The Human W42R γD-Crystallin Mutant Structure Provides a Link between Congenital and Age-related Cataracts**

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Background: The mechanism of cataract formation by the recently discovered γD-crystallin W42R mutant is unknown.

Results: Structural, biochemical, and biophysical studies revealed a partially unfolded species of the W42R mutant.

Conclusion: Partially unfolded species serve as nuclei for aggregation.

Significance: The properties of the W42R mutant γD-crystallin provide the link to the pathogenesis of age-related cataract caused by photodamaged wild-type γD-crystallin.

Some mutants of human γD-crystallin are closely linked to congenital cataracts, although the detailed molecular mechanisms of mutant-associated cataract formation are generally not known. Here we report on a recently discovered γD-crystallin mutant (W42R) that has been linked to autosomal dominant, congenital cataracts in a Chinese family. The mutant protein is much less soluble and stable than wild-type γD-crystallin. We solved the crystal structure of W42R at 1.7 Å resolution, which revealed only minor differences from the wild-type structure. Interestingly, the W42R variant is highly susceptible to protease digestion, suggesting the presence of a small population of partially unfolded protein. This partially unfolded species was confirmed and quantified by NMR spectroscopy. Hydrogen/deuterium exchange experiments revealed chemical exchange between the folded and unfolded species. Exposure of wild-type γD-crystallin to UV caused damage to the N-terminal domain of the protein, resulting in very similar proteolytic susceptibility as observed for the W42R mutant. Altogether, our combined data allowed us to propose a model for W42R pathogenesis, with the W42R mutant serving as a mimic for photodamaged γD-crystallin involved in age-related cataract.

Cataract, referred to as opacification of all or part of the crystalline lens of the eye (1), can be broadly divided into two classes: early onset (congenital or juvenile) and age-related cataract (2). The congenital cataracts occur at an estimated prevalence of 2.2–2.5 cases per 10,000 live births (3), and ~50% of congenital cataracts are inherited (4). Cataract-associated mutations in the crystallin genes are known to occur in all three crystalline lens domains: early onset (congenital or juvenile) and age-related cataract.

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The atomic coordinates and structure factors (code 4GR7) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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Cataract Formation by the W42R Mutant of Human γD-Crystallin

todamage in the crystallins may be one of the factors in the etiology of cataract formation (25).

Recently, a novel mutation in HGD was discovered in a Chinese family, with the affected individuals exhibiting nuclear congenital cataracts (26). A single T/C change at position 127 in the CRYGD gene was mapped, translating into a tryptophan to arginine change at position 42 (W42R) in the amino acid sequence of the mature protein, i.e., the loss of one of the four conserved tryptophans from the protein. The W42R mutant protein was reported to be less stable and more prone to aggregate when subjected to environmental stresses, such as heat and UV irradiation, when compared with HGD (26). However, no structural or other biochemical characterizations were carried out to elucidate how this mutation could lead to cataract formation.

To address whether the loss of the tryptophan residue in the mutant alters the protein such as to make it more susceptible to aggregation and precipitation, we investigated W42R γD-crystallin by NMR spectroscopy and determined its high resolution x-ray structure. In addition, the thermodynamic behavior was probed by guanidinium hydrochloride (GdnHCl)-induced unfolding, and a comparative proteolysis study was performed for W42R and HGD. We show that W42R exhibits the HGD fold with no major, discernable conformational differences between mutant and wild type, although the relative domain orientation in the two x-ray structures is slightly different. Interestingly, however, a small, but noteworthy fraction of partially unfolded protein was detected for the W42R mutant in solution by NMR spectroscopy. Based on our overall results, we conclude that the W42R mutation introduces partial unfolding in the N-td, mimicking the effect of photodamage caused by UV exposure of HGD. This partially folded species may act as the seed for aggregation and precipitation of the mutant protein, nucleating cataract formation.

