Association Properties of βB1- and βA3-Crystallins: Ability To Form Heterotetramers[†]

May P. Chan,[‡] Monika Dolinska,[‡] Yuri V. Sergeev,[‡] Paul T. Wingfield,[§] and J. Fielding Hejtmancik*[,‡]

National Eye Institute and National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, Maryland 20892

Received July 1, 2008; Revised Manuscript Received August 25, 2008

ABSTRACT: As major constituents of the mammalian lens, β-crystallins associate into dimers, tetramers, and higher-order complexes to maintain lens transparency and refractivity. A previous study has shown that dimerization of βB2- and βA3-crystallins is energetically highly favored and entropically driven. While heterodimers further associate into higher-order complexes in vivo, a significant level of reversibly associated tetrameric crystallin has not been previously observed in vitro. To enhance our understanding of the interactions between β-crystallins, we characterized the association of βB1-crystallin, a major component of large β-crystallin complexes (β-high), with itself and with βA3-crystallin. Mouse βB1-crystallin and human βA3-crystallin were expressed in *Escherichia coli* and purified chromatographically. Their association was then characterized using size-exclusion chromatography, native gel electrophoresis, isoelectric focusing, and analytical sedimentation equilibrium centrifugation. When present alone, each β-crystallin associates into homodimers; however, no tetramer formation is seen. Once mixing has taken place, formation of a heterocomplex between βB1- and βA3-crystallins is observed using size-exclusion chromatography, native gel electrophoresis, isoelectric focusing, and sedimentation equilibrium. In contrast to results previously obtained after βB2- and βA3-crystallins had been mixed, mixed βB1- and βA3-crystallins show a dimer−tetramer equilibrium with a K_d of 1.1 μM, indicating that these two β-crystallins associate predominantly into heterotetramers in vitro. Thus, while each purified β-crystallin associates only into homodimers and under the conditions studied mixed βB2- and βA3-crystallins form a mixture of homo- and heterodimers, mixed βB1- and βA3-crystallins associate predominantly into heterotetramers in equilibrium with heterodimers. These findings suggest a unique role for βB1-crystallin in promoting higher-order crystallin association in the lens.

Crystallins, which exist at extremely high concentrations in the eye lens, are structural proteins critical for transmitting and focusing light. The human lens contains α-, β-, and γ-crystallins, of which β-crystallins, taken as a whole, represent the greatest part. Seven different subtypes of β-crystallins have been identified in the human lens, four of which are relatively acidic (βA1, βA2, βA3, and βA4) and three of which are relatively basic (βB1, βB2, and βB3). β- and γ-crystallins, which have highly similar core sequences, form the $\beta\gamma$-crystallin superfamily. Structurally, both β- and γ-crystallins contain two homologous domains connected by a short connecting sequence. Each domain contains two Greek-key motifs, and each motif comprises four antiparallel β-sheets (*1*). β-Crystallins contain N-terminal extensions ranging in length from 12 to 57 amino acids. In addition, the basic β-crystallins contain C-terminal extensions ranging from 11 to 16 amino acids. β-Crystallins are highly conserved across species with βA3-crystallin being more than 95% identical between mice and humans. In contrast to β-crystallins, γ-crystallins have either no terminal extensions or short extensions containing only a few amino acids (*2*).

β-Crystallins are known to associate into dimers, tetramers, and higher-order complexes in vivo. Size-exclusion chromatography of bovine or human lens extracts shows three size classes of β-crystallin complexes: βH (160−200 kDa, primarily octamers), βL1 (70−100 kDa, primarily tetramers), and βL2 (46−50 kDa, primarily dimers) (*3, 4*). The distribution of β-crystallins among different size classes is dependent on protein concentration, pH, and ionic strength (*5, 6*). Despite the sequence homology between β- and γ-crystallins, γ-crystallins are observed in the lens strictly as monomers, which have prompted investigations into the role of terminal extensions in crystallin association. Previous studies have shown that although the terminal arms of β-crystallins are not required for association, the N-terminal extension of βA3-crystallin assists in self-association into homodimers, while the N-terminal extension of βB2-crystallin actually decreases the level of association into homodimers (*7, 8*). Several studies have also demonstrated a correlation between the length of the N-terminal extension of βB1-crystallin in lens extracts and the size class in which it migrates on size-exclusion chromatography (*9−11*). Bateman et al. have previously shown that truncated forms of βB1- and βA3-

---

[†] M.P.C. was supported by the Howard Hughes Medical Institute Research Scholars Program.

* To whom correspondence should be addressed: OGVFB/NEI/NIH, 10/10B10, 10 Center Dr., Bethesda, MD 20892. Telephone: (301) 496-8300. Fax: (301) 435-1598. E-mail: f3h@nei.nih.gov.

[‡] National Eye Institute.

[§] National Institute of Arthritis and Musculoskeletal and Skin Diseases.

10.1021/bi8012438 CCC: $40.75

crystallin migrate at a size of approximately 100 kDa on gel permeation chromatography and have a similar size as estimated by laser light scattering (*12*).

While β-crystallins are known to form oligomers in vivo, a significant level of tetramers or higher-order complexes generally has not been observed in previous in vitro studies. This can be explained in part by the lower protein concentration being examined in vitro, compared with the extremely high concentration in the eye lens. It has previously been demonstrated that both mouse βB2-crystallin and human βA3-crystallin form reversible homodimers when present alone and that these readily form heterodimers upon mixing and incubation at room temperature in the absence of denaturants (*13*). However, no heterotetramers or higher-order complexes were observed between these two crystallins. This study employs a similar strategy for studying the association properties of another pair of β-crystallins: mouse βB1-crystallin and human βA3-crystallin. When present alone, each protein associates into homodimers; however, neither forms homotetramers. When mixing occurs in the absence of denaturants, formation of a heterocomplex between these two β-crystallin subtypes was observed by size-exclusion chromatography, native gel electrophoresis, isoelectric focusing, and sedimentation equilibrium. In contrast to the results obtained previously with βB2- and βA3-crystallins, both size-exclusion chromatography and sedimentation equilibrium of the mixture of βB1- and βA3-crystallins show a dimer−tetramer equilibrium, with a K_d of 1.1 μM calculated from the latter. These results show that mixed βB1- and βA3-crystallins associate predominantly into heterotetramers in vitro.

