Differences in solution dynamics between lens β -crystallin homodimers and heterodimers probed by hydrogen-deuterium exchange and deamidation

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ABSTRACT

BACKGROUND

Lens transparency is due to the ordered arrangement of the major structural proteins, called crystallins. β B2 crystallin in the lens of the eye readily forms dimers with other β -crystallin subunits, but the resulting heterodimer structures are not known and were investigated in this study

METHODS

Structures of β A3 and β B2 crystallin homodimers and the β A3/ β B2 crystallin heterodimers were probed by measuring changes in solvent accessibility using hydrogen-deuterium exchange with mass spectrometry. We further mimicked deamidation in β B2 and probed the effect on the β A3/ β B2 heterodimer. Results were confirmed with chemical crosslinking and NMR.

RESULTS

Both β A3 and β B2 had significantly decreased deuterium levels in the heterodimer compared to their respective homodimers, suggesting they had less

solvent accessibility and were more compact in the heterodimer. The compact structure of β B2 was supported by the identification of chemical crosslinks between lysines in β B2 within the heterodimer that were inconsistent with β B2's extended homodimeric structure. The compact structure of β A3 was supported by an overall decrease in mobility of β A3 in the heterodimer detected by NMR.

In β B2, peptides 70-84 and 121-134 were exposed in the homodimer, but buried in the heterodimer with $\geq 50\%$ decreases in deuterium levels. Homologous peptides in β A3, 97-109 and 134-149, had 25-50% decreases in deuterium levels in the heterodimer. These peptides are probable sites of interaction between β B2 and β A3 and are located at the predicted interface between subunits with bent linkers. Deamidation at Q184 in β B2 at this predicted interface led to a less compact β B2 in the heterodimer.

The more compact structure of the β A3/ β B2 heterodimer was also more heat stable than either of the homodimers.

CONCLUSIONS

The major structural proteins in the lens, the β -crystallins, are not static, but dynamic in solution, with differences in accessibility between the homo- and heterodimers. This structural flexibility, particularly of β B2, may facilitate formation of different size higher-ordered structures found in the transparent lens.

GENERAL SIGNIFICANCE

Understanding complex hetero-oligomer interactions between β -crystallins in normal lens and how these interactions change during aging is fundamental to understanding the cause of cataracts.

KEY WORDS: β -crystallins, deamidation, lens, cataracts, solution dynamics, hydrogen-deuterium exchange, mass spectrometry

INTRODUCTION

The lens is unique from other organs in that it is optically transparent. This is in order for the lens to focus light onto the retina. Lens transparency is due to the short-range ordered arrangement of the major structural proteins, the α , β and γ -crystallins that are present in the cytosol at 300-500 mg/ml ¹. At these high concentrations, short-range interactions between nearby atoms contribute to the compact structures of the crystallins and to their close packing giving the lens a

dense liquid or glass-like structure^{2, 3}. The dynamic polydispersity of the oligomeric α -crystallins and possibly the β -crystallins prevent crystallization or precipitation of the soluble crystallins in the normal lens^{4, 5}.

The two-domain compact structures of the related monomeric γ -crystallins and oligomeric β -crystallins have been well characterized, with several structures solved from the members of the β/γ -crystallin family⁶⁻¹⁰. There are extended or compact main structures for the β -crystallin monomers depending on the conformation of the connecting peptide (extended or bent, respectively) between the two domains within each crystallin subunit.

In β B2, which has an extended structure, the N- and C-terminal domains are joined together by an extended connecting peptide⁶. In the homodimer, this allows swapping of the domains between subunits where the N-terminal domain of one subunit interacts with the C-terminal domain of the partner subunit. This interdomain interaction is across a pseudo 2-fold axis of symmetry called the P dyad (PQ interface) in the crystal structure. This PQ interface is stabilized by hydrophobic interactions and salt bridges and has been shown by our laboratory to be extremely solvent inaccessible by hydrogen-deuterium exchange (HDX)¹¹.

In contrast to β B2, the connecting peptide in β B1 is bent allowing the N- and C-domains to be paired intrachain, giving the dimer a compact structure with both inter- and intradomain interfaces. The intradomain interface in β B1 is equivalent to the interdomain (PQ) interface in β B2. The additional interface between the domains of the partner β B1 subunits is the RQ interface in the crystal structure⁷. Both interfaces are stabilized by hydrophobic interactions and salt bridges and contribute to β B1's more compact structure and greater thermodynamic stability compared to β B2¹²⁻¹⁴. More recently, the crystal structures of β A4 and β B3 have also been solved (PDB:3LWK and 3QK3, respectively, Structural Genomics Consortium, 2010) and predict compact structures like β B1 with bent linkers that result in two interfaces within the homodimers. The structure of β A3 is not known, but it has been predicted to undergo a concentration-dependent equilibrium between extended and bent conformations^{15, 16}.

