Published in final edited form as:

J Mol Biol. 2011 January 21; 405(3): 840–850. doi:10.1016/j.jmb.2010.11.005.

Characterization of a transient unfolding intermediate in a core mutant of γ S-crystallin

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Abstract

In many age-related and neurological diseases, formerly native proteins aggregate via formation of a partially unfolded intermediate. γ S-crystallin is a highly stable structural protein of the eye lens. In the mouse *Opj* cataract, a non-conservative F9S mutation in the N-terminal domain core of γ S allows adoption of a native fold but renders the protein susceptible to temperature and concentration-dependent aggregation, including fibril formation. Hydrogen/Deuterium exchange and denaturant unfolding studies on this mutant (Opj) have suggested the existence of a partially unfolded intermediate in its aggregation pathway. Here we have used NMR and fluorescence spectroscopy to obtain evidence for this intermediate. In 3.5 M urea Opj forms a stable and partially unfolded entity, characterized by an unstructured N-terminal domain and a largely intact C-terminal domain. Under physiologically relevant conditions, Carr-Purcell-Meiboom-Gill (CPMG) T_2 -relaxation dispersion experiments showed that the N-terminal domain residues were in conformational exchange with a loosely structured intermediate with a population of 1-2%, which increased with temperature. This provides direct evidence for a model in which proteins of native fold may explore an intermediate state with an increased propensity for formation of aggregates, such as fibrils. For the crystallins, this also shows how inherited sequence variants or environmentally induced modifications can destabilize a well-folded protein allowing the formation of intermediates able to act as nucleation sites for aggregation and the accumulation of light scattering centers in the cataractous lens.

Keywords

Relaxation dispersion; γ S crystallin; protein unfolding; cataract

Introduction

Cataract, a leading cause of blindness world-wide, is often associated with the aggregation of crystallins, structural proteins of the lens¹. In some cases, such aggregation includes fibril

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formation, reminiscent of other diseases of aging². Elucidation of the pathways at a molecular level by which a native-folded crystallin could undergo structural and/or dynamic changes to form toxic aggregates could give insights into preventative or therapeutic strategies.

γ S-crystallin, a member of the $\beta\gamma$ -crystallin superfamily, is a major structural component of the lenses in most vertebrates and is one of the most abundantly expressed proteins in adult mammalian lens^{1;3}. This monomeric protein is highly stable and can retain native structure and short range order at the high concentrations required for the functional transparency and refractivity of the eye lens. Like other $\beta\gamma$ -crystallins, γ S adopts a global architecture with two topologically similar domains constructed from paired Greek key motifs and stabilized by extensive hydrophobic interactions^{4;5;6;7;8;9;10}. In the murine *Opj* cataract, one of the core residues of the N-terminal domain Phe9 is non-conservatively replaced with a serine. This mutation destabilizes the protein and results in progressive opacity, disruption of the architecture of the lens cortical fiber cells, and the appearance of fibrillar aggregates¹¹.

Structural, hydrogen/deuterium exchange, and GdnHCl-induced unfolding studies showed that the *Opj* F9S mutation has a native fold, but exhibits a much shallower free-energy minimum in the folded N-terminal domain¹². Together with the biphasic unfolding transition monitored by fluorescence emission, our previous results suggested the existence of a partially unfolded intermediate that could act as the precursor for aggregation in cataract formation¹². The current work focused on the identification and characterization of this entity in the presence of chemical denaturant and, most importantly, under physiologically relevant conditions. In particular, we used the CPMG T_2 relaxation dispersion approach, which in the past several years has emerged as an excellent tool for studying protein minor states invisible to other biophysical techniques^{13;14;15;16}. This method can provide a wealth of information on the interconversion in the millisecond timescale between the ground state and the minor state, from which the nature of the latter and its population can be inferred.