EXPERIMENTAL PROCEDURES

Expression and Purification of βB1-Crystallin. Mouse cDNA encoding βB1-crystallin (consistent with NCBI sequence NM_023695) was cloned into a pET-20b(+) vector (Novagen) utilizing the ATG codon of its NdeI site (CATATG) as a start codon (generous gift of H. Mchaourab). The recombinant pET/βB1 plasmid was then used to transform competent BL21(DE3)pLysS cells according to the manufacturer's protocol (Invitrogen). Bacterial cultures were grown to an OD$_{600}$ of 0.5−0.6 and induced for 2 h with 1 mM IPTG. The harvested cell pellet was resuspended in buffer A [50 mM Tris-HCl, 1 mM EDTA, 0.15 M NaCl, 1 mM DTT, and 50 μM TCEP (pH 7.5)] with added Complete Protease Inhibitor cocktail (Roche). Lysis was performed by sonication. The presence of βB1-crystallin was confirmed by SDS−PAGE. The lysate was centrifuged, and the supernatant was dialyzed overnight against 2 L of buffer B [50 mM sodium phosphate, 1 mM EDTA, 1 mM DTT, and 50 μM TCEP (pH 6.8)]. The dialysate was then loaded on a HiTrap SP FF cation exchange chromatography column (GE Healthcare) and eluted with a gradient from 0 to 1 M NaCl in buffer B. Fractions containing βB1-crystallin were pooled, concentrated, and chromatographed on a Superdex 75 HR16/60 size-exclusion chromatography column with buffer A. The Superdex 75 column was precalibrated with the following low-molecular mass standards: bovine serum albumin, ovalbumin, chymotrypsinogen, ribonuclease A, and acetone (Amersham Biosciences; Sigma-Aldrich). Fractions containing βB1-crystallin were pooled with a final purity of >95% as assessed by SDS−PAGE.

Table 1: Starting Concentrations of βB1- and βA3-Crystallins for Association Experiments

starting concentration of each protein in mg/mL (μmol/L)	A_{280} of βB1-crystallin (ou)[a]	A_{280} of βA3-crystallin (ou)[a]
0.5 (18)	0.54	0.48
1 (36)	1.14	1.04
2 (72)	2.09	1.85

[a] Arbitrary optical units (ou).

Expression and Purification of βA3-Crystallin. Human cDNA encoding βA3-crystallin (consistent with NCBI sequence NM_005208) was cloned into a pET-20b(+) vector utilizing the ATG codon of its NdeI restriction site as a start codon. Recombinant pET/βA3 plasmid was then used to transform competent BL21(DE3) cells according to the manufacturer's protocol (Invitrogen). Bacterial cultures were grown to an OD$_{600}$ of 0.5−0.6 and induced for 2 h with 0.5 mM IPTG. The harvested cell pellet was resuspended in buffer A. Lysis was performed by sonication. The presence of βA3-crystallin was confirmed by SDS−PAGE and Western blots using antibodies that target the first Greek-key motif of βA3-crystallin [amino acids 37−68 (DQEN-FQGKRMEFTSSCPNVSERNFDNVRSLKV)] (*6*). The lysate was centrifuged, and the supernatant was dialyzed overnight against 2 L of buffer C [50 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, and 50 μM TCEP (pH 8.1)]. The dialysate was then loaded on a HiTrap DEAE FF anion exchange chromatography column (GE Healthcare) and eluted with a gradient from 0 to 1 M NaCl in buffer C. Fractions containing βA3-crystallin were pooled, concentrated, and eluted on a Superdex 75 HR16/60 size-exclusion chromatography column with buffer A. Fractions containing βA3-crystallin were pooled and showed a final purity of >95% as assessed by SDS−PAGE.

Association of βB1- and βA3-Crystallins. Purified βB1- and βA3-crystallins in buffer A were adjusted to three sets of concentrations according to Table 1 (approximately 0.5, 1, and 2 mg/mL each), to give a 1:1 molar ratio when present in equal volumes. Concentrations were estimated from A_{280} readings on a spectrophotometer. At each concentration, equal volumes of βB1- and βA3-crystallins were mixed and incubated at room temperature in buffer C. Aliquots of 300 and 30 μL were taken 0, 0.5, 1, 2, 4, 6, 12, and 24 h (all or some of these time points) after mixing, immediately frozen in an ethanol/dry ice bath, and stored at −80 °C until they could be analyzed.

Chromatography and Electrophoresis. Each 300 μL aliquot of the mixture of βB1- and βA3-crystallins was subsequently thawed and loaded on a Superdex 75 HR10/30 size-exclusion chromatography column (analytical grade) precalibrated with the same low-molecular mass standards mentioned above. Each sample was eluted with 1 bed volume (25 mL) of buffer A, and the A_{280} was monitored throughout the run. For SDS−PAGE analysis, selected fractions were electrophoresed on ReadyGels (Tris-HCl, 4 to 15% gradient; BioRad) in Tris/glycine/SDS buffer at 200 V for 30 min, using a Benchmark Protein Ladder (Invitrogen) as molecular mass markers. For native gel electrophoresis, the 30 μL aliquots were electrophoresed on ReadyGels in the same manner but in the absence of SDS.

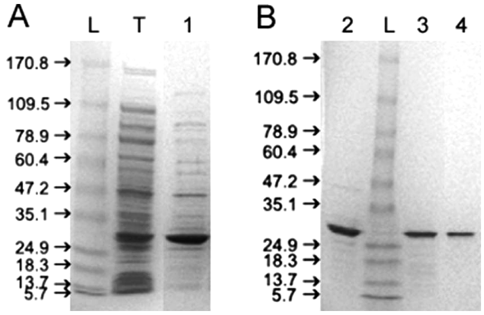

FIGURE 1: SDS−PAGE showing stepwise purification of mouse βB1-crystallin: L, protein ladder; (A) T, total lysate of bacteria expressing βB1-crystallin; 1, sample after cation exchange; (B) 2, sample after size-exclusion chromatography; 3, sample after partial unfolding by urea and subsequent refolding by dialysis; 4, sample after a second size-exclusion chromatography. A discrete band at 28 kDa is seen with a final purity of >95% (Figure 1B, lane 4).