EXPERIMENTAL PROCEDURES

Expression and Purification of Proteins—Cloning, expression, and purification of the W42R mutant was essentially similar to that previously reported for wild-type HGD and the R76S mutant (27, 28). Briefly, Escherichia coli cells that harbored pET-14b with the mutant γD-crystallin gene were grown at 37 °C, induced with 1 mM isopropyl-1-thio-pET-14b with the mutant similar to that previously reported for wild-type HGD and the R76S mutation, and purification of the W42R mutant was essentially similar to that previously reported for wild-type HGD and the R76S mutation. To purify the protein, we harvested bacteria and added antibiotics to growth medium (50 μg/ml) and lysed by passage through a Microfluidizer (Microfluidics, Newton, MA). The cell lysate was clarified by centrifugation at 100,000 × g for 1 h, and the supernatant was loaded onto a HiTrap Q XL anion exchange column (GE Healthcare), or 1 M Tris-HCl (pH 8.5). Subsequent optimization was performed by adding 1% (w/v) of octyl β-D-glucopyranoside to the protein solution (8, 12, and 16 mg/ml) and by varying both Li2SO4 (0.14, 0.18, 0.22, and 0.26 M) and PEG 4000 (18, 20, 22, 24, 26, and 28%) concentrations. The ratio of protein to mother liquor was kept constant at 2.2 μl.

The final purification step was a Mono Q HR5/5 column (GE Healthcare) in 20 mM sodium phosphate buffer (pH 6.2), 1 mM EDTA, 5 mM DTT as the final purification step.

All purified proteins were characterized by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry on a Voyager-DE PRO instrument, operated in a linear mode with external calibration. HGD and W42R exhibited experimental molecular masses of 20,592.10 and 20,558.05 Da, respectively (the theoretical molecular masses are 20,606.94 and 20,576.91 Da, respectively). Protein concentrations were determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific), using extinction coefficients of 42.86 and 37.36 mM⁻¹ cm⁻¹ at 280 nm for HGD and W42R, respectively.

Crystallography—Small and low quality crystals of W42R were initially grown using the sitting drop vapor diffusion method from a solution containing 0.17 M Li2SO4, 0.085 M Tris-HCl (pH 8.5), 25.5% (v/v) PEG 4000, and 15% (v/v) glycerol. Subsequent optimization was performed by adding 1% (w/v) of octyl β-D-glucopyranoside to the protein solution (8, 12, and 16 mg/ml) and by varying both Li2SO4 (0.14, 0.18, 0.22, and 0.26 M) and PEG 4000 (18, 20, 22, 24, 26, and 28%) concentrations. The ratio of protein to mother liquor was kept constant at 2.2 μl.

Chemical Denaturation—Chemical unfolding by GdnHCl was monitored by tryptophan fluorescence, using an excitation wavelength of 295 nm, and emission was measured over 305–420 nm. Spectra were recorded at 37 °C on a Cary Eclipse fluo-
Cataract Formation by the W42R Mutant of Human γD-Crystallin

TABLE 1
Data collection and refinement statistics (molecular replacement) for W42R

| Data collection | P3,21 |
|----------------|-------|
| Space group    |       |
| Cell dimensions| 68.61, 68.61, 133.38 |
| a, b, c (Å)    | 90, 90, 120 |
| Resolution (Å) | 33.22–1.60 (1.66–1.60) |
| Rmerge (I/σI)  | 0.115 (0.574) |
| Completeness (%) | 11.2 (2.1) |
| (Redundancy)   | 92.1 (84.0) |
| Refinement     | 10.59 (6.33) |

Structure Assessment Using NMR

To assess the W42R mutant structure, we recorded 1H-15N HSQC spectra of the mutant and the wild-type protein under identical buffer conditions. Because backbone 1H-15N amide group resonances are very sensitive to conformation and electronic environment, 1H-15N HSQC spectra can be used as a fingerprint of the conformation of a protein. In the W42R mutant spectrum, 166 of 168 possible amide resonances were assigned. As evidenced by the superposition of the 1H-15N HSQC spectra of W42R and HGD shown in Fig. 1A, the mutant exhibits equally well dispersed resonances of uniform line widths as the wild-type protein, with most resonances overlapping or shifted only by a small amount. This suggests that the structure of the mutant is very similar to that of HGD. A quantitative comparison of the backbone chemical shifts between W42R and HGD reveals that all effects of the mutation are confined to the N-td, and perturbed resonances of uniform line widths as the wild-type protein, with most resonances overlapping or shifted only by a small amount.
associated with Ile-3, Ser-39, Cys-41, and Ile-81, with a maximum value of ~1.25 ppm. As expected, removal of the tryptophan side chain is expected to affect amide resonances of residues located above or below the large aromatic ring because ring current shifts will no longer be present in the mutant. Indeed, such changes are observed for Arg-2–Lys-5, Val-75, Cys-78–Ile-81, and Ser-84.

