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Changes in solvent accessibility of wild-type and deamidated β B2-crystallin following complex formation with α A-crystallin

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Abstract

Aberrant protein interactions can lead to aggregation and insolubilization, such as occurs during cataract formation. Deamidation, a prevalent age-related modification in the lens of the eye, decreases stability of the major lens proteins, crystallins. The mechanism of deamidation altering interactions between α A-crystallin and β B2-crystallin was investigated by detecting changes in solvent accessibility upon complex formation during heating. Solvent accessibility was determined by measuring hydrogen/deuterium exchange levels of backbone amides by high-resolution mass spectrometry. Deuterium levels in wild type β B2-crystallin increased 50-60% in both domains following complex formation with α A-crystallin. This increased solvent accessibility indicated a general loosening along the backbone amides. Peptides with the greatest deuterium increases were located at the buried monomer-monomer interface, suggesting that the β B2 dimer was disrupted. The only region where the deuterium levels decreased was in β B2 peptide 123-139, containing an outside loop, and may be a potential site of interaction with α A. Mimicking deamidation at the β B2 dimer interface prevented complex formation with α A. When temperatures were lowered, an α A/ β B2 Q70E/Q162E complex formed with similar solvent accessibilities as α A/WT β B2. Deamidation did not disrupt specific α A/ β B2 interactions but favored aggregation before complex formation with α A. We conclude that deamidation contributes to cataract formation through destabilization of crystallins before they can be rescued by α -crystallin.

1. Introduction

The transparency of the lens of the eye is maintained by ordered arrangement of the major proteins within the lens, the crystallins (Delaye and Tardieu, 1983). The ordered structure of the lens is disrupted by the aggregation and precipitation of partially unfolded crystallins, leading to cataract formation (Harding, 1969; Waley, 1964). Because the lens has a low protein turnover rate (Lynnerup et al., 2008; Waley 1964), mechanisms to prevent the unfolding and aggregation of lens crystallins are important in maintaining lens transparency. One proposed mechanism for maintaining lens transparency is the chaperone activity of α -

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Conflicts of Interest

None declared.

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crystallin that protects other lens crystallins from unfolding pathways that result in aggregation and precipitation (Horwitz et al., 1999; Horwitz, 2000).

α -crystallin exists in the lens as a polydisperse assembly of α A- and α B-crystallins undergoing rapid subunit exchange (Bova et al., 1997). The assembly has an average size of 40-45 subunits and approximately doubles in size following thermal treatment at 60°C and again increases in size at 66 °C (Putilina et al., 2003). While, the crystal structure of the α -crystallin domain of α -crystallin has been solved (Bagneris et al., 2009; Laganowsky et al., 2010), the N- and C- terminal regions of α -crystallin, are probably unstructured. The significance of the large assembly of α -crystallin or of the N- and C- terminal regions are not known, but may be important in maintaining its own solubility or maintaining the solubility of other crystallins in the lens during chaperone action.

Several laboratories including ours have extensively reported that α -crystallin prevents aggregation of thermally-denatured proteins of the *in vivo* target/client proteins β B1, β B2, and β A3-crystallins (Lampi et al., 2002, 2006; Takata et al., 2007). For example, when exposed to heat, α -crystallin and β B2-crystallin form a large, multimeric complex (Evans et al., 2008). Increasing the β B2: α -crystallin ratio during complex formation increases the overall size as well as the mass of the complex (Michiel et al., 2010), indicating that β B2 does not simply fill empty spaces within the α -crystallin assembly as was previously proposed (Regini et al., 2007). However, the interactions between α -crystallin and β B2 have yet to be characterized, and it is not known if β B2 covers the surface of the α -crystallin assembly, is incorporated into the assembly by subunit exchange, or both.

Age-related cataracts are the leading cause of low vision in the United States and the leading cause of blindness in developing countries (Congdon et al., 2004). Post-translational modifications of crystallins in the aged lens lead to aggregation, resulting in decreased transparency and cataract (Hains and Truscott, 2007). High levels of deamidated species have been identified in aged lenses by two-dimensional gel electrophoresis and mass spectrometry (Lampi et al., 1998; Wilmarth et al., 2006), with several sites associated with cataracts (Wilmarth et al., 2006). About half the potential sites of deamidation are deamidated *in vivo*. An accumulation of even low levels of deamidation at several sites could disrupt the structure leading to unfolding or increase the susceptibility to other modifications like oxidation.