Analytical Ultracentrifugation. Prior to centrifugation, purified proteins were treated with 10 mM DTT and 1 M urea, incubated at room temperature for 30 min, then dialyzed against 2 L of buffer A without DTT at 4 °C for 24 h. Only samples showing a single peak on size-exclusion chromatography and >95% purity on SDS−PAGE were used for analytical ultracentrifugation. The sample concentration was adjusted to 0.6 mg/mL. Centrifugation was conducted in a Beckman Optima XL-I analytical centrifuge. Absorption optics, an An-60 Ti rotor, and standard double-sector centerpiece cells were used. All analyses were performed using duplicate protein samples. Data were collected after centrifugation for 16 h at 16500 rpm and 20 °C. The baselines were established by overspeeding at 45000 rpm for an additional 4 h. Equilibrium profiles were analyzed by standard Optima XL-I Origin-based data analysis software. Solvent density was estimated as previously described (*14*).

Dissociation constants were determined by fitting either a monomer−dimer equilibrium curve (for βB1-crystallin) or a dimer−tetramer equilibrium curve (for the βB1−βA3 heterocomplex) to the concentration gradient profile established at each temperature.

Isoelectric Focusing. Samples taken at various time points after mixing of βB1- and βA3-crystallins were electrofocused on nondenaturing PhastGels IEF 3-9 (Pharmacia). One microliter of sample was applied in each lane. A PhastSystem (Pharmacia) was set up to run the following protocol: (1) prefocusing at 2000 V, 2.5 mA, 3.5 W, and 15 °C for 75 V h, (2) sample application at 200 V, 2.5 mA, 3.5 W, and 15 °C for 15 V h, and (3) focusing at 2000 V, 2.5 mA, 3.5 W, and 15 °C for 410 V h. Broad-range pI 3−10 markers (Pharmacia) were applied as standards. After isoelectric focusing, gels were fixed with 20% trichloroacetic acid, stained with 0.02% PhastGel Blue R solution (Coomassie) in 30% methanol and 10% acetic acid with 0.1% (w/v) $CuSO_4$, and finally destained with 30% methanol and 10% acetic acid.

RESULTS

Expression of Mouse βB1-Crystallin. Mouse βB1-crystallin is expressed as a soluble protein with high yield in *E. coli*. Figure 1 shows the SDS−PAGE results of the sample following purification by ion exchange (A) and size-exclusion chromatography. A discrete band at 28 kDa is seen with a final purity of >95% (Figure 1B). This band reacts strongly with anti-βH-crystallin antibodies on the Western blot (data not shown). At 1 mg/mL, βB1-crystallin elutes on a Superdex 75 column as a single peak with an apparent molecular mass of 37 kDa, intermediate between the predicted masses of monomeric (28 kDa) and dimeric (56 kDa) βB1-crystallin (Figure 2A). After incubation at room temperature for 24 h, βB1-crystallin elutes again as a single peak, but at a slightly larger apparent mass of 37.8 kDa (data not shown). This difference is small and of uncertain significance. The peaks are somewhat broad and slightly skewed, as expected for a protein in rapid monomer−dimer equilibrium (*7*). Mass spectrometry shows the molecular mass to be 27871.16 Da, consistent with the expected monomeric mass of 27870 Da. The purified proteins were intact as assessed by SDS−PAGE, and no contaminating peptides were detected via mass spectrometry.

Expression of Human βA3-Crystallin. Human βA3-crystallin was expressed as a soluble protein with high yield in *E. coli* as previously described (*7*). After purification by ion exchange and size-exclusion chromatography as previously described (*15*), SDS−PAGE shows a discrete band at 25 kDa with a final purity of >95%. This band reacts strongly with anti-βA3 antibodies on Western blots (data not shown). At 1 mg/mL, βA3-crystallin elutes on a Superdex 75 column as a single peak with an apparent molecular mass of 41.3 kDa (Figure 2A). This is intermediate between the predicted masses of monomeric (25 kDa) and dimeric (50 kDa) βA3-crystallin. After incubation at room temperature for 24 h, βA3-crystallin elutes as a single peak and shows minimal if any increase in its apparent molecular size (data not shown). The purified proteins were intact as assessed by SDS−PAGE, and no contaminating peptides were detected via mass spectrometry.

Size-Exclusion Chromatography of Mixed βB1- and βA3-Crystallins. When βB1- and βA3-crystallins are mixed at 36 μM (equivalent to approximately 1 mg/mL) each, allowed to stand at room temperature for varying periods of time, and chromatographed on a Superdex 75 column, one or two peaks are observed depending on the incubation time. These two distinct peaks have averaged apparent molecular masses of 71.1 and 37.5 kDa, respectively (Figure 2B). At longer incubation times, there is a clear trend toward an increasing amount of the higher-molecular mass species (71.1 kDa), coupled with a decreasing amount of the lower-molecular mass species (37.5 kDa) with an initial half-life of ∼5 h. SDS−PAGE shows that at the beginning of the incubation (0 h), the lower-molecular mass peak comprises βB1- and βA3-crystallins that are asymmetrically distributed with βA3-crystallin appearing slightly before βB1-crystallin (Figure 2B, gel a). At the end of the incubation (24 h), the higher-molecular mass peak comprises samples with a 1:1 ratio of the two crystallins in the high-molecular mass peak and some excess βB1-crystallin in lower-molecular mass fractions [Figure 2B, gel b, confirmed by scanning of the electrophoresed bands (data not shown)].

When the initial concentration is doubled to 72 μM (equivalent to approximately 2 mg/mL), a similar trend is observed, with both species showing slightly higher molecular masses (76.4 and 41.7 kDa, respectively) (Figure 2C). A similar trend is also observed when the initial concentration is lowered to 18 μM (equivalent to approximately 0.5 mg/

