Biophysical investigations of the molecular basis of cataract associated with the R76S mutation in human gammaD-crystallin

Vurghun Ahmadov
University at Albany, State University of New York, vahmadov@albany.edu

The University at Albany community has made this article openly available. Please share how this access benefits you.

Follow this and additional works at: https://scholarsarchive.library.albany.edu/legacy-etd

Part of the Biochemistry Commons, and the Chemistry Commons

Recommended Citation

This Master’s Thesis is brought to you for free and open access by the The Graduate School at Scholars Archive. It has been accepted for inclusion in Legacy Theses & Dissertations (2009 - 2024) by an authorized administrator of Scholars Archive. Please see Terms of Use. For more information, please contact scholarsarchive@albany.edu.
BIOPHYSICAL INVESTIGATIONS OF THE MOLECULAR BASIS OF CATARACT ASSOCIATED WITH THE R76S MUTATION IN HUMAN γD-CRYSSTALLIN

By

Vurghun M. Ahmadov

A Thesis

Submitted to the University at Albany, State University of New York

in Partial Fulfillment of

the Requirements for the Degree of

Master of Science

College of Arts & Sciences

Department of Chemistry

2011
Acknowledgements

Foremost, I would like to express my sincere gratitude to my advisor Prof. Jayanti Pande for the continuous support of my study and research, for her patience, motivation, enthusiasm, and immense knowledge. Her guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my study.

Especially I would like to thank Dr. Ajay Pande for his guidance during my research and study. He has always been accessible and willing to help me with my research and studies. As a result, research life became smooth and rewarding for me.

I sincerely acknowledge my committee member Prof. Carla Theimer for her support and encouragement.

I would like to thank all my lab members Dr. Priya Banerjee, Dr. Kalyan Ghosh, Dr. Geo Rajan, Michelle Audi, who made it a convivial place to work. In particular, I would like to thank Dr. Priya Banerjee for his friendship and help in the past two years. Lastly, and most importantly, I wish to thank my parents, Mammad Ahmadov and Gulnara Ahmadova and my brother Toghrul Ahmadov. They bore me, raised me, supported me, taught me, and loved me. To them I dedicate this thesis.
Table of Contents

Chapter-1: Introduction ........................................................................................................... 1
  1.1 Human eye lens and its transparency ......................................................................... 1
  1.2 The crystallins ............................................................................................................. 2
  1.3 Genetic mutations and hereditary cataract .............................................................. 3
  1.4 The R76S mutation ..................................................................................................... 4

Chapter-2: Experimental methods ...................................................................................... 4
  2.1 Cloning and expression ............................................................................................ 8
  2.2 Purification of proteins ............................................................................................ 8
  2.3 Preparation of the R76S mutant .............................................................................. 9
  2.4 Gel Electrophoresis (SDS-PAGE and Isoelectric focusing) and Western blotting ... 10
  2.5 Circular Dichroism (CD) Spectroscopy .................................................................. 11
  2.6 Fluorescence Spectroscopy .................................................................................... 11
  2.7 Thermal Denaturation of HGD and R76S .............................................................. 12
  2.8 Liquid-liquid phase separation ............................................................................. 13
  2.9 Quasi-elastic light scattering ................................................................................ 14
  2.10 NMR spectroscopy ............................................................................................... 15

Chapter-3: Results ................................................................................................................... 17
  3.1 Structure and stability ............................................................................................. 17
  3.1.1 Near-UV CD Spectroscopy ............................................................................... 17
  3.1.2 Far-UV Spectroscopy ....................................................................................... 17
  3.1.3 Fluorescence studies ......................................................................................... 19
    3.1.3.A Tryptophan Fluorescence Emission Spectroscopy ..................................... 19
Table of Figures

Chapter-1

| Figure 1 | 1 |
| Figure 2 | 5 |
| Figure 3 | 6 |
| Figure 4 | 7 |

Chapter-2

| Figure 5 | 14 |

Chapter-3

| Figure 6 | 18 |
| Figure 7 | 19 |
| Figure 8 | 20 |
| Figure 9 | 21 |
| Figure 10 | 22 |
| Figure 11 | 23 |
| Figure 12 | 24 |
| Figure 13 | 25 |
| Figure 14 | 27 |
| Figure 15 | 28 |
| Figure 16 | 29 |
| Figure 17 | 31 |
| Figure 18 | 32 |
| Figure 19 | 33 |
**List of Abbreviations and Symbols Used**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bis-ANS</td>
<td>4,4'-bis(anilinonaphthalene-8-sulfonate)</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>CRYGD</td>
<td>human γD-crystallin gene</td>
</tr>
<tr>
<td>Ct-HGD</td>
<td>C-terminal domain of HGD</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>HGD</td>
<td>human γD-crystallin</td>
</tr>
<tr>
<td>HSQC</td>
<td>heteronuclear single quantum coherence</td>
</tr>
<tr>
<td>IEF</td>
<td>isoelectric focusing</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl 1-thio-D-galactopyranoside</td>
</tr>
<tr>
<td>LLPS</td>
<td>liquid-liquid phase separation</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>Nt-HGD</td>
<td>N-terminal domain of HGD</td>
</tr>
<tr>
<td>Nt-R76S</td>
<td>N-terminal domain of P23T</td>
</tr>
<tr>
<td>PDB</td>
<td>protein data bank</td>
</tr>
<tr>
<td>pI</td>
<td>isoelectric point</td>
</tr>
<tr>
<td>R14C</td>
<td>Arg14 to Cys mutant of HGD</td>
</tr>
<tr>
<td>R36S</td>
<td>Arg36 to Ser mutant of HGD</td>
</tr>
<tr>
<td>R58H</td>
<td>Arg58 to His mutant of HGD</td>
</tr>
<tr>
<td>Rₜh</td>
<td>hydrodynamic radius</td>
</tr>
<tr>
<td>SE</td>
<td>size exclusion</td>
</tr>
<tr>
<td>Tₜc</td>
<td>critical temperature</td>
</tr>
<tr>
<td>Tₜcloud</td>
<td>cloud point temperature</td>
</tr>
<tr>
<td>Tₜm</td>
<td>melting temperature</td>
</tr>
<tr>
<td>Tₜph</td>
<td>phase separation temperature</td>
</tr>
</tbody>
</table>
ABSTRACT

Cataract disease results when the eye lens becomes opaque and scatters a significant part of the incoming light into the eye. The lens contains very high concentrations of the lens proteins, called crystallins, which are present at concentrations comparable to those found in protein crystals (about 400-600 mg/mL). Chemical modifications of the crystallins, such as oxidation and deamidation, or genetic mutations are known to result in increased light-scattering \textit{in vitro}, and are implicated in cataract formation \textit{in vivo}. Here we present the \textit{in vitro} work on a mutant protein of human γD crystallin (HGD), namely R76S (i.e. Arg 76 to Ser substitution) which is associated with juvenile cataract. Our spectroscopic studies show that the mutant protein has secondary and tertiary structures identical to those of the native protein. Its thermal stability also appears to be unaltered by the mutation. The observed differences between the wild-type and mutant proteins are: (a) the pI of R76S is lower by 0.4 pH units relative to HGD, which is expected since the basic Arg residue is replaced by a neutral Ser, (b) small chemical shift differences in the HSQC spectra of the two proteins; which indicate that there are minor differences in their structure that are localized near the mutation site, and (c) the R76S mutant shows a slightly higher level of Ca$^{2+}$ ion binding than the wild type, although the difference is less than 10%, and is unlikely to lead to lens opacity.

