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Tryptophan Cluster Protects Human γD-Crystallin from Ultraviolet Radiation-Induced Photo-Aggregation In vitro

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ABSTRACT

Exposure to ultraviolet radiation (UVR) is a significant risk factor for age-related cataract, a disease of the human lens and the most prevalent cause of blindness in the world. Cataract pathology involves protein misfolding and aggregation of the primary proteins of the lens, the crystallins. Human γD-crystallin (HγD-Crys) is a major γ-crystallin in the nucleus of the human lens. We report here analysis of UVR-induced damage to HγD-Crys in vitro. Irradiation of solutions of recombinant HγD-Crys with UVA/UVB light produced a rise in solution turbidity due to polymerization of the monomeric crystallins into higher molecular weight aggregates. A significant fraction of this polymerized protein was covalently linked. Photo-aggregation of HγD-Crys required oxygen and its rate was protein concentration and UVR dose dependent. To investigate the potential roles of individual tryptophan residues in photo-aggregation, triple W:F mutants of HγD-Crys were irradiated. Surprisingly, despite reducing UVR absorbing capacity, multiple W:F HγD-Crys mutant proteins photo-aggregated more quickly and extensively than wild-type. The results reported here are consistent with previous studies that postulated that an energy transfer mechanism between the highly conserved pairs of tryptophan residues in HγD-Crys could be protective against UVR-induced photo-damage.
INTRODUCTION

Protein misfolding and aggregation are hallmarks of the pathology of many human diseases (1). Cataract is the leading cause of blindness in the world, projected to affect 20-30 million people in 2020, primarily the elderly, and is associated with misfolding and aggregation of the lens proteins (2). Despite the widespread prevalence of cataract, the relative contributions of identified risk factors to cataract have not been determined (3). One of the several risk factors identified is exposure to ultraviolet radiation.

Ultraviolet radiation (UVR) is a ubiquitous environmental hazard for life on Earth. Although the development of the ozone layer 2.4 billion years ago limited terrestrial UVR exposure to the UVA (400-315 nm) and UVB (315-280 nm) ranges, UVR still exerts selective pressure on extant creatures (4). DNA photo-damage and its downstream impacts on the cellular level have been well studied (5-9). The accumulation of UVR-induced DNA lesions leads to mutations, the obstruction of DNA replication, and, if unaddressed, cell death. Several DNA repair pathways have been identified and characterized that target UVR-induced DNA lesions.

Unlike DNA photo-damage, most types of protein photo-damage cannot be repaired by cell processes. In cells, damaged proteins can be poly-ubiquitinylated and degraded by the ubiquitin proteasome pathway or assembled into large aggresomes and disposed of through autophagocytosis (10). In some
specialized tissues, such as the lens, where cataract occurs, neither option is available (11-13).

The human lens focuses light onto the retina; to do so, it must remain translucent (12). As the epithelial cells in the lens terminally differentiate and produce large quantities of the crystallin proteins, they degrade their organelles and ribosomes (14). Due to the lack of protein turn over in the lens, the damaged and aggregated crystallins are not cleared from the lens fibers and accumulate as cataracts. Covalently damaged lens proteins have been shown to have decreased stability and solubility, and tend toward aggregation (15).

The three crystallin families, α, β, and γ, comprise 90% of total lens protein and are present at ~400 mg/ml (16). α-Crystallins are ATP-independent chaperones of the small heat shock protein (sHSP) family. These bind partially unfolded or damaged proteins, sequestering them, but cannot refold them (17, 18). The β- and γ-crystallins are globular, two domain structural proteins of approximately 20 kDa, related by sequence and structure homology (16, 19). Each domain is composed of two Greek Key motifs and contains a number of highly conserved aromatic residues. The β-crystallins form oligomers through domain swapping; the γ-crystallins are monomeric (20).