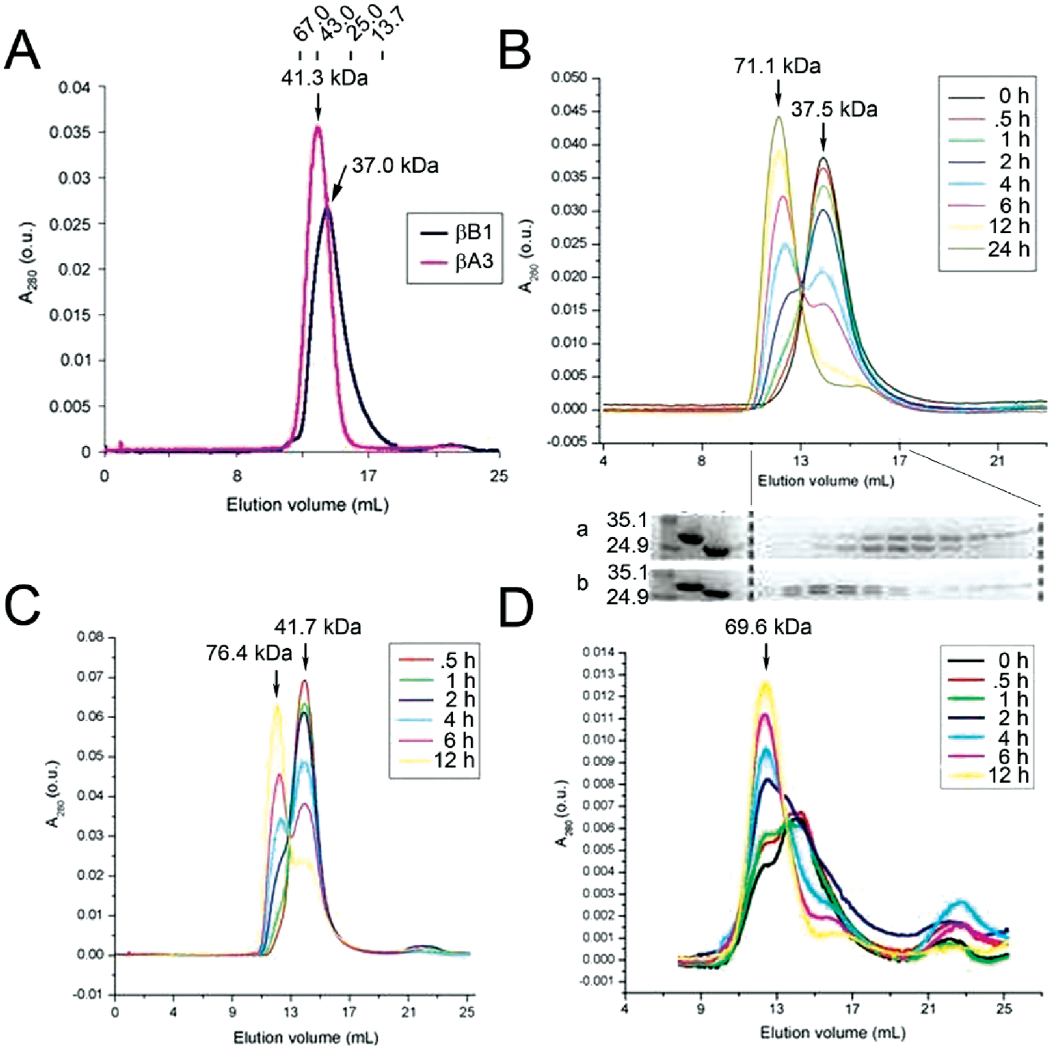

FIGURE 2: Size-exclusion chromatography of mouse βB1-crystallin and human βA3-crystallins. (A) Size-exclusion chromatography of mouse βB1-crystallin and human βA3-crystallin, each at 1 mg/mL. The elution points of molecular mass standards [albumin (67.0 kDa), ovalbumin (43.0 kDa), chymotrypsinogen (25.0 kDa), and ribonuclease A (13.7 kDa)] are shown at the top for reference. Molecular masses of βB1- and βA3-crystallins were calculated to be 37.0 and 41.3 kDa, respectively. (B) Size-exclusion chromatography of βB1- and βA3-crystallins mixed at 1 mg/mL. Protein concentrations were monitored by absorbance at 280 nm. Chromatograms obtained for each time point after initial mixing of the two crystallins are overlaid to show the trend. Molecular masses of the two peaks at different time points were calculated from the standard curve of the sizing column, and the averages were 71.1 and 37.5 kDa, respectively. A stepwise increase in the amount of the higher-molecular mass species (71.1 kDa) and a stepwise decrease in the amount of the lower-molecular mass species (37.5 kDa) were observed. SDS−PAGE of the fractions from chromatography runs at (a) 0 and (b) 24 h are aligned on the bottom to show contents of the peaks. The first lane refers to protein markers (35.1 and 24.9 kDa). The second and third lanes refer to βB1- and βA3-crystallin standards, respectively. (C and D) Size-exclusion chromatography of βB1- and βA3-crystallins mixed at (C) 2 and (D) 0.5 mg/mL. The averaged molecular mass of each peak is indicated, except for the lower-molecular mass species in panel D, which is indistinct. The small peak observed at the end of each run corresponds to a molecular mass that is smaller than the column volume and is therefore not analyzed.

mL), but the peaks become less distinct as the levels approach the detection limit of the monitor (Figure 2D). A peak that corresponds to the higher-molecular mass species has an averaged molecular mass of 69.6 kDa, which is slightly lower than that observed at higher concentrations, suggesting a rapid dimer−tetramer equilibrium for βB1-crystallin similar to that of βA3-crystallin monomers and dimers described in ref *7*.

Native Gel Electrophoresis of Mixed βB1- and βA3-Crystallins. Figure 3A shows native gel electrophoresis of βB1- and βA3-crystallin samples mixed at 1 mg/mL. The migration of βB1-crystallin is markedly retarded, with most of the protein retained at the loading position of the gels. Conversely, βA3-crystallin migrates well into the gels. Both concentrations exhibit qualitatively similar patterns. No interaction between βB1- and βA3-crystallins is observed at time zero, followed by an increasing level of formation of the intermediate species, and decreasing amounts of the starting proteins at successive time points. The most prominent intermediate band is located closer to the βB1-crystallin position than to the βA3-crystallin position.

