

Function and Aggregation in Structural Eye Lens Crystallins

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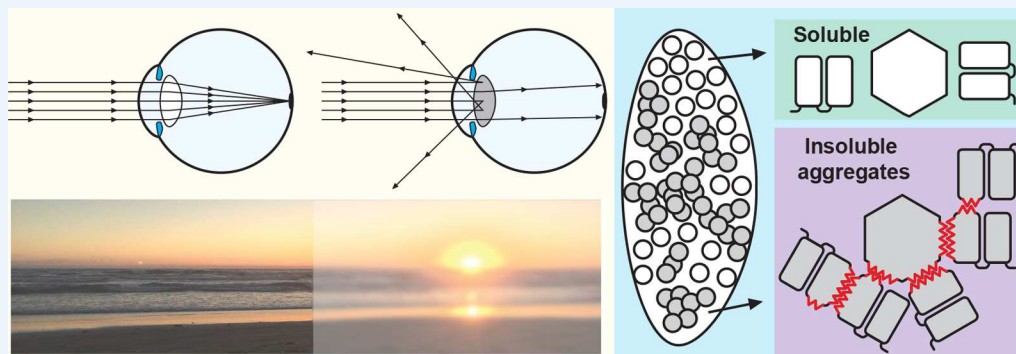
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CONSPECTUS: Crystallins are transparent, refractive proteins that contribute to the focusing power of the vertebrate eye lens. These proteins are extremely soluble and resist aggregation for decades, even under crowded conditions. Crystallins have evolved to avoid strong interprotein interactions and have unusual hydration properties. Crystallin aggregation resulting from mutation, damage, or aging can lead to cataract, a disease state characterized by opacity of the lens.

Different aggregation mechanisms can occur, following multiple pathways and leading to aggregates with varied morphologies. Studies of variant proteins found in individuals with childhood-onset cataract have provided insight into the molecular factors underlying crystallin stability and solubility. Modulation of exposed hydrophobic surface is critical, as is preventing specific intermolecular interactions that could provide nucleation sites for aggregation. Biophysical measurements and structural biology techniques are beginning to provide a detailed picture of how crystallins crowd into the lens, providing high refractivity while avoiding excessively tight binding that would lead to aggregation.

Despite the central biological importance of refractivity, relatively few experimental measurements have been made for lens crystallins. Our work and that of others have shown that hydration is important to the high refractive index of crystallin proteins, as are interactions between pairs of aromatic residues and potentially other specific structural features.

This Account describes our efforts to understand both the functional and disease states of vertebrate eye lens crystallins, particularly the γ -crystallins. We use a variety of biophysical techniques, notably NMR spectroscopy, to investigate crystallin stability and solubility. In the first section, we describe efforts to understand the relative stability and aggregation propensity of different γ S-crystallin variants. The second section focuses on interactions of these proteins with the holdase chaperone α B-crystallin. The third, fourth, and fifth sections explore different modes of aggregation available to crystallin proteins, and the final section highlights the importance of refractive index and the sometimes conflicting demands of selection for refractivity and solubility.

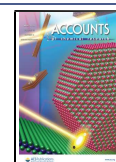
■ INTRODUCTION

The crystallins of the vertebrate eye lens are structural proteins that maintain the transparency and high refractive index of the lens, enabling it to focus light on the retina. Because of the unique functional requirements of this tissue, the fiber cells elongate and lose their organelles during development. The resulting mature fiber cells contain exceptionally high concentrations of crystallin proteins, comprising nearly 90% of the dry weight of the human lens.¹ Lens crystallins must function throughout an organism's lifetime, despite frequent exposure to UV light, which can cause irreversible and deleterious photochemistry. As demonstrated by King and

co-workers, the ability of $\beta\gamma$ -crystallins to resist photoinduced damage depends on two highly conserved pairs of Trp residues, one in each domain.² Mutating these key Trp residues to Phe reduces the UV absorption of these proteins but greatly increases their aggregation propensity upon UV

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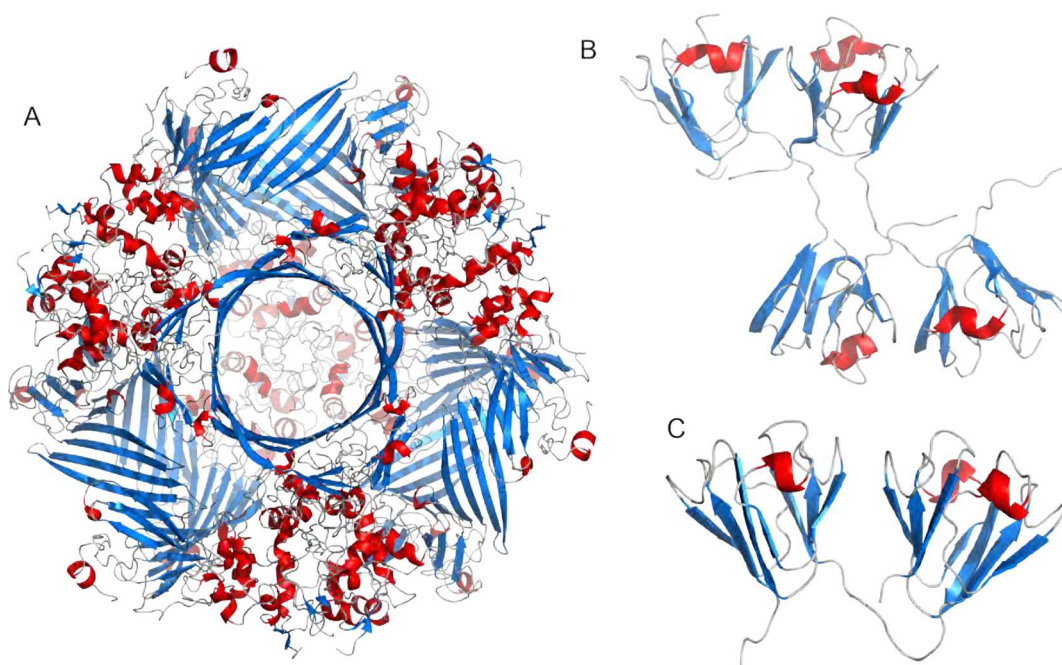


Figure 1. Representative structures of the three major crystallin types in the vertebrate eye lens. (A) Human α B-crystallin multimer (PDB ID 2YGD).¹³ (B) Bovine β B1-crystallin dimer (PDB ID 1BLB).¹⁴ (C) Homology model of monomeric human γ S-crystallin based on the murine structure (PDB ID 1ZWO).¹⁵

irradiation.³ The effect is especially pronounced for mutation of the more strongly quenched Trp residues, suggesting a photoprotective quenching mechanism involving multiple tryptophans.⁴ Under UV irradiation, Trp residues can also undergo photochemistry to produce products such as kynurenine, which provide additional UV filtering in the lens⁵ but can accelerate oxidative damage when covalently attached to crystallin proteins.⁶ In addition to their obvious biomedical relevance, the extraordinary biophysical properties of the lens crystallins make them a fertile ground for investigating the fundamentals of protein solubility and aggregation. Many studies in this topic space have focused on particularly aggregation-prone proteins, but understanding these phenomena also requires detailed studies of proteins that resist aggregation, even under difficult conditions.

