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Partially Folded Aggregation Intermediates of Human γ D-, γ C- and γ S-Crystallin Are Recognized and Bound by Human α B-crystallin Chaperone

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Abstract

Human γ -crystallins are long-lived unusually stable proteins of the eye lens exhibiting duplicated, double Greek key domains. The lens also contains high concentrations of the small heat shock chaperone α -crystallin, which suppresses aggregation of model substrates *in vitro*. Mature-onset cataract is believed to represent an aggregated state of partially-unfolded and covalently damaged crystallins. Nonetheless, the lack of cell or tissue culture for anucleate lens fibers and the insoluble state of cataract proteins has made it difficult to identify the conformation of the human γ -crystallin substrate species recognized by human α -crystallin. The three major human lens monomeric γ -crystallins, γ D, γ C, and γ S, all refold *in vitro* in the absence of chaperones, on dilution from denaturant into buffer. However, off-pathway aggregation of the partially folded intermediates competes with productive refolding. Incubation with human α B-Crystallin chaperone during refolding suppressed the aggregation pathways of the three human γ -crystallin proteins. The chaperone did not dissociate or refold the aggregated chains under these conditions. The α B-crystallin oligomers formed long-lived, stable complexes with its γ D-crystallin substrates. Using α -crystallin chaperone variants lacking tryptophans, we obtained fluorescence spectra of the chaperone/substrate complex. Binding of substrate γ -crystallins with two or three of the four buried tryptophans replaced by phenylalanines, showed that the bound substrate remained in a partially folded state with neither domain native-like. These *in vitro* results provide support for protein unfolding/protein aggregation models for cataract, with α -crystallin suppressing aggregation of damaged or unfolded proteins through early adulthood, but becoming saturated with advancing age.

Keywords

α -crystallin; cataracts; small heat shock protein; chaperone; aggregation

INTRODUCTION

The vertebrate lens has a unique architecture necessary for its transparency and refractive properties.¹ Its nuclear region is composed of elongated, organelle-free, anucleate, fiber-like

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cells that contain high concentrations of the crystallin proteins and lack the capability of new protein synthesis.² The ubiquitous crystallins, α -, β -, and γ -crystallin, account for approximately 90% of the total protein in lens fiber cells.

α -crystallin is a member of the small heat shock protein (sHsp) family of ATP-independent chaperones.^{3, 4} In adult human lenses, α -crystallin is a polydisperse oligomer (20–40 subunits) of two subunits αA - and αB -crystallin (αA - and αB -Crys) present in a 3 αA :1 αB ratio.² In young human lenses, approximately 28% of the crystallin content is α -crystallin hetero-oligomers.⁵ α -crystallin has a dual role as the main protein chaperone system in mature fiber cells and as a structural protein.^{6–10}

The β - and γ -crystallins are structural proteins whose high concentrations and native state interactions are important in maintaining the transparency and refractive index gradient of the lens. They are members of the same family of proteins, the $\beta\gamma$ -crystallin. They are organized into two domains, with each domain composed of two intercalated Greek-key motifs.^{8, 11}

γC -, γS - and γD -crystallin (γC -, γS - and γD -Crys) are the most abundant γ -crystallins (~21 kDa) and comprise 7, 9 and 11%, respectively, of the total protein content in young human eye lenses.⁵ Human γC - and γD -Crys are expressed *in utero* and localized to the nuclear region of the lens, while γS -Crys is expressed solely in the cortical region. Compared to other mesophilic proteins, the γ -crystallins are extremely stable with melting temperatures above 70°C. The high thermodynamic stability of their native states along with kinetic barriers to unfolding allow them to remain stably folded for long periods of time, a necessary prerequisite for maintaining lens transparency.¹² Their buried tryptophan fluorescence is quenched in the native state relative to the unfolded state in the absence of metal ligands or cofactors.^{13–15} This quenching mechanism is believed to protect Trp residues from photo-damage (e.g. photolysis) caused by constant exposure to environmental UV radiation during our lifetime, thus contributing to the stability of the native protein.¹⁶

Disturbances to the proteins of fiber cells lead to cataract, a high molecular weight state of the lens crystallins that scatters visible light. Cataract is the leading cause of blindness in the world.¹⁷ Age-related or mature-onset cataract is the prevalent form of this disease, while congenital hereditary cataract occurs in early childhood and is relatively rare.¹⁸

Characterization of cataracts removed by surgery revealed that they are insoluble aggregated states, requiring denaturation for solubilization.¹⁹ β - and γ -crystallins isolated from water insoluble fractions of cataractous lenses contain a high percentage of glutamine and asparagine deamidation, methionine and tryptophan oxidation, and truncations.^{20, 21} Destabilization of β - and γ -crystallins resulting from the accumulation of these covalent damages could cause these proteins to partially unfold and populate aggregation-prone species. *In vitro* studies of deamidation-mimicking mutants have shown that these modifications can greatly affect the stability of these proteins.^{22, 23}

Such pathways, in which partially folded species form high molecular weight aggregates, either within cells or outside them, underlie the pathology of many protein deposition diseases including Parkinson's disease, Light chain amyloidosis, Alzheimer's disease, and many other protein deposition diseases.^{24–26} For the well characterized chaperonins, for example GroEL-ES or TriC, such aggregation-prone species are their substrates.²⁷

Horwitz⁶ showed that bovine α -crystallin had chaperone activity since it inhibited the thermally-induced aggregation of different enzymes and bovine β - and γ -crystallins. α -crystallin has also been shown to suppress the aggregation of different non-physiological substrates such as insulin, citrate synthase, α -lactalbumin and alcohol dehydrogenase *in*

vitro.^{6, 28-31} α A- and α B-Crys subunits (~ 20 kDa) can also form polydisperse homo-oligomers with comparable structural and chaperone properties as the hetero-oligomeric α -crystallin.³² α -crystallin does not require ATP hydrolysis for chaperone function, although ATP binding has been shown to induce structural changes and enhance chaperone function.³³

Sathish *et al.* investigated the interaction between human α A- and α B-crystallin and destabilized multimeric β B2-crystallin mutants. They showed that α A- and α B-crystallin recognized and bound mutants with altered equilibrium unfolding patterns observed using the fluorescence emission of a bimane probe attached to β B2-crystallin proteins.³⁴ These mutants had similar thermodynamic stability as WT, but—unlike wild type—aggregated at high protein concentrations under native conditions. The authors proposed that changes in the stability of the substrate have to be accompanied by the population of a folding intermediate for α -crystallin to recognize the β B2-crystallin substrate.³⁴ In addition, Evans *et al.*³⁵ have shown that calf α -crystallin will bind to partially folded species of human and calf β B2-crystallin that structurally resemble an unfolding intermediate of human β B2-crystallin. α -crystallin is able to discriminate between species that are productive intermediates versus those intermediates involved in off-pathway aggregation of model substrate.^{29, 31, 35-37} This mode of chaperone function seems to be a conserved mechanism for other members of the sHsp family.³⁸

Since primary lens fiber cells are terminally differentiated and enucleated, it has not been possible to culture them. In the absence of appropriate cell or tissue culture of the lens, it has been difficult to identify the actual crystallin conformers that are recognized and bound by α -crystallin in the lens. The characterization of the composition of cataractous material removed by surgery has required denaturing conditions such that the identity of the chaperone substrates and their conformation are lost. In the protein aggregation model for cataract, human α -crystallin would be expected to recognize partially unfolded molecules of the human lens γ -crystallins. Horwitz⁶, Carver *et al.*³¹, and Putilina *et al.*³⁹ have previously shown that human α B-Crys is able to suppress the aggregation of a mixture of γ -crystallins from lens' cells lysates. Most of these studies utilized heat denaturation to initiate competitive aggregation and the substrates usually consisted of all γ -crystallins from lenses purified via size exclusion chromatography (SEC). We report here experiments to identify the conformation of species of monomeric human γ C-, γ D- and γ S-Crys that are recognized by the human α B-Crys chaperone.

Unfolding and refolding studies *in vitro* of human γ D-Crys monitored the fluorescence emission of four tryptophan residues (two Trps per domain), each buried in a quadrant of the protein.⁴⁰ These residues are very efficiently quenched in the folded state of the protein so that their fluorescence emission quantum yield increases upon unfolding.^{13, 14} The efficient quenching of Trp68 and Trp156 depends on an unusual conformation of the Trp ring with respect to its backbone amide, as well as the presence of two tightly bound H₂O molecules with oppositely oriented dipoles. The other two tryptophans, Trp 42 and Trp 130, are quenched by Förster transfer to their counterpart Trps.¹⁴⁻¹⁶ As a result, the Trp fluorescence from the γ -crystallins is a very sensitive reporter of the protein's conformation.