At first sight, it seemed surprising that the replacement of the large hydrophobic side chain (Trp) by a long, positively charged side chain (Arg) did not cause a significant structural change. Given that in the HGD structure the Trp residue is positioned in the hydrophobic core of the N-td, one would have assumed that the positive charged Arg side chain would perturb the structure considerably. However, this appeared not to be the case.

X-ray Structure—Conformation of our NMR findings was obtained by crystallography. Superposition of the N-td (residues 1–81) and C-td (residues 89–171) domains of the W42R mutant (Fig. 2A, colored red and magenta, respectively) onto those of HGD (Fig. 2A, colored cyan) yielded average pairwise r.m.s.d. values of 0.363 and 0.280 Å, respectively, for the backbone atoms. These low r.m.s.d. values confirm that the domain structures of the W42R mutant and HGD are extremely similar (Fig. 2A). Note that the two molecules of W42R in the asymmetric unit exhibit an average pairwise backbone atom r.m.s.d. value of 0.205 Å.

Quite surprisingly, superposition of the overall W42R mutant and HGD structures (residues 1–171) yielded a somewhat larger, average backbone r.m.s.d. value of 0.795 Å. Indeed, the relative orientation between the N-td and C-td in the W42R mutant and HGD is slightly different with a tilt angle of 9.1° between the domains (Fig. 2B). This either could be caused by the Trp-Arg substitution or may simply be the result of crystal packing. Further detailed analysis of the W42R and the HGD structures revealed that there is some minor structural readjustment around the Arg side chain, involving hydrogen bonding and hydrophobic interactions, as detailed below.

The final refined electron density map of the W42R mutant reveals well defined electron density for the Arg residue (Fig. 2C). Surprisingly, a similar positioning of the Arg side chain
within the hydrophobic core of the N-td to that of the Trp residue in the HGD structure is observed (Fig. 2D). Several interactions of the Arg residue with the surrounding atoms are identical to those seen for the Trp side chain, with several minor differences as detailed below. The backbone amide and carbonyl groups of Arg-42 form hydrogen bonds with the carbonyl and amide groups of Leu-57, respectively, and these are identical in wild-type HGD. Hydrogen bonds are formed between the Hε of the Arg-42 side chain and the backbone carbonyl oxygen atom of Ser-39, mediated by a buried water molecule inside the cavity, between one of the Arg-42 guanidino Hη atoms and the carbonyl group of Gly-1 and between the other Arg-42 guanidino Hη and the carbonyl group of Ser-20. There is only one hydrogen bond formed by the corresponding Trp-42 residue in the HGD structure (PDB ID: 1HK0), namely between the Hε of Trp-42 side chain and the backbone carbonyl group of Gly-1. Hydrophobic interactions involve the aliphatic portion of the Arg-42 side chain (Hβ, Hγ, and Hδ) and the side chains of Ile-3 and Leu-5 (Fig. 2D). In the HGD structure, the side chains of Ile-3 and Leu-5 also form similar hydrophobic contacts with the aromatic ring of the Trp-42 side chain. However, the hydrophobic contact between the side chain of Leu-80 and the six-membered ring of the Trp-42 side chain in the HGD structure is no longer observed in the W42R mutant because the guanidino group of the Arg-42 side chain occupies that position.

The presence of a buried water molecule inside the cavity of W42R is intriguing. As described above, it forms two hydrogen bonds: first, to the amide Hε of the Arg-42 side chain and, second, to the backbone carbonyl oxygen atom of Ser-39, bridging Arg-42 and Ser-39. In addition to these hydrogen bonds, it is also engaged in hydrogen bonding to the backbone carbonyl oxygen atom of Gly-40. In the HGD structure, such a water molecule is not present, and the backbone carbonyl oxygen of Gly-40 is directly hydrogen-bonded to the amide proton of Arg-59. In the W42R structure, however, the amide proton of Arg-59 is now hydrogen-bonded to the carbonyl oxygen atom (Oδ2) of the Asp-171 side chain. Thus, the additional contact from the Arg-59 backbone to Asp-171 located in the C-td of the