In contrast to most proteins, which have turnover half-lives as short as minutes for ornithine decarboxylase⁷ or up to a month for some immunoglobulins,⁸ the crystallins are not replaced after their initial expression during early development because of the loss of organelles in the lens fiber cells. This extraordinary longevity places lens crystallins, along with most other proteins in the lens,⁹ in a unique class known as extremely long-lived proteins (ELLPs). Other well-known examples are collagen and elastin, the structural proteins of the extracellular matrix, which form a cross-linked polymeric structure, and the scaffold proteins of the nuclear pore complex, which exist within massive cellular machinery structures.¹⁰ Compared with other ELLPs, the lens α - and β -crystallin oligomers are relatively small, while the γ -crystallins are monomeric. In the crowded lens fiber cells, crystallin–crystallin interactions are critical for maintaining solubility, as relatively few types of proteins are present. Surprisingly, the attractive interactions of the γ -crystallins¹¹ do not lead to aggregation despite their high concentration, longevity, negligible protein turnover, and frequent exposure to damaging UV radiation. The failure of this system causes crystallin

aggregation, which is central to the etiology of cataract, the leading cause of blindness worldwide and a World Health Organization priority eye disease.¹²

Vertebrate lens crystallins belong to two major families, α and $\beta\gamma$. The two are grouped together on the basis of their common function rather than structural similarity, as they have different folds and separate evolutionary histories. Representative structures of each type are shown in Figure 1. The α -crystallins (Figure 1A) are small heat shock proteins that function as holdase chaperones, which bind to damaged structural proteins but lack ATPase activity and therefore cannot refold them. In addition to the lens, they are expressed in several different tissues, including heart, brain, and kidney.¹⁶ Structurally, α -crystallins are composed of mostly unstructured N- and C-terminal domains flanking a 90-residue immunoglobulin-like α -crystallin domain. Monomers come together to form hetero- and homodimers, which serve as the building blocks for high-molecular-weight complexes. These complexes are dynamic and heterogeneous, with the most common species ranging in size from 24-mers to 33-mers.^{13,17–19} α A-Crystallins form smaller oligomers assembled from tetrameric subunits, with associations mediated by cross-dimer domain swapping. Unlike α B-crystallin, which has no cysteines, this protein can transfer disulfide bonds to aggregation-prone client proteins to help keep them in solution,²⁰ a role that is complementary to the function of glutathione as the primary redox buffer of the lens. Binding interactions with both α A- and α B-crystallin can also proceed via contact of exposed hydrophobic surfaces on the client protein with specific regions of the chaperone. Subsequent analysis and characterization of these α -crystallin peptide sequences, named mini- α A/B-crystallin (MaAC/MaBC), have shown that they retain chaperone activity in vitro.²¹

The lens β - and γ -crystallins have evolved from an ancestral Ca^{2+} -binding protein.²² Members of this family are characterized by the $\beta\gamma$ -crystallin domain, a β -sandwich composed of

a double Greek key motif containing four antiparallel strands within each sheet. A conserved feature of the $\beta\gamma$ -crystallin superfamily is a (F/Y/W)-x-x-x-x-(F/Y)-x-G sequence found across the first β -hairpin of each Greek key. The aromatic side chains in this motif participate in π -stacking interactions, while the glycine is capable of adopting unconventional backbone torsion angles. Lens β - and γ -crystallins have in common two characteristic $\beta\gamma$ -crystallin domains, but there are several important differences as well: β -crystallins (Figure 1B) are typically dimeric and have longer interdomain linkers and N- and C-terminal extensions than the more compact, monomeric γ -crystallins (Figure 1C). Both the structure of the $\beta\gamma$ -crystallin fold and the two-domain architecture of the structural lens crystallins are important for their stability. Hydrophobic residues are almost exclusively packed into the core of the β -sandwich in each domain and at the interdomain interface. Externally, the γ -crystallin surface is a patchwork of acidic, basic, and neutral polar residues that form a highly interconnected network of hydrogen bonds and salt bridges. Interspersed internally and externally are pairs and collections of aromatic residues that form π - π -stacking interactions. Systematic removal of key residues has shown that each contributes to protein stability but that modifications of N-terminal residues result in greater destabilization than the equivalent C-terminal domain mutations.²³ The two domains are structurally very similar, though they are not identical in sequence; the interdomain interface is also critical for maintaining stability.²⁴

This Account focuses on our efforts to understand the molecular factors underlying the extraordinary stability and solubility of the crystallin proteins. We have used a variety of biophysical techniques to elucidate the molecular determinants of human crystallin solubility and aggregation.

■ IDENTIFYING KEY SEQUENCE DETERMINANTS OF γ S-CRYSTALLIN STABILITY

One way to separate the effects of folding and intermolecular interactions on protein aggregation is to investigate variant proteins with reduced solubility or stability. Surprisingly, many crystallin mutations causing autosomal dominant cataract result in proteins that are fully folded at room temperature but have reduced thermal stability and aggregate at physiological temperatures, either alone or in mixed precipitates with one or more α -crystallins. These known mutations provide a clear starting point; however, it is essential to test hypotheses about the molecular basis of protein solubility using site-directed mutagenesis.

We began by studying the G18V variant (γ S-G18V), on the basis of its role in childhood-onset cataract,²⁵ and the replacement of a highly conserved glycine in a tight turn with a bulky hydrophobic residue. We then designed the symmetry-related variant γ S-G106V, which occupies the homologous position in the C-terminal domain, in order to determine whether the increased aggregation propensity is due to the G to V mutation per se or structural factors specific to the N-terminal domain. This comparison would elucidate whether the reduced solubility of G18V is due to gross misfolding, altered intermolecular interactions due to exposure of a previously buried hydrophobic residue, or more subtle structural effects necessitating further characterization.

Our experimental results and molecular dynamics simulations performed by our collaborators demonstrated that these two superficially similar mutations have quite different effects

on folding stability and solubility.²⁶ At room temperature, comparison of the circular dichroism (CD) spectra of wild-type γ S-crystallin (γ S-WT), γ S-G18V, and γ S-G106V indicated that all three are folded, with a primarily β -sheet secondary structure. However, interesting differences became apparent upon heating. Although both variants are less stable than γ S-WT, γ S-G106V is substantially less stable than the disease-related γ S-G18V. Monitoring the aggregates that form using dynamic light scattering (DLS) gave the opposite result: γ S-G18V is by far the more aggregation-prone variant. This result was somewhat surprising because in many discussions of protein misfolding and aggregation, a decrease in thermodynamic stability is used as a proxy for increased aggregation propensity. Along the way to structure determination, NMR chemical shifts indicated that γ S-G18V has significant conformational differences in much of the N-terminal domain,²⁷ whereas the effects of γ S-G106V are limited to the area directly surrounding the mutation site (Figure 2).²⁸ An increase in conformational flexibility was also recently observed for aggregation-prone γ S-crystallin variants mimicking deamidation in age-related cataract.²⁹