In equilibrium and kinetic refolding experiments, a stable, partially folded intermediate was identified with the N-terminal domain unfolded and C-terminal domain folded. When the protein was refolded from high to low concentrations of denaturant, off-pathway aggregation was observed that competed with productive refolding of human γ D-Crys.⁴¹ Recombinant human γ C-Crys behaved similarly under matching assay conditions (Dr. Yongting Wang, personal communication). Human γ S-Crys, on the other hand, had a more cooperative unfolding/refolding transition during equilibrium experiments and aggregation

was not detected during refolding.¹² In this study, we have taken advantage of the *in vitro* aggregation reactions that compete with productive folding to examine individually the interactions of human α -crystallin with aggregating, partially unfolded, purified human γ -crystallins that are likely candidates for physiological chaperone substrates within the aging lens.

RESULTS

Purification and characterization of recombinant wild-type, human α B-Crys

Human recombinant α A- and α B-Crys form large (400-600 kDa), polydisperse homo-oligomers when expressed in *E. coli* cells.^{32, 42} Sun *et al.*³² have shown that these homo-oligomers have similar structural characteristics as endogenous bovine α -crystallin, with small differences in surface hydrophobicity between recombinant homo-oligomers. Further, both human α A- and α B-Crys oligomers showed chaperone-like activity with α B-Crys oligomers suppressing aggregation at lower substrate to chaperone weight ratios. The multimeric state, heterogeneous character and absence of catalytic activity make it difficult to compare preparations of α -crystallin from different sources. Figure 1 describes the characteristics of the α B-Crys oligomers from our expression and purification protocols.

Recombinant, WT human α B-Crys was expressed at low temperature (18°C) and purified via anion exchange and SEC. Fractions collected during the final, polishing size-exclusion chromatography step were electrophoresed through a 14% SDS-PAGE gel to confirm purity of protein samples (Figure 1A and B). The secondary, tertiary and quaternary structures of purified WT α B-Crys were characterized using far-UV CD spectroscopy, fluorescence emission spectroscopy and SEC methods. Far-UV CD spectrum (Fig. 1C) of native α B-Crys showed a minimum at 214 nm consistent with previously published spectra.³² Human α B-Crys has two tryptophans at amino acid positions 9 and 60 in its N-terminal region. Comparable to previously published results,³² the fluorescence emission spectra showed maxima at wavelengths 339 and 349 nm for native and unfolded protein respectively (Fig. 1D). Quaternary structure characteristics were examined using a Superose 6 SEC column. Figure 1A shows a representative size-exclusion chromatogram. From elution profiles, recombinant WT α B-Crys oligomers had apparent molecular weights between 500-600 kDa (25-30 subunits per oligomer). This size distribution is consistent with previously published results³².

WT human γ C-, γ D- and γ S-Crys aggregate upon refolding out of high concentrations of denaturant into buffer with low concentrations of denaturant

Previous work by Kosinski-Collins and King⁴¹ showed that wild-type human γ D-Crys can refold to its native state at 37°C after unfolding in high concentrations of GdnHCl in equilibrium refolding experiments that tracked tryptophan fluorescence changes between the folded and unfolded states. Upon dilution out of denaturant, refolding was already significant within the mixing dead-time of the experiment. The C-terminus reached its native-like state first, with a $t_{1/2}$ of 35 sec, while the folding of the N-terminal, with a $t_{1/2}$ of 130 sec, took significantly longer.²² Thus, there is a partially folded intermediate that is significantly populated during the first few minutes of refolding even at 37°C.²² However, when diluting to buffer or low denaturant concentrations (less than 1 M GdnHCl) early intermediates of γ D-Crys aggregated under the experimental conditions (10 μ g/ml, pH 7.0). Early, partially folded intermediates of WT human γ C-Crys displayed analogous aggregation behavior in similar experiments (Dr. Yongting Wang, personal communication). On the other hand, partially folded intermediates of wild-type human γ S-Crys did not aggregate under similar experimental refolding conditions during equilibrium refolding experiments.¹² Given the structural and sequence similarity between these three γ -

crystallins (~ 50% identity between human γ S-Crys and γ D-Crys),¹² it seemed likely that partially folded species of γ S-Crys would aggregate during refolding if the protein concentration was increased.⁴³

Recombinant, WT human γ D- and γ S-Crys containing an N-terminal His₆ tag were expressed in *E. coli* and purified via Ni-NTA affinity chromatography. Wild-type human γ C-Crys, without any affinity tags, was also expressed in *E. coli*, but purified via SEC. Kosinski-Collins *et al.*^{13, 41} have shown no difference in protein stability and aggregation behavior between γ D-Crys with or without a His₆ tag.

Wild-type γ D-, γ C-, and γ S-Crys were unfolded in 5.0 M GdnHCl at pH 7.0 and 37°C for 24 hours. Refolding at 37°C was initiated by a ten-fold dilution with Refolding Buffer (without GdnHCl) and thorough mixing of the sample. Aggregation was followed by monitoring solution turbidity measured as optical density at 350 nm (O.D._{350 nm}) without continuous stirring of the sample. The final protein concentration was 10 times higher than that used in the aforementioned equilibrium unfolding/refolding experiments. Figure 2 shows the O.D._{350 nm}, which is a direct measure of static light scattering due to formation of macromolecular aggregates, for each γ -Crys protein sample (1 γ :0 α B traces). The first recorded O.D._{350 nm} values (at t=0) were higher than the buffer baseline due to the rapid formation of light-scattering species within the mixing dead-time of the experiment (~ 5 sec). Aggregates formed for all three γ -crystallins were confirmed to be amorphous by transmission electron microscopy (Fig. S1) and were visible to the naked eye minutes after initiation of refolding and aggregation.

WT γ D-Crys had the highest increase in solution turbidity (~ 1.15 AU) followed by γ C-Crys (~ 0.7 AU) and γ S-Crys (~0.35 AU) (Fig. 2 and S2). At 37°C, O.D._{350 nm} traces for all three γ -crystallins displayed two phases. The traces increased sharply in the first 40 seconds and reached a plateau 200 seconds after initiation of refolding and aggregation (Fig. 2 and S2). The two-phase aggregation process observed in these traces for all partially folded γ -Crys was probably due to an initial, fast growth phase of the small-globular aggregates into fibril-like, amorphous structures. Eventually, the light scatter traces reached a plateau due to the formation of large aggregates within the first 30 minutes of the reaction. These aggregate species have been observed by Kosinski-Collins and King⁴¹ using atomic force microscopy.

α B-Crys chaperone suppresses the aggregation of partially folded WT γ D-, γ C- and γ S-Crys

To test whether partially folded crystallins were substrates of α -crystallin, wild-type γ D-, γ C- or γ S-Crys protein was allowed to refold and aggregate in the presence of WT α B-Crys chaperone at different mass-based ratios of γ -Crys to α B-Crys (Fig. 2). All γ -Crys to α B-Crys ratios described hereafter were mass-based ratios. Refolding buffer with α B-Crys chaperone was added to unfolded γ -Crys solutions, mixed thoroughly, and aggregation was monitored by measuring changes in O.D._{350 nm} in a UV-Vis Spectrophotometer without subsequent, continuous stirring. Unless otherwise stated, this mixing regime was followed for all of the short-term suppression experiments mentioned hereafter.

α B-Crys chaperone suppressed the aggregation of partially folded intermediates of all three γ -crystallins in assays measuring solution turbidity. Increasing the concentration of α B-Crys chaperone, from 1 γ :1 α B to 1 γ :5 α B, led to further reduction in light scattering (Fig. 2). WT γ D- and γ C-Crys aggregation was suppressed to similar levels (Fig 2A and C), while γ S-Crys aggregation was not suppressed as well under identical experimental conditions (Fig. 2B). Further, we observed that only the partially folded γ -Crys substrate was incorporated into the aggregate while the α B-Crys chaperone was present as either free oligomers or bound to γ -Crys in high molecular weight complexes (data not shown). We also

performed the same aggregation suppression assays in the presence of α B-Crys chaperone with different concentrations of γ D-Crys ranging from 50-150 μ g/ml. We did not observe significant differences in the suppression of aggregation levels at the same ratios within this range (data not shown).

