Modeling the early, aging-related changes in human recombinant gammas-crystallin in vitro

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MODELING THE EARLY, AGING-RELATED CHANGES IN HUMAN RECOMBINANT γS-CRYSTALLIN IN VITRO

By

Christiana O. Salami

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 2014
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Abstract

Cataract is the leading cause of blindness in the world. Cataractous lenses scatter a significant part of the light entering the eye. Cataract can be of several types: Age-related cataract, secondary cataract, traumatic cataract, congenital cataract, and radiation cataract. Age-related cataract is the most common type of cataract. Several factors contribute to age-onset cataract; among them are the Post-Translational Modifications (PTMs) to the major lens proteins or crystallins, which constitute about 80-90% of the protein content of the lens. Predominant PTMs of the crystallins are oxidation, deamidation, phosphorylation, glycation, and disulfide crosslinking, which tend to increase as the lens ages.

In the work presented in this thesis, two major PTMs—deamidation and disulfide crosslinking were selected for study using a model system in vitro, to determine whether or not they occurred concurrently in a short period of time. The model system selected was human recombinant γS-crystallin solution, treated under conditions known from the literature to undergo oxidation and deamidation. The samples were monitored over time using a variety of spectroscopic techniques such as Nuclear Magnetic Spectroscopy (NMR), Circular Dichroism (CD) and Tryptophan fluorescence. Although residues participating in disulfide formation in HGS were identified, and possible sites of deamidation were examined, the data showed no evidence of deamidation of the Asparagine (Asn) and Glutamine (Gln) residues after 30 days of incubation. Further, extended incubation leads to a significant loss of protein solubility. These preliminary data suggest that, in the initial stages of the aging process of the γS-crystallin model system, protein oxidation leading to disulfide crosslinking precedes the deamidation of
Asn and Gln residues. At this stage, the secondary structure of the protein is essentially unaltered, and the tertiary structure of the protein in both the soluble and insoluble forms is only marginally altered and the protein is still folded. As aging progresses, protein aggregation sets in leading to insolubilization which is likely to be the source of the light scattering and opacity and eventually cataract formation.
## List of Abbreviations and Symbols Used

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD</td>
<td>Circular Dichroism</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>GDn-HCl</td>
<td>Guanidinium-Hydrochloride</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl)-1-piperazineethanesulfonic Acid</td>
</tr>
<tr>
<td>HGC</td>
<td>Human γC-crystallin</td>
</tr>
<tr>
<td>HGD</td>
<td>Human γD-crystallin</td>
</tr>
<tr>
<td>HGS</td>
<td>Human γS-crystallin</td>
</tr>
<tr>
<td>HPLC-ESI-MS</td>
<td>High Performance Liquid Chromatography-Electrospray Mass Spectrometry</td>
</tr>
<tr>
<td>HSQC</td>
<td>Heteronuclear Single Quantum Coherence</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl 1-thio-D-galactopyranoside</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>SE</td>
<td>Size Exclusion</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>HGS$_{30d}$-soluble</td>
<td>Soluble Fraction of HGS after 30-day Incubation</td>
</tr>
<tr>
<td>HGS$_{30d}$-insoluble</td>
<td>Insoluble (resolubilized) Fraction of HGS after 30-day Incubation</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1 Human Lens and Cataract

Cataract is defined as clouding of the lens in the eye which leads to a significant scattering of the light entering the eye (Hejtmancik, 2008). The lens and cornea of the eye are components of the anterior part of the eye. The lens and the cornea (Figure 1) focus light on the retina which then transforms the light energy into an electric signal. Anterior to the lens is the iris which controls the amount of light that enters the eye (Figure 1).

The human lens contains highly soluble crystalline proteins at high concentrations (above 350 mg/mL) which are tightly packed to have a short-range order (Delaye and Tardieu, 1983) critical for the maintenance of a high refractive index, essential for lens transparency (Barton et al., 2009 and Andley et al., 2009).

\[ \alpha, \beta, \text{and } \gamma - \text{crystallins make up the majority of lens proteins. }\]

\[\alpha-\text{Crystallins are a member of the family of small heat-shock proteins which constitute 30\% of lens proteins (Barton et al., 2009 and Bhattacharyya et al., 2006). }\]

\[\alpha-\text{Crystallins are oligomers with an average molecular weight of 800 kDa and consist of } \alpha A - \text{and } \alpha B- \text{subunits}\]
α-Crystallins bind to partially unfolded proteins and prevent their denaturation during the normal course of lens aging (Barton et al., 2009, Andley et al., 2009, and Kapphahn et al., 2003). β-Crystallins are oligomers with an average molecular weight of 200 kDa (Zhang et al., 2003). β-Crystallins consist of four acidic (βA1-βA4) and three basic (βB1-βB3) subunits (Liu et al., 2007 and Slingsby and Bateman 1990). The γ-crystallin family consists of seven distinct proteins: γA, γB, γC, γD, γE, γF, and γS. γ-Crystallins are monomers with an approximate molecular weight of 20 kDa (Slingsby and Bateman, 1990). Among the γ-crystallins, γC-, γS-, γD-crystallin are the most abundant in the lens, constituting 7, 9, and 11% respectively of the total protein found in young human lenses (Acosta-Sampson and King, 2010). Human γS-crystallin (HGS) is abundant in the outer layer of the cortex and human γD-crystallin (HGD) is found in the lens nucleus.