The effect of deamidation is specific to the location in the structure (Harms et al., 2004; Takata et al., 2008). Deamidations at the buried dimer interface residues have been found *in vivo* and appear to have the greatest affect on the stability of the protein (Takata et al., 2007, 2008; Wilmarth et al., 2006). Studies by our laboratory and others have demonstrated that deamidation decreases crystallin stability (Flaugh et al., 2006; Harms et al., 2004; Takata et al., 2007). Specifically, we have shown that deamidation at the dimer interface in β B1, β B2, and β A3 crystallins destabilizes the dimers and causes structural perturbations both locally and distant from the dimer interface (Lampi et al., 2002, 2006; Takata et al., 2007, 2010). In addition, deamidation increases the amount of α -crystallin required to effectively chaperone β -crystallins (Lampi et al., 2002; Michiel et al., 2010). However, the mechanism by which deamidation interferes with the ability of α -crystallin to chaperone β -crystallin is unknown partly because the interactions between α - and β -crystallin are not well described.

Understanding the conformation of wild-type and deamidated β B2 in complex with α A, the major α -crystallin in the lens, will bring insight to the current model of chaperone activity of α A and stabilizing crystallin interactions in the lens. This study aimed to (1) determine the conformation and location of β B2 in complex with α A and (2) determine how deamidation of β B2 affected α A/ β B2 interaction within the complex. We used heat to form a complex

containing α A and either wild-type (WT) β B2 or the double mutant (DM), Q70E/Q162E, mimicking deamidation of β B2. Hydrogen/deuterium exchange coupled to mass spectrometry (HDMS) yielded information regarding solvent accessibilities of localized regions in α A/WT β B2 complex, α A/DM β B2 complex, and WT β B2 dimer. We show that upon thermal stress, there was a generalized increase solvent accessibility of β B2 except for a region located on an outside loop that was protected. The greatest increase in exposure occurred at the interfaces. These results support a model of partially, but not completely unfolded β B2 monomers associating with α A multimer and suggest a possible region of interaction with α A. We also demonstrate that deamidation of β B2 does not alter specific α A/ β B2 interactions in complex but instead promotes aggregation over complex formation. In addition, we show that α A amino acid segments 1 to 53 and 121 to 156 have multiple modes in deuterium uptake distributions, indicating that they are sites with multiple conformations that may promote polydispersity in α A.

2. Materials and methods

2.1 Production of crystallins

Wild type α A, wild-type (WT) β B2 and deamidated mutant, Q70E/Q162E, (DM) β B2 were recombinantly expressed in *E. coli* and purified as described previously (Lampi et al., 2006; Takata et al., 2007). The DM β B2 was chosen because deamidation at both Gln70 and Gln162 at the predicted monomer-monomer interface disrupted the structure and stability of β B2 more than deamidation at other sites (Lampi et al., 2006; Michiel et al., 2010; Takata et al., 2010). Our previous HDMS results confirmed that there was an increase in solvent accessibility at this interface in both the N- and C-terminal domains (Takata et al., 2010). Proteins were dialyzed into 25 mM sodium-phosphate (pH 7.4), 1 mM EDTA, 1 mM DTT, and 100 mM KCl (HDX buffer). All proteins were flash-frozen and stored at -70° C until used.

2.2 α A/WT β B2 and α A/DM β B2 complex formation and isolation

For α A/WT β B2 and α A/DM β B2 complex formation, 2 or 4 mg/mL α A and 1 mg/mL of either WT β B2 or DM β B2 were incubated in HDX buffer for 18 hours at 55 and 50 °C, respectively. The samples were then filtered at 0.1 μ m and applied to a Superose 6 10/300GL gel filtration column (GE Biosciences, Piscataway, NJ) at 0.4 mL/min. Protein molecular weights were monitored by a Dawn Heleos (Wyatt Technology, Santa Barbara, CA) multi-angle light scattering (MALS) instrument. Complex formation was confirmed by coelution of α A and β B2 during SDS-PAGE analysis of individual fractions and control experiments where WT β B2 or DM β B2 were incubated without α A and formed no soluble high molecular weight species. MALS calculations for β B2 used a theoretically determined Abs280 extinction coefficient of 1775 mL g⁻¹ cm⁻¹.

The heat stability of α A/WT β B2 and α A/DM β B2 complexes was determined by measuring the turbidity of mixtures in a 96-well plate placed in a shaker incubator set at 55 °C or 60 °C at 150 rpm. Actual temperatures were monitored with a temperature probe and were confirmed to be ± 2 °C. The time for samples to reach set temperature was 20-25 min. Both proteins were mixed at a final concentration of either 0.25 or 0.5 mg/ml in the HDX buffer. The turbidity of the solutions was measured by the change in O.D. at 405 nm (Lampi et al., 2002). Standard error of four independently prepared samples was reported.