Samples from these suppression reactions were further analyzed via SEC—performed at 4°C in SEC Buffer—2 h after samples were prepared. At this temperature, we did not observe any further aggregation after the samples had been filtered with a 0.2 μ m filter prior to SEC. Chromatograms from the 1 γ D:5 α B and 1 γ C:5 α B samples showed the presence of a high molecular weight complex that eluted in the void volume (V_0 , ~7.6 ml) of the Superose 6 GL 10/300 SEC column, along with the signature peaks for α B-Crys chaperone and γ D- or γ C-Crys monomers (Fig. 3A and B, gray traces). The high molecular weight complexes constitute the γ — α B complexes. This early-eluting peak was only observed in samples from suppression reactions. Chromatograms from samples of native α B-Crys chaperone and native γ D-Crys incubated together under final refolding conditions (0.5 M GdnHCl and Refolding Buffer), for 2 h at 37°C, lacked this early-eluting peak (Fig 3A and B, black traces). Chromatograms from 1 γ :0 α B aggregation reactions, native α B-Crys chaperone only, and native γ -Crys only also lacked this peak (data not shown). Interestingly, when 1 γ S:5 α B samples were applied to the Superose 6 column, no early-eluting peak was observed (Fig. 3C). This result along with turbidity data shown in Fig. 2 indicates that α B-Crys did not suppress the aggregation of partially folded γ S-Crys species as efficiently as the aggregation from partially folded γ D- and γ C-Crys. These results also show that the N-terminal His₆ tag of γ D- and γ S-Crys does not seem to affect the chaperoning interactions between α B-Crys and that the changes in suppression efficiency observed for different, partially folded γ -Crys were due to particular characteristics of these proteins. This differential interaction with γ S-Crys could be due to a shorter lifetime of the aggregation-prone species populated during γ S-Crys refolding.¹² Another possibility is that amino acid sequence differences in γ S-Crys make it a poorer substrate for the α B-Crys chaperone.

Destabilization of the C-terminal domain is required for the formation of the partially folded, aggregation-prone intermediate of γ D-Crys

Since the crystal structure of γ D-Crys has been solved to 1.25 Å resolution and its folding pathway has been previously described in detail, we chose γ D-Crys to further study interactions between α B-Crys chaperone and this physiological substrate.^{13: 40: 41: 44: 45} Long-lived intermediates have been implicated as species responsible for the aggregation of several proteins because they might give rise to aggregation-prone species during refolding.^{43: 46} A stable intermediate is populated during WT γ D-Crys unfolding/refolding equilibrium experiments and is manifested as a slight inflection in the equilibrium traces at 2.3 M GdnHCl.^{13: 41} This species is well-populated during the unfolding/refolding equilibrium experiment of a variety of crystallins carrying single amino acid substitutions.^{22: 44: 45} This species has its N-terminal domain unfolded and the C-terminal domain folded, hence the hydrophobic patches in the domains' interfaces are exposed to solvent.¹³ Consequently, these hydrophobic patches could be candidate sites for intermolecular aggregate interactions.

Previous studies have shown that the two transitions observed during equilibrium unfolding/refolding experiments correspond to the successive unfolding of each domain, with the first transition (~ 2.2 M GdnHCl) corresponding to the unfolding/refolding of the N-terminal domain and the second transition (~ 2.8 M GdnHCl) to the unfolding/refolding of the C-terminal domain ^{13: 45}. Therefore, it is possible to partially unfold γ D-Crys at different concentrations of GdnHCl encompassing both transitions and to populate stable, partially-unfolded species.

To explore the structural requirements for aggregation, two step reactions were performed. Wild-type γ D-Crys was unfolded at different concentrations of GdnHCl in order to investigate whether the long-lived folding intermediate was the aggregation-prone species (2.49, 2.82, 3.09 and 5.03 M GdnHCl). γ D-Crys unfolded at these concentrations does not self-associate and aggregate prior to dilution of the denaturant.⁴⁴ After equilibrium conditions were reached, unfolded γ D-Crys was diluted to lower concentrations of GdnHCl (0.5 M GdnHCl, final protein concentration was 50 μ g/ml) (Fig. 4). The O.D._{350 nm} amplitudes were 0.2 and 0.25 AU for 2.82 M and 3.02 M GdnHCl unfolded samples respectively, while the final O.D._{350 nm} amplitude was 0.45 AU for 5.03 M GdnHCl unfolded samples (Fig. 4). This lower level in final O.D._{350 nm} was due to a higher proportion of protein refolding productively when diluted out of these intermediate GdnHCl concentrations (data not shown). These results indicate that the C-terminal domain of γ D-Crys must be destabilized and partially unfolded for the protein to aggregate *in vitro*. Further, these result suggests that the stable folding intermediate, with its C-terminal domain folded and N-terminal domain unfolded, observed in unfolding/refolding equilibrium experiments, is likely not the aggregation-prone intermediate.

Monitoring the conformation of the bound substrate

γ D-Crys has four buried Trp residues, two in the N-terminal domain (Trp42 and Trp68, Fig. 6A) and two in the C-terminal domain (Trp130 and Trp156, Fig. 6A).⁴⁰ Rapid electron transfer from the excited Trp indole ring to its amide backbone results in the efficient quenching of the weakly fluorescing, strongly quenched Trp68 and Trp156.¹⁴ The intra-domain mechanism of quenching of Trp fluorescence in the native state of γ D-Crys is due to partial resonance energy transfer (FRET) from the strongly fluorescing tryptophans (Trp42 and Trp130) to their weakly fluorescing counterparts (Trp68 and Trp156). We took advantage of the structural information provided by the fluorescence emission of buried tryptophan residues in γ D-Crys to further elucidate the structural nature of the substrate that is recognized by α B-Crys chaperone during aggregation.

Wild-type α B-Crys contains two tryptophan residues at positions 9 and 60. Mutating both residues to phenylalanine resulted in the elimination of fluorescence emission compared to wild-type protein when the excitation wavelength (λ_{EX}) was set to 300 nm to selectively excite tryptophan residues (Fig. 5A). Liang and colleagues⁴⁷ have shown that individually mutating these tryptophan residues to phenylalanine does not result in secondary structural changes to α B-Crys. Structural changes in quaternary structure between wild-type and mutant proteins were not detected during SEC (Fig. 5C). Suppression assays using γ D-Crys as the substrate showed that the chaperone activity was conserved in the W9F/W60F α B-Crys mutant (noTrp α B) (Fig. 5B).

1 γ D:5 noTrp α B suppression reactions were collected and loaded onto a Superose 6 SEC column (Fig. 5C). The fluorescence emission spectrum was recorded for the fraction corresponding to the high molecular weight complex (Fig. 5C, gray bar) at a λ_{EX} of 300 nm. Since α B-Crys contains no tryptophan residues, only the tryptophan residues from γ D-Crys present in this complex would contribute to the fluorescence spectrum. The spectrum of the γ D— α B-Crys complex (max. emission wavelength $\sim$ 335 nm) did not coincide with either the spectra of the native or unfolded WT γ D-Crys suggesting that the substrate might be in a partially-folded state (Fig. 5D).

To further investigate the conformation of γ D-Crys in these complexes, we studied double- and triple-Trp γ D-Crys mutants with the Trps replaced by phenylalanines.¹⁴ Double-Trp mutants had either the N-terminal (W130F/W156F γ D-Crys, Fig. 6A) or C-terminal domain (W42F/W68F γ D-Crys, Fig. 6A) buried tryptophan pair conserved in order to preserve the tryptophan fluorescence quenching mechanism within each domain. These mutants provided

a more detailed picture of the folding status of each domain of γ D-Crys when bound to α B-Crys during chaperoning interactions. Both W130F/W156F and W42F/W68F γ D-Crys recapitulated the behavior of WT γ D-Crys: their tryptophan fluorescence was quenched in the native state and, upon unfolding in high concentrations of denaturant, showed an increase in fluorescence intensity with a concurrent red-shift in their maximum emission wavelength (λ_{EM}) to ~ 350 nm.¹⁴

Figures 6B and C show the relative fluorescence emission spectra for W130F/W156F and W42F/W68F γ D-Crys after unfolding these proteins in increasing concentrations of GdnHCl. The red-shift in maximum λ_{EM} coincided with previously published transition GdnHCl concentrations that correspond to the unfolding of the N-terminal and the C-terminal domains of the wild-type protein.^{13, 41, 45} W130F/W156F γ D-Crys (N-terminal Trp pair) retained the native-like fluorescence spectrum at 1.6 M GdnHCl when the λ_{EX} was 300 nm. Increasing the denaturant concentration to 2.0 M GdnHCl resulted in a red-shift in the max. λ_{EM} to 350 nm (Fig. 6B). The emission spectrum for W42F/W68F γ D-Crys (C-terminal Trp pair), on the other hand, remained native-like at 2.3 M GdnHCl (λ_{EX} was 300 nm). At higher concentrations of GdnHCl, the spectra were red-shifted until reaching the maximum λ_{EM} for the fully unfolded protein, approximately 350 nm (3.1 M and 5.2 M GdnHCl traces, Fig. 6C).