Isoelectric Focusing of Mixed βB1- and βA3-Crystallins. Figure 3B shows that βB1-crystallin electrofocuses at an isoelectric point slightly lower than 7.35, consistent with its predicted pI of 7.28. βA3-Crystallin electrofocuses slightly lower than pI 6.55, also consistent with its predicted pI of 6.20. Isoelectric focusing immediately after mixing essentially produces an overlay of the two patterns observed with the individual proteins. At increasing incubation times, less protein is observed at the pI's of the individual crystallins

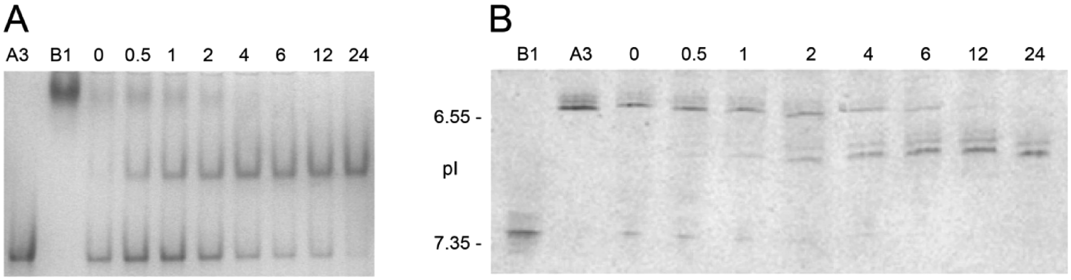

FIGURE 3: Native gel electrophoresis and isoelectric focusing of βB1- and βA3-crystallins. (A) Native gel electrophoresis of βB1- and βA3-crystallins mixed at 1 mg/mL each. B1, βB1-crystallin only; A3, βA3-crystallin only; 0−24, aliquots taken at corresponding time points (in hours) after initial mixing of the two crystallins. (B) Isoelectric focusing of a mixture of βB1- and βA3-crystallins at 1 mg/mL. Locations of two pI markers, human carbonic anhydrase B (pI 6.55) and horse myoglobin basic band (pI 7.35), are indicated at the left: B1, βB1-crystallin; A3, βA3-crystallin; 0−24, aliquots taken at corresponding time points (in hours) after mixing.

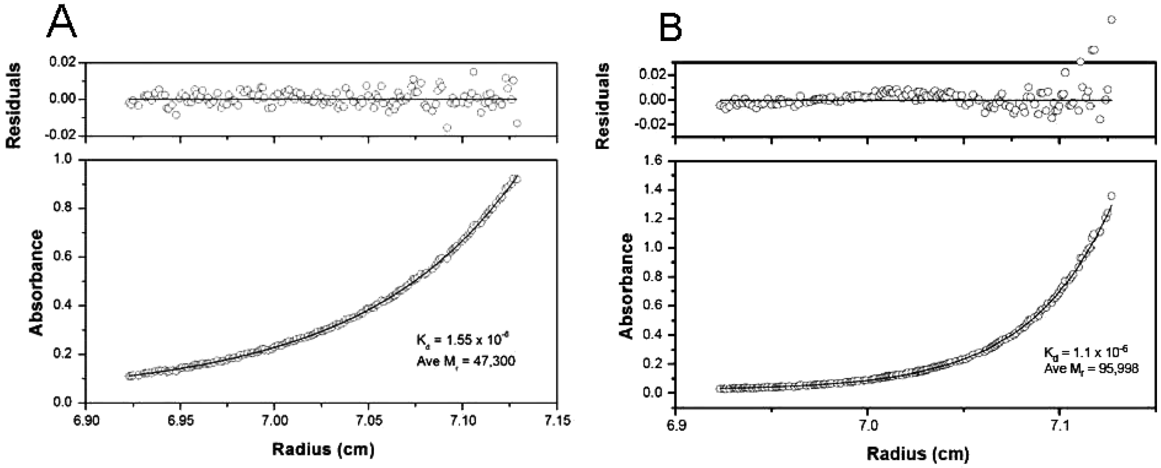

FIGURE 4: Sedimentation equilibrium of purified mouse βB1-crystallin and of the mouse βB1-crystallin and human βA3-crystallin complex. (A) Sedimentation equilibrium of purified mouse βB1-crystallin at 0.6 mg/mL and 20 °C. The absorbance (280 nm) gradient in the ultracentrifuge cell after sedimentation equilibrium had been attained is shown in the bottom panel. The solid line indicates the predicted monomer−dimer association model, and the white circles represent the experimental values. The top panel shows the difference between the predicted and experimental values as a function of radial position (residuals). (B) Sedimentation equilibrium of the mouse βB1-crystallin and human βA3-crystallin complex at 0.6 mg/mL and 20 °C. The solid lines indicate the predicted heterodimer−heterotetramer association model, and the white circles represent the experimental values.

while more protein is found at an intermediate pI, with two additional lighter and slightly more acidic bands also appearing as the incubation progresses. Formation of the intermediate species becomes noticeable by 0.5 h after mixing and appears to be approaching equilibrium at 12 h, at which point most protein is present as the intermediate species.

Sedimentation Equilibrium of βB1-Crystallin. Sedimentation equilibrium analysis of purified mouse βB1-crystallin shows that the experimental values fit closely with the predicted monomer−dimer model at 20 °C (Figure 4A). Assuming a monomer−dimer equilibrium, the dissociation constant (K_d) is 1.55 ± 0.20 μM and the weight-average molecular mass (M_r) is determined to be 47295 ± 276 Da. Comparison of the average K_d and M_r of mouse βB1-, βA3-, and βB2-crystallins is shown in Table 2.

Sedimentation Equilibrium of the βB1−βA3 Heterotetramer. Figure 4B shows that at 20 °C, βB1- and βA3-crystallins form heterocomplexes that fit well with the predicted dimer−heterotetramer equilibrium, with a K_d of 1.1 μM. The average molecular mass of the complex is determined to be 95998 ± 714.9 Da at 20 °C, which is close to the predicted mass of the heterotetramer containing two subunits of each crystallin (106.2 kDa).

Table 2: Comparison of Homodimer Dissociation Constants (K_d) and Weight-Average Molecular Masses (M_r) of Mouse βB1-, βA3-, and βB2-Crystallins Determined by Sedimentation Equilibrium at 20 °C

protein	K_d (μM)	average M_r (kDa)	predicted M_r of the dimer (kDa)
βB1	1.55	47	56
βA3[a]	0.8	47	50
βB2[a]	5.0	39	46

[a] Values previously determined (*13*).

DISCUSSION

Here we describe the association of mouse βB1-crystallin and human βA3-crystallin. When present alone, each protein associates into homodimers; however, neither forms homotetramers. This result is similar to those obtained with βB2- and βA3-crystallins. However, in contrast to the previous results obtained with βB2- and βA3-crystallins, mixed βB1- and βA3-crystallins show a dimer−tetramer equilibrium, with a K_d of 1.1 μM. Thus mixed βB1- and βA3-crystallins associate predominantly into heterotetramers in vitro.

