Association properties of βB2- and βA3-crystallin: ability to form dimers

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Introduction

High concentrations of closely packed crystallins undergoing appropriate intermolecular interactions are required for transparency and refraction in the eye lens. Aberrations in expression or structure of lens crystallins have been shown to cause cataracts in the Philly mouse (Chambers and Russell, 1991), guinea pig strain 13/N (Rodriguez et al., 1992) and the ELO mouse (Cartier et al., 1992). Understanding the mechanism by which lens crystallins associate to form stable aggregates will provide insight into the molecular mechanisms by which mutations in crystallin genes cause inherited cataracts, both in animal models and in humans.

There are three major classes of crystallins in the mammalian lens. α-Crystallins have an aggregate molecular mass of up to 800 kDa, β-crystallins range from 45 to 200 kDa, and γ-crystallins exist as monomers of 20–25 kDa. β-Crystallins are the most varied in aggregate size, forming several distinct classes of aggregates: βH (primarily octamers of 160–200 kDa), βL1 (primarily tetramers of 70–100 kDa), and βL2 (primarily dimers of 46–50 kDa). The distribution of β-crystallin polypeptides among these size classes is dependent on protein concentration, pH and ionic strength (Asselbergs et al., 1979; Li, 1978, 1979; Bessem and Hoenders, 1986) (J.N. Hope, Y.V. Sergeev, and J.F. Hejtmancik, unpublished observation).

The β- and γ-crystallins together form a superfamily of crystallin polypeptides based on their conserved sequences and tertiary structures. β- and γ-crystallins have a common core structure comprising two homologous domains. Each domain comprises two ‘Greek key’ motifs, each formed by a four stranded antiparallel β-sheet (Wistow et al., 1983). The two domains are joined by a connecting peptide of 9–10 amino acids. All β-crystallins have N-terminal extensions ranging from 12 to 57 amino acids in length. The ‘basic’ β-crystallins (βB1, βB2 and βB3) also have C-terminal extensions of 11–16 amino acids, which the ‘acidic’ β-crystallins (βA1, βA2, βA3 and βA4) lack. For example, βA3-crystallin has a 30 amino acid N-terminal arm, whereas βB2 has a 15 amino acid N-terminal arm and an 11 amino acid C-terminal arm. The γ-crystallins either have no extensions or short terminal extensions containing only a few amino acids (Hejtmancik and Piatigorsky, 1994). Earlier we reported that the N-terminal arm of βA3-crystallin assists in self dimer-formation but is not required to form heterodimers (Hope et al., 1994b). However, βB2-crystallin lacking both N- and C-terminal extensions is reported still to behave as a dimer (Kroone et al., 1994; Trinkl et al., 1994). NMR spectroscopy of βB2-crystallin indicates that the arms are conformationally flexible, unlike the arms of other β-crystallins, suggesting that they are not essential for stabilizing homodimers (Cooper et al., 1993). The sequence of the peptide connecting the two domains is not critical for higher association of βA3-crystallin into dimers or higher aggregates (Hope et al., 1994a). Furthermore, replacement of the connecting peptide of γB-crystallin with the corresponding peptide from βB2-crystallin does not allow dimer formation of the recombinant hybrid γB-crystallin (Mayr et al., 1994). In contrast, Trinkl et al. (1994) replaced the normal connecting peptide sequence of βB2-crystallin with the corresponding sequence of γB-crystallin and found that the chimeric βB2-crystallin was monomeric, suggesting that the sequence of the βB2-crystallin linker is important for dimerization.

