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A Single Destabilizing Mutation (F9S) Promotes Concerted Unfolding of an Entire Globular Domain in γ S-Crystallin

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Abstract

Conformational change and aggregation of native proteins are associated with many serious age-related and neurological diseases. γ S-Crystallin is a highly stable, abundant structural component of vertebrate eye lens. A single F9S mutation in the N-terminal domain of mouse γ S-crystallin causes the severe *Opj* cataract, with disruption of cellular organization and appearance of fibrillar structures in the lens. Although the mutant protein has a near-native fold at room temperature, significant increases in hydrogen/deuterium exchange rates were observed by NMR for all the well-protected β -sheet core residues throughout the entire N-terminal domain of the mutant protein, resulting in up to a 3.5-kcal/mol reduction in the free energy of the folding/unfolding equilibrium. No difference was detected for the C-terminal domain. At a higher temperature, this effect further increases to allow for a much more uniform exchange rate among the N-terminal core residues and those of the least well-structured surface loops. This suggests a concerted unfolding intermediate of the N-terminal domain, while the C-terminal domain stays intact. Increasing concentrations of guanidinium chloride produced two transitions for the *Opj* mutant, with an unfolding intermediate at ~ 1 M guanidinium chloride. The consequence of this partial unfolding, whether by elevated temperature or by denaturant, is the formation of thioflavin T staining aggregates, which demonstrated fibril-like morphology by atomic force microscopy. Seeding with the already unfolded protein enhanced the formation of fibrils. The *Opj* mutant protein provides a model for stress-related unfolding of an essentially normally folded protein and production of aggregates with some of the characteristics of amyloid fibrils.

Keywords

H/D exchange; γ S-crystallin; amyloid; denaturation; cataract

Introduction

Unfolding of previously well-ordered proteins with formation of insoluble and potentially pathogenic oligomers, aggregates, fibrils, and amyloid plaques is a feature of many serious protein deposition diseases, including Alzheimer's disease, prion disease, type II diabetes, and systemic amyloidosis.¹ Some forms of cataract are also associated with formation of light-scattering centers derived from crystallins, the major soluble proteins of the lens.² These proteins are extremely long lived and are required to retain native structure and short-range

order at high protein concentrations to maintain the functional transparency and refraction of eye lens.³

In mammals, there are three major families of crystallins: α , β , and γ .^{3,4} The multigene β -crystallins and γ -crystallins are evolutionarily related and belong to the same $\beta\gamma$ -crystallin superfamily.^{4,5} Crystal structures of $\beta\gamma$ -crystallins, including γ B,^{6,7} γ D,^{8,9} γ E, γ F,¹⁰ and β B2,¹¹ and the solution NMR structure of mouse γ S-crystallin^{12,13} revealed a common, highly symmetrical two-domain architecture, with each domain consisting of two anti-parallel β -sheets arranged as two intercalated four-stranded “Greek key” motifs (Fig. 1). Each motif is built around a β -hairpin, consisting of the first two strands, that is stabilized by an unusual and characteristic loop that folds back onto the hairpin.^{6,7}

Several autosomal-dominant human and mouse cataracts have been identified as the consequence of frameshift and single-residue mutations in γ -crystallins.^{14–18} The resulting crystallin may form light-scattering aggregates either by nonfolded/misfolded aggregation, by simple condensational precipitation, or through conformational disorder. Sequences derived from frameshift are likely to never adopt any tertiary fold, thus following the first mechanism. Mutants of human γ D-crystallin, such as R14C,^{19,20} R58H, R36S,²¹ and P23T,^{22–24} follow the second pathway with reduced solubility, resulting in progressive juvenile-onset cataracts. In the third mechanism, mutation increases protein conformational dynamics, allowing partial unfolding under physiological conditions,^{14,25} as has been proposed for T5P of human γ C-crystallin.²⁶ It has been recently demonstrated that partially unfolded human γ D-crystallin is capable of forming high-molecular-weight fibrils *in vitro*.²⁷ Several structurally impaired truncation mutants of murine γ B-crystallin and γ E-crystallin also appear to form amyloid in cataractous lenses.²⁸ Furthermore, studies on bovine crystallins have indicated that characteristic amyloid fibril formation can occur in all α -crystallins, β -crystallins, and γ -crystallins *in vitro*.²⁹