Given the structural information provided by these γ D-Crys mutants, we utilized W42F/W68F and W130F/W156F γ D-Crys as substrates in suppression of aggregation reactions with Trp-less α B-Crys chaperone. Both double-Trp mutants aggregated to similar levels as the wild-type protein and Trp-less α B-Crys chaperone showed similar suppression efficiency as with the wild-type γ D-Crys (data not shown). The substrate-chaperone complexes were isolated from 1 γ D:5 noTrp α B suppression reactions via SEC using a Superose 6 SEC column (Fig. 6D). Fluorescence emission spectra ($\lambda_{EX} = 300$ nm) were recorded for the fraction corresponding to the substrate-chaperone peak for both double-Trp mutants (Fig. 6E and F). The fluorescence emission spectra for both double-Trp mutants in complex with Trp-less α B-Crys were similar (max. $\lambda_{EM} \sim 333$ nm). However, these spectra did not coincide with the spectra for their respective native or unfolded states (Fig. 6E and F). These results point to similar environments for both the N-terminal domain and C-terminal domain of γ D-Crys in complex with α B-Crys. The tryptophan residues of γ D-Crys in the complex did not seem to be exposed to solvent nor retained the conformation necessary for the quenching phenomenon observed in the native state. The tryptophans were either in buried but in non-native conformations, or they were directly interacting with the chaperone subunits.

Given the similarity in fluorescence spectra between wild-type and the double-Trp mutants of γ D-Crys, it is likely that it is due to interaction of regions surrounding the Trp residues with α B-Crys in the chaperone-substrate complexes. In order to monitor binding interactions at specific regions of γ D-Crys more closely, we took advantage of triple-Trp mutants that conserved the highly-fluorescent, buried Trp residues in either the N-terminal (W42only γ D-Crys) or the C-terminal domain (W130only γ D-Crys), while the remaining three tryptophans were changed to phenylalanines. Chen *et al.*¹⁴ previously characterized the buried tryptophan fluorescence behavior of these proteins and found that the quenching phenomenon observed in the native state of wild type and double-Trp mutants described above was absent in these triple-Trp γ D-Crys mutants.

Since these tryptophan residues (Trp42 and Trp130) emit a robust fluorescence emission signal, we used these mutants in suppression aggregation reactions with Trp-less α B-Crys chaperone to reveal general features of the environment around these residues in the γ D— α B complex. Both W42only and W130only γ D-Crys aggregated after dilution out of

denaturant and Trp-less α B-Crys suppressed their aggregation efficiently (at 1 γ :5 α B ratios). Figure 7A shows the emission spectrum of W42only γ D-Crys in the γ D— α B complex excited at 300 nm; the max. λ_{EM} was approximately 334 nm and the spectrum did not coincide with the spectra for the native and unfolded W42only γ D-Crys controls. The fluorescence emission spectrum for W130only γ D-Crys in the complex had a max. λ_{EM} at approximately 333 nm and also did not coincide with either the native or fully-unfolded tryptophan fluorescence spectra of controls (λ_{EX} =300 nm; Fig. 7B). These results indicate that the residues in close proximity to Trp42 and Trp130 are not solvent exposed in the γ D— α B complex and might be involved in binding interactions with α B-Crys chaperone.

α B-Crys chaperone forms stable complexes with partially folded γ D-Crys

α -crystallin is the major chaperone system in differentiated lens fiber cells. Since there is no protein turnover, aggregation of covalently damaged crystallins is prevented through interactions with α -crystallin.² Therefore, the complex between α -crystallin and its β/γ -crystallin substrates must be long-lived in order for lens transparency to be maintained. We investigated the lifetime of this complex *in vitro* by initiating the aggregation of γ D-Crys in the presence of α B-Crys at a 1 γ D:5 α B ratio. The suppression reaction was allowed to proceed at 37°C with moderate shaking and aliquots were removed at different times and applied to a Superose 6 SEC column. During this incubation period, we did not confirm whether any chemical modifications were introduced into γ D-Crys or α B-Crys chaperone during the 4-day period.

Figure 8 shows the set of chromatograms collected within a time period of 30 min to 4 days after aggregation was initiated. The high molecular weight peak eluted at the V_0 , ~7.6 ml (Fig. 8, Inset). This peak was still present 4 days after the suppression reaction was initiated. Further, the absorbance at 280 nm of this peak at 7.6 ml increased after 30 min, decreased and remained constant thereafter for later time points. The WT α B-Crys chaperone peak (eluted at 13.5 ml) remained constant throughout the duration of the experiment. These results showed that the γ D— α B complexes were stable for long periods of time supporting α -crystallin's role as a chaperone in the lens.

α B-Crys chaperone interacts with early and late aggregate species in the aggregation pathway of partially folded γ D-Crys

Despite its chaperone function, α -crystallin is often found in cataractous inclusions in significant proportions.^{19; 20; 48; 49} Further, α A-Crys knock-out mice developed cataract at an early age in which α B-Crys was localized to the insoluble fraction of lens fiber cell cytoplasm.⁵⁰ Therefore, we were interested in investigating the lifetime of the substrate species that is recognized by α B-Crys chaperone.

To do so, we added Trp-less α B-Crys chaperone at very early time points (2, 6, and 10 sec) after dilution of γ D-Crys out of denaturant. Wild-type γ D-Crys was added to Refolding Buffer that was constantly stirred with a spin-bar; the solution was stirred only for the first 30 sec after fully unfolded γ D-Crys was added. The final O.D._{350 nm} levels did not change significantly using this method. Trp-less α B-Crys was added 0, 2, 6 or 10 sec after refolding and aggregation of γ D-Crys were initiated. Solution turbidity increased the later Trp-less α B-Crys was added after refolding and aggregation were initiated (Fig. 9A and B). Controls were performed by adding H₂O after 6 and 10 sec and showed no significant change in the kinetics or the level of aggregation of γ D-Crys (data not shown).

Using Trp-less α B-Crys chaperone allowed us to record the fluorescence emission spectrum of the WT γ D-Crys species bound to α B-Crys chaperone in the complexes isolated via SEC (Fig. 9C). All spectra had the same max. λ_{EM} (~336 nm), however, the fluorescence

intensity decreased in samples from later addition time points indicating a decrease in the concentration of γ D— α B complexes (Fig. 9D). These results indicate that the species recognized and bound to α B-Crys chaperone is a transient intermediate populated at very early times of refolding and aggregation.

Adding α B-Crys chaperone any time after 15 sec resulted in no suppression of γ D-Crys aggregation (Fig. S3). Interestingly, when α B-Crys was added 5 minutes after the aggregation reaction was started there was an increase in the scatter of the aggregate compared to controls (Fig. S3). Similar results were also observed when α B-Crys was added 30 minutes after initiation of aggregation of partially folded γ D-Crys. Given the structures of γ D-Crys aggregates observed at these time points by Kosinski-Collins and King,⁴¹ it seems that α B-Crys chaperone is being incorporated into or binds to these preformed large fibril-like aggregates of γ D-Crys, but does not disaggregate them. Similar behavior has been reported for IbpA and IbpB, sHsps in *E. coli*, which are known to be present in inclusion bodies formed during overexpression of heterologous protein.^{51, 52}

DISCUSSION

The native states of the γ -crystallins are very soluble, very stable and among the longest lived proteins in the human body.² They show little tendency to aggregate or associate even at high concentrations. Except for special cases such as sickle cell anemia, most well characterized protein aggregation reactions represent the polymerization of partially folded species.^{46, 53, 54} The examples of the well-characterized protein aggregation diseases, including Parkinson's disease, prion protein encephalopathies, and Huntington's disease, suggest that partially folded or unfolded intermediates are also the key species in the crystallin aggregation reactions that lead to cataract.^{24, 25} Extensive characterization of α -crystallins with model substrates support models in which partially folded species are the substrates.^{29, 31} However, the absence of a high resolution structure for the full-length protein chaperone, and the difficulty in characterizing the substrate conformations recognized within normal or cataractous lens, has limited our ability to define the conformation of the physiological substrates of α -crystallins.