Despite what we know about the homodimers, the lack of structural information about the hetero-oligomers—which are found in vivo—remains a major obstacle to understanding lens transparency. Stable hetero-oligomers readily form between the basic and acidic β -crystallin subunits^{12, 16-20}. β B1 and β A3 preferentially form a tetramer, while β B2 and β A3 preferentially form a heterodimer^{18, 19}. Changes in the conformation of the polypeptide chains have been observed by changes in the spectroscopic properties of the proteins, but the exact nature of the conformational changes have not been described. The β A3/A1, β B1, and β B2 crystallins are the most abundant β -crystallins in the human lens, with β B1 and β A3 the most modified during aging and cataracts^{21, 22}. The simpler β A3/ β B2 heterodimer was chosen to investigate in this study by HDX, with future experiments planned to investigate the more complex tetramer.

Our laboratory has previously determined the solution dynamics of the β B2 homodimer by probing the solvent accessibility using HDX measured by mass spectrometry¹¹. Regions with low deuterium incorporation are inaccessible to the solvent, suggesting either buried locations or surface regions involved in protein-

protein interactions. Using these methods, we have observed that the protein-protein interface region is particularly solvent inaccessible ¹¹, less than 15% deuterium was exchanged, suggesting a “tight” interaction and a slow rate of subunit exchange between stable dimers.

During both aging and cataracts, crystallins accumulate many modifications that are associated with an increase in insoluble proteins isolated from donor lenses ²². These modifications are expected to disrupt normal interactions between crystallins. We have identified deamidation of Asn and Gln residues as a major age-related modification in lens ^{21, 22}. By mimicking deamidation in vitro, we have determined that deamidation increases solvent accessibility between interacting domains in β B2, providing a mechanism for the deamidation-induced decreases in its stability ^{11, 14}. In this study, we characterize the β A3/ β B2 heterodimer using HDX and mimicked deamidation in β B2 to probe its effect on interface interactions in the heterodimer structure.

METHODS AND MATERIALS

Protein expression and purification. The recombinant expression and purification of untagged wild type (WT) β A3, WT β B2 crystallin, and the deamidated mimics of β B2 (β B2Q70E and β B2Q184E) was carried out using *E. coli* as previously described^{14, 23}. Confirmation of purity was estimated by SDS-PAGE to be 90-95% and mass spectrometry confirmed the predicted masses. Crystallins were then concentrated and dialyzed into buffer specific to experimentation. Protein concentrations were determined by absorbance at 280 nm using a NanoDrop UV/Vis spectrophotometer (Thermo Scientific, Waltham, MA). A theoretical Abs₂₈₀ extinction coefficient of 2.6 (mg/mL)⁻¹ cm⁻¹ was used for β A3, and 1.7 (mg/mL)⁻¹ cm⁻¹ for β B2.

Formation of β A3/ β B2 heterodimer. Following purification, proteins were buffer exchanged into 100 mM sodium-phosphate (pH 7.4), 1 mM EDTA, 2.5 mM TCEP, and 100 mM KCl (HDX buffer). In order to form a heterocomplex from β B2 and β A3 homodimers, both proteins were mixed together at equal molar concentrations at 37°C for 18 hours. The polydispersity of the resulting mixtures was determined by size-exclusion chromatography in-line with multi-angle laser scattering (MALS). Polydispersity was measured as the weight-averaged molecular weight divided by the number-averaged molecular weight measured by light scattering. This confirmed that β A3 and β B2 preferentially form heterodimers after overnight incubation as previously reported¹⁹, and that the selected deamidations did not prevent the subunit exchange.

Hydrogen deuterium exchange of β -crystallins. In order to probe changes in solvent accessibility between homodimeric and heterodimeric structures, HDX was performed for both β B2 and β A3 crystallin homodimers and these results compared to the HDX of β A3/ β B2-crystallin heterodimers. Deuterium labeling was done as previously described with the following modifications: a 12 μ L sample from an overnight incubation of the crystallin oligomer containing 80 μ M of each protein was diluted 10-fold by combining with 108 μ L of HDX buffer, prepared with D₂O. HDX was performed at 4°C for 0, 10, 100, 1000, and 10,000 sec²⁴. At each time point, the deuterated sample was combined with an equal volume of quenching buffer (0.42% phosphoric acid). These samples were then immediately flash-frozen in liquid nitrogen and stored at -70°C for less than one week before mass spectrometry was performed.

Frozen samples were thawed and immediately analyzed using proteolytic digestion and mass spectrometry. A 40 μ L quenched sample described above was added to 18 μ L of 8 M urea, a 2 μ L sample of saturated protease from *Aspergillus saitoi*, Type XIII (Sigma, St. Louis, MO) in 50 mM sodium citrate buffer, pH 3, was added, and digestion performed for 2 min in an ice bath²⁵. Liquid chromatography/mass spectrometric (LC/MS) analysis was then immediately performed as previously described using an LTQ-Orbitrap Discovery Instrument (Thermo Scientific, San Jose, CA) with peptide trap/chromatography column on ice, except MS scans were collected at a resolution of 30,000 with an

m/z range of 400-1500 ¹¹. One tandem mass spectrometry analysis of each sample was performed using identical chromatographic conditions to identify peptides by alternating survey MS scans in the Orbitrap mass analyzer with three data-dependent MS/MS scans in the linear ion trap using collision-induced dissociation, and the dynamic exclusion feature of the instrument's control software.