These differences were observed when both proteins were known to be monomeric and in their normally-folded state. However, other differences between the
two proteins relate to the aggregation behavior of these proteins: These are: (a) the R76S mutant at pH 7 and 37 °C gives rise to large aggregates (~200 times larger than HGD) even under reducing conditions (i.e. in the presence of DTT) — conditions under which HGD remains almost totally monomeric, and (b) SDS-PAGE analysis of R76S shows a distinct band corresponding to a tetramer, in addition to the monomer band. The tetramer band is not observed for HGD. When excised and re-electrophoresed in the presence of an excess of DTT, the tetramer band is partially monomerized. Notably however, the tetramer band does not form if the mutant sample is boiled briefly prior to the SDS-PAGE analysis. Heating the protein sample under reducing conditions prior to analysis, leads to monomerization. These data led to our current working hypothesis that minor structural perturbations near the mutation site in R76S render the nearby Cys residue(s) vulnerable to intermolecular S-S crosslinks. Such interactions are likely to be followed by other attractive (for example, hydrophobic) interactions, culminating in the formation of a stable tetrameric species observed in the SDS-PAGE analysis. It is possible that these oligomers are stabilized by SDS as described above; while in its absence, much larger aggregates are observed using dynamic light scattering. These types of large protein aggregates could be responsible for light scattering in vivo. Further studies would be required to substantiate these findings.
Chapter – 1

1. INTRODUCTION

1.1 Human eye lens and its transparency

The human eye lens is a transparent structure that refracts light entering through the cornea and focuses it on the retina. It has three main parts: (1) the lens capsule which separates the lens from its surroundings, (2) the lens epithelial layer which is located between the capsule and the lens fiber cells and is present only in the anterior part of the lens, and (3) the lens fibers, which form—the bulk of the lens. (Figure-1) The transparency of the lens results from the appropriate architecture of the lens cells and the unique tight packing of their proteins. The high protein content (protein concentrations above 400 mg/mL) is essential for maintaining a high refractive index thereby minimizing light scattering and keeping the lens transparent. [1]

Figure-1: Structure of mature human lens. This image is adapted from J. F. Hejtmanick, Seminars in Cell and Developmental Biology 19 (2008) 134-149
1. The crystallins

Over 90% of the weight of the lens is due to water soluble proteins called the crystallins [2]. There are three types of crystallins in the vertebrate eye, α-, β-, and γ-crystallins. This classification is based on their elution from gel filtration chromatography. The α-crystallins elute first from gel filtration chromatography with an average molecular weight of 800 kDa, followed by the β-crystallins which emerge from the column with molecular weights ranging from 46 kDa to 200 kDa and γ-crystallins elute last in the monomeric fraction with a molecular weight around 20 kDa [38]. α-crystallins consist of many subunits with each subunit having an approximate molecular weight of 20 kDa. They can be found in other tissues besides the eye. They belong to the small heat-shock protein family and function like molecular chaperones. β- and γ-crystallins are primarily found only in the lens and are similar in structure and sequence and belong to the β-/γ-superfamily. The β-crystallins, naturally exist as oligomeric complexes, and can be classified as either “acidic” or “basic” β-crystallins. The apparent subunit molecular weight for the β-crystallins ranges from 23 kDa to 32 kDa [38]. The γ-crystallin family is composed of seven distinct proteins: γA, γB, γC, γD, γE, γF, and γS. They usually exist in the lens in monomeric form and have a molecular weight of 20 kDa as well [38].
2. Genetic mutations and hereditary cataract

Cataract is a disease that is caused by a clouding in the lens of the eye, which obstructs the passage of light to varying degrees [3, 33]. These lens opacities arise due to fluctuations in the refractive index of the lens milieu, often due to the formation of high molecular weight protein aggregates of 1000 Å or greater in size [4]. Cataract can be classified into the following types based on its etiology: age-related cataract, congenital cataract, and trauma-induced cataract [4]. Congenital cataracts can be defined by the age at onset with infantile cataracts presenting within the first year of life and juvenile cataracts presenting within the first decade of life. Between 8.3 and 25 percent of congenital cataracts are believed to be inherited (hereditary cataracts) [35]. Most congenital cataracts are associated with mutations in specific genes, half of which occur in the crystallin genes. These mutations occur in all three crystallin gene families: α-, β-, and γ. Among the seven γ-crystallin genes (CRGA (γA), CRGB (γB), CRGC (γC), CRGD (γD), CRDE (γE), CRGF (γF), and CRGS (γS)), the γ-crystallin genes that are most abundantly expressed in the human lens are CRGC, CRGD, and CRGS [41]. While mutations linked to childhood cataracts have been found in all three genes, those affecting CRGD seem to be more numerous [28]. It is well documented that single-point mutations in γD-crystallins are associated with congenital cataract [7, 8, 9, 13, 19]. In recent years, several mutations in the human γD-crystallin (HGD) gene have been reported which show a variety of cataract phenotypes. For example, a point mutation that results in substitution of Arg-14 to Cys in the human γD-crystallin gene causes
progressive juvenile-onset hereditary cataract [5]. Previous studies from this laboratory have shown that the mutant protein aggregates even at pH 4.5, by means of intermolecular disulfide crosslinking triggered by the introduction of the highly reactive Cysteine-14 residue [9]. The R36S and R58H mutations of the human γD-crystallin gene lead to a dramatic lowering of the solubility and rapid crystallization of the mutant proteins [6, 19, 42]. The P23T mutant of human γD-crystallin (HGD) also causes a dramatic lowering of protein solubility, but in addition, the mutant exhibits retrograde solubility relative to the wild-type protein, i.e. the solubility has an inverse dependence on temperature [7, 18]. This appears to be responsible for the macroscopic protein aggregate formation in vivo [7]. In all these cases, the change in solubility is due to altered γ-γ-interactions, and occurs without a significant change in protein structure or stability. In contrast, the E107A mutation not only shows a structure, and stability nearly identical to that of the wild-type protein, its solubility is also not altered relative to HGD. However, it was found that a change of one pH unit in the pI value of the mutant protein alters its interaction with a different crystallin – viz., α-crystallin, which is responsible for the increased light scattering and opacity in this case [8].