1.2 Types of Cataract

Types of cataract include: Age-related cataract, Secondary cataract, Traumatic cataract, Congenital cataract, and Radiation cataract (NEI, “Facts about Cataract”). One-fifth of blindness from cataract is due to Ultraviolet light (UV) radiation exposure (Chen et al., 2009). The retina is susceptible to photodamage, so the lens absorbs the incident UV radiation to protect the retina. Overtime, damage to the lens crystallins due to exposure to UV accumulates and can ultimately lead to cataract (Chen et al., 2009). Congenital cataract (Figure 2) is the major cause of loss of vision in children worldwide. Congenital cataract is characterized by clouding of the lens present at early stages of child development (Deng and Yuan, 2013 and Chacon-Camacho et al., 2014).
Traumatic cataract develops as a result of traumatic injury to the eye. The significance of traumatic cataract relies on the injury agent, impact point, and presence of pre-existing ocular diseases (Tabatabaei et al., 2012 and Moreschi et al., 2013). Secondary cataract is cataract that develops after glaucoma surgery or in people with health issues like diabetes (Srinivasan et al., 2013 and Patel et al., 2011). In this thesis, I will focus on a specific aspect of age-related cataract—i.e. an aspect that pertains to protein modifications occurring as a function of aging and environmental factors.

1.3 Age-Related Cataract

A major contributor to lens opacity or cataract is the aggregation of the major lens proteins, or crystallins. As the lens ages, proteolytic fragments of the crystallins in the water-insoluble fraction increase, also contributing to aggregation, increase in light scattering and opacity (Udupa et al., 2005). Combinations of aging, environmental, and genetic stresses are considered to be the main cause of aggregation in the lens (Elanchezhian et al., 2012). A significant factor in lens aging is the occurrence of several distinct post-translational modifications (PTMs) of the lens crystallins (Sharma and
Santoshkumar, 2009). Evidence in the literature suggests that some of these modifications are measurably enhanced in the cataractous lens leading to the speculation that they may be directly responsible for the age-related opacity. In the following section, I discuss the specific PTMs prevalent in the aging lens and their implications for the formation of age-onset cataract formation.

### 1.4 Post-Translational Modifications (PTMs) of Crystallins

Studies show that over time, extensive post-translational modifications (PTMs) of crystallins, that appear to be amplified in cataractous lenses and may lead to the formation of the observed cataract (Goulet et al., 2011). Accumulations of a number of PTMs (Table 1) such as glycation, truncation, phosphorylation, acetylation, methylation, oxidation of cysteine and methionine residues, disulfide bridges, and deamidation have been shown to be enhanced in aged and cataractous lenses compared to young lenses (Ji et al., 2012, Goutlet et al., 2011, Hains et al., 2007, and Kapphahn et al., 2003). Due to these PTMs, as the lens ages the fraction of water-insoluble protein increases (Hains et al., 2007). Among the PTMs that feature prominently in aged and cataractous lenses are deamidation of Asn and Gln residues, and oxidation-mediated changes, especially disulfide-crosslinking (Hooi et al., 2012). It has been found that individual members of the three crystallin families undergo different extents of deamidation at each Asn or Gln site, with some sites being significantly more deamidated in cataract (Lampi et al., 2014). Furthermore, as the lens ages, crystallins are also progressively oxidized and the cysteine residues and others are found to be significantly oxidized in cataract (Sharma and Santoshkumar, 2009). These findings are discussed in some detail in the following
sections. Since γ-crystallins are the main focus of the Pande lab, I have discussed these two PTMs with respect to these proteins.

**Table 1**: Common PTMs in crystallins and some sites where they occur in crystallins

<table>
<thead>
<tr>
<th>PTM Type</th>
<th>Description</th>
<th>Sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphorylation</td>
<td>Addition of a phosphate group</td>
<td>αB-crystallin: Ser 45, Ser 59, and Ser 19 or Ser 21 (Smith et al., 1992)</td>
</tr>
<tr>
<td>Truncation</td>
<td>Shortening of the protein</td>
<td>Usually in N-terminus of crystallins and can also occur in C-terminus of αA-crystallin (Biswas et al., 2006, Liao et al., 2002)</td>
</tr>
<tr>
<td>Glycation</td>
<td>Covalent bonding of protein and sugar molecule</td>
<td>Lysine amino group or N-terminal amino acid of crystallins (Hains et al., 2007, Kim et al., 2003)</td>
</tr>
<tr>
<td>Disulfide formation</td>
<td>Covalent linking of two sulfur groups in cysteine residues</td>
<td>γS-crystallin: Cys 22, Cys 24, Cys 114, and Cys 129 (Hanson et al., 2002), αA-crystallin: Cys 131 and Cys 142 (Takemoto, 1996)</td>
</tr>
</tbody>
</table>
1.5 Disulfide cross-links in HGS

γ-Crystallins contain 6-8 cysteine residues, which is more than in any other lens crystallin (Srikanthan et al., 2004). The high number of cysteine residues in γ-crystallins increases its susceptibility for disulfide bond formation (Srikanthan et al., 2004, Hanson et al., 1998). A single-point mutation in γD-crystallin gene resulting in the substitution of Cys 14 for Arg 14, leads to the formation of a disulfide bond, possibly with Cys 110, resulting in oligomerization (Pande et al., 2000). Increase in protein disulfide content in the lens nucleus has been associated with age-related cataract (Srikanthan et al., 2004). Disulfide-bonded cysteines have also been associated with juvenile onset cataract formation (Srikanthan et al., 2004).