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**FIGURE 2. Crystal structure of human W42R γD-crystallin.** A, superposition of the N-td (red, residues 1–81) and C-td (magenta, residues 82–171) coordinates of W42R onto those of full-length HGD (cyan, PDB ID: 1HK0). B, best-fitting the W42R N-td-only coordinates (magenta, residues 1–81) onto full-length HGD (cyan, PDB ID: 1HK0). C, the electron density of residue Arg-42 in W42R, contoured at 1.0 electron density standard deviation. D, contacts around the Arg-42 side chain in the W42R mutant structure (left) and around Trp-42 in the HGD structure (PDB ID: 1HK0, right). Hydrogen bonds are labeled with black dots, and hydrophobic contacts are labeled with black semicircles.
Cataract Formation by the W42R Mutant of Human γD-Crystallin

The W42R mutant of γD-crystallin appears to pull the C-terminal tail closer to the bottom of the N-td domain, causing the top part of the C-td to be slightly tilted away relative to the top of the N-td (Fig. 2B).

Whether the above interaction is responsible for the slightly different domain orientation or whether it is caused by a bound phosphate ion located between the two protein molecules in the crystal of the W42R mutant cannot be established unambiguously. This phosphate is coordinated by the side chains of Arg-59 and Arg-168 from each polypeptide, with each of the four oxygen atoms held by one guanidino group.

Because solubility of globular proteins may be associated with the extent of protein-protein interactions in solution, we carefully assessed whether the Arg-42 substitution could cause changes in the solvent-accessible surface of the protein. However, both the wild-type HGD and the W42R structures possess essentially identical solvent-accessible surface areas (8894.32 and 8669.40 Å², respectively, as calculated by VMD (40), or 8894.32 and 8520.40 Å², respectively, as calculated by NACCESS (41)). Therefore, no features of the globular structure of the W42R mutant protein can explain its involvement in cataract formation, and other properties needed to be considered and examined.

Equilibrium Unfolding of W42R Mutant—Because there were no noticeable changes in the structure that could explain the low solubility of the mutant, we analyzed the thermodynamic stability of W42R and compared this with HGD under close to physiological conditions (37 °C, pH 7.0). We performed equilibrium unfolding/refolding experiments and monitored the denaturation by tryptophan fluorescence. The unfolding curve of W42R clearly shows two-step unfolding (Fig. 3), indicating that a stable, highly populated unfolding intermediate exists. The first unfolding transition occurs with a GdnHCl half-point of 0.19 M followed by a prominent plateau throughout the interval of GdnHCl concentrations from 1.2–2.4 M (Fig. 3). The second transition exhibits a GdnHCl half-point of 3.1 M. The presence of an intermediate was previously observed for the Q54E mutant of HGD, for which it was shown that the unfolded portion of the protein comprised the N-td, whereas the C-td remained in a native-like structure (22). The unfolding isotherm for W42R was fit to a three-state model. This yielded a ΔG°N-U of 2.4 ± 1.1 kcal/mol for the first transition and ΔG°I-U = 9.8 ± 0.8 kcal/mol for the second transition. Under identical conditions, HGD unfolds with a midpoint at 3.1 M GdnHCl, and no intermediate state is observed. Therefore, the unfolding isotherm was fit to a two-state model with an apparent ΔG°N-I = 6.0 ± 0.3 kcal/mol. This demonstrates that the substitution of Trp-42 by Arg-42 results in a dramatic destabilization of the N-td, consistent with the location of the amino acid substitution.

Because the GdnHCl unfolding curve revealed that the W42R mutant was already starting to unfold at the lowest GdnHCl concentration employed, we repeated the unfolding experiment with urea as the chaotropic agent under identical conditions. These data are provided in the inset of Fig. 3. As can be seen, at low urea concentrations (<1 M), the W42R protein starts to unfold, whereas HGD displays no sign of unfolding up to 3 M urea. The above results demonstrate that although no significant changes in the overall three-dimensional structure of the W42R protein when compared with HGD are observed, dramatic stability changes can occur. In the context of γD-crystallins, this may well relate to the aggregation and precipitation properties of destabilizing mutants that are associated with cataract formation. Indeed, protein aggregation and precipitation prevented us from extracting accurate data for thermal denaturation; precipitation occurred prior to the completion of the unfolding, and therefore, unfolding was irreversible.