It has been postulated that acidic crystallins (βA1, βA2, βA3 and βA4) preferentially interact with basic β-crystallins (βB1, βB2 and βB3) (Slingsby and Bateman, 1990a). Slingsby and Bateman have demonstrated that mixing βB2- and βA3-crystallin in the presence of 6 M urea followed by dilution to remove the urea produced βB2–βA3 heterodimers and have hypothesized that these dimers can further associate into tetramers (dimers of heterodimers). They speculated that the higher order association of dimers requires a β-crystallin peptide with a long N-terminal arm such as that found in βA3-crystallin. On size chromatography bovine βA3-crystallin is...
Expression and purification of recombinant β\textsubscript{2}-bovine
the C-terminal extension of mouse reported CPPLQLEP (Chambers and Russell, 1993). Therefore, was determined to be AFHPSS rather than that previously investigation, the C-terminal extension of mouse
and sequence integrity of the (Invitrogen), generating pBB\textsubscript{β} Hin\textsubscript{III} ligated and subcloned into the corresponding
Nco\textsubscript{I} 357 bp
frugiperda Sf9 insect cells and the recombinant (gal
with wild-type linearized AcMNPV DNA into
Transfer plasmids pBB\textsubscript{β}\textsubscript{H} and cloned into the vector pKK233-2 (Pharmacia). The
cDNA sequence from pKK233-2 as 403 bp
Bateman, 1990b) and
β-crystallin were isolated (Slingsby and Bateman, 1994). It has been suggested that
β- and other
β-crystallins to form dimers and higher-order aggregations. When expressed separately, both recombinant
β-crystallins exist as homodimers. However upon mixing the two β-crystallins, β\textsubscript{A3}/β\textsubscript{B2}-crystallin heterodimers are formed. The heterodimer formation did not require denaturants, and significant levels of higher aggregates, such as tetramers, were not observed. Association of the β\textsubscript{A3}- and β\textsubscript{B2}-crystallin monomer into a β\textsubscript{B2}/β\textsubscript{A3}-crystallin heterodimer appears to be preferred slightly rather than homodimer formation.

To further our understanding of the interactions necessary for the β-crystallins to form dimers and higher-order aggregates, we have expressed recombinant mouse β\textsubscript{B2}-crystallin and β\textsubscript{A3}-crystallin in Sf9 insect cells infected with recombinant baculoviruses. When expressed separately, both recombinant crystallins exist as homodimers. However, upon mixing the two β-crystallins, β\textsubscript{A3}/β\textsubscript{B2}-crystallin heterodimers are formed. The heterodimer formation did not require denaturants, and significant levels of higher aggregates, such as tetramers, were not observed. Association of the β\textsubscript{A3}- and β\textsubscript{B2}-crystallin monomer into a β\textsubscript{B2}/β\textsubscript{A3}-crystallin heterodimer appears to be preferred slightly rather than homodimer formation.

Materials and methods

Cloning the mouse β\textsubscript{B2}-crystallin into pBlueBacIII
cDNA clones coding for the mouse β\textsubscript{B2}-crystallin were isolated from a mouse lens cDNA library (Chambers and Russell, 1991) and cloned into the vector pKK233-2 (Pharmacia). The restriction enzymes Nco\textsubscript{I} and HindIII were used to cut out the cDNA sequence from pKK233-2 as 403 bp Nco\textsubscript{I}–Nco\textsubscript{I} and 357 bp Nco\textsubscript{I}–HindIII fragments. The two fragments were religated and subcloned into the corresponding HindIII and Nco\textsubscript{I} sites in the baculovirus transfer vector pBlueBacIII (Invitrogen), generating pBB\textsubscript{BB2}. Plasmid pBB\textsubscript{BB2} DNA was bi-directionally sequenced to confirm the correct orientation and sequence integrity of the β\textsubscript{B2} cDNA insert in the pBlue BacIII vector. As a result of this sequencing and further investigation, the C-terminal extension of mouse β\textsubscript{B2}-crystallin was determined to be AFHPSS rather than that previously reported CPLEQLEP (Chambers and Russell, 1993). Therefore, the C-terminal extension of mouse β\textsubscript{B2}-crystallin is identical to bovine β\textsubscript{B2}-crystallin.

Expression and purification of recombinant β-crystallins

Transfer plasmids pBB\textsubscript{BB2} and pBB\textsubscript{BB3} were co-transfected with wild-type linearized AcMNPV DNA into Spodoptera frugiperda Sf9 insect cells and the recombinant (gal\textsuperscript{+} occ\textsuperscript{-}) virus plaques purified as described for rβA3-crystallin (Hope et al., 1994b). Recombinant clones were selected for study based on the level of recombinant protein expressed in Sf9 cells infected with purified recombinant viruses as observed by SDS–PAGE and Western blots of soluble extracts. Sf9 cells were infected with the selected recombinant virus, harvested and lysed as previously described (Hope et al., 1994b). The molecular weight of rβB2 in the soluble extract was determined by gel filtration on a Pharmacia FPLC Superdex 75 HR 10/30 column calibrated with the protein standards bovine serum albumin, ovalbumin, carbonic anhydrate, chymotrypsin, soybean trypsin inhibitor and lysozyme. Recombinant proteins (rβA3 and rβB2) were purified from soluble extracts of Sf9 cells infected with rβB2-recombinant AcMNPV and rβA3-recombinant AcMNPV by DE-52 ion exchange and Sephacryl S-200 gel filtration chromatography as previously described (Hope et al., 1994a).