Our results are significant in the following respects: we showed that *Opj*, like some other crystallins, can form a partially unfolded intermediate in the presence of urea; and more importantly we revealed under physiologically relevant conditions that the N-terminal residues of *Opj* are conformationally more dynamic in millisecond timescale with respect to the WT, allowing the accumulation of a persistent intermediate characterized by a loosely structured N-terminal domain with the C-terminal domain largely intact. This intermediate may trigger aggregation through nucleation-dependent polymerization. It is important to note that, unlike previous biophysical studies on cataract-associated crystallins¹, the current work mostly by NMR methodology focused on detailed site-specific information. As such, this study sheds light on general processes of unfolding in proteins that are capable of adopting native-like conformation but have severely reduced structural stability leading to progressive formation of cytotoxic aggregates.

Result

Partially unfolded *Opj* intermediate can be populated by 3.5 M Urea

Previous fluorescence study of *Opj* suggested the presence of an unfolding intermediate by the observation of a biphasic transition under denaturing condition¹². Therefore we carried out titration studies on a 0.2 mM ¹⁵N-labeled sample with urea aiming to capture the unfolding intermediate amenable for NMR studies. Figure 1 shows the overlay of 2D HSQC spectra of *Opj* in the presence and absence of 3.5 M urea. Even though sequence-specific resonance assignments pertaining to C-terminal domain appear to be readily transferred, a 3D ¹⁵N-edited NOESY spectrum was recorded to confirm the assignments. Clearly, most of

the signals from the residues in the C-terminal domain appear to be unperturbed, including those with intrinsic line-broadening in both WT and Opj, e.g. Cys129, G134 and Ile137. Together with the conservation of NOE patterns, these results suggest a largely intact C-terminal domain in 3.5 M urea. On the other hand, the N-terminal residues lost all of its characteristic upfield and downfield peaks, which likely collapse into the region between 7.5 to 8.5 ppm in the ^1H dimension, signaling the unfolding of the N-terminal domain. This observation directly confirms previous unfolding results that crystallin mutants may form a partially unfolded intermediate in the presence of weak denaturant^{17; 18; 19; 20; 21; 22}. Notably, the WT protein as a control appears to be immune to the addition of urea under an identical condition.

Mutation in Opj promotes ms conformational exchange in the N terminal domain

The most important question in this work is whether there exists such an intermediate of Opj under physiologically relevant conditions. Given the increased motion in the timescale of millisecond to hours characterized by H/D exchange, it was conceived that such a minor state, if exists, may experience a μs -ms conformational exchange with the native state, a dynamic event well suited for the backbone ^{15}N T_2 -relaxation dispersion studies. Therefore, parallel experiments were performed with variable refocusing delays on both Opj and WT. Figure 2 shows the residues in Opj that exhibit dispersion profiles characteristic of μs -ms conformational exchange dynamics. In comparison with the WT, these residues can be broadly classified into three groups: Group I, II and III, exemplified by Ile7, Gln70, and Lys158, respectively.

Group I (e.g. Ile7, Figure 2B) includes most of the residues located throughout the first Greek-Key motif (GK1), such as Lys6-Ser9 on $\beta 1$, Arg19-Tyr20 and Cys22 on $\beta 2$, Asp23-Cys26 on $\beta 3$, Ser38 and Val41 on $\beta 4$ and Phe29-Arg30 in the loop between $\beta 3$ and $\beta 4$. These residues demonstrate small but significant exchange term of relaxation rate (R_{ex}) in Opj whereas virtually none were observed in their WT counterparts. The residues of Group II (e.g. Gln70, Figure 2C) mainly reside on $\beta 6$, $\beta 8$ and the loop between $\beta 7$ and $\beta 8$ in the second Greek-Key motif (GK2). Unlike Group I, they already experience substantial intrinsic ms motion in WT protein, but display marked increase in exchange-related relaxation rate upon mutation. Taken together, F9S mutation apparently promotes slow motion at ms timescale within the N-terminal domain of Opj.

