CONCLUSIONS

This study represents the first attempt to determine the solution structure of both $d(GCAATTGC)_2$ and its complex with berenil by NMR spectroscopy. Data presented above for the oligonucleotide both alone and complexed with berenil demonstrate that the duplex is in a B conformation. NOE experiments provide evidence for binding of the drug in the minor groove of the oligonucleotide at the AATT sequence. Molecular models for this drug-DNA interaction that are consistent with our NMR data were generated on a computer graphics system and analyzed by CORMA. We are currently extending the graphical analysis of this system by carrying out energy minimization calculations.

ACKNOWLEDGMENTS

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Quaternary Interactions in Eye Lens β-Crystallins: Basic and Acidic Subunits of β-Crystallins Favor Heterologous Association†

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ABSTRACT: β-Crystallins are complex eye lens proteins made up of several related basic and acidic subunits that combine to form differently sized oligomers each displaying extensive polydispersity. As the sequences are homologous to the X-ray-determined bilobal structure of γ-crystallin, β-subunits are visualized as having a similar structure with additional N- and C-terminal extensions. Two basic (βB2 and βB3) and two acidic (βA3 and βA4) subunits have been isolated in deaggregating media, refolded, and reassociated in various combinations to determine which components favor dimers or higher oligomers. Homopolymers were compared with βB2 homodimer in terms of charge, using Mono Q fast protein liquid chromatography, and size, using Superose 12 chromatography. Heterooligomeric formations were monitored by their intermediate charge properties compared with homooligomers. βB2 associates with either βB3- or βA4-forming heterodimers whereas a larger oligomer is formed with βA3. Naturally occurring β-crystallin oligomers were analyzed by Mono Q chromatography and PhastGel electrophoresis. Whereas βB2, βB3, and βA4 can each be reassociated to homodimers, βA4 dimers are not found in native β-crystallins. βB2-βA3 is a major component of intermediate-sized βL_1-crystallin and is absent from dimeric βL_2-crystallin. It is suggested that the pH dependence of the size of βL_1-crystallin is due to a dimer to tetramer equilibrium. By following dimer interactions using Superose 12 chromatography, βB2-βA4 was shown to interact with βB2-βA3. A model of β-crystallin structure is proposed based on β-subunits forming dimers with the next level of organization requiring an acidic subunit, βA3, with a long N-terminal extension.

Refractive index is the fundamental property which determines the focal power of lenses. In animal lenses, heterogeneous populations of globular oligomeric and monomeric crystallins are packed together inside cells (Harding & Crabbe, 1984; Wistow & Piatigorsky, 1988), forming protein concentration gradients from which optical characteristics are

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derived (Philipson, 1969). In mammalian lenses, there are three classes of proteins, α-, β-, and γ-crystallins, which are clearly distinct in terms of size. Control of the size distribution of lens crystallins is probably a major factor in determining the packing on which refractive index and transparency depend. γ-Crystallins are a family of monomers of molecular weight 20 000 with each member composed of two closely interacting similar globular domains (Blundell et al., 1981; Wistow et al., 1983; Chirgadze et al., 1986; Sergeev et al., 1988; White et al., 1989). In the case of α-crystallin, which can be isolated as a heteropolymer of molecular weight 1 000 000, the transparency of concentrated solutions has been calculated to be very sensitive to variations in the compactness of the aggregate (Vérêtout et al., 1989). β-Crystallins comprise a range of sizes in between α- and γ-crystallins being oligomers formed from a wide variety of subunits (Zigler & Sidbury, 1973; Herbrink et al., 1975; Berbers et al., 1984).

β-Crystallins are the most complex of the lens proteins as the many subunits combine in a variety of ways to form several populations of aggregates with each size class displaying extensive polydispersity (Zigler et al., 1980; Bindels et al., 1981; Chiou et al., 1989). Sequencing studies showed that bovine β-crystallin subunits could be divided into a basic (βB1, βB2, βB3) and an acidic (βA2, βA3, βA4) family with apparent molecular weights derived from sodium dodecyl sulfate (SDS)[1] gel electrophoresis of 32 000, 26 000, 27 000, 23 000, 25 000, and 23 000, respectively, and that they shared a common ancestry with γ-crystallins (Driessen et al., 1981; Berbers et al., 1984; Gorin & Horwitz, 1984; Lubsen et al., 1988). Model-building β-crystallin sequences using the coordinates of γ-crystallin led to the prediction that all β-crystallin subunits would have a two-domain structure with each domain comprising two Greek key motifs organized in a symmetrical way (Wistow et al., 1981; Inana et al., 1983; Slingsby et al., 1988). However, whereas only basic subunits had an extension of sequence at the C-terminus, all β-subunits had an N-terminal extension, though of different lengths (Berbers et al., 1984). The observed heterogeneity of β-crystallin aggregates could be generated partly at the combinatorial level as a much larger number of oligomers can be derived from a limited number of subunits if most associations are allowed. However, subunit heterogeneity is increased by proteolytic processing (bovine βB1b is derived from βB1; Berbers et al., 1983), through alternative usage of mRNA initiation codons (βA1, molecular weight 23 000 from βA3; Quax-Jeuken et al., 1984; Hogg et al., 1986) and through phosphorylation of βB2 (Kleiman et al., 1988) and βB3 (Voorter et al., 1989). Aging of lenses is associated with greater polydispersity of β-crystallin aggregates (Bessems et al., 1986) and increased subunit cleavage (Zigler, 1978; Alcala et al., 1988). In human lens, the N-terminal extension of βB2 is cleaved with aging (Takemoto et al., 1987).

Earlier work has shown that although many β-subunits are common to all β-aggregates there are restrictions. Bovine βB1 is specifically associated with βH-crystallin, molecular weight 160 000–200 000, and absent in both the dimeric βL_2-crystallin, molecular weight 46 000, and the intermediate βL_1-crystallin, molecular weight approximately 70 000 (Bindels et al., 1981; Berbers et al., 1982; Siezen et al., 1986). The higher molecular weight acidic β-subunit βA3 is more predominant in βH- and βL_1-crystallins than in the smaller βL_2-crystallins (Berbers et al., 1982; Slingsby & Bateman, 1990). These observations indicated that in order to construct higher molecular weight aggregates, specific β-crystallin subunits were required.