Due to the lack of protein synthesis or degradation in the lens fiber cells, a model of cataract has been put forth in which aggregation prone species accumulate over the lifetime of an individual and gradually titrate away free α-crystallin; when no free chaperone remains, aggregation occurs, causing cataract (21). HγD-Crys, the γ-crystallin chosen here for study, is extremely stable and
one of the more abundant γ-crystallins in the human lens nucleus (22). Several mutations in the gene for HγD-Crys are known to be associated with congenital cataract (23, 24).

Earlier studies have investigated how bovine lenses, extracted mixtures of bovine crystallins, and extracted mixtures of human crystallins have responded to photo-sensitizers and UVR, which generate reactive oxygen species (ROS) that can mediate photo-damage to proteins (25-27). One photosensitizer studied, N-formylkynurenine, is similar to the tryptophan-based UV filters abundant in the lens (28). Extracted lenses and lens protein extracts grew cloudy when exposed to photo-sensitizers and UVR, with an increase in the insoluble protein fraction population and disruptions to crystallin structure. A rise in turbidity, cross-linked products, non-tryptophan fluorescence, and the presence of ROS was also reported when mixtures of bovine and human crystallins were irradiated in the absence of photo-sensitizers (29). More recently, Estey et al. showed that UVR causes non-disulfide cross-linking and non-native aggregation in the corneal crystallin ALDH3A1 (30).

Other studies found exposure to UVR caused cataract to develop in laboratory animals (31-33). When Ayala et al. exposed rats to short bursts of 300 nm UVR, they observed the development of light scattering in exposed lenses in the weeks and months following irradiation (32). Other work has found light scattering develops in guinea pig lens after UVA exposure in vivo in the lab (34). Further in vivo work by Giblin et al. found that UVA and UVB blocking contact lenses prevented UVR-induced cataract in rabbits (35, 36).
The availability of high resolution crystallin X-ray structures from Basak et al. enabled the discovery of a distinctive energy transfer mechanism at work in HγD-Crys between the conserved tryptophan pairs within the N- and C-terminal domains (Fig. 1a) (37, 38). By examining fluorescence spectra and quantum yields of mutant HγD-Crys constructs, Chen et al. found evidence that one tryptophan of a pair (W68 or W156) has its fluorescence extremely quenched, while the other (W42 or W130, respectively) is moderately fluorescent and was shown to transfer its excited state energy to its quenched partner, resulting in anomalous native state Trp quenching (Fig. 1b). It was hypothesized that the mechanism could have evolved as a form of resistance to photo-damage (39).

To investigate the molecular mechanism underlying photo-aggregation of the crystallins, solutions of recombinant purified HγD-Crys were irradiated with a mixture of UVA/UVB, and the resulting photo-aggregation monitored via solution turbidity, absorption spectroscopy, SDS-PAGE, and TEM. Based on the fact that tryptophans absorb UVR the strongest and on the previously characterized energy transfer mechanism, we tested an initial hypothesis that photo-aggregation proceeded through direct or indirect photo-damage to one or more of the four Trp residues. Unexpectedly, we show here that damage to the Trp residues is unlikely to be on the pathway leading to the photo-aggregated high molecular weight state. Rather the results reveal that the Trp residues may play a protective role.
MATERIALS AND METHODS

Mutagenesis, Expression, and Purification of HγD-Crys: N-terminally 6x-His tagged wild-type (WT) HγD-Crys expression constructs were modified via site-directed mutagenesis to introduce quadruple and triple W:F mutants (40). Constructs were confirmed via sequencing (Genewiz).

Recombinant WT HγD-Crys and mutant proteins were expressed and purified as described previously (40) with several modifications. Cells were grown to OD$_{600}$ ~1 in Super broth media at 37 °C with shaking. IPTG was added to 1 mM and cultures were transferred to 18 °C followed by shaking overnight. Cells were pelleted by centrifugation for 20 minutes at 17000 x g and resuspended in 30 ml Ni-NTA Lysis Buffer (300 mM NaCl, 50 mM NaPO$_4$, 18 mM imidazole, pH 8) containing 2 tablets of Roche Complete EDTA-free protease inhibitor. After addition of lysozyme to 3 mg/mL and DNase to 3 µg/mL, pellets were lysed via ultrasonication, and centrifuged at 17000 x g for 45 minutes. Supernatants were filtered and applied to a Ni-NTA column (GE Healthcare). Protein was eluted using a linear gradient of increasing imidazole concentration. Fractions containing the protein of interest were pooled and dialyzed three times against storage buffer (10 mM ammonium acetate, pH 7.0).