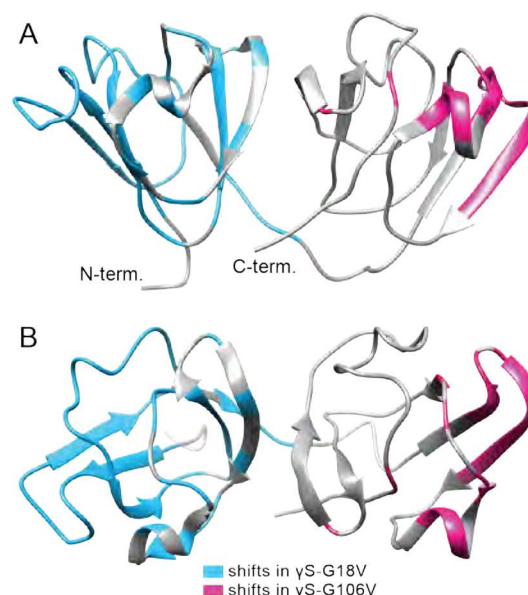


Figure 2. (A) “Front” and (B) “top” views of γ S-WT (PDB ID 2M3T), indicating that the residues that have significantly different chemical shifts in γ S-G18V (light blue) and γ S-G106V (magenta) relative to γ S-WT. NMR chemical shift perturbations are a sensitive probe of the local chemical environment and hence serve as a reporter for local changes in conformation. Adapted from ref 28. Copyright 2013 American Chemical Society.

Contrary to initial hypotheses, the solution NMR structures of γ S-WT and γ S-G18V (Figure 3A) revealed that the first 17 residues of γ S-G18V are largely unaffected by the mutation. V18, at odds with the molecular dynamics simulations, the original hypotheses, and conventional wisdom regarding backbone torsion angles, remains in almost the same position as G18 in γ S-WT (Figure 3B). Instead of being exposed to solvent, the V18 side chain remains buried, wedged tightly between two β -strands. As a result, the amide proton of R19 is locked between the V18 terminal methyl groups, avoiding A-1,3 strain and resulting in a drastic difference in the dihedral angles of R19 relative to the wild type (Figure 3C). This 180°

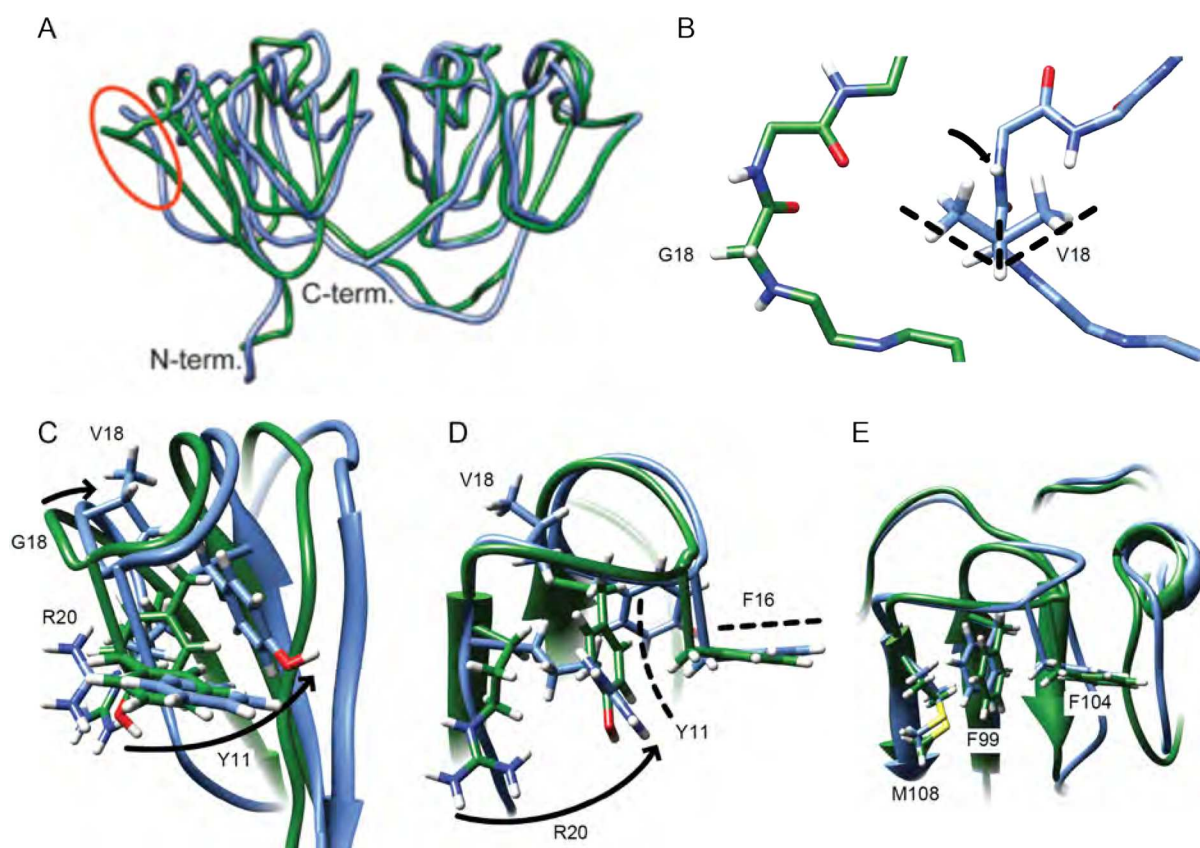


Figure 3. Ribbon diagrams showing the structural differences between γ S-WT (green) and γ S-G18V (blue). (A) Overlay of the two structures. (B) The backbone near the mutation site undergoes subtle structural changes. In γ S-G18V, the backbone dihedral angles for residue 18 occupy a disallowed region for non-glycine residues. In γ S-WT, the G18 torsion angles are $\phi = 73.7^\circ$, $\psi = 163.0^\circ$, while for V18 in γ S-G18V they are $\phi = 129.8^\circ$, $\psi = 134.3^\circ$. (C) The addition of V18 and its effect on the backbone angles the β -strand outward. The torsion angles of R20 shift from $\phi = -58.6^\circ$, $\psi = -177.2^\circ$ in γ S-WT to $\phi = -110.2^\circ$, $\psi = -17.9^\circ$ in γ S-G18V. (D) R20 displaces Y11 and forces the phenol ring away from F16 and against the β -strands. Here R20 appears to also adopt the same highly conserved perpendicular ring orientation with F16 that Y11 occupies in γ S-WT, possibly forming a stabilizing interaction. (E) C-terminal outer corner loop region of γ S-WT and γ S-G18V. F99, F104, and M108 side chains are shown. M108 lacks a planar side-chain group to replace F99 in the perpendicular ring motif, making a similar stabilizing rearrangement impossible in γ S-G106V. Panels A–D adapted with permission from ref 30. Copyright 2013 Cell Press.

rotation of the ψ angle of R19 of γ S-G18V disrupts the R20–C23 β -strand, causing a conformational change that is propagated throughout the backbone of the N-terminal domain. In γ S-G18V, the R20 C β is rotated inward, which in turn displaces Y11. In γ S-WT, this residue participates in an edge–face π -stacking interaction with F16. In γ S-G18V, the planar guanidinium group from R20 replaces the ring of Y11 in a π -stacking interaction stabilizing the first β -strand and corner loop (3D). This stabilizing rearrangement is not available to γ S-G106V: the three corresponding residues in the C-terminal domain are F99, F104, and M108 (Figure 3E). M108 is unable to participate in a similar stability-enhancing π -stacking interaction because it lacks a planar side-chain group containing delocalized π electrons. This suggests that the outer corner loop of the C-terminal domain is disrupted as a result of the G106V mutation, leading to a hypothesis about why γ S-G106V is thermally less stable than both γ S-WT and γ S-G18V.