γ S-crystallin is one of the most abundantly expressed crystallins in adult mammalian lens.^{5,30,31} In contrast to the closely related γ A-crystallins to γ F-crystallins, which are preferentially localized in the highest-protein-concentration central regions of the lens, γ S-crystallin is the major γ -crystallin of the somewhat less dense cortical regions of the lens and in lens epithelial cells.³² In the mouse cataract *Opj*, a nonconservative mutation replacing hydrophobic Phe9 in the core of the N-terminal domain of γ S-crystallin with serine leads to a temperature-dependent and concentration-dependent loss of structure and solubility at close to physiological temperature, despite its native-like behavior at low temperature.³³ In the lens of *Opj/Opj* mice, the architecture of cortical fiber cells is seriously disrupted, and transmission electron microscopy reveals striking fibrillar aggregates in affected cells.³³

The *Opj* mutant of γ S-crystallin provides a model for a protein that is fully capable of adopting a native conformation but has severely reduced structural stability, leading to unfolding and formation of potentially cytotoxic aggregates. This study investigates the processes of destabilization and unfolding in this protein to gain insight into possible mechanisms for disease-associated conformation change.

Results

The *Opj* mutant protein has a native fold

The tertiary fold of the F9S mutant protein (designated *Opj*) was investigated by multidimensional NMR methods. Comparison of backbone chemical shifts with those of wild type (WT) indicates that chemical shift perturbation caused by Phe-to-Ser mutation is exclusively located in the N-terminal domain. As expected, the largest combined amide ¹H–¹⁵N chemical shift differences, comprising Lys6-Tyr10, Asp21, Phe29, and Gly80,

are observed around the site of mutation, with the maximum value of ~3.3 ppm. The pairwise rmsd values for the N-terminal and C-terminal domains are 0.77 ppm and 0.06 ppm, respectively, implying that the structure of the C-terminal domain is essentially unperturbed. A similar effect was also observed in the P23T mutant in human γ D-crystallin.²² Since residual dipolar coupling (RDC) is exquisitely sensitive to small structural variation, we fitted the measured $^1D_{\text{HN}}$ RDC restraints to the WT structure (Protein Data Bank entry 1ZWM). Figure 2 shows a nice correlation between the measured and the predicted $^1D_{\text{HN}}$ values, with a Q -factor of 19%. When the N-terminal and C-terminal domains were fitted individually, similar quality factors were obtained, strongly suggesting that Opj maintains the native fold. Next, we determined the solution structure of Opj using a total of 1009 experimental restraints, including 353 RDCs, a limited set of nuclear Overhauser enhancement distance restraints (247 $\text{H}^{\text{N}}\text{-H}^{\text{N}}$ and $^{13}\text{CH}_3\text{-}^{13}\text{CH}_3$), and 409 backbone and side-chain dihedral angles. As expected, the global fold of Opj closely resembles that of the WT protein, with backbone rms differences of 0.8 Å, 0.9 Å, and 1.4 Å for the N-terminal domain, C-terminal domain, and full-length protein, respectively. It is noteworthy that these values are very likely to underestimate the similarity that is truly present in the real proteins, as they are dominated by noise in the structure determination process. Consistent with its WT behavior at room temperature, this result suggests that the F9S mutant is capable of folding correctly with an intact surface property unlike those intrinsically insoluble truncation or surface residue mutants.¹⁴⁻²¹