1.6 Deamidation of Asparagine (Asn) and Glutamine (Gln) residues

Deamidation is the removal of an amide functional group from Asn and Gln side chain in proteins. Nonenzymatic deamidation in proteins begins with the formation of an imide intermediate (Figure 3) between a terminal amide and the carboxyl group of an Asn or Gln residue (Takata et al., 2009). The imide intermediate is then rapidly hydrolyzed to aspartic or isoaspartic acid at a ratio of 1:3 for Asn residues and glutamic acid for Gln residues.
Studies have shown that enzymatic deamidation via transglutamination of Gln residues can occur, but enzymatic deamidation of Asn residues is not known (Takata et al., 2009). Deamidation can be induced by the use of high pH and high temperature (Takata et al., 2009) and as a result a negative charge is introduced which can lead to structural changes that can potentially affect the transparency of the lens (Takata et al., 2009, Takemoto et al. 2001, Gupta and Srivastava, 2004, and Hains and Truscott, 2010). Amino acids adjacent to Asn and Gln residues affect the deamidation process in proteins. Glycine, histidine, glutamine, serine, and tyrosine have been shown to promote deamidation (Hains and Truscott, 2010, Hooi et al., 2012). HGS in the cataractous lens shows preferential deamidation of Asp-143, Gln-170, and Gln-92 (Takemoto et al., 2001, Flaugh et al., 2006). Deamidation of Gln residues occurs 50 times slower than that of Asn.

**Figure 3:** Mechanism for the cyclization of Asn to form an imide intermediate (Wright, 1991)
residues, which is due to the fact that a stable five membered ring intermediate forms easier than a six membered ring intermediate (Takata et al., 2009, Robinson et al., 2005).

It appears that the most common PTMs found in cataractous lenses are deamidation (Takemoto et al., 2001). By converting Gln to glutamic acid and Asn to aspartic acid, deamidation increases the acidity of the crystallin. Increase in acidity and aggregation due to deamidation and disulfide crosslinking may be the reason why the lens proteins become highly unstable, which could possibly lead to cataract formation.

In view of these findings, it is intriguing that while the two types of major PTMs (deamidation and disulfide crosslinking) are known to occur over time and in cataract, it is not evident whether the onset of one precedes that of the other or if they occur concurrently.

In the work shown in the thesis, I have attempted to answer this question using HGS as a model system since HGS is one of the two crystallins expressed at high levels in the human lens the other being HGD (Wistow, 2012). I used this model system to study the effects of deamidation and disulfide formation in lens crystallins in-vitro to model the aging process in the early stages. I have tried to address the question of which of these PTMs occurs first in the early stages of aging in the model HGS solution in vitro, using NMR spectroscopy.
Chapter 2: Experimental Section

2.1 Materials

Sodium Dodecyl Sulfate (SDS), tetramethylethylenediamine (TEMED), 30% acrylamide/bis, Tris-HCl buffer (pH 8.8), ammonium persulfate, Laemml buffer, bromophenol blue, and Tris were purchased from Bio-Rad. Ampicillin, isopropyl β-D-1 thiogalactopyranoside (IPTG), and dithiothreitol (DTT) were purchased from GoldBio. Guanidinium Hydrochloride, lysozyme, and Triton X-100 were purchased from Sigma Aldrich. Hydrochloric acid was purchased from Pharmaco.

2.2 Experimental Methods

2.2.1 General procedure for cloning, expression, isolation, and purification of proteins

2.2.1.1 Cloning and Expression

HGS cDNA cloned in pET3a vector was a gift from Dr. Nicolette Lubsen (Radboud University, Nijmegen, Netherlands). Procedures for protein expression and purification were almost identical as for HGD (Pande et al., 2000). Recombinant HGS was prepared as described in the published procedure. The recombinant protein was expressed in E. coil. An overnight culture of 25 mL E.coil (BL21(DE3)Gold) was transferred into a 1 L LB medium and then grown at 37°C until an OD of 0.7-0.8 at 600 nm was reached. Isopropyl 1-thio-D-galactopyranoside (IPTG) (1 mM final
concentration) was then added to induce overexpression of HGS. The cells were then grown for an additional 6-7 hours and harvested by centrifugation at a speed of 5840 X g.

2.2.1.2 Protein isolation and Purification

The harvested cell pellets were then re-suspended in lysis buffer (5 mM Tris.HCl, 25 mM NaCl, and 2 mM EDTA, pH 8). A protease inhibitor tablet (Roche Molecular Biochemicals) (1 tablet per 25 mL) and lysozyme (250 µg/mL) were then added. The lysed suspension was then rapidly frozen in liquid nitrogen and thawed in a water bath at 30°C (4-5 cycles), after which DNAse (1 mg/mL) and MgSO₄ (1 mM) were added to the cell lysate. The cell lysate was incubated for 1 – 1.5 hours and centrifuged at 48,000 X g. SDS-PAGE analysis of both the pellet and supernatant showed that most of the γS-crystallin is fractionated into the supernatant. The protein was purified by first loading the supernatant into a Sephacryl S 200 HR size exclusion (SE) column using 0.275 M sodium acetate buffer (pH 4.5) at a flow rate 2.5 mL/min, which was then followed by cation-exchange chromatography on a SP-Sepharose fast flow column. Column chromatography for protein purification was executed using the GE AKTA prime plus system. The protein was eluted with a NaCl gradient of 0 – 0.3 M in 0.275 M sodium acetate buffer (pH 4.8) containing 0.02% sodium azide. Fractions containing γS-crystallin were tested for the presence of γS-crystallin using SDS-PAGE. The concentration of HGS was determined using an extinction coefficient of 42, 860 M⁻¹ cm⁻¹ at 280 nm (Somireddy et al., 2013).
2.2.2 Gel Electrophoresis

SDS-PAGE was performed on polyacrylamide gels using a Bio-Rad Power Pac 1000 electrophoresis system in the presence of SDS detergent. The protein samples were prepared in a Laemmli sample buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) at a ratio of 1:1 protein to buffer. The samples were loaded into a 12% gel and run at a constant voltage of 180 V for 45 mins. PAGEmark Tri-color markers were used as a standard. The protein bands were detected using 0.1% Coomaisse Blue G-250 staining solution and images were captured using the ChemiDoc™ MP System imager from Bio-rad.