Peptides and their retention times were identified using Sequest software and peptide lists and retention times exported for use by either HD Desktop as previously described ²⁴ or by HD Examiner (version 1.2.0) with similar results. The sequences of β A3 and β B2 crystallin peptides identified following protease digestion were used to calculate their expected isotopic distributions and average masses. The sample immediately quenched at 0 sec was used as the non-deuterated control to determine the shift in peptide masses following HDX at different time points. The exchange rates of peptides derived from β A3 and β B2 homodimers were each compared to their respective exchange rates in peptides derived from β A3/ β B2 heterodimer. Peptide fragments identified by MS/MS analysis were manually selected based on the quality of mass spectra across samples, and average masses of exchanged peptides calculated by HD Examiner or HD Desktop. Three independent repeats were performed for each sample. The number of deuterons exchanged into each peptide at different time points was exported into either Microsoft Excel or Prism (version 6) to identify peptides with statistically different exchange rates using a t-Test (Student's t-Test or the Holm-Sidak method, with $\alpha=5.000\%$).

NMR analysis of β □□-crystallins. A uniformly ¹⁵N-labeled β A3-crystallin homodimer was produced from E. coli grown in ¹⁵N medium. 1H-15N HSQC spectra were collected both from this ¹⁵N labeled β A3-crystallin homodimer and from a β A3/ β B2-heterodimer, but containing a ¹⁵N labeled β A3 subunit. Data were collected on a 700 MHz Bruker spectrometer at 5 and 37°C.

Disordered regions were characterized by spectra with limited dispersion in the amide region spanning 8-8.5 ppm instead of 7-11 ppm expected for folded proteins (Barbar, Biopolymers 1999). In addition, disordered segments with sharp peaks were distinguished from the extensively broad peaks arising from the large ordered domains. Large folded proteins have long correlation time due to slow tumbling but disordered segments within the same protein tumble at much higher rate (Morgan et al 2011).

Crosslinking of heterodimers. WT β A3 and β B2 crystallin homodimers or β A3/ β B2 mixtures were incubated overnight at 37 °C, dialyzed into 20 mM sodium phosphate, 150 mM NaCl (pH 7) and subjected to chemical crosslinking using an equalmolar mixture of undeuterated and deuterated forms of CyanurBiotinDimercaptoPropionylSuccinimide (CBDPS) crosslinker with a 14Å span (Creative Molecules, Inc. Vancouver, CAN). CBDPS was dissolved immediately before use in anhydrous DMSO at a 1.25 mM concentration and diluted 10 fold into the crystallin solutions that were at approximately 1 mg/ml

concentration. Following incubation at room temp for 30 min, the reaction was quenched by addition of an equal volume of 100 mM ammonium bicarbonate and samples dried by vacuum centrifugation. The samples were then dissolved in 8 M urea, reduced, alkylated, urea diluted to 2 M concentration, and digested overnight with trypsin as previously described ²⁶. Peptides reacting with CBDPS were purified using cation exchange and avidin affinity chromatography (ICAT labeling kit, AB SCIEX, Redwood City, CA). Purified peptides were then separated using a Dionex NCS-3500RS UltiMate RSLCnano UPLC system and PepMap RSLC C18, 2 μ m, 75 μ m x 50 cm EasySpray column (Thermo Scientific), 2–30% ACN gradient over 100 min at a 300 μ l/min flow rate in a mobile phase containing 0.1% formic acid. Mass spectrometric analysis used an EasySpray nanosource and Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific). Survey scans from m/z = 350-1600 at 120,000 resolution and MS/MS scans at 60,000 resolution were both collected in the Orbitrap mass analyzer, using a 1.6 Da quadrupole isolation, collision-induced dissociation in the ion trap, 40 sec dynamic exclusion time with a repeat count of 1, charge state selection between 3-7, monoisotopic precursor selection filter on, detection properties set to top speed, and a 5-sec dwell time between survey scans. The user defined lock mass feature was also used by monitoring a m/z = 445.12 polysiloxane ion. Results were analyzed using StavroX software (v3.4.9) ²⁷. A maximum mass deviation of 10 ppm for precursor and 20 ppm for fragment ions between theoretical and experimental mass was allowed. Searches used a database consisting of β A3 and β B2 crystallin sequences, required score values in excess of matches to sequence reversed peptides, and were manually inspected to assess assignment of fragment ions to both crosslinked peptides.

Heat stability of heterodimers. β A3 and β B2 homodimers and β A3/ β B2 heterodimers were produced as described above and heated to 55°C. The turbidity of each solution was measured as the change in optical density at 405 nm and the percent of the maximum OD was reported ²⁸.

RESULTS

Formation of β A3/ β B2 heterodimer. Our goal was to characterize the heterodimer of β A3/ β B2 to shed light on the complex protein-protein interactions that determine transparency in the lens. Following the bacterial expression of β A3 and β B2 crystallins, the last step of their purification used size-exclusion chromatography with in-line determination of molar masses by light scattering. We have previously reported that the homodimers eluted with symmetrical peaks and molar masses of 49 ± 2 and 45 ± 3 kDa for β A3 and β B2, respectively ^{14, 23}. The theoretical molecular weights are 50.3 kDa for β A3 and 46.5 kDa for β B2. After 90 min at 37°C, the β A3/ β B2-heterodimer eluted in a nonsymmetrical peak with a sharp leading edge of 64 kDa and a sloped tailing edge of 45 kDa (Takata Mol Vis 2009). Results were repeated in this study. Immediately upon mixing, β A3- and β B2-crystallins eluted in a broad peak with a polydispersity of 1.03. After 18 h at 37°C, the β A3/ β B2-heterodimer eluted in a symmetrical peak with a polydispersity index of 1.000 (Fig 1A. The weight-average molecular weight of this peak is 51.1 kDa (0.1%). The distribution of the species was 90% between 49-52 kDa.