Protease Susceptibility of W42R—During purification of the W42R mutant protein, we noticed that degradation products were present, whereas this was not the case for HGD or other mutants. In particular, an ~11-kDa species was observed, and its size was accurately determined by MALDI as 11,313.54 Da. Adding protease inhibitor mixture to the cell lysate, as well as including PMSF and EDTA in all purification buffers, suppressed the degradation. This suggested that the W42R mutant protein was particularly susceptible to proteases.

Therefore, we subjected intact W42R protein to trypsin cleavage and compared the mutant behavior with that of HGD. Overnight incubation of the W42R protein with trypsin generated an ~11-kDa species (Fig. 4, inset, lanes 4 and 5), whereas no such band was observed with HGD under identical conditions. The molecular mass of the ~11-kDa band from the 15N-labeled sample was determined by MALDI as 11,453.78 Da (when compared with 11,313.54 Da from the unlabeled sample; see above). These masses suggest that the final cleavage occurred between Arg-79 and Leu-80 because the calculated molecular masses for a fragment from Leu-80 to Ser-173 are 11,323.69 and 11,472.69 Da for natural abundance and 15N-labeled W42R, respectively. Comparison of the 3H-15N HSQC spectra prior to and after trypsin treatment shows that all resonances of N-td have disappeared, whereas those belonging to...
the C-td are present with the same chemical shifts (Fig. 4). Several smaller fragments (<2000 Da) were also detected by MALDI (data not show), and the $^1H$-$^{15}N$ HSQC spectrum of the trypsin-treated sample displays resonances in the random coil region, indicating that N-td is degraded into small peptides. These results indicate that W42R contains a small population of a species with an unfolded N-td that can be effectively proteolyzed by trypsin.

Unfolded Species Determined by NMR Spectroscopy—It has been hypothesized that cataracts may be caused by an off-pathway aggregation under (un)folding equilibrium conditions, and a few studies showed that $\gamma$D-crystallin is prone to aggregation under partially denaturing conditions (42). Evidence for a partially folded species was obtained by NMR spectroscopy. $^1H$-$^{15}N$ HSQC spectra of W42R were recorded at 900 MHz with a large number of scans at 25, 37, and 42 °C. Several small, well resolved resonances that were not observed at 600 MHz were detected. For assignment of these resonances, this spectrum was compared with that of the V75D mutant of HGD (V75D) that is known to possess an unfolded N-td and folded C-td at 3.6 M urea and 37 °C, as shown by fluorescence spectroscopy (15) and solution NMR spectroscopy. Comparing the $^1H$-$^{15}N$ HSQC spectra of native W42R, trypsin-digested W42R, HGD C-td, and V75D in 3.6 M urea, many minor resonances were assigned to residues that belong to the C-td (Fig. 5A).

To estimate the amount of the partially folded species of the W42R mutant under native conditions, we selected the Trp-68 H and Leu-145 amide resonances as representative resonances for the N-td and the C-td. The relative peak volumes in the 900-MHz $^1H$-$^{15}N$ HSQC spectrum at 37 °C for the natively folded, major state (N) of W42R and for the partially folded, minor state (I) (Fig. 5B) yielded 92:8 for the Trp-68 H resonance and 91:9 for the Leu-145 amide resonance. Given the identical percentages, it seems likely that these small resonances belong to the same chemical species. With increasing temperature, the amount of the I-state increased, implying that an unfolding process is involved.

To further confirm that the small resonances do not arise from an unrelated chemical species, but belong to the partially folded minor I-state that can exchange with the major N-state, we also performed H/D exchange experiments for $^{15}N$-labeled HGD and the W42R variant. After the addition of 50% (v/v) D$_2$O to the $^{15}N$-labeled samples, $^1H$-$^{15}N$ HSQC spectra were recorded as a function of time. In the folded state, slow exchange of amide hydrogens with deuterons occurs (43), whereas in the unfolded state, exchange is fast. Therefore, if any changes in H/D exchange for the folded state are observed over time, this would support the notion that chemical exchange between the partially folded and native state takes place. As shown in Fig. 6, the W42R protein exhibited pronounced H/D exchange of amide protons, throughout the entire N-td, whereas residues in the C-td displayed no detectable changes. Thus, the H/D exchange also supports the view that the I-state possesses an unfolded N-td and a folded C-td.