Group III residues (e.g. Lys158, Figure 2D) are located throughout the C-terminal domain, where comparatively larger dispersion profiles are observed in both WT and Opj. It was noticed that several amide signals such as Lys94 and Gln96 in $\beta 1'$, Ser128 in $\beta 4'$ and Lys153-Arg157 in $\beta 7'$ were vanishingly weak in 2D ^1H - ^{15}N HSQC, consistent with the presence of ms timescale motion in the center of the β -sheet. While most of the C-terminal residues in Opj show similar dispersion profiles to the WT, a few residues in close proximity to the domain interface, Gln148 and Val176 in particular, demonstrate substantial increases in R_{ex} (Figure 2A). These changes may result from motions propagated from the N-terminal domain via the domain interface.

The N-terminal domain of Opj exchanges with an unfolding related intermediate

To probe the nature of the exchanging minor states of the N- and C-terminal domains, dispersion experiments were performed at several temperatures on both WT and Opj. Notably, in Opj, the temperature dependence of the dispersion profiles varied among the aforementioned three groups of residues, as exemplified by the relaxation dispersion profiles of Cys22, Gln70, Ala84 and Lys158 (Figure 3). Specifically, the majority of Group I (e.g. Cys22, Figure 3A) exhibited an increased conformational exchange with temperature, as evidenced by increased ΔR values ($\Delta R = R_2^{\text{eff}}(\text{lowest } \nu_{\text{CPMG}}) - R_2^{\text{eff}}(\text{highest } \nu_{\text{CPMG}})$) (Figure

S1), whereas those in Group III (e.g. Lys158, Figure 3D) showed an opposite effect, suggesting that these two groups may be associated with different nature of conformational exchange processes. This temperature-dependence of Group III residues in Opj is similar to the counterpart in WT, indicating that the intrinsic motion associated with the C-terminal core was essentially unperturbed by the mutation. Interestingly, while most of Group II residues in WT follow a similar temperature dependence of their motions to the C-terminal domain, many of them in Opj demonstrated positive correlation with temperature (e.g. Gln70 in Figure 3B), reminiscent of those in Group I. This opposite temperature response again implies that the ms motions observed in this very same region are fundamentally different between the Opj and WT γ S-crystallins. As shown below, the relaxation profiles of the most of Group II residues in Opj can be simultaneously fitted with Group I residues, suggesting that the F9S mutation disrupted the motion intrinsically present in the GK2 in WT protein, and that the motions within both GK1 and GK2 of Opj are becoming correlated. It was further noticed that the Trp46 N^ε1 also exhibits a positive correlation with temperature. This residue is a key structural component stabilizing the β -turn linking the GK1 and GK2 via an H-bond formation from its N^ε1 to the carbonyl of Gly43⁹. It should be noted that there are a few exceptions in Group II including Leu61, Ala84 (Figure 3C) and His86-Leu87 located in close proximity to the domain interface. Their motions are likely influenced by dual effects from the N- and C-terminal domains.

Analysis of above temperature dependent relaxation profiles revealed important insight into the nature of the minor conformations the N-terminal domain is exchanging with. Initial fitting of most of the residues in Group I and II individually indicated that the exchange processes pertaining to the N-terminal domain of Opj confer positive enthalpy ($\Delta H > 0$) and entropy ($\Delta S > 0$). This result suggests that the N-terminal domain is in exchange with a less bonded minor state, consistent with an unfolding process. Attempts to fit 32 out of 39 N-terminal relaxation profiles simultaneously to a model of a two-state exchange process led to a good fit with a reduced χ^2 value of 1.1, assuring that these motions are mutually correlated (Table 1). The increase in the amplitude of the dispersion profiles from 25 to 37 °C (Figure 3) at least in part resulted from the population increase of the minor conformation ranging from $1.1 \pm 0.1\%$ at 25 °C to $2.0 \pm 0.1\%$ at 37 °C (Figure 4A). Based on the temperature dependence of the equilibrium constant K_{eq} and forward exchange rate k_a (Figure 4B and 4C), an endothermic process was obtained for this domain with a ΔH of 8.8 ± 1.4 kcal/mol and a ΔS of 87.0 ± 6.0 J/mol•K. The percentage of the minor state at 25 °C corresponds to a free energy ΔG_{eq} of ~ 2.6 kcal/mol, a value comparable to the ΔG_{N-I} of 3.1 kcal/mol for the first unfolding transition observed in an equivalent L5S mutation of γ D-crystallin²¹. The presence of a low population of a loosely structured state may explain a slightly lower β -sheet content observed by circular dichroism¹¹ as well as a small red-shift of the tryptophan fluorescence of Opj with respect to the WT protein under native condition (Figure S2).