Further insight into the kinds of quaternary interactions used for building and controlling the size of β-crystallins required subunit analysis of monodisperse oligomers. One approach is to isolate individual subunits in dissociating media and then investigate how they reassociate either on their own or in combination with other β-subunits. To this end, we have developed rapid procedures for isolation of two similar-sized basic subunits, βB2 and βB3, each with an N- and C-terminal extension, and two acidic subunits, βA3 and βA4, which have different lengths of N-terminal extension (Slingsby & Bateman, 1990). In this paper, we have refolded and reassociated various combinations of subunits and analyzed the resulting oligomers in order to determine whether homodimers or heterodimers are preferentially formed and which components favor further aggregation.

Materials and Methods

Preparation of βH-, βL_1-, and βL_2-Crystallins. Soluble proteins from calf lens cortical extracts were subjected to rapid gel filtration on Pharmacia Sephacryl S300 HR (column size 1000 × 70 mm) in 0.05 M sodium phosphate, 0.2 M KCl, 1 mM EDTA, 1 mM EGTA, and 0.02% NaN_3, pH 6.7, as described previously (Slingsby & Bateman, 1990). Filtration was also carried out at pH 8.5 in which case 0.05 M Tris-HCl replaced phosphate buffer and the column was equilibrated with several bed volumes of the new elution buffer.

Protein Concentration and Estimation. Solutions were concentrated under nitrogen by ultrafiltration in a 50-mL Amicon cell equipped with a YM 10 membrane. Protein concentration was estimated by using an absorbance (1%, 1 cm) coefficient of 22 at a wavelength of 280 nm for all β-subunits and oligomers.

Preparation of β-Crystallin Subunits. βB2, βB3, βA3, and βA4 were prepared in 6 M urea by ion-exchange fast protein liquid chromatography (FPLC) using an 8-mL Mono Q column (Pharmacia–LKB) as described previously (Slingsby & Bateman, 1990).

Reassociaton of β-Crystallin Subunits. Each subunit was individually concentrated in 0.05 M Tris-HCl, pH 8.5, 6 M urea, and 0.1% (v/v) 2-mercaptoethanol to 1 mg/mL. Refolding of each subunit was achieved by dilution × 10 volumes with 0.05 M Tris-HCl, pH 8.5, and 0.1% (v/v) 2-mercaptoethanol followed by concentration back to starting volume. The dilution and concentration procedure was repeated and then once more except this time with 0.05 M Tris-HCl, pH 8.5 (buffer A). Combinations of β-subunits in deaggregating buffer were also refolded: 5 mL of βB2 and 3.6 mL of βB3, 2 mL of βB2 and 2 mL of βA4, 2 mL of βB2 and 2 mL of βA3. Each of the three mixtures was then subjected to the same dilution and equilibration procedure as described above.

Ion-Exchange Chromatography of Reassociated β-Crystallins. Solutions of individually reassociated β-subunits and mixtures of β-subunits were filtered through a 0.45-μm Millipore membrane and applied to an 8-mL Mono Q column equilibrated with 0.05 M Tris-HCl, pH 8.5 (buffer A). The elution program consisted of 16 mL of buffer A, a linear NaCl gradient ranging from 0 to 30% of buffer B (buffer A, 1 M NaCl) in a volume of 80 mL, 16 mL of buffer B, and 16 mL of buffer A. The flow rate was 2 mL/min. Reassociated βB2, βB3, and βA4 eluted at around 11%, 15%, and 25% of buffer B, respectively. Reassociated βA3 eluted broadly between 13 and 14% of buffer B. Protein products from reassociated mixtures of β-subunits were chromatographed under the same

[1] Abbreviations: FPLC, fast protein liquid chromatography; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propandiol; MES, 2-(*N*-morpholino)ethanesulfonic acid; IEF, isoelectrofocusing; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

conditions, and the main protein peaks were pooled as indicated in the figures and subsequently analyzed by gel electrophoresis. In each case, the column was calibrated with a βB2 dimer solution which had been equilibrated with the same batch of buffer A which was used for equilibrating the column and was also the final buffer used to reassociate the mixture of β-subunits.

Ion-Exchange Chromatography of βL_1- and βL_2-Crystallins. Freshly prepared βL_1- and βL_2-crystallins from gel filtration on Sephacryl S-300 HR were separately concentrated to 5 mg/mL followed by equilibration in the Amicon with buffer A ensuring that this protein concentration was not exceeded. Each preparation was subjected to ion-exchange chromatography on an 8-mL Mono Q column using the same elution and calibration procedures as described above.

Analytical Gel Filtration of β-Crystallins. Two hundred microliter samples were loaded onto a column of Superose 12 prepacked HR 10/30 equilibrated in 0.05 M MES, pH 6.7, and 0.2 M NaCl; the column was eluted at a flow rate of 0.5 mL/min, and 250-μL fractions were collected. All protein solutions were filtered through a 0.45-μm Millipore membrane prior to column loading.

(A) Reassociated β-Subunits. βB2 (0.75 mg/mL), βB3 (0.75 mg/mL), and βA3 (1 mg/mL), each having been reassociated into buffer A as described previously, were separately loaded on the column and their elution volumes observed. Reassociated βB2 was also equilibrated with the Superose 12 pH 6.7 elution buffer using the Amicon, and samples were loaded at 0.3 and 3 mg/mL. Reassociated βA4 was equilibrated with pH 6.7 elution buffer and loaded at 1 mg/mL. Reassociated βB2–βA4 was equilibrated into pH 6.7 elution buffer and loaded at 3 mg/mL. Equal volumes of reassociated βB2–βA4 and reassociated βB2, each having been equilibrated at pH 6.7, were mixed, and a 200-μL sample was loaded. Reassociated βB2–βB3, purified by FPLC on 8-mL Mono Q, was applied to the Superose 12 column at 0.75 mg/mL when equilibrated with buffer A and at 0.3 and 3 mg/mL when equilibrated with pH 6.7 elution buffer. Reassociated βB2–βA3 was purified by FPLC on 8-mL Mono Q and loaded at 1.5 mg/mL. One hundred fifty microliters of reassociated βB2 (1 mg/mL) in buffer A and 150 μL of FPLC-purified βB2–βA3 (1 mg/mL) in buffer A were mixed, and a 200-μL sample was loaded on the Superose 12 column.

