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The Cataract-associated R14C Mutant of Human γ D-Crystallin Shows a Variety of Intermolecular Disulfide Crosslinks: A Raman Spectroscopic Study[†]

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Abstract

The Arg14 to Cys (R14C)1 mutation in the human γ D-crystallin (HGD)1 gene has been associated with a juvenile-onset hereditary cataract. We showed earlier (1) that rapid oxidation of Cys14 in the mutant leads to the formation of intermolecular, disulfide-crosslinked aggregates at physiological pH. Here we present Raman spectroscopic analysis of R14C and HGD and show that R14C forms such aggregates even at pH 4.5. The lower pH enabled us to monitor the evolution of a variety of disulfide crosslinks with distinct conformations around the CC-SS-CC dihedral angles. At least three cysteine residues are involved, forming protein-protein crosslinks through disulfide-exchange reactions. From the pattern of the S-S and Trp Raman bands, we infer that Cys32 is likely to be involved in the crosslinking. The data suggest that protein precipitation in the mutant may not be the direct result of disulfide crosslinking, although such crosslinking is the initiating event. Thus, our Raman data not only enhance the understanding of the reactivity of Cys14 in the R14C mutant and the mechanism of opacity, but also shed light on the mechanism of oxidative degradation during long-term storage of thiol-containing pharmaceuticals.

Among the rapidly growing class of inherited cataracts, a sizeable number have been associated with mutations in the γ -crystallin genes (2,3). One such example is the Arg 14 to Cys mutation in the HGD gene, observed in juveniles and young adults, and shown to exhibit distinct phenotypes (4) (5). In this form of hereditary cataract, the lens is clear at birth and gradually develops punctate opacities over a period of time as the affected individual reaches early childhood or young adulthood. Stefan et al. (4) were the first to identify the mutation in the HGD gene, and speculated, based on molecular modeling alone, as to how the mutant protein could lead to the observed cataract.

In a previous publication (1), we showed that in contrast to HGD, the R14C mutant rapidly forms protein aggregates composed of intermolecular, disulfide-crosslinks. More importantly, the R14C mutant forms such aggregates even at protein concentrations that are two orders of magnitude lower than that of the wild-type. Whereas HGD remains predominantly monomeric and highly soluble up to protein concentrations of 400–500 mg/ml — values typical of crystallin concentrations within the lens fiber cell *in vivo* (6) — the R14C mutant forms dimers

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¹**Abbreviations:** HGD, recombinant human γ D-crystallin protein; R14C, Arg14 → Cys mutant of HGD; BGB, recombinant bovine γ B-crystallin protein; DTT, dithiothreitol; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); HPLC, high-performance liquid chromatography.

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and higher aggregates at concentrations of 1–2 mg/ml at pH 7. Aggregates of R14C eventually become insoluble and precipitate. We concluded that at physiological pH, the molecular basis for the observed light scattering and opacity due to the mutation was the thiol oxidation-mediated protein aggregation triggered by the highly reactive Cys14 residue in R14C. Our results suggested that the thiol group of the surface-localized Cys14 residue (which replaces the solvent-accessible Arg14) was ionized at physiological pH to the thiolate (S^-) form and was extremely reactive, readily forming protein-protein disulfide crosslinks. From the extensive crosslinking of R14C it was evident that in addition to Cys14, at least one other cysteine residue was involved. Based on homology modeling we proposed that the second cysteine residue was likely to be Cys 110 (now considered Cys 111) (7) with a 10% exposed surface area. These two cysteine residues acting together could produce the observed pattern of aggregation in the mutant protein.

Besides the dramatic changes observed in the aggregation state, liquid-liquid phase separation behavior and protein solubility observed in R14C at pH 7 (1), another intriguing observation about R14C prompted us to undertake the present study. We found that R14C solutions stored at pH 4.5 and 4°C for several days also showed disulfide-crosslinked aggregates, suggesting a more complex mechanism of thiol-mediated aggregation than was apparent from our original study. Therefore, we decided to systematically examine the thiol to disulfide conversions in R14C using Raman spectroscopy, which is a proven method to probe the chemical state of protein thiols and disulfide (8–10). The $-SH$ stretching frequency of cysteine is generally not coupled with any other vibrational mode, and typically appears in an isolated region of the Raman spectrum between 2500–2600 cm^{-1} (9,10). The position of the $-SH$ stretch is an indicator of the hydrogen bonding state of the thiol group (10,11), and thus may reflect upon its reactivity. The disulfide (S-S) Raman frequency arising from the oxidation of protein thiol groups typically appears in the 500–550 cm^{-1} region at the opposite end of the Raman spectrum, also where there is little overlap with other protein contributions. In model compounds and peptides, some lower-frequency S-S modes below 500 cm^{-1} , have also been reported (12)