The N-terminal unfolded intermediate may be loosely structured

The structure of the minor state can be probed by the ¹⁵N chemical shift changes, $\Delta\omega_{exp}$ between the two exchanging states derived from the relaxation dispersion experiments. The values of these chemical shift differences of the Opj N-terminal domain range from -4.7 to 2.6 ppm. The signs of these $\Delta\omega$ coincide with the direction expected for unfolding of a native structure towards a random coil, however, their amplitudes are somewhat smaller than those $\Delta\omega_{cal}$ assuming a completely unfolded minor state²³. The ratio of $\Delta\omega_{exp}/\Delta\omega_{cal}$ is a good indication on how similar in structure an on-pathway intermediate is compared to a completely unfolded state^{13; 14; 16}. As shown in Figure 4D, while most of the N-terminal β -sheet residues in the exchanging minor state of Opj contain some structure (low $\Delta\omega_{exp}/\Delta\omega_{cal}$ values), relatively larger values were observed for residues 68-76 encompassing the helical-loop between $\beta 7$ and $\beta 8$, suggesting that this region is least structured. Overall, upon

mutation, the N-terminal domain of Opj appears to be in conformational exchange with a loosely structured state even though it appears to be scarcely populated.

Previous hydrogen/deuterium exchange result indicated that F9S mutation decreases the stability of the N-terminal domain of Opj from 8.5 to 6.3 kcal/mol¹². It is noteworthy that the exchange equilibrium ΔG_{eq} determined in the CPMG-relaxation experiment is only ~40% of the opening free energy ΔG_{op} of Opj¹², further supporting an intermediate distinctly different from a fully unfolded state. Similar observation has been reported in mutational variants of human lysozyme, which can conformationally access a partially unfolded state without crossing the major energy barrier of unfolding for the fibril formation²⁴. Currently, it is not clearly whether the Opj intermediate under native condition is the same as the aforementioned partially unfolded species in the presence of urea. However, the fact that both denaturant and elevated temperature can trigger formation of ThT-sensitive fibrillar materials¹² argues that these two partially unfolded species share at least some degree of structural similarity.

The C-terminal domains of WT and Opj share a similar intrinsic ms motion

The molecular nature of the exchange processes involving Group III residues of Opj is similar to the motion observed in the WT protein. Notably, individual fitting of the dispersion data revealed that many of them have similar exchange rate k_{ex} values. A global analysis using 25 out of 29 dispersion profiles resulted in a nice fit with a χ^2_{red} value close to 1, implying that the C-terminal residues are dynamically coupled and exhibit a high degree of cooperativity (Table 1). In contrast to the N-terminal domain of Opj, the C-terminal intrinsic motion is associated with much smaller amide ^{15}N $\Delta\omega$ between the two exchanging conformers, ranging from 0.2 to 1.3 ppm. However, the minor state is surprisingly more populated at a level of 14-17%, inversely correlated with temperature. The exothermic nature of this exchanging process from the Van't Hoff plot (Figure 4C and Table 1) undoubtedly is incompatible with an unfolding mode observed with the N-terminal domain of Opj.