(B) βL_1- and βL_2-Crystallins. Two hundred microliters of βL_2 (10 mg/mL) was mixed with 300 μL of βL_1 (10 mg/mL) both in Sephacryl HR elution buffer, and 500 μL of Superose 12 elution buffer was added. A 200-μL sample was applied to the Superose 12 column.

(C) Oligomer Interactions. A 100-μL sample of reassociated βB2–βA4 (2.5 mg/mL) in buffer A was added to 200 μL of Superose 12 pH 6.7 elution buffer; a 200-μL sample was loaded, and the elution volume of the protein peak was estimated. βB2–βA3 oligomer was purified from undissociated βL_1-crystallin by Mono Q FPLC in buffer A as described earlier and concentrated to 2.5 mg/mL. To a 100-μL sample of βB2–βA3 oligomer was added 200 μL of pH 6.7 elution buffer and the elution volume of the protein peak estimated. Equal volumes (100 μL) of reassociated βB2–βA4 (2.5 mg/mL) and βB2–βA3 (2.5 mg/mL) were mixed at pH 8.5, and 100 μL of pH 6.7 elution buffer was added. A 200-μL sample was applied to the column, and the relative proportions of the two protein peaks were estimated. The Superose 12 column was calibrated with reassociated βB2 dimer (1 mg/mL).

Subunit Analysis by Gel Electrophoresis. Protein solutions (0.5 mg/mL) were boiled with Laemmli buffer, and 1-μL samples were loaded onto a Pharmacia 20% homogeneous SDS PhastGel, run for 150 Vh followed by automatic Coomassie staining using the Phast (Pharmacia-LKB) development system. For IEF–urea gels, a PhastGel IEF 3–9 was washed free of Pharmalytes and rehydrated for 2 min in ampholines (pH 3.5–9.5) in 6 M urea containing 0.0025% Nonidet P-40. One-microliter protein samples (5 mg/mL) were loaded near the anode; the gel was run for 800 Vh followed by automatic Coomassie staining.

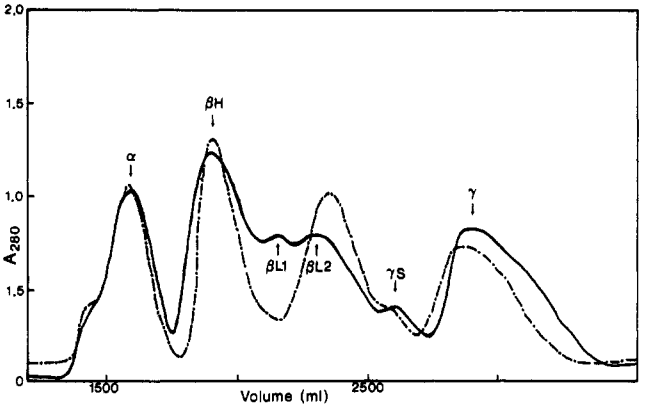


FIGURE 1: Sephacryl S-300 HR gel filtration chromatography of calf cortical lens soluble proteins eluted at a flow rate of 130 mL/h at pH 6.7 (—) or pH 8.5 (-·-). The elution positions of the main crystallin classes are indicated.

RESULTS

Isolation of β-Crystallin Subunits. The three size classes, βH-, βL_1-, and βL_2-crystallins, were prepared by rapid gel filtration on Sephacryl S300 HR at pH 6.7 (Figure 1) as described previously (Slingsby & Bateman, 1990). The basic subunits βB2 and βB3 were isolated from βL_2-crystallin, and the acidic subunits βA3 and βA4 were isolated from either βL_1 or βH by ion-exchange FPLC in 6 M urea as described earlier (Slingsby & Bateman, 1990). It has been shown spectroscopically that βB2 reversibly denatures in 6 M urea (Horwitz et al., 1986) and undergoes a reversible order–disorder transition around 60 °C in nondeaggregating media (Maiti et al., 1988).

Reassociation of Basic β-Crystallin Dimers. (A) βB2 Homodimer. βB2 subunit was refolded at pH 8.5 in the presence of 2-mercaptoethanol and finally equilibrated in reducing agent free buffer. The resulting oligomer elutes sharply on anion-exchange chromatography in nondissociating buffer at pH 8.5 (Figure 2A). This preparation of βB2 eluted on the Superose 12 column in the same position as βL_2-crystallin when elution was performed at pH 6.7 (Figure 5). The elution volume was unaffected by a 10-fold difference in protein concentration or whether the protein was initially applied at pH 6.7 or pH 8.5. βB2 prepared by FPLC has been crystallized in the same form as βB2 prepared by the method of Herbrink et al. (1975) when it was shown to be a dimer (Slingsby et al., 1982). It is concluded that this new preparation of βB2 yields a homodimer.

(B) βB3 Homodimer. βB3 subunit was refolded into pH 8.5 buffer following the same procedure as for βB2. The resulting reassociated oligomer eluted as a more acidic protein than βB2 dimer on chromatography on Mono Q at pH 8.5 (Figure 2A). The reassociated βB3 oligomer eluted in the same position as βB2 dimer on chromatography on Superose 12 at pH 6.7. It is concluded that βB3 subunit self-associates to form a homodimer.