3. The R76S mutation

Causal single point mutation, namely R76S, in CRGD gene is associated with childhood cataract in patients from India [16]. Figure 2 shows a slit lamp image of an individual with anterior polar coronary cataract phenotype along with the underlying anterior cortex [29].
The R76S mutation in HGD, besides its association with human cataract, had other features of special interest to us. (1) Pande et al. [19] had shown previously that another Arg to Ser mutation in HGD (namely, R36S) lowered the solubility and led to rapid crystallization of the protein even at very low concentrations. Thus, we wondered if the same mutation in another region of the protein would have a similar outcome. Notably, in the HGD crystal structure, residues R36 and R76 are both surface-exposed; but R36 is in the middle of a β-strand, while R76 is in a loop just preceding a strand. (figure-3). (2) Yogendra Sharma and coworkers have long argued that β-γ crystallins are potentially calcium-binding proteins [26], although many members of this family have partially or totally lost this property due to the disabled calcium-binding motif. The authors argue that N/D-N/D-X-X-S/T-S is the original microbial canonical Ca\(^{2+}\)- binding motif, which in vertebrates is often present in a degenerate form. That motif in HGD is
SDSVRS, and in the mutant R76S becomes SDSVSS. Thus, the mutation renders the motif much closer in sequence to the motif that is implicated in Ca\(^{2+}\)-binding. Clearly therefore, we were interested in comparing the Ca\(^{2+}\)-binding ability of HGD and R76S.

Figure-3: Crystal structure of HGD showing residues Arg36 and Arg76 as well as the Ca binding motif SDSVSS.

Prior to undertaking the experimental work, we also tried to understand what changes in the properties of the mutant we could expect on the basis of the crystal structure of HGD. In the motif described above (Figure 3, residue 72 to 77), the R76S mutation gives rise to a cluster of Ser residues (4 out of 6 serines). This could enable new interactions of the mutant with water molecules due to the H-bonding potential of the Ser-OH. Specifically, we noted that in HGD, the NH(2) of R76 shares a water
molecule with the Ne2 of Q47. This water molecule is 3.56 Å away from the R76 NH(2) and 2.55 Å away from Q47 Ne2. This interaction would clearly be disrupted in the mutant. Furthermore, there is a cluster of 4 or 5 water molecules (within 2-5 Å) of the Ser 74 OH and Ser 72 OH, which would most likely undergo reorganization in the mutant. Therefore, we reasoned that these reorganizations could potentially alter protein solubility and the phase diagram, or possibly also disrupt the protein structure. Therefore, to evaluate if these considerations were valid, we performed a series of experiments to compare the phase diagram, stability and solubility of the mutant with wild-type HGD.

Figure-4: Crystal structure of the cluster with four Serine residues, Arginine and water molecules.
Chapter 2

2. EXPERIMENTAL METHODS

2.1. Cloning and expression

Recombinant human γD crystallin (HGD) and its R76S mutant were prepared according to established procedure [9]. The plasmid containing HGD coding sequence was a gift from Dr. Nicolette Lubsen from the University of Leiden. The recombinant protein is expressed in *E. coli*. For the overexpression of the protein, 25 ml overnight *E. coli* cultures (BL21 (DE3) Gold) were transferred into 1 L of LB medium and grown at 37 °C to an absorbance of 0.7-0.8 at 600 nm. Expression of the γ-crystallins was induced by the addition of isopropyl 1-thio-D-galactopyranoside (IPTG) to a final concentration of 1 mM, and then grown for additional 6-7 hours.

2.2 Purification of proteins

Cells were harvested by centrifugation and the cell pellet is resuspended in lysis buffer (10 mL buffer per one liter cell culture, 50 mM Tris.HCl containing 25 mM NaCl and 2 mM EDTA, pH 8) to which a “Complete” protease inhibitor cocktail tablet (Roche Molecular Biochemicals) was added at 1 tablet per 25 mL of cell suspension. The cell suspension was lysed with lysozyme (250 mg/mL) followed by five cycles of a rapid freezing in liquid nitrogen and then thawing in a water bath at 30 °C. DNase (1 mg/mL) was added to the cell lysate, incubated for 1-1.5 hours, and then the cell-pellet was
centrifuged at 48,000 X g. Both the supernatant and pellet were tested for the presence of γ crystallin by SDS-PAGE and Western Blotting. The proteins are fractionated almost exclusively into the supernatant. The purification of the protein from the supernatant is done in two steps. First, the supernatant is subjected to size exclusion (SE) chromatography which was performed on a Sephacryl S 200 HR column using 0.275 M sodium acetate buffer (pH 4.5) at a flow rate 2mL/min, followed by cation exchange chromatography on a SP-Sepharose fast flow column [10]. The protein is eluted during SE and then cation exchange with NaCl gradient of 0 – 0.3 M in 0.275 M sodium acetate buffer (pH 4.8). Both buffers contained 0.02% sodium azide. The purity of HGD is analyzed using electrospray ionization mass spectrometry (ESI-MS) at the Center for Functional Genomics at the University at Albany. The ESI-MS showed an average mass of 20609±2 units for five independent protein preparations, consistent with the published mass of HGD, which is 20 kDa. The concentration of HGD was determined using an extinction coefficient of 41.4 mM⁻¹cm⁻¹ at 280 nm [7].

2.3 Preparation of the R76S mutant

Site directed mutagenesis technique was used to introduce Serine in place of Arginine-76. Following oligonucleotide primers were made: GCG ACT CGG TCA GCT CCT GCC GCC TCA TCC CC as the forward primer and GGG GAT GAG GCG GCA GGA GCT GAC CGA GTC GC as the reverse primer. Site-directed mutagenesis was performed with the QuickChange site-directed mutagenesis kit (Stratagene). Primers were designed
(synthesized by MWG Operon) to introduce Ser in place of Arg76 to generate R76S mutant. The plasmid DNA obtained after mutagenesis was sequenced at the Biomolecular Core facility of the University at Albany and was found to contain the desired mutation but no other sequence changes. The rest of the procedures for the expression, isolation and purification were the same as described above for the wild type HGD. The ESI-MS showed an average mass of 20540±1 units for five independent protein preparations, consistent with the mass difference between Arginine and Serine.

2.4 Gel Electrophoresis (SDS-PAGE and Isoelectric focusing) and Western Blotting

SDS-PAGE, Western blotting and Isoelectric focusing were performed on polyacrylamide gels using the Bio-Rad Power Pac 1000 electrophoresis system. Polyacrylamide Gel Electrophoresis (PAGE) is done in the presence of detergent Sodium Dodecyl Sulfate (SDS). Protein samples were mixed with 1:1 ratio of Laemmli sample Buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) before loading into the gel. 12% gels were run at constant 200V voltage for 40 mins. Visible proteins markers from Bio-Lyte were used as a standard. Protein bands were detected using 0.1% Coomassie Blue G-250 staining solution. Gel images were captured using the Chemi-doc imager from Bio-rad.

Western Blotting was performed by transferring the protein bands from Polyacrylamide gels onto PVDF membrane using Bio-Rad Power Pac 1000
electrophoresis system. Immuno-Specific detection was performed by adding antibody solutions and proteins were detected by chemiluminescence using Bio-Rad Chemi-doc imager.