2.3 Local hydrogen/deuterium exchange measurements

Complexes used for H/D exchange measurements were formed using an α A concentration of 3 mg/ml. α A/ β B2 complexes eluting between 9.2 and 14.0 ml during size exclusion chromatography (SEC) were concentrated and analyzed by SDS-PAGE. SDS-PAGE gel

densitometry using β B2 protein standards was used to determine concentrations of β B2 within the complex and then diluted to an estimated β B2 concentration of 0.4 mg/mL. In a separate experiment, α A/WT β B2 and α A/DM β B2 complexes were prepared and isolated as described above, incubated at 4 °C for 10 days, concentrated to 1.77 mg/mL, and analyzed by SEC monitored by MALS to ensure that the complex was stable for an extended period of time.

Hydrogen/deuterium exchange reactions were initiated by diluting 17 μ L of the α A/ β B2 complexes 5-fold with 68 μ L of HDX buffer made with D₂O (99.9 atom %D, Sigma-Aldrich, St. Louis, MO). For each species, three independent reactions were performed at 20°C. At each time point (7 s, 4 min, 60 min), 24 μ L of the reaction solution were added to 24 μ L of quenching buffer (0.42% phosphoric acid made with H₂O). Nondeuterated samples were also prepared for use as 0 s time points by adding HDX buffer made with H₂O and quenching soon after in an identical manner. Following the addition of quenching buffer, samples were immediately flash-frozen with liquid nitrogen, and mass spectrometry was performed within 48 h.

Protease was coupled to Poros 20AL resin (Applied Biosystems, Carlsbad, CA) using previously published methods (Wang et al., 2002), except Type XIII protease from *Aspergillus saitoi* (Sigma-Aldrich, St. Louis, MO) was used instead of pepsin. Immobilized protease was then slurry packed into a 1 $\times$ 20 mm column (IDEX Health & Science, Oak Harbor, WA) to form an in-line digestion column. Deuterium incorporation was measured using an LTQ-Orbitrap Discovery instrument (Thermo Scientific, San Jose, CA) as previously described (Takata et al., 2010) using a resolution of 30,000. Peptides were digested in-line with the type XIII protease-coupled Poros column before reaching a desalting column. Three independent experiments were performed for WT β B2 dimer, α A/WT β B2 complex, and α A/DM β B2 complex, and standard errors were reported. MS/MS analysis was also performed under identical conditions for samples of undeuterated WT β B2 and DM β B2 for peptide identification.

2.4 Data analysis for deuterated peptides

Data acquisition for HDMS was performed using Xcalibur software (Thermo Scientific, San Jose, CA). Deuterated peptides were identified based on measured masses and peptide assignments made during MS/MS analysis of undeuterated WT β B2 dimer and DM β B2 samples using Sequest (Thermo Scientific, San Jose, CA). Data files were converted from .RAW format to .mzXML format and uploaded to the web-based program HD Desktop (Pascal et al., 2009). HD Desktop was used to find centroid mass/charge values for specific peptides. Peptides showing a low signal-to-noise ratio or interference from coeluting peptides were filtered from the list of monitored peptides. All peptides that were selected for analysis eluted between 2.5 and 3.5 minutes, and retention time ranges were adjusted manually for each peptide. The mass/charge centroid values were exported from HD Desktop and multiplied by the charge state of each peptide to find the centroid mass values, and centroid mass values for peptides with multiple charge states were calculated. The number of residues containing exchangeable backbone hydrogens was calculated for each peptide by adding up the number of residues, excluding proline and the N-terminal residue. The relative deuterium incorporation per peptide was calculated by subtracting the mass centroid value of the peptide at the 0 s time point from the mass centroid value at each other timepoint. Peptides were considered statistically different based on the results of the t-test at a confidence level of 99%.

3. Results

3.1 Formation of a stable α A/ β B2 complex for HDMS analysis

A complex was formed by incubating 1 mg/mL WT β B2 with increasing concentrations of α A (either 2 mg/mL or 4 mg/mL) overnight at elevated temperatures. During analysis of the complex by size exclusion chromatography (SEC) there was a dramatic reduction of the β B2 dimer peak area, confirming interaction between α A and β B2 (Figure 1). SEC analysis of WT β B2 incubated alone as a control showed a major monodisperse peak with a molar mass of 45 ± 1 kDa, corresponding to β B2 homodimer (predicted molar mass of 46.5 kDa), and a minor peak eluting at a retention time corresponding to β B2 tetramer (Figure 1A). The sample was primarily dimer, with a dimer:tetramer ratio calculated to be 9:1. The tetramer peak did not have sufficient signal for determination of molecular weight by multi-angle light scattering (MALS).