In practical terms, the Raman measurements at pH 7 were problematic because of the ready aggregation of R14C as stated above, and in Pande et al. (1). Thus, it was not possible to determine the initial state of the thiol groups (i.e. when all seven Cys residues would be fully protonated) in R14C at physiological pH. Since this was a key first step in tracking the $-SH$ to S-S conversion, we decided to make Raman measurements at pH 4.5 where, *as a rule*, all seven $-SH$ groups in R14C would be expected to be fully protonated. It should be mentioned that we generally store γ -crystallin solutions at pH 4.5 and 4°C in sealed and degassed amber-colored ampoules for extended periods of time with minimal oxidation and degradation. However, as the Raman data at pH 4.5 show (this report), contrary to our expectations, we found that the R14C mutant was susceptible to thiol oxidation even at pH 4.5. Furthermore, although the rate of disulfide crosslinking was much slower at pH 4.5 than at pH 7, the data reveal the presence of multiple crosslinks which probably form via a process of disulfide exchange with neighboring protein molecules. These intermolecular S-S crosslinks apparently assume all possible, distinct conformations about the C-C-S-S-C-C torsion angles predicted by other groups based on their Raman studies of a number of model disulfides (13–17). The Raman data also reveal the involvement of at least three and possibly four Cys residues in the oxidation-mediated aggregation of R14C. More importantly, at both pH values (4.5 and 7), the disulfide profiles of the products eventually appear to be identical in the soluble, as well as insoluble-aggregate phase, suggesting that the final redox status of the cysteine residues in both cases is the same.

In this study we attempt to show the potential of Raman spectroscopy in characterizing the thiol oxidation-mediated spectral and phase changes, in a biologically important crystallin

mutant. The measurement of the pK_a of Cys14, and other related studies will be published separately.

MATERIALS AND METHODS

Expression and purification of recombinant proteins

All the procedures used here to express, isolate and purify recombinant wild-type HGD and R14C have been described in detail earlier (1). Protein solutions that were eluted from the cation-exchange column at pH 4.8 were further dialyzed into 0.275M sodium acetate buffer, pH 4.5, and concentrated to about 2–5 mM using an extinction coefficient of $41.4 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 280 nm for both HGD and R14C (1,18). For the data at pH 7, protein solutions were dialyzed into 0.1M sodium phosphate buffer, pH 7.1 (1). The purity of the recombinant proteins was checked using electrospray-ionization mass spectrometry at the Center for Functional Genomics at the University at Albany. The masses of HGD and R14C obtained were $20,608 \pm 1$ and $20,555 \pm 1$ respectively, which are consistent with our previous determinations for the two proteins (1).

Measurement of thiol content

The thiol contents of HGD and R14C were determined using the 5,5'-dithiobis(2-nitrobenzoic acid, DTNB) assay as described in (19). These values agree with those calculated from the areas under the thiol envelopes in the Raman spectra, to within 10%.

Raman spectroscopy

Raman spectra were measured on a state-of-the-art inVia Raman microscope system from Renishaw Inc. (Gloucestershire, U.K), equipped with a Leica microscope, an air-cooled CCD detector and a 785 nm diode laser. Power levels at the sample for 785 nm laser excitation typically averaged about 10 mW. Spectral resolution at this wavelength was 5 cm^{-1} . 10–20 μL of the protein sample at a concentration of 2–5 mM was loaded into quartz capillaries (i.d. = 1.8 mm) secured on a microscope slide, and scanned with 785 nm laser excitation. Since the excitation wavelength is far removed from any protein absorption bands, we did not expect photo-bleaching or heat-induced sample deterioration effects. However, to ensure sample integrity throughout the measurement, we measured the Raman signal from different spots on the sample by moving the sample stage after every few scans, i.e. less than 10 minutes of signal accumulation. We compared each data set carefully and found no changes in the Raman signals from scan to scan. The individual data sets were then added and processed further.

Contributions of buffer bands to the Raman spectra were eliminated using standard subtraction methods. Spectra were calibrated using the 520 cm^{-1} line of silicon. Raman intensities in the -SH region ($2500\text{--}2800 \text{ cm}^{-1}$) were normalized with respect to a protein band near 2727 cm^{-1} (see inset to Fig. 5) which has been shown to be conformation-insensitive (11,20). Previous papers have shown this band to appear around $2730\text{--}2732 \text{ cm}^{-1}$. Spectra in the lower frequency region containing -S-S- bands ($495\text{--}635 \text{ cm}^{-1}$) and the spectral region typical of protein side chains and secondary structure ($700\text{--}1700 \text{ cm}^{-1}$), were normalized with respect to the conformation-insensitive phenylalanine bands at 622 and 1003 cm^{-1} respectively.

Raman spectra of the insoluble aggregates were measured as follows: 100 μL aliquots of the sediment from suspensions of R14C samples which were stored at 4°C for several months was repeatedly washed with double distilled water to minimize the concentration of acetate buffer. A 10 μL drop was placed on a glass slide and allowed to evaporate to dryness. Spectra were measured from different points of the sample from 400 to 2800 cm^{-1} , carefully compared and added together, as described above.