Isoelectric focusing was performed using the Model 11 Mini IEF Cell by Bio-Rad, with polyacrylamide being as a choice of support matrix. Ampholyte and catalyst solutions for running the IEF Gel were prepared by diluting Bio-Lyte stock solutions. Focusing was carried out under constant voltage in a stepped fashion: 100 V for 15 minutes, 200 V for 15 minutes and 450 V for 60 minutes. Visible marker proteins of known pl by Bio-Lyte were used as a standard. Proteins were detected using Coomassie blue G-250 staining solution containing 0.025% dye. Gel images were captured using Chemi-doc XRS+ system imager by Bio-rad.

2.5 Circular Dichroism (CD) Spectroscopy

CD spectra were recorded on a JASCO J-815 spectropolarimeter equipped with a Peltier type temperature controller. For Near UV CD spectra, a protein concentration of 0.5mg/mL in 0.1 M sodium phosphate buffer (pH 7) was used and spectra were obtained in a 10 mm path length cuvette. Data were normalized with respect to the protein concentration. For far UV CD spectra, a protein concentration of 0.1 mg/mL in 0.1 M phosphate buffer was used and spectra were obtained in a 1.0 mm path length cuvette. Data were normalized with respect to the concentration of peptide bonds.
2.6 Fluorescence Spectroscopy

Tryptophan and Bis-ANS fluorescence spectra were obtained with a Horiba Jobin Yvon flurolog-3 spectrometer. Tryptophan fluorescence emission was scanned with an excitation wavelength of 290 nm and emission recorded in the 250-350 nm region.

Bis-ANS fluorescence emission spectra were scanned between 460 and 560 nm with an excitation wavelength of 390 nm. The excitation and emission slits were set to 5 nm for all measurements. Spectra were measured using the same cuvette and identical protein concentrations (0.1 mg/mL) for HGD and R76S in 0.1 M phosphate buffer (pH 7). The spectra were corrected by subtracting the contribution of the buffer. Bis-ANS binding fluorescence emission spectra were recorded at a protein concentration of 0.1 mg/mL (5 μM) and a Bis-ANS concentration of 100 μM. A stock solution of Bis-ANS was prepared in methanol and the final alcohol concentration was maintained at less than 7% v/v, when the reagents were mixed with proteins. The concentration of Bis-ANS was measured using an extinction coefficient of 16.8 mM$^{-1}$ cm$^{-1}$ at 385 nm.

Terbium binding experiments to investigate Ca$^{2+}$ binding were done by exciting the protein and terbium chloride mixtures at 290 nm and recording the emission in the 450-550 nm region. Proteins at concentrations of 75 μM were titrated with terbium chloride solutions, with a final concentration of the latter being 0.75 mM, 1.5 mM and 5 mM. Samples were prepared in 50 mM Tris-HCl, pH 6.4 buffer. Aliquots of terbium chloride were added to the protein solution, and spectra were recorded.
2.7 Thermal Denaturation of HGD and R76S

To compare the thermal stability of HGD and R76S, near UV CD spectroscopy was used as a probe and the change in ellipticity at 290 nm as a function of temperature was recorded. Data were recorded on a JASCO J-815 CD spectropolarimeter equipped with a Peltier type temperature controller. 10mm path length cuvettes of were used, and protein solutions were prepared in 0.1 M phosphate buffer (pH 7) with a protein concentration of 0.5 mg/mL. Spectra were collected at 5 °C intervals from 30 °C to 65 °C and 2 °C intervals from 66 °C to 90 °C. Samples were equilibrated before each scan and each spectrum was taken as an average of 4 scans. The loss of ellipticity at 290 nm at higher temperatures was interpreted as thermal unfolding of the proteins and the melting point ($T_M$) of the proteins was obtained from the thermal unfolding profiles.

2.8 Liquid-liquid phase separation

γ-crystallins undergo liquid-liquid phase separation in concentrated proteins solutions [38] A plot of the phase separation temperature, $T_{ph}$, as a function of protein concentrations is called the liquid-liquid coexistence curve (see figure-4).

The proteins were concentrated using an ultrafiltration technique (Amicon Centricon). Protein solutions were stored at above the phase separation temperature of 3 °C (see figure-7), but well below the denaturation temperature for γ-crystallins, which is 65-70 °C [11]. Protein concentrations were determined using UV absorption spectroscopy. Small aliquots (5-10 μL) were diluted in a known volume of 100mM
phosphate buffer (pH 7) and the resulting UV absorbance was measured. A positive displacement pipette (Gilson Microman) was used to pipette highly concentrated (viscous) protein solutions. The phase separation temperatures were measured by using previously described cloud-point method [11]. In this method, a tube (Kimax-51, glass, 6mmX50mm from Kimble Glass Inc) containing the protein solution is kept in a water-bath. As the temperature of the bath is lowered, the solution becomes cloudy, at T1 for example. Then the bath-temperature is raised until the protein solution becomes clear again at a temperature T2. The average of T1 and T2 is then taken as the phase-separation temperature.

Figure-5: Schematic representation of a coexistence curve for liquid-liquid phase separation. The phase boundary (curved line) separates the single-phase region from the two-phase region.
2.9 Quasi-elastic light scattering

The hydrodynamic radii ($R_H$) of proteins were determined at 25 °C and 37 °C using dynamic light scattering (DLS) in a Zetasizer-Nano analyzer (Malvern instruments). Protein samples were prepared in 100 mM phosphate buffer (pH 7), containing 20 mM DTT for the measurements at room temperature, and in another series of experiments at 37 °C in 100 mM phosphate buffer (pH 7.5), in the absence and presence of 10 mM DTT. $R_H$ was measured as a function of concentration, and as a function of time in the presence of Sodium Dodecyl Sulfate (SDS). Samples were filtered through a 0.22 μM filter (Millipore, Bedford, MA). Protein concentrations were determined using published extinction coefficients as described above.

2.10 NMR spectroscopy

For NMR experiments, *Escherichia coli* cells were grown in LB broth until induction. Subsequently, cells were transferred to the M9 minimal media that contains 1g/L [U-¹⁵N] NH₄Cl and 2 g/L D-glucose (for [U-¹⁵N]-labeled protein). After harvesting the cells, the protein was purified and obtained in soluble form as described for the unlabeled protein [12]. Protein samples in the concentration range 0.3–0.5 mM were dissolved in the NMR buffer (10 mM KPO₄ (pH 7.0), 100 mM NaCl, 0.02% NaN₃, 90% H₂O and 10% D₂O).

NMR spectra were collected at 25 °C in a Bruker Avance spectrometer, operating at a $^1$H frequency of 700 MHz, and equipped with a z-axis gradient TCI cryoprobe. A standard double resonance NMR experiment, the 2D $^1$H–¹⁵N HSQC was used in order to
compare backbone chemical shifts with assigned HGD shifts [13]. R76S spectra were processed using TOPSPIN 2.1 (Bruker, Inc.) and the spectral analysis was carried out using CARA [14]. Chemical shift differences (shown in Fig. 26), are weighted averages for each $^1$H/$^{15}$N pair, and calculated as $\left(\frac{\Delta \delta_{NH} + \Delta \delta_{N}}{25}/2\right)^{1/2}$, as in [15], where $\Delta \delta_{NH}$ and $\Delta \delta_{N}$ are differences in the amide $^1$H and $^{15}$N chemical shifts for HGD and R76S, respectively. These experiments were carried out by Priya Banerjee in Prof. Alex Shekhtman’s laboratory.
Chapter 3

1. RESULTS

In order to determine the molecular basis of lens opacity due to the Arg76 to Ser mutation in HGD as discussed in the Introduction (page -2), I carried out the experiments described below.