Protein Concentration Measurement: Stock protein sample concentration was determined using absorbance at 280 nm with the following extinction coefficients
(determined using SIB’s ProtParam): WT \( \gamma\)D-Crys 42,860 M\(^{-1}\) cm\(^{-1}\), triple W:F H\( \gamma\)D-Crys 26,360 M\(^{-1}\) cm\(^{-1}\), quadruple W:F H\( \gamma\)D-Crys 20,860 M\(^{-1}\) cm\(^{-1}\).

**Photo-aggregation Experiments:** Samples of H\( \gamma\)D-Crys were prepared at 0.25 mg/ml or 1 mg/ml in 1x Reaction Buffer (100 mM Na\(_2\)PO\(_4\), 1 mM EDTA, pH 7). Samples were irradiated at room temperature in a quartz cuvette (Starna Group) using a UVP Inc. UVLMS-38 lamp equipped with a 302 nm midrange bulb delivering a range of UVA/UVB light. UVR dose delivery was set to 2 mW/cm\(^2\), varied by adjusting the cuvette’s distance to the lamp, and determined before each experiment by a UVX Radiometer with midrange UVX-31 sensor. Turbidity readings at OD\(_{600}\) on a Cary UV/Vis Spectrometer were taken at regular time points during irradiation. Samples removed and analyzed via SDS-PAGE were reduced and boiled and electrophoresed through 14% acrylamide gels at 170 V for 1 hour; gels were stained using Krypton Fluorescent Protein Stain (Thermo Fisher Scientific) and imaged on a Typhoon 9400 (Amersham Biosciences). Samples removed and analyzed using the bicinchoninic acid assay for protein concentration were filtered with a 0.2 \( \mu \)m membrane to remove aggregated protein and treated following the kit manufacturer’s protocol (Thermo Scientific Pierce). The results were read on a Fluostar Optima plate reader (BMG Technologies). Aggregation rates were measured by calculating the steepest linear slope of the OD\(_{600}\) versus exposure time curve.

Oxygen-free irradiation experiments were conducted using a Coy anaerobic chamber under nitrogen. After an overnight incubation, samples were
sealed into screw-top quartz cuvettes (Starna Group) with rubber stoppers before removal from the anaerobic chamber, and immediately used in photo-aggregation experiments. An oxygen sensitive dye solution (7.5 mM methyl viologen, 9 mM dithionite) in an identically sealed cuvette was used to confirm anaerobic conditions.

Action spectrum analysis of photo-aggregation was achieved using small Newport Stabilife UVR cutoff filters to construct a shielding cage around the sample cuvette, and photo-aggregation experiments were conducted as above.

**Transmission Electron Microscopy:** Five-microliter samples of irradiated and unirradiated 0.1 mg/ml H$_2$D-Crys sample in storage buffer were directly applied onto glow-discharged, carbon-coated, Formvar-filmed 400 mesh copper grids (Ted Pella). They were subsequently negatively stained with 1% uranyl acetate and blotted dry with filter paper. Sample grids were viewed in a transmission electron microscope (1200 XII; JEOL) and images were taken using an Advanced Microscopy Techniques XR41S side-mounted charge-coupled device camera.

**Absorbance Spectra Measurements:** Samples were collected from photo-aggregation experiments and diluted into reaction buffer and 5 M guanidine hydrochloride (GuHCl) in black-walled tubes to minimize light scatter interference by aggregated protein. Samples were then incubated at 37 °C for 6 hours before
scanning absorbance. Absorbance spectra of irradiated and unirradiated HγD-Crys samples were collected using a Cary UV/Vis Spectrometer.