Computational method

To model the effect of a Trp residue located within $\sim 3\text{--}4\text{\AA}$ of a disulfide crosslink, we computed the Raman spectra of 1) a cystine residue alone, 2) a tryptophan residue alone and 3) a cystine together with a tryptophan using Density Functional Theory (DFT) in the Gaussian 03 program (21). A B3LYP functional was employed along with the 6-31G (d) basis set to calculate the Raman spectra. Frequencies obtained from the Gaussian calculation were not scaled for comparison with those observed experimentally.

RESULTS

The thiol and disulfide spectral regions

Initial stages at pH 4.5—In Fig. 1A we compare the Raman spectra of R14C and HGD at pH 4.5, in the $2500\text{--}2600\text{ cm}^{-1}$ region typical of the --SH stretching frequency. The corresponding spectra in the S-S frequency region are shown in Fig. 1B. The --SH band intensities in Fig. 1A are normalized with respect to the conformation-insensitive protein mode at 2727 cm^{-1} (Fig. 5 inset, (11), (22)).

We now make the assumption that the area under the Raman --SH band of HGD corresponds to 6 Cys residues — i.e. the total number of Cys residues in HGD — all of which are known to be in the reduced form. The justification for this assumption comes from two sources: 1) We find that in the highly homologous bovine γB -crystallin (BGB)¹, the Raman intensity of each of the seven Cys residues is almost identical, even though the individual --SH frequencies are slightly different (20), and 2) Raso et al (23) also found that the eight Cys residues in the P22 tail spike protein contribute equally to the composite Raman intensity of the --SH band, even though their individual --SH frequencies were quite distinct. Thus, experimentally, while the precise frequency of the --SH stretching mode depends upon the H-bonding of the --SH group, its Raman cross section appears to be constant.

Fig. 1A shows that the --SH bands of R14C and HGD appear as an asymmetric doublet with a main band near 2580 cm^{-1} and a distinct shoulder around $2562\text{--}2565\text{ cm}^{-1}$. In freshly prepared solutions of R14C (red curve), the area under the thiol band envelope corresponds to about 6.8 cysteine residues relative to HGD (black curve) with 6 cysteine residues. Thus, the ratio of the areas R14C/HGD is $6.8/6$ or 1.13 , which scales closely with $7/6$ or 1.17 , the ratio of the theoretical number of cysteines in R14C (7 Cys) and HGD (6 Cys), indicating that each cysteine residue in R14C contributes equally to the --SH band envelope. The area under the thiol envelope of R14C is $<10\%$ lower than the theoretical value, suggesting that the Cys14 thiol is oxidized, albeit minimally, even in freshly prepared solutions.

Within a matter of 48–72 hours, the area under the thiol band envelope of R14C (green curve) falls below that of HGD, and the reduced area corresponds to the loss of about 1.7 ± 0.2 thiol groups. This pattern continues as the samples age further at pH 4.5 such that after several weeks (Fig. 1A, pink curve), about 3.0 ± 0.2 of the seven protein thiols are oxidized. Thus, contrary to expectations, R14C clearly shows thiol oxidation and aggregation also at pH 4.5 in a manner reminiscent of its behavior at pH 7, although at a slower rate.

In Fig. 1B the corresponding spectra in the S-S region for HGD and R14C are shown, with intensities normalized to the phenylalanine band around 623 cm^{-1} . Again, even in freshly prepared solutions (Fig. 1B, red curve), the data show a shoulder at 508 cm^{-1} , which is consistent with the small amount of thiol oxidation evident from the --SH region (Fig. 1A, red curve), and indicates that disulfide crosslinking has begun. Raman bands near 510 cm^{-1} are typical of disulfide bonds in proteins (8,9). In parallel, the bands spanning $547\text{--}571\text{ cm}^{-1}$ appear to be lower in intensity relative to HGD, even though the band profile is essentially unchanged. Within 48–72 hours, the 508 cm^{-1} shoulder is replaced by a broad band centered at 526

cm⁻¹ (Fig. 1B, green curve), which corresponds to the loss of 1.7 ± 0.2 thiol groups (Fig. 1A and previous paragraph). This change is accompanied by a further lowering of intensity in the 547–571 cm⁻¹ region. As the sample ages further to several weeks, there is another shift in the pattern of evolution of the S-S frequency (Fig. 1B, pink curve). The broad band centered at 526 cm⁻¹ (Fig. 1B, green curve) is replaced by two disulfide bands centered at 518 and 538 cm⁻¹. Simultaneously, several marked changes occur in the 547–571 cm⁻¹ region with a reduction in intensity of the 547 cm⁻¹ band and an increase in intensity of the 571 cm⁻¹ band, and possibly the 555 cm⁻¹ band. As mentioned above, although about 3 of the protein thiols are lost in R14C (Fig. 1A, pink curve), the aggregates are still largely soluble and reversible to the monomer form with DTT treatment. There is little sedimentation occurring in the solution at this stage.