Protein unfolding probed by proton/deuterium chemical exchange

To assess global protein folding/unfolding process, we measured amide hydrogen/deuterium (H/D) exchange rates for ^{15}N -labeled WT and Opj γ S-crystallin. As expected from the inherent high stability and tight folding of γ S-crystallin, the exchange rates of WT fall into two major classes. Amide protons that belong to interstrand hydrogen bonds in the β -sheets showed very slow exchange rates (Fig. 3a and b, open circle), whereas those located on the edges of β -strands and in more loosely structured loops exhibited much faster exchange rates. Interestingly, several residues exhibit an unusual H/D exchange pattern as their peak intensities increase initially, followed by the expected decay (Fig. 3c, open circle). This counter-intuitive exchange pattern is likely due to a much faster exchange amide in very close proximity, whose fast depletion of proton significantly enhances the relaxation properties of the nearby slow-exchanging amide, resulting in the observed initial signal increase. As shown in Fig. 3c, the increasing rate of Phe15 can be nicely correlated with the exchange rate of Asn14, which is about 2.6 Å away. After the exchange of Asn14 has been completed, the intensity of Phe15 is then dominated by its own H/D exchange. Interestingly, these residues, including (Phe15 and Ser38), (Arg51, Phe54, and Ser81), (Ser128), and (Leu141, Tyr144, and Ser171), are consistently clustered around the β -hairpin between strands *a* and *b* in each Greek key (Fig. 3d). This observation strongly suggests that the folded hairpin motif, which is well conserved even in outlying members of the $\beta\gamma$ superfamily, is a highly stable structural unit.

Mutation of Phe9 to serine in Opj increased the H/D exchange rate of amide protons not only close to the mutation site but also throughout the entire N-terminal domain as compared to WT protein, while residues in the C-terminal domain displayed no detectable changes (Fig. 3b). An increase on the order of $2 \times 10^{-3} \text{ min}^{-1}$ was observed for most of the N-terminal β -sheet residues. Such increases in H/D exchange rates in Opj correspond to a higher fractional decrease in protection factors³⁴ for those well-protected core β -sheet residues such as Ile7 (Fig. 3a, filled circle), but to a relatively lower fractional change for those on the edge of β -sheets, such as Lys6, that already exhibit a substantial amount of H/D exchange in WT. Since H/D exchange rate is indicative of the fraction of time that an amide makes a hydrogen bond,³⁴ the observed decrease in protection factors from WT to Opj suggests that for every second that an H-bond remains intact, there are longer periods when it is “open” in Opj than in WT protein. Furthermore, those residues that are believed to stabilize the conserved β -hairpin structures no

longer exhibit any unusual H/D exchange pattern due to a significant increase in their own exchange rate (Fig. 3c, filled circle). Remarkably, these residues, such as structurally equivalent Phe15 and Phe54, are more than 10 Å away from the mutation site. Overall, F9S mutation on strand β 1 imposed profound effects on the stability of the core β -sheet residues and the characteristic β -hairpin motifs in both Greek keys in the N-terminal domain of Opj, allowing the entire folded N-terminal domain to be much more solvent accessible.³⁴ It thus appears that the stability of the fold of every part of the two core β -sheets is surprisingly interdependent, suggesting that the entire domain structure of two Greek key motifs may fold as a unit. This destabilization of the N-terminal domain has no knock-on effect on the C-terminal domain, at least at this temperature.

Under neutral pH and room temperature, H/D exchange of WT and Opj can be described by a bimolecular exchange mechanism, where the protein refolding rate is much faster than the intrinsic exchange rate.^{35,36} This assumption was validated by a linear correlation between the exchange rate and the solution pH value. Therefore, the free energy of breaking/forming the H-bonds of each amide can be derived from the measured exchange rate: $\Delta G_{\text{op}} = -RT \ln(R_{\text{obs}}/R_{\text{int}})$, where R_{int} is the intrinsic exchange rate of hydrogen in the exposed form in the context of the protein sequence. As shown in Fig. 4, the ΔG_{op} of opening each amide from H-bonding in the N-terminal domain becomes less positive for Opj than for the WT, implying that breaking the hydrogen bonds of these amides is much less energetically unfavorable for Opj. Even at 25 °C when the protein is macroscopically indistinguishable from WT, the amplitude of the decrease in ΔG_{op} observed was as high as 3.5 kcal/mol for Cys82, with an average of 8.5 kcal/mol and 6.3 kcal/mol for the core residues of WT and Opj, respectively. Increasing the temperature to 32 °C further decreased ΔG_{op} for those residues by ~0.6 kcal/mol to an average of 5.7 kcal/mol, whereas the C-terminal domain maintained the stability of WT, indicating that increasing temperature has a much greater impact on the conformational equilibrium of the N-terminal domain of Opj. Again, for amides on the edge of a secondary structure such as Lys6 and Leu33, little change occurred from WT to Opj at different temperatures. It thus appears that the free energy of the local opening/closing events of well-protected β -sheet amides approaches the free energy of those located in loosely structured regions in Opj, a phenomenon reminiscent of a concerted global unfolding of the entire supersecondary structure of the domain.³⁷