3.1 Structure and Stability

In the initial survey to determine the effects of the Arg76 to Ser mutation, changes in the secondary and tertiary structure of the protein were, probed by comparing the near- and far- UV CD and tryptophan fluorescence emission spectra of HGD and R76S. These spectroscopic methods have not only been successfully applied by many laboratories working on different proteins, but also specifically to the γ-crystallins [7, 8, 9, 13, 19].

3.1.1 Near-UV CD

The CD spectrum in the near-UV spectral region (250-350 nm) is sensitive to the tertiary structure of a protein, as it probes in particular, chirality due to the aromatic amino acid residues and disulfide bonds. The CD spectra in the near-UV region of γ-crystallins arise primarily due to the Trp residues. These are 4 invariant Trp residues – two each in the N- and C-terminal domains. Even in the primary sequence they are evenly dispersed – Trp 42 and 68 forming a part of the 2nd “greek key” motif and Trp 130...
and 156, in a more-or-less equivalent position in the C-terminal domain, forming a part of the 4\(^{th}\) greek-key motif. The significance of this distribution, for our purpose here, is that tryptophan residues in \(\gamma\)-crystallins are an excellent intrinsic probe of the overall protein structure.

The spectra for HGD and R76S are compared here to determine if there are any large changes in the tertiary structure of the mutant protein. Figure-6 shows that the near-UV CD spectra of HGD and R76S are closely similar, suggesting that this cataract-causing mutation does not significantly alter the tertiary structure of the mutant protein.

![Figure 6: Near-UV CD spectra of HGD and R76S in 0.1 M phosphate buffer at pH 7.1. Protein concentration is 0.5 mg/mL.](image-url)
3.1.2 Far-UV CD Spectroscopy

Far-UV CD Spectroscopy was used to examine the secondary structure of the wild-type and mutant proteins. The CD spectrum in the far UV region (190-250 nm) provides an empirical measure of the overall secondary structure of a protein. Thus, β-helix, β-sheet, and random coil structures each give rise to a characteristic shape and magnitude of the far-UV CD spectrum. Figure-7 shows that the secondary structures of the two proteins are nearly indistinguishable. Both proteins have far-UV CD profiles typical of predominantly β–sheet rich proteins.

![Graph showing Far-UV CD spectra of HGD and R76S in 5mM phosphate buffer at pH 7.1.](image)

Figure 7: Far-UV CD spectra of HGD and R76S in 5mM phosphate buffer at pH 7.1. Protein concentration was 0.1 mg/mL.
3.1.3 Fluorescence studies

1. Tryptophan Fluorescence Emission Spectroscopy

Gamma crystallins contain four buried tryptophan residues [17], two in each domain and the overall structural integrity of the protein can be determined by measuring the intensity and the $\lambda_{\text{max}}$ values for HGD and R76S. Figure-8 shows that the fluorescence emission spectra of HGD and R76S are comparable. The intensities are almost equal and the $\lambda_{\text{max}}$ values are 326 nm and 325 nm, respectively, indicating that the tryptophan residues are buried in the mutant as in the wild-type protein. The slight difference in quantum yield is observed among different members of gamma-crystallin family. [17]

![Fluorescence spectra](image)

Figure-8: Tryptophan fluorescence emission spectra of HGD and R76S in 0.1M phosphate buffer at pH 7.1 at a protein concentration 0.02 mg/mL.
2. **Bis-ANS fluorescence to determine surface hydrophobicity**

The surface hydrophobicites of HGD and R76S were determined by reacting both proteins with Bis-ANS, a well-tested fluorescent probe [18]. Figure-9 shows the fluorescence emission spectra of both proteins when bound to Bis-ANS. The spectra are shown after subtraction of the emission spectrum of Bis-ANS alone. The Bis-ANS data does not show significant changes in the net surface hydrophobicity for R76S, compared to HGD, since both proteins have emission intensity maxima at 515 nm, and comparable emission intensities. The difference seen here is negligible compared to the difference observed in another mutant of HGD, namely P23T. [18].

![Figure-9: Fluorescence emission spectra of HGD and R76S in mixtures with Bis-ANS. Spectra are corrected for Bis-ANS by subtraction of its contribution. The protein concentration was ~5 μM, and the Bis-ANS concentration was 100 μM. The excitation wavelength was 390 nm.](image-url)
3.2 Thermal stability

Loss of ellipticity at 290 nm in the near-UV CD spectrum of gamma crystallins in 0.1M sodium phosphate buffer, pH 7, upon heating is associated with unfolding of the protein. Figure 10 shows the change in the near-UV CD of R76S as the protein unfolds in the temperature range of 30-80 °C. Spectral changes observed here, are very similar to the published spectra for HGD [8]. From this data, if we now plot the change in ellipticity at 290 nm as a function of temperature, we obtain the data shown in Figure 11.

Figure-10: Change in the near-UV CD of R76S due to thermal unfolding.

Figure 11 therefore shows the thermal stability profile of R76S (in 0.1 M sodium phosphate buffer, pH 7) measured as change in ellipticity at 290 nm, versus temperature. The midpoint of the transition corresponds to the melting point ($T_m$) of the protein. The protein appears to unfold in a single step without forming a stable
intermediate and the melting temperature does not significantly vary from that of HGD. 

T_m values for R76S is 77.7±3 °C as compared to 79.3±2 °C for HGD. Since a 2-4 °C difference in T_m is typically observed among members of the γ–crystallin family, the reduction in the case of R76S mutant is not significant and the mutant protein appears to be virtually as stable as the wild type. [17, 18].

Figure-11: Thermal stability profile of R76S (red) and HGD [17, 18] (black) in 0.1 M sodium phosphate buffer, pH 7, measured as change in ellipticity at 290nm, versus temperature.

3.6 Isoelectric Focusing

The mutation from Arg76 to Ser is expected to lower the pl, as a basic residue is replaced by a neutral residue. Indeed, as shown by the isoelectric focusing gel (IEF) in Figure 12, R76S and HGD have pl values of 6.9 and 7.3, respectively. The pl values were assigned based on the pl standard (Bio-rad) run alongside.
3.7 Comparison of the solubility of HGD and R76S at pH 7

In order to determine the lowest protein concentration at which the R76S mutant formed visible aggregates relative to HGD, we exchanged both proteins from 0.275 M acetate buffer at pH 4.5 to 0.1 M sodium phosphate buffer pH 7, containing 20 mM DTT, and concentrated them using ultrafiltration devices. We found that R76S could be concentrated up to 300 mg/mL without forming any visible aggregates, just like the wild type protein [8]. Thus, we concluded that the R76S mutant was comparable to the wild type not only in structure and stability, but also in its high solubility as well. This observation is in dramatic contrast to that of the R36S mutant published earlier from this laboratory and shown to be only marginally soluble and
prone to rapid crystallization, while essentially maintaining its protein structure.