Incubations used to generate α A/ β B2 complexes for analysis with hydrogen/deuterium exchanged monitored by mass spectrometry were performed at 1 mg/ml of either WT β B2 or DM β B2 and 3 mg/ml of α A and were again analyzed by MALS during SEC to confirm complex formation. Incubation of WT β B2 or DM β B2 with α A at 55 or 50 °C, respectively, for 18 h showed polydisperse peaks eluting between approximately 8 and 13 mL (Figure 1B). Different incubation temperatures were used because no temperature supported effective complex formation for both WT and DM β B2. Specifically, incubation at temperatures of approximately 55 °C or higher were required for α A/WT β B2 complex formation while incubation at temperatures higher than 50 °C resulted in aggregation and precipitation of DM β B2, even in the presence of α A.

The α A/WT β B2 and α A/DM β B2 complexes ranged in size from approximately 2×10^6 to 8×10^6 Da and from approximately 2×10^6 to 1×10^7 Da, respectively. The incubations also produced peaks eluting at the void volume, indicating the presence of high-molecular-weight aggregate. These peaks were not selected for HDMS analysis because of their high polydispersity and the potential for the complexed β B2 to be buried in a large heterogeneous aggregate. The difficulty of detecting a potentially small number of surface exposed regions compared to a large number of buried regions in the complexes would be further complicated by the large number of different potential conformations. Improved methodology will be needed to analyze these soluble aggregates, which most likely correlate to species found in aged and cataractous lenses.

Within the elution volumes collected for HDMS (9.2-14 mL), SDS-PAGE analysis showed coelution of α A and β B2, confirming complex formation with both WT and DM β B2 (Figure 1C). SDS-PAGE densitometry showed an α A/ β B2 ratio of approximately 3:1 in both α A/WT β B2 and α A/DM β B2 complexes. Analysis of the α A/WT β B2 complex and α A/DM β B2 complex by SEC 10 days after complex formation showed minimal aggregation and no elution of additional free β B2, indicating that the complex was stable over extended periods of time (Supplementary Figure 1).

The different stabilities of the WT and DM β B2 complexes with α A were compared during heat-induced denaturation (Figure 2). During heating at 55 °C, DM β B2, but not WT β B2 precipitated, increasing the turbidity of the solution (Figure 2a). The temperature at 55 °C was just below that reported for the thermal denaturation of WT β B2 and above the denaturation for DM β B2 (Michiel et al., 2010). Precipitation was concentration dependent and was not detected when half the concentration was used (Figure 2a). α A was only able to partially prevent the increase in turbidity of DM β B2 (Figure 2b). After an overnight incubation at 55 °C, the turbidity of DM β B2 incubated with α A was the same as with DM β B2 incubated alone. The increased error in the turbidity measurements reflected the protein

precipitating and is similar to the spiking in light scattering measurements previously reported (See Figure 5 in Michiel et al., 2010).

During heating at 60 °C, near the thermal-denaturation temperature for WT β B2, there was a sharp increase in turbidity of the WT β B2 samples after 60-90 min, not observed at 55 °C and that was not prevented by α A-crystallin (Figure 2c). This is in contrast to what we previously reported during shorter incubation times (Michiel et al., 2010). Interesting, even at 55 °C there was an increase in turbidity after overnight incubation of α A and WT β B2, not observed for either protein alone (Figure 2b).

3.2 β B2 showed higher solvent accessibilities in both domains following complex formation

The masses of proteolytic peptides of β B2 dimer alone and in complex with α A were analyzed. There was 80% sequence coverage for the overlap of peptides with acceptable mass signals for WT β B2 dimer alone and WT β B2 in complex with α A. These peptide regions were used to determine changes in β B2 solvent accessibility following complex formation. Peptides with acceptable signals in WT β B2 in complex with α A and DM β B2 in complex with α A provided 62% coverage for studies used to determine the effects of deamidation on α A/ β B2 interaction in complex.

Since back exchange during analysis can occur, we measured masses during in-line digestion with protease XIII in order to decrease back exchange. This led to slightly higher deuterium levels than previously observed. The levels of deuterium incorporation were well within the 30,000 mass accuracy of the high-resolution mass spectrometer and allowed for direct comparison of the same peptides between samples.

Deuterium uptake values in complexed β B2 and β B2 dimer incubated alone were used to probe differences in the structure of free versus complexed β B2 and to determine possible interaction sites between β B2 and α A (Figure 3). The relative deuterium uptake per peptide at 7 s for each covered peptide of WT β B2 dimer and α A/WT β B2 complex is shown in Figure 3A. The relative deuterium uptakes per exchangeable residue in β B2 for selected peptides are shown in Figure 3B. For β B2 alone, the relative deuterium levels followed a similar pattern as previously published (Figure 3 and Takata et al., 2010), with 10-35% of backbone amide hydrogen exchanged in individual peptides, suggesting an overall low accessibility of β B2 in solution. Deuterium levels increased with time, except for in peptides containing the N- and C-terminal extensions. These peptides had similarly high exchange at all time points measured, as would be expected for exposed regions. The N-terminal extension of β B2, comprised of residues 1-15, and the C-terminal extension of β B2, comprised of residues 189-204, are structurally disordered and have been proposed to be important in the dimerization of β B2-crystallin (Trinkl et al., 1994).