Overall, it seems that the highly constrained structural elements of the N-terminal domain in γ S-crystallin, principally organized β -sheets, are less tightly organized in Opj even at room temperature, and that they become more conformationally disordered with increasing temperature, breaking hydrogen bonding more readily and converging on dynamic characteristics more similar to those on the edge of the β -strands. It is plausible that the whole N-terminal domain loosens up to the point that it may eventually 'pop open.' Even before complete transition, a prolonged lifetime of the transiently unfolded state results in the buildup of a higher concentration of such species, allowing them to explore other conformations, including polymerization into fibrils.^{1,38}

Fluorescence titration of WT and Opj γ S-crystallin

Next, we investigated the unfolding of both WT and Opj using tryptophan fluorescence emission spectra under increasing concentrations of guanidinium chloride (GndCl) (Fig. 5). While WT displayed a monophasic transition at ~2.8 M GndCl, Opj gave a biphasic denaturing curve, with one transition at ~0.5 M and the other transition at 2.3 M GndCl. A similar biphasic unfolding, believed to correspond to sequential unfolding of the two domains, has been observed for other β -crystallins and γ -crystallins,^{27,39,40} including an equivalent mutation (L5S) in human γ D-crystallin.⁴¹ As suggested by the increased conformational disorder observed above, it seems clear that the N-terminal domain of Opj is likely to unfold first,

resulting in a partially unfolded intermediate. This intermediate appears to be relatively stable at 1.0–1.5 M GndCl, followed by unfolding of the C-terminal domain at a higher denaturant concentration. The second unfolding transition of Opj requires a lower concentration of denaturant than WT (2.3 M *versus* 2.8 M), suggesting that N-terminal and C-terminal domains in WT costabilize, but this effect is lost in Opj. This is consistent with the idea that evolution from one-domain to two-domain proteins confers benefits of extra stability.⁴²

Amyloid fibril formation of Opj

As has been reported for γ D-crystallin, it seems likely that the partially unfolded intermediate of Opj is capable of forming amyloid-like fibrils.²⁷ At >0.5 mM Opj, the amide peaks in two-dimensional (2D) heteronuclear single quantum coherence (HSQC) were significantly damped with an increase in GndCl concentration and eventually vanished at ~1.0 M. Concurrently, a translucent gel sample was formed. The disappearance of peak intensities is consistent with the formation of large aggregates that broaden the NMR signals beyond detection. When using the thioflavin T (ThT) assay for fibril formation, we detected a significant increase in ThT fluorescence for Opj incubated under 0.5 M and 1.0 M GndCl, where the first transition of fluorescence emission and a stable intermediate take place. Under the same condition, no fluorescence change was noticed for WT. Next, the ThT assay was performed on WT and Opj incubated at 32 °C and 37 °C (Fig. 6). As expected, a higher temperature significantly promoted the formation of a ThT-sensitive structure in Opj, while the WT crystallin showed no detectable change in ThT fluorescence within the same time period. When Congo red was introduced into the above samples, the characteristic redshift expected from an interaction with amyloid-like fibrils was observed. Furthermore, Fig. 6 also indicated that the lagging period during fibril formation was markedly reduced from 40 h to 20 h when the temperature was increased from 32 °C to 37 °C. This is consistent with the H/D exchange results showing that a higher temperature allows Opj to unfold much more readily and increases the population of the partially unfolded species. The cooperative nature of fibril formation by unfolded Opj was tested by adding a small amount of pre-heat-treated protein to serve as an aggregation nucleus. Indeed, this produced a much shorter lag time for the production of ThT-sensitive fibril. Overall, the partially unfolded intermediate of Opj produced either by denaturant or by elevated temperature is able to trigger a cascade of amyloid-like fibril structure formation.