Circular dichroism analysis

Secondary structural analysis of the recombinant crystallin was performed by circular dichroism using a JASCO J-720 spectropolarimeter in the far UV (186–250 nm) range (Figure 1). Purified recombinant crystallin was dialyzed against 50 mM potassium phosphate, pH 6.8, and concentrated to 0.2–0.3 mg/ml prior to analysis. Each spectrum is the average of 10 scans and is noise reduced. Secondary structural predictions were calculated from CD spectra using both the PROSEC program with reference spectra from Yang et al. (1986) and the LINCOMB method using the four reference curve set: α-helix, β-turn, β-sheet and random coil (Perczel et al., 1992).

Sequence analysis of rβB2-crystallin

Purified rβB2-crystallin was subjected to Edman degradation on a Applied Biosystems Procise model 494A protein sequenator. Peptides generated by cyanogen bromide (CNBr) cleavage of βB2-crystallin in 70% formic acid were separated by 14% SDS–PAGE and electrotransferred to PVDF membrane (Immobilon-P) prior to N-terminal sequencing.

Association of rβB2- and rβA3-crystallin

Purified rβA3-crystallin (50 µg, 2×10\textsuperscript{-9} moles) was mixed with an equal amount of rβB2-crystallin in 0.5 ml of 50 mM Tris–HCl, pH 7.1, 1 mM EDTA, 1 mM DTT (buffer A) in the presence of 6 M urea. After incubating for 1 h at 4°C samples were diluted 10-fold with 10 mM Tris–HCl, 1 mM DTT and concentrated to 0.2 ml by Centricon-10 (Amicon) ultrafiltration concentrators. The dilution and concentration procedure was repeated once. Samples were analyzed on a Superdex 75 10/30 gel filtration column (void volume 7 ml) and by IEF gels. The Pharmacia PhastSystem was used to run nondenaturing pH 3–9 IEF PhastGels for 45 min with 2×10\textsuperscript{-9} moles of each in 50 µl, 12 µM and incubated at room temperature in the absence of denaturants. Samples taken at 0, 0.2, 1, 2, 4, 6, 12 and 18 h after mixing were electrofocused on nondenaturing IEF PhastGels (Pharmacia). Gels stained with Coomassie blue were scanned with a LKB Ultrascan XI laser densitometer and the relative absorption of the protein bands at 633 nm were

![Fig. 1. Far UV circular dichroic spectra of rβB2-crystallin (—) and rβA3-crystallin (—). [q], mean residue ellipticity in degrees cm\textsuperscript{2} dmol\textsuperscript{-1}.](image-url)
determined. Additional samples (6.25 µg or 2.5×10⁻¹⁰ moles of each in 25 µl total volume) were freshly mixed and applied directly or after a 6 h incubation at room temperature to a Superdex 75 HR 10/30 column with a void volume of 7 ml and chromatographed at 1 ml/min.