Mouse βB1-crystallin was faithfully expressed with high yield in BL21(DE3)pLysS, which is the same system used previously for expressing human βB1-crystallin by Lampi et al. (*16*). The identity of the expressed βB1-crystallin is supported by its correct molecular mass via SDS−PAGE and mass spectrometry. Human βA3-crystallin was previously expressed using the baculovirus system (*6*). This study utilized the BL21(DE3) bacterial system, which is a more convenient and equally productive method for the expression of relatively stable proteins, such as human βA3-crystallin. The identity of the expressed βA3-crystallin is confirmed by its molecular mass on SDS−PAGE and its strong reactivity with antibodies that specifically target the first Greek-key motif of bovine βA3-crystallin (data not shown). While mouse and human βB1-crystallins are only 80.9% identical (90% similar), mouse and human βA3-crystallins are 95.3% identical (100% similar), suggesting that the use of human and mouse crystallins provides a reasonably accurate model of crystallin behavior, especially in the mouse lens. Also, while the conserved PAPA sequence is more evident in mouse βB1-crystallin, both Chou-Fasman and Garnier-Robson algorithms predict that human βB1-crystallin shares similar structural characteristics in this region (data not shown).

Size-exclusion chromatography of mouse βB1-crystallin at 1 mg/mL shows a somewhat broad and asymmetrical single peak with an apparent molecular mass that is intermediate between the monomeric and dimeric forms, consistent with a monomer−dimer equilibrium (*17*). An intermediate molecular mass is observed as monomers constantly associate and dissociate in a rapidly reversible manner as the protein is chromatographed (*17*). The homodimer formation observed in this study agrees with other reports of βB1-crystallin (*16, 18*) and most other β-crystallins, including βA3- and βB2-crystallins (*6, 13*). However, while size-exclusion chromatography in these studies has suggested that βB1-crystallin might behave as a dimer as concentrations increase above 10 mg/mL (*18*) or at 0.7 mg/mL (*16*), light scattering suggests molecular masses consistent with a dimer with a small population in aggregates of 264 kDa (*16*) or varying between 48 and 125 kDa depending on the protein concentration (*18*). While the nature of the larger aggregates is unclear from the data presented, they do not appear to represent the reversible high-affinity association described here. Rather, the data suggest either a stable high-molecular mass complex coexisting with a smaller dimeric form in some cases and a gradual smooth increase in molecular mass in others.

The stability of βB1-crystallin over time is supported by its molecular mass, which remains relatively constant after incubation for 24 h at room temperature. Similar results were obtained for βA3-crystallin, which also displays a persistent monomer−dimer equilibrium. Despite having a smaller predicted molecular size, βA3-crystallin shows a higher apparent molecular mass on size-exclusion chromatography than βB1-crystallin. Delayed elution of βB1-crystallin from size-exclusion chromatography has been reported previously (*16, 18*) and has been attributed to interaction between βB1-crystallin and the column matrix, which is seen with amino-truncated βA3-crystallin (*8*). However, it is also consistent with the higher affinity for self-dimerization of βA3-crystallin relative to βB1-crystallin. The increase in the apparent molecular mass with an increase in βB1-crystallin concentration on size-exclusion chromatography suggests that the delayed elution of βB1-crystallin is due to a reversible monomer−dimer equilibrium rather than interactions with the column or unusual effects of an unusual shape of the molecule, or perhaps both might contribute to some degree.

Sedimentation equilibrium of mouse βB1-crystallin at 0.6 mg/mL and 20 °C again confirms a monomer−dimer equilibrium. In contradistinction to some previous reports (*18*), no evidence for homotetramer formation was seen with analytical ultracentrifugation or size-exclusion chromatography in these studies. The apparent average molecular mass estimated from sedimentation equilibrium at 20 °C (47 kDa) is significantly higher than that estimated from size-exclusion chromatography (37 kDa). This discrepancy may be attributed to the fact that the association of crystallins is dependent on protein concentration but also might reflect a tendency of βB1-crystallin to stick to the gel matrix in a size-exclusion column as discussed above. Unlike the sedimentation analyses, size-exclusion chromatography subjects protein samples to dilution by the running buffer. As the protein concentration decreases, the monomer−dimer equilibrium is expected to shift toward monomer, thus resulting in a lower apparent molecular mass.

When mouse βB1-crystallin and human βA3-crystallin are incubated together at room temperature, formation of the heterocomplex is readily appreciable within 30 min by size-exclusion chromatography, native gel electrophoresis, and isoelectric focusing. In Figure 2, the higher-molecular mass species that increases in amount with incubation time represents a dimer−tetramer equilibrium, as it corresponds to an apparent molecular mass that is intermediate between that of a dimer (either homodimer or heterodimer) and that of a tetramer. A number of observations suggest that this peak represents tetramer rather than trimer formation. As the protein concentration of βA3:βB1 mixtures increases, the apparent mass approaches that of the predicted tetramer size (summarized in Figure 2).

SDS−PAGE of the eluted fractions shows that this peak contains equal amounts of βB1- and βA3-crystallins (Figure 2B). This 1:1 ratio suggests that each tetramer is composed on average of two subunits of βB1-crystallin and two subunits of βA3-crystallin. Conversely, the lower-molecular mass species corresponds to a monomer−dimer equilibrium that decreases in amount over time. SDS−PAGE of this peak shows an asymmetric distribution of βB1- and βA3-crystallins, suggesting a lack of interaction at the beginning of the incubation, and perhaps a tendency of βB1-crystallin to stick to the column matrix. In theory, three types of dimers can be present: βB1 homodimers (56 kDa), βA3 homodimers (50 kDa), and βB1−βA3 heterodimers (53 kDa). It is likely that the proximity in molecular masses of these three species has exceeded the resolution capacity of the Superdex 75 column, resulting in a broad single peak. While the exact mechanism of heterotetramer formation is unknown, it seems likely that it occurs via the association of two βB1−βA3 heterodimers rather than association of a βB1 homodimer with a βA3 homodimer. Ultimately, one would expect the mixture to reach a steady state of heterodimer−heterotetramer equilibrium. These data are in agreement with those of Bateman et al. (*12*). As expected, the final amount of heterotetramer formed is dependent on crystallin concentration. As the protein concentration increases, the percentages of protein at the molecular masses representing both monomer−dimer and dimer−tetramer equilibria increase, and vice versa (Figure 2B−D). This indicates that higher concentrations shift the equilibria toward association.