Next, atomic force microscopy (AFM) was used to examine the ThT-positive Opj aggregates. Figure 7 shows a snapshot of Opj fibrils induced by incubation at 37 °C. Apparently, physiological temperature allowed Opj to form long tangles of fibrils, reminiscent of the inclusions observed by electron microscopy in the cataractous lens of *Opj* mice³³ and the putative amyloid fibrils of WT human γ D-crystallin prepared *in vitro*.²⁷ Under the same condition, no fibrils were detected for the WT protein. In the presence of 0.5–1.0 M GndCl, fibrillar structures, much shorter in length however, were also readily observed. A similar fibril formation has also been observed for mouse γ B-crystallin,²⁸ human γ C-crystallin,⁴³ and γ D-crystallin.⁴⁴

Opj can form fibrils *in vitro*, but what about *in vivo*? In addition to the electron microscopy images of fibrils in *Opj* cataractous lenses,³³ immunofluorescence (IF) localization shows distinct clumping of γ S immunoreactivity in the cortical fibers of *Opj* lenses, in contrast to a more even distribution in WT lens (Fig. 8a and b). These clumps appear to be Opj aggregates, but are they amyloid-like fibrils? Congo red staining of *Opj* lens shows fairly large clumps of intensely fluorescent material in cortical fibers (Fig. 8c), suggestive of plaque formation.²⁸ (Similar Congo-red-positive inclusions have also been reported for γ B^{nop}-crystallin protein located in lens fiber cell nuclei.) However, in *Opj* lens sections, these clumps do not appear to be significantly birefringent, a classic test for amyloid plaques. It is possible that in the high protein concentrations of the mouse lens, with many other protein components present, fibrils

are not formed with sufficient order to allow the proper alignment of bound dye for birefringence to be observed. Nevertheless, the *Opj* mutant protein clearly gives rise to large fibrillar aggregates in the cataractous lens.

Discussion

The majority of known cataractogenic mutations in β -crystallins and γ -crystallins, particularly in mouse models, involve truncations that prevent the protein from ever adopting a native conformation.^{17,45,46} Others, such as R14C,^{19,20} R58H,²¹ and P23T^{24,47} in human γ D-crystallin, cause changes in surface residues, but have no effect on core structure. Such mutants have a native tertiary structure, but their disrupted surface properties lead to protein oligomerization. Indeed, recent studies have suggested that the solution properties of γ -crystallins are highly dependent on organized molecular dipoles and that single-residue changes can significantly affect these dipoles and drastically reduce solubility.⁴⁸

The current studies indicate that the *Opj* mutant of mouse γ S-crystallin is different. It has a normal molecular surface and, at lower temperatures, can adopt and maintain a native fold. However, as shown here, the free-energy minimum occupied by the folded N-terminal domain is much shallower for the mutant than for the WT protein. As the consequence, the amides involved in hydrogen bonds of the β -sheets of the mutant N-terminal domain show significantly higher H/D exchange rates even at room temperature. As temperature rises, the exchange rates increase to a level comparable with those structurally loosely restrained residues, possibly leading to a concerted domain unfolding. Furthermore, since H/D exchange rates are indicative of the lifetime of an amide that is protected by a hydrogen bond,³⁷ a significant reduction in lifetimes in the N-terminal domain of *Opj* strongly suggests that the transiently unfolded state can be much more populated than in the WT. α -Crystallins, another major component of the lens, are small heat shock proteins with chaperone-like properties that are thought to have a role in preventing the aggregation of unfolded lens proteins.⁴⁹ The high concentration of *Opj* protein in the mutant lens would likely overwhelm the protective capacity of this system.