**Circular Dichroism Thermal Unfolding Measurements:** CD spectra of the WT and mutant proteins were obtained using an AVIV model 202 CD spectrometer (Lakewood, NJ). Protein samples were prepared at a concentration of 0.1 mg/ml in 10 mM sodium phosphate, pH 7.0. Data were collected at 218 nm in a 1 cm quartz cuvette. Sample temperature was increased from 25°C to 95°C in 1°C steps with 1 minute of equilibration time per °C, followed by 5 second reads. Data were buffer-corrected, and mean residue ellipticity was calculated. The mean residue ellipticity versus temperature data were fit to a sigmoidal curve using Kaleidagraph (Synergy Software), and the unfolding midpoints were calculated. The unfolding temperatures reported are averages of 3 thermal unfolding experiments.

**RESULTS**

**HγD-Crys photo-aggregation under UVR**

We exposed purified HγD-Crys to UVR to investigate the underlying mechanism of photo-damage in this highly stable lens protein. When WT HγD-Crys was exposed for 2 hours to 2 mW/cm² of UVR, solution turbidity rose dramatically after a lag period and then plateaued, consistent with previous studies and indicative of protein aggregation (Fig. 2). A lag period is often interpreted as
evidence of a nucleation step in polymerization kinetics. However, we observed a shorter lag period when aggregation was monitored at 280 and 350 nm than those observed at 600 nm (Fig. S1a, see Supporting Materials). This behavior suggests that the different lag times observed using different wavelengths of light represent detection of differently sized aggregates, and that, for the reaction under study, the apparent lag period is a consequence of initial aggregating species being too small to scatter light significantly.

As an additional approach to whether the lag phase represents a true nucleation step, we examined the lag time versus HyD-Crys concentration (Fig. S1b, see Supporting Materials). Unlike well-documented nucleation reactions, the lag time was relatively insensitive to protein concentration (41).

At longer exposure times, the dose of UVR delivered to the sample may be lower than initially measured due to scattering in the sample. However, the OD$_{600}$ continued to rise steadily even after the OD$_{280}$ plateaued (Fig. S1a), indicating continuing photo-aggregation despite potentially lower dose delivered.

The concentration of WT HyD-Crys detected dropped steadily throughout UVR exposure to less than 40% its original concentration (Fig. 2), indicating more than 60% of the original sample’s protein had entered an aggregated state by the time the OD$_{600}$ had ceased increasing, but a significant population remained in solution.
Photo-aggregation dependencies

To understand the parameters governing the photo-aggregation of HγD-Crys, the irradiation and turbidity monitoring experiment was repeated, varying the concentration of WT HγD-Crys (Fig. 3a) and the dose of UVR (Fig. 3b).

Increased rate of photo-aggregation correlated with increased concentration of WT HγD-Crys and with UVR dose. The dependence of aggregation on temperature and pH was also examined. Photo-aggregation did not show any clear dependence on either parameter (Fig. S2 and S3, see Supporting Materials).

To determine whether oxygen played a role in the in vitro photo-aggregation of HγD-Crys, buffer and protein samples were prepared anaerobically and then exposed to UVR doses as before (Fig. 3c). No change in turbidity was observed over an hour of UVR exposure under anaerobic conditions. At 60 minutes, oxygen was reintroduced to the reaction. As UVR exposure continued, solution turbidity developed robustly. These results indicated oxygen is required to mediate photo-damage for the in vitro photo-aggregation of HγD-Crys.

Glass filters that sharply block all wavelengths shorter than specific thresholds were used to determine the action spectrum of photo-aggregation (Fig. 3d). When wavelengths below 280 nm were blocked, there was no difference observed in the development of turbidity over exposure time. However, cutting off UVR at 295 nm and below, 305 nm and below, and 320 nm and below
progressively and dramatically slowed photo-aggregation. This indicated an 
action spectrum encompassing the UVB range of approx. 280-320 nm, but not 
the UVA range; this overlapped with the Tyr and Trp absorption spectra, as well 
as that of Trp photo-products like kynurenine.