Late stage spectra at pH 4.5—The effects of prolonged aging (samples kept at pH 4.5 and 4°C for several months to a year), are seen in the spectra shown in Fig. 2A and B. Here the protein aggregates are no longer soluble and sediment out of solution. We found no further significant change in the Raman spectra of solutions of R14C aged over a year in which the soluble protein coexists with the aggregated protein in the solid phase. A notable feature of these data is that while the -SH region of the soluble (supernatant) phase of R14C shows that about half the cysteine residues are oxidized (3.5 ± 0.2 , Fig. 2A, red curve), there is little change in the pattern of evolution of the disulfide bands, or the 547–571 cm⁻¹ bands (Fig. 2B, red curve) beyond what was observed in Fig. 1B (pink curve). The area under the -SH band in the solid aggregates (Fig 2A, green curve) is nearly equal to that in the supernatant (red curve) but the band profile differs in that it is broader, and unlike the supernatant, has no defined shoulder around 2563–65 cm⁻¹. The data in Fig. 2 lead us to conclude that at pH 4.5 only about half the cysteine residues of R14C are susceptible to oxidation and intermolecular disulfide-crosslinking. Furthermore, protein thiols contributing to both bands in the doublet (2563 cm⁻¹ and 2580 cm⁻¹) seem to be involved in the aggregation of R14C, with those contributing to 2580 cm⁻¹ being preferentially oxidized first. In the disulfide frequency region (Fig. 2B, green curve), the sedimented aggregates show a somewhat featureless spectrum, although the S-S frequencies, while rather broad, appear to be at about the same location as in the supernatant.

Spectral profiles at pH 7—In Fig. 3A we present the Raman spectra in the thiol region, and in Fig. 3B, the disulfide region for HGD and R14C at pH 7. At this pH, consistent with our previous observation that R14C undergoes rapid thiol oxidation-mediated aggregation at pH 7 (1), we were unable to track the progressive loss of thiols and pattern of evolution of the disulfides which we observed at pH 4.5 (Fig. 1 and Fig. 2). Thus, the spectrum of R14C shown in Fig. 3A (red curve) corresponds to the depletion of approximately half the protein thiols and is comparable to the data shown in Fig. 2A. It is notable that the two spectra of the R14C supernatant (pH 7, Fig. 3A, red curve) and (pH 4.5, Fig. 2A red curve) are nearly identical, strongly suggesting that while the rate of oxidation at pH 7 is considerably higher than at pH 4.5, the final redox status of the protein in the two cases is identical.

The 545–575 cm⁻¹ region—We noted in the beginning of the Results section that the emergence of disulfide frequencies in R14C is accompanied by intensity changes in the 547–571 cm⁻¹ bands (Fig. 1B, Fig. 2B). These bands initially show a drop in intensity (Fig. 1B, red and green curves), followed by a recovery in the intensity of the 555 and 571 cm⁻¹ bands, but not the 547 cm⁻¹ band (Fig. 1B, pink curve). Raman bands in the 547–571 cm⁻¹ region have been shown to arise from some of the numerous ring modes of tryptophan (24). Lord and Yu (25,26) were the first to assign the relatively weak band near 547 cm⁻¹ to a tryptophan ring breathing mode in lysozyme and α -chymotrypsin, but not much has been reported about the 547 cm⁻¹ band in proteins probably because of overlapping disulfide frequencies in this

spectral region. These changes in intensities of the tryptophan ring modes due to disulfide crosslinking suggested a possible interaction between an S-S crosslink and a neighboring Trp residue, and led us to investigate this further. Due to the proximity of Cys32 to Arg14 in the x-ray crystal structure (Fig. 4A, (27)) it seemed probable that an intermolecular disulfide crosslink formed by Cys32 could be involved in such S-S...Trp interactions. We examine this possibility further in the Discussion section.

In an attempt to understand the intensity changes in the 547–571 cm^{-1} bands, we modeled the Raman spectra of a tryptophan residue alone, and a tryptophan residue together with a cystine residue within $\sim 3\text{--}4\text{\AA}$ of the S-S bond, using Density Functional Theory implemented in the Gaussian 03W program (21). These results are shown in Fig. 4B in which the black curve represents the calculated Raman spectrum for Trp alone, and the red curve is the combined Trp plus S-S spectrum. Here we see that a band at 534 cm^{-1} in the spectrum for Trp alone (which is likely to be at 547 cm^{-1} in the protein), is absent in the combined spectrum. At the same time, the intensity of the bands near 565 and 572 cm^{-1} (which are close to the 555 and 571 cm^{-1} bands in R14C) are enhanced in the combined spectrum. We recognize that the calculated model spectra cannot be directly extrapolated to proteins, and the absolute intensities may not be reliable. However the model spectra do show the same trends in the intensity changes for Trp modes observed in our R14C spectra due to the formation of disulfide crosslinks.

