisingly, the calcium-binding ability of γ-crystallin is not yet known, although it is the representative model of the superfamily, whose three-dimensional structure is very well known. Studying the calcium binding to γ-crystallin not only points to the inherent characteristic of the superfamily but would also help in identifying the orphan motif in the members of βγ-crystallin superfamily. In this work, we report that γ-crystallin does bind calcium. We also show that the Greek key crystallin fold forms the calcium-binding site. Since members of this superfamily share a homologous crystallin fold, we suggest that other members also bind calcium and thus represent a novel class of calcium-binding proteins.

**EXPERIMENTAL PROCEDURES**

All chemicals used were of the analytical grade. Terbium chloride was obtained from Aldrich and Stains-all from Sigma. Radioactive calcium chloride ($^{45}\text{CaCl}_2$, specific activity 11.7 mCi/mg) was purchased from PerkinElmer Life Sciences.

Preparation of Crystallins—Bovine eyes were collected from animals (age 3–5 years) from a local slaughterhouse and brought to the laboratory on ice. Lenses were excised and homogenized in the gel filtration buffer. γ-Crystallin from bovine lenses was purified on a Bio-Gel A-1.5m column (2.5 × 90 cm) in 50 mM Tris buffer, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.02% sodium azide at 4°C. Solutions of crystallins were rendered calcium-free by dialyzing first against EDTA at pH 2 and then against calcium-free water. Protein solutions were concentrated using an Amicon ultrafiltration unit with YM-10 membrane. Finally, protein and buffer solutions were passed through Chelex-100 columns before use, so as to remove any calcium that might be present as a contaminant and stored in plasticware. Protein concentrations were calculated from their absorption coefficients.

Design and Synthesis of Four-stranded Greek Key β-Sheet Peptides—We have selected a stretch of 42 amino acids corresponding to the third motif of the bovine γ1-crystallin (residues 90–131) to have a complete geometry of the Greek key crystallin fold. We have synthesized the wild type and its variants by modifying the potential residues coordinate with it. Optimization and molecular dynamics operations were performed in this assembly.

**RESULTS**

Probing Calcium Binding to γ-Crystallin by Direct $^{45}\text{Ca}$ Binding—The calcium binding to γ-crystallin was performed by gel filtration method of Hummel-Dreyer (see Ref. 30). A representative Hummel-Dreyer gel elution chromatogram is

| Peptides | Amino acid sequence |
|----------|--------------------|
| g3       | KKKMRIRYRDFRFGKMS| |
| g3a      | KKKMRIRYRDFRFGKMS| |
| g3b      | KKKMRIRYRDFRFGKMS| |
| s3a      | GEVFLYKHNFPQDG| |

The abbreviations used are: Stains-all, 1-ethyl-2-[3-(1-ethylnaph-tho[1,2-d][lazol-2-yldiene]-2-methylpropenyl]naphtho[1,2-d][lazoli- lium bromide; MOPS, 3-(N-morpholino)propanesulfonic acid; HPLC, high pressure liquid chromatography.
plot analysis of data collected from the Hummel-Dreyer chromatography of the lanthanide probe, TbCl₃·Tb₃⁺/H₂O binds to the c-myc family (Table II).


ductive runs and found to be 90 μM, was calculated by an inverse plot (31) from several chromatography runs, carried out at different free calcium concentrations (25–100 μM calcium chloride) containing ⁴⁰Ca. A represents free calcium concentration;  in is the ratio of bound calcium to the total protein.

shown in Fig. 1A. In a calcium-equilibrated column, a high radioactivity count at the protein elution peak (void volume), which quantitated calcium bound to the protein, was followed by a trough of low radioactivity count, which represented calcium depletion following calcium binding to the protein (Fig. 1A). The gel chromatography runs were repeated at ⁴⁰Ca concentrations of 25, 50, 75, and 100 μM, keeping protein concentration constant. The dissociation constant,  for γ-crystallin, was calculated by an inverse plot (31) from several chromatographic runs and found to be 90 μM, with 4 sites for metal binding (Fig. 1B). The calcium-binding affinity of γ-crystallin (90 μM) was comparable to that of other members of this superfamily (Table II).