**Analytical ultracentrifugation**

Analytical ultracentrifugation was performed using a Beckman Optima XL-A analytical ultracentrifuge with an An-60 Ti rotor and standard double sector centerpiece cells. For equilibrium measurements, samples (90 µl) were centrifuged for 14–20 h at 20°C at either 15 000 r.p.m. for βA3-crystallin or 16 500 r.p.m. for βB2-crystallin. The buffer was 25 mM Tris–HCl, pH 7.5, 0.15 M NaCl, 1 mM EDTA and 5 mM DTT. Data was analyzed using the Beckman-Origin software (v.2.0 for Windows). Protein partial specific volumes were calculated from the amino acid compositions (Cohn and Edsall, 1943). Molar extinction coefficients of 25 000, 65.83 mM⁻¹ cm⁻¹ and 23 235, 40.4 mM⁻¹ cm⁻¹ were used for βA3 and βB2 crystallins, respectively. Dissociation constants, monomeric molecular weights and CD analysis of these crystallins were determined using both the PROSEC and LINCOMB curve fitting programs (Hope et al., 1991). Values (g/ml) of 0.72 and 0.718 were used for the βA3 and βB2 crystallins, respectively. Solvent density was calculated as described by Laue et al. (1992). For the calculation of dissociation constants, monomeric molecular weights and molar extinction coefficients of 25 000, 65.83 mM⁻¹ cm⁻¹ and 23 235, 40.4 mM⁻¹ cm⁻¹ were used for βA3 and βB2 crystallins, respectively.

**Results**

**Expression of recombinant mouse βB2-crystallin**

The expressed recombinant βB2 (rβB2) elutes from the Superdex 75 gel filtration column with an apparent size larger than a monomer but slightly less than a dimer, with minimal tetramer formation. The rβB2 elutes from the Superdex 75 gel filtration column 2 ml downstream of βA3-crystallin (Figure 2). From the elution volume a molecular mass of 34 kDa was estimated, assuming a globular shape, although as discussed below the elution point changes with varying protein concentration or ionic conditions. SDS–PAGE and western blotting of the fractions, developed with antibodies to mouse βB2-crystallin, confirms the identity of the peak as rβB2-crystallin (data not shown). The rβB2-crystallin band on SDS–PAGE corresponds to a molecular mass of 24 kDa, compared with a predicted mass of 23 380 Da. The rβA3-crystallin band has an apparent molecular mass of 25 kDa (predicted molecular mass 25 206), slightly higher than rβB2 crystallin.

**Sequence of rβB2-crystallin**

No amino acids were detected by several cycles of N-terminal sequencing of the intact rβB2-crystallin, indicating that the N-terminus is blocked (data not shown). Sequences of N-termini of two fragments (residues 47–122 and 123–193) generated by CNBr digestion are consistent with the predicted sequences of mouse βB2-crystallin (Chambers and Russell, 1991).

**CD analysis of βB2-crystallin**

The far UV circular dichroic spectrum shows a minimum at 218 nm and a maximum at 195 nm (Figure 1), consistent with a predominantly β-sheet structure. These results closely resemble those seen with rβA3-crystallin, which showed a minimum at 215 nm and maximum at 197 nm, and with a mouse lens total β-crystallin fraction which showed a double negative peak with a minimum at 216 nm and a maximum at 194 nm (Hope et al., 1994b). Secondary structure prediction using both the PROSEC and LINCOMB curve fitting programs indicated the recombinant βB2-crystallin is 67% β-sheet. The percentages of β-sheet predicted by the same programs for rβA3 (85%) and mouse total β-crystallins (75%) were similarly high.

**Dimer formation by βB2- and βA3-crystallin**

Chromatography of purified rβB2-crystallin on Superdex 75 yields a peak in fractions 21–22 (Figure 2), corresponding to an apparent molecular mass of 34 kDa, similar to that seen with Sf9 total cell lysates. Purified rβA3-crystallin elutes slightly ahead of rβB2, in fractions 17–18, corresponding to a molecular mass of 44 kDa. As discussed below, the association properties of these β-crystallins result in shifts of their elution peaks corresponding to several kDa depending on the specific conditions of the chromatography. Similarly, while the ultracentrifugation data are accurate to within 5–10%, the estimated molecular masses are dependent on ionic strength and temperature. The quaternary structure of the recombinant βB2-crystallin was independently assessed using analytical ultracentrifugation. The dimerization of the two crystallins was confirmed by sedimentation equilibrium (Figure 3). Weight average molecular masses of 39 000 and 47 000 Da were determined for the βB2 and βA3 crystallins, values which are close to the respective predicted dimer masses of 46 000 and 50 000. When the data were fitted assuming reversible monomer dimer equilibria, dissociation constants (K_d) of about 0.8×10⁻⁶ and 5.0×10⁻⁸ M were determined for the βA3 and βB2 crystallins, respectively. In practical terms this means that at a protein concentration of 1 mg/ml in this buffer βA3-crystallin is about 91% dimer whereas βB2-crystallin is only about 80% dimer.
Fig. 3. Sedimentation equilibrium of murine crystallin proteins. Measurements were made using an Optima XL-A analytical ultracentrifuge. The absorbance gradients in the centrifuge cell after attaining sedimentation equilibrium are shown in the bottom panels: (A) βA3-crystallin and (B) βB2-crystallin. Solid lines are results of fits to monomer–dimer association model and the open circles are the experimental values. The corresponding top panels show the difference in the fitted and experimental values as a function of radial position (residuals).