(C) βB2–βB3 Heterodimer. When βB3 subunit was mixed with a slight excess of βB2 in dissociating media in the presence

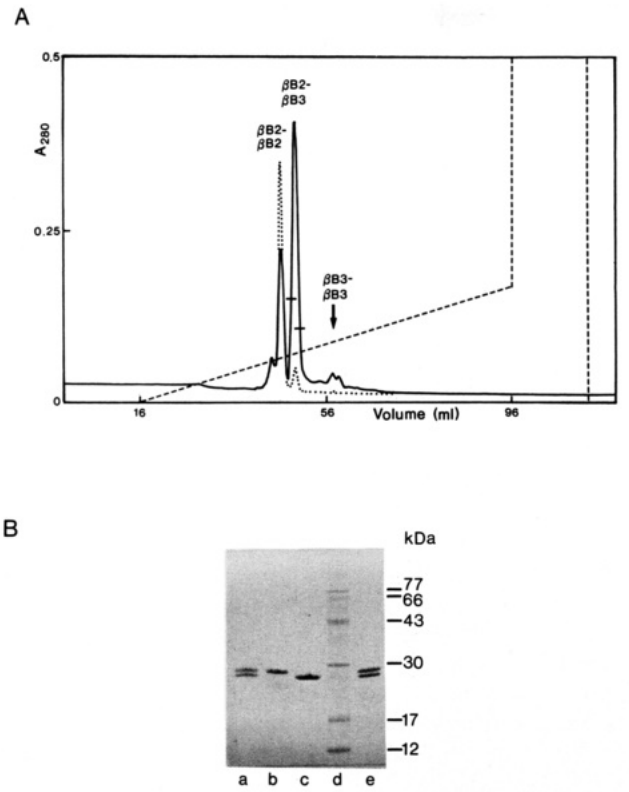


FIGURE 2: (A) Mono Q column chromatography following reassociation of βB2 and βB3 subunits. Five hundred microliters of the reassociated products (1.5 mg/mL) was loaded onto an 8-mL column and eluted with a linear NaCl gradient as described under Results. The flow rate was 2 mL/min. Five hundred microliters of reassociated βB2 dimer (0.7 mg/mL) was chromatographed under identical conditions. (—) Absorbance at 280 nm of the products of reassociation of βB2 with βB3 subunits; (···) absorbance at 280 nm of reassociated βB2 dimers; (- - -) NaCl. The arrow marks the position of elution of βB3 dimer. The horizontal bars indicate fractions of βB2–βB3 which were collected. (B) Electrophoresis of reassociated βB2–βB3 on 20% SDS homogeneous PhastGel. (Lanes a and e) βB2–βB3 taken from Mono Q column; (b) βB3; (c) βB2; (d) molecular weight markers.

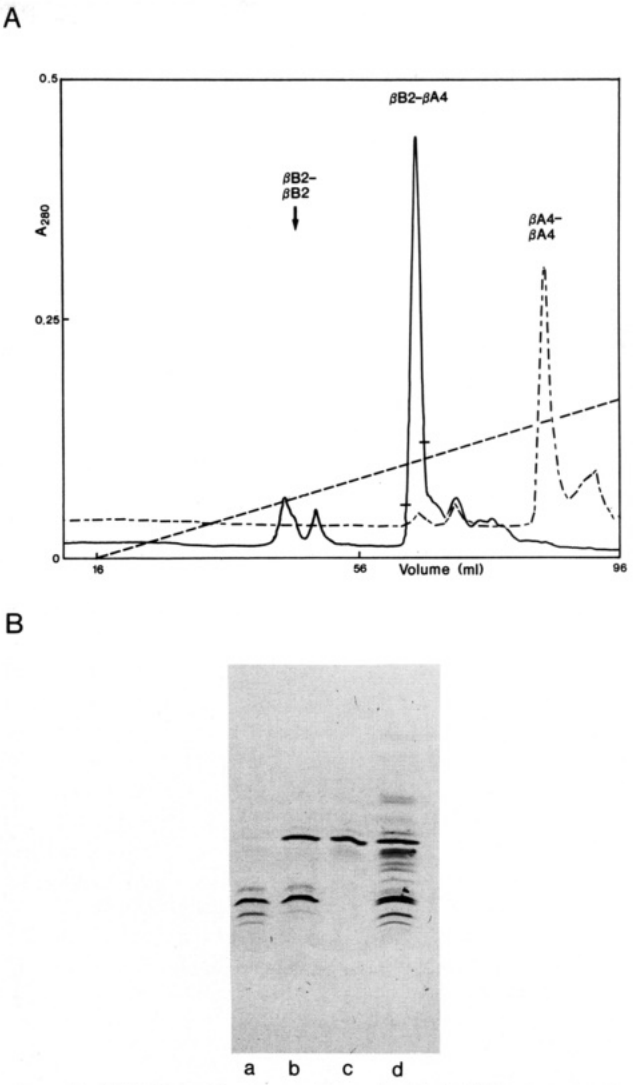


FIGURE 3: (A) Mono Q column chromatography following reassociation of βB2 and βA4 subunits. One milliliter of the reassociated products (1 mg/mL) was loaded onto an 8-mL column and eluted wiht a linear NaCl gradient at a flow rate of 2 mL/min. One milliliter of reassociated βA4 (1 mg/mL) and 1 mL of reassociated βB2 (1 mg/mL) were each chromatographed under identical conditions. (—) Absorbance at 280 nm of the products of reassociation of βB2 with βA4; (- - -) absorbance at 280 nm of reassociated βA4; (– –) NaCl. The arrow marks the position of elution of reassociated βB2 dimer. The horizontal bars indicate fractions of βB2–βA4 which were collected. (B) IEF–urea gel electrophoresis, pH 3–9, of reassociated βB2–βA4. The anode is at the bottom. (a) βA4; (b) βB2–βA4 taken from Mono Q column; (c) βB2; (d) βL_1-crystallin.

of 2-mercaptoethanol followed by dilution and equilibration into nondissociating media using the same procedure as for the reassociation of βB2 dimer, the predominant reassociated product is an oligomer with a charge intermediate between βB2 and βB3 homodimers. Following preparative chromatography at pH 8.5 on a Mono Q column (Figure 2A), the protein peak of intermediate charge was isolated and analyzed for subunit composition by SDS–PAGE electrophoresis. The oligomer clearly contained equal amounts of 27 000 and 26 000 molecular weight components (Figure 2B), consistent with it comprising βB2 and βB3 subunits (Slingsby & Bateman, 1990). The heterooligomer eluted on the Superose 12 column at pH 6.7 with the same elution volume as βB2 dimer and was unaffected by a 10-fold difference in protein concentration. It is concluded that βB2 and βB3 subunits readily reassociate to form heterodimers.