Competitive off-pathway aggregation of γ D-Crys during refolding conditions

Productive kinetic refolding experiments showed that two partially folded intermediates were populated during WT γ D-Crys refolding.²² Significant refolding, as measured by changes in tryptophan fluorescence, occurred within the mixing dead time that initiated these kinetic refolding experiments. It is unlikely that a true random coil, as found in the fully denatured state, is significantly populated after the first seconds of the refolding reaction. The first two transitions, with $t_{1/2}$ values of 8 and 35 sec, corresponded to the folding of C-terminal domain, while the third transition, with a $t_{1/2}$ value of 130 sec, was significantly longer with and corresponded to the folding of the N-terminal domain.²² Flaugh *et al.*^{44, 45} showed that the residues on the interface of the C-terminal domain nucleated the folding of the N-terminal domain. Previously published work on deamidated γ D-Crys mutants of the interface glutamine Page 16 of 43 residues²² and molecular dynamics simulations of γ D-Crys unfolding⁵⁶ suggest that the folding nucleus of the protein is located in the Greek key motif in the C-terminal domain closest to the domain interface of the protein (Motif IV).

Aggregation experiments with partially unfolded γ D-Crys showed that the C-terminal domain must be partially unfolded for the protein to aggregate after dilution into low concentrations of denaturant (Fig. 4). Further, when γ D-Crys was fully unfolded, α B-Crys recognized and bound to partially folded, aggregation-prone species populated during the first 10 sec after refolding and aggregation were initiated (Fig. 9A and B). The C-terminal

domain also productively refolds during this time period.^{12, 13, 45} Experiments with the isolated single domain constructs of the N-terminal or C-terminal domain of γ D-Crys showed that the single C-terminal domain of γ D-Crys aggregated during dilution out of high concentrations of denaturant, while the single N-terminal domain of γ D-Crys did not aggregate under similar conditions (unpublished results). Therefore, it is likely that the aggregation-prone region is located in the C-terminal domain and was recognized by the α B-Crys chaperone during these aggregation reactions.

Conformation of the partially folded γ -crystallin bound to α B-Crys chaperone oligomers

The long-lived nature of the γ D— α B complex allowed us to characterize the conformation of the substrate bound to the chaperone (Fig. 8). The isolation using SEC of γ D— α B-Crys complexes from 1 γ D:5 noTrp α B suppression reactions indicated that γ -crystallin was present in the high molecular weight oligomeric complex. Since all tryptophans in α B-Crys were changed to phenylalanines and the excitation wavelength was set to preferentially excite tryptophan residues, the fluorescence emission spectra of these complexes provided structural information for bound WT γ D-Crys (Fig. 5D). We compared this spectrum to the spectra from native and unfolded WT γ D-Crys. The fluorescence emission spectrum for each conformation is distinct (i.e. the max. λ_{EM} is unique to each state). The fluorescence emission spectrum of γ D-Crys from these complexes did not resemble the spectra for either native or fully unfolded γ D-Crys (Fig. 5D).

Suppression reactions with double-Trp mutants of γ D-Crys as substrates and Trp-less α B-Crys as the chaperone provided information on the folding status of each domain of γ D-Crys in the γ D— α B complex (Fig. 6). These double-Trp γ D-Crys mutants only had the tryptophan pairs within the N-terminal (W130F/W156F γ D-Crys) or C-terminal domain (W42F/W68F γ D-Crys), so that the intra-domain native state quenching mechanism was conserved. If γ D-Crys had either domain folded in the γ D—noTrp α B complex, then fluorescence emission spectra of the substrate would be similar to the spectra of the native protein. Our results showed that both double-Trp mutants in γ D—noTrp α B complexes had similar maximum λ_{EM} , and that their spectra did not resemble either their respective native or unfolded states (Fig. 6E and F). Further, these spectra were similar to the spectrum of the wild-type protein in complex with Trp-less α B-Crys chaperone (Fig. 5D). These results indicate that the intra-domain tryptophan pairs of γ D-Crys were in similar environments in the γ D— α B complexes.

The maximum λ_{EM} for tryptophan fluorescence is sensitive to local environment and changes in response to various degrees of solvent exposure.⁵⁵ Chen *et al.*¹⁴ studied the fluorescence behavior of the buried tryptophans in the double- and triple-Trp mutants described previously (*vide supra*). For the native states of the triple-Trp γ D-Crys mutants, they found that the highly fluorescing Trps (Trp42 and Trp130) had a max. λ_{EM} at ~322 nm, while the maximum λ_{EM} was 332 nm for the quenched Trps (Trp68 and Trp156). They compared these values to the max. λ_{EM} from fluorescence emission spectra of 3-methylindole (3-MI), a tryptophan indole ring analog, in different solvent mixtures. The maximum λ_{EM} for Trp42 and Trp130 native spectra closely matched the spectrum for 3-MI in a less polar solvent mixture (cyclohexane-dioxane, 83:17), while the fluorescence spectra and max. λ_{EM} for 3-MI in a more polar solvent mixture (methanol-dioxane, 25:75 and 10:90) were similar to native spectra from Trp68 and Trp156. Within the context of the quenching mechanism in the native state, these results bolstered the model of intra-domain partial resonance energy transfer from the highly-fluorescent Trps (donors) to the quenched Trps (acceptors) proposed by Chen and colleagues.¹⁴

This comprehensive investigation of the buried Trp fluorescence of γ D-Crys allowed us to refine our model on the conformation of the aggregation-prone γ D-Crys species that is

bound to α B-Crys during the chaperoning process. We performed aggregation suppression assays with W42only and W130only γ D-Crys triple-Trp mutants as substrates (Fig. 7). These mutants retained the highly fluorescing tryptophan from each domain thus allowing us to assess the environment surrounding these regions in both domains in the γ D—noTrp α B complex. The fluorescence emission spectra of W42only or W130only γ D-Crys in the substrate-chaperone complex did not coincide with their respective native and unfolded spectra. Therefore, Trp42 and Trp130 were neither fully exposed to solvent nor did they remain buried in the hydrophobic core of the native state (Fig. 7).

These results point to a possible interaction between the Trp residues, or the immediate regions surrounding them, and α B-Crys chaperone in the complex. One possible model to explain these results is that α B-Crys could initially recognize the exposed, buried aggregation-prone region in Motif 4 (which includes Trp130 and Trp156) in the C-terminal domain, bind to it, and keep it from interacting with other aggregation-prone regions. Binding to the homologous hydrophobic region in the N-terminal domain could occur after the initial recognition step. An exposed state of the Trp rings, which are intended to be buried in the native state, offers an attractive model for how the chaperone discriminates between partially folded intermediates and the fully folded state.

Another possible model is that the distinct fluorescence emission spectra of double- and triple-Trp γ D-Crys mutants in complex with Trp-less α B-Crys could be due to their tryptophan residues being exposed to polar environments between γ D-Crys chains in small, soluble aggregates formed in the first 10 sec after dilution out of denaturant. α B-Crys chaperone would bind these small γ D-Crys oligomers prevent further growth into fibril-like aggregates. If this were the case, however, we would see similar or increased efficiency of suppression within these first 10 sec, rather than a progressive decrease in suppression of aggregation as judged by the increase in light scattering at 2, 6, or 10 sec (Fig. 9A and B).

As mentioned above, α B-Crys suppression activity was confined to early species along the aggregation pathway of γ D-Crys, which may still be monomeric (Fig. 9). Adding α B-Crys at later times showed that the chaperone no longer suppressed aggregation, but may have been interacting with insoluble aggregates as judged by increases in solution turbidity (Fig. S3). Kosinski-Collins and King⁴¹ have shown that during this time period small fibrillar aggregates form. Small Hsps IbpA and B from *E. coli* are integrated into inclusion bodies when the amount of substrate exceeds the amount of sHsp in the cell.^{51: 52}

Human γ C-, γ D-, and γ S-Crys share a common aggregation-prone species that is recognized by α B-Crys chaperone

We have shown that the partially folded forms of all three human γ -crystallins, γ C, γ D and γ S, will aggregate during refolding when diluted out of denaturant into buffer (Fig. 2 and Fig. S2). Their aggregation propensity points to a common species populated during refolding that is aggregation-prone. This species is populated very early during dilution out of high concentrations of denaturant (Fig. 9).^{13: 45}

The lens is a protected environment lacking blood supply and with low metabolic activity, it is not clear what agents in that environment destabilize proteins, but the leading candidates are oxidative and photo-oxidative damage.²¹ Many of the crystallins found in insoluble inclusions from mature-onset cataractous lenses contain a host of covalent damages with deamidation of glutamine and asparagines, and oxidation of methionine and tryptophan residues being the most abundant.⁵⁶ Lampi and coworkers have shown that deamidation-mimicking changes at interface residues of β -crystallins lower protein stability and stabilize intermediates during equilibrium unfolding/refolding experiments.^{23: 57} In the case of human γ D-Crys, Flaugh *et al.*²² showed that the introduction of negative charges at the

interface in gln→glu deamidated mutants led to the preferential destabilization of the N-terminal domain as shown by the population of the folding intermediate at lower concentrations of denaturant in equilibrium unfolding/refolding experiments. γ -crystallins, with their four buried tryptophans, also absorb UV-radiation and photo-oxidized tryptophans have been reported in cataractous lenses.^{14-16; 21} These damages would reduce the kinetic barrier to unfolding, facilitating further unfolding of the protein and exposure of the aggregation-prone region in the C-terminal domain. Once this region is exposed, the high concentrations of γ -crystallins in the lens would favor second-order aggregation reactions over productive refolding.