**Heterodimer formation by rβB2- and rβA3-crystallins**

Chromatography of equal amounts of rβA3- and rβB2-crystallin immediately after mixing results in two peaks corresponding to the elution positions of the individual purified proteins alone (Figure 2). When the rβA3- and rβB2-crystallin fractions are allowed to stand at room temperature for 6 h before chromatography a single peak eluting at an intermediate position between those of pure βB2- and βA3-crystallin results. Similarly, chromatography of equal amounts of rβA3 and rβB2 which had been disassociated by mixing in the presence of 6 M urea followed by reassociation through removal of the urea by dialution resulted in a peak which eluted intermediate between those of pure βB2- and βA3-crystallins (data not shown). All the peaks, but especially the combined peak, show a slight asymmetry, with a sharp frontal region and a trailing back region typical of self associating systems (Winzor and Scheraga, 1963). The observation that an intermediate peak is formed upon reassociation of the βB2- and βA3-crystallin suggests that heterodimers are present, and this is supported by the presence of both βA3- and βB2-crystallin in the intermediate peak demonstrated by SDS–PAGE and Western blot analysis with antibodies to βA3- and βB2-crystallins.

The formation of heterodimers between rβA3- and rβB2-crystallin was confirmed by isoelectric focusing. Isoelectric focusing of rβB2-crystallin alone gives a single band with a pI of 6.4, while isoelectric focusing of rβA3 gives a major band at pI 6.1 as well as several bands which presumably represent different oxidation states of this crystallin as has been reported previously for β-crystallins (Zigler, 1994). When rβA3- and rβB2-crystallin are mixed without denaturants, rβA3–rβB2 heterodimers are observed within 10 min (Figure 4). Isoelectric focusing of reassociated rβA3/rβB2-crystallin heterodimers demonstrates that the new species has a pI of 6.25, intermediate between rβB2 (pI 6.4) and rβA3 (pI 6.1). The intermediate band increases in intensity as the rβB2- and rβA3-crystallin bands decrease (Figure 2). Equilibrium between rβA3 and rβB2 and the heterodimer is reached by 4–6 h, at which time approximately 50% of the rβA3- and rβB2-crystallin is in the heterodimer form.

**Discussion**

Recombinant βB2-crystallin is faithfully expressed in a baculovirus system and appears to associate as a homodimer or as a heterodimer by associating with βA3-crystallin in a biologically
appropriate fashion. The murine C-terminal sequence is identical with those of human and cow and shares five of six amino acids with the chicken (Sergeev and Hejtmancik, 1997). The N-termini of the recombinant crystallins are blocked, so that appropriate fashion. The murine C-terminal sequence is identical with those of human and cow and shares five of six amino acids with the chicken (Sergeev and Hejtmancik, 1997). The N-termini of the recombinant crystallins are blocked, so that previous results with recombinant β from bovine lenses (Berbers et al., 1984), and our own previous results with recombinant βA3-crystallin expressed in a baculovirus system (Hope et al., 1994a,b). The identity of the expressed protein is, however, confirmed by the sequences of two internal CNBr cleaved peptides which match the deduced sequences for βB2-crystallin, as well as immunoreactivity of the recombinant protein to antibodies specific to βB2-crystallin. The overall conformation of the βB2-crystallin core domain structure was assessed by comparing its far UV circular dichroism spectrum to recombinant βA3-crystallin and to mixed β-crystallins isolated from the mouse lens. The overall spectrum is extremely similar to rβA3 (Hope et al., 1994b), indicating that rβB2 has a high β-sheet content, similar to rβA3-crystallin and naturally occurring β-crystallins isolated from the mouse lens.