**Liquid-liquid Phase separation measurements**

Unlike most other cataract associated mutations that show higher phase separation temperature, R76S shows a similar liquid-liquid coexistence curve with that of HGD. The phase diagram (Fig. 7) was obtained by using published methods, as described in the Methods section (page 14) [9]. The average of $T_{\text{cloud}}$ and $T_{\text{clarify}}$ was taken as a phase separation temperature. In figure 13, the liquid-liquid phase diagram of R76S and HGD are shown.

![Liquid-liquid coexistence curves of HGD and R76S in 0.1M sodium phosphate buffer, pH 7, containing 20mM DTT. The data obtained in the present study (green triangle, R76S; red circle, HGD), are compared with the published HGD* data (black square). [9]](image.png)
The coexistence curve for HGD used as a control in our experiments (red curve, HGD\textsubscript{control}) closely follows the published curve for the wild type (black curve, HGD). The coexistence for the mutant is comparable to that of HGD within the limits of experimental error. The apparent lowering of the coexistence curve for R76S (green curve) relative to HGD may not be significant given the uncertainly in the measured data.

3.9 Measurement of $R_H$, the hydrodynamic radius using Quasi-elastic light scattering (QLS)

In order to compare the sizes of HGD and R76S, we used QLS to measure their hydrodynamic radii which reveals their size distribution in solution, the apparent $R_H$ was measured as a function of concentration and is shown in Figure 14. The $R_H$ of both proteins is around 2.3 nm, which is consistent with previously published data [22], and confirms that the mutant is monomeric just like the wild type. The $R_H$ for both proteins increases slightly as they are concentrated, probably due to a concomitant increase in the viscosity of the solution.
Figure 14. Concentration dependence of the apparent hydrodynamic radius, $R_{\text{H}}$, of HGD and R76S.

Figure 15 shows the size distribution of both proteins across a wide concentration range. It can be seen that the intensities of the scattered light and width of the peaks for wild type and mutant protein are comparable. Both proteins show monodisperse size distribution even at higher concentrations.
Figure 15: Size distribution of HGD and R76S as determined by dynamic light scattering at different protein concentrations. The protein samples were prepared freshly each time for size measurements in order to prevent aggregation.

Figure 16 shows the size distribution of HGD and R76S over a period of time in 0.1 M sodium phosphate buffer, pH 7, containing 20 mM DTT. Every 24 hours, samples were buffer-exchanged against the same buffer containing fresh reducing agent, in order to account for the air oxidation. This experiment was also done without a reducing agent in the buffer. Both proteins, in that case, form relatively similar amounts of larger aggregates showing a second distribution, with ~10% percent of higher aggregates.
Figure 16: Size distribution of HGD and R76S determined by dynamic light scattering at constant protein concentration over a period of time.

Figure 17 shows time-dependent study of the wild type and the mutant protein and the mixture of both proteins at physiological conditions, namely 37 °C, pH 7.5, 100 mM sodium phosphate buffer. The motivation for testing the 1:1 mixture of HGD and R76S comes from the fact that R76S mutation is known as an autosomal-dominant type, (see introduction, page 2), which would result in one copy each of the normal and the mutated protein in vivo. Furthermore, at the molecular level, we argued that the loss of charge (Arg 76) in the mutant protein might favor the electrostatic attractive interaction between the normal and the mutant protein.
Protein concentrations were the same for individual proteins and the mixture. After few hours of incubation, the mutant protein and the mixture start forming higher molecular aggregates, not present initially. Both the mixture and the mutant protein show the presence of higher aggregates very rapidly, while HGD remains monomeric. That is the case at least until 48h; eventually in a week, no more monomeric form remained in any solution. It should be emphasized that these studies were conducted at 37 °C and a slightly alkaline pH, namely 7.5, in contrast to the previous results (Figs. 15 and 16), which were obtained at ~25 °C and pH 7.0. These conditions were adopted to evaluate if we could distinguish between the aggregation profiles of the two proteins, since the slightly alkaline pH should increase the rate of possible disulphide-mediated protein aggregation. As the results show, there was no significant difference between the two proteins also in these studies.
Figure-17: Size distribution of HGD, R76S and their 1:1 mixture as determined by dynamic light scattering at constant protein concentration over a period of time.

Figure 18 shows the results for the above experiment in the presence of 10mM DTT. After few hours of incubation, the mutant protein forms higher molecular aggregates and the intensity of the monomeric peak decreases, whereas the wild type protein and the mixture remain mainly in monomeric form. In these experiments, the mutant protein showed a more pronounced aggregation with time compared to the mixture. HGD remained largely monomeric.
Figure-18: Size distribution of HGD, R76S and their 1:1 mixture as determined by dynamic light scattering at constant protein concentrations over a period of time.

Figure 19 shows the results from the DLS experiments of the N-terminal domain of R76S and HGD (Once you define the mutant and wild type, use those symbols as much as possible rather than “mutant protein” and wild-type protein) and a 1:1 mixture of the two proteins at under the same conditions and concentrations as with the full length protein. From the data shown it is very clear that the N-terminal domain of R76S starts forming higher molecular aggregates after a few hours of incubation under these conditions, whereas the 1:1 mixture of the N-terminal domains of these two proteins forms smaller aggregates with less scattering intensity, and the N-terminal domain of HGD remains mainly in the monomeric form.
3.10 Calcium binding properties

The normal mammalian lens has around 0.2 mM calcium, of which the concentration of free Ca$^{2+}$ is only in the order of a few microMolar (μM) [24]. Cataracts can occur both under hypo-calcemic and hyper-calcemic conditions [25]. A localized increase in the calcium ion concentration is associated with increased light scattering in the lens [40]. This is the reason for the overall interest in calcium-binding vis-à-vis cataract.
As stated in the introduction, Sharma et al. [26], reported that γ-Crystallin binds calcium, but this observation remains controversial because others (Slingsby and Jaenicke) find no evidence of calcium binding by the γD-crystallins, even though binding has been observed in microbial proteins that are homologous to the γD-crystallins [37]. One problem is that Ca\(^{2+}\)-binding is very weak, and not detectable by ITC (isothermal titration Calorimetry) for any of the γD-crystallins. Indirect methods such as measurement of Terbium binding in place of Calcium, are somewhat questionable. For example, the Terbium-ion fluorescence method used here depends on the fluorescence resonance energy transfer (FRET) between the Tb-binding site (generally regarded as equivalent to Ca-binding sites) and a nearby Trp. Thus, in this method, one excites Trp residues and looks for Tb emission, along with a corresponding lowering in the Trp emission. But this is not observed, presumably because it is below the detection limit of the instrument. Therefore, we consider the difference observed in the Tb-emission for the mutant relative to the wild type, to be insignificant at present. These data are therefore preliminary and in order to gain a better understanding of calcium binding, a careful analysis of calcium-binding with positive and negative controls, would be required, as well as additional methods to confirm such binding. That is beyond the scope of this thesis.