2.2.3 Circular Dichroism (CD) Spectroscopy

Far UV CD spectra were acquired using a JASCO J-815 spectropolarimeter. A protein concentration of 10 µM in 5mM sodium phosphate buffer (pH 7.1) was used. A 1.0 mm path length cuvette was used. Data were normalized to factor in the concentration of peptide bonds.

To determine the mean residue ellipticity, the following equation was used:

\[
\Delta \varepsilon_{MR} = \frac{\varepsilon_{LCP} - \varepsilon_{RCP}}{CMR \times l} = \frac{\Delta A}{CMR \times l}
\]

Where \(\varepsilon_{LCP}\) and \(\varepsilon_{RCP}\) are the molar extinction coefficients for left and right circularly polarized light respectively. CMR is the mean residue concentration and \(l\) is the pathlength of the cuvette in centimeters. CMR was determined by multiplying the number of amino acids in HGS (177) by the concentration of the sample (10 µM).
2.2.4 Fluorescence Spectroscopy

Tryptophan fluorescence spectra were acquired using a Horiba Jobin Yvon flurolog-3 spectrometer. Excitation and emission slits were set for 2.5 nm for all measurements. A protein concentration of 10 µM (0.2 mg/ml) in 5mM sodium phosphate buffer (pH 7.1) was used. The spectra were acquired using an excitation wavelength of 290 nm and emission was recorded in the 250 – 350 nm regions. The spectra were corrected by subtracting the contribution of the buffer.

2.2.5 Preparation of $^{15}$N labeled HGS

$^{15}$N labeled HGS was prepared by growing E-Coli cells until induction. The cells were then transferred to an M9 minimal media containing 1 g/L [U- 15N] NH$_4$CL and 2 g/L D-glucose. After the cells were harvested, the protein was purified as described above in section 2.1 for the unlabeled protein. To study the aging process of HGS, the HGS samples were incubated at 42°C in 0.1 M Na$_2$HPO$_4$ buffer (pH 7.1) for 30, 72, and 198 days.

2.2.6 Solubilization of insoluble fractions

After 30 days the protein sample was centrifuged 10,000 X g for 15 mins and the soluble and insoluble fractions were separated. The insoluble fraction was resolubilized by slowly adding increasing amounts of guanidinium hydrochloride (GuHCl) (concentration of 0 – 7 M) in phosphate buffer (10 mM Na$_2$HPO$_4$, 5 mM DTT, and 1 mM EDTA, pH 7.1) as described by King et al. (2003). The samples were incubated at 42°C for 6 hours. The GuHCl was removed through buffer exchange using Millipore Amicon®
Ultra-15 centrifugal filter with Ultracel® 10 Regenerated Cellulose Membrane with a 10 kDa molecular weight cutoff.

### 2.2.7 NMR Spectroscopy

Protein samples for NMR measurements in the concentration range of 50 µM – 120 µM were prepared in NMR buffer (0.1 M Na₂HPO₄ (pH 7.1), 100mM NaCl, 0.02% NaN₃, 90% H₂O, and 10% D₂O). A Heteronuclear single quantum coherence (HSQC) spectra were acquired at 25°C in a Bruker Avance 700 MHz spectrometer with a z-axis TCI cryoprobe, in Prof. Shekhtman’s laboratory. The spectra were processed using TOPSPIN 2.1 and analysis was carried out using the software Computer Aided Resonance Assignment (CARA). The chemical shifts were assigned by Dr. Bharat Sommireddy by conducting triple resonance experiments (Somireddy et al., 2013). Spectra with and without 25 mM DTT were attained. Differences in chemical shift and intensities between the HGS control and the after 30 days incubation sample were determined by overlaying the spectra of ¹⁵N HGS and after 30 days incubation ¹⁵N HGS.

### 2.2.8 PBS and Tris-HCl Buffer Preparation

0.1 M PBS buffer was prepared by mixing Monobasic Sodium Phosphate (NaH₂PO₄) and Dibasic Sodium Phosphate (Na₂HPO₄) and then dissolved in deionized water. The pH was adjusted using sodium hydroxide and phosphoric acid. 0.1 M Tris-HCl buffer was prepared by dissolving Tris-HCl in deionized water. The pH was adjusted using sodium hydroxide.
Chapter 3: Results

In order to study the use of HGS as a model system to monitor aging-related modifications in vitro, I carried out the following experiments:

Time and temperature were used as parameters to study the aging process. Far-UV CD and tryptophan fluorescence emission were used to determine the effects of the aging process on the secondary structure of HGS. A solution of the HGS control sample was compared to the soluble and insoluble (and resolubilized) fractions of an HGS sample incubated for 30 days at 42°C (soluble fraction, HGS\textsubscript{30d-soluble}, and insoluble fraction, resolubilized, HGS\textsubscript{30d-insoluble}).

3.1 Far-UV CD

The CD spectrum in the far UV region (190-250 nm) reveals important details of the protein secondary structure. α-helices, β-sheets, and random coils give rise to characteristic shape and magnitude in the far-UV region of the CD spectrum (Chen et al., 2009). HGS typically shows a characteristic β-sheet profile in the far-UV region of the CD spectrum. Figure 4 shows that HGS control, HGS\textsubscript{30d-soluble}, and HGS\textsubscript{30d-insoluble} have virtually indistinguishable secondary structures.
Figure 4: Far-UV CD spectra of HGS control (Blue), HGS\textsubscript{30d-soluble} (Red), and HGS\textsubscript{30d-insoluble} (Purple). Protein concentration in each of the case was 10 µM (0.2 mg/mL) in 5mM PBS buffer (pH 7.12), cell path length 1.0 mm. All spectra were recorded at 25°C, corrected for background buffer signal, and each spectrum is an average of 5 independent runs.