These β A3 and β B2 crystallin homodimers were analyzed using BN:PAGE (Fig. 1B, lanes 1 and 2). The incubated β A3 homodimer (lane 3, arrow a), β B2 homodimer (lane 4, arrow b), and β A3/ β B2 heterodimer (lane 5, arrow c) each migrated on BN:PAGE with a major band distinct from each other. This result confirmed the previous finding that β A3 and β B2 homodimers spontaneously undergo subunit exchange when mixed to form a heterodimer ¹⁹.

Based on migration distances, BN:PAGE indicated that β A3 had the most compact structure, β B2 the least compact, and that β A3/ β B2 heterodimer adopted a structure of intermediate size, migrating to a position close to β A3 homodimer.

Changes in solvent accessibility between homodimers and heterodimer.

The different migration patterns on BN:PAGE suggested that β B2 and β A3 had conformations in the heterodimer (Fig. 1, lane 5) that were different from their homodimer conformations (lanes 3 and 4). To test this, we compared the HDX rates between homodimers and heterodimers for β A3 and β B2. Exchange times that ranged from 10 to 10,000 sec were used so that we could compare differences in both fast and slow exchanging residues. These data are shown in Supplemental Figs. 1 and 2, and demonstrated that for most regions of the protein, the greatest differences were observed after 1000 sec of exchange.

The results following 1000 sec of exchange for β B2 are shown in Fig. 2, where the extent of deuterium incorporation in peptides in the homodimer (black bars) and heterodimer (white bars) are compared. The β B2 regions analyzed were restricted to peptides that were identified in digests of both homodimers and heterodimers. We will use residue numbers starting from the actual protein N-

terminus (initial methionine lost), which differs from residue numbers in UniProt (www.uniprot.org). We found the greatest deuterium incorporation (>25%) in the β B2 homodimer in peptides from the N- and C- terminal extensions, peptide 4-21, peptide 191-204, as well as in peptides at exposed loops (peptide 34-52 and peptide 126-139). Additional peptides with high exchange were peptide 70-84 and peptide 170-184, located on the exposed RQ surface; and peptide 95-102, containing part of the connecting peptide between the N-terminal domain (N-td) and C-terminal domain (C-td) ⁸. The RQ surface is at the interface between two β B2 homodimers when they form a tetramer, but remains exposed in the β B2 homodimer.

We found the lowest deuterium incorporation (<10%) in the C-td peptide 151-163, which is located at the predicted PQ interface between the N-td and C-td in the β B2 homodimer (Fig. 3, blue regions). We also found low incorporation in peptide 103-110, which contains the first β -strand of the third Greek Key in the C-td. Peptides with deuterium uptake between 10-25% are highlighted in yellow in Fig. 3.

The HDX results were compared to analysis of the β B2 homodimer structure in the Protein Data Base (PDB:1YTQ) using the Protein Interfaces, Surfaces, and Assemblies server (PDBePISA, http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html) and were found to be in good agreement. Three peptides, 34-52, 70-84, and 126-134 (labeled in Fig. 3), had high deuterium exchange, and contained highly solvent-accessible residues. Residues within peptide 34-52 were all exposed with P36, K41, E42, and K47 having accessible surface areas equal to or greater than 100 Å². The 70-84 peptide included the exposed residues, 76-83, with E77 and R80 having accessible surface areas greater than 100 Å². Residues within peptide 126-134 were exposed and included two of the three adjacent Asp residues, D125, D126 and D127. Residue D126 has a calculated surface area of 94.5 Å². Therefore, these three peptides contained residues with large calculated solvent accessibilities that also agreed with our previous HDX experimental results ¹¹.

Since the structure of β A3 is unknown, the crystallographic structure of β A4 homodimer (PDB:3LWK) was used to predict the locations of peptides in β A3. β A3 and β A4 share 68% sequence homology overall and 75% homology in the C-td alone. We again use residue numbers starting from the actual protein N-terminus (initial methionine lost). Deuterium incorporation in the β A3 homodimer (Fig. 4) reached 50% at the flexible N-td extension in residues 1-36 (red in Fig. 5). Other regions with high levels of deuterium included the predicted connecting peptide and loop regions (peptides 48-67, 97-109, 113-123, and 140-162 in Fig. 4 and highlighted in red in Fig. 5). Within the peptide 97-109, several homologous residues (102-106) were not resolved in the β A4 crystal structure suggesting they were highly flexible. This supports the high level of deuterium incorporation that we found in this peptide. Adjacent to this flexible region in the structure are three peptides containing residues 68-96 with less than 10% deuterium incorporation (Fig. 4). These residues are located at the PQ interface between N-td and C-td regions in β A4 homodimer and extend into the RQ interface between the partner subunit (Fig. 5, blue regions). This region in β A3

homodimer had lower rates of deuterium incorporation compared to the homologous regions in β B2 homodimer, suggesting that this region was less solvent accessible in β A3 homodimer. Additional deuterium incorporation below 10% was also observed in residues 163-183 (Fig. 5, blue regions). These results provide the first structural information for β A3 homodimer in solution and support a homodimer similar to β A4 with a bent linker between the domains (compact structure) ¹⁵.