Native gel electrophoresis, which separates proteins in their native states, provides additional information about the shapes of the complexes. Of interest, the migration of βB1-crystallin by itself is markedly retarded with some protein not entering the gel (Figure 3A). One possible explanation is that the N-terminal extension of βB1-crystallin, being the longest among all β-crystallins, severely hinders the migration of monomeric/dimeric βB1-crystallin through the polyacrylamide gel matrix. The intermediates formed over time, representing formation of a heterocomplex with βA3-crystallin, migrate further down the gel compared to βB1-crystallin alone. This suggests the interaction between βB1- and βA3-crystallins gives rise to a conformation that is more compact and thus penetrates the matrix more easily. One possibility is that the N-terminal arm of βB1-crystallin may be involved in holding the heterocomplex in place and thus becomes less flexible in the tetramer (*19*), protruding less and allowing migration of the complex into the gel. Alternatively, the βB1-crystallin N-terminal arm might interact with the gel matrix less in heterotetramers, which would also allow the heterocomplex to migrate further than the βB1-crystallin dimer.

Heterocomplex formation is also confirmed by isoelectric focusing. As expected, the heterocomplex has a pI that is intermediate between that of βB1-crystallin and that of βA3-crystallin (Figure 3B). While this technique does not distinguish between heterodimer and heterotetramer formation, the absence of homodimers or homotetramers, which would have identical isoelectric points, after longer incubation times suggests that most of the intermediate band is composed of heterotetramers. This can be seen in comparison to isoelectric focusing of mixed βB2- and βA3-crystallins, in which the heterodimer and two homodimers coexist in a 2:1:1 ratio (*13*). That is, at equilibrium, there is twice as much heterodimer as there is of each homodimer, suggesting that none of the three possible dimers is energetically favored over the other.

Finally, analytical ultracentrifugation provides thermodynamic characterization of the heterocomplex equilibrium. The experimental data fit well with the expected gradient of the heterodimer−heterotetramer equilibrium, with a K_d of 1.1 μM. The observed molecular mass (96 kDa) is consistent with the predicted molecular mass of the heterotetramer consisting of two βB1-crystallin and two βA3-crystallin subunits (106.2 kDa) but significantly higher than the apparent molecular mass estimated from size-exclusion chromatography (69.6−76.4 kDa). Again, this may be due to the dilution effect during chromatography, adherence of the βB1-crystallin protein to the size-exclusion chromatography gel matrix, or both.

The formation of heterotetramers by mixed βB1- and βA3-crystallin contrasts with the lack of tetramers seen under similar conditions with mixed βB2- and βA3-crystallins (*7, 8*). However, this is consistent with the high βB1-crystallin content observed in βH aggregates synthesized in vivo (*5, 20*). βB2-Crystallin, on the other hand, is present in all size classes and is the primary constituent of βL2-crystallin (*20*), suggesting βB2-crystallin might have a low affinity for higher-order association with other β-crystallins. These observations might relate to the structural difference between βB2- and βB1-crystallins. The major factor that distinguishes βB1-crystallin from other β-crystallins, and especially basic β-crystallins, is its extremely long N-terminal extension containing 57 residues, including a PAPA sequence (*21*). We hypothesize that βB1-crystallin may promote higher-order complex formation with other β-crystallins in the lens through the action of its long N-terminal extension. While it cannot, by itself, account for the formation of βH-crystallin oligomers, this does agree with the presence of βB1-crystallin preferentially in the βH complexes and truncated βB1-crystallins in βL1 and βL2 complexes previously described (*9*).

It is important to realize that under physiological conditions, where the protein concentration exceeds 300 mg/mL, higher-order association between β-crystallins would be favored. Therefore, βB1-crystallin might have a crucial role in associating with other β-crystallins in higher-order complexes. We have not been able to measure the dissociation constant of the putative βB1-crystallin−βA3-crystallin heterodimer. However, if it has a dissociation constant similar to that of the βB1 homodimer, the fraction of heterotetramer at 300 mg/mL would be greater than 99.99%, and this fraction would still approach 99.9% at 1 mg/mL. Thus, as previously suggested, the rapid interchange of crystallins might have more physiological implications than the existence of monomer or even dimer forms of the protein. This being said, the findings in this study have implications for cataractogenesis of the aging lens where N-terminal truncation of βB1-crystallin has been well-documented (*10, 22*). In this regard, the size distribution of β-crystallins is dependent on the age of the lens, which is itself correlated with crystallin modifications, including truncation of the terminal arms (*10, 11*). Finally, the lack of homotetramer formation in vitro indicates that higher-order association between different β-crystallin subtypes is favored over self-association. This supports a previous suggestion that acidic β-crystallins may preferentially associate with basic β-crystallins (*23*), although this may well result from a heterotetramer−heterodimer equilibrium with βB1- and βA3-crystallins as opposed to the heterodimer in which βB2- and βA3-crystallins associate into βB2 homodimers, βB2−βA3 heterodimers, and βA3 homodimers in a roughly 1:2:1 ratio (*13, 15*). Consistent with this concept, there have been suggestions of βB2- and βA3-crystallin heterotetramer formation at high concentrations on analytical contrifugation (*24*). This differs from the condition in our study, in which βB1-βA3-crystallin heterotetramers appear at both high and low concentrations.

In summary, this study has demonstrated for the first time reversible spontaneous in vitro formation of heterotetramers by β-crystallins. When present alone under these conditions, βB1- and βA3-crystallin associate into homodimers; however, neither forms homotetramers. When they are mixed under physiological conditions, formation of the heterocomplex between these two β-crystallin subtypes was observed by size-exclusion chromatography, native gel electrophoresis, isoelectric focusing, and sedimentation equilibrium. In contrast to the previous results obtained from βB2- and βA3-crystallins, which did not show tetramer formation, analytical centrifugation shows a dimer−tetramer equilibrium with a K_d of 1.1 μM, suggesting that βB1- and βA3-crystallins associate predominantly into heterotetramers in vitro. Although we suspect that heterotetramers are formed preferentially through the interaction of two heterodimers (βB1−βA3 + βB1−βA3), these studies are unable to discriminate this mechanism from that involving the interaction between two homodimers (βB1−βB1 + βA3−βA3) or mixtures of both hetero- and homodimers. Future studies will address this question as well as elucidate the molecular mechanisms of association of βB1-crystallin into both dimers and heterotetramers.