The –SH frequency of the Cys14 thiol group

From the data in Fig. 1A, where thiol oxidation is minimal (red curve), it is possible to determine the –SH frequency of the thiol group of Cys14. In Fig. 5 we present the Raman difference spectrum obtained by subtracting the spectrum of HGD from that of R14C (the inset to Fig. 5 shows the full spectra in the 2500–2800 cm^{-1} region for HGD and R14C with intensities normalized to that of the 2727 cm^{-1} band). The –SH stretching frequency of Cys14 appears predominantly as a single band centered around 2575 cm^{-1} . A minor conformation may be present as indicated by the shoulder at 2592 cm^{-1} , but given that this shoulder is weak in the difference spectrum, it cannot be identified with certainty. The shift in the Cys14 –SH frequency from 2580 cm^{-1} in R14C to 2575 cm^{-1} indicates that Cys14 is more strongly hydrogen bonded than the cysteine residues contributing to the 2580 cm^{-1} band in R14C. Since Cys14 is located on the protein surface, it is likely to be hydrogen-bonded to either the solvent water or a neighboring H-bond donor.

Region specific to secondary structure and side-chain modes

Fig. 6 shows the Raman spectra of HGD and R14C in the spectral region sensitive to secondary structure and side-chain modes. In the 1000–1800 cm^{-1} region (also referred to as the “fingerprint region”) vibrational modes sensitive to the secondary structure and specific side chains, some of which are conformation sensitive, are seen. The data show that despite the marked changes in the –SH and –S-S- stretch regions, bands in the fingerprint region of the mutant protein (red, green and pink curves) are generally within $\pm 3\text{ cm}^{-1}$ of the wild-type (black curve) even in the solid phase containing aggregated protein (pink curve). This includes the amide-I and amide-III bands at 1672 and 1238 cm^{-1} that are typical of antiparallel beta sheet-containing proteins (9), the bands assigned to phenylalanine (623, 1003, 1032 cm^{-1}), and tyrosine (833, 856, 1211 cm^{-1}), and the higher frequency modes of tryptophan (758, 879 and 1340 cm^{-1}). In proteins, a sharp band near 1360 cm^{-1} which is part of the Fermi doublet consisting of the 1360 and 1340 cm^{-1} bands (28) has been assigned to Trp and is reported to be indicative of buried tryptophan residues (29). Interestingly, although the Trp residues in HGD are known to be buried (7), this band appears only as a weak shoulder in the data shown in Fig. 6. The absence of a sharp 1360 cm^{-1} band has also been noted in other proteins in which Trp residues are buried in a hydrophobic environment (29). Therefore, contrary to existing beliefs, the 1360 cm^{-1} band may not be a good indicator of the environment of tryptophan

residues in proteins. We should state that in this spectral region, due to the varying backgrounds for individual protein samples, changes in peak intensities are not quantitative and cannot be used to identify differences between the two proteins. Nevertheless, the data shown in Fig. 6 indicate that the secondary structure of R14C is largely preserved during the various stages of aggregation, including the formation of the solid phase.

DISCUSSION

The Raman spectroscopic data presented here clearly show that the thiol group of Cys14 is sufficiently ionized to the thiolate form not only at physiological pH, consistent with our original conclusion, but also at pH 4.5, which accounts for the formation of disulfide bonds at the lower pH as well. Such aggregation *per se* is not unusual for a cysteine residue located on the protein surface. We have observed a similar pattern of aggregation also in the case of the bovine ortholog, BGB at pH 7, in which the intrinsic Cys15 residue on the surface forms intermolecular, disulfide crosslinked aggregates (19). However, what is unusual in the case of R14C is that the Cys14 thiol forms such crosslinks also at pH 4.5, a phenomenon not observed in BGB. The Raman frequency assigned to Cys14 in this work (Fig. 5) at 2575 cm^{-1} indicates that the reactive -SH group acts as a weak to moderate hydrogen-bond donor or acceptor (10) either to solvent water, or to an internal protein group. Since the 3D structure of R14C is not available, we have modeled the R14C mutation on the HGD structure, and find that Glu7 or Tyr28 are the likely residues for H-bonding, depending on the orientation of the -SH group in Cys14. Clearly, the ionization of Cys14 will be influenced by neighboring amino acid residues, which could explain why Cys14 is ionized also at the lower pH and may have a pK_a value well below 8.5, the normal pK_a of cysteine. We are in the process of determining the pK_a of Cys14 and will present the data elsewhere.