Additional variants (γ S-G18V/R20A, γ S-G18V/R20M, and γ S-G106V/M108R) were constructed to test our hypotheses about how structural factors affect stability. CD and DLS measurements were performed as in our previous studies of γ S-crystallin variants.²⁶ Table 1 summarizes the midpoint temperatures of unfolding (T_m) for all of the variants studied;

Table 1. Thermal Unfolding Temperatures for γ S-Crystallin Variants

protein variant	T_m ($^\circ\text{C}$)
γ S-WT	72.0 ± 0.1
γ S-G18V	63.6 ± 0.1
γ S-G106V	58.9 ± 0.1
γ S-G18V/R20A	67.1 ± 0.1
γ S-G18V/R20M	63.1 ± 0.1
γ S-G106V/M108R	62.7 ± 0.1

the thermal unfolding curves are shown in Figure 4A,B. The M108R substitution confers stability to γ S-G106V as expected: the T_m of γ S-G106V/M108R is 62.7°C , which is several degrees higher than that of γ S-G106V and similar to that of γ S-G18V. Unexpectedly, the substitution of R20 with alanine increases the stability of γ S-G18V, while substituting it with methionine has little effect on the stability. Although substituting M108 with arginine does restore the thermal stability of γ S-G106V to the same level as γ S-G18V, the data for the variants with mutations in the N-terminal domain suggest that R20 does not confer stability to the N-terminal domain of γ S-G18V in the manner hypothesized above: when it is removed, the resulting structures are not destabilized.

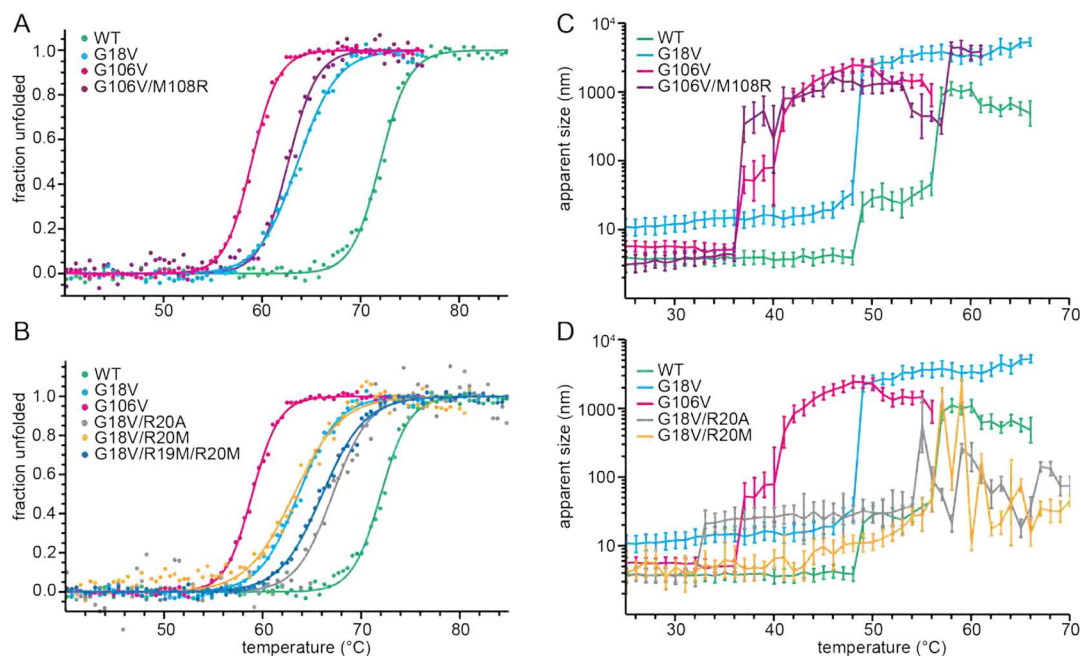


Figure 4. (A) Thermal unfolding data for variants of human γ S-crystallin: γ S-WT (green), γ S-G18V (light blue), γ S-G106V (magenta), and γ S-G106V/M108R (purple). Unfolding was measured by monitoring the CD signal at 218 nm; lines are best-fit unfolding curves. Replacing M108 with R accounts for nearly the entire stability difference between γ S-G18V and γ S-G106V. (B) Thermal unfolding data for γ S-WT (green), γ S-G18V (light blue), γ S-G106V (magenta), γ S-G18V/R20A (orange), γ S-G18V/R20M (gray), and γ S-G18V/R19M/R20M (dark blue). Contra the initial hypothesis, replacing R20 with M has little effect on the stability, while replacing R20 with A or R19 and R20 with M increases the stability relative to γ S-G18V. (C) Thermal aggregation measured using DLS for the γ S-crystallin variants described in (A). As previously described, G18V is the most aggregation-prone.²⁶ The aggregation propensity of γ S-G106V/M108R is higher than that of γ S-G106V; their aggregation onset temperatures are similar, but γ S-G106V/M108R forms larger aggregates. (D) DLS measurements during thermal aggregation for the γ S-crystallin variants described in (B). Overall, these data illustrate that the relationship between thermal stability and aggregation propensity is not easily predictable.

To gauge the effects these mutations have on the aggregation propensity of γ S-crystallin, the formation of thermally induced aggregates was also monitored by DLS, and the results are shown in Figure 4C,D. In contrast to the stability results, the M108R mutation of γ S-G106V increases the overall aggregation propensity of the protein. In γ S-G106V, intermediate-sized aggregates are formed at 36 °C, whereas γ S-G106V/M108R instead forms large aggregates directly. Unlike γ S-G18V, which often forms oligomers below 30 °C, both γ S-G18V/R20A and γ S-G18V/R20M begin as monomers at 25 °C. At 32 °C, γ S-G18V/R20A forms intermediate-sized aggregates before developing much larger aggregates at 54 °C. γ S-G18V/R20M gradually forms intermediate aggregates near 42 °C that grow rapidly beginning at 56 °C.

Overall, observation of the thermal denaturation of these γ S-crystallin variants does not support the hypothesis that R20 provides extra stability to the N-terminal domain of γ S-G18V: when the functionality of R20 was removed, the resulting variants experienced an increase in thermal stability. However, as in the initial set of experiments, the aggregation propensity of these γ S-crystallin variants does not follow the same trend as their thermal stabilities. Replacing R20 with either A or M decreases the aggregation propensity of γ S-G18V, whereas replacing M108 with R in the corner loop of the C-terminal domain of γ S-G106V leads to the elimination of intermediate aggregate formation in favor of larger aggregates of γ S-G106V/M108R at a higher temperature. It appears that while R20 participates in a favorable interaction, it also locks the N-terminal domain of γ S-G18V in a conformation that promotes self-aggregation. These results support the idea that

aggregation propensity is not correlated to thermal stability in a straightforward manner.