Decreased solvent accessibility of β B2 upon incorporation into the β A3/ β B2 heterodimer. The faster migration of β B2 observed by BN:PAGE (Fig. 1) when it was in complex with β A3 suggested a more compact structure that would be less solvent-accessible than when in its homodimeric form. This was confirmed by the 50-80% decrease in deuterium incorporation in β B2 peptides 53-63, 70-84, 121-125, 126-134, and 151-163 in β B2/ β A3 heterodimer (Fig. 2). Data for all time points for peptides 70-84 and 126-134 are shown in Fig. 6. These peptides are solvent exposed in the crystallographic structure of β B2 homodimer described above (PDB:1YTQ, Fig. 3). The decrease in deuterium incorporation in these peptides suggests that β B2 underwent a structural change, with these residues becoming less solvent accessible in the β A3/ β B2 heterodimer.

To determine if the above results are expected if β B2 adopts a compact structure in the β A3/ β B2 heterodimer, we compared peptides 70-84 and 126-134 to homologous regions of the β B1 homodimer structure (PDB:1OKI). These residues in the β B1 homodimer structure are intrachain interface residues and are highlighted in purple in Fig. 7A. While, residues 76-83 in β B2 are accessible, the homologous residues in the β B1 homodimer, 118-125, are partially inaccessible with four residues involved in hydrogen bonding with the partner subunit. Residues D126 and D127 in β B2 are also accessible with no buried surface areas, while the homologous residues in β B1, D168 and D169, have partially buried surface areas. These observations support that β B2 changes conformation in the β A3/ β B2 heterodimer and may adopt a structure similar to β B1 with a bent linker and alternate intermolecular interface.

Deuterium incorporation was not significantly altered in the N- and C-terminal extensions of β B2 (peptides 4-21 and 191-204, respectively) in the β A3/ β B2 heterodimer (Fig. 2). This confirmed previous NMR results supporting that they remain unstructured in the heterodimer and thus, may not be necessary for dimer formation ²⁹.

Decreased solvent accessibility of β A3 upon incorporation into the β A3/ β B2 heterodimer. Since the NB:PAGE results in Fig. 1 suggested that the β A3 homodimer had the most compact structure, we predicted that some β A3 peptides in the β A3/ β B2 heterodimer would exhibit higher rates of exchange than in the β A3-homodimer. However, β A3 peptides in the heterodimer either showed no change in deuterium incorporation or, like peptides in β B2, had decreased rates of exchange.

As in β B2, differences in β A3 exchange at 10 to 10,000 sec were compared between β A3 homodimer and β A3/ β B2 heterodimer (Supplemental Fig. 2). Figure 4 summarizes the differences in exchange at 1000 sec between β A3 homodimer (black bars) and β A3/ β B2 heterodimer (white bars). The high deuterium incorporation in the N-td extension of β A3 (residues 1-36) in both the β A3 homodimer and β B2/ β A3 heterodimer, suggest that the N-td extensions are not involved in dimer formation.

Decreased deuterium incorporation in the β A3/ β B2 heterodimer was localized to the C-td with the exception of a decrease in the N-td at peptide 97-109 (Fig. 4). These peptides were mapped to the locations of homologous peptides in the β A4 structure (PDB:3LWK) and are highlighted in purple in Fig. 7B. These peptides were predicted to be at the β A4 RQ interchain interface, and in the C-td at the PQ intrachain interface. The decreased deuterium incorporation in both β B2 and β A3 in the β A3/ β B2 heterodimer and spontaneous heterodimer formation suggests increased stabilizing protein-protein interactions in heterodimers compared to homodimers.

NMR spectra confirmed less mobility of β A3 in the β B2- β A3 heterodimer than in the homodimer. NMR of heavy-labeled β A3 was performed because the structure of β A3 in solution was not known, and to confirm changes in its structure during heterodimer formation. In Figure 8, the spectra at the top show the peaks from sharper signals due to greater mobility. The only peaks in the spectra are of labeled β A3. These spectra verified that there is a region in the protein, presumably the long extensions, that were of higher mobility than the rest of the protein. The spectra were the same in the homo- and heterodimer confirming the hydrogen deuterium exchange results that the flexibility of the extensions did not change in the heterodimer.

The middle and bottom NMR spectra of labeled β A3 were recorded at 5°C and 35°C, respectively. The middle spectra are plotted at a lower contour level to show the peaks that are of minor intensity and were subtracted out in the top panel. The minor peaks correspond to regions of slower tumbling and most likely to the intact protein dimer. The bottom spectra were collected at 35°C to increase the tumbling rate of the protein, and thus the signal intensity. While more peaks were detected at 35°C than at 5°C for the homo- and heterodimers, the intensity of the minor peaks was less in the heterodimer. This difference was more apparent at 35°C. Regions where the intensity of the minor peaks in the heterodimer (right) was lower and even disappeared are circled in red. This suggested that the intact β A3 in the heterodimer was more compact and less mobile than in the homodimer.