The Raman data at pH 4.5, obtained by monitoring oxidation-mediated changes over extended periods of time, reveal how the pattern of intermolecular disulfide crosslinks evolves in R14C. These changes occur at a faster rate at pH 7 – hence only the final product is seen at this pH. The data at both pH values suggest that at least three or possibly four Cys residues may be involved in the oxidation-mediated aggregation of R14C. The uneven loss in the two -SH bands of R14C as oxidation progresses, clearly suggests that the Cys residues contributing to the shoulder around 2565 cm^{-1} (indicative of stronger H-bonding), are much less diminished compared to the ones contributing to the Cys frequency around 2580 cm^{-1} (which represents a weaker H-bonding of the -SH group). We and others have found that the Raman -SH frequency of individual Cys residues indicates how a single Cys residue can populate two or more distinct hydrogen-bonding states ((23), Pande et al., unpublished data). Therefore it is likely that in the Cys residues in R14C contributing to the strongly H-bonded state (i.e. the shoulder around $2562\text{--}2565\text{ cm}^{-1}$), the S^- ion is better stabilized and hence less reactive. If that is generally true, the position of the Raman SH band may become a good indicator of its reactivity. Based on our high-resolution x-ray crystal structure of HGD (27) we previously invoked the participation of the partially exposed Cys110 (now designated as Cys111) in addition to Cys14 in intermolecular disulfide crosslinking and aggregation (1). In addition, we now suggest that Cys32, in the N-terminal domain, which is not solvent-accessible in HGD (7) may also be implicated in intermolecular crosslinking together with Cys14 and Cys111.

The x-ray crystal structure of HGD (27) consists of two domains of almost equal size, each consisting of two Greek key motifs. In HGD, Arg 14 is extensively H-bonded to Pro27, Tyr28 and Ser30 and may play a role in maintaining the local structure in the first Greek key motif. Replacement of Arg 14 with Cys, clearly results in a diminution of this H-bonding network since Cys14 cannot form all these H-bonds. The overall effect of a weakened H-bonding may be an increased flexibility of the local structure surrounding residue 14. Thus, the long loop (residues 19 to 33) containing Cys32 is less constrained, making the Cys32 thiol solvent-

accessible, and amenable for intermolecular disulfide formation (Fig. 4A). Such a disulfide bond would be less than 4 Å from Trp 68. It should be noted that Cys78 and Trp 42 are also about 4 Å apart, but that region of the structure in R14C is more distant and not likely to be affected by the mutation at residue 14. Thus, from our earlier HPLC data (1), which showed that progressively larger disulfide-linked aggregates form in solutions of R14C over time, and our current data which shows that as oxidation progresses, there is an inverse correlation between the intensities of the S-S Raman bands with a Trp Raman band (Fig. 2B), we conclude that Cys32 is likely to be involved in *intermolecular* disulfide crosslinking. While an *intramolecular* S-S bond involving Cys18 and Cys78, which would be close to Trp42, is possible, we reject this possibility since it cannot explain the formation of progressively larger aggregates over time. Furthermore, Hains and Truscott (30) report that Cys18 remains in the reduced form even in very advanced cases of nuclear cataract in which all other Cys residues appear to be oxidized. Hence *intramolecular* S-S crosslinking involving Cys18 is highly unlikely. Finally, we recognize that the unambiguous identification of intermolecular disulfides would require other analytical techniques such as mass spectrometric analysis of peptides derived from the crosslinked products. That type of analysis is however, beyond the scope of the present work.

For the assignment of Raman bands between 547–571 cm⁻¹, we defer to the work of two groups of investigators who have assigned experimentally (24) and theoretically (31) several Raman bands of indole and tryptophan, including those around 544, 554 and 571 cm⁻¹ to indole ring deformation modes. From their work we conclude that the Raman band near 540 cm⁻¹, in HGD and R14C, most likely arises from a benzene ring deformation mode in tryptophan. Our calculated spectra of a tryptophan residue with and without a cystine disulfide bridge within 3–4 Å of the indole ring (Fig. 4B), show that the Trp ring mode at 534 cm⁻¹ is absent when coupled with an S-S linkage in its vicinity, even as the bands at 565 and 572 cm⁻¹ are considerably enhanced.

Interactions such as those described above between aromatic rings and disulfides are quite common in proteins and are often invoked to understand their biological function. In a recent review of such interactions, Meyer et al (32) highlight the finding (33) that in a survey of over 60 structures of immunoglobulin (Ig) proteins, that a highly conserved (Cys SS)...Trp interaction emerges within the Ig fold motif. Clearly therefore, such Trp-disulfide interactions are biologically important and Raman spectroscopy may be a good technique to detect them. In fact, ultraviolet resonance (UV-resonance) Raman spectroscopy with an excitation wavelength close to 280 nm (the absorption maximum of tryptophan), may be a better tool to identify such interactions definitively, but so far, the UV-resonance Raman studies of Trp have only focused on the tryptophan Raman lines above 700 cm⁻¹ (34), and to our knowledge, no data is currently available in the lower frequency region.