Reassociation of Oligomers Containing Acidic β-Subunits. (*A*) *βA4 Homodimer.* βA4 when isolated from either βL_1- or βH-crystallin shows four differently charged components when analyzed by IEF–6 M urea electrophoresis (Figure 3B). Whereas the major protein has been shown to be a primary translation product (Berbers et al., 1984), the three minor components are uncharacterized and may be posttranslational modifications of βA4. βA4 subunit was refolded and reassociated from 6 M urea in the same way as was described for basic β-subunits. The reassociated oligomer was subjected to anion-exchange chromatography in nondissociating media at pH 8.5 on a Mono Q column (Figure 3A), where it eluted near the end of the salt gradient. The reassociated βA4 oligomer eluted as a dimer when subjected to gel filtration on Superose 12 at pH 6.7. It is concluded that βA4 self-associates to a dimer.

(*B*) *βB2–βA4 Heterodimer.* When equal amounts of βB2 and βA4 subunits were mixed together in 6 M urea in the presence of 2-mercaptoethanol followed by dilution and equilibration into nondissociating media, then the major product was a protein with a charge intermediate between βB2 and βA4 (Figure 3A). The product was isolated and its subunit composition estimated on IEF–urea electrophoresis (Figure 3B), showing it to comprise βB2 and βA4. The reassociated product comprised a greater proportion of the primary translation product compared with the minor contaminating proteins. The reassociated oligomer eluted with the same elution volume as βB2 dimer on the Superose 12

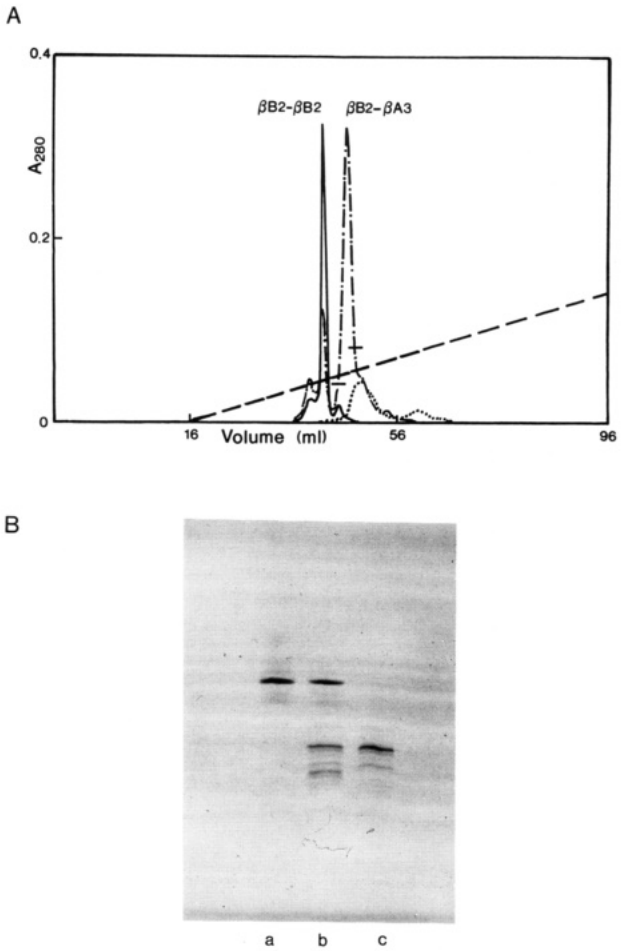


FIGURE 4: (A) Mono Q column chromatography following reassociation of βB2 and βA3 subunits. One milliliter of the reassociated products (1 mg/mL) was loaded onto an 8-mL column and eluted with a linear NaCl gradient at a flow rate of 2 mL/min. Five hundred microliters of reassociated βA3 (1 mg/mL) and 500 μL of reassociated βB2 (1 mg/mL) were each chromatographed under identical conditions. (–·–) Absorbance at 280 nm of the products of reassociation of βB2 with βA3; (—) absorbance at 280 nm of reassociated βB2; (···) absorbance at 280 nm of reassociated βA3; (– –) NaCl. The horizontal bars indicate fractions of βB2–βA3 which were collected. (B) IEF–urea gel electrophoresis at pH 3–9 of reassociated βB2–βA3. The anode is at the bottom. (a) βB2; (b) βB2–βA3 taken from Mono Q column; (c) βA3.

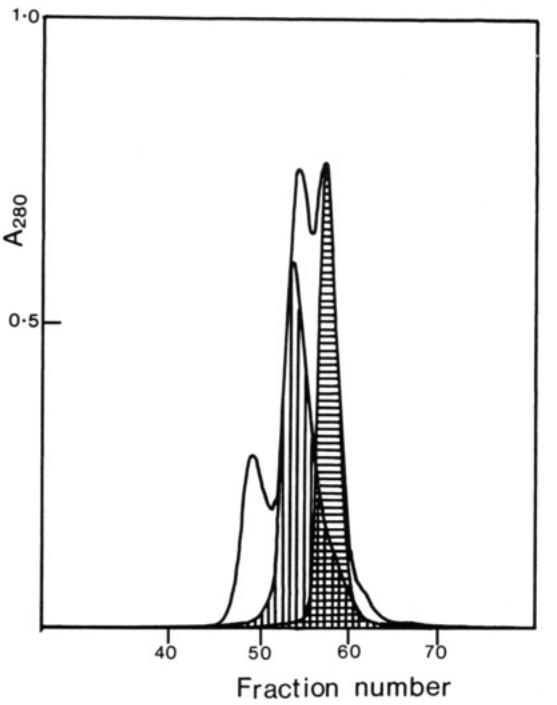


FIGURE 5: Gel filtration chromatography of β-crystallin oligomers on Superose 12 HR 10/30 eluted in 0.05 M MES, pH 6.7, and 0.2 M NaCl. Two hundred microliter samples were loaded, and 250-μL fractions were collected. (Open curves) Absorbance at 280 nm of βL_1- + βL_2-crystallins; (horizontally lined curve) reassociated βB2 dimer applied at 1.5 mg/mL; (vertically lined curve) reassociated βB2–βA3 applied at 1.5 mg/mL. The first peak eluting from the mixture of βL_1- and βL_2-crystallins is βH-crystallin which was incompletely separated from βL_1-crystallin on Sephacryl S-300 HR.

column gel filtration at pH 6.7. When reassociated βB2–βA4 equilibrated at pH 6.7 was mixed with βB2 dimer at pH 6.7 and then subjected to gel filtration, a single peak emerged with the elution volume of βB2 dimer. It is concluded that when βB2 and βA4 are refolded together then they preferentially form a heterodimer.