The effect of mutations Q70E and Q184E in β B2 on interface interactions in the β A3/ β B2 heterodimer. To probe how deamidation, a major age-related modification in lens, alters heterodimeric interactions, we created β A3/ β B2 heterodimers with β B2 mutants mimicking deamidations at the putative

interfaces. Our laboratories have identified Gln at residues 70 and 184 in β B2 as *in vivo* sites of deamidation ^{22, 30, 31}.

We have previously shown that Q70E mutation increases the solvent accessibility across the PQ interface in β B2 homodimer ¹¹. However, the Q70E mutation would not be predicted to disrupt the new RQ interface in a bent-linker model of β B2, whereas the Q184E mutation, located near the RQ interface, would be predicted to disrupt this interface ⁸. WT β A3/ β B2 and heterodimers formed between WT β A3 and either β B2 Q70E or β B2 Q184E mutants were separated by BN:PAGE (Fig. 9A). Both mutants formed heterodimers that migrated just below the WT heterodimer, suggesting a slightly more compact structure.

HDX rates were determined for the β A3/ β B2 heterodimers containing mutant β B2. Small, but significant, increases in solvent exposure due to the Q184E mutation, were observed for both the β B2 homodimer and β A3/ β B2 heterodimer (Fig. 9B). However, as expected, the solvent exposure was more pronounced in the β A3/ β B2 heterodimer. Deuterium incorporation in β B2 peptides 72-81 and 126-136 increased 17 and 24%, respectively, in the β A3 heterodimer with β B2 Q184E compared to 9 and 18% with β B2 WT (Fig. 9B, 1000 sec). Both of these peptides are located on the RQ interface, although only peptide 126-134 would be predicted to be affected by the mutation at Q184 due to its close proximity of less than 4 Å to H132 within the same polypeptide chain.

As expected, Q70E mutation in β B2 did not lead to any significant changes in solvent accessibility in β B2 within the β A3/ β B2 heterodimer.

HDX rates were also determined for β A3 within the β A3/ β B2 heterodimers containing mutant β B2s (Fig. 9C). Only the mutation at Q184 affected the solvent accessibility in β A3, and instead of increasing accessibility, the accessibility was decreased. The decreased accessibility was in the long peptide 134-162 from the C-td. While, it is not clear why Q184E led to decreased accessibility, these data fit with its greater thermal stability described below.

Chemically cross-linking Lys residues in β A3/ β B2 heterodimers. In order to provide further evidence of a change in conformation of β B2 in the β A3/ β B2 heterodimer, experiments were performed using the chemical cross linking reagent, CyanurBiotinDimercaptoPropionylSuccinimide (CBDPS) (Supplemental Fig. 3A). This reagent crosslinks Lys residues that are within distances of 14 Å. There are 12 Lys residues in β B2. Five of these Lys are highlighted in Fig. 10 due to their close proximity to the RQ interface. Other Lys residues that have their R groups oriented on the opposite surface of the predicted RQ interface would not be expected to form crosslinks with β A3 in the β A3/ β B2 heterodimer.

Mass spectrometry identified a crosslinked peptide between K75 and K119 in β B2 within the β A3/ β B2 heterodimer (supporting MS/MS spectra shown in Supplemental Fig. 3B). The location of these crosslinked residues are shown in the extended structure of the β B2 homodimer (Fig. 10). The distance between K75 and K119 is 26 Å. This distance is too great to be compatible with the extended β B2 homodimer structure. No crosslinks were identified between any of the Lys on the opposite surface of β B2 heterodimer, consistent with this surface

being solvent exposed in the heterodimer. Unexpectedly, no cross-linked peptides were identified between β B2 and β A3 in the heterodimer. The lack of identified crosslinks may reflect a low number of available Lys groups at the interacting surfaces.

Differences in heat stability of β B2, β A3, β A3/ β B2, β A3/ β B2Q70E, and β A3/ β B2Q184E. In order to determine if the more compact β A3/ β B2 heterodimer was also more stable than the respective homodimers, dimers were heated and the rate at which the solutions reached their maximum turbidity was measured as an indication of denaturation and precipitation. The β A3/ β B2 heterodimer reached its maximum turbidity at a slower rate than did the β A3 or β B2 homodimers (Fig. 11). The β A3/ β B2Q184E heterodimer reached its maximum turbidity at an even slower rate than the WT β A3/ β B2 heterodimer. The β A3/ β B2Q184E dimer was the most resistant dimer to heat denaturation and may reflect the more compact C-td of β A3 in the β A3/ β B2Q184E heterodimer. These data are consistent with more compact dimers being more heat stable.

DISCUSSION

In this study, we have examined the solution structure of a β -crystallin heterodimer and identified, for the first time, that β B2 crystallin adopts a structure quite unlike that predicted by its extended-linker structure as a homodimer. This suggested that like the resolved crystallographic structures of β B1, β B3, and β A4 homodimers, β -crystallin heterodimers may adopt a bent linker conformation containing an intramolecular PQ interface and intermolecular RQ interface. This is consistent with the conformational changes in the β B2 subunit being driven by new and favorable interactions with its heterologous partner when subunit changes occur.