3.2 Tryptophan Fluorescence

HGS contains four tryptophan residues, two in the N-terminal domain, and two in the C-terminal domain (Chen et al., 2009). Overall tertiary structure of the protein samples can be determined by measuring the intensity and $\lambda_{\text{max}}$ values. After 30 days of incubation, the tryptophan fluorescence (Figure 5) show a decrease in fluorescence intensity and a 7 to 8 nm blue shift in the emission maximum. In the case of the HGS\textsubscript{30d-soluble} and HGS\textsubscript{30d-insoluble}, the slight blue shift and lower intensity suggest that the tryptophan residues may be somewhat more buried in these samples relative to the wild-
type. This is consistent with change in $\lambda_{\text{max}}$ observed in the tryptophan residues of HGS that are buried compared to surface tryptophans (Xu et al., 2009).

![Figure 5: Tryptophan fluorescence emission spectra of HGS control (blue), HGS$_{30d}$-soluble (red), and HGS$_{30d}$-insoluble (purple) samples, emission intensity in arbitrary units. Protein concentration in each of the case was 10 µM (0.2 mg/ml) in 5mM PBS (pH 7.12). Spectra were recorded at 25°C, using an excitation wavelength of 295 nm, with 2.5 nm slits.]

3.3 Analysis of the HSQC spectra for the HGS$_{30d}$-soluble and HGS$_{30d}$-insoluble

Figure 6 shows an overlay of the $^{15}\text{N}^{1}\text{H}$ HSQC spectra of HGS control and after 30 days incubation samples. HSQC spectra provide information about the backbone amide groups and the 3D structure of HGS. The amide cross-peaks seen in the spectra are due to each backbone and side chain amide group except proline, and the N-terminal amide group which is typically present as $\text{NH}_3^+$. The amide cross-peaks for HGS were
assigned in our laboratory by Dr. Bharat Somireddy. The HSQC spectra in Figure 6 show that the protein is fully folded, since all cross-peaks are well dispersed in both $^{15}\text{N}$ and $^1\text{H}$ dimensions. The overlay of the spectra of the wild-type (red contours) and the 30-day aged samples (green contours) with (A) and without (B) 25mM DTT clearly suggest that at this stage in the aging of HGS, the protein is still fully folded.

The intensities of each corresponding peak were compared to determine which region of the HGS had the most significant change due to disulfide crosslinking when compared to the wild-type. To take into account the difference in concentration of the NMR samples, the intensity values were normalized. To normalize the intensity, the intensity values of the peaks from the 30-day aged samples that had minimal change in chemical shift compared to the HGS wild-type, were divided by the intensity value of the same residues from the control spectra. All the intensity values from the 30-day aged spectra were then divided by the resulting quotient. A sample calculation is shown below.

HE1/NE1 W136 intensity from HGS$_{30\text{d-soluble}}$ $^{15}\text{N-}^1\text{H}$ HSQC spectrum: 157525

HE1/NE1 W136 intensity from HGS wild-type $^{15}\text{N-}^1\text{H}$ HSQC spectrum: 181323

$$\frac{157525}{181323} = 0.869$$

All intensity values from HGS$_{30\text{d-soluble}}$ $^{15}\text{N-}^1\text{H}$ HSQC spectrum was then divided by 0.869.
Figure 6: Overlay 2D $^{15}$N-$^1$H HSQC spectra of HGS control (Red contour) and after HGS$_{30}$d-soluble (Green contour) with 25 mM DTT (A) and without 25 mM DTT (B). All cross-peaks are well dispersed in both $^{15}$N and $^1$H dimensions indicating that the protein is completely folded.
Figure 7A: Normalized Intensity plot of HGS Control (Red lines) and HGS$_{30\text{d-soluble}}$ (Green lines) with DTT. There are no significant changes in intensity compared to the plot without DTT (Figure 7B).
Figure 7B: Normalized Intensity plot of HGS Control (Red lines) and HGS30d-soluble (Green lines) without 25 mM DTT. Significant changes in intensity in the plot without DTT are limited to the regions of sequence 1 to 84.
3.3.1 Disulfide crosslink formation