With this caveat, we performed the studies described in Figure 20 as follows:

Calcium binding to γD-crystallin was probed using the luminescent lanthanide probe TbCl\(_3\). Tb\(^{3+}\) is known to bind to Ca\(^{2+}\) binding sites and shows enhanced
luminescence in the visible spectral region [27]. Following this procedure, we have observed Tb\(^{3+}\) binding as shown in Figure 20. It shows that both HGD and R76S bind the Ca\(^{2+}\)-mimic lanthanide ion. The difference in the emission intensities of both proteins in increasing TbCl\(_3\) concentrations is comparable. Specifically, we note \(~5-10\%\) higher binding for the mutant compared to HGD. We believe this increase is not significant enough to have any functional implication.

Figure-20: Terbium ion binding to HGD and R76S. 75M proteins were reacted with 0.75mM and 1.5mM TbCl\(_3\) in 50mM Tris.HCl, pH 6.4. The excitation wavelength was 290 nm. Thus the luminescence signal observed is in fact excited by protein-Trp emission. This mechanism of fluorescence excitation is generally called Foerster Resonance Energy Transfer or FRET.
3.11 SDS-PAGE ANALYSIS

A major difference between HGD and R76S is observed in the SDS-PAGE analysis. R76S shows monomeric and tetrameric bands in 12% Polyacrylamide gels, whereas HGD does not. As shown below both proteins show the monomer protein band around 20K at pH 4.5 (left panel, Figure 21) and pH 7 (right panel, Figure 21). In addition, R76S clearly shows a band, which corresponds to a tetramer, at low pH as well as physiological pH. These tetramer bands are not observed for HGD.

Figure-21: SDS-PAGE gels showing R76S tetrameric aggregates formed at both low (left) and physiological (right) pHs. The amount of protein samples loaded and the pH conditions differ in these two gels, which is reflected in the band intensities.
Figure-22: SDS-PAGE gel showing that if the tetramer band shown in Figure 21 at pH 7 is extracted and rerun on an SDS-PAGE gel, it once again shows a distribution of monomeric R76S and tetrameric R76S.

In order to determine whether the teramer band seen for R76S represented disulfide-linked (i.e. reducible) oligomers, this gel band was excised, and incubated with excess DTT in a slurry form, and reloaded for SDS-PAGE analysis. The resulting gel is shown in Figure 22. A significant part of the tetramer now appears to have dissociated into monomers, although some of the tetramer band still persists. We believe this band may represent tetramers resulting from non-reducible attractive interactions. The nature of these putative attractive interactions remains to be determined. The gel (Figure 22) also shows the presence of other bands, which appear to be dimers and trimers. Usually when disulfide-linked oligomers are reduced they lead to the formation of various “smaller” oligomers formed by intermolecular disulfide
crosslinking, by the rearrangement or “scrambling” of the remaining disulfides and sulphydryls [39].

This difference is not observed between HGD and R76S when the protein samples are boiled for 60 seconds at 90 °C before loading on the gel, as can be seen from Figure-23. This suggests that the tetramer state in the mutant is stabilized in the absence of boiling, probably because the mutant is only partially denatured by SDS alone. Interestingly, this small variation in procedure leads to the first tangible difference between the wild-type and mutant proteins.

Figure-23: SDS-PAGE gels showing no difference between R76S and HGD when boiling samples before loading.
3.12 HSQC Spectra of HGD and R76S

Figure 24 shows an overlay of the $^{15}$N-$^1$H HSQC spectra of HGD and R76S. It provides a fingerprint of the 3D structure of protein in 2-dimensions. Each backbone amide group, except proline, is expected to contribute a single cross peak. In our laboratory, ~90% of these amides cross peaks have been assigned to the contributing amide residues. The data confirm that both proteins are fully folded and closely similar in tertiary structure as shown by the CD spectra. The small displacements in some cross peaks, quantified as average chemical shift differences, are plotted in Figure 25 as a function of residue number,

![Figure-24. Overlay of $^{15}$N-$^1$H HSQC spectra of HGD and R76S. The red contours represent HGD and the blue contours represent R76S.](image-url)
The data reveal that most of these changes, greater than 0.1 ppm, are localized in the N-terminal domain, especially close to the mutation site. These chemical shift differences are mapped in the high-resolution crystal structure of HGD in the next page (Figure-28).

Figure-25. Weighted average chemical shift differences in HGD and R76S calculated using \([([Δδ_{NH}^2+Δδ_N^2}/25)/2]^{1/2}\)
3.13 A Computational model for the binding of R76S to SDS

As described in the Experimental Section, we found that SDS binds to the mutant protein in a manner distinct from that of the wild type. In an attempt to determine the binding interactions of SDS on the surface of R36S which could help us understand how the tetramer could be stabilized by SDS, we modeled SDS binding where the NMR affected residues are located, close to the mutation site, R36. Figure 26 shows the docking results of SDS binding to HGD and R76S. The sulfate group of SDS is close to the 76th residue in both results, however layout of the hydrophobic chain of SDS is different in two different protein binding sites.

Figure 26. Docked conformation showing the SDS binding conformation around the mutation site (blue spheres, represent the SDS binding conformation to HGD, green spheres are the SDS binding conformation to R76S).
Chapter 4

3. DISCUSSION

A recent report [29] on the DNA sequencing analysis of the CRGD gene of patients from India with childhood cataracts shows a novel heterozygous missense mutation C>A at position 229 in CRYGD in three affected members of family C-35 with anterior polar coronary cataract. This variation, C229A, results in the substitution of arginine at position 76 by serine (R76S). The study was performed on patients with familial non-syndromic bilateral childhood cataracts. Among these patients, zonular cataract was most frequent (46%), followed by total (13%) posterior subcapsular (10%), and nuclear cataracts (8%), and the age at onset being within the first decade of life. The proband underwent a surgery for cataract removal at the age of 11 years. The right eye showed the phenotype as anterior polar with coronary cataract and the left eye with only anterior polar cataract with a progressive loss of vision.

In recent years, a number of studies have been published in an effort to understand the underlying molecular mechanism of lens opacity associated with several mutants of HGD. The substitution of Arg14 by Cys, for example, leads to rapid protein aggregation via intermolecular disulfide crosslinking due to the highly reactive Cys14 [5]. The Pro23 to Thr mutation dramatically lowers the protein solubility, due to the association of the P23T mutant to form a new condensed phase that contains clusters of the mutant protein [7]. R58H mutation leads to crystal formation which is responsible
for the lowered protein solubility and enhanced light scattering and lens opacity [6]. Another Arg to Ser mutation at residue 36 (also in the N-terminal) results in the mutant protein readily forming micro-crystals. In fact, it has been shown [6] that this mutation leads to the crystallization of the protein in vivo as well, which is the actual cause of light scattering and cataract. Most of these reported mutants involve arginine at different positions [9, 13, 19]. This suggests that arginine residues are critical in maintaining the solubility of the γ-crystallins. Most arginine residues in γ-crystallins are on the protein surface and are known to be involved in forming salt-bridges. Thus the R76S mutation is not only important for its association with cataract, but also to understand the consequences of substituting arginine for another residue in HGD. It also affords us an opportunity to compare the results of this substitution with a similar cataract-associated mutant, namely R36S.