Conformations of disulfides

Our Raman data also reveal an intriguing variety of disulfide crosslinks. The first discernible S-S band at 508 cm⁻¹ appears as a small shoulder at the onset of aggregation in R14C when the concentration of aggregates detected by HPLC is minimal (≤3%). An S-S frequency around 510 cm⁻¹ has been routinely observed in proteins containing *intra*-molecular disulfide bonds arising from cystine residues (9). For R14C however, this band seems to be an indicator of an *inter*-molecular S-S crosslink at the early stage of protein aggregation. Wild-type HGD does not have cystine residues and no *intra*-molecular S-S bonds have been detected (27). The disappearance of this band and the emergence of a broad S-S band near 526 cm⁻¹ as aggregation proceeds further suggests that disulfide exchange reactions and rearrangements between neighboring protein molecules may be taking place until stable and distinct disulfide

frequencies emerge around 518 and 538 cm^{-1} , both in the soluble protein as well as the solid phase (Fig. 1B, Fig. 2B).

Based on a comprehensive examination of model compounds containing a variety of primary, secondary and tertiary disulfide bonds, Sugeta et al. (13,14) and van Wart and Scheraga (15, 16) have correlated the observed S-S stretching frequencies in the Raman spectra to the conformation of the C-C-S-S-C-C moieties in these compounds. Their work collectively suggests that in the case of primary disulfides (such as cystine), (a) a $\nu(\text{S-S})$ around $510 \pm 5 \text{ cm}^{-1}$ arises from a gauche-gauche-gauche conformation or a dihedral angle, $\chi(\text{SS-CC})$ between $50\text{--}180^\circ$ on either side of the S-S bond, (b) a $\nu(\text{S-S})$ of $525 \pm 5 \text{ cm}^{-1}$ has a trans-gauche-gauche conformation or a $\chi(\text{SS-CC})$ of $0\text{--}50^\circ$ and $50\text{--}180^\circ$ on either side, and (c) a $\nu(\text{S-S})$ near $540 \pm 5 \text{ cm}^{-1}$ has a trans-gauche-trans conformation or a $\chi(\text{SS-CC})$ of $0\text{--}50^\circ$ (13,14,17). These assignments have generally been applied to proteins containing *intra*-molecular disulfides, but have recently been used to interpret the *inter*-molecular disulfide frequencies in the Raman spectra of peptides of a lung surfactant (12). Our data show that in R14C, the conformations of the intermolecular, primary disulfide crosslinks in the $500\text{--}540 \text{ cm}^{-1}$ region fall mainly into the three recognized classes first proposed by the two schools of thought.

It should be mentioned that for R14C, we have observed what appear to be additional disulfide bands below 500 cm^{-1} that are not present in HGD (data not shown). These bands are not shown because they are superimposed on a broad, moderately intense protein mode of unknown origin in HGD around $490\text{--}496 \text{ cm}^{-1}$ (35) which makes it difficult to accurately detect and assign them. However, their presence suggests that disulfide conformations with strained S-S bonds as originally suggested by van Wart and Scheraga (16) may be formed during protein-protein disulfide crosslinking in R14C. Strained disulfides have also been reported in lung surfactant peptides by Biswas et al. (12).

Aggregation and overall protein structure

The Raman data shown here also support our view that global protein unfolding is not required to effect the pathological changes due to a point mutation in proteins (1). Even at advanced stages of thiol oxidation, the secondary structure of the mutant protein is maintained. Li et al (36) have studied the effect of intermolecular disulfide bond formation on the structure and stability of a member of the TGF- β family of proteins, and shown that these characteristics are not altered by intermolecular disulfide-crosslinking. To our knowledge, the Raman data presented here are the first to reveal that multiple conformational isomers of intermolecular disulfide crosslinks are formed by disulfide exchange reactions in a disease-associated mutant of human γD -crystallin. In an interesting study using Raman spectroscopy, Schlucker et al (37) have also identified conformational isomers of protein disulfides due to a disease-linked genetic mutation in hair shafts.

As shown in the Results section only about half the cysteine residues in R14C are involved in intermolecular disulfide-crosslinking in both solution and solid phases. Thus the aggregation of R14C to the insoluble phase must proceed by secondary mechanisms following disulfide crosslinking. Earlier (1) we observed the temporal evolution of aggregation in dilute ($1\text{--}2 \text{ mg/ml}$) solutions of R14C at neutral pH, and found that dimers and higher oligomers are formed that could be quantitatively reversed to monomers with DTT. However when the protein concentration was higher than $\sim 100 \text{ mg/ml}$, the soluble oligomers formed initially, rapidly lead to protein precipitation but the precipitate could not be quantitatively monomerized with DTT. Therefore, at high protein concentrations, protein precipitation is likely to result from a mechanism other than disulfide crosslinking even though it is initiated by such crosslinking. Based on these observations, it is tempting to suggest a mechanism for the observed irreversible aggregation, which is similar to the one put forth by Guo and Eisenberg (38) for amyloid formation in which intermolecular disulfide crosslinks facilitate "runaway domain-swapping".