(C) βB2–βA3 Oligomer. These subunits were mixed in equal amounts in 6 M urea at pH 8.5 and then allowed to refold and associate using the same dilution procedures as described previously. The refolded oligomers were analyzed at pH 8.5 on a Mono Q column in nondissociating conditions, showing that the major product eluted at a more acidic position than βB2 homodimer (Figure 4A). This protein was isolated and analyzed by SDS–PAGE and IEF–urea electrophoresis (Figure 4B), which confirmed that the oligomer comprised both βB2 and βA3, although a more negatively charged form of βA3 was beginning to be generated (Figure 4B). When βA3 subunit was refolded and reassociated in the absence of βB2, then the resulting products gave a broad distribution when analyzed on the Mono Q column (Figure 4A). Reassociated βB2–βA3 oligomer, isolated by FPLC on Mono Q, was subjected to gel filtration on Superose 12 at pH 6.7. It eluted with a higher molecular weight than βB2 dimer and in a similar position to βL_1-crystallin (Figure 5). When βB2–βA3 oligomer was coeluted with βB2 dimer, the heterooligomer was clearly distinguishable from the dimer peak. Reassociated βA3 oligomers gave a broad distribution of sizes. The ability of βA3 subunit to reassociate with βB2 subunit to give a heterooligomer bigger than βB2 dimer suggests that at pH 6.7 it forms either a trimer or a tetramer.

Correspondence of Reassociated Oligomers with βL_1- and βL_2-Crystallins. βL_1- and βL_2-crystallins, freshly prepared by gel filtration at pH 6.7 (Figure 1), can each be resolved into several component oligomers when chromatographed on Mono Q at pH 8.5 in nondissociating buffers (Figure 6A,B). As these are the same conditions that were used for the chromatography of reassociated β-crystallins, the elution profiles of "native" β-crystallins can be correlated with reassociated oligomers. βL_1- and βL_2-crystallins can each be resolved into six major protein peaks (labeled 1–6 in Figure 6) together with many minor components. Peaks 1, 3, and 4–6 from both size classes of β-crystallins have the same elution position whereas peak 2 from βL_1-crystallin elutes as a more acidic protein than peak 2 from βL_2-crystallin. The subunit constituents of peaks 1, 2, 3, and 5 from βL_2-crystallin were examined by IEF–urea electrophoresis (Figure 7A) and compared with peaks 1–6 from βL_1-crystallin which were analyzed by IEF–urea and SDS–PAGE electrophoresis (Figure 7B,C). SDS–PAGE electrophoresis was useful for distinguishing βA1 (molecular weight 23 000) from βB3 (27 000) and βA4 (23 000) from βA3 (25 000) as these pairs of subunits have similar isoelectric points.

Reassociated βB2 homodimer coelutes with peak 1 from βL_1- and βL_2-crystallin. Peak 1 is the predominant fraction from βL_2-crystallin, and βB2 is the predominant subunit

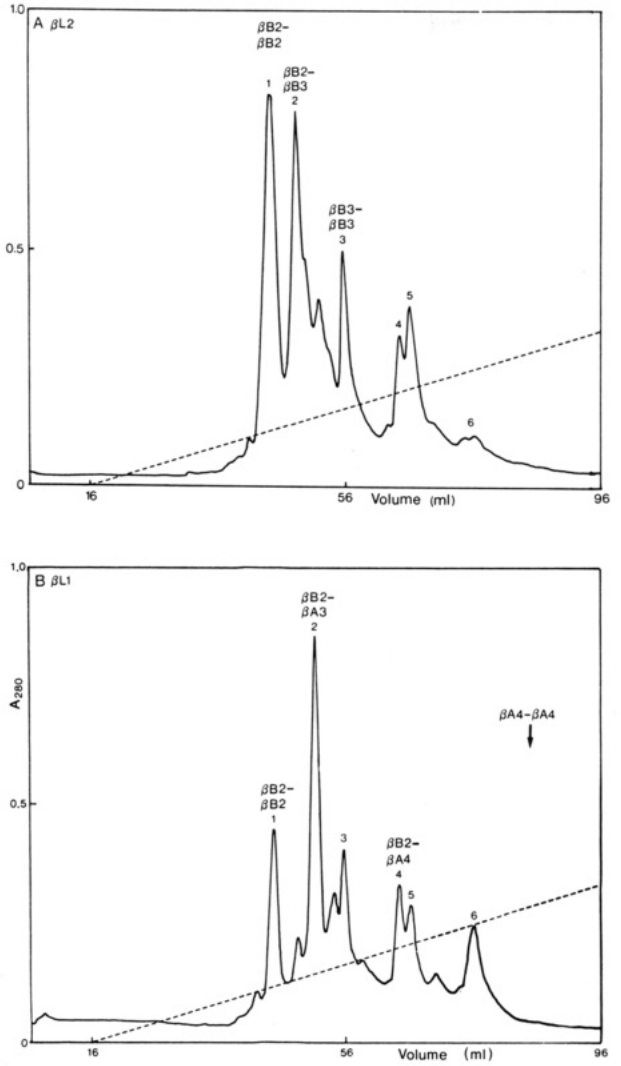


FIGURE 6: Mono Q column chromatography of (A) βL_2-crystallin and (B) βL_1-crystallin. Five milligrams in 2 mL of 0.05 M Tris-HCl, pH 8.5, was applied to an 8-mL column using the same elution conditions as described in Figure 2A. The elution position of peak 1 from both samples coincides with reassociated βB2 dimer. The arrow marks the elution position of reassociated βA4 dimer. (—) Absorbance at 280 nm; (---) NaCl.