HGS contains 7 Cys residues, all of which are capable of forming disulfide crosslink. HSQC spectra with and without DTT were measured. A comparison of the backbone amide chemical shifts and intensity of the HGS control, HGS\textsubscript{30d-soluble}, and HGS\textsubscript{30d-insoluble} samples show that the changes are confined to the areas surrounding Cys 22, Cys 26, Cys 36, and Cys 82 (Figure 7). Figure 7A shows an intensity plot comparing the intensities of the HGS\textsubscript{30d-soluble} (Green lines) sample with the HGS Control (Red lines) in the presence of DTT. Figure 7B shows an intensity plot comparing the intensities of the HGS\textsubscript{30d-soluble} (Green lines) sample with HGS Control (Red lines) in the absence of DTT. In the absence of DTT there are significant differences in intensity between the HGS\textsubscript{30d-soluble} and HGS Control. The changes are localized in the N-terminal of the protein. Significant changes to backbone amide chemical shifts and intensity were not observed in the presence of 25 mM DTT (Figures 8 and 11). Slight changes to Cys 82 were observed in the presence of DTT (Figure 8) but without DTT peaks corresponding to Cys 82 and Cys 26 was not observed (Figure 9) in the HGS\textsubscript{30d-soluble} sample, which suggests that the changes observed are mostly due to disulfide formation. Cys 22 and Cys 36 in the HGS\textsubscript{30d-soluble} show significant changes to backbone amide chemical shifts in the absence of 25mM DTT compared to HGS control (Figure 9). For the HGS\textsubscript{30d-insoluble} sample, a peak for all Cys residues were observed in the presence and absence of 25 mM DTT, but in the absence of DTT Cys 22, Cys 82, Cys 26, and Cys 36 show significant changes to backbone amide chemical shifts compared to the HGS control (Figure 10), which suggests that the changes observed are mostly due to disulfide formation.
Figure 8: Overlay of HSQC spectra of HGS control (red contour) and after 30 days incubation soluble fraction (green contour) showing (A) Cys 22, (B) Cys 26, (C) Cys 36, (D) Cys 82 in the presence of 25 mM DTT. Compared to the spectra without DTT, there are no significant differences in chemical shifts.
Figure 9: Overlay of HSQC spectra of HGS control (red contour) and HGS_{30d-soluble} (green contour) showing (A) Cys 22, (B) Cys 26, (C) Cys 36, (D) Cys 82 in the absence of 25 mM DTT. There are significant differences in chemical shifts between the control and HGS_{30d-soluble} peaks. No Cys 26 cross peak was seen in the HGS_{30d-soluble} spectra.
Figure 10: Overlay of HSQC spectra of HGS control (red contour) and HGS$_{30d}$-insoluble (green contour) showing (A) Cys 22, (B) Cys 26, (C) Cys 36, (D) Cys 82 in the absence of 25 mM DTT. Compared to the spectra with DTT there are significant differences in chemical shifts between the cysteine residues. The Cys 26 cross-peak was seen in the HGS$_{30d}$-insoluble spectra but there was no significant difference in the chemical shift compared to the control.
Figure 11: Overlay of HSQC spectra of HGS control (red contour) and HGS30d-insoluble (green contour) showing (A) Cys 22, (B) Cys 36, (C) Cys 82, (D) Cys 26 in the presence of 25 mM DTT. Compared to the spectra without DTT, there are no significant differences in chemical shifts. The peak shape of Cys 26 for HGS30d-insoluble is significantly different from the peak shape of the Cys 26 peak in the HGS control.
Most of the changes observed were confined to the N-terminal domain (Figure 12); and essentially no changes were detected in the C-terminal domain. Figure 13, provides a list of residues in the absence of DTT which showed significant differences in chemical shifts and intensity (yellow shaded) and residues that did not show any significant changes in chemical shifts and intensity.

**Figure 12:** (A) Structural Mapping showing positions of residues in yellow after 30 days of incubation with major changes in chemical shift and intensity when compared to the HGS control. The changes are confined to the N-terminus. (B) 3D structure of HGS showing regions with the most chemical shift and intensity differences after 30 days incubation without DTT in yellow as determined by NMR. Cys residues involved in disulfide crosslinks are highlighted.
Figure 13: HGS sequence showing residues with significant chemical shift differences (shaded in yellow) as a result of disulfide crosslinks.

Figure 14: Molecular model showing Cys residues involved in disulfide crosslink formation.
3.4 SDS-PAGE Analysis

Usually, a decrease in the mobility of the monomer band of the protein sample on the SDS-PAGE after reduction with DTT is an indication of intramolecular disulfide bonds (Xu et al., 2009). When intermolecular disulfide crosslinks are being formed, a reduction of the oligomers leads to the formation of smaller oligomers which can be seen on an SDS-PAGE gel. Therefore to determine whether intermolecular or intramolecular disulfide bonds are being formed, an SDS-PAGE analysis was conducted. The resulting gel is shown in Figure 15. The SDS-PAGE data showed no obvious signs of intermolecular disulfide crosslinks which suggest that predominately intramolecular disulfide links are being formed in both the soluble and insoluble (re-solubilized) fractions after 30 days of incubation.

Figure 15: SDS-PAGE gel of HGS control, after 30 days soluble fraction, and after 30 days insoluble (re-solubilized) with and without DTT. Lane 1 PAGEmark Tri-color marker. Lanes 2 and 3 HGS control without and with DTT respectively. Lanes 4 and 5 HGS30d-soluble sample without DTT and with DTT respectively. Lanes 6 and 7 HGS30d-insoluble sample without DTT and with DTT. This figure shows that predominately intramolecular disulfide crosslinks are formed in the HGS samples.
3.5 Deamidation

Figure 16: Overlay of HSQC spectra of side-chain peaks of Asn and Gln residues showing, A) HGS control (red contour) and HGS_{30d}-insoluble (green contour) in the presence of 25 mM DTT. B) Overlay of HSQC spectra of HGS control (red contour) and HGS_{30d}-soluble (green contour) in the presence of 25 mM DTT. No significant changes in chemical shift was observed in both the soluble and insoluble samples.
In HGS, four sites of deamidation have been identified: Asn 143, Asn 76, Gln 102, and Gln 92. Asn 143 has been shown to be wholly deamidated (Takemoto et al., 2001). Asn 14 and Gln 16 are more deamidated in cataractous lenses compared to age-matched normal lenses (McDermott et al., 1988). To determine whether any deamidation occurred after 30 days of incubation, $^{15}$N – $^1$H HSQC spectra for side chain Asn and Gln were analyzed. In a typical $^1$H-$^{15}$N HSQC spectrum, the amide peaks of Asn and Gln residues are found in the upper right quadrant.