Interestingly, two C-terminal residues of the domain interface, Gln148 and Val176 (Figure 2A), also possess increased conformational exchange with temperature in Opj, surprisingly demonstrating positive values of ΔH and ΔS . In fact, both residues gave better fit with the N-terminal residues than the C-terminal ones, although worse than their individual fit. It appears that partial unfolding of the N-terminal domain may be accompanied by loosening of the domain-domain packing interaction and, as a consequence, the direct contact residues in the C-terminal domain may experience a much more complicated motion. Interestingly, the equivalent residue to Gln148 in human γD , Gln143, has been shown to be critical for interaction with the N-terminal domain, and its deamination is associated with mature-onset cataract formation^{19; 20}.

Probing the unfolded state by ANS under native condition

It is well known that 8-Anilino-1-naphthalene sulfonic acid (ANS) can be used to probe the population of compact partially unfolded intermediate of proteins²⁵. We compared the ANS accessibility of both WT and Opj proteins at various temperatures. As shown in Figure 5, no ANS fluorescence change was observed with WT protein, suggesting that the hydrophobic core of WT is well protected at both experimental temperatures. Opj, in contrast, revealed a small increase in the ANS fluorescence under identical temperatures, indicating that the Opj mutant somewhat exposed its hydrophobic cluster accessible to ANS. Furthermore, the mutant allows a steady increase in ANS fluorescence from 25 to 37 °C, consistent with the population increase of the partially unfolded intermediate as addressed above. In conclusion,

this result reaffirms the presence of a partially unfolded state of Opj but not in WT at physiologically relevant condition.

Fast internal dynamics (ps-ns) of WT and Opj γ S-crystallins are unchanged

Both H/D exchange and the T_2 -relaxation dispersion experiments have revealed significant dynamics differences between WT and Opj in the timescale of millisecond or slower. What about fast internal motions? To address this issue, ^{15}N relaxation experiments were conducted, including transverse relaxation rate $R_{1\rho}$ and $\{^1\text{H}\}$ - ^{15}N heteronuclear NOEs. As shown in Figure 6, within experimental error, the backbone amides in Opj shared comparable residue-specific values with the WT protein. Apparently the mutation did not induce significant changes of fast end (picoseconds-nanoseconds) dynamics, and both N-terminal and C-terminal core residues remain rigid with similar relaxation properties, consistent with a globally tumbling molecule. However, it is interesting to note that the inter-domain linker in both WT and Opj, unlike the counterpart in human γD crystalline ²⁶, appears to possess fast internal motion. Overall, we have explored the changes upon mutation in dynamic events occurring over a wide range time scale, and the fast internal dynamics do not contribute to Opj aggregation behavior under physiologically relevant condition.

Discussion

Many proteins associated with deposition diseases share a common feature of formation of a partially unfolded or non-native conformation ^{27; 28; 29; 30}. Such species have been suggested to be aggregation-prone, leading to formation of insoluble and potentially pathogenic aggregates and fibrils. However, direct observation and characterization of these intermediates under physiological conditions is difficult because of their low populations and partial homogeneity. CPMG-dispersion methodology thus offers excellent opportunities for investigating such intermediate, provided that it exchanges with the native state on the millisecond time-scale and its population is in excess of 0.5%. In this work, we have successfully applied this technique on the mouse γS -crystallin. The parallel experiments on both WT and Opj allowed us to extract the mutation-induced changes in kinetic, thermodynamic and structural properties of the unfolding process in the context of population, exchange rate constant, and chemical shift differences between the exchanging states. Most important, this work revealed an on-pathway unfolding intermediate populated around 1-2% under physiologically relevant conditions.