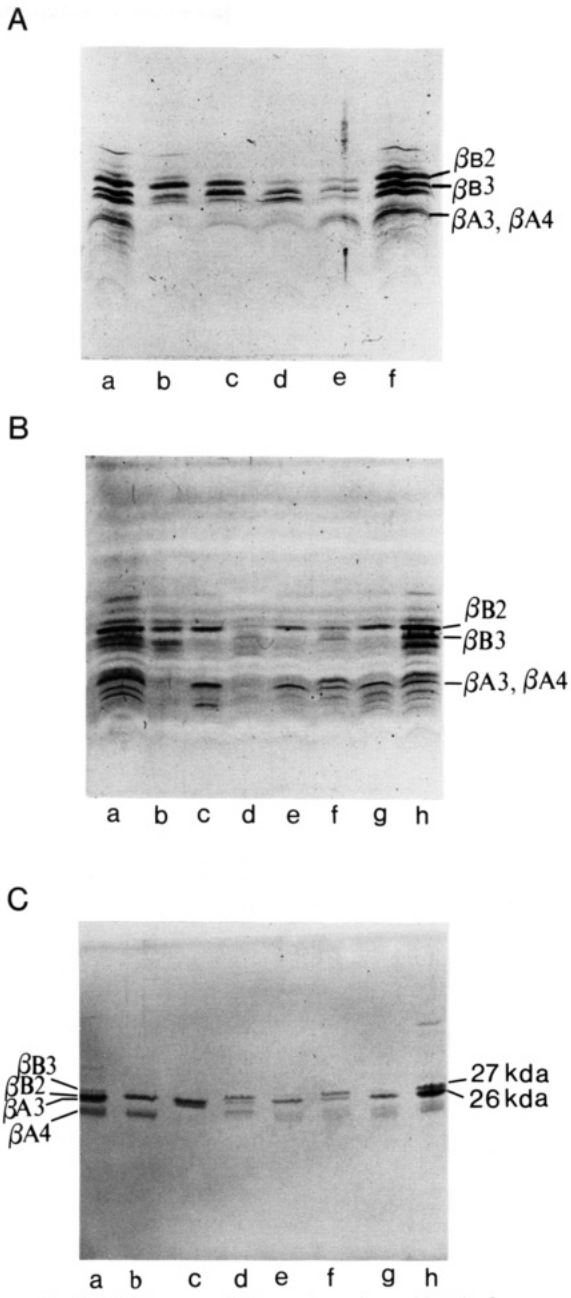


FIGURE 7: (A) IEF–urea gel electrophoresis at pH 3–9 of components from βL_2-crystallin resolved on Mono Q as described in Figure 6A: (a) βL_2; (b) peak 1; (c) peak 2; (d) peak 3; (e) peak 5; (f) βL_2. The anode is at the bottom. (B) IEF–urea gel electrophoresis at pH 3–9 of components from βL_1-crystallin resolved on Mono Q as described in Figure 6B: (a) βL_1; (b) peak 1; (c) peak 2; (d) peak 3; (e) peak 4; (f) peak 5; (g) peak 6; (h) βL_2. The anode is at the bottom. (C) Electrophoresis on 20% SDS homogeneous PhastGel of components from βL_1-crystallin resolved on Mono Q as described in Figure 6B. Lane samples (a–h) the same as in (B).

constituent (Figure 7A); therefore, βB2 homodimer will be a prominent component of βL_2-crystallin. Subunit analysis of peak 1 from βL_1-crystallin shows that a 23 000 molecular weight component (Figure 7C), which is more acidic than βB2 (Figure 7B), contributes significantly to this protein peak. Therefore, peak 1 from βL_1-crystallin comprises a smaller proportion of βB2 homodimers than peak 1 from βL_2-crystallin.

Peak 2 from βL_2-crystallin coelutes with reassociated βB2–βB3 heterodimer, and this assignment is consistent with βL_2 peak 2 subunit analysis (Figure 7A). By contrast, peak 2 from βL_1-crystallin, the major protein peak, coelutes with reassociated βB2–βA3 oligomer. The subunit composition of peak 2 from βL_1-crystallin indicates the presence of βB2 and a 25 000 molecular weight acidic subunit (βA3) together with a presumptive more negative form of βA3 (Figure 7B,C). It is concluded that βB2–βA3 heterooligomer is the predominant component of βL_1–crystallin.

βB3 homodimer coelutes with peak 3 from βL_1- and βL_2-crystallin. However, the subunit composition of these peaks shows other components, leaving it unresolved as to whether the homodimer is a native component. Peaks 4 and 6 from βL_1-crystallin have subunit compositions comprising mainly βB2 and βA4. Peak 4 from both βL_1- and βL_2-crystallin has the same elution position as reassociated βB2–βA4 heterodimer whereas peak 6 elutes as a more acidic oligomer. Significantly, in neither βL_1- nor βL_2-crystallin is there a protein fraction which corresponds to βA4 homodimer.

The predominant subunit βB2 appears to be used extensively in heterologous interactions although βL_2-crystallin comprises significant amounts of βB2 homodimer. In the higher molecular weight βL_1-crystallins, the bulk of the component oligomers are composed of both basic and acidic subunits. Although βA4 can be separated from basic subunits and be shown to reassociate to homodimers, there was no evidence

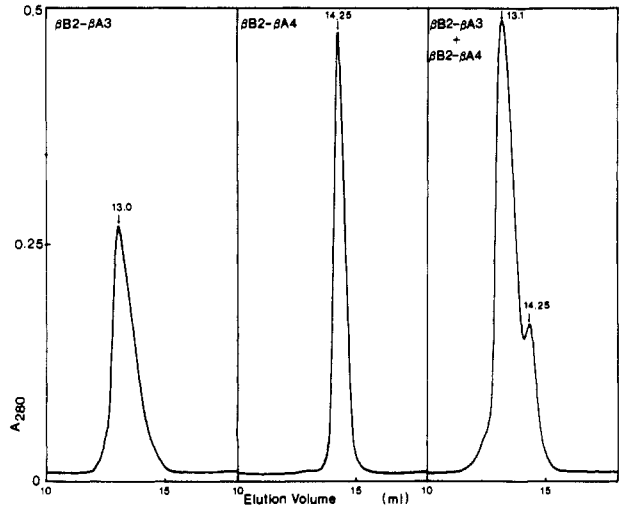


FIGURE 8: Demonstration of the interaction between βB2–βA3 and βB2–βA4 by Superose 12 gel filtration chromatography using 0.05 M MES/0.2 M NaCl, pH 6.7, as eluant. The peak heights of βB2–βA3 and βB2–βA4 were estimated when each sample was applied independently on the column and compared with the relative proportions of the two peaks when they were applied together.

that this subunit occurs as a homodimer in βL_1- or βL_2-crystallins. The major component of βL_1-crystallin is βB2–βA3 heterooligomer.