Features of α B-Crys chaperone involved in substrate recognition and aggregation suppression *in vitro*

Small heat shock proteins are divided at the level of their primary structure into three distinct regions: the conserved α -crystallin and the variable N-terminal domain and C-terminal extension.⁴ All three regions contribute to and are important for chaperone function in this family of heat shock proteins.²⁸ In the case of α B-crystallin, Sharma *et al.*⁵⁸ have shown that residues 57-69 in the N-terminal domain and residues 93-107 in the α -crystallin domain of bovine α B-crystallin are involved in chaperoning interactions with alcohol dehydrogenase. Further, Ghosh *et al.*⁵⁹ using protein pin arrays identified seven regions in α B-Crys that were involved in chaperone-substrate interactions. Two regions were in the N-terminal domain, 4 regions in the α -crystallin domain and one region in the C-terminal extension. Two regions in the α -crystallin domain of α B-Crys interacted and prevented the thermal aggregation of β _H-crystallin.⁵⁹

Recently, Bagnris *et al.*⁶⁰ have solved the crystal structure of the α -crystallin domain of human α B-Crys to 2.9 resolution. They found that the homo-dimers formed an extended β -sheet with a shared groove. Based on known crystal structures of non-metazoan sHsps, they postulated that regions of the N-terminal of α B-Crys could bind to this groove in the full-length oligomers. They hypothesize that, in some vertebrate sHsps, disassembly of the oligomers into dimers could expose the groove and other pockets in the dimer where substrates, like the γ -crystallins, could bind during chaperoning interactions *in vivo*.

Many structural characteristics have been implicated in contributing to the chaperone function of α -crystallin. Hydrophobic regions in the N-terminal region of small heat shock proteins, for one, seem to play an important role.⁶¹ The α -crystallin domain and the C-terminal extension of sHsps are also important for chaperone function and interactions with the substrate.³⁸ Subunit-exchange and oligomeric state of α -crystallin are also important factors in chaperone activity.²⁸

Implications for cataract disease

The high molecular weight γ D— α B-Crys complexes formed during the early stage of the suppression reactions were stable after four days at 37C (Fig. 8). Given the incubation conditions, these proteins could have accumulated some non-enzymatic modifications (e.g. deamidation). However, as the SEC absorbance traces in Fig. 8 show, α B-Crys remained an oligomers during the four-day incubation period, which is an indication that the α B-Crys complexes remained stable despite the long incubation conditions. Nonetheless, these results show that α B-Crys forms stable complexes with aggregation-prone, partially folded species of γ D-Crys *in vitro*. Similarly, water-soluble, high molecular weight species from normal aged lenses also consist of α -crystallin and γ -crystallin and it has been suggested that these complexes form when α -crystallin binds destabilized γ -crystallin proteins.^{19; 62}

The finding that α -crystallin oligomers recognize and bind early, partially folded intermediates of the three human γ -crystallins is consistent with models of cataract formation from the aggregation of partially unfolded crystallins (Fig. 10). How these are generated in the protected environment of the lens remains to be determined, but multiple pathways of oxidative and photo-oxidative damage are all candidates for the etiology.^{16, 23, 57, 63} Since there is little protein degradation in lens fiber cells, and formation of high molecule aggregates needs to be avoided, it is not surprising that α -crystallin oligomers form long-lived complexes with their substrates. If this is true it may explain the age distribution of cataract, very rare below age of 50, then sharply increasing subsequently, reaching 50% of the population at age 80.⁶⁴ In fact, α -crystallins binding many molecules of partially folded and/or covalently damaged β - and γ -crystallins may themselves end up aggregating, thus contributing to the growth of the cataract in the aged lens. Analysis of the water-soluble fraction from normal and cataractous age-matched lenses shows that the percentage of water-soluble high molecular weight species, consisting mostly of α -crystallin along with β - and γ -crystallins, increases not only with age but also in cataractous lenses. It is believed that these complexes are the precursors to water-insoluble crystallin aggregates responsible for cataract.⁶⁵⁻⁶⁷

MATERIAL AND METHODS

Expression and Purification of human α B-Crys and γ -crystallins

Human γ D-Crys cloning into pQE.1 was previously described in Kosinski-Collins *et al.*¹³ Human γ S-Crys construct was previously described in Mills *et al.*¹² Double- (W130F/W156F and W42F/W68F γ D-Crys) and triple-Trp (W42only- and W130only γ D-Crys) mutants were previously described in Chen *et al.*¹⁴ These recombinant proteins were expressed by transforming pQE.1 plasmids into *E. coli* M15 [pREP4] cells (Qiagen). Both constructs contained an N-terminal His₆ tag. Proteins were purified following procedures described in Kosinski-Collins *et al.*¹³ using Ni-NTA (Qiagen) media for affinity chromatography. After purification, proteins were dialyzed into 10 mM ammonium acetate (pH 7.0) and stored at 4°C.

Human α B-Crys pAED4 plasmid was a generous gift from Dr. Jack Liang at Harvard Medical School. The wild-type α B-Crys containing plasmid was transformed into BL21 Gold (DE3) *E. coli* cells (Stratagene). Protein expression was induced by addition of IPTG and allowed to proceed over night at 18°C. Cells were harvested and stored at -80°C until future use. The α B-Crys purification protocol was modified from a previous protocol published by Horwitz and colleagues.⁶⁸ Modifications included performing all steps for cell lysis at 4°C. Two rounds of ion-exchange chromatography were performed using HiPrep 16/10 Q Sepharose FF column (GE Lifesciences) followed by size-exclusion chromatography (SEC) in Superose 6 10/300 GL (GE Lifesciences). Protein was stored in 50 mM sodium phosphate and 150 mM sodium chloride buffer, pH 7.0 (SEC Buffer). α B-Crys aliquots were dialyzed into 10 mM ammonium acetate and concentrated using 10 kDa MWCO Amicon Ultra-4 concentrators (Millipore) prior to use in aggregation suppression assays. All protein batches were tested for chaperone efficiency in aggregation suppression assays at 1:1 and 1:5 γ - α B ratios, with WT γ D-Crys as the substrate. Double tryptophan substitutions of residues 9 and 60 to phenylalanines were constructed using mutant primers (IDT-DNA) to amplify the α B-Crys gene in the pAED4 vector during site-directed mutagenesis. Amplified plasmid DNA was sequenced to confirm the substitutions (DNA Sequencing Facility, Massachusetts General Hospital). W9F/W60F α B-Crys was purified using the same procedures described above for the WT α B-Crys protein.

Wild-type human γ C-Crys pET-15b plasmid was transformed into BL21 (DE3) *E. coli* cells (Stratagene). The plasmid and purification protocol were also kindly provided by Dr. Jack

Liang 69. Briefly, protein expression was induced by addition of IPTG. Cells were lysed, centrifuged at high speeds, and the supernatant was subjected to two rounds of ammonium sulfate fractionation. The supernatant was centrifuged at high speeds and the pellet was resuspended in Lysis Buffer (50 mM Tris, 1 mM EDTA, 100 mM sodium chloride, pH 7.0). The resuspended pellet was run on a HiPrep 26/60 Sephacryl S-100 size-exclusion column connected to an AKTA FPLC (GE Lifesciences). Protein was concentrated to approximately 2 mg/ml and stored at 4°C until further use.

Protein concentrations were calculated using UV absorbance at 280 nm of unfolded proteins and the following extinction coefficients: 42,860 (γ D-Crys, γ S-Crys and γ C-Crys), 31,860 (W42F/W60F and W130F/W156F γ D-Crys), 26,735 (W42only and W130only γ D-Crys) and 13,980 (α B-Crys) $M^{-1}cm^{-1}$. 70: 71 Purity of all proteins mentioned herein was determined to be greater than 90% by SDS-PAGE.