Surprisingly, although demonstrated to be highly stable by our previous H/D exchange measurement ¹², the C-terminal domain of both the WT and Opj protein exhibits substantial conformational exchange, through a process fundamentally different from the mutated N-terminal domain of Opj. Perhaps, in combination with the aforementioned ps-ns fast internal motion, these protein motions may make an entropic contribution to the high solubility of the crystallins within the protein-dense lens ⁹. Consistent with this notion, mutation of residue Pro23 located in the loop between $\beta 3$ and $\beta 4$ in the GK1 of human γD reduced the higher than average motion in the residues near this position observed in WT protein, a change which causes reduced solubility and cataract ²⁶. In addition, the fact that several C-terminal domain residues proximal to the domain interface were dynamically affected upon mutation suggests that the destabilizing effect of mutation in the GK1 may be propagated into the domain interface through interaction with the GK2, allowing the two domains of Opj unfold more independently than their WT counterparts.

Previous studies indicated that Opj is capable of forming fibrils ¹². The presence of a partially unfolded state in the mutant but not in the WT protein reinforces the emerging view that polymerization of a native-like protein may originate from a partial unfolded

intermediate. Consistently, the aggregation of Opj is dependent on temperature, protein-concentration and denaturant-concentration, factors that may radically shift the equilibrium of the folded and partially unfolded states. Once the intermediate population reaches a critical concentration for nucleation, a cascade of self-assembly may follow. This mechanism of aggregation, in analogous to a nucleation-dependent polymerization, is supported by a growing list of evidence. For example, a low population of an unfolding intermediate at the level of 1% was observed for the N-terminal domain of the *Escherichia coli* HypF under mild denaturing conditions, and such partially unfolded state was shown to be responsible for triggering formation of ThT-sensitive proto-fibrils *in vitro*³¹. Furthermore, the importance of residual structures in amyloidogenic intermediates has been suggested for human prion protein for the formation of an infectious scrapie-like state²⁸. The Opj F9S mutant of γ S-crystallin apparently follows a similar pathway.

Like Opj, several γ -crystallin mutants have been shown to be able to adopt a native-fold and are also capable of forming a partially unfolded intermediate containing one unfolded and one intact domain in the presence of denaturant^{17; 18; 19; 20; 21; 22}. Such partially structured species have been further demonstrated to be amyloidogenic *in vitro*^{18; 32; 33}, and may give rise to the formation of fibril plaques in cataractous lenses³⁴. The mechanism of aggregation of Opj is consistent with those previous results but, importantly, demonstrated the presence of a low populated, partially unfolded intermediate under a non-denaturing condition. Similar intermediates may be shared by some other cataract-associated crystallins, as has been demonstrated in human γ D-crystallin²¹ and G18V mutation of human γ S-crystallin²². The latter mutation disrupts a highly conserved hairpin structure that forms a highly stable structural unit¹² and thus compromises the stability of the N-terminal domain, allowing formation of a partially unfolded state in the presence of denaturant²². It is conceivable that this mutant may also transiently access a partially unfolded state under native condition, and slow nucleation of such intermediate and possible saturation of α -crystallin chaperon capacity¹ may together contribute to the progressiveness of G18V cataract. Evidently, the free energy of partial unfolding of G18V (2.6 kcal/mol) is comparable with the value determined for Opj by the NMR dispersion experiment.

In conclusion, our studies of Opj mutant of γ S-crystallin may provide a generalized model for aggregation or polymerization of a natively-folded protein which undergoes conformational sampling with a loosely-structured or partially unfolded intermediate due to reduced stability induced by mutation or external insults.