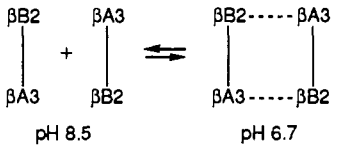
Dimer–Dimer Interactions. In order to isolate βL_1-crystallin, the gel filtration must be carried out over a restricted pH range. In this work, βL_1-crystallin was prepared at pH 6.7. When gel filtration was performed at pH 8.5, βL_1-crystallin coeluted with βL_2-crystallin (Figure 1). If it is assumed that βL_1-crystallin dissociates into dimers at pH 8.5, then the protein fractions observed when βL_1-crystallin was analyzed by ion-exchange chromatography on Mono Q at pH 8.5 (Figure 6B) are also mainly dimers. Further experiments were performed to investigate how dimers might interact to form higher aggregates.

βB2–βA4 heterodimer was prepared by reassociating βB2 and βA4 subunits followed by purification of the heterodimer by chromatography on FPLC at pH 8.5 (Figure 3A). A solution of βB2–βA4 heterodimer was subjected to gel filtration on Superose 12 equilibrated in pH 6.7 buffer. The protein eluted with the same volume as βB2 dimer, 14.25 mL (Figures 5 and 8). βB2–βA3 oligomer isolated from βL_1-crystallin on Mono Q in nondissociating buffer at pH 8.5 (peak 2, Figure 6B) was subjected to gel filtration at pH 6.7 whereupon it eluted in a volume (13.0 mL) typical of βL_1-crystallin (Figures 5 and 8). However, when the same amounts of βB2–βA4 dimer and βB2–βA3 oligomer were mixed at pH 8.5 and applied to the gel filtration column at pH 6.7, then the dimer peak was considerably diminished with a corresponding increase in the higher molecular weight peak (Figure 8). This observation indicates that βB2–βA4 dimer becomes part of a higher molecular weight oligomer at pH 6.7, having been mixed with βB2–βA3 at pH 8.5. If it is assumed that βB2–βA3 is a dimer at pH 8.5, then as the pH is changed to 6.7 during gel filtration, not only can βB2–βA3 self-associate to form a larger oligomer but also it can associate with βB2–βA4 dimer to form larger oligomers whereas βB2–βA4 alone can only form dimers.

Discussion

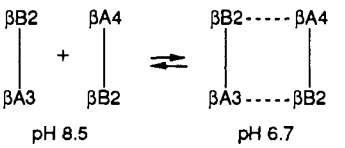
Populations of aggregates comprising βL_1 and βL_2-crystallins have been dissociated in 6 M urea, allowing the isolation by chromatography of subunits βB2, βB3, βA3, and βA4. These components have been refolded and allowed to associate either individually or in various combinations. The predominant subunit, βB2, can associate either with βB3 or with βA4 to form heterodimers. Both of these heterodimers have been shown to be present in undissociated β-crystallins. βB2, βB3, and βA4 can each be unfolded, refolded, and reassociated to form homodimers although βA4 homodimers are not present in native β-crystallins.

βA3 was shown to reassociate with βB2 to form an oligomer which appeared bigger than βB2 dimer on gel filtration at pH 6.7 and in this respect is similar to the higher molecular weight βL_1-crystallin. An important question concerns the size of this βB2–βA3 oligomer at neutral pH. Molecular weight estimates of isolated βL_1-crystallin would suggest a trimer (Bindels et al., 1981). However, the simplest hypothesis to explain the pH-dependent transition of βL_1 to βL_2 would be a tetramer to dimer dissociation. If this were so, then the βB2–βA3 heterodimer purified in this work at pH 8.5 would simply further associate to form a dimer of dimers at pH 6.7 of the same stoichiometry of subunits yet with a new contact site:



βB2–βA3 oligomer is a major component of βL_1-crystallin and not βL_2-crystallin. As βA3 is the striking discriminator between βL_1 and βL_2, these data indicate that the βA3 subunit has a principal role in the higher organization of β-crystallins. It is significant that βB2 forms higher molecular weight aggregates with βA3 but not with βA4. All β-crystallin subunits are predicted to have two similar domains each formed from two symmetrically organized "Greek key" motifs based on their sequence homology with γ-crystallins (Slingsby et al., 1988). The complete sequence of βA3 is known, showing that it comprises an extension of 29 amino acid residues beyond the predicted compactly folded N-terminal domain (Berbers et al., 1984). Partial sequence data are available for βA4 from the region of the C-terminal domain showing that it is closely related (77% identical) to βA3 (Berbers et al., 1984). As βA4 is a shorter polypeptide than βA3, it is most likely that the N-terminal extension of βA4 is shorter than that of βA3. These data suggest that a long N-terminal arm is critical for higher organization of dimers.

However, by mixing βB2–βA4 dimer and βB2–βA3 dimer at pH 8.5 followed by lowering the pH, the βB2–βA4 dimer appears to form half of a tetramer:



This would suggest that one long N-terminal arm is sufficient for higher aggregation. This model would also explain why a substantial proportion of βB2–βA4 dimer was present in βL_1-crystallin following fractionation at pH 8.5.

These observations indicate that β-crystallin quaternary structure is largely based on interactions between different subunits. The first level of organization is to have a versatile component, βB2, capable of interacting with many other β-crystallin subunits to form dimers. Although βL_1-crystallin comprises high levels of acidic subunits, they appear to be interacting principally with βB2 subunits and not with other acidic components. Evolution of the major division of β-

crystallins into basic and acidic subunits appears to be one of structural divergence such that interface regions are heterologous and more complementary than truly isologous contacts. The next level of organization appears to be dependent on the amount and kind of acidic β-crystallin subunits. If there are low levels of acidic subunits synthesized, then there are higher levels of purely basic dimers including homodimers, whereas increased synthesis of acidic subunits will favor basic–acidic heterodimers. The more heterodimers there are with long N-terminal arms, then the equilibrium will favor tetramers. Presumably evolution has secured diversity by maintaining genes for several β-crystallin subunits yet selected some strong interaction sites, particularly the dimer interface of basic and acidic heterodimers, while allowing many opportunities for further polydispersity through a dimer–dimer interface that is sensitive to the environment.

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