REFERENCES

1. Wistow, G., Turnell, B., Summers, L., Slingsby, C., Moss, D., Miller, L., Lindley, P., and Blundell, T. (1983) X-ray analysis of the eye lens protein γ-II crystallin at 1.9 Å resolution. *J. Mol. Biol. 170*, 175–202.
2. Hejtmancik, J. F., and Piatigorsky, J. (2000) Lens Proteins and their Molecular Biology. In *Principles and Practice of Ophthalmology* (Alpert, D. M., Jakobiec, F. A., Azar, D. T., and Gragoudas, E. S., Eds.) pp 1409−1428, W. B. Saunders Co., Philadelphia.
3. Bindels, J. G., Koppers, A., and Hoenders, H. J. (1981) Structural aspects of bovine β-crystallins: Physical characterization including dissociation-association behavior. *Exp. Eye Res. 33*, 333–343.
4. Zigler, J. S., Jr., Horwitz, J., and Kinoshita, J. H. (1980) Human β-crystallin. I. Comparative studies on the β1-, β2- and β3-crystallins. *Exp. Eye Res. 31*, 41–55.
5. Siezen, R. J., Anello, R. D., and Thompson, J. D. (1986) Interactions of lens proteins. Concentration dependence of β-crystallin aggregation. *Exp. Eye Res. 43*, 293–303.
6. Hope, J. N., Chen, H. C., and Hejtmancik, J. F. (1994) βA3/A1-Crystallin association: Role of the amino terminal arm. *Protein Eng. 7*, 445–451.
7. Sergeev, Y. V., Wingfield, P. T., and Hejtmancik, J. F. (2000) Monomer-dimer equilibrium of normal and modified βA3-crystallins: Experimental determination and molecular modeling. *Biochemistry 39*, 15799–15806.
8. Sergeev, Y. V., Hejtmancik, J. F., and Wingfield, P. T. (2004) Energetics of Domain-Domain Interactions and Entropy Driven Association of β-Crystallins. *Biochemistry 43*, 415–424.
9. Ajaz, M. S., Ma, Z., Smith, D. L., and Smith, J. B. (1997) Size of human lens β-crystallin aggregates are distinguished by N-terminal truncation of βB1. *J. Biol. Chem. 272*, 11250–11255.
10. Lampi, K. J., Ma, Z., Hanson, S. R., Azuma, M., Shih, M., Shearer, T. R., Smith, D. L., Smith, J. B., and David, L. L. (1998) Age-related changes in human lens crystallins identified by two-dimensional electrophoresis and mass spectrometry. *Exp. Eye Res. 67*, 31–43.
11. Ma, Z., Hanson, S. R., Lampi, K. J., David, L. L., Smith, D. L., and Smith, J. B. (1998) Age-related changes in human lens crystallins identified by HPLC and mass spectrometry. *Exp. Eye Res. 67*, 21–30.
12. Bateman, O. A., Sarra, R., Van Genesen, S. T., Kappe, G., Lubsen, N. H., and Slingsby, C. (2003) The stability of human acidic β-crystallin oligomers and hetero-oligomers. *Exp. Eye Res. 77*, 409–422.
13. Hejtmancik, J. F., Wingfield, P., Chambers, C., Russell, P., Chen, H.-C., Sergeev, Y. V., and Hope, J. N. (1997) Association properties of βB2- and βA3-Crystallin: Ability to form dimers. *Protein Eng. 10*, 1347–1352.
14. Laue, T. M., Shah, B. D., Ridgeway, T. M., and Pelletier, S. L. (1992) Computer-aided interpretation of analytical sedimentation data for proteins. In *Analytical Ultracentrifugation in Biochemistry and Polymer Science* (Harding, S. E., Rowe, A. J., and Horton, J. C., Eds.) pp 90−125, Royal Society of Chemistry, Cambridge, United Kingdom.
15. Hope, J. N., Chen, H.-C., and Hejtmancik, J. F. (1994) Aggregation of βA3-crystallin is independent of the specific sequence of the domain connecting peptide. *J. Biol. Chem. 269*, 21141–21145.
16. Lampi, K. J., Oxford, J. T., Bachinger, H. P., Shearer, T. R., David, L. L., and Kapfer, D. M. (2001) Deamidation of human βB1 alters the elongated structure of the dimer. *Exp. Eye Res. 72*, 279–288.
17. Winzor, D. J., and Scheraga, H. A. (1963) Studies of chemically reacting systems on sephadex: Chromatographic demonstration of the Gilbert theory. *Biochemistry 2*, 1263–1267.
18. Bateman, O. A., Lubsen, N. H., and Slingsby, C. (2001) Association behaviour of human βB1-crystallin and its truncated forms. *Exp. Eye Res. 73*, 321–331.
19. Lapatto, R., Nalini, V., Bax, B., Driessen, H., Lindley, P. F., Blundell, T. L., and Slingsby, C. (1991) High resolution structure of an oligomeric eye lens β-crystallin: Loops, arches, linkers and interfaces in βB2 dimer compared to a monomeric γ-crystallin. *J. Mol. Biol. 222*, 1067–1083.
20. Slingsby, C., and Bateman, O. A. (1990) Rapid separation of bovine β-crystallin subunits βB1, βB2, βB3, βA3 and βA4. *Exp. Eye Res. 51*, 21–26.
21. Hejtmancik, J. F., Thompson, M. A., Wistow, G., and Piatigorsky, J. (1986) cDNA and deduced protein sequence for the βB1-crystallin polypeptide of the chicken lens: Conservation of the PAPA sequence. *J. Biol. Chem. 261*, 982–987.
22. David, L. L., Lampi, K. J., Lund, A. L., and Smith, J. B. (1996) The sequence of human βB1-crystallin cDNA allows mass spectrometric detection of βB1 protein missing portions of its N-terminal extension. *J. Biol. Chem. 271*, 4273–4279.
23. Slingsby, C., and Bateman, O. A. (1990) Qarternary interactions in eye lens β-crystallins: Basic and acidic subunits of β-crystallins favor heterologous association. *Biochemistry 29*, 6592–6599.
24. Werten, P. J., Lindner, R. A., Carver, J. A., and de Jong, W. W. (1999) Formation of betaA3/betaB2-crystallin extensions. *Biochim. Biophys. Acta 1432*, 286–292.

BI8012438