UV-C Irradiation—Given that Trp-42 appears to be important for protein stability and that the loss of this residue leads to aggregate formation, possibly causing cataract, we probed whether the loss of a Trp could be associated with photodam-

FIGURE 4: Superposition of the $^1H$-$^{15}N$ HSQC spectra of untreated (red) and trypsin-digested (blue) W42R. Trypsin cleavage reactions were performed overnight at 37 °C in 20 mM sodium phosphate, pH 7.0, 5 mM DTT, 0.02% NaN$_3$ with an enzyme-to-substrate ratio of 1:100 (w/w). A comparison between trypsin-digested wild-type HGD and the W42R mutant by SDS-PAGE is shown in the inset. Note that lane 1 contains protein standards, lanes 2 and 3 contain HGD in the absence and presence of trypsin, respectively, and lanes 4 and 5 contain W42R in the absence and presence of trypsin, respectively.
age of HGD. UV is an environmental factor that damages tryptophans, and Trp-42 is the most UV-sensitive tryptophan in H9253D-crystallin (44). Covalent damage of Trp residues leads to ring cleavage, originating from a short-lived Trp cation radical (45, 46) that, upon photooxidation, turns to kynurenine or derivatives. Therefore, the mutation of tryptophan to arginine in the W42R mutant may serve as a model for photodamaged H9253D-crystallin and UV-induced cataract. To evaluate whether UV damage in the tryptophans of HGD results in similar properties as seen in the W42R mutant, we exposed HGD to different doses of UV.

15N-labeled wild-type protein was exposed to UV-C (254 nm) light, and 1H-15N HSQC spectra were recorded before and after irradiation with increasing energy doses until new resonances appeared (data not shown). After irradiation, the intensity of the Trp-42 Hε1 resonance was reduced by 50%, that of Trp-68 was reduced by 37%, whereas that of Trp-156 was only decreased by 5% (Fig. 7A). Due to overlap of Trp-130 Hε1 and Phe-115 amide resonances, the intensity decrease of the Trp-130 Hε1 resonance could not be calculated reliably. Overall, many new resonances appeared in the 1H-15N HSQC spectrum after irradiation, indicating that new protein species had appeared. We estimated which parts (amino acids) of HGD experienced damage by measuring the intensities of resonances that were associated with undamaged protein over the time course of the irradiation. These data for a UV exposure of 1J/cm² are provided in Fig. 7B. The largest changes occurred in resonances belonging to residues in N-td, with changes observed for resonances of amino acids 2–5, 16–25, 37–46, 55–61, and 74–80. Some changes were also seen in the C-td, namely for residues ~144 and 170–173. Interestingly, the UV-affected regions are essentially identical to those for which differences in the spectra between the W42R mutant and HGD were noted (Fig. 1). This suggests that the changes observed in the irradiated HGD spectrum were caused by damage of Trp-42, rather than by damage to all the residues that experience changes.

We further investigated whether the Trp-42-damaged HGD protein behaved similar to the W42R mutant protein in the trypsin digestion experiment. Not surprisingly, the HGD pro-
tein became susceptible to degradation by trypsin after having been irradiated at 254 nm (Fig. 8A). The trypsin digestion product (indicated by the lower arrow on the SDS-PAGE gel) was also analyzed by MALDI, and this fragment had a molecular mass of 11,464.82 Da, very similar (≤ 120 Da difference) to that found for the trypsin-generated W42R C-td fragment. The UV-irradiated 15N HGD NMR sample was also subjected to trypsin cleavage and analyzed by SDS-PAGE as well as MALDI, revealing that a fragment of 11,468.34 Da is generated (Fig. 8B) whose molecular mass is in agreement with the previously observed and predicted mass of a 15N-labeled fragment, comprising residues Leu-80 to Ser-173. Although the native HGD protein is resistant to trypsin digestion (Fig. 4), UV irradiation appears to damage Trp-42, as shown by NMR, and initiates the formation of a partially folded species that is easily cleaved by protease. At UV-C doses above 5 J/cm², the irradiated HGD was completely digested by trypsin, suggesting more widespread damage also to the C-td.