As the name implies, it is distinct from the domain swapping which leads to homologous dimer formation in some proteins, such as in β B2 crystallin, a member of the β - γ crystallin family (39). Cho et al (40) have looked into a large number of cases of domain swapping in a variety of proteins and have provided an interesting computational analysis of this process, especially the involvement of disulfide bonds. Their results suggest that intermolecular disulfide bonds may be critical to facilitating domain swapping in some proteins.

Implications for pharmaceutical applications and cataract formation *in vivo*

Our Raman data also highlight how the long-term storage of protein solutions often leads to oxidation and disulfide crosslink formation. Such oxidation-mediated changes brought about by the aging of protein solutions are an important challenge for the pharmaceutical industry as well (41,42), where it is essential that protein-based drugs stored for long periods of time remain viable. Our studies suggest that Raman spectroscopy can be a useful technique to track and quantify the evolution of disulfide crosslinks over time, in both the solution and solid phases of protein-based drugs.

The observation that aggregation of R14C can be suppressed by DTT in the early stages ((1), this report) clearly suggests that the deleterious effects of this mutation can be inhibited under reducing conditions. Since the lens is known to have strong redox regulation mediated by several enzymes including thiol transferase and thioredoxin (43), it is rather puzzling that individuals with this mutation do get cataract, although the progression of opacity appears to vary with the individual even within a family (4). This suggests the possibility that the redox systems may be compromised to different degrees in these individuals. The recent work of Lofgren et al. (44) shows that in thiol transferase knockout mice, the susceptibility of lens epithelial cells to oxidative stress is significantly enhanced. It is conceivable that the reduced level of redox regulating enzymes in these individuals, may affect the progression of the cataract. We propose that the levels of redox regulating enzymes in the cataractous lens materials from affected individuals be carefully compared, which would be an important step towards providing a plausible mechanism of cataract formation *in vivo* due to this mutation.

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Fig. 1A

Fig. 1B

FIGURE 1.

Raman spectra of HGD and R14C at pH 4.5 in the (A) -SH frequency region and (B) in the S-S frequency region. (HGD, black curve; R14C when freshly prepared, red curve; R14C 48–72 hours later, green curve; R14C several weeks later, pink curve). Intensities in the -SH region are normalized with respect to the conformation-insensitive band at 2727 cm^{-1} (shown in the inset to Fig. 5), and in the S-S region with respect to the phenylalanine band at 623 cm^{-1} .

Fig. 2A

Fig. 2B

FIGURE 2.

Raman spectra of HGD and R14C showing long-term effects at pH 4.5 when insoluble protein aggregates are formed. (A) The $-SH$ region showing no change in HGD (black curve), the R14C supernatant showing that only about half the protein thiols remain (red curve), and the solid phase also showing about half the protein thiols remaining (green curve). (B) The $S-S$ region with the same color scheme as in (A). The overall pattern of disulfides in the supernatant and sediment is nearly identical.

Fig. 3A

Fig. 3B

FIGURE 3.

Raman spectra of HGD and R14C at pH 7. (A) The -SH region showing freshly prepared HGD (black curve, 6 -SH groups), R14C in the same time period (red curve, 3.5 ± 0.2 -SH groups). (B) The S-S region for the same samples with the same color scheme.

Fig. 4A

Fig. 4B

FIGURE 4.

(A) A part of the N-terminal domain of HGD (PDB ID: 1hk0) is shown here. The proximity of Cys 32 and Trp 68 is shown (3.7 Å) in the foreground. The extensive H-bonding network of Arg14 with Glu7, Pro27 and Ser30 is indicated in the upper left-hand corner. In the R14C mutant, this H-bonding is considerably diminished which in turn may allow Cys32 to be solvent-accessible and amenable to oxidation (see text for details). The picture was drawn using DSVIEWERPro (Accelrys). (B) Raman spectra of model tryptophan with and without a disulfide bond in the vicinity, generated using the Gaussian software: Tryptophan residue alone (black curve), Tryptophan with a cystine S-S within ~3–4 Å of the indole ring (red curve). The bands

at 534, 565 and 572 cm^{-1} closely approximate the bands around 547, 555 and 571 cm^{-1} in HGD and R14C which are typically assigned to tryptophan ring modes in proteins.

FIGURE 5.

Raman difference spectrum of the data shown in Fig. 1A showing the -SH frequency of the Cys14 residue in R14C. The inset shows the spectra of HGD and R14C (with minimal thiol oxidation at pH 4.5) used to determine the -SH frequency of the Cys14 residue.

FIGURE 6.

Raman spectra of HGD and R14C in the fingerprint region at pH 4.5. Compared to HGD (black curve), the secondary structure of R14C is essentially unchanged in all states of thiol oxidation: R14C solution with little or no oxidation (red curve), R14C solution with less than half the thiols oxidized (green curve) and R14C aggregates in the solid phase with about half the thiols oxidized (pink curve). Intensities in this region are normalized with respect to the phenylalanine band at 1003 cm^{-1} , but cannot be compared quantitatively for the different samples due to variations in their individual backgrounds.