There are significant differences between R76S and R36S. Although both mutants show similar spectral characteristics and stability, comparable to that of the wild type protein, the R36S mutant shows a severe drop in solubility, by almost two orders of magnitude with respect to HGD [19]. In contrast, the solubility of R76S appears comparable to that of HGD. There is also very little difference in the liquid-liquid phase separation (LLPS) characteristics between HGD and R76S. LLPS is a hallmark of the thermodynamics of aqueous solutions of the gamma crystallins [30].

The major difference between HGD and R76S was observed by SDS-PAGE experiments. The R76S mutant showed the presence of a significant fraction of the protein as tetramer. When the tetramer band was excised and incubated in DTT, and
reanalyzed by SDS-PAGE, a significant amount of the protein was found as monomer. This indicates that the tetramers are predominantly formed by intermolecular disulfide crosslinks. Notably, the tetramer band persists even after the DTT-reduction, suggesting that in addition, the aggregate also consists of non-reducible oligomers.

The Arg76 to Ser mutation in CRYGD have been associated with autosomal dominant juvenile cataract [16]. Considering this, it is conceivable that there is an interaction between the mutant and wild-type proteins. As this mutation involves the substitution of a highly basic and charged Arg for a neutral Ser residue, it is likely that there is an attractive electrostatic interaction between R76S and HGD. Consistent with the above, the isoelectric point (pI) was found to be 7.3 for the wild type and 6.9 for the mutant (see Fig. 20). These observations suggest that aside from the intermolecular disulfides, there are attractive electrostatic interactions between R76S and HGD. However, as Figure-21 shows, when the aggregation of an equimolar mixture of HGD and R76S was compared with the individual proteins for aggregation, it did not consistently show increased aggregation compared to the R76S alone. Therefore, this putative electrostatic interaction does not seem to play a direct role in aggregation.

As already indicated, one important difference between HGD and R76S is that intermolecular disulfides appear to form in R76S due to oxidation in a time-dependent manner. This means that although spectroscopic studies such as circular dichroism and tryptophan fluorescence and thermodynamic probes such as solubility and phase diagrams cannot distinguish between HGD and R76S, somehow one or more Cys residues in R76S becomes “more susceptible to oxidation”. Thus, we hypothesize that
upon mutation, minor structural changes in R76S lead to some Cys residues becoming more reactive. As shown in the Results (Figure - 22), disulfide-linked oligomers, R76S also shows the formation of non-reducible aggregates as well. It is not clear whether these non-reducible aggregates are somehow triggered by the formation of disulfide crosslinks, or are formed independently. The evidence for the non-reducible aggregates comes from the SDS-PAGE analysis shown in Figure-22 and the dynamic light-scattering data presented in Figure-18 (see the 3h and 18h data).

In order to get a better understanding of the structural differences between HGD and R76S, the NMR (HSQC) spectrum was measured both for HGD and R76S. As shown in Figures 24 and 25, even though most of the backbone residues in the two proteins appear to be in the same position, there are indeed distinct changes observed between their backbone amides. The main affected residues are Asp21, His22, Tyr28, Glu46, Asn49, Gly52, Ser74, Val75, Arg76 and Ser77. Thus besides immediate neighbors of Arg76, namely 74 to 77, there are two other clusters of residues that are affected, namely 21, 22 and 28 in one cluster, and 46, 49, 52 in another. From the 3D structure it can be clearly seen that all these residues are quite close to each other.
Figure 27. 3D structure of HGD with Arg76 to Ser mutation site shown in yellow, and affected residues determined by the NMR (HSQC) experiment shown in light orange.

Two important outcomes emerge from the NMR data: (1). It is clear that the residues that are affected by the R76S mutation are almost exclusively localized in a small region of the N-terminal domain, and the C-terminal domain remains virtually unaffected. (2). There are several Cys residues that are in close proximity to the residues that show a major change.

Let us first discuss the Cys residues. Cys32 is ~3.6 Å from Val75, and Cys18 is 4.5Å away from His22. Cys 18 is also ~4 Å away from Cys78. Thus it is clear that at least His22 and Val75 are each close to a specific Cys residue. Since HGD forms disulfide-crosslinked dimers upon aging, it is likely to already have a moderately reactive Cys residue ( ). The NMR data shown here suggest that at least one other Cys residue becomes reactive in
R76S. This could explain the formation of higher oligomers beyond a dimer in R76S solutions. Further verification of this issue might require a study of specific double mutants.

The NMR observation that the effects of mutation are localized in the N-terminal domain, prompted us to study the effect of this mutation on the N-terminal domain. In an earlier study, we observed amplified effects of mutation in the N-terminal domain of the P23T mutant [31, 32]. There it was shown that the rapid aggregation of the P23T mutant relative to the wild type occurs in the N-terminal domain [18]. We therefore expected “an amplification” of the effects of the R76S mutation in studies conducted using only the N-terminal domain. However that did not turn out to be the case, as the N-terminal domains for HGD and R76S behaved the same in their aggregation potential under the conditions we tested, as shown in Figure-19.

Finally, we have tried to build a model, using the Expasy server, of how monomers of R76S could be organized into a tetramer. The protein is represented as lines and the main residues affected by the mutation, and identified as such by NMR studies, are shown as ribbons. Each residue group as well as each monomer is shown in a different color.
This provides us with a working model of the tetramer, in which the affected domains of each monomer are shown to interact, giving rise to a tetramer. We believe that such a tetramer may well be stabilized by SDS, in the partially unfolded state, as discussed in the Results section, page --. In the absence of SDS, aggregation proceeds further and we observe much larger aggregates which are detected by dynamic light scattering.
Building a γD-crystallin tetramer model

In order to visualize the tetramers of the R76S mutant protein, obtained under certain experimental conditions, a molecular model of a tetramer was generated in the following manner. Non-crystallographic symmetry was built by transforming MTRIX records of protein from the PDB files using the Swiss PDB Viewer computer program. A full tetramer is built from a pdb file containing only a monomer of a protein. Below is a step by step procedure how a functional unit was built from a monomer. A copy of file is opened from the “Open PDB file” menu and the orientation is reset from the “Edit” menu. Then three more copies of the pdb file are opened the same way and program will automatically load each copy into a new layer. All residues in all layers are selected by holding down the shift key while invoking the Select All item of the “Select” menu. This is done for each layer by making each layer active using the control panel popup menu to switch among layers. By clicking on the little text icon in the main display window will let the user consult the pdb file as a text file, which is useful to have a look at the annotations. By scrolling down the pdb file until MTRIX lines (they are just before the ATOM lines) 9 MTRIX lines can be seen. They represent three transformation matrices, and allow the user to build the non-crystallographic symmetries of the protein. By clicking on the first line of the first MTRIX record will load the transformation matrix into a dialog. Simply by clicking on OK the transformation will be applied on the current layer. This is done for each layer. After applying transformation into all four
layers, each layer is colored into different colors from the color menu and the final functional unit is saved into a single pdb file from the “Save project (All layers)” menu.