Probing Calcium Binding to γ-Crystallin by Stains-all Interactions—We have used the calcium-mimic dye Stains-all as a reporter to study the calcium binding properties of γ-crystallin. Stains-all displays distinct absorption and induced CD bands in the 480–660 nm region when it binds to Ca²⁺-binding sites in proteins (34–37). We have reported earlier that Stains-all binds to β- and δ-crystallin, which induce the J and γ bands of the dye, respectively (33, 35). Fig. 2A shows that Stains-all binds to γ-crystallin and induces an intense J band at 670 nm. The intensity of this band increases if the dye/protein ratio is increased. The binding is strong enough to be able to induce optical activity in the otherwise achiral dye. Upon addition of 50 μM calcium, the J band intensity decreases due to the conformational rearrangement, also shown by the individual EF-hand motif of calcium-binding proteins (38). Upon further addition of calcium, the J band intensity decreases and is finally abolished in the presence of excess calcium (300 μM) (Fig. 2B). The abolition of the J band in the presence of calcium shows that the dye binds at the calcium-binding sites in γ-crystallin (Fig. 2B).

Probing Calcium Binding to γ-Crystallin by Terbium Fluorescence—Calcium binding to γ-crystallin was probed using the luminescent lanthanide probe, TbCl₃. Tb³⁺ is known to bind to Ca²⁺-binding sites and, as a consequence, displays enhanced luminescence in the visible region (39). The ionic size and binding characteristics of Tb³⁺ are similar to those of the Ca²⁺ ions (39). Fig. 3 shows that γ-crystallin enhances Tb³⁺ fluorescence, suggesting that the protein binds the Ca²⁺-mimic lanthanide ion. The dissociation constant for terbium binding to γ-crystallin was found to be about 300 μM. The presence of aromatic residues in the vicinity of the calcium-binding site is of interest, and one would expect an interaction between the aromatic rings and the bound metal ions. Indeed, when the Ca²⁺-mimic Tb³⁺ is used, we see the luminescence of the lanthanide activated upon excitation at 285 nm (aromatic absorption region), indicative of energy transfer at an estimated distance of 5 Å (40).

Identification of Greek Key Motif as Calcium-binding Sites in γ-Crystallin—The above results clearly demonstrate that γ-crystallin is a calcium-binding protein with four sites for ion binding. Since it has no EF-hand or any such known motif for calcium binding, we thought that four calcium-binding sites may be located in the four Greek keys. Based on sequence homology with protein S, we have identified Greek keys as calcium-binding sites in γ-crystallin, as shown in Fig. 4. γ-Crystallin shows 47% sequence homology as well as similarity in the structure of 4-fold repeats (13). The residues involved in calcium ligation in protein S are known from its crystal structure (27). The motifs 1 and 3 of γ-crystallin are similar to motifs 2 and 4 in protein S, whereas motifs 2 and 4 of γ-crystallin are similar to motifs 1 and 3 of protein S (13). The alignment of individual βγ motifs of these proteins was used to identify the residues involved in calcium ligation, which shows that calcium-binding sites of γ-crystallin are located within Greek key motifs. Based on sequence alignment, we find that the first calcium ligates at Glu-7, Arg-31, Asn-33, and Ser-30, whereas the second calcium ligates at Glu-46, Arg-76, Ser-74, and Asp-73 (Fig. 4). All these residues are located at the surface of the structure, and their side chains are available for calcium binding (Fig. 5). Similarly, two other calcium-binding sites located at the C-terminal domain were also identified. The residues Glu-95, Thr-120, Glu-121, and His-123 form site 3, whereas Glu-136, Asn-162, Ala-163, and Gly-165 constitute site 4. The propensity of these residues for calcium ligation was investigated by molecular modeling. These residues were found to possess bond length and bond angles suitable for forming a bond for calcium binding to the oxygen atoms of the side chains of these residues (Fig. 5).

| Proteins         |  | Number of binding sites | Ref.  |
|------------------|---|-------------------------|-------|
| γ-Crystallin     | 90| 4                       | This work |
| βγ-Crystallin    | 170| 4                       | 18    |
| Protein S        | 27, 76| 4                       | 6     |
| Spherulin 3a     | 9, 200| 2                       | 9     |

TABLE II

KD and number of binding sites calculated for γ-crystallin

For comparison, the stoichiometry of calcium binding to other members of the βγ-crystallin superfamily are shown.