Materials and Methods

Nuclear Magnetic Resonance Spectroscopy

Uniformly ¹⁵N-labeled WT and Opj proteins were overexpressed and purified according to the protocol as described previously^{9; 12}, and dissolved in the NMR buffer containing 25 mM imidazole (pH 6.5), 25 mM NaCl and 0.04% NaN₃ (92% H₂O/8% D₂O). NMR spectra were recorded on Bruker Avance DRX 800 MHz (¹H) and 600 MHz (¹H) NMR spectrometers equipped with cryogenic probehead. Backbone ¹⁵N T_{1ρ} and steady-state heteronuclear Overhauser effects (NOE) were collected at 25 °C and 32 °C for both Opj and WT. The chemical exchange contribution to the backbone ¹⁵N transverse relaxation rate was measured by relaxation dispersion experiments^{35; 36; 37} with a series of 2D ¹⁵N-¹H spectra with the values of CPMG field strength ranging from 33.3 to 966 Hz and a total constant time relaxation delay of 30 ms. Each series of data contains one reference spectrum and two duplicate points that are used for error analysis. Dispersion profiles were recorded at multiple temperatures for both Opj (25, 29, 32 and 37°C) and WT (25, 29 and 32°C) proteins. All experiments were collected in an interleaved manner to minimize variation in sampling condition. Direct observation of partially unfolded protein was achieved in the

presence of 3.5 M urea, and the assignment of the C-terminal domain was confirmed by the NOE pattern from 3D ^{15}N -edited HSQC-NOESY.

Data Analysis

All data were analyzed with NMRPipe package software³⁸. Effective relaxation rates at each CPMG frequency, R_2^{eff} , were extracted from the intensities of the cross peaks according to $R_2^{\text{eff}}(\nu_{\text{CPMG}}) = -\ln(I(\nu_{\text{CPMG}})/I(0)) \cdot (1/T_{\text{relax}})$, where $I(\nu_{\text{CPMG}})$ and $I(0)$ are the peak intensities in the presence and absence of the constant-time relaxation³⁹. Error of R_2 was estimated from two repeated measurements at each serial of data using the method described¹⁴ with a minimum error of 0.5 /s. Relaxation dispersion profiles were selected only if $\Delta R = R_2^{\text{eff}}(\text{lowest } \nu_{\text{CPMG}}) - R_2^{\text{eff}}(\text{highest CPMG}) > 3$ /s at the field strength of 18.8 T, and subsequently fitted to a model of two-state exchange ($A \rightleftharpoons B$) using an in-house program and a fitting routine kindly provided by Dr. Lewis Kay's group with variable parameters including: 1) chemical shift difference $|\Delta\omega_N|$ which is independent of temperature; 2) the population (mole fraction) of the minor state P_B and the exchange rate k_{ex} ($k_{\text{ex}} = k_A + k_B$) which are constant at each temperature under different magnetic fields; and 3) intrinsic R_2 relaxation rate for each dispersion curve. In the case of a global fit, a group of correlations were assumed to have the same values of population P_B and the exchange rate k_{ex} , and the goodness of the fitting were evaluated by comparing the χ^2 values obtained through both individual and global fits. The changes of enthalpy (ΔH) and entropy (ΔS) were derived from the temperature dependence of K_{eq} ($K_{\text{eq}} = P_B/(1-P_B)$) using $\ln(K_{\text{eq}}) = -\Delta H/RT + \Delta S/R$.

Fluorescence spectroscopy

Horiba FluoroMax-3 fluorimeter equipped with a circulating water bath was used to collect the fluorescence emission spectra of ANS in the presence of Opj and WT at multiple temperatures. The buffer used in this experiment was 50 mM sodium phosphate (pH 7.0). All spectra were collected in the range of 400-600 nm after excitation at 380 nm. For each measurement, the spectrum of free ANS (Sigma Aldrich) in the concentration of 40 μM was first recorded. After addition of WT or Opj γ S-crystallin to a final protein concentration of 6 μM , the sample was stirred and incubated for 10 minutes prior to data collection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are very grateful for useful discussion with Dr. Lewis Kay and the software support from his laboratory. ZW is funded by NIH R21EY018423, GW is funded by the NEI intramural program, and IK is supported by NIH R016M077234 for Dr. Mark Foster.

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Figure 1.