Instead, we hypothesize that a major driver of room-temperature aggregation in γ S-G18V appears to be the solvent exposure of the previously buried residues C23, C25, and C27 (Figure 5), which allows the formation of intermolecular disulfides as a starting point for aggregation. This is consistent with observations in the literature for both γ S- and γ D-crystallin: intramolecular disulfide bonding apparently traps the W42R and W42Q variants of γ D-crystallin in a partially misfolded conformation that promotes aggregation,³¹ while intermolecular disulfide bond formation is essential for dimer formation in γ S-WT.³² In γ S-G18V, this type of disulfide bonding may be facilitated by the increased exposure of C23, C25, and C27 relative to their positions in γ S-WT. Of course, dimerization is only the first step in aggregation, and detailed solid-state NMR measurements, such as those performed for the native-like aggregates of γ D-P23T,³³ are needed to elucidate the structural basis for the disease state.

■ RECOGNITION OF AGGREGATION-PRONE CLIENTS BY THE HOLDASE CHAPERONE α B-CRYSTALLIN

Point mutations are frequently assumed to have only a very local effect on protein structure; our work and that of other groups have indicated that this is often not the case for crystallins.³⁵ As discussed in the previous section, the relatively minor conformational change caused by the G18V mutation nevertheless propagates down the polypeptide chain through more than half of the N-terminal domain.³⁰ In collaboration

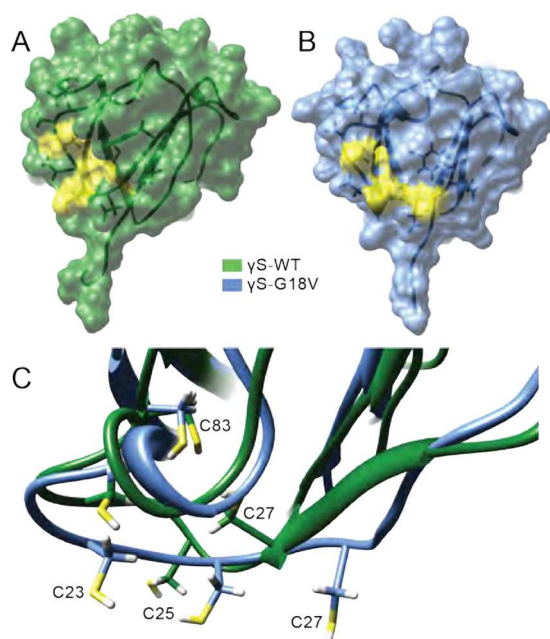


Figure 5. Molecular surfaces of (A) γ S-WT (PDB ID 2M3T) and (B) γ S-G18V (PDB ID 2M3U)³⁴ showing the exposure of additional cysteines C23, C25, and C27 (yellow) in γ S-G18V. (C) Overlaid ribbon structures of γ S-WT (green) and γ S-G18V (blue), showing the positions of the relevant cysteine side chains.

with Oschkinat and co-workers, we performed solution-state NMR experiments to identify the particular surface of G18V that interacts with the molecular chaperone α B-crystallin. This protein binds to misfolded structural crystallins in the lens and keeps them in solution but is unable to refold them. In this role, it binds only weakly and transiently to wild-type γ S, even under heat stress (Figure 6A,B). In contrast, it binds γ S-G18V strongly and specifically (Figure 6C,D). To characterize the interactions between α B and γ S, heteronuclear single-quantum coherence (HSQC) spectroscopy was performed on mixtures of 15 N-labeled γ S-WT and γ S-G18V with natural-abundance α B. Because NMR spectroscopy is sensitive to small changes in the electronic environment of the detected nuclei, binding interactions between α B and γ S result in shifts or disappearances of relevant cross-peaks representing specific N–H moieties, providing exquisitely specific information about the interaction in solution. To our knowledge, this was the first binding interface between α B-crystallin and an aggregation-prone structural crystallin to be characterized.

We also directly probed the exposed hydrophobic surface on γ S-G18V that is recognized by α B-crystallin. In a concerted experimental and computational study performed with the Tobias group, we explored the binding of γ S-WT and γ S-G18V to the fluorescent dye 8-anilidonaphthalene-1-sulfonic acid (ANS). Binding of this dye, measured via an increase in fluorescence intensity relative to the unbound state, serves as an imperfect but useful proxy for exposed hydrophobic surface area. Although ANS fluorescence provides quantitative information about hydrophobic surface exposure, it lacks molecular-level detail. Therefore, we investigated specific binding sites using NMR chemical shift perturbations and docking simulations performed by Tobias and co-workers, yielding a detailed picture of how the dye interacts with the protein. We then tied these results back to α -crystallin binding by demonstrating that ANS competitively interferes with the

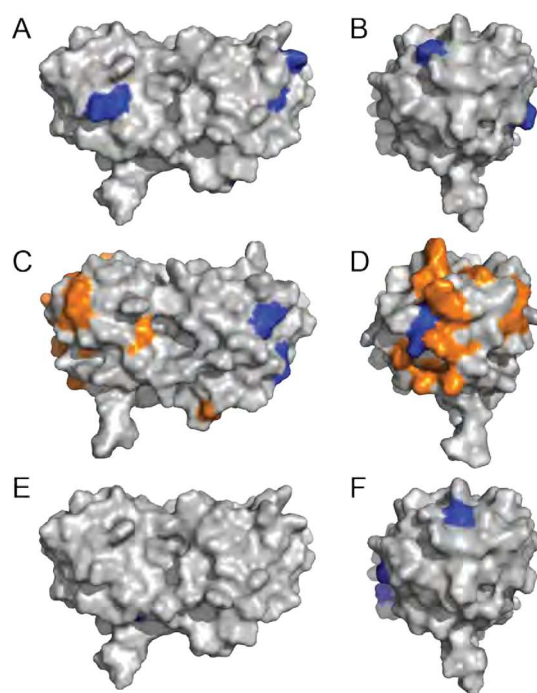


Figure 6. Surfaces of γ S-WT colored to show residues involved in interactions with α B-crystallin, as measured by solution-state NMR spectroscopy. Two views are shown: (A) “front” and (B) with the N-terminal domain rotated forward. Noninteracting residues are shown in gray, while those involved in weak, transient interactions are shown in blue. (C) and (D) show the same views of γ S-G18V with noninteracting residues in gray, weakly interacting residues in blue, and strongly interacting residues in orange. (E) and (F) show the same views of γ S-G18A with noninteracting and weakly interacting residues shown using the same color coding. Panels A–D adapted with permission from ref 30. Copyright 2013 Cell Press. Panels E and F generated from data reported by Sprague-Piercy et al.³⁶

α B- γ S-G18V interaction.³⁷ In contrast to the results for γ S-G18V, replacing G18 with alanine produces a much smaller, more local structural perturbation that does not produce a substantial increase in exposed hydrophobic surface area. The γ S-G18A variant has decreased thermal stability and increased aggregation propensity relative to γ S-WT, albeit to a lesser extent than γ S-G18V. We initially hypothesized that a protein of intermediate aggregation propensity between γ S-WT and γ S-G18V would also recruit α B-crystallin, although perhaps with lower affinity than the disease-related variant. However, this was not the case for γ S-G18A, which α B-crystallin does not bind (Figure 6E,F). This result indicates that α B-crystallin is able to distinguish among structurally similar variants of its client proteins.³⁶