**HγD-Crys photo-aggregate structure**

WT HγD-Crys photo-aggregates were visualized using uranyl-acetate negative 
stain transmission electron microscopy (TEM) (Fig. 4). Aggregates observed 
were from 100 to ~1000 nm in length. They were non-amyloid in structure but, 
while irregularly arranged, appear to have a rough, globular repeating unit 
approximately 40-80 nm in size.

>Figure 4<

Samples of photo-aggregation reactions of WT HγD-Crys were collected 
as a function of irradiation time and were analyzed by SDS-PAGE (Fig. 5a). All 
the samples exhibited a strong 20 kDa band, representing monomeric WT HγD-
Crys (iv). After 25 minutes of UVR exposure an ~40 kDa band could be seen (iii) 
appropriate in size to be a HγD-Crys dimer. In addition a series of high molecular 
weight bands (ii) appeared near the top of the gel, presumably multimeric 
species. By 45 minutes, a thin band at the top edge of the resolving gel could be 
seen, indicative of species too large to enter the gel (i). A series of lower 
molecular weight degradation products can be seen below the monomer band (v). 
Image analysis was used to quantify changes in band density between lanes, 
and showed that the monomer band diminished over time to half its original
intensity over exposure time, while the dimer band increased until ~45 minutes then diminished (Fig. 5b). This is consistent with the formation of an initial covalent dimeric cross-linked photo-product that accumulates but is consumed by further photo-cross-linking and incorporated into larger aggregates.

When the density of the dimer band is examined at earlier UVR exposure times and compared with solution turbidity, we saw that the dimer appeared before OD$_{600}$ increased, and began to wane as turbidity plateaued (Fig. 6a). At the earliest time points, with only 2 minutes of UVR exposure, the dimer band was detected (Fig. 6b). This suggests the formation of a covalent dimer is an early step in the photo-aggregation of HyD-Crys, and that solution turbidity measurements monitor the presence of later aggregation products.

**The role of aromatic residues in photo-aggregation**

We initially assumed that UVR absorption by tryptophans was a key step in photo-damage, and that the photo-oxidized indole moiety was participating in free-radical polymerization. We therefore examined the absorbance spectra of HyD-Crys samples after varying UVR exposure times to assess whether photo-damage occurred to aromatic residues (Fig. 7a). We observed no significant change in absorbance spectra in samples from 0 minutes to 36 minutes. However, over that same time period, aggregation proceeded robustly (Fig. 7b). The lack of significant changes in the absorbance spectra indicates an overall lack of damage to HyD-Crys’s Trp’s and Tyr’s during photo-aggregation.
To determine the role of Trp residues in photo-aggregation we examined triple and quadruple W:F mutant constructs of HyD-Crys, with a single Trp remaining or no Trp’s remaining, respectively. Previous studies established that these mutants folded into native-like structures (40). We confirmed that the mutant HyD-Crys proteins were stably folded by measuring far UV circular dichroism as a function of increasing temperature. Though all the mutant proteins were less stable than wild type, they retained their folded conformation to 60°C or above (Table 1).

In photo-aggregation experiments with all four triple mutants and the NoTrp quadruple mutant, turbidity rose dramatically faster and reached a higher plateau than WT. This indicated the W:F mutations made HyD-Crys more vulnerable to UVR-induced photo-aggregation, not less, and that the absence of Trp residues did not retard photo-aggregation (Fig. 8a).

We examined the concentration dependence of the rate of photo-aggregation for WT and mutant HyD-Crys and found that the multiple W:F mutant HyD-Crys’s diverge significantly in the concentration dependence of their photo-aggregation rate (Fig. 8b). This implied that the W:F mutations significantly alter the photo-aggregation pathway with respect to wild-type.
DISCUSSION

The $\gamma$D-crystallins are very stable, resistant to denaturation both by chemical denaturants and heat, and are among the longer-lived proteins in the human body (16). However, exposure to UVR \textit{in vitro} results in rapid aggregation into high molecular weigh complexes. Since this reaction requires oxygen, it presumably involves photo-oxidation of certain residues.