We also identified potential sites of protein-protein interactions between these β -crystallin complexes. The ability of β -crystallins to take on different conformations could facilitate formation and stability of different size crystallin oligomers, potentially disrupting detrimental long-range order at the high protein concentrations found in the lens¹. Our findings suggested that the individual crystallins are, in fact, dynamic and not just static structural proteins.

Structures of four of the seven β -crystallin subunits (crystallized as homodimers) are known. They all have homologous interfaces between the N- and C-terminal domains within the same polypeptide, or between partner subunits. Two distinct conformations have been solved: a less compact conformation (β B2) with an extended linker, and a more compact conformation with a bent linker connecting the N- and C-terminal domains (β B1, β B3, and β A4). In the domain swapped β B2 dimer, the interface is between domains from partner subunits. In the compact dimers, this same interface is between domains from the same polypeptide chain (as in all γ -crystallins) with an additional interface between the two subunits.

The crystal structures of β -crystallins heterodimers are not known. We report here for the first time that β B2 takes on a more compact and less solvent accessible structure with β A3 in the β A3/ β B2 heterodimer. The domain-domain interface (equivalent to the paired domain interface in γ -crystallins) in the extended β B2 homodimer is between partner subunits. In a compact conformation of the β A3/ β B2 heterodimer, this interface would now be intrachain within β B2, leaving other β B2 residues to interact with β A3. Our study identified residues in these protein-protein interaction regions from their decreased deuterium incorporation in the β A3/ β B2 heterodimer. Our data were consistent with β B2 changing from an extended linker conformation in homodimers to a more β B1-like bent-linker conformation in the heterodimers with β A3. In other words, the new heterodimer interface is similar to the monomer-monomer interface of the β B1 homodimer. Our results do not preclude that the β B2 homodimer can also take on more compact dimer conformations in solution. The flexibility of β B2 in solution is an important property that would facilitate dynamic interactions with different β -subunit partners.

The structure for β A3 crystallin has not yet been solved; however, our data are in agreement with a compact β A3 structure, similar to β A4. In complex with β B2, the β A3 structure is less solvent accessible than in homodimers, most notably in the C-td. NMR spectra confirmed that β A3 was less mobile in the heterodimer than in the homodimer.

There were no changes in solvent accessibility determined in the N-td extensions of either β B2 or β A3 upon heterodimer formation, implying these extensions do not play a role in formation of the β A3/ β B2-dimer. Although not determined, similar results would be expected for β A1-crystallin, which is homologous to β A3, only with an alternate start site at Met-18 in β A3. The extension of β A1 includes residues 18-30 of β A3, which were exposed in the heterodimer.

Taken together, these findings suggest that β B2 may facilitate the spontaneous formation of the heterodimer by interacting with the C-td of β A3.

Our results also highlight a potential role for flexible loop regions in the predicted buried interface interactions between β B2 and β A3. In both β B2 and β A3, two regions containing predicted (from crystal structures) surface loops were exposed in the homodimers, but less solvent accessible in the heterodimer (Figs. 3, 5, and 7). The C-td loops contain several charged residues, including three adjacent Asp residues in β B2 and two in β A3. In the heterodimer, these residues may participate in interactions similar to structures in β B1, β A4, and β A3 homodimers with the potential for charge interactions between one of the Asp residues and a near by Arg across the interface (PDBePISA analysis). Our HDX results suggest that these loops are sites of interaction in the β A3/ β B2 heterodimer.

In conclusion, the ability of β B2 to take on different conformations depending on its partner subunit suggests that the β/γ crystallins are not just static structural proteins, but are dynamic in solution. More compact, less solvent accessible structures of β -crystallin hetero-oligomers may slow their denaturation caused by age-related modifications like deamidation. The more compact, less solvent

exposed structures may also serve to slow age-related modifications. While we have shown that deamidation is capable of altering solvent accessibility in β -crystallin hetero-oligomers, crystallographic or NMR based structures of these proteins are needed to fully understand the cause of these structural perturbations that lead to crystallin aggregation and light scatter in cataract.

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FIGURE LEGENDS

Figure 1. β A3 and β B2 preferentially form a heterodimer. (A) Light scattering of the β A3/ β B2 heterodimer during size-exclusion chromatography inline with multiangle laser scattering. Molar masses were determined from the light scattering under the protein peak detected by the change in refractive index. **(B)** Blue-native polyacrylamide electrophoresis of β A3 (lane 1) and β B2 (lane 2). The same samples were incubated overnight at 37 °C, β A3 (lane 3), β B2 (lane 4), plus mixed to form β B2/ β A3 (lane 5). Each lane contains 5 μ g.

Figure 2. Deuterium incorporation in β B2 peptides from the β B2 homodimer and the β A3/ β B2 heterodimer. Deuterium uptake per exchangeable residue after 1000 s of hydrogen deuterium exchange measured by mass spectrometry showing regions of solvent accessibility of β B2 alone (black bars) and in complex with β A3 (white bars). Data for representative peptides are shown from the N-terminal through the C-terminal extensions. Error bars are the standard error from an N of 3, Students t-Test, $p < 0.01$ (★★), $p < 0.001$ (★★★).