Circular Dichroism Spectroscopy

WT α B-Crys was first incubated at a concentration of 100 μ g/ml in 10 mM sodium phosphate buffer (pH 7.0) for 1 hour at 37°C. Far-UV CD spectra were then collected from 198 to 250 nm in a 1 mm cuvette in an AVIV model 202 CD spectrometer (Lakewood, NJ). The temperature was kept at 37°C using an internal Peltier thermoelectric temperature controller. Mean residual ellipticity was calculated after buffer signal had been subtracted from spectra.

Fluorescence Emission Spectroscopy

Native WT α B-Crys was incubated at a concentration of 100 μ g/ml in Refolding Buffer (100 mM sodium phosphate, 1 mM EDTA, 5 mM DTT, pH 7.0) for 4 h at 37°C prior to collecting spectra. For the unfolded samples, α B-Crys was unfolded in Unfolding Buffer (100 mM sodium phosphate, 1 mM EDTA, 5 mM DTT, 5.0 M GdnHCl, pH 7.0) for 24 h at 37°C. The λ_{EX} was 295 nm (PMT voltage was 700 V) and the emission scans were collected from wavelengths 310–400 nm. The excitation and emission slit width were set to 10 nm and the temperature was kept at 37°C using a circulating water bath. For experiments comparing native WT and noTrp α B-Crys, the protein concentration was 20 μ g/ml, the λ_{EX} was 300 nm, the PMT voltage was 950V, and the slit widths were 10 nm (at 25°C).

For experiments comparing the fluorescence emission spectra of WT γ D-Crys—noTrp α B-Crys SEC fractions, the λ_{EX} was 300 nm and the emission scans were collected from wavelengths 316–400 nm (PMT voltage was 950 V). The excitation and emission slits were set to 10 nm and the temperature was kept at 25°C. These were the same conditions for experiments measuring the emission spectra of the W42only γ D—noTrp α B and W130only γ D—noTrp α B complex-containing fractions and their respective native and unfolded γ D-Crys controls. However, the high molecular weight peak fractions were pooled (1 ml total), concentrated to 250 μ l, and their fluorescence emission spectra were recorded with the λ_{EX} set to 300 nm. Fluorimeter settings were the same for experiments with W42F/W68F and W130F/W156F γ D-Crys except the PMT voltage was set to 700 V. WT, double- and triple-Trp γ D-Crys controls were fully unfolded in Unfolding Buffer for 24 h at 37°C and pre-equilibrated to 25°C prior to recordings. The native γ D-Crys controls were equilibrated to 25°C for 1 h in Refolding Buffer prior to recording spectra. Protein concentration for WT and double Trp γ D-Crys controls was 50 μ g/ml. The concentration for W42only γ D-Crys controls was 50 μ g/ml, and for W130only γ D-Crys controls was 25 μ g/ml.

For experiments showing the emission spectra for double-Trp γ D-Crys mutants at different concentrations of GdnHCl, the protein was unfolded in Unfolding Buffer with the matching concentration of GdnHCl for 24 h at 37°C. All fluorimeter settings were the same as

described above except the PMT voltage was set to 700 V. Protein concentration for both double-Trp mutants was 20 µg/ml. Fluorescence spectra for respective buffers were collected and subtracted from the sample spectra for all experiments described herein. Spectra were collected using a Hitachi F-4500 fluorescence spectrophotometer equipped with a circulating water bath. All experiments described in this section were repeated as three separate trials.

Aggregation and Aggregation Suppression Assays

For aggregation experiments where γ D-Crys was unfolded at intermediate GdnHCl concentrations, γ D-Crys was unfolded in Unfolding Buffer at the target concentration (2.49, 2.82, 3.02, and 5.03 M GdnHCl) and incubated at 37°C for 24 hours. Aggregation was initiated by diluting the GdnHCl concentration to 0.5 M with Refolding Buffer and thorough mixing of the samples. The final protein concentrations were the same for all samples (50 µg/ml). Light scatter was recorded as O.D._{350 nm}. All aggregation reactions were carried out at 37°C without continuous stirring.

For all aggregation suppression assays, unfolded γ -Crys was diluted ten-fold to initiate refolding and aggregation; α B-Crys chaperone was already added to the Refolding Buffer and equilibrated to 37°C. Samples were mixed thoroughly upon addition of Refolding Buffer. The final γ -Crys protein concentration was 100 µg/ml for assays with WT γ D-, γ C-, γ S- and double-Trp γ D-Crys protein substrates. The γ -Crys protein concentration was 50 µg/ml for assays with triple-Trp γ D-Cry as substrates. Final GdnHCl concentration for all assays described above was 0.5 M GdnHCl. Different mass-based ratios of γ -Crys to α B-Crys were used to assay the chaperone activity of the α -crystallin oligomers. Turbidity of the solutions was measured at 37°C without continuous stirring using a Cary 50 UV-Vis spectrophotometer.

For the early time point addition experiments, WT γ D-Crys was unfolded in Unfolding Buffer for 24 h at 37°C. Unfolded protein was added to constantly stirred Refolding Buffer ($\pm$ noTrp α B-Crys) at 37°C (dead time $\sim$ 1sec); unfolded protein was added 60 sec after O.D._{350 nm} measurements were started. Samples were stirred only for the first 30 sec after addition of γ D-Crys. For the 0 sec addition, α B-Crys chaperone had already been added to the Refolding Buffer, which had been equilibrated to 37°C for 1 h. For subsequent time point additions, γ D-Crys was added followed by addition of noTrp α B-Crys after 2, 6, or 10 sec. NoTrp α B-Crys had been equilibrated for 1 h at 37°C in 10 mM ammonium acetate buffer at pH 7.0. Data collection was not paused during addition of either γ D- or α B-Crys. Suppression reactions were performed at a 1 γ D:5 α B ratio and a γ D-Crys protein concentration of 50 µg/ml. All experiments described in this section were repeated as three separate trials.

Size-exclusion chromatography

Aggregation and aggregation suppression samples (0.5 ml) were collected after UV spectrophotometer measurements, incubated at 37°C for 2 h. Samples for double-Trp and triple-Trp γ D-Crys suppression experiments and early time point addition suppression experiments were incubated for 24 h at 37°C. One-hundred µl of 6X SEC Buffer (300 mM sodium phosphate, 900 mM sodium chloride, pH 7.0) were added and samples (500 µl) were filtered using Ultrafree-MC Centrifugal filter units with a 0.22 µm membrane (Millipore) and then equilibrated to 4°C. Samples were injected into a 0.5 ml Teflon loop (GE Lifesciences) and loaded onto a Superose 6 GL 10/300 (CV=24 ml, V_0 = 8 ml, GE Lifesciences) attached to an AKTA FPLC (GE Lifesciences). Chromatography runs were performed at a flow rate of 0.5 ml/min in SEC Buffer (50 mM sodium phosphate, 150 mM sodium chloride, pH 7.0, degassed) at 4°C. For the native mixture samples, native γ -Crys at

100 µg/ml and native αB-Crys at 500 µg/ml were mixed together and incubated at 37°C for 2 h in Refolding Buffer with 0.5 M GdnHCl.

For experiments assaying the lifetime of the γD—αB-Crys complexes, γD-Crys was unfolded in Unfolding Buffer for 24 h at 37°C. Aggregation was initiated by ten-fold dilution of the unfolded protein solution with Refolding Buffer plus αB-Crys (ratio was 1 γD:5 αB) at 37°C; the final protein concentration for γD-Crys was 100 µg/ml and the final GdnHCl concentration was 0.5 M. The final volume for this reaction was 5.0 ml and the sample was left at 37°C in a Nutator shaker (BD Diagnostics) for 4 days. Aliquots (0.5 ml) were removed, 100 µl of 6X SEC Buffer were added, samples were filtered using Ultrafree-MC Centrifugal units with a 0.22 µm filter and equilibrated at 4°C. Filtered samples were loaded onto a Superose 6 GL SEC column equilibrated in SEC Buffer and run at a flow rate of 0.5 ml/min at 4°C. All experiments described in this section were repeated as three separate trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

AU	Arbitrary Units
sHsp	small heat shock protein
Crys	crystallin
γD-Crys	γD-crystallin
DTT	dithiothreitol
WT	wild-type
Trp	tryptophan
SEC	size-exclusion chromatography
GdnHCl	guanidinium hydrochloride

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Fig. 1.

Purification and structural characterization of recombinant Human α B-Crys. Human WT α B-Crys was expressed and purified from *E. coli* cells (see Experimental Procedures). A) Representative size-exclusion chromatogram showing the final, polishing step of the purification procedure. WT α B-Crys eluted as a single peak (from 11 to 14.5 ml) with apparent molecular weight between 500-600 kDa. B) Fractions corresponding to the α B-Crys elution peak were run in a 14% SDS-PAGE gel. C) Far-UV CD spectra (198-250 nm) of native α B-Crys at pH 7.0 and 37°C. D) Fluorescence emission spectra of native (●) and unfolded (●) α B-Crys at 37°C. Protein concentration for far-UV CD and fluorescence emission measurements was 100 μ g/ml.