Figure 16, shows an overlay of wild-type HGS control and samples after 30 days of incubation. The spectra show no significant differences in chemical shift or a disappearance of the Asn and Gln residues as is expected when these residues are deamidated. The $^{15}$N- $^1$H HSQC data after 30 days incubation, showed no obvious changes to the side chain Gln and Asn in both the HGS$^{30d}$-soluble and HGS$^{30d}$-insoluble fractions. The data suggest that no deamidation occurred after 30 days of incubation.
Chapter 4: Discussion

Studies by Takemoto et al. (2001), Clarke et al. (1987), Hooi et al. (2012), Hanson et al. (1998), and Santhoshkumar et al. (2012) have shown the effects of PTMs on crystallin solubility and consequently lens opacification. The work presented in this thesis focuses on age-related PTMs in-vitro, using HGS as a model system. In this model system, set up to mimic the early stages of this aging process, I have attempted to track the effects of two major PTMs, deamidation and disulfide formation.

After 30 days of incubation, most of the protein was found to be aggregated and only 14% of the starting protein was found in the soluble fraction. Despite this significant insolubilization the data showed that the protein in both the HGS\textsubscript{30d-soluble} and HGS\textsubscript{30d-insoluble} fractions remained fully folded. After 72 and 198 days of incubation, no protein was found in the soluble fraction. This suggests that as the crystallin ages under physiological temperature the propensity of the protein to aggregate increases significantly. This result is consistent with previous studies by Santhoshkumar et al. (2008) which showed that the fraction of water insoluble proteins increases with age.

We hypothesized at the outset of this study that aggregation after 30 days of incubation may be a result of intermolecular (protein-protein) disulfide crosslinking and/or deamidation, and furthermore that the two PTMs may or may not be occurring in parallel. This led us to carry out NMR studies on both the HGS\textsubscript{30d-soluble} and HGS\textsubscript{30d-insoluble} fractions with and without DTT.
One observation that stood out from our HSQC data is that significant differences in chemical shift and intensities were only observed in the absence of DTT. HSQC spectra with DTT showed no differences in chemical shift and intensities. The differences were also restricted to regions surrounding Cys 22, Cys 26, Cys 36, and Cys 82. When considering cys oxidation, there is a possibility that the sulfhydryl groups in the cysteines are being oxidized to sulfones. If this were the case, no NMR cross-peak would have been seen in the residues forming sulfones. In the HGS$_{30d}$-soluble HSQC spectra without DTT (Figure 9), cross-peaks corresponding to Cys 26 and Cys 82 are not observed. This suggests that Cys 26 and Cys 82 are more oxidized in the HGS$_{30d}$-soluble sample compared to the other Cys residues. The spectra data indicates that the sites with differences in chemical shifts and intensities are seen as a consequence of disulfide formation. Based on these findings, I propose that there is a disulfide crosslinking between Cys 22 and Cys 26. This is consistent with studies done by Hanson et al. (1998).

Figure 17: A model of a monomer of $\gamma$-crystallin. Residues affected by disulfide crosslink formation are shown in yellow and cysteine residues involved in the disulfide crosslink are shown in red. The residues were identified by NMR.
We also propose that there is disulfide crosslinking between Cys 36 and Cys 82. Hanson et al. (1998) do not report any disulfide formation between Cys 36 and Cys 82 as well.

Hanson et al. (1998) identified sites of disulfide formation and deamidation by isolating HGS crystallin from 32 weeks gestation, 4 day old, and 55 years old lenses and then used HPLC-ESI-MS with a 0.3 Da mass accuracy over a mass range of 300 – 2000 Da and a 2 Da mass accuracy for a 20 kDa protein to determine the molecular weight of the peptide digest. Smith et al. reports a mass decrease of 2 Da of the peptides due to hydrogen loss as a result of disulfide bonding and a mass increase of 1 Da as a result of deamidation. Hanson et al. (1998) showed that Cys 22, Cys 24, Cys 114, and Cys 129 participated in the formation of two disulfides in HGS. Hanson et al. (1998) also reported that Cys 26 is not involved in disulfide formation. The data presented in this thesis also show the presence of two disulfides as reported by these authors. The residues participating in the disulfide formation we determined using NMR is different from what Hanson et al. obtained using HPLC-ESI-MS. The residues participating in disulfide formation according to our data are Cys 22, Cys 26, Cys 36, and Cys 82. Evidence of disulfide formation was not seen anywhere else in our NMR data.
The distance between Cys 22 and Cys 26 is 4.8 angstroms (Figure 18) and the distance between Cys 82 and Cys 36 is 10.9 angstroms (Figure 19). Hanson et al. reported a distance of 6 Angstroms between Cys 22 and Cys 24 and a distance of 11
Angstroms between Cys 114 and Cys 129. According to Hanson et al., even though the distances between the cysteine residues do not favor disulfide formation, a disulfide crosslink forms due to conformational change as a result of increased acidity due to the deamidation of Gln 53, Gln 63, Gln 70, Gln 92, Gln106, Asn 120, and Gln 170. Our model system data showed no evidence of deamidation, therefore the disulfide formation is not a result of deamidation. Hanson et al. also reports deamidation of Gln 53, Gln 63, Gln 70, and Gln 106 in 32 weeks gestation lenses, identified using mass spectrometry. Using site-directed mutagenesis, Takemoto et al., reports deamidation of Asn 143 of γS-crystallin (Takemoto et al., 2001), Hanson et al. (1998) do not report deamidation of Asn 143.

After incubation for 30 days under physiological conditions, we did not observe any deamidation in both the HGS\textsubscript{30d-soluble} and HGS\textsubscript{30d-insoluble} fractions. After 72 and 198 days of incubation, there was no protein in the soluble protein fraction and we were unable to re-solubilize the insoluble fraction. Therefore we were unable to determine if deamidation occurs after 30 days.

