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^1H , ^{13}C , and ^{15}N assignments of wild-type human γS -crystallin and its cataract-related variant γS -G18V

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Abstract

We present the backbone and sidechain NMR assignments and a structural analysis of the 178-residue wild-type γS -crystallin and the cataract-related point mutant, γS -G18V. γS -crystallin is a structural component of the eye lens, which maintains its solubility and stability over many years. NMR assignments and continued structural investigations of γS -crystallin and aggregation-prone variants will advance understanding of cataract formation.

Keywords

crystallin; eye lens; cataract; protein aggregation; protein stability; solution-state NMR assignments

Biological Context

The structural proteins in the eye lens, known as crystallins, are interesting structural targets because of their very high stability and solubility. These proteins are classified into two broad families; the α -crystallins form polydisperse complexes and have a solubilizing function (Horwitz, 1992, Tanaka et al, 2008), binding to other crystallins via a pH-dependent mechanism (Jehle et al, 2010). By contrast, the $\beta\gamma$ -crystallins play a purely structural role. Crystallins of either type can aggregate due to either genetic mutation or post-translational modification, forming a cataract. Cataract, listed as a priority eye disease by the World Health Organization, is responsible for 48% of blindness worldwide. Epidemiological studies have shown that even in progressive age-related cataract, heredity is a major factor, possibly affecting 70% of cases. This indicates that hereditary features of the crystallin proteins affect their solubility, with point mutations causing congenital cataract being merely the most dramatic examples of a widespread phenomenon. Determining the molecular mechanisms of cataract formation will require structural studies of the native protein and cataract-related variants.

γS -crystallin is the most highly expressed crystallin in the cortex of the lens during development (Wang et al, 2004). All the crystallins are highly conserved, however γS -crystallin has the most highly conserved sequence among mammalian species, for example

between human and murine γ S there is 89% sequence identity and 96% sequence homology. A clinically-discovered polymorphism in the γ S-crystallin gene (γ S-G18V) that causes congenital cortical cataract in early childhood (Sun et al, 2005) provides a useful model system for investigation of cataract formation. Subsequent investigation of the biophysical properties of γ S-G18V indicated that while the point mutation causes a significant thermodynamic destabilization of the protein fold (Ma et al, 2009), the aggregation of γ S-G18V appears to be due to alteration of the local intermolecular forces between γ S monomers, rather than a bulk unfolding of the protein (Brubaker et al, 2011). Solution-state structures of the wild-type and variant proteins will provide insight into the extent and type of the local structural perturbations. Previous NMR studies on γ S include a solution structure of the highly homologous murine protein (Wu et al, 2005), characterization of its destabilizing variant F9S (Mahler et al, 2011), and extensive backbone and sidechain assignments of wild-type human γ S (γ S-WT) at pH 6.0 (Baraguey et al, 2004). Although structures of γ S-WT and its variants are not yet available, analysis of the assignments reveals chemical shift changes suggesting substantial structural perturbation in the region of the point mutations. Here we report the backbone and sidechain resonance assignments of γ S-WT and γ S-G18V at 22 °C and pH 4.5 and the differences between them.

Methods and Experiments

Sample Preparation:

Recombinant human wild-type human γ S-crystallin and variant human γ S containing the G18V substitution were expressed and purified as described previously (Brubaker et al, 2011). Briefly, a plasmid containing either the wild-type or G18V human γ S-crystallin sequence was amplified with primers containing flanking restriction sites for NcoI and XhoI, an N-terminal 6x His tag, and a TEV protease cleavage sequence (ENLYFQG). The PCR product was cloned into the pET28a(+) vector (Novagen, Darmstadt, Germany). For expression in minimal media, an appropriate volume of overnight culture of Rosetta *E. coli* such that the final OD₅₅₀ would be approximately 0.9 units was spun down and resuspended in 1 L of M9 minimal media with U-¹³C glucose and ¹⁵NH₄Cl. The cells were thereafter grown at either 37 °C (γ S-WT) or 25 °C (γ S-G18V). The cells were allowed to grow for an additional hour before induction of protein expression. IPTG was added to a final concentration of 1 mM, and the cells were grown for either an additional 5–6 hours (γ S-WT) or 16–24 hours (γ S-G18V), and harvested by centrifugation. The His-tagged protein was then purified on a nickel column and the 6x His tag removed by cleavage with TEV protease; in both proteins the N-terminal methionine is replaced with glycine as a consequence of the TEV protease cleavage sequence. After purification, the samples were dialyzed against 10 mM acetate buffer (pH 4.5), concentrated, and supplemented with 0.05% sodium azide, 2 mM TMSP, and 10% D₂O. The final sample concentrations used in experiments were 2.11 mM and 1.50 mM for γ S-WT and γ S-G18V, respectively.

NMR Experiments:

Experiments were performed at 22 °C on a Varian *UnityINOVA* system operating at 800 MHz equipped with a ¹H/¹³C/¹⁵N 5mm tri-axis PFG triple-resonance probe. Resonance assignments were made using the following experiments: 2D ¹H-¹⁵N HSQC, 2D ¹H-¹³C

HSQC, 3D HNCACB, 3D HNCO, 3D CBCA-(CO)NH, 3D H(C)CH-COSY, 3D H(C)CH-TOCSY, 3D ^1H - ^1H - ^{13}C NOESY-HSQC (140 ms mixing), 3D ^1H - ^1H - ^{15}N NOESY-HSQC (150 ms mixing). Aromatic side-chain resonances were assigned using aromatic 2D ^1H - ^{13}C HSQC and 3D ^1H - ^1H - ^{13}C NOESY-HSQC (140 ms mixing) spectra. Decoupling of ^{13}C and ^{15}N nuclei were performed by WURST (Kupce and Freeman, 1995), and GARP (Shaka et al, 1985), sequences, respectively. ^1H shifts were referenced to TMSP, and ^{13}C and ^{15}N were referenced indirectly to TMSP. NMR data were processed using NMRPipe (Delaglio et al, 1995), and analyzed using Sparky (Goddard and Kneller, 2011).