The Greek Key β-Sheet Peptides, Structure and Conformation—In order to verify if the Greek key crystallin fold is a motif for calcium ion binding, we have synthesized four-stranded 42-residue β-sheet peptide (peptide g3) corresponding to the third Greek key motif of γ-crystallin, and the first Greek key motif of spherulin 3a (residues 14–56) as control (Table I). To ascertain if the residues identified by alignment (above, and Fig. 4) participate in ligation, we have also synthesized several mutants of Greek key peptides (Table I). The two putative residues (Glu-95 and Glu-121), which were identified for cal-
Calcium binding by homology with protein S, were modified to Lys in the peptide g3a (Table I). We have also investigated whether other acidic residues are involved in calcium binding (such as two aspartates at positions 108 and 109 are replaced by Lys in the peptide g3b).

We have characterized and evaluated the secondary structure and aggregational propensity of these peptides by CD and HPLC. The wild type peptide g3 and its mutants are fairly

**FIG. 2.** Stains-all binding to \( \gamma \)-crystallin. CD spectra of Stains-all induced upon binding to \( \gamma \)-crystallin were recorded in 2 mM MOPS buffer, pH 7.2, containing 30% ethylene glycol, path length 1 cm. Dye concentration in all experiments was 16 \( \mu \)M. The ellipticity data are represented in millidegrees. A, CD spectra recorded with increasing concentration of \( \gamma \)-crystallin: 1, 0.026 mg/ml (----); 2, 0.078 mg/ml (- - -); 3, 0.13 mg/ml (-----); and 4, 0.156 mg/ml (----). B, effect of calcium on Stains-all-\( \gamma \)-crystallin complex spectra. Calcium was added in the dye-protein mixture (\( \gamma \)-crystallin concentration was 115 \( \mu \)g/ml), and CD was recorded. Calcium concentrations used are as follows: 1, no calcium (----); 2, 50 \( \mu \)M calcium (----); 3, 100 \( \mu \)M calcium (-----); 4, 200 \( \mu \)M calcium (-----); and 5, 300 \( \mu \)M calcium (-----). The J band was abolished with 300 \( \mu \)M calcium.

**FIG. 3.** Terbium ion binding to \( \gamma \)-crystallin. A solution of \( \gamma \)-crystallin (440 \( \mu \)g/ml) was titrated with terbium chloride, and fluorescence emission spectra were recorded at excitation 285 nm. The fluorescence emission intensity at 545 nm was measured and plotted against terbium chloride concentration. Inset shows the increase in terbium fluorescence upon binding to \( \gamma \)-crystallin, spectra 1 and 2 for terbium and protein only. Spectra 3–8 are with increasing concentrations of terbium chloride.

**FIG. 4.** Putative calcium-binding sites in \( \beta \)-\( \gamma \)-crystallin superfamily. Multiple sequence alignment of the crystallin domain of members of the superfamily is shown. Residues proposed to be involved in calcium binding are indicated by solid arrows showing the calcium-binding sites in members of the \( \beta \)-\( \gamma \)-crystallin superfamily. The conserved residues are shown in the boxes. Since the sequences of the domains are the repeats, the sequence of only one domain is aligned. PS, protein S; g, \( \gamma \)-crystallin; BB2, \( \beta B2 \)-crystallin.

two aspartates at positions 108 and 109 are replaced by Lys in the peptide g3b). We have characterized and evaluated the secondary structure and aggregational propensity of these peptides by CD and HPLC. The wild type peptide g3 and its mutants are fairly
soluble in aqueous medium. However, the peptide s3a is not soluble in water; it was solubilized in trifluoroethanol. These peptides form aggregates of various sizes as seen in their gel filtration profiles on HPLC (the chromatogram for g3 is shown as Fig. 6). All these peptides show a poorly organized, unfolded structure in aqueous solution. However, in increasing concentration of methanol, these peptides adopt β-sheet conformation as shown by increasing ellipticity at 216 nm (Fig. 7, a–d). These peptides form well-defined β-sheet conformation at >80% methanol/water mixture. The s3a peptide also exhibits β-sheet conformation (Fig. 7d). The designed β-sheet peptides have been shown earlier to possess unfolded structure in aqueous medium, which readily form β-sheet in organic medium (41).

**Probing Calcium Binding to Synthetic Greek Key Peptides by Direct 45Ca Binding**—The calcium binding to these Greek key peptides was performed by direct calcium binding using 45Ca overlay method. Fig. 8 shows the peptide dot-blot for three peptides, g3, g3a, and g3b. To validate the procedure, we have used the peptide, corresponding to the EF4 of neuronal calcium sensor-1 (36 residues, residues 144–179) and the control peptide s3a. Fig. 8 shows that wild type peptide g3 binds calcium as strongly as the EF-hand peptide of neuronal calcium sensor-1. However, the two modified peptides (g3a and g3b) do not show detectable binding of 45Ca. These results show that an individual peptide corresponding to a Greek key is able to bind calcium and forms a complete unit in the same way as the EF-hand does, although the binding site geometry is different. The g3a peptide in which Glu-95 and Glu-121 residues were modified to Lys does not show any binding, indicating their participation in the binding. These residues were also investigated on the molecular model and were found to be able to show the geometry suitable for binding. The other peptide, g3b, also does not show any significant signal since the affinity was decreased due to the modification of aspartic acid residues 108 and 109 to Lys (Fig. 8).

**Probing Calcium Binding to Synthetic Greek Key Peptides by Calcium Probe Stains-all**—The calcium binding to individual Greek key peptides was further evaluated using the more sensitive assay of Stains-all binding. This assay is suitable and convenient for comparing closely related calcium-binding proteins and peptides. A similar approach was used previously to study and compare the calcium binding properties of individual EF-hand peptides of calmodulin (38). The magnitude of the induced J or γ band is a direct indicator of the affinity toward calcium. The g3 peptide binds Stains-all and induces a strong J band and a weak γ band (Fig. 9a). The addition of calcium decreases the J band intensity. There is no induction of CD band in the peptide g3a and Stains-all complex at the higher dye:peptide ratio. However, the CD band induction is seen when higher concentrations of peptide are used (Fig. 9c). The binding of Stains-all to modified peptide g3a is decreased to several fold indicating the role of these two amino acid residues in calcium binding (Fig. 9c). When dye binding was performed with peptide g3b, we found that this peptide binds the dye and induces the γ band indicating that it has lost the calcium-binding ability completely, although the geometry and microenvironment of the binding site is altered due to modifications of acidic residues to basic residues (Fig. 9d). The addition of calcium in g3a and g3b decreases the CD band intensity indicating the replacement of the dye (data not shown). The control peptide, S3a, also binds the dye and induces a calcium-sensitive band at 470–475 nm (data not shown). These results show that although the 45Ca binding was not seen in the dot-blot assay for peptides g3a and g3b, we were able to see the dye binding indicating the decreased affinity. The affinity of these peptides calculated based on induced CD band are in the order of g3 > g3b > g3a.

**Calcium Binding Does Not Influence Conformational Changes in Secondary Structure**—Structural changes upon calcium binding to γ-crystallin were monitored by CD and fluorescence. γ-Crystallin shows β-sheet structure in the far-UV CD with a minimum at 218 nm. Addition of calcium does not induce any significant changes in the secondary and tertiary
structures (data not shown). Similar observations were also obtained for individual Greek key peptides (data not shown). These results were confirmed by the inability of calcium to induce any change in protein Trp fluorescence (data not shown). These results are relevant for a compact and stable protein such as \( \beta \)-crystallin, which does not undergo conformational changes in tertiary structure upon calcium binding. It is relevant to mention that other proteins of the superfamily, protein S and \( \beta \)-crystallin, also do not undergo any conformational changes upon binding calcium.

**DISCUSSION**

We have probed calcium binding to \( \gamma \)-crystallin both by direct (Hummel-Dreyer method using \(^{45}\)Ca) and indirect (terbium fluorescence and Stains-all interaction) methods. \( \gamma \)-Crystallin binds \( \text{Tb}^{3+} \) and induces fluorescence through energy transfer from the two Trp present, one in each domain. It binds the calcium probe Stains-all in a calcium-dependent manner. We have also shown that a peptide corresponding to a Greek key crystallin fold is able to bind calcium, indicating this to be a site for ion binding. By mutating the potential residues, we have identified the amino acids, which participate in ligation. These results clearly show that \( \gamma \)-crystallin is a calcium-binding protein, with binding affinity in the range of 90 \( \mu \)M and with 4 sites for calcium binding. The binding affinity of calcium to \( \gamma \)-crystallin (90 \( \mu \)M) is more than that of \( \beta \)-crystallin (170 \( \mu \)M), another calcium-binding protein of the eye lens (6).

**Greek Key Crystallin Fold Is a Calcium-binding Motif**

For the first time, we have shown that the four-stranded Greek key \( \beta \)-sheet peptide corresponding to crystallin fold forms an individual calcium-binding site. These peptides adopt \( \beta \)-sheet conformation in water/methanol mixture and form aggregates producing anti-parallel \( \beta \)-sandwich motif as shown by far-UV CD (Fig. 7). Our results of alignment of relevant regions of proteins that contain this fold and of four-stranded Greek key peptides used in this study (Fig. 4) show that the first calcium ligates at the residue next to the conserved aromatic amino acid of the sequence Y/F/WXXXXXXG, which is located at the end of the first \( \beta \)-strand ("a" strand). The amino acid at this position is generally Asp, Asn, Glu, Gln, Ser, Tyr, and rarely Lys. Other
concentration was 57 mg/ml. Calcium was added to the dye-peptide mixture (peptide concentration of the wild type peptide g3; 0.013 mg/ml (57), 0.07 mg/ml ( ), and 0.057 mg/ml ( ), b, effect of calcium on Stains-all- peptide complex. Calcium was added to the dye-peptide mixture (peptide concentration was 57 mg/ml) and calcium concentrations used were: no calcium ( ), 0.7 mM calcium ( ), and 7.5 mM calcium ( ). c and d represent the Stains-all binding of the modified peptides, g3a and g3b. Identical concentrations of dye and peptides were used.

three residues needed for calcium ligation lie just before the beginning of the fourth β-strand (before the conserved Ser) and are usually Asp, Asn, Ser, Thr, Val, or Ala (Fig. 5). Clout et al. (42) have shown similarity in the calcium-binding sites in spherulin 3a and protein S and pointed out the role of these residues in ligation. Our results with Greek key peptides directly demonstrate the importance of these residues in ion binding. The role of conserved Ser in calcium ligation has already been shown earlier (14). Although there is a stretch of acidic residues in the “b” and “z” strands, they do not directly participate in the ion binding as shown in our results with peptide g3b. However, modifying the aspartate pair (Asp-108 and Asp-109) partially decreases the affinity toward calcium and alters the microenvironment of the peptide g3b as shown by Stains-all binding, since these residues are known to form a part of the cluster of alternate sign affecting the molecular surface (12).

βγ-Crystallin Superfamily Represents a Novel Class of Calcium-binding Protein Family—Our results demonstrate that Greek key crystallin fold forms a motif for calcium ion binding in βγ-crystallin superfamily. The calcium binding properties of protein S, β-crystallin, and spherulin 3a have already been reported (6, 14, 17). Our results suggest that other proteins of the superfamily, which have not been shown so far to bind calcium, would also bind the cation. In fact, we have confirmed this point by studying another non-lens member of the superfamily, AIM1 (absent in melanoma). We have found that a single crystallin domain of AIM1 (1022–1117 residues), comprising two Greek keys, binds calcium with a comparative affinity to γ-crystallin, which further corroborate our results (data not shown).

The presence of topologically homologous residues in members of the superfamily makes us suggest that this family represents a novel class of calcium-binding proteins. Conformationally, these are all-β proteins, and the binding site is located within the Greek key topology. As the geometry of this motif is distinct from that of the other calcium-binding motifs, we propose that the crystallin fold is a novel calcium-binding motif.

It is interesting to note that calcium binding does not induce marked changes in crystallin conformation, which may be due to the stable and compact structure of the fold. However, the stability of γ-crystallin is increased in the presence of calcium ions, which suggests that the presence of calcium restricts unfolding. Two other proteins of the superfamily, protein S and spherulin 3a, also exhibit a similar enhancement in the stability upon calcium binding (43, 44). Members of this family may act as a buffer in the set processes dealing with the uptake, sequestration, and transport of calcium ions, since calcium-binding changes are larger in the sensor proteins than in buffer proteins.

In the physiological context, the calcium binding properties of β- and γ-crystallins would ensure that cytosolic levels of calcium in the lens are maintained in a steady or homeostatic condition, since any change in free calcium levels can lead to opacification (1, 2). As the eye lens ages or turns cataractous, structural alterations and damage occur to its components, including the crystallins. These would be expected to lead to alterations in the Ca2+-binding and -storing ability of these molecules and to the release of Ca2+ ions, which would in turn trigger lens opacification.

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