Significantly, this ‘melting’ of the core β -sheets is essentially global. The modified Greek key motifs that make up each domain have distinct and highly characteristic supersecondary elements. In particular, in each motif, strands *a* and *b* form a hairpin with a loop that folds back onto the hairpin and also interacts with strand *d* of the same motif.^{3,6,7} This highly conserved element is thought to be a key part of the stability of a $\beta\gamma$ superfamily domain. Indeed, H/D exchange data in the WT protein show unusual effects on residues in these conserved structures that may reflect on their role as a structural unit contributing to domain stability. However, the H/D exchange results of *Opj* show that the stability of the whole β -sheet sandwich structure of the N-terminal domain is surprisingly codependent and that unfolding is a concerted process, with loosening of the main-chain hydrogen-bond networks throughout the domain. Throughout this ‘melting’ process, the C-terminal domain is unaffected. Indeed, chemical denaturation curves show that the mutant protein unfolds in two phases, with an intermediate that very likely consists of an unfolded N-terminal and a folded C-terminal domain.

The concerted unfolding of a whole domain is also observed for the familial amyloidosis variants of human lysozyme, I56T and D67H. These mutations significantly reduce the stability of the β -domain in the native structure and promote a transient, locally cooperative unfolding of the β -domain under physiologically relevant conditions.^{1,38} It has been proposed that this partially unfolded state allows formation of amyloid fibrils by the unfolded domain, while the rest of the protein may retain native conformation. WT lysozyme can also undergo the same transition into amyloid, but only at nonphysiological temperatures. Mutation can allow the domain to explore unfolded states, even if only transiently, possibly giving access to a new amyloidforming conformation. The *Opj* F9S mutant of γ S-crystallin apparently follows a

similar pathway, but this is all the more remarkable since the canonical $\beta\gamma$ -crystallin domain, with its sandwiched β -sheets and defined supersecondary elements, is a much more discrete, tightly folded, globular structure than the lysozyme β -domain, which consists of a single β -sheet leading to a C-terminal helix.

Like other models of protein misfolding or unfolding,¹ the partially denatured intermediate of Opj is able to form amyloid-like fibrils *in vitro*. Indeed, electron microscopy has shown the presence of fibrous structures within the lens fiber cells of the cataractous Opj lens,³³ and both IF localization of γ S-crystallin and Congo red staining show very uneven distributions in the cataract.

Since the unfolding of Opj occurs close to physiological temperatures, it is reasonable to ask whether the mutant protein is able to adopt a native conformation at all *in vivo*. Evidence that it can do so is provided by the progressive nature of the Opj cataract. Although apparent at birth, the cataract becomes much more severe with age,³³ suggesting that aggregates build up over a time course of weeks and months. As such, Opj provides a model for some of the processes that are thought to contribute to age-related cataract in humans.

Turnover of crystallins in the lens is extremely slow and ceases in mature enucleated fiber cells. Crystallins then survive for years while exposed to light and other insults.² Many modifications, such as deamidation, sulfur oxidation, glycation, and carbamylation, have been detected in crystallins from aged or cataractous lenses, and these may contribute to unfolding or aggregation.^{2,50–52} Opj shows that modification of even a single residue can be sufficient to destabilize a $\beta\gamma$ -domain to the point that it can unfold, even if only transiently, to allow formation of amyloid-like aggregates. Such aggregates, once seeded, may recruit additional molecules and grow until they begin to degrade cellular functions. In the lens, this may amount to light scattering; however, when similar processes occur in amyloidoses and other protein conformation diseases in neural and other tissues, even more serious consequences can arise.