Assignments and Data Deposition

In general the data for both γS -WT and γS -G18V were of good quality, with γS -WT having higher sensitivity than γS -G18V at comparable concentrations. Because γS -G18V is sensitive to thermal stress, NMR experiments were performed at 22 °C. At this temperature, improved sensitivity was observed at pH 4.5. Signal from residues between L151 and R158 were weak or undetectable in both datasets, and in general backbone amides from the N-terminal domain were much stronger and more complete than those from the C-terminal domain, as is indicated by the relative peak intensities in the ^1H - ^{15}N HSQC (Figure 1). Final assignments for both γS -WT and γS -G18V (all numbers given respectively) included 90.5% (153) and 87.6% (148) of the 169 expected backbone amides (excluding prolines and G1). 96.1% (171) and 94.4% (168) of 178 sidechains had complete aliphatic resonance assignments. Aromatic sidechain assignments included all histidines, all tryptophans, all phenylalanines except F10 in γS -G18V, and 8 of 14 tyrosines in both variants. All sidechain primary amides were assigned except for N38 in γS -G18V. The assignments for γS -WT are mostly consistent with the existing assignments deposited in the BioMagRes-Bank for pH 6.0 (Baraguey et al, 2004).

Assignment Strategies:

The high degree of structural symmetry between the N-terminal and C-terminal domains of γS -crystallin allowed easily backbone assignment of residues that shared homologous positions on the two domains. In addition to this interdomain symmetry, each double Greek key domain has conserved residues at certain positions, and can be divided into symmetric subdomains (Figure 2b). This intradomain symmetry allows the rapid assignment of residues with “partners” at other positions in the structure in the absence of obvious inter-residue connectivity, but also has a tendency to crowd these regions of the spectra. In particular, the sidechain resonances of N15, N54, D103, and N144 occur at these positions and show significant peak overlap in several experiments.

Chemical Shift Variation:

The G18V substitution appears to cause widespread disruption throughout the N-terminal domain of γS -crystallin rather than only the residues immediately around it. However, the protein remains folded as indicated by chemical shift dispersion and other biophysical data (Brubaker et al, 2011). In general, chemical shift variation between γS -WT and γS -G18V occurs largely between residues 1 and 83, which show significant shifts in backbone and sidechain chemical shift frequencies (Figure 1, Figure 2a), while the C-terminal domain

shows little difference in either the amide or aliphatic chemical shift frequencies between the two variants. Furthermore, the largest variation in chemical shifts appears to be primarily in the first subdomain of the N-terminal domain of γ S-G18V (residues 1–39).

Multiple Conformers:

In the process of assigning γ S-G18V it became apparent that some residues in the N-terminal domain around V18 seemed to be populating two discrete conformations with different backbone chemical shifts (Figure 3). In particular residues S2, G5, R19, T32–C37, I61, Q64, R72, H87 and L88 appear to exist in two conformations according to the NMR data (Figure 1), while residues T32–C37 appear to have the strongest signal, with the secondary chemical shifts easily identifiable in backbone through-bond experiments (Figure 4). These residues correspond with the region predicted by molecular dynamics simulations to shift, allowing water into the interior of the N-terminal domain as a result of the local destabilization caused by the G18V substitution (Brubaker et al, 2011). It is interesting to note that the weaker secondary backbone amide resonances often are closer or even identical to the chemical shifts for γ S-WT, suggesting that γ S-G18V may be able to access both the wild-type conformation and an alternate conformation that may be related to cataract formation.

Data Deposition:

The assigned chemical shifts for γ S-WT and γ S-G18V have been deposited in the BioMagResBank (www.bmrb.wisc.edu) under accession numbers 17576 and 17582, respectively.

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Fig. 1. 2-dimensional ^1H - ^{15}N HSQC of γS -WT (black) and γS -G18V (blue) showing assignments. Peaks that do not shift between γS -WT and γS -G18V are labeled in green. Arginine $\text{N}\epsilon\text{H}\epsilon$ cross-peaks are folded in the ^{15}N dimension and appear in the lower-right portion of the spectrum. Experimental parameters: ^1H : center frequency (CF) 4.8 ppm, spectral width (SW) 10.0 kHz; ^{15}N : CF 118.7 ppm, SW 3.8 kHz.

Fig. 2.

a) Comparison of γS -WT and γS -G18V $\text{C}\alpha$ and $\text{C}\beta$ chemical shifts by residue number. **b)** Alignment of γS -WT subdomains, with homologous residues highlighted in blue (first subdomains) and red (second subdomains). Residues which are homologous across all four subdomains are highlighted in purple.

Fig. 3. Model of γ S-G18V from molecular dynamics simulation (Brubaker et al, 2011) with residues showing evidence of multiple conformations in γ S-G18V in NMR spectra highlighted. Residues are colored by type: positive (blue), polar (green), negative (red), other (yellow), and V18 (orange).

Fig. 4.

Backbone walk of γ S-G18V showing slices of HNCACB and CBCA(CO)NH experiments corresponding to residues displaying evidence of two conformations. Experimental parameters: ^1H : center frequency (CF) 4.8 ppm, spectral width (SW) 13.0 kHz; ^{13}C : CF 46.0 ppm, SW 16.1 kHz; ^{15}N : CF 118.7 ppm, SW 3.0 kHz.