UV irradiation appears to cause other changes in the protein as well because increased molecular masses were observed for the HGD protein, namely 20,559.77 Da (1 J/cm²), 20,736.57 Da (2 J/cm²), 21,197.66 Da (3 J/cm²), 21,630.29 Da (4 J/cm²), and 21,525.03 Da (5 J/cm²). All the above findings strongly suggest that UV-mediated protein damage, in particular Trp-42 oxidation, plays a critical role in destabilizing γD-crystallin, contributing to the etiology of age-related cataract.

DISCUSSION

Many proteins are associated with deposition diseases in humans, including Alzheimer disease, prion-related Creutzfeldt-Jakob disease, type II diabetes, and cataract. The proteins involved exhibit a common feature: the presence of a partially unfolded or non-native conformation (47–50). Elucidating how these proteins lose their structure by environmental factors is crucial for understanding the pathology of protein deposition diseases. One of the prevailing models for cataract formation evokes off-pathway folding and aggregation. In this model, unfolded protein escapes from sequestration by α-crystallin and forms aggregates. Support for this model is based on results with hydrophobic core mutants of γD-crystallin under denaturing conditions (15). Although suggestive, there was no direct evidence linking protein damage to aggregation under physiological conditions up to now.

Our current research appears to supply this missing link since it revealed that the mutant protein contains a population of partially folded protein, although no significant difference in the overall structure from HGD can be discerned for the major conformer. At 37 °C, a noticeable amount (≤ 10%) of partially folded species is present, similar to related observations for a F9S mutant of mouse γS-crystallin that is associated with Opj cataract in mice and for which an unfolding intermediate was found to be populated to ≤ 1–2% at physiological conditions (19, 20).

It is also worth noting that the structures of other HGD mutants, such as R36S and R58H (51, 52), do not exhibit major structural changes when compared with wild type. As to causes for their solubility changes, for the R36S mutant, it has been suggested that the elimination of the surface charge by the substitution of the Arg-36 side chain by Ser induces the mutant protein to engage in protein-protein contacts that are not possible in the wild-type protein (52). Similarly, for the R58H mutant, it has been proposed that the reduced solubility is mainly due to an effect of the mutation on the solution phase (51). Altogether, our current results and combined previous data clearly show that no major conformational changes of
**Cataract Formation by the W42R Mutant of Human γD-Crystallin**

HGD are necessary, as evidenced by the very similar x-ray structures, but that it is solution behavior and in particular (un)folding dynamics of the different mutants that play critical roles in cataract formation. Indeed, this notion is unambiguously borne out by the results of the study presented here.

Most importantly, our findings link the behavior of the cataract-associated W42R mutant to UV-damaged HGD. Although effects of UV damage on crystallins have been studied previously (44), no biophysical data about the structural consequences have been analyzed. Here we investigated whether the W42R mutant protein and UV-damaged HGD share common properties. Our NMR data revealed that the UV-induced damage was confined to the area around Trp-42, essentially limited to the N-td, supporting previous observations on the susceptibility of this Trp-42 residue upon UV exposure (53).

The combined data on the W42R mutant and UV-irradiated HGD demonstrate that modification of even a single residue is sufficient to destabilize γD-crystallin enough to induce protein aggregation in vitro, even if the x-ray structure of the mutant is essentially identical to that of HGD.

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**FIGURE 8. UV-C exposure and trypsin digestion of HGD and 15N HGD.** A and B, SDS-PAGE of UV-C-exposed and trypsin-digested natural abundance HGD for increasing energy doses (1.0, 2.0, 3.0, 4.0, 5.0 J/cm²) (A) and UV-C-exposed and trypsin-digested 15N HGD (B). In B, lane 1, protein standard; lane 2, 15N HGD; lane 3, 15N HGD incubated at 37 °C overnight without trypsin; lane 4, 15N HGD incubated at 37 °C overnight with trypsin. Nonexposed HGD samples are loaded in the lanes marked by asterisks.
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