Figure 3. Deuterium uptake in β B2 peptides highlighted in the β B2 homodimer structure. Deuterium uptake levels from Figure 2 are highlighted in the β B2 homodimer structure (PDB:1YTQ). The dashed line in the dimer to the left shows the interface between the two monomers, the PQ interface in the crystal structure. The dashed line to the right shows the exposed surface when the dimer is rotated down 90°, the RQ interface in the crystal structure. The C₁-td and the N₂-td are hidden from view in the structure on the right. Arrows point to peptides 70-84 and 126-134.

Figure 4. Deuterium incorporation in peptides in β A3 homodimer and in β A3 in the β A3/ β B2 heterodimer. Deuterium uptake per exchangeable residue after 1000 s of hydrogen deuterium exchange measured by mass spectrometry showing solvent accessibility of β A3 alone (black bars) and in complex with β B2 (white bars). Data for representative peptides are shown from the N-terminal through the C-terminal extensions. Error bars are the standard error from an N of 3, Students t-Test, $p < 0.05$ (★), $p < 0.01$ (★★), $p < 0.001$ (★★★).

Figure 5. Deuterium uptake in β A3 peptides highlighted in the β A4 homodimer structure. Deuterium uptake levels from Figure 4 are highlighted on the β A4 homodimer structure (PDB:3LWK). The dashed line in the dimer to the left shows the interface between the two monomers. The dashed line to the right shows the buried surface when the dimer is rotated down 90°. Arrows point to peptides 89-96 and 140-149.

Figure 6. Differences in deuterium uptake in β A3 and β B2 homodimers compared to the β A3/ β B2 heterodimer. Deuterium levels with increasing time were compared between peptides 70-84 (upper graph) and 126-134 (lower graph) in β B2 to the closely related peptides of 89-96 (upper graph) and 140-149 (lower graph) in β A3. Deuterium levels are compared for both the homodimers (left) and the heterodimers (right).

Figure 7. β B2 and β A3 peptides with decreased deuterium uptake in the β A3/ β B2 heterodimer. (A) β B2 peptides with >40% decrease in the β A3/ β B2 heterodimer compared to the β B2 homodimer are highlighted in the homologous peptides in the β B1 structure (PDB:1OKI). (B) β A3 peptides with >40% decrease in the β A3/ β B2 heterodimer compared to the β A3 homodimer are highlighted in the homologous peptides in the β A4 structure (PDB:3LWK). The PQ interface between domains in the same monomer is drawn with a vertical line. The additional RQ interface present in the compact model is drawn in a horizontal dashed line. Q70 in β B2 at the PQ interface and Q184 at the RQ interface in β B2 are highlighted in yellow.

Figure 8: NMR spectra of 15 N-labeled β A3 in the β A3 homodimer (left) and the β A3- β B2 heterodimer (right). (A) NMR spectra of β A3 at 5°C. (B) NMR spectra are shown at a lower contour level than in A to allow observation of the low intensity peaks close to the noise level. (C) NMR spectra are for the same proteins, but collected at 35 °C. Peak intensities that are significantly decreased for the spectrum on the right compared to the homodimer on the left are circled in red.

Figure 9. Changes in deuterium uptake in β A3/ β B2 heterodimer due to deamidation in β B2. (A) Blue-native polyacrylamide electrophoresis of β A3/ β B2 (lane 1), β A3/ β B2Q70E (lane 2), and β A3/ β B2Q184E (lane 3) after 18 h at 37 °C as in Figure 1. Arrows denote migration of the major species in lane 1(a) and lanes 2 and 3 (b). (B) Deuterium uptake in β A3 within β A3/ β B2 (red), β A3/ β B2Q70E (black) and β A3/ β B2Q184E (green). (C) Deuterium uptake in β B2 within β A3/ β B2 (orange), β A3/ β B2Q70E (black) and β A3/ β B2Q184E (green). Only peptides where differences were observed between the heterodimers are shown. Error bars are the standard error from an N of 3, * = $p < 0.001$.

Figure 10: Crosslinked residues support a compact β B2 in the heterodimer with β A3. The crosslinker reagent, CBPDS, was used to crosslink near by residues. Lys residues oriented near the surface of the RQ interface are highlighted in red. Crosslinked residues on or near this surface were identified with mass spectrometry as K75 to K119 in the heterodimer.

Figure 11: Thermal-induced denaturation of β B2, β A3, β A3/ β B2, β A3/ β B2Q70E, and β A3/ β B2Q184E. Proteins were incubated overnight at 37°C and then subjected to 55°C and the turbidity measured at 405 nm. Data is a percent of the OD at 180 min. N=3. Students t-Test, p-value = <0.05

β A3 and β B2 are more compact in the heterodimer than in their homodimers.
Deamidation at Q184, but not Q70 in β B2 partially prevents this change.

Regions with decreased deuterium uptake are highlighted in purple on the known β A4 structure.

Figure 1A

Figure 1B

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9**A** **B** **C**

Figure 10

Figure 11