Fig. 2.

Suppression of the aggregation of partially folded WT γ D-, γ S- and γ C-crystallins by α B-Cryst chaperone. A) γ D-Cryst, B) γ S-Cryst, and C) γ C-Cryst aggregate during refolding out of high concentrations of GdnHCl at a protein concentration of 100 μ g/ml and a final GdnHCl of 0.5 M. The suppression reactions were done at 1 γ :1 α B and 1 γ :5 α B ratios.

Fig. 3.

Size-exclusion chromatograms for α B-Crys and γ D-, γ C-, or γ S-Crys suppression reactions.

A) 1 γ D:5 α B suppression reaction (—) and 1 γ D:5 α B mixture of native proteins (—) chromatograms are shown. B) 1 γ C:5 α B suppression reaction (—) and 1 γ C:5 α B mixture of native proteins (—) chromatograms are shown. C) 1 γ S:5 α B suppression reaction (—) and 1 γ S:5 α B mixture of native proteins (—) chromatograms are shown. The suppression reactions shown in Fig. 2 were applied to a Superose 6 size-exclusion column (for the native mixture samples' preparation see Materials and Methods) Arrows correspond to elution volume of standards. Numbers represent standards' molecular weights in kDa.

Fig. 4.

Aggregation of partially unfolded γ D-Crys during refolding. 1 γ D:0 α B reactions for γ D unfolded at GdnHCl concentrations encompassing the intermediate to unfolded protein transition. γ D was unfolded at 2.49, 2.82, 3.02, or 5.03 M GdnHCl for 24 h at 37°C to ensure equilibrium conditions. Protein was refolded by diluting the GdnHCl concentration to 0.5 M at 37°C. The final concentration protein concentration was 50 μ g/ml for all samples.

Fig. 5.

Tryptophan fluorescence of γ D-Crys in complex with α B-Crys chaperone. Tryptophan

residues at positions 9 and 60 in α B-Crys were substituted to phenylalanines. A) Fluorescence emission spectra for native WT α B-Crys (●) and W9F/W60F α B-Crys (●) at 25°C. λ_{EX} was set to 300 nm.

B) W9F/W60F α B-Crys suppressed the aggregation of γ D-Crys under the same refolding and aggregation described previously (see Materials and Methods). Final γ D-Crys concentration was 100 μ g/ml and final GdnHCl concentration was 0.5 M at 37°C.

C) A high molecular weight complex could be isolated from the 1 γ D:5 noTrp α B suppression reactions during SEC. The volume highlighted by the gray bar corresponds to the fraction collected for fluorescence measurements.

D) Relative fluorescence emission spectrum of the fraction containing the γ D— α B complex (●). For comparison purposes, the fluorescence emission spectrum of native (○) and fully unfolded (●) WT γ D-Crys (20 μ g/ml) are shown. The λ_{EX} was set to 300 nm for all samples. Traces were normalized to the fluorescence intensity of the maximum λ_{EM} for unfolded γ D-Crys.

Fig. 6.

Tryptophan fluorescence spectra from double-Trp γ D-Crys mutants in complex with α B-Crys chaperone. A) Crystal structure of γ D-Crys (PDB: 1HK0) showing Trp pairs conserved within the N-terminal domain (W130F/W156F γ D-Crys) or the C-terminal domain (W42F/W68F γ D-Crys).

B) Relative fluorescence emission spectra of W130F/W156F γ D-Crys unfolded at different concentrations of GdnHCl. W130F/W156F γ D-Crys (20 μ g/ml) was incubated in Refolding buffer with 0, 1.6, 2.0, 2.6 and 5.1 M GdnHCl for 24 h at 37°C. Fluorescence emission spectra were recorded using a λ_{EX} set to 300 nm. All traces were normalized to the fluorescence intensity of the max. λ_{EM} for W130F/W156F γ D-Crys unfolded at 5.1 M GdnHCl.

C) Relative fluorescence emission spectra of W42F/W68F γ D-Crys unfolded at different concentrations of GdnHCl. W42F/W68F γ D-Crys (20 μ g/ml) was incubated in Refolding buffer with 0, 2.3, 2.6, 3.1 and 5.2 M GdnHCl for 24 h at 37°C. Fluorescence emission spectra were recorded using a λ_{EX} set to 300 nm. All traces were normalized to the fluorescence intensity of the max. λ_{EM} for W42F/W68F γ D-Crys unfolded at 5.2 M GdnHCl.

D) SEC chromatograms for 1 γ D:5 noTrp α B suppression reactions using W130F/W156F (—) or W42F/W68F (—) γ D-Crys as substrates. The shaded area on the trace indicates the fraction used for fluorescence measurements.

E) Relative fluorescence emission spectrum of the fraction containing the γ D- α B complex from 1 W130F/W156F γ D: 5 noTrp α B suppression reaction (●). For comparison purposes, the fluorescence emission spectrum of native (○) and fully unfolded (●) W130F/W156F γ D-Crys (50 μ g/ml) are shown. The λ_{EX} was set to 300 nm for all samples. Traces were normalized to the fluorescence intensity of the maximum λ_{EM} for unfolded γ D-Crys.

F) Relative fluorescence emission spectrum of the fraction containing the γ D- α B complex isolated from 1 W42F/W68F γ D: 5 noTrp α B suppression reaction (●). The λ_{EX} was set to

300 nm for all samples and the protein concentration for the native (○) and fully unfolded (●) W42F/W68F γ D-Crys protein samples was 50 μ g/ml. Traces were normalized as described above.

Fig. 7.

Fluorescence emission spectra of triple-Trp γ D-Crys mutants in complex with α B-Crys. A) Relative fluorescence emission spectra for W42only γ D-Crys in complex with noTrp α B-Crys (●). B) Fluorescence emission spectra for W130only γ D-Crys in complex with noTrp α B-Crys (●). Spectra for their respective native (○) and unfolded (●) controls is also shown (protein concentration for W42only γ D-Crys was 50 μ g/ml and for W130only γ D-Crys was 25 μ g/ml). The λ_{EX} was 300 nm and the traces were normalized as described previously in Figure 6. Complexes were isolated from 1 γ D:5 α B suppression reactions in which the final concentration of γ D was 50 μ g/ml.

Fig. 8.

SEC chromatograms of α B— γ D complexes at different time points after suppression reaction. SEC chromatograms of 0.5 ml aliquots removed from a 1 γ D:5 α B suppression reaction incubated at 37°C with continuous agitation. Time points at which aliquots were collected are shown on the chromatogram. The peak that eluted at 7.6 ml is the designated high molecular weight complex and eluted in the void of the column (V_0 = 8 ml). The peak that eluted at 13.5 ml corresponded to wild-type α B-Crys. Inset shows the high molecular weight peak that eluted at 7.6 ml in detail.

Fig. 9.

Addition of α B-Crys at very early time points after initiation of refolding and aggregation of partially folded WT γ D-Crys. A) O.D. $_{350\text{nm}}$ data for no addition of α B-Crys (1 γ D:0 α B ratio) and addition 0, 2, 6, and 10 sec after WT γ D-Crys was added to Refolding buffer (1 γ D:5 α B ratio). Buffer was constantly stirred with a magnetic spin bar and γ D-Crys was added 60 sec after light scattering data collection was initiated. Samples were allowed to mix for the first 30 sec after γ D-Crys addition. B) Light scattering data presented in (A) showing traces from 50-100 sec after recordings were initiated. C) SEC traces for suppression reactions shown in (A). Samples were loaded onto a Superose 6 SEC column. The fraction corresponding to the γ D— α B complex was collected for fluorescence emission measurements (gray bar). D) Raw fluorescence emission spectra for fractions highlighted in (C) and containing the γ D— α B complex formed during addition of α B-Crys 0, 2, 6, and 10 sec after addition and initiation of γ D-Crys refolding and aggregation. λ_{EX} was set to 300 nm.

Fig. 10.

A molecular model for age-related cataract formation in the lens. Environmental stresses could lead to covalent damage, such as deamidation or photo-oxidation, to the crystallins. This damage could destabilize γ -crystallins, which are otherwise very stable, populating aggregation-prone species (I^*). α -crystallin would sequester such species in younger individuals (green arrow). But as we age, the finite levels of free α -crystallin in the mature lens fibers will be diminished leading to aggregation (orange arrows).