Taken together, these results suggest that the intermolecular interactions of α B-crystallin are precisely tuned to bind only aggregation-prone variants of its client proteins. This is consistent with a view of cataractogenesis in which the finite allotment of α -crystallin in the lens is used up by damaged or mutated structural crystallins; the highly aggregation-prone γ S-G18V variant occupies a lifetime’s worth of binding capacity in a few short years, causing childhood-onset cataract. Cataract disease progression may also be accelerated by the large complexes formed between human α B-crystallin and its client proteins, particularly if exposed hydrophobic areas are incompletely protected. These mixed crystallin particles may be more prone to aggregation and precipitation as a result of

exposure of aggregation-prone regions on their surfaces, an idea that we call the “sticky side out” hypothesis. To date, the exact mechanism of aggregation in the lens is not fully understood, but it appears likely that in the case of severely aggregation-prone structural crystallin variants, holdase chaperone activity may result in recruitment of ever-larger amounts of α B-crystallin to the complexes, eventually exhausting the holdase chaperone capability of the lens.

■ INTERACTIONS OF HUMAN EYE LENS PROTEINS WITH SOLVENT

Dehydration is thought to play an important role in protein aggregation, disrupting solvent–solute interactions and causing irreversible interprotein interactions. Despite the importance of such interactions, there is a dearth of mechanistic detail on the relationship between the binding of water at the protein surface and initiation of aggregation. One important set of questions relates to the relative importance of environmental factors versus characteristics of the protein itself. Are protein surface dehydration and subsequent aggregation effects that occurs as a function of crowding, confinement, and pressure, or is the aggregation propensity actually dictated by the hydration shell, whose stability and compressibility are intrinsic properties of the protein surface? In collaboration with Han and co-workers, we found the latter to be the case: wild-type human γ S-crystallin harbors a dynamic yet exceptionally stable hydration shell. In contrast, in γ S-G18V this stability is greatly altered, suggesting that maintaining a robust, mobile water layer is essential to maintaining the extraordinary solubility of γ S-WT. Indeed, a critical function of γ S-crystallin in the eye lens may be precisely its capacity to preserve this stable hydration shell.

Our study showed that upon crowding of γ S-WT, the population of the stable surface hydration water is increased, but no new water population with slower dynamics or otherwise altered properties is generated. Using a combination of Overhauser dynamic nuclear polarization (ODNP) and solution-state NMR spectroscopy, we directly observed the link between the stability of this protein’s hydration shell and its aggregation propensity. The Han lab has pioneered ODNP methods for observing the relaxation behavior and hence the mobility of water near the protein surface.³⁸ Here, ODNP experiments showed that the hydration water of γ S-WT retains its rapid bulklike mobility up to extremely high protein concentrations (greater than 500 mg/mL), while that of γ S-G18V dramatically and irreversibly slows down at much lower protein concentrations, as is typical for most proteins.³⁹

In order to gain detailed molecular-level insight into this phenomenon, we measured the amide temperature coefficients, which directly report on site-specific protein–solvent interactions. Because hydrogen bonding, either intramolecular or to the solvent, greatly influences the local electronic environment of amide protons, the temperature dependence of the ^1H chemical shifts reveals whether the amide proton of a given residue participates in intramolecular hydrogen bonding. For globular proteins, the temperature coefficients of the backbone resonances are usually linear over a temperature range up to about 15 K below the thermal denaturation temperature. Typical values range from -16 to $+2$ ppb/K. Below the denaturation temperature, increasing the temperature increases the magnitudes of local fluctuations in the protein structure, causing lengthening of each hydrogen bond and hence movement of the chemical shift toward its random coil

value. At the mutation site (residue 18), there is a large difference in chemical shift, but the difference in chemical shift as a function of temperature, $\delta\Delta/\Delta T$, for G18 of γ S-WT (-4.98 ppb/K) versus V18 of γ S-G18V (-3.00 ppb/K) is relatively subtle, indicating that V18 is involved in an intermolecular hydrogen bond, consistent with our NMR structures. Our analysis revealed that several other residues in the N-terminal domain do have a different hydrogen-bonding status in γ S-WT than in γ S-G18V. Overall, the cataract-related variant has more hydrogen bonds to solvent than γ S-WT, underscoring the point that increasing protein–water interactions does not necessarily lead to greater solubility: greater solvent interaction with normally buried hydrophobic groups can lead to fragility of the hydration shell and hence aggregation.

■ DIFFERENT AGGREGATION PATHWAYS OF γ S-CRYSTALLIN AND ITS VARIANTS

The observations that aggregation-prone γ S-G18V has altered interactions with water and α B-crystallin led us to investigate the character of the particular aggregates formed. A variety of aggregation mechanisms have been proposed, with experimental evidence supporting each. Given the different cataract phenotypes and genetic and environmental factors as well as by analogy to other protein condensation diseases, it is likely that cataractogenesis can occur through a variety of pathways. Some researchers have observed the presence of fibrillar aggregates similar to those seen in Alzheimer’s, Huntington’s, and Parkinson’s diseases,⁴⁰ while others have reported amorphous-looking aggregates in which the monomers retain native-like structure.³³ Our observations of the aggregation of γ S-WT and γ S-G18V driven by pH extremes, high temperatures, and UV exposure also indicate multiple pathways leading to different products: all of the aggregation conditions tested produced a mixture of aggregate types. Both low and high pH favored amyloid fibrils, while amorphous aggregates were the dominant species in heat-treated and UV-exposed samples.⁴¹ Other researchers have observed a variety of aggregation mechanisms in structural crystallins, including disulfide-induced dimerization,³² domain swapping,^{42,43} loss of key interactions in the interdomain interface,⁴⁴ partial⁴⁵ or complete⁴⁶ unfolding of the N-terminal domain, and bridging via metal chelation.^{47,48}

■ METAL ION BINDING AND AGGREGATION

In addition to mutation and oxidative or photochemical damage, crystallin aggregation can also be caused by interactions with other species in the lens, including metal ions. Metal-ion-induced aggregation is particularly interesting in light of the evolutionary origin of the vertebrate lens $\beta\gamma$ -crystallins. These proteins evolved from an ancestral protein similar to the Ca^{2+} -binding $\beta\gamma$ -crystallin from the tunicate *Ciona intestinalis* (*Ci- $\beta\gamma$*).⁴⁹ Tunicates are believed to have diverged from cerebrates before the development of the eye. In metal-ion-binding crystallins, including several from microbial species and *Ci- $\beta\gamma$* , binding of divalent cations is essential for maintaining protein stability. *Ci- $\beta\gamma$* contains a double-clamp Ca^{2+} -binding motif, which is characterized by the sequence D/N–N/D–X–X–S/T–S, and is typical of microbial $\beta\gamma$ -crystallins.⁵⁰ We determined that *Ci- $\beta\gamma$* binds Ca^{2+} with very high affinity ($K_d \approx 0.05 \mu\text{M}$), resulting in very high thermal and chemical stability: the Ca^{2+} -bound form tolerates temper-

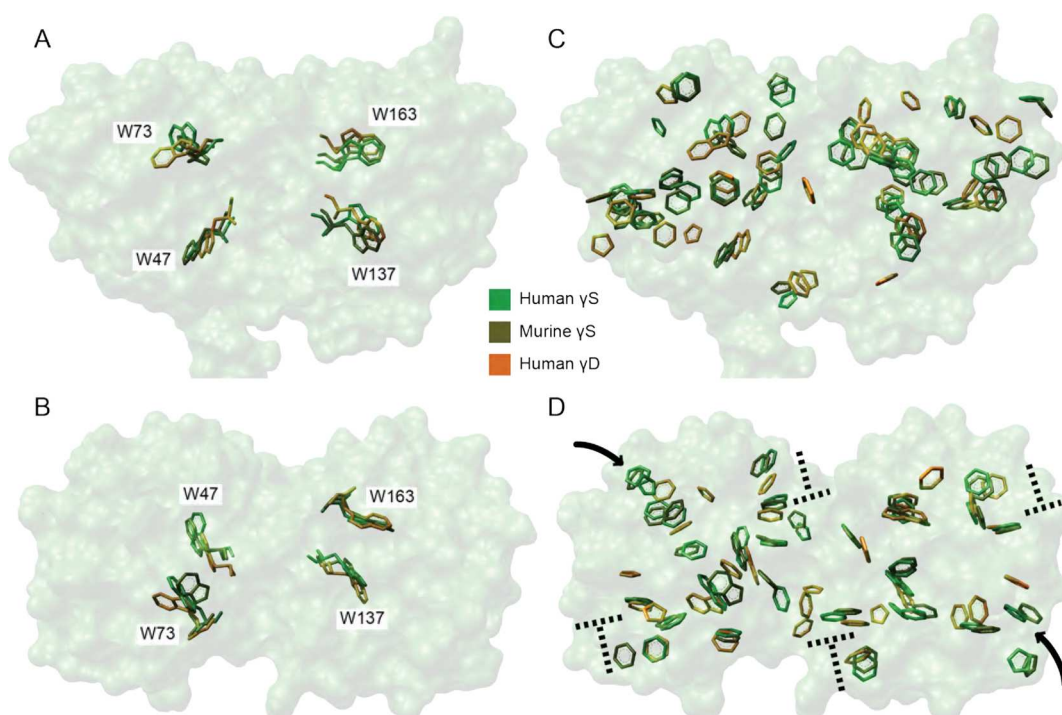


Figure 7. Molecular surfaces of human γ S- (green), murine γ S- (khaki, PDB ID 2ASM⁵⁹), and human γ D-crystallins (orange, PDB ID 2KLJ⁶⁰), with aromatic residues highlighted. (A) “Front” and (B) “top” views showing the positioning of the four conserved tryptophans. W47 and W163 occupy very similar orientations in all three structures, W73 is highly variable, and the indole ring of W137 is flipped in the structure of human γ S. (C) “Front” and (D) “top” views showing all of the aromatic residues. A perpendicular ring motif is consistent across all of the corner loops (dotted lines), and ring stacking in the outer bottom loops is also fairly consistent across the three structures (arrows).

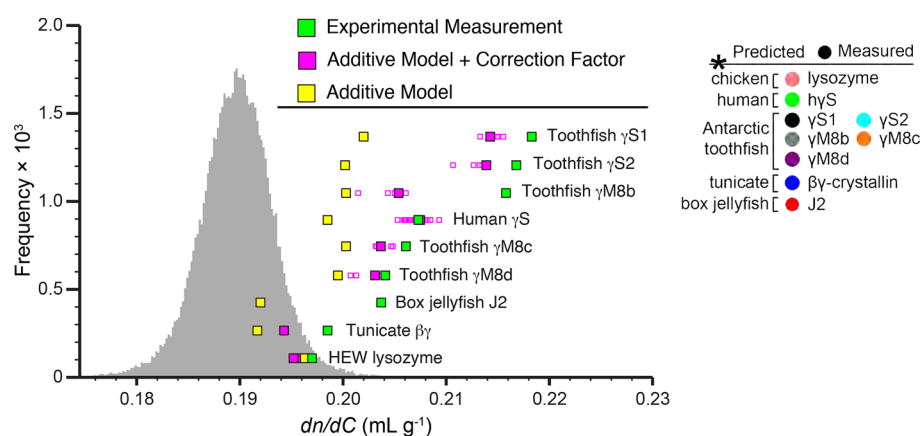


Figure 8. Predicted and measured dn/dc values for toothfish γ S1-, γ S2-, γ M8b-, γ M8c-, and γ M8d-crystallins, hen egg white (HEW) lysozyme, human γ S-crystallin, box jelly J2-crystallin, and tunicate $\beta\gamma$ -crystallin. Experimentally measured (green), additive Gladstone–Dale model predictions (yellow), and predictions using the π -pair correction (magenta) are shown for each protein. The histogram depicts the predicted dn/dc values for the human proteome generated using the additive model (gray). In cases where multiple conformations are available, e.g., human γ S-crystallin, corrected predictions are shown with solid squares for the lowest-energy structure and open squares for alternate conformations. For HEW lysozyme, two solid squares representing two crystal structures are shown, while no predictions are shown for J2-crystallin, which lacks known homologues that could be used for homology modeling.

atures up to 94 °C and 8 M urea, in contrast to the much less stable apo form.⁵¹ We identified specific cation-binding residues using NMR chemical shift perturbations.⁵¹ For the first time, we observed binding of *Ci*- $\beta\gamma$ to several other divalent cations, with varying effects on its stability.⁵²

In contrast, vertebrate lens crystallins lack this feature and bind Ca^{2+} only weakly and nonspecifically if at all: many of them have positively charged residues at key positions, abrogating cation binding. Some researchers have suggested that the loss of Ca^{2+} -binding affinity was required for the

vertebrate crystallins to evolve their high refractivity,⁵³ while others have observed that some human β - and γ -crystallins have weak Ca^{2+} binding activity.^{54,55} We did not observe any changes to the structure or solubility of human γ S-crystallin upon addition of Ca^{2+} ,⁵¹ whereas other divalent cations promote aggregation.⁵²

REFRACTIVE INDEX

Most studies of structural crystallins to date have focused on their extraordinary stability and solubility, which are indeed

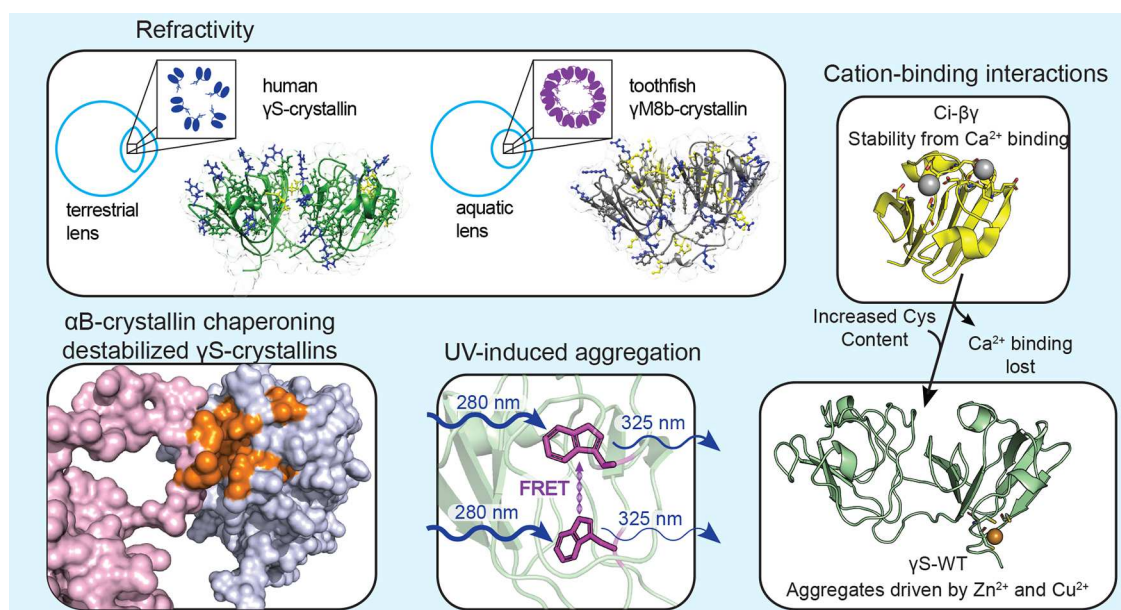


Figure 9. Summary of the major themes related to $\beta\gamma$ -crystallins discussed in this Account. Clockwise from top left: Aquatic lenses are more refractive than terrestrial ones because of the shape of the lens, the protein concentration, and the refractivity of the crystallins themselves (illustrated here by highlighting highly refractive residues, including aromatics, arginines, and methionines.) Divalent cations stabilize tunicate $\beta\gamma$ -crystallin, which resembles microbial cation-binding crystallins, but either do not bind or do not cause aggregation in human γ S-crystallin. UV light causes chemical modification of tryptophan residues, as observed by other researchers, including King and Truscott. We observe that UV-induced aggregates are similar to those induced by thermal denaturation rather than those produced by extreme pH conditions. We observe that human α B-crystallin recognizes aggregation-prone variants of human γ S-crystallin that have significant differences in hydrophobic surface area but not those with only minor and local structural disruption. Overall, the structural and refractive functions of the $\beta\gamma$ -crystallins are sometimes in tension, providing a fascinating system for investigating the biophysical chemistry of protein stability.

critical to their function in the lens. However, they are also strongly selected for high refractivity, which entails a high concentration of polarizable amino acids and is sometimes at odds with selection for solubility. This effect is particularly pronounced in the lenses of aquatic organisms, which do not derive focusing power from an air–water interface at the cornea, leading to much more stringent selection for refractivity. The refractive index is defined as the ratio of the velocity of light in a vacuum to its velocity in a given isotropic medium. When traversing between media, the extent to which light is bent is a function of the refractive index, which can be readily determined using Snell's law. The contribution to the change in refractive index with concentration for proteins is known as the refractive index increment (dn/dc). A conventional value of 0.185 is often used for all proteins, as relatively few dn/dc values have been experimentally measured. A more refined model estimates the dn/dc value on the basis of sequence.⁵⁶ However, our experimental dn/dc measurements on recombinant γ -crystallins show that such additive models substantially underestimate the refractivity of lens proteins, particularly those from aquatic animals.⁵⁷

We performed the first measurements of the refractive index increment for seven crystallins from aquatic organisms: tunicate Ci - $\beta\gamma$ -crystallin, Antarctic toothfish γ S1-, γ S2-, γ M8b-, γ M8c-, and γ M8d-crystallins, and box jelly J2-crystallin. All of the measured values were much higher than their predicted ones. We found that the factors correlating most strongly with anomalous dn/dc values were exposure of hydroxyl groups on the protein surface and pairs of highly polarizable amino acids with π systems in close proximity, a feature that is common in lens crystallins (Figure 7). The latter observation led to our π -pair correction to the additive model

of dn/dc , yielding better agreement with experimental results.⁵⁷ Figure 8 shows a comparison between experimentally measured dn/dc values and those calculated using the additive model alone as well as with the π -pair correction, which accounts for much of the discrepancy between the additive model and the experimental data. Another potential source of error in the additive model is failure to account for the protein's hydration shell, which is quite different from that of a solution of its component amino acids. Intriguingly, zebrafish γ M-crystallins have been observed to have very weak interactions with water,⁵⁸ consistent with our own observations of the hydration shell of human γ S-crystallin.³⁹ We observed that the *Dissostichus mawsoni* γ M-crystallins predicted by molecular modeling to have the most exposed hydroxyl groups also have the greatest deviations in dn/dc from the additive model, suggesting that the unusual hydration behavior of γ -crystallins is important for their core refractive function as well as avoiding aggregation. Solving the structures of these proteins and investigating their hydration shells in detail will allow us to begin to disentangle the complex relationships among surface hydration, solubility, and refractivity.

SUMMARY AND OUTLOOK

Crystallins are a unique class of ELLPs that remain soluble and transparent for the life of the organism, even in the absence of protein turnover and under frequent UV light exposure. Numerous mutations and post-translational modifications are known to impact crystallin solubility, but the molecular-level details of the aggregation process and sometimes even the aggregates formed are highly variable. Our work on crystallin aggregation focuses on using a variety of biophysical techniques in order to explore as many aspects of the

phenomenon as possible. In order to fully understand crystallin stability, solubility, and transparency, more experimental data are required to inform generalizations about the underlying molecular properties. In particular, more structures of aggregation-prone variants, both disease-related and engineered to test specific hypotheses, are needed, as well as solid-state NMR and cryo-EM structures of the disease-related aggregates. Another exciting direction is the structural and biophysical study of fish crystallins, which have evolved to withstand temperature and pressure extremes as well as concentrations even higher than those found in the human lens. Experimental measurements of protein refractivity and intermolecular interactions are also essential for elucidating the physical chemistry underlying lens function. Lessons learned from studies of these fascinating aggregation-resistant proteins will not only guide future cataract treatments and improve the design of artificial lenses but also be broadly applicable to understanding protein solubility and aggregation more generally. Figure 9 summarizes the major themes of our work on $\beta\gamma$ -crystallins.

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Notes

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Carolyn N. Paulson is a research scientist in the Institute for Therapeutics Discovery and Development at the University of Minnesota. She earned her Bachelor of Science in Biochemistry at UCLA and her Ph.D. in Chemistry at UC Irvine. At UC Irvine, she worked with Prof. Rachel Martin to study intermolecular interactions between eye lens crystallins using solution-state NMR spectroscopy and other biophysical techniques. She did her postdoctoral research at the University of Illinois at Chicago with Prof. Michael Caffrey, where she performed cell-based screening of peptides and fragment-based screening by NMR spectroscopy for influenza entry inhibitors, and at the University of Minnesota with Dr. Jon Hawkinson, where she developed and validated biophysical and biochemical assays for

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