Following complex formation, there was a generalized loosening of the β B2 structure along the backbone with deuterium uptake increasing 33-200% in individual peptides from both the N- and C-terminal domains (Figure 3A). The deuterium per exchangeable residue was between 30-40% (Figure 3B). For most peptides, differences in deuterium uptake upon complex formation were greatest at the 7 s time point with no further increase at 4 min or at 60 min as was seen for β B2 alone (see Supplementary Figure 2 for data at all time points). An exception to this is peptide 140-164 containing the buried C-terminal interface residues, with the greatest difference at the later time points. These data suggested that β B2 in complex with α A had undergone a loosening of the backbone structure, without complete unfolding. These changes in solvent accessibility are summarized in Figure 4 by overlaying percent changes onto the ribbon representation of the crystal structure (PDB:1YTQ). The

overall increase in accessibility is represented by the prevalence of the yellow, orange, and red regions of the ribbon structure.

3.3 Complex formation resulted in increased solvent accessibility in all dimer interface peptides, decreased solvent accessibility in residues 123 to 139, and no change in the C-terminal extension

Figure 5 shows deuterium uptake with time for the N-terminal dimer interface (Figure 5 A), an outside loop (Figure 5B), and the C-terminal interface (Figure 5C). In β B2 alone, peptide 53-69 in the N-terminal domain at the buried monomer-monomer interface was moderately protected along with the nearby peptide 72-81 with residues in the buried N-terminal domain (Figure 5A and Supplementary Figure 2). Peptide 148-164 at the buried C-terminal interface was protected from exchange (Figure 5C). Upon complex formation, deuterium incorporation in β B2 increased significantly in both the N- and C-terminal interface peptides compared to WT β B2 dimer alone, suggesting increased exposure of the interface residues in the complex. Peptides 53-69 and 72-81 are depicted in orange in the N-terminal domain and peptide 148-164 in red in the C-terminal domain in Figure 4.

Residues 123-139 composed the only region with significantly lower deuterium incorporation by about 20% in the α A/WT β B2 complex than in the WT β B2 dimer (Figure 5B). These differences in residues 123-139 were significant at longer exchange time points and are represented in blue on the ribbon structure in Figure 4. These residues contain a surface loop with a short helical region, but also a beta-strand that makes up part of the Greek Key motif in the C-terminal domain, which may contribute to its being moderately protected from exchange in β B2 alone.

In agreement with our previous study (Takata et al., 2010), the β B2 extensions had relatively high deuterium levels (peptide 4-21 containing the N-terminal extension and peptide 191-204 containing the C-terminal extension), as would be expected by their flexible structure. In peptide 191-204, the deuterium uptake was the same regardless if β B2 was associated with α A or not and did not change with time (Figure 3), suggesting the C-termini were flexible in both forms.

3.4 Deamidated and WT β B2 showed similar deuterium uptake in all peptides

WT β B2 in complex with α A and DM β B2 in complex with α A had similar deuterium incorporation values for all identified peptides, except for peptide 140-164 which had a 20% increase in deuterium in the α A/DM β B2 complex compared to the α A/WT β B2 complex (Figure 6). However, this difference was also observed between WT and DM β B2 alone as has previously been reported (Takata et al., 2010).

3.5 α A showed bimodal distributions in peptides within amino acid regions 1-53 and 121-156

Deuterium uptake into α A was similar to what has previously been reported by Hasan et al., (2004) (Figure 7). There were 17 overlapping peptides identified between residues 1 and 63 from the N-terminal region and all showed increasing deuterium incorporation with time. Peptides containing residues 146-173 in the C-terminal tail had maximum uptake at all time points, reflecting the greater exposure of the tail region. There were 21 different peptides detected spanning residues 56-145. This region contains the α -crystallin domain and was the most protected with the least exchange of deuterium. This region also included the previously reported protected residues of 72-75 (Hansan et al., 2004).

Analysis of α A revealed no differences between α A incubated alone and α A in complex with β B2 in the α -crystallin domain and the C-terminal tail regions (Figure 7). However, in

the N-terminal domain region of residues 25-52, deuterium incorporation in α A was greater in the complex with β B2 than in α A alone. This peptide within α A also showed bimodal distributions in deuterium uptake (Figure 8). At longer time points, the bimodal distributions became more apparent. Bimodal distributions were only observed in particular regions of α A, peptides within amino acid regions 1 to 53 and 121 to 156 and may suggest multiple conformations of these regions (Supplementary Figure 4).