$\gamma$D-Crys contains many aromatic amino acids capable of UVR absorption and radical photochemistry - four tryptophans, fourteen tyrosines and six phenylalanines. Tryptophan has the highest specific absorption of protein amino acids at 280 nm, and $\gamma$D-Crys’ four highly conserved tryptophan residues contribute 51.3% of its absorptivity, the remaining 48.7% coming from its fourteen tyrosines (42, 43). The tryptophan excited state can transfer its energy to other side chains or the peptide backbone, or scission of the indole ring of the excited residue can occur creating photo-products, among them, kynurenine (44). In either path, absorption of UV photons by tryptophan could be an initial step in UVR-induced photo-damage.

We monitored photo-aggregation of $\gamma$D-Crys by two main methods, turbidity and SDS-PAGE. The inability to dissociate the aggregated state by boiling in SDS in the presence of reducing agents suggests covalent bonds between the subunits in the aggregated state. This suggests that aggregation of $\gamma$D-Crys was occurring via a radical polymerization mechanism (42).

SDS-PAGE revealed the formation of covalent dimeric photo-products immediately after UVR exposure began. These subsequently decreased in
intensity, consistent with a role as intermediates in the polymerization reaction. When aggregation was monitored by turbidity, an apparent lag phase was present. While a lag in an aggregation reaction could be indicative of a nucleation step in the aggregation mechanism (41), it was more likely a consequence of early aggregates being too small to scatter light at 600 nm; this is supported by the shorter lag times when turbidity was monitored at shorter wavelengths, as well as the absence of a lag in production of dimeric photo-products. The presence of distinct lower molecular weight bands of photo-products suggests that photochemical scission of particular peptide bonds is also occurring. UVR-induced photochemical scission of peptide bonds (45) and degradation of crystallins (46) have been described previously. Though we cannot exclude the possibility that the fragments are incorporated into the covalent aggregated state, their steady increase during the course of UVR exposure is consistent with a photo-product that is off the aggregation pathway. We were surprised to find that in NoTrp HγD-Crys, the quadruple W:F mutant, photo-aggregation occurred; this indicated that Trp was not necessary for UVR-induced aggregation, despite being the strongest UVR absorber in HγD-Crys. Counter-intuitively, removing half the UVR absorbing capacity of HγD-Crys actually increased the rate of photo-aggregation. This suggests that the tryptophan residues, which are highly conserved among β/γ-crystallins (16), have a photo-protective role in HγD-Crys, and their replacement with phenylalanine made HγD-Crys more vulnerable to photodamage. It also implicates the tyrosines
of HyD-Crys, the remaining significant UVR absorbers, as playing a role in photo-
aggregation.

Such a photoprotective mechanism had been proposed to account for the
super-quenched fluorescence emission found for the tryptophans of HyD-Crys as
well as other crystallins (37). The human retina is very sensitive to UVR and the
lens acts as a UV filter protecting the retina (47, 48). Bova et al proposed that
kynurenine and related metabolites served as UVR filters in the lens (28). The
work of Chen et al. makes the point that the β/γ-crystallins themselves--with their
four conserved buried tryptophans – would also serve as UVR filters (49). The
rapid quenching would then represent protection of the protein itself from UVR
photo-damage.

The occurrence of photo-aggregation in the absence of tryptophan at first
may appear inconsistent with several previous studies linking tryptophan photo-
oxidative damage with the development of aggregation and/or cataract (50-53).
Previous studies, however, often examined protein mixtures from human or
animal lenses, or purified tryptophan in solution as opposed to a single
recombinantly purified γ-crystallin protein. Such situations differ from the
conditions under study here in their oxygen levels, the presence of other proteins,
and redox regulators. Other studies have also utilized photo-sensitizers to
produce ROS and initiate oxidative damage to crystallins (26, 27). UVR-induced
damage has also been studied using laser radiation sources at differing
wavelengths and sometimes of much higher power than the comparably
physