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Calcium-binding to lens βB2- and βA3-crystallins suggests that all β-crystallins are calcium-binding proteins

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Keywords

βA3-crystallin; βB2-crystallin; βγ-crystallins; calcium-binding crystallin; Greek key motif

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Crystallins are the major proteins of a mammalian eye lens. The topologically similar eye lens proteins, β- and γ-crystallins, are the prototype and founding members of the βγ-crystallin superfamily. βγ-Crystallins have until recently been regarded as structural proteins. However, the calcium-binding properties of a few members and the potential role of βγ-crystallins in fertility are being investigated. Because the calcium-binding elements of other member proteins, such as spherulin 3a, are not present in βB2-crystallin and other βγ-crystallins from fish and mammalian genomes, it was argued that lens βγ-crystallins should not bind calcium. In order to probe whether β-crystallins can bind calcium, we selected one basic (βB2) and one acidic (βA3) β-crystallin for calcium-binding studies. Using calcium-binding assays such as ^{45}Ca overlay, terbium binding, Stains-All and isothermal titration calorimetry, we established that both βB2- and βA3-crystallin bind calcium with moderate affinity. There was no significant change in their conformation upon binding calcium as monitored by fluorescence and circular dichroism spectroscopy. However, ^{15}N-^{1}H heteronuclear single quantum correlation NMR spectroscopy revealed that amide environment of several residues underwent changes indicating calcium ligation. With the corroboration of calcium-binding to βB2- and βA3-crystallins, we suggest that all β-crystallins bind calcium. Our results have important implications for understanding the calcium-related cataractogenesis and maintenance of ionic homeostasis in the lens.

Crystallins are abundant proteins found in the eye lens of vertebrates that belong to two superfamilies named as α-crystallins and βγ-crystallins [1]. α-Crystallins are known to play an important role as molecular chaperone [2]. On the other hand, βγ-crystallins are thought to play structural role in the mammalian eye lens. Their nonstructural functions, which appear to be very important, have not been elucidated [3].

β-Crystallins from vertebrate eye lens are a group of seven proteins broadly classified into four acidic (βA1/A3, βA2 and βA4) and three basic β-crystallins (βB1, βB2, and βB3). β-Crystallins have high sequence similarity and identity [4]. Acidic β-crystallins have both N- and C-terminal extensions, whereas basic β-crystallins have only N-terminal extensions. All β-crystallins have four Greek key motifs organized into two crystallin domains. In this respect, β-crystallins are similar to γ-crystallins, which also have a similar domain organization and structure [5,6]. The major difference between the β- and γ-crystallins is their oligomeric state. γ-Crystallins are monomeric, whereas β-crystallins exist as dimers to octamers in solution [7]. β- and γ-crystallins are the prototype and founding members of the βγ-crystallin superfamily [8,9].

Abbreviations

AIM1, protein absent in melanoma 1; HSQC, heteronuclear single quantum correlation; ITC, isothermal titration calorimetry; PDB, protein databank; TCEP, Tris(2-carboxyethyl) phosphine hydrochloride.

βγ-Crystallin superfamily consists of members from various taxa having the characteristic crystallin-type Greek key motifs [8,10]. Some well studied members of the superfamily are Protein S [11,12], spherulin 3a [8,13], protein absent in melanoma 1 (AIM1) [14,15], geodin [16], ciona crystallin [17], yersinia crystallin [18] and cargo proteins from *Tetrahymena* [19].

Except for some conserved residues present at crucial positions, there is not much sequence similarity among the diverse proteins of the βγ-crystallin superfamily. Recently, it has been proposed that these βγ-crystallins might play unknown and unconceived noncrystallin roles [3]. These 'noncrystallin roles' have not been elucidated to date. We are interested in understanding the nonstructural functions of βγ-crystallins. Previously, we reported that γ-crystallins bind calcium [20], and therefore, might be involved in maintaining calcium homeostasis in lens. Recently, βB2-crystallin has been implicated in the subfertility of mice expressing mutant βB2-crystallin [21]. Some proteins of the superfamily, Protein S, spherulin 3a [10], βγ-crystallin domains of AIM1 [14,15], yersinia crystallin [18], geodin [22] and ciona crystallin [17] are known to bind calcium ions.

However, the binding of calcium to β-crystallins is inconclusive and highly debatable [10,23], even though the aggregated form of β-crystallins, β_H-crystallin, isolated from bovine lens homogenate was shown to bind calcium [24,25]. Sequence D/NXXS, which is involved in calcium-binding in Protein S, spherulin 3a and in an invertebrate ciona crystallin [17,23,26], is not conserved in vertebrate lens β-crystallins. Furthermore, the calcium-ligating side chains and the backbone conformation of spherulin 3a are structurally not conserved in βB2-crystallin [23]. Accordingly, it has been argued that β-crystallins should not bind calcium. In the light of these contradictory observations, it is important to investigate whether β-crystallins from vertebrate lenses bind calcium or not.

In this context, to establish calcium-binding to the individual β-crystallins, we have selected a basic (βB2-crystallin) and an acidic (βA3-crystallin) subunit as representative members of β-crystallins. Using number of assays for proving specificity of calcium-binding, we have conclusively demonstrated that both acidic and basic β-crystallins bind calcium with varying affinity, thus suggesting that all β-crystallins would bind calcium. Calcium-binding does not influence protein conformation, a property exhibited by some of the calcium-binding members of the βγ-crystallin superfamily [14,15,20]. Based on our results, together with the published data on calcium-binding to a few other members, we suggest that calcium-binding is a prevalent property of the βγ-crystallin superfamily. Demonstration of calcium-binding to β-crystallins would fill an important and missing link in our existing knowledge about βγ-crystallins as calcium-binding proteins and understanding their function in maintaining calcium homeostasis in the lens, which is implicated in cataracts.

Results and Discussion

Selection of β-crystallins

The sequence alignment of seven β-crystallins [four acidic (A1–A4) and three basic (B1–B3) crystallins] is shown in Fig. 1. There is 45–60% sequence identity between different β-crystallins [4]. We have selected one acidic (βA1/A3-crystallin) and one basic (βB2-crystallin) subunit as representatives of all β-crystallins for probing the calcium-binding properties. We have selected βB2-crystallin because it is the major crystallin among all β-crystallins and its 3D structure is known [27]. βA1- and βA3-crystallins are identical in sequence except for N-terminal extension of 17 amino acids in βA3-crystallin. Moreover, these β-crystallins have been widely studied for structural properties and hetero- and homo-domain interactions with each other as well as with other β-crystallin subunits [7,28]. These proteins have been predicted not to bind calcium [10,17,23]. We believe that studying these two β-crystallins would provide an insight into the calcium-binding properties of all β-crystallins.

Overexpression and purification

Bovine βB2- and βA3-crystallin were cloned in expression vector and overexpressed in *Escherichia coli* as recombinant proteins. Proteins were purified using a combination of chromatographic methods. The purity of each batch of protein was confirmed by examining the samples on SDS/PAGE (supplementary Fig. S1). Protein solutions were treated with Chelex-100 for removing divalent ions and used as fresh as possible for further calcium-binding studies, otherwise the proteins were stored frozen at −80 °C.

Calcium-binding to βB2- and βA3-crystallins

Because there is no known motif for calcium-binding in βB2- and βA3-crystallins, it was therefore necessary that calcium-binding should be assayed by several specific methods. We used well-known calcium probes, Stains-All (Sigma-Aldrich, St Louis, MO, USA) and terbium binding to assess the calcium-binding. We also

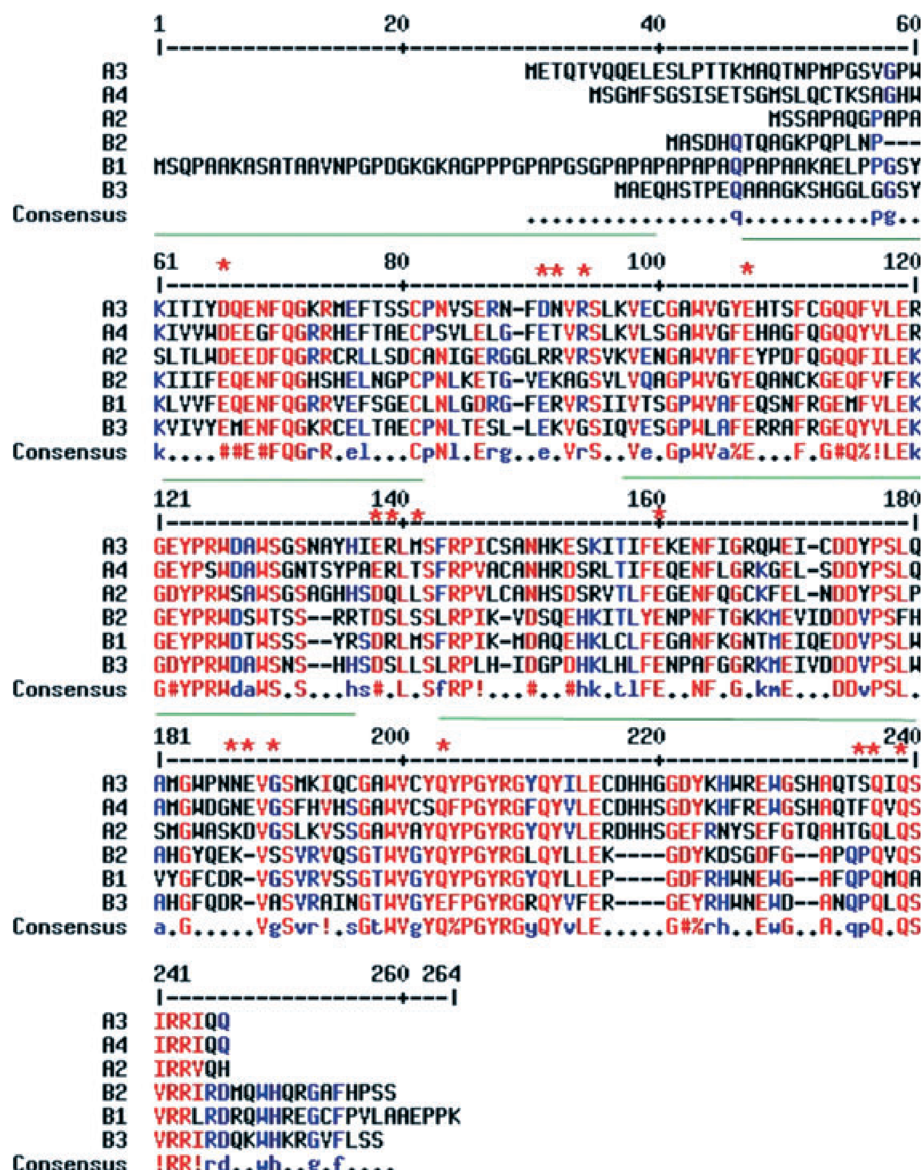

Fig. 1. Sequence alignment and putative calcium-binding sites: Amino acid sequences of six bovine β-crystallins were aligned using Multialin. Putative calcium-binding residues are indicated by asterisks. Green line marks the Greek key motif.

used direct calcium-binding on membrane using ^{45}Ca. The binding constants and other thermodynamic parameters were determined using isothermal titration calorimetry.

Probing calcium-binding by Stains-All assay

Calcium-binding to βB2- and βA3-crystallins was evaluated by calcium probe Stains-All, a carbocyanine dye [29]. The dye binds the recombinant βA3- and βB2-crystallins and induces a strong J band at 660 nm (Fig. 2). The intensity of the circular dichroic band decreases upon addition of calcium ions because calcium displaces the dye bound to calcium-binding sites of the protein. Other proteins of this superfamily, namely γ-crystallin [20] and AIM1-g1 [15] also induce the J band of the dye indicating similarity in the microenvironment of the dye-binding site [30]. Calcium saturated proteins exhibited no binding to Stains-All dye, suggesting higher affinity of the cation for the calcium-binding site than the dye. Calcium displaced Stains-All to a lesser extent from βA3-crystallin than from βB2-crystallin, indicating lower affinity of calcium for the former compared to the latter.

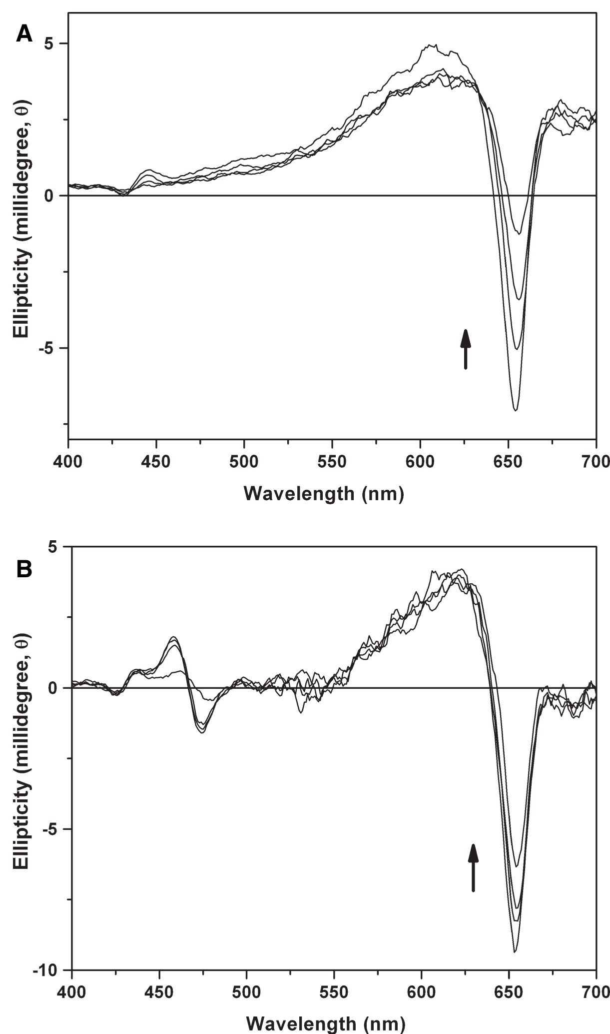

Fig. 2. Stains-All binding to (A) βB2- and (B) βA3-crystallins: 100 μg of either βB2- or βA3-crystallin protein was added to Stains-All dye in 2 mM Mops/NaOH (pH 7.2) and 30% ethylene glycol and CD spectra were recorded from 400–700 nm. (A) Calcium was added to a final concentration of 25, 300 and 5300 μM. (B) calcium was added to a final concentration of 0.5, 1.5 and 8.5 mM. Arrows indicate increasing concentrations of calcium.

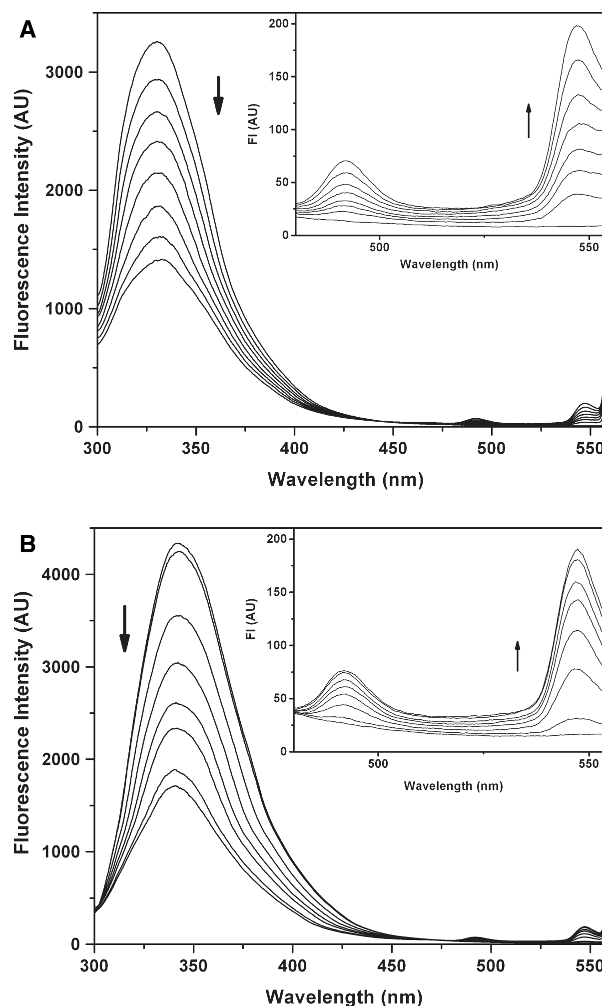

Fig. 3. Terbium binding to β-crystallins: (A) 7.68 μM of βB2- and (B) 22.68 μM of βA3-crystallin were excited at 285 nm and emission spectra recorded from 300–560 nm. Terbium was added to a final concentration of 0, 5, 25, 45, 65, 85, 300, 700 μM to βA3-crystallin and 0, 15, 35, 55, 85, 500, 1200 and 3200 μM to βB2-crystallin. Inset shows the region from 480–555 nm. Arrows indicate increasing concentrations of terbium.

Probing calcium-binding by terbium

We also probed calcium-binding using another calcium probe, terbium. The ionic radius of terbium is similar to that of calcium, thus making it an ideal choice for use as a calcium mimic probe [31]. Terbium ions bind to the calcium-binding sites in proteins and induce luminescence peaks at 492 nm and 547 nm via energy transfer from Trp and Tyr residues [32]. Terbium binds to βB2- and βA3-crystallins and induces luminescence peaks at 492 and 547 nm (Fig. 3). The enhanced luminescence of terbium in the presence of these crystallins indicates that Tyr and Trp residues are in the vicinity of the calcium-binding site. The sequence of β-crystallins has several Tyr and Trp residues distributed around the putative calcium-binding residues of both crystallins, resulting in the observed increase in intensity (Fig. 1). Similar results were observed with the D2 domain of yersinia crystallin [18], which also had a Trp residue near the second calcium-binding site. We also carried out a terbium–calcium competition assay. βB2- and βA3-crystallins presaturated with calcium showed increased fluorescence intensity upon adding increasing concentrations of terbium, which indicated that terbium displaced the bound calcium. This is expected because terbium ions have a higher affinity

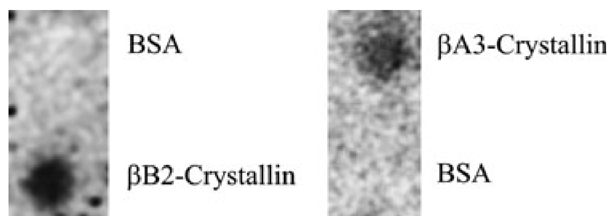

Fig. 4. ^{45}Ca overlay: 50 μg of BSA, βB2- and βA3-crystallins were spotted on a nitrocellulose membrane. The processed membrane was exposed to imaging plate before scanning in a phosphor imager (Fuji FLA-3000).

than calcium for calcium-binding sites in the protein due to the higher positive charge of terbium than calcium [31].

Calcium-binding by ^{45}Ca overlay method

Calcium-binding was also demonstrated by direct ^{45}Ca-binding using the membrane overlay method [33]. This simple and direct assay has been widely used to ascertain the cation binding to calcium-binding proteins. Both β-crystallins immobilized on nitrocellulose membrane bound calcium, whereas the negative control BSA did not show any binding (Fig. 4). The buffer used for this assay contained $MgCl_2$, another divalent cation that usually competes for calcium-binding sites in proteins, despite which we observed positive signal from βA3- and βB2-crystallin immobilized on the membrane. This demonstrates the specificity of these proteins for calcium unlike EF-hand proteins, which bind both calcium and magnesium. In control experiments, we have carried out ^{45}Ca-binding to these crystallins in the presence of cold $CaCl_2$ and found that the signal was abolished (data not shown).

Calcium-binding by isothermal titration calorimetry

The cation-binding constants of both crystallins were determined by isothermal titration calorimetry (ITC) measurements. Calcium-binding to βB2-crystallin is an exothermic reaction (Fig. 5A). The integrated heats of injection of calcium titration to βB2-crystallin best fitted to a sequential binding model with four sites. By varying the initialization parameters of the fitting procedure, it was determined that the fit was stable and no other model and parameter set could provide a

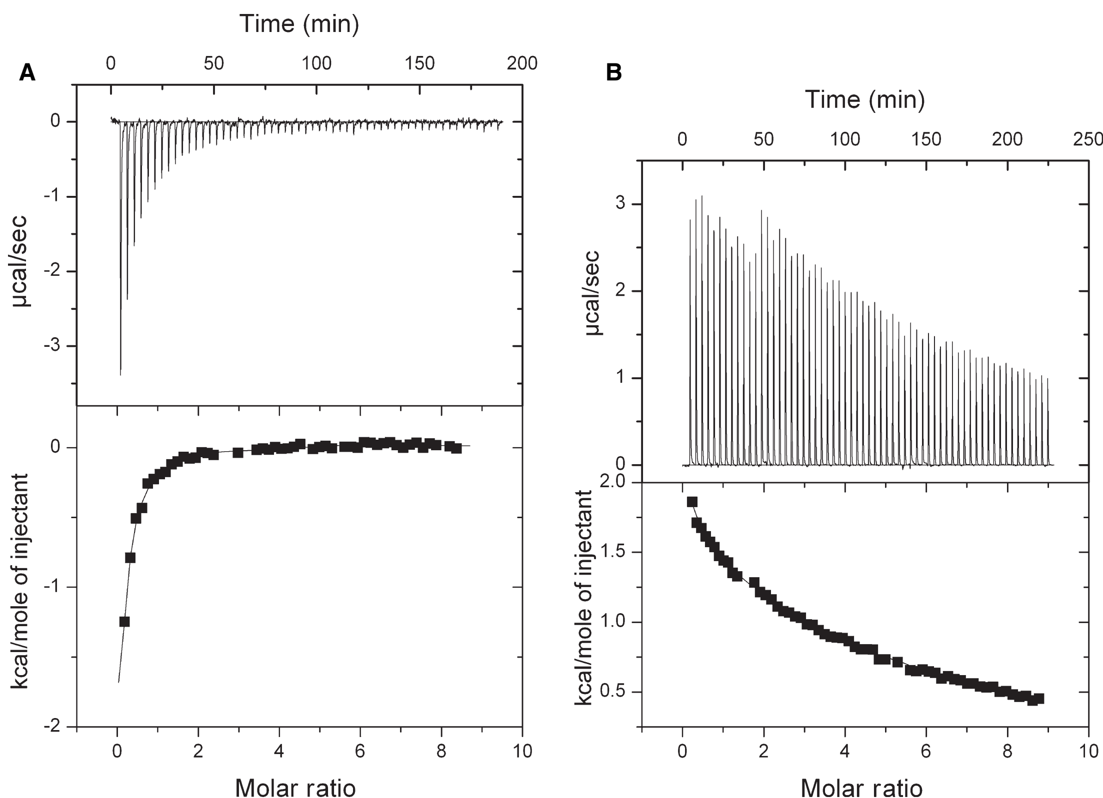

Fig. 5. Isothermal titration calorimetry: (A) calcium-binding isotherm of βB2-crystallin. (B) Terbium binding isotherm of βA3-crystallin. The best fit to four-site sequential binding model is shown in the lower panels.

Table 1. Binding constants and the enthalpy change of calcium- and terbium-binding to βB2- and βA3-crystallins. *K*, dissociation constant (M); Δ*H*, enthalpy change of binding (kcal·mol^{-1}).

Parameters	βB2-Crystallin (calcium-binding)	βA3-Crystallin (terbium binding)
K_1	(2.15 ± 1.3) × 10^{-4}	(1.08 ± 0.08) × 10^{-4}
K_2	(1.65 ± 0.98) × 10^{-4}	(1.46 ± 0.09) × 10^{-4}
K_3	(8.33 ± 7.63) × 10^{-5}	(4.03 ± 0.3) × 10^{-5}
K_4	(5.71 ± 4.89) × 10^{-4}	(2.72 ± 0.13) × 10^{-3}
ΔH_1	2.75 ± 0.65	2.76 ± 0.07
ΔH_2	4.0 ± 1.0	2.15 ± 0.23
ΔH_3	−2.4 ± 0.86	−0.98 ± 0.26
ΔH_4	0.61 ± 0.28	13.5 ± 0.34

better fit. The dissociation constants of calcium-binding to βB2-crystallin range from 0.16 mM to 83 μM (Table 1). These results reveal the presence of four calcium-binding sites with moderate to low affinity.

Stains-All and terbium-binding studies indicated that βA3-crystallin has relatively lower affinity for the cation than βB2-crystallin. Calcium-binding to βA3-crystallin studied by ITC resulted in poor signal as expected and, thus, this method was unsuitable for determining the binding constants of calcium to βA3-crystallin (data not shown). We, therefore, carried out terbium binding to this crystallin by ITC and determined the binding constant for the calcium mimic probe. Terbium is believed to bind strongly to calcium-binding sites of proteins compared to calcium due to its higher charge ratio than calcium, even though both ions have similar ionic radii [31]. The dissociation constants of terbium-binding to βA3-crystallin range from 2.7 mM to 40 μM (Table 1). The low affinity might explain the nonsaturating nature of binding thermogram (Fig. 5B). Calcium is thus likely to bind to βA3-crystallin with lower affinity than terbium.

The above results using specific assays for calcium-binding, suggest that both βB2- and βA3-crystallins bind calcium with moderate affinity. We have observed that these proteins lose the calcium-binding ability upon storage and specific precautions, such as the use of freshly prepared protein, are required to perform calcium-binding experiments.

Effect of calcium on protein conformation

We further studied the effect of calcium-binding on the conformation, stability and hydrodynamic radii of these crystallins using fluorescence spectroscopy, CD spectroscopy, differential scanning calorimetry, analytical gel filtration and dynamic light scattering.

Trp fluorescence emission spectra

Trp fluorescence emission spectrum is an important tool in probing the microenvironment of Trp residues in proteins. We used this to probe the changes in the polarity of Trp residues upon calcium-binding. βA3- and βB2-crystallins exhibited emission maxima at 342 and 333 nm, respectively, indicating that Trp residues in both proteins are in nonpolar environment (Fig. 6). Calcium-binding does not induce any significant changes in the emission spectra of both crystallins; however, only minor changes were seen in case of βA3-crystallin (Fig. 6B).

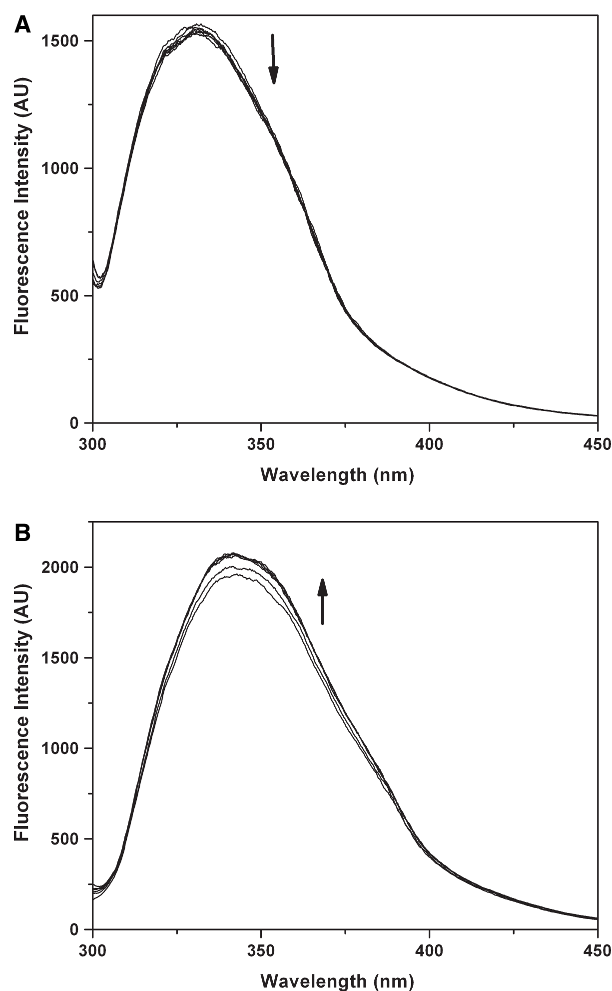

Fig. 6. Fluorescence spectroscopy: 7 μM of each protein was excited at 295 nm and emission recorded from 300–450 nm. Calcium was added to the desired concentration and incubated for 5 min before recording the emission spectra. (A) Emission spectra of βB2-crystallin: Final concentration of calcium added was 0, 0.5, 2, 12, 30, 100, 1000 μM (B) Emission spectra of βA3-crystallin: final concentration of calcium added was 0, 0.5, 1,6, 24, 80, 1000, 2000, 3000 μM. Arrows indicate an increasing concentration of calcium.

Far- and near-UV CD spectroscopy

The native state of the recombinant proteins as well as structural changes upon calcium-binding were monitored by far- and near-UV CD spectroscopy (Fig. 7). Far-UV CD spectra of both crystallins have a minima around 218–220 nm characteristic of β-sheet conformation. There is a slight change in the spectra in the region below 200 nm upon addition of calcium; however, secondary structure fractions of apo and holo forms calculated using the program CDNN [34] indicated no significant changes in both the proteins.

The near-UV CD spectra of βB2- and βA3-crystallins are dominated by a broad band in the 255–285 nm region, indicating the contribution from aromatic amino acids and Cys (there are 5 Trp, 9 Tyr, 8 Phe and 2 Cys in βB2-crystallin and 9 Trp, 11 Tyr, 8 Phe and 8 Cys in βA3-crystallin) (Fig. 8). There is no significant change in the near-UV CD spectra of both proteins upon titration with calcium, corroborating our results of far-UV CD and Trp fluorescence spectroscopy.

2D NMR spectroscopy

Each crosspeak in the ^{15}N-^{1}H heteronuclear single quantum correlation (HSQC) spectrum of a protein represents an amide bond of amino acids in the

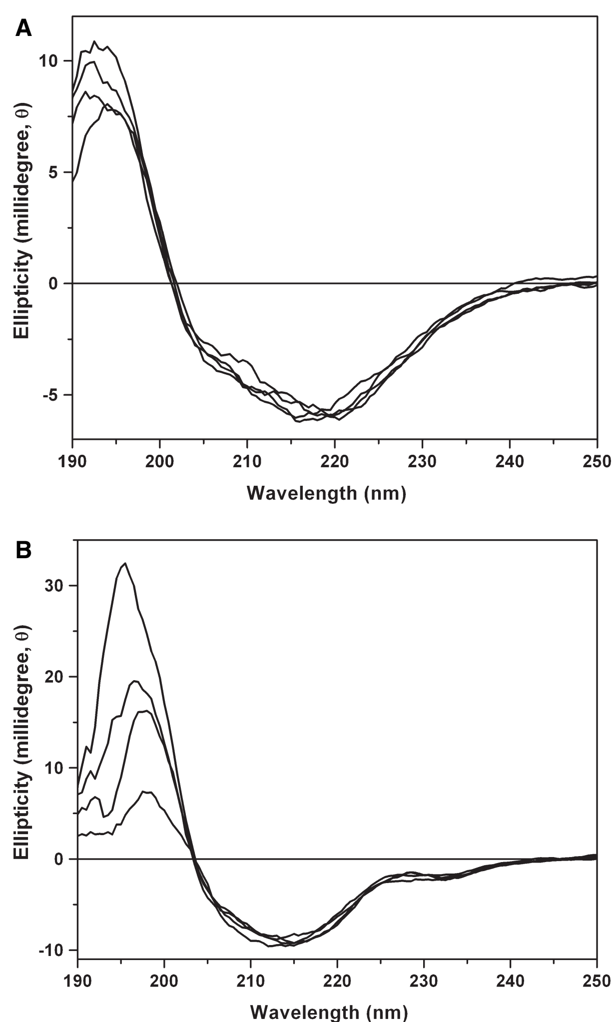

Fig. 7. Far-UV CD spectroscopy: (A) 0.71 mg·mL^{-1} of βB2-crystallin and (B) 2.1 mg·mL^{-1} of βA3-crystallin in 10 mM Tris-Cl (pH 7.5) and 30 mM KCl was used for recording the far-UV CD spectra. Calcium aliquots were added from a standard stock solution to a final concentration of 0, 0.1, 1 and 10 mM to βB2-crystallin and 0, 0.5, 1 and 5 mM to βA3-crystallin.

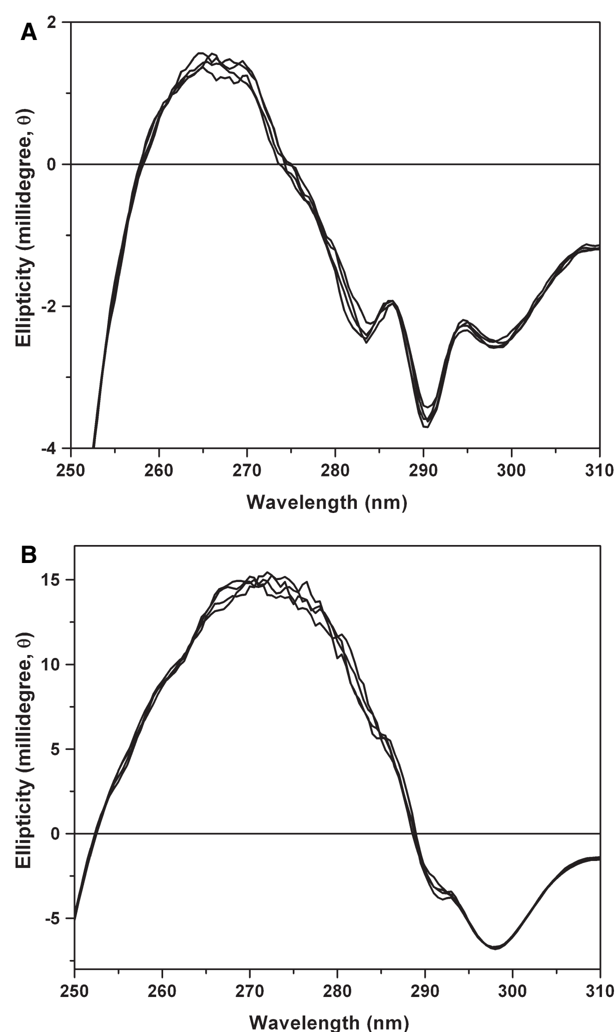

Fig. 8. Near-UV CD spectroscopy: (A) 1.1 mg·mL^{-1} of βB2- and (B) 0.65 mg·mL^{-1} of βA3-crystallin was used for recording the far-UV CD spectra. Calcium was added from a standard stock solution to a final concentration of 0, 0.1, 0.5, 1.5 and 3.5 mM each to either βB2- or βA3-crystallin.

protein. Perturbation of these crosspeaks upon ligand-binding is an indication of changes in the microenvironment of that residue. Sensitivity enhanced 2D [^{15}N-^{1}H] HSQC spectra were recorded. We used this technique to determine the changes in ^{15}N-^{1}H HSQC spectra of the βB2-crystallin upon calcium-binding (Fig. 9). Three spectra corresponding to apo, half-saturated and saturated proteins have been overlapped for comparison. Some of the residues marked in the box underwent changes in peak intensity and position in the 2D ^{15}N-^{1}H HSQC spectrum upon calcium titration, suggesting calcium ligation. The large size of the protein due to known homodimerization and higher oligomer formation with increasing protein concentration makes it difficult to carry out the necessary 3D NMR experiments for assignment of residues of this protein [35]. Also, a number of structures for βB2-crystallin are available in protein databank (PDB) structures solved by X-ray crystallography [6,27,36,37].

We also carried out the differential scanning calorimetry, analytical gel filtration and dynamic light scattering of the apo and holo forms of βA3- and βB2-crystallins. There was no significant change in the stability and hydrodynamic radius of the both forms of proteins (data not shown).

These properties are similar to the results on few other proteins of this superfamily such as γ-crystallin [20], AIM1-g1 [15], AIM1-g5 [14] and D2 domain of yersinia crystallin [18], in which calcium-binding does not cause significant changes in protein conformation. This might suit to their function as calcium buffers because they are not expected to transduce signals as calcium sensors by conformational change upon calcium-binding.

All β-crystallins are calcium-binding proteins

We have for the first time evaluated the calcium-binding properties of two widely studied representative proteins of β-crystallins, βB2- and βA3-crystallin, both by direct (^{45}Ca-binding to protein on membrane and by ITC) and methods using calcium-mimic probes (terbium and Stains-All binding). Our results conclusively demonstrate that both proteins bind calcium with moderate affinity with no change in their conformation, stability and hydrodynamic radii. Proteins with moderate to low affinity for calcium are also known, such as calsequestrin (with a dissociation constant of approximately 1 mM) [38] and calreticulin [39] belonging to the EF-hand superfamily. There is high sequence similarity in all β-crystallins, and we therefore suggest that all seven β-crystallins would bind calcium.

Putative calcium-binding sites

Each Greek key motif of spherulin 3a and Protein S contains a D/ND/NXXSS sequence element at the loop between c–d strands, and the elements in two motifs combine to form two symmetrical calcium-binding sites in each crystallin domain [23,26]. This sequence element is not exactly present in β-crystallins, which could explain the comparatively moderate affinity of these proteins as shown by our data. It has been proposed that similar calcium-binding sites are also present in the γ-crystallins [20]. A peptide corresponding to the third Greek key motif of γ-crystallin was shown to bind calcium whereas mutation of binding residues abolished binding, suggesting that the motif is the minimal entity required for calcium ligation [20]. The first Greek key motif of βA3/A1-crystallins has the sequence signature 'DNVRS', similar to the 'D/NXXS' sequence of microbial crystallins, whereas others are diverse (Fig. 1). Based on the comparison with Protein S and spherulin 3a, we suggest that homologous residues in βA3- and βB2-crystallins, would participate in calcium ligation, as indicated in Fig. 1. We used 3D coordinates of βB2-crystallin (PDB id 1BLB) to identify the putative calcium-binding site via the WEBFEATURE interface [40] (supplementary Fig. S2). It will be of great interest to define this binding motif more precisely by detailed structural analyses from the diverse members of this superfamily, particularly from vertebrate homologues.

Low levels of contaminating calcium ions are usually found in laboratory solutions. Although the crystal structures of βB2-, βB1- and γ-crystallins have been solved, calcium ion was not noticed in their solved structures [6,41,42]. This could be due to several technical reasons. However, the most probable reasons are the acidic pH inconducive for calcium-binding, the use of calcium chelating phosphate buffer or protein modification during the long course of incubation resulting in loss of calcium-binding ability. The prolonged time required for crystallization may result in loss of the labile, moderate to low affinity cation-binding ability of these proteins. *In vitro*, we have observed that purified protein looses its calcium-binding ability upon storage. We encountered difficulties in carrying out ITC of several batches of βB2-crystallin, which were not used fresh after purification. As seen in supplementary Fig. S3, the signal was abolished to a large extent and extraction of any meaningful binding parameters was difficult. Such problems are not unusual and have been observed in the case of several other calcium-binding proteins. We have also shown previously that, despite the absence of a clear and divergent D/ND/NXXSS sequence, γ-crystallin and

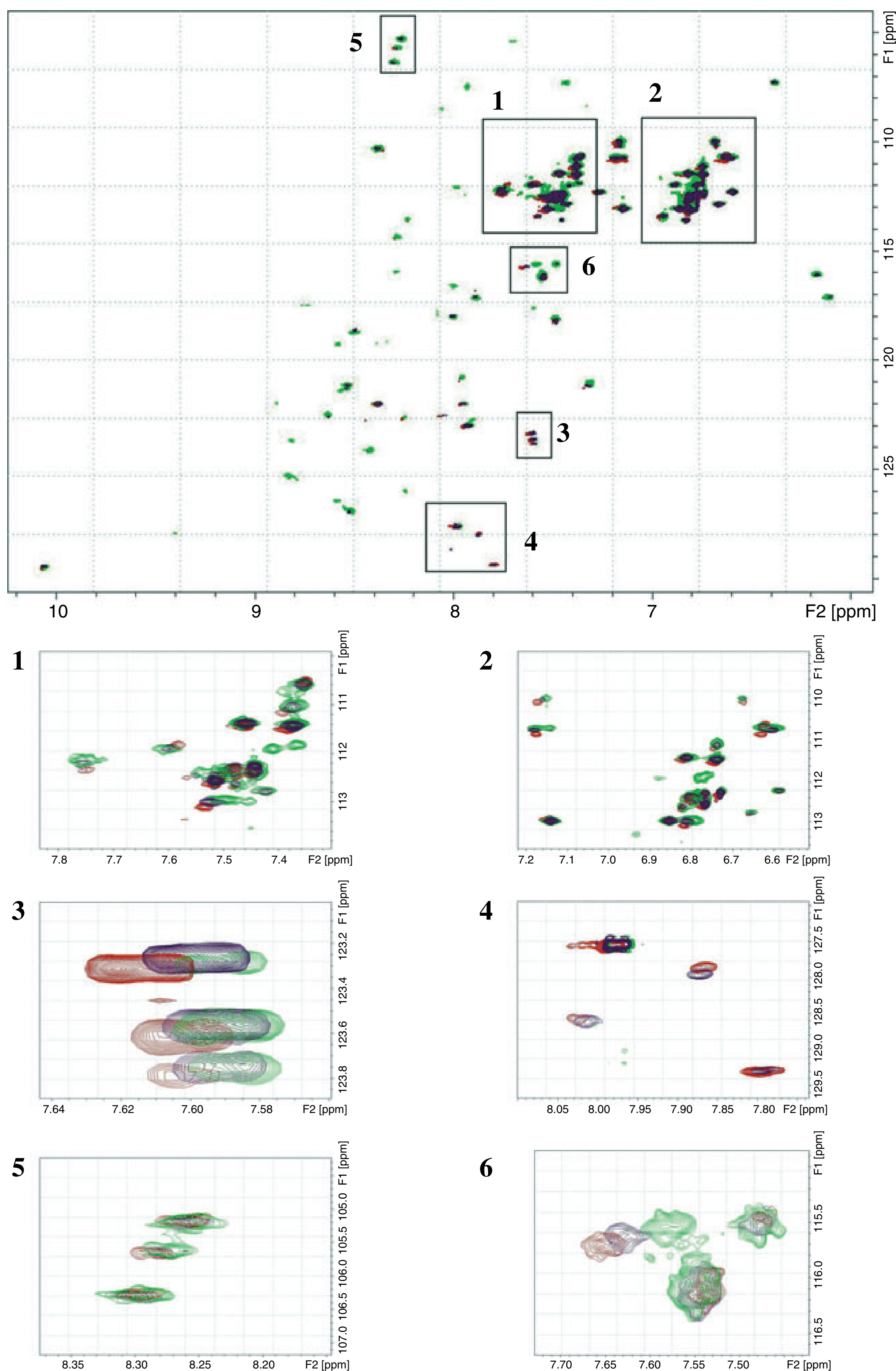

Fig. 9. 2D ^{15}N-^{1}H HSQC spectra. The figure represents the overlap of apo, half-saturated and calcium-saturated (green, purple and red colored contours, respectively) HSQC spectra of ^{15}N-labelled βB2-crystallin. Boxes in the lower panel are magnified for ease of visualization.

AIM1-g1 bind calcium with affinity equivalent to microbial crystallins [15,20].

Implications of calcium-binding to crystallins in cataract – a noncrystallin function

It has been known for a long time that abnormal levels of free calcium are deleterious for the transparency of the lens [43,44]. The mechanisms and components involved such as sensors, buffers and modulators for maintaining calcium homeostasis in the lens are not known. Electron tomographic studies [45] have indicated that most of the calcium in lens is bound to the targets in fiber cell cytoplasm, with very little bound to phospholipids near the membranes. They have suggested the presence of proteins as calcium buffer in lens fiber cells. The moderate millimolar affinity and high capacity calcium-binding of β-crystallins owing to their high concentration in the lens indicate their potential role in calcium sequestration. In other words, these calcium-binding crystallins appeared to have been recruited for this specialized function in the lens during evolution. However, the physiological relevance of calcium-binding to lens-crystallins remains to be experimentally established. Earlier studies have linked βB2-crystallin expression in extra-lenticular tissues to calcium dependent stress management [46–48]. Recently, mice harboring Philly mutation in βB2-crystallin were found to be subfertile [21]. These studies implicate the importance of βB2-crystallin expression for normal physiological functions in nonlenticular tissues.

In conclusion, our data demonstrate that all β-crystallins are moderate affinity calcium-binding proteins. These results add one more calcium-binding protein to a growing list of βγ-crystallin superfamily. Our work lays a strong foundation for the identification and study of more proteins for calcium-binding properties of this understudied superfamily.

Experimental procedures

Materials

All restriction enzymes and molecular biology enzymes were from New England Biolabs Ltd (Hitchin, UK). Fine biochemicals were from Sigma-Aldrich, Calbiochem (Nottingham, UK) or SRL Fine Chemicals, Mumbai, India. Plastic wares were obtained from Tarsons Industries, Kolkata, India.

Cloning and overexpression

Cloning and overexpression of bovine βB2-crystallin has been described previously [49]. PCR amplified βA3-crystallin gene from the cDNA of bovine lens epithelial cells was ligated to pBSK cloning vector and the insert was released using *Nde*I and *Bam*HI restriction enzymes. The insert with cohesive ends was ligated to *Nde*I and *Bam*HI digested pET-21a using T4 DNA ligase (New England Biolabs) followed by transformation to *E. coli* to select for positive clones. The positive plasmids were sequenced to confirm the insert sequence.

pET-21a-A3 construct was transformed to expression host *E. coli* BL 21(DE3). The strain was grown in terrific broth to mid log phase at 37 °C. When the A_{600} was between 0.6 and 1.0, isopropyl thio-β-D-galactoside was added to the final concentration of 1 mM to induce protein overexpression. The cultures were harvested after 3 h and cell pellet was stored at −80 °C.

Purification

Recombinant βB2-crystallin was purified using hydrophobic interaction chromatography as described earlier [49]. βA3-Crystallin was purified using anion exchanger Q-Sepharose FF (GE Life Sciences, Piscataway, NJ, USA) using a modified method of steady state elution [50]. The *E. coli* cell pellet containing overexpressed βA3-crystallin was lysed by ultrasonication in 50 mM Tris-Cl (pH 7.0) containing 1 mM EDTA, 5 mM dithiothreitol and 5 mM phenylmethanesulfonyl fluoride. The clarified cell lysate was loaded on a Q-Sepharose FF column equilibrated in 50 mM Tris-Cl (pH 7.0) and 1 mM EDTA. Under these conditions, βA3-crystallin does not bind to the resin. The eluate was collected and again passed through the same column. After two passages through the column, the protein was further purified on a Sephadex G-75 (GE Life Sciences) column equilibrated in 50 mM Tris-Cl (pH 7.5) containing 100 mM KCl and 1 mM dithiothreitol. Fractions containing the pure protein were collected and buffer exchanged with Chelex-treated buffer to remove calcium. Proteins were either used fresh or stored in plasticwares at −80 °C after quantitating by absorption at 280 nm.

Stains-All binding assay

The calcium mimic dye, Stains-All, was used to probe the calcium-binding properties of βB2- and βA3-crystallins as described previously [29]. Briefly, 100 μg protein was mixed with the 100 μM dye solution made in 2 mM Mops/NaOH (pH 7.2) containing 30% ethylene glycol, and incubated for 5 min. CD spectra were then recorded between 400 and 700 nm with a 1 cm pathlength cell.

Terbium binding

Terbium-binding to both β-crystallins was monitored on a Hitachi F-4500 spectrofluorimeter (Hitachi Corp, Tokyo,

Japan). The excitation wavelength was 285 nm with band-passes of 5 nm for excitation and emission. The buffer used was 20 mM Tris-Cl (pH 7.5) containing 100 mM KCl. Increasing concentrations of terbium chloride from a stock solution (10 mM) were added to the protein solution in the cuvette and incubated for 5 min before recording the spectra from 300–560 nm.

^{45}Ca overlay assay

Calcium-binding to βB2- and βA3-crystallins was evaluated by ^{45}Ca membrane overlay method originally described by Maruyama *et al.* [33]. Proteins (50 µg each) were spotted onto a nitrocellulose membrane using a dot-blot apparatus. The membrane was washed with a solution containing 10 mM imidazole-HCl (pH 6.8), 60 mM KCl, 5 mM $MgCl_2$ and then incubated for 15 min at 25 °C in the same buffer containing 1 µCi·mL^{-1} of ^{45}Ca (New England Nuclear, Boston, MA, USA). The membrane was then rinsed twice in 45% ethanol, dried and signal was read with a Phosphorimager (Fuji Bas-3000, Stamford, CT, USA).

Fluorescence spectroscopy

Fluorescence emission spectra were recorded on a Hitachi F-4500 spectrofluorimeter. The cuvettes were soaked in 10 mM EDTA solution, rinsed with Chelex-100 treated MQ-water (Millipore, Bedford, MA, USA) and dried before use. The buffer used was 10 mM Tris-Cl (pH 7.5) containing 20 mM KCl. The spectra were recorded in the correct spectrum mode of the instrument using excitation and emission band passes of 5 nm.

CD spectroscopy

Far- and near-UV CD spectra of both crystallins were recorded at room temperature, on a Jasco-715 (Jasco Inc., Tokyo, Japan) spectropolarimeter using 0.01 cm and 1 cm path length cuvettes, respectively. The buffer used was 10 mM Tris-Cl (pH 7.5) containing 30 mM KCl. Secondary structure fractions from far-UV CD spectra were calculated using CDNN based on neural networks [34].

Isothermal titration calorimetry

Calcium- and terbium-binding isotherms for βB2- and βA3-crystallins were determined using a Microcal VP-ITC (MicroCal Inc., Northampton, MA, USA). Freshly prepared βB2-crystallin was used at a concentration of 341 µM in 10 mM Tris-Cl (pH 7.5) containing 50 mM KCl and 0.2 mM TCEP [Tris(2-carboxyethyl) phosphine hydrochloride]. The ligand $CaCl_2$ was prepared in the same buffer at a concentration of 20 mM. The titration was carried out at 25 °C using 57 injections of 4 µL each. Similarly, freshly prepared βA3-crystallin in 20 mM Hepes/NaOH (pH 7.0), 100 mM KCl and 0.2 mM TCEP at a concentration of 265 µM was used in the sample cell at 20 °C. The ligand terbium chloride (10 mM) in the same buffer was loaded in the syringe and a total of 62 injections were made. The first 13 injections were of 4 µL each and the rest of 5 µL each. The integrated heat of each injection was used for fitting to binding models using the program MICROCAL ORIGIN 7.0 (Microcal Inc., Northampton, MA, USA) after subtraction with the appropriate buffer blank.

NMR spectroscopy

$^{15}NH_4Cl$ (Cambridge Isotopes, Cambridge, MA, USA) was used to label the recombinant βB2-crystallin overexpressed in M9 minimal media using the protocol of Marley *et al.* [51]. NMR experiments were carried out on a Bruker Avance II 600 MHz Ultrashield high resolution NMR spectrometer (Bruker, Ettlingen, Germany) equipped with a pulsed field gradient unit and a triple resonance probe with actively shielded Z-gradient. Sensitivity enhanced 2D [^{15}N-^{1}H] HSQC spectra of the protein sample (300 µM, pH 7.5, 25 °C) were recorded. Spectra were processed using TOPSPIN software (Brucker).

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Supplementary material

The following supplementary material is available online:

Fig. S1. A sample of recombinant βB2- and βA3-crystallin resolved on 15% SDS/PAGE to determine the purity.

Fig. S2. The putative calcium-binding sites visualized on the crystal structure of βB2-crystallin.

Fig. S3. ITC thermogram of an inactive preparation of βB2-crystallin.

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