Overlay of 2D ^1H - ^{15}N HSQC spectra of Opj in the absence (black) and presence (red) of 3.5 M Urea recorded at 800 MHz at 25 °C. Peaks are labeled according to the assignment in the absence of denaturant. The signals of the N-terminal domain are significantly altered while those of the C-terminal domain are largely unperturbed. The three residues mentioned in the text with substantial line-broadening are colored in blue.

Figure 2.

Comparison of the ^{15}N -dispersion profiles of WT and Opj crystallins recorded at 800 (filled) and 600 (open) MHz at 32 °C. The measured effective transverse relaxation rate R_2^{eff} is plotted as a function of the CPMG frequency ν_{cpmg} . (A) Ribbon representation of γS crystallin (1ZWM) showing three groups of ^{15}N transverse relaxation dispersion profiles with Group I color coded in red, Group II in blue and Group III in green, each represented by (B) Ile7, (C) Gln70 and (D) Lys158 with the same color scheme as (A) and black for WT protein. Each domain of γS -crystallin is constructed by two Greek-Key motifs, namely GK1 and GK2 in the N-terminal domain, and GK3 and GK4 in the C-terminal one.

Figure 3.

Temperature dependence of the backbone ^{15}N relaxation dispersion profiles collected at magnetic field strengths of 14.1 (lighter color) and 18.8 T (darker color) of (A) Cys22 from Group I, (B) Gln70 and (C) Ala84 from Group II, and (D) Lys158 from Group III of Opj, indicating both positive and negative correlation with respect to temperatures.

Figure 4.

Temperature dependence of the population of the minor state of the N-terminal domain of Opj (A), the Arrhenius plot (B) and Van't Hoff plot (C) of both the N-terminal (red) and C-terminal (green) domains. (D) Ratio of $\Delta\omega_{exp}/\Delta\omega_{cal}$ for the backbone ¹⁵N nuclei of the N-terminal domain of Opj. Only those residues with $|\Delta\omega_{cal}| > 2.0$ ppm have been plotted. Arrows indicate the β -stranded secondary structures.

Figure 5. ANS fluorescence upon binding to γ S proteins: ANS alone (solid black), WT protein (dotted lines), and Opj mutant (solid lines) collected at 25 °C (red) and 37 °C (blue). All data were normalized according to ANS alone spectrum at 32 °C.

Figure 6.

Comparison of $\{^1\text{H}\}\text{-}^{15}\text{N}$ heteronuclear NOE (top) and $T_{1\rho}$ (bottom) values of Opj (red) and WT γS (black) recorded at 600 MHz at 32 °C, indicating similar ps-ns motion in both proteins. A spin lock field of 2.5 KHz with 8 durations ranging from 0.02 to 30 ms was used for $T_{1\rho}$, and a 5s-proton saturation was employed for the NOE spectrum.

Table 1

Kinetic and thermodynamic parameters of the N- and C-terminal domains of WT and Opi

Group	25 °C			29 °C			32 °C			37 °C			Equilibrium		Arrhenius Barrier (kcal/mol)
	P _B (%)	k _{ex} (S ⁻¹)	P _B	k _{ex} (S ⁻¹)	P _B	k _{ex} (S ⁻¹)	P _B	k _{ex} (S ⁻¹)	P _B	k _{ex} (S ⁻¹)	P _B	k _{ex} (S ⁻¹)	ΔH (kcal/mol)	ΔS (J/mol•K)	
N (Opi)	1.1±0.1	671±39	1.3±0.1	897±38	1.5±0.1	1041±34	2.0±0.1	1179±34	8.8±1.4	87±6	-17.2±2.1				
C (Opi)	16.9±1.4	748±16	15.6±1.4	986±19	14.8±1.3	1224±25	14.1±1.4	1978±56	-3.2±2.0	-58±28	-12.1±3.3				
C (WT)	16.7±1.3	809±18	15.9±1.4	1127±23	14.6±1.3	1337±29	-4.0±1.8	-69±25	-9.7±3.5						