4. Discussion

Stabilizing interactions between proteins, particularly lens structural proteins called crystallins, contribute to the order and transparency in the lens of the eye. The lens acts as an incubator, accumulating numerous modified crystallins over time that eventually unfold and precipitate, causing light scattering and cataracts (Truscott and Augusteyn, 1977). One of the most prevalent modifications in the aged cataractous lens is deamidation (Lampi et al., 1998). We have previously reported deamidation of β B2 leads to subtle structural changes (Takata et al., 2010) that decreased stability, enhanced aggregation (Lampi et al., 2006) and increased the amount of α A required to prevent its heat-induced precipitation (Michiel et al., 2010). In this study, we have characterized the nature of the β B2 substrate associated with α A and proposed a model for the mechanism by which deamidation may contribute to cataract.

Hydrogen/deuterium exchange with mass spectrometry support a model of a partially unfolded β B2 monomer in complex with α A-crystallin. The total deuterium incorporation from either the N-terminal domain or C-terminal domain of β B2 was higher by 50-60% in the α A/ β B2 complex than the heat-treated β B2 dimer alone, indicating increased solvent accessibility of both domains in complex, not just the N-terminal domain as previously reported (Evans et al., 2008). The average exchange per residue in β B2 was less than 0.5 deuterium suggesting that β B2 in complex with α A retained significant structure, being only partially exposed to the solvent.

These differences in solvent exposure between peptides from the WT β B2 alone and β B2 in complex with α A were superimposed against the three dimensional structure of the β B2 dimer shown in Figure 4 (PDB:1YTQ, Smith et al., 2007). The regions of lowest solvent accessibility in both β B2 dimer alone and β B2 in complex with α A were found in residues 121 to 164 of the core of the C-terminal domain, including the buried interface peptides. The lower solvent accessibility supports the C-terminal domain having greater stability than the N-terminal domain (Wieligmann et al., 1999). The C-terminal domain also had the greatest increase in solvent accessibility in the α A/WT β B2 complex than the WT β B2 dimer alone (Shown in red in Figure 4). Of note, then, was peptide 123-139 with lower deuterium incorporation in the complex, especially at the later exchange times (Figure 5B and shown in blue in Figure 4). This lower deuterium incorporation could be due to changes in either intramolecular or intermolecular interactions. We hypothesize that intermolecular interactions are involved, because this region in β B2 is a likely candidate to interact with α A. This region is similar in location to the proposed α A bindings sites in β A3 crystallin (Gupta et al., 2009), is a highly charged peptide with the sequence KMEIHHDDVPSFHA, and is located on an outside loop. This loop region on β B2 would be accessible to interact with α A.

Analysis of deuterium incorporation in peptides at the buried dimer interface from both the N- and C-terminal domains in β B2 revealed that these peptides were exposed in the α A/ β B2 complex (Shown in orange and red in Figure 4). This finding suggests that the interface in the β B2 dimer has been disrupted and is compatible with a β B2 monomer, rather than the dimer interacting with α A in the heat-induced complex (Evans, et al., 2008).

Our findings are distinct from the urea-induced unfolding intermediates of β B2 (Acosta-Sampson and King, 2010; Fu and Liang, 2002; Wieligmann et al., 1999). Attempts to form a complex between α A and β B2 at a urea concentration of 1.2 M equivalent to β B2 with an unfolded N-terminal domain (Lampi et al., 2006), were unsuccessful. The heat-induced complex formation described here would have occurred between a β B2 partially unfolded in both domains and native α A. Further studies are needed to characterize the exact species of β B2 recognized by α A and what conformational changes occur after complex formation.

In the α A/ β B2 complex, it has not been determined where the β B2 is located in the α A multimer. We have previously shown that increasing the β B2: α -crystallin ratio during complex formation increases the size of the complex, indicating that β B2 does not simply fill empty space within the α -crystallin assembly, which would not increase the apparent molar mass (Michiel et al., 2010). The high solvent accessibility of β B2 in complex with α A observed in this study indicates that the majority of β B2 is exposed and covers the surface of the α A-crystallin assembly following complex formation as shown in Figure 9 and is not initially incorporated into the assembly through subunit exchange.

The lack of large differences in deuterium uptake patterns between α A alone and α A in complex with β B2 is not surprising considering the high observed α A: β B2 ratio in complex (~3:1), which suggests that only a minority of monomers within the α A multimer would have direct interaction with β B2. Furthermore, the large polydispersity of α A and of the complex would mask differences in α A due to interaction with β B2. Of interest then, is the region where increased solvent accessibility in α A in the complex was detected in the N-terminal domain, indicating that upon interaction with β B2, the N-terminal region of α A becomes more exposed. This region is also where bimodal distributions were observed.

Bimodal distributions of specific peptide masses were observed for α A. Bimodal distributions have been observed in deuterium uptake levels as a result of cooperative exchange, in which a number of residues become exposed to solvent simultaneously (Ferraro et al., 2004; Houliston et al., 2002; Maier et al., 1999; Roder et al., 1985; Sivaraman and Robertson, 2001). However, cooperative exchange is promoted by non-physiological conditions such as altered pH or the presence of denaturants and is not common for stable proteins (Weis et al., 2006) suggesting that such kinetics would not be found in this study considering the mild conditions used during hydrogen/deuterium labeling and stable nature of the lens crystallins. The existence of external and internal α A subunits within the α A multimer could also create two modes in the α A deuterium uptake distribution. However, the bimodal distribution in deuterium uptake was seen only in specific regions of α A (residues 1-53, 121-156), indicating that the bimodal distribution was primarily due to variations in specific regions of α A. This observation matches closely with recent findings regarding the polydispersity of α A through specific mechanisms such as bidirectional binding of the C-terminal extension, multiple registration sites in specific regions, and a flexible hinge loop (Laganowsky et al., 2010). The bimodal distribution found at residues 121-156 is likely due to varying conformations of the flexible hinge loop (residues 147-155). The bimodal distribution in residues 1-53, which have not yet been characterized by x-ray crystallography, indicates that the region contains multiple conformations in subunits of the α A multimer and contributes to the polydispersity of α A.

The region in the N-terminal domain, residues 25-52, where bimodal distributions were observed is of interest because this region is also where α A becomes less protected in the complex with β B2. Since the bimodal distributions were observed in peptides from both α A alone and α A in complex, it is possible that the flexibility in this region plays a role in interaction with target/client proteins. Further studies are needed to determine how flexibility in this region would facilitate complex formation.

Since our recent studies showed that mimicking deamidation at the dimer interface in β B2 caused structural perturbations throughout the protein (Takata et al., 2010) and led to incomplete chaperoning by α A (Michiel et al., 2010), we hypothesized that deamidation could alter α A: β B2 interactions in complex. Our initial finding showed that under conditions used to produce α A/WT β B2 complex, DM β B2 was not rescued by α A but instead precipitated. When WT β B2 was heated at temperatures above its denaturation temperature, α A was also not able to completely rescue it from precipitating. After prolonged heating, both α A and WT or DM β B2 precipitated as was previously observed for β B1 (Lampi et al., 2002). Because turbidity of α A alone did not increase, the denatured β B2 most likely saturated α A, causing both proteins to precipitate.

After adjusting the conditions to use a milder temperature, DM β B2 formed a complex with α A. Hydrogen/deuterium exchange measurements in β B2 complexed with α A indicated that deamidation of β B2 did not significantly alter β B2 solvent exposure in complex. This suggests that deamidation in β B2 lowers the stability of β B2 leading to a higher proportion of unfolded intermediates that escape α A during heat-denaturation, rather than disrupting specific interactions with α A (Figure 9).

Given the enhanced rate of denaturation in deamidated β B2, deamidation could decrease the chaperone ability of α A by decreasing the amount of time α A has to recognize and bind partially folded β B2 before aggregation and precipitation occur. A similar process may occur in vivo during aging in human lens, since deamidations were more abundant in water-insoluble crystallins than water-soluble ones (Wilmarth et al., 2006).

5. Conclusions

Using hydrogen/deuterium exchange with mass spectrometry, we have determined that when β B2 is in complex with α A, it is more solvent exposed in both domains, with the exception of an outside loop in the C-terminal domain that is more protected. The greatest increases are at the dimer interface. The only change in α A is an increase in solvent accessibility in the N-terminal domain. These data are consistent with a model of 1) β B2, with both domains partially unfolded, interacting with native α A under stress conditions and of 2) a more solvent exposed β B2 interface in complex with α A, possibly due to a β B2 monomer coating the surface of the α A oligomer depicted in Figure 9 (Carver et al., 2002; Evans et al., 2008; Haley et al., 2000; Horwitz et al., 1998 and 2003; Linder et al., 1997; Rajaraman et al., 1998; Regini et al., 2010). The more protected outside loop in β B2 and the less protected N-terminal domain in α A in the α A/ β B2 complex may be potential sites of interaction in the complex.

Introducing deamidation at the dimer interface of β B2 does not alter the α A: β B2 interactions in this complex, but decreases the ability of α A to chaperone β B2 through mechanisms that occur before complex has been formed. We postulate that because β B2 destabilized by deamidation has solvent exposures similar to wild-type β B2 following complex formation, deamidation of β B2 interferes with the chaperone ability of α A through an increased tendency toward the unfolding and aggregation pathway prior to recognition of the partially unfolded intermediate by α A (Figure 9).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

C-td	C-terminal domain
DM	double mutant
HDX	Hydrogen-deuterium exchange
MALS	Multi-angle light scattering
N-td	N-terminal domain
SEC	Size exclusion chromatography
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis
WT	wild-type

Highlights

- β B2 in complex with α A has greater exposure than β B2 alone along the backbone amides in both domains.
- The interface in β B2 dimer is more exposed in the α A/ β B2 complex, suggesting the dimer is disrupted.
- The only decrease in exposure in β B2 is in an outside loop, which may be important in the α A/ β B2 complex.
- There is increased exposure in a region of the N-terminal domain of α A, suggesting increased flexibility of this region in the complex.
- Deamidation of β B2 decreases rescue by α A without altering α A/ β B2 interactions in complex.

Figure 1.

Detection and isolation of α A/ β B2 complex. (A) WT β B2 incubated at 55° C for 18 h with 0, 2, or 4 mg/ml of α A. β B2 incubated alone was found to exist primarily as a dimer. Increasing the concentration of α A resulted in decreased concentrations of β B2 dimer, demonstrating an interaction between α A and β B2. (B) WT and DM β B2 incubated with α A formed complexes eluting between approximately 8 to 14 mL with aggregate eluting at the void peak and uncomplexed dimer eluting at approximately 17.5 mL. (C) SDS-PAGE analysis of fractions collected for both WT and DM β B2 complexes showed coelution of α A and β B2.

Figure 2.

Relative changes in turbidity of proteins during thermal-denaturation. (A) Turbidity of α A, WT β B2, and DM β B2 at 0.25 and 0.5 mg/ml during heating at 55 °C. DM β B2, at 0.5 mg/ml, had the greatest increase in turbidity. (B) Turbidity of β B2-crystallins at 55 °C with a 1:1 ratio of α A at 0.5 mg/ml. α A was not able to prevent the increase in turbidity of β B2-crystallins after overnight incubation. (C) Turbidity of WT β B2 at 60 °C with a 1:1 ratio of α A at 0.5 mg/ml. At the higher temperature of 60 °C, WT β B2 also had increased turbidity.

Figure 3. Deuterium incorporation for β B2 WT dimer and WT β B2 in complex with α A. (A) Deuterium uptake per peptide at 7 s. Peptides with statistically different amounts of deuterium uptake are marked (*). (B) Deuterium uptake per exchangeable residue of select peptides at 7 s, 4 m, and 60 m.

Figure 4. Differences in deuterium incorporation between WT β B2 dimer and WT β B2 in complex with α A at the 7 s time point overlayed onto the human β B2 crystal structure (PDB:1YTQ). Differences were calculated by subtracting D-uptake per exchangeable residue in the WT β B2 dimer from the D-uptake per exchangeable residue in the WT β B2 in complex with α A.

Figure 5.

Deuterium incorporation for peptides of interest at 7 s, 4 m, and 60 m in WT β B2 dimer and WT β B2 in complex with α A. (A) Peptides in the N-terminal dimer interface showed higher solvent exposure following complex formation with α A. (B) Peptides in amino acid region 123-139 showed a decrease in solvent-exposure after complex formation. (C) Peptides in the C-terminal dimer interface showed an increase in solvent exposure following complex formation. Time points with statistically different amounts of deuterium between β B2 dimer and β B2 in complex uptake are marked (*).

Figure 6. Deuterium incorporation at 7 s in WT and DM $\beta B2$ peptides in complex with αA . WT $\beta B2$ in complex with αA had deuterium incorporation values similar to DM $\beta B2$ in complex with αA .

Figure 7.

Deuterium incorporation per exchangeable residue for peptides of interest at 7 s, 4 m, and 60 m in αA and αA in complex with WT $\beta B2$. Of note are peptides containing residues 25-52 that become more exposed in the complex.

Figure 8.

Relative intensity of mass spectra at increasing times showing deuterium uptake into a representative peptide, α A residues 32-52. The second centroid appearing with time illustrates the bimodal distribution of deuterium uptake found in this region of α A. After longer times of D_2O exposure, the two populations with differing solvent exposure became more apparent.

Figure 9.

A model for αA/βB2 complex formation in WT and deamidated βB2. As found in this study, αA binds to βB2 that is partially unfolded in both the N-td and C-td, and bound βB2 is located in a solvent-exposed region of the complex. Wild type βB2 is more likely to form complex with αA, while deamidated βB2 is destabilized and has a higher propensity for the unfolding pathway.