Materials and Methods

NMR spectroscopy

Uniformly labeled WT and Opj proteins were overexpressed and purified according to the protocol described previously, with the exception that the temperature for Opj was lowered to 25 °C before induction.¹² NMR spectra were recorded on Bruker Avance DRX 800-MHz (¹H) and 600-MHz (¹H) NMR spectrometers equipped with a cryogenic probehead. Backbone assignments were made by conventional triple-resonance experiments: HNCA, HNCACB, and CBCA(CO)NH.^{53–55} Side-chain assignments were determined by three-dimensional (3D) HCCH total correlated spectroscopy and ¹⁵N total correlated spectroscopy experiments.⁵⁶ Interamide distance restraints were determined from 3D ¹⁵N-edited nuclear Overhauser enhancement spectroscopy ($\tau_{\text{mix}}=120$ ms) on a ¹⁵N-labeled protein, and methyl–methyl interactions were obtained from a 3D ¹³C-edited nuclear Overhauser enhancement spectroscopy ($\tau_{\text{mix}} = 120$ ms)⁵⁷ on a fully deuterated methyl-protonated sample. Side-chain χ_1 angles for aromatic and C γ methyl-carrying residues were measured from ³J_{C γ C γ and ³J_{NC γ by quantitative *J* correlation experiments,^{58–60} as well as from 2D {¹³C γ } spin-echo difference experiments.⁶¹ For H/D exchange experiments, lyophilized ¹⁵N-labeled samples were quickly dissolved in 99.9% D₂O and immediately subjected to a series of HSQC spectra with 2 scans/fid for 2 h, followed by 4 scans/fid for 18 h, and, finally, 12 scans/fid for 1 day. All data were processed with NMRPipe.⁶² The normalized peak intensities in H/D exchange experiments were fitted to a single-exponential decay curve for most of the residues in WT and all amides in Opj. Residues that exhibit an unusual exchange pattern were fitted by a double-exponential function.}}

RDCs were measured in 3 mg/ml filamentous Pf1 whose orientation was stabilized in a gel matrix.¹² $^1D_{NH}$ RDCs were obtained from 2D HSQC transverse relaxation optimized spectroscopy spectra, recorded in an interleaved mode.⁶³ $^1D_{NC}$ and $^1D_{C\alpha C}$ RDCs were measured from 3D HNCO⁶⁴ and 2D HNCOC⁶⁵ experiments, respectively, utilizing a quantitative J correlation. $^1D_{C\alpha C}$ RDCs were obtained from C^α -coupled HNCO experiments recorded at 600 MHz (1H frequency) to minimize C' relaxation.

Fluorescence spectroscopy

A Horiba FluoroMax-3 fluorimeter equipped with a circulating water bath was used to collect the fluorescence emission spectra of Opj and WT at 30 °C under different GndCl concentrations. The buffer condition for all experiment was 25 mM sodium phosphate (pH 7). Three milliliters of 5 μ M protein samples was prepared in a cuvette and, at each titration, 100 μ l of sample solution was withdrawn and replaced with 100 μ l of 7.5 M GndCl solution. After addition of GndCl, the sample was stirred and incubated for 5 min, and the emission of intrinsic tryptophan fluorescence was measured in the range of 310–420 nm after excitation at 295 nm. Baseline data were collected in the same way in the absence of the protein and subtracted from the sample emission spectra. The unfolding processes of WT and Opj were plotted by the fluorescence intensity ratio between 360 nm and 320 nm *versus* the concentration of GndCl.

ThT assays

Protein samples (5 mg/ml) in 25 mM sodium phosphate buffer (pH 7.0) were incubated at 0.5 M or 1.0 M GndCl or under the specified temperature. During each measurement, 10 μ l of the above samples was introduced into 3 ml of 50 μ M ThT solution. An excitation wavelength of 450 nm was used for bound ThT, and the emission spectra at 482 nm were recorded. A blank spectrum was recorded for 3 ml of 50 μ M ThT in the absence of protein, and the difference between the two intensities at 482 nm was plotted against the time incubated.

Atomic force microscopy

AFM experiments and analysis were provided by the AFM facility at The Ohio State University. All reagents were prefiltered by a 0.1- μ m filter to remove any large particles. WT and Opj solution at 5 mg/ml were first incubated either at 37 °C for 48 h or in the presence of 0.5 M and 1.0 M GndCl overnight. Ten microliters of each sample was allowed to nonspecifically bind to a clean mica surface, which was then dried by a vacuum for 1 h before imaging.