In conclusion, I have presented evidence of disulfide formation as one of the possible PTMs occurring in HGS at the early stages of aging in our model system. I have identified the residues participating in disulfide formation in HGS. I have identified Cys 26 as one of the residues involved in disulfide crosslinking \textit{in vitro}. Cys 26 is not involved in disulfide crosslinking in crystallins isolated from human lenses. The fact that Cys 26 is involved in disulfide crosslinking in HGS crystallin \textit{in vitro} and not in HGS crystallin isolated from human lenses may suggest that Cys 26 is methylated or otherwise modified \textit{in vivo}. I have also shown that \textit{in vitro}, disulfide crosslinking occurs before
deamidation of Asn and Gln residues. These findings are contrary to previous studies on HGS crystallin isolated from human lenses that proposed that deamidation occurs first and structural changes resulting from the increase negative charge facilitate disulfide formation (Hanson et al., 1998). The data also shows that under these experimental conditions in vitro, deamidation of Asn and Gln residues does not occur in the model system.

Formation of these disulfide crosslinks and other PTMs is likely to enhance protein aggregation, this can in turn affect light scattering in the lens and lead to cataract formation.
Chapter 5: Suggestions for Future Work

5.1 Tris-HCl vs PBS buffer

For the studies described in this report, PBS buffer was used. To determine whether PBS or Tris-HCl buffer is better for this \textit{in vitro} study, I incubated HGS samples with concentrations ranging from 1 mg/mL to 5 mg/mL in both Tris-HCl and PBS buffer. The samples were incubated as shown in Table 2.

\textit{Table 2:} pH, temperature, and concentrations of protein samples incubated at 42°C in Tris-HCl and PBS.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Buffer</th>
<th>pH</th>
<th>Temperature (°C)</th>
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</thead>
<tbody>
<tr>
<td>5</td>
<td>PBS</td>
<td>7.2</td>
<td>42</td>
</tr>
<tr>
<td>5</td>
<td>PBS</td>
<td>7.2</td>
<td>42</td>
</tr>
<tr>
<td>5</td>
<td>Tris-HCl</td>
<td>7.2</td>
<td>42</td>
</tr>
<tr>
<td>5</td>
<td>Tris-HCl</td>
<td>7.2</td>
<td>42</td>
</tr>
<tr>
<td>1</td>
<td>PBS</td>
<td>7.2</td>
<td>42</td>
</tr>
<tr>
<td>1</td>
<td>PBS</td>
<td>7.2</td>
<td>42</td>
</tr>
<tr>
<td>1</td>
<td>Tris-HCl</td>
<td>7.2</td>
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<tr>
<td>1</td>
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<td>5</td>
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<td>42</td>
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</tbody>
</table>
The samples were checked every day for signs of precipitate formation. The pH values of the samples were also checked every day to ensure that all the samples stayed at the desired pH. Regular CD data was also taken to ensure that there were no changes to the secondary structure of the protein. After 2 days, the 5 mg/mL samples incubated in PBS started to show signs of precipitation but none of the Tris-HCl samples showed signs of precipitation. The first signs of precipitation in the samples incubated in Tris-HCl were seen after 10 days of incubation. For future studies, to reduce the amount of precipitation, I propose that Tris-HCl buffer be used.

### 5.2 Site-directed mutagenesis

Takata et. al (2009) mimicked deamidation by site-directed mutagenesis and determined the effects of deamidation on the secondary and tertiary structure of βA3-crystallin using molecular modeling, CD, and Fluorescence. The effects of deamidation on the structure of crystallins has yet to be determined using NMR. Using HGS as a
model system, we can mimic deamidation by site-directed mutagenesis and then use NMR to determine the effects of the increase acidity in the sites of deamidation. Information obtained from the NMR spectra can be used to create a molecular model which we can use to answer the question whether deamidation induces a conformational change in the protein that may potentially lead to increase disulfide formation.

5.3 Effects of other Crystallins on Increased Deamidation in Older Lenses

In younger lenses, HGC is found to be the most abundant γ-crystallin, but in older lenses HGS makes up majority of the crystallin fraction (Santhoshkumar et al., 2008). The extent of deamidation also increases as the lens ages (Santhoshkumar et al., 2008). The direct correlation between an increase in deamidation and decrease in HGC has not yet being made. Using HGS as a model system in vitro, we can study the effects of HGS on HGC. We can incubate $^{15}$N- HGS with HGC at physiological temperature and pH and then use NMR to track PTMs of HGS in the presence of HGC. We can also monitor changes to the secondary structure of HGS using CD and fluorescence. Dynamic light scattering can also be used to track the effects of HGS light scattering properties in the presence of HGC over time.

5.4 Solubilization of longer term samples

Longer term samples (72 and 120 days) were not successfully solubilized using the methods described in Chapter 2. In addition, the samples were heated to 100°C.
I propose that 1% SDS be added to the unfolding buffer described in section 2.2.6. SDS is a very strong denaturant that is widely used in protein chemistry. The pH of the unfolding buffer should also be increased to pH 8. To refold the SDS-denatured protein, amphipathic co-solvents should be added to the refolding buffer as described by Michaux et al. (2008).

5.5 Effects of PTMs on Secondary Structure of Crystallins

The changes due to disulfide crosslinking were seen on both the strands and loop regions of the protein. It is not clear whether this is the case for other PTMs, other than disulfide crosslinking, occurring in γ-crystallins. The loop regions of the protein connect two domains. For future studies, I propose that the effects of PTMs on the loop and strand regions of γ-crystallins be studied. By determining which regions in the secondary structure of the γ-crystallins are affected by the different PTMs, the secondary structure rather than the sequence of the crystallins can be used to predict which residues will be affected by the PTMs.
Bibliography