Both sieve chromatography and sedimentation equilibrium show that βA3-crystallin and βB2-crystallin associate into dimers, consistent with previous results (Hope et al., 1994a,b; Kroone et al., 1994; Trinkl et al., 1994; Wertten et al., 1996). Molecular mass estimates for βB2- and βA3-crystallin from sieve chromatography (34 and 44 kDa respectively) agree with the weight average estimates from centrifugation (39 and 47 kDa respectively), but are somewhat smaller than the calculated dimer molecular masses (46 and 50 kDa respectively). These intermediate molecular weight estimates, i.e. larger than a monomer and less than a dimer, are best explained by a reversible monomer–dimer association. The dissociation constants we have estimated (see Results section) indicate that under the conditions used, βA3-crystallin is a more stable dimer than the βB2-crystallin, and this is reflected by a higher weight average molecular weight relative to the calculated dimer molecular weight. It is of interest to compare the association of the murine crystallins with the analogous bovine-derived proteins. Molecular masses of 51.4 and 47.7 kDa have been determined for bovine βA3- and βB2-crystallin, respectively, both values being within 3% of the theoretical dimer masses of 50 and 47 kDa. While it is difficult to be sure there is no self-association or aggregation from the data presented (McRorie and Voelker, 1993), the difference between the estimated molecular masses of the cow and mouse β-crystallin dimers might well relate to tighter association of the bovine crystallins relative to the murine.

We have observed that most of the β-crystallins isolated from the mouse lens migrate in the βL-crystallin peak while under similar ionic conditions most bovine β-crystallins are found as higher order aggregates (Hope and Hejtmancik, unpublished results). This also suggests that β-crystallin tetramers may be less stable in the mouse than in the cow. Lower association strengths for mouse β-crystallins might contribute not only to the lower size estimates relative to calculated masses estimated by sieve chromatography and by equilibrium centrifugation, but also to the slight asymmetry of the chromatographic peaks seen in Figure 2. The presence of a sharp leading edge followed by a broader trailing edge also suggests that these proteins are best described by a reversible monomer–dimer association (Winzor and Scheraga, 1963).

The rapid subunit exchange by mouse β-crystallins might also reflect the difference between the associative tendencies of bovine and mouse β-crystallins (Hope et al., 1994b). Berbers et al. (1982) have shown that it is possible to dissociate β-crystallin aggregates reversibly without denaturing their core domains by placing them in 6 M urea. Here it is shown that mouse βB2-crystallin and βA3-crystallin homodimers will exchange monomers to form heterodimers at room temperature in the absence of denaturing agents. This exchange is apparent within minutes and reaches equilibrium within 4 to 6 h. The distribution of the species at equilibrium indicates equivalent association or possibly a slight preferential association of acidic and basic monomers into heterodimers as previously reported by Slingsby and Bateman (1990a). Precise quantitative analysis was difficult due to the multiple bands resulting from isoelectric focusing of rβA3-crystallin.

The association of crystallins into higher order aggregates is thought to be of critical importance for maintaining lens transparency (Hejtmancik et al., 1995). At the high protein concentrations found in the lens (over 400 mg/ml), essentially all the β-crystallin would exist as higher order aggregates, consistent with studies of light scattering at high crystallin concentrations (Delaye and Gromiec, 1983). The Ks found for murine βA3- and especially murine βB2-crystallin, however, suggest that even at high concentrations these crystallin aggregates might still undergo some monomer exchange rather than existing as static structures. This is particularly true since subunit exchange would be much more rapid at physiological temperatures than at room temperature, at which the exchange experiments were performed (Joss and Rabton, 1996).

In summary, we have expressed mouse βB2-crystallin in the baculovirus system and demonstrated that the recombinant βB2-crystallin folds appropriately and associates as a homodimer. The βB2-crystallin is capable of associating with rβA3-crystallin to form heterodimers and does so rapidly in the absence of denaturants. The dissociation constant of murine βB2-crystallin (5×10⁻⁶M) is slightly greater than that of βA3-crystallin (0.8×10⁻⁶ M). These studies using baculovirus-expressed recombinant crystallins form the foundation for further investigation of associative properties of normal and modified β-crystallins.

References

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