Protein localization

γ S-Crystallin localization by IF used the peptide antibody described previously.⁶⁶ Frozen sections of 6-month-old lenses were prepared and labeled as described by Wyatt *et al.*⁶⁷ For Congo red staining, paraffin sections of 18-month-old *Opj/Opj* lens were used. Congo red dye was obtained from Sigma (St. Louis, MO), and staining was performed as described by Meloan and Puchtler.⁶⁸

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Abbreviations used

WT	wild type
RDC	residual dipolar coupling
H/D	hydrogen/deuterium
GndCl	guanidinium chloride
2D	two-dimensional
HSQC	heteronuclear single quantum coherence
ThT	thioflavin T
AFM	atomic force microscopy
IF	immunofluorescence
3D	three-dimensional

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Fig. 1. Ribbon representation of murine WT γ S-crystallin (1ZWM) showing a highly symmetrical two-domain architecture. The mutation site Phe9 is located on the first β -strand of the N-terminal domain.

Fig. 2. Correlation of $^1D_{NH}$ measured from the N-terminal domain (●) and the C-terminal domain (▲) of Opj with the predicted values based on the WT structure. The RDCs of both domains of Opj agree very well with the WT structure.

Fig. 3.

H/D exchange decays of (a) Ile7, (b) Tyr149, (c) Asn14 (square), and Phe15 (circle) from WT γ S-crystallin (open symbols) and Opj (filled symbols) at 25 °C, best fitted with single-exponential function, except for Phe15 in WT (fitted with a double-exponential function). All N-terminal core residues in Opj exhibit a faster H/D exchange rate, while all C-terminal residues remain unchanged. The initial increase in the intensity of Phe15 correlates with the rate of decrease in Asn14 in WT, while in Opj, Phe15 is dominated by its own H/D exchange rate. (d) Residues (Phe15, Ser38, Arg51, Phe54, and Ser81 denoted by spheres) that exhibit a Phe15-like H/D exchange pattern are located in close proximity to the conserved β -hairpins, formed by *a* and *b* β -strands in both Greek key motifs in the N-terminal domain (1ZWM). The mutation site Phe9 is shown in stick representation.

Fig. 4.

Comparison of the opening free energy (ΔG_{op}) of WT (25 °C, black) and Opj (25 °C, blue; 32 °C, red) assuming a bimolecular exchange H/D exchange mechanism. The three dotted lines represented with the same coloring scheme show the average ΔG_{op} values of the N-terminal core residues, suggesting that the entire N-terminal domain of Opj becomes less stable and much more temperature sensitive. The arrows represent β -stranded secondary structures.

Fig. 5. Equilibrium unfolding of WT γ S-crystallin (open) and Opj (filled) by increasing GndCl concentration at 30 °C monitored by tryptophan fluorescence.

Fig. 6.

Fluorescence change in ThT when bound to Opj preincubated at 37 °C (●) and 32 °C (■), and to WT γS-crystallin preincubated at 37 °C (▲), indicating that a higher temperature can promote fibril formation in Opj, but not in WT γS-crystallin.

Fig. 7. AFM snapshot of Opj fibril induced at 37 °C, exhibiting tangled fibrils reminiscent of those observed in γ D-crystallin.²⁷

Fig. 8.

Evidence for protein aggregation in the *Opj* mouse lens. (a) IF localization of γ S-crystallin in the frozen section of adult WT mouse lens. γ S immunoreactivity is shown in green, and 4',6-diamidino-2-phenylindole staining of nuclei is shown in blue. γ S-Crystallin is distributed evenly throughout epithelial and fiber cells. (b) Similar IF of γ S localization in *Opj* lens. γ S-Crystallin distribution (green) is very uneven, apparently in clumps. (c) Congo red stain of a paraffin section of *Opj* lens. Irregularly distributed clumps of intense stain are seen in secondary lens fibers (SF), but not in the lens nucleus (LN), the embryonic lens region with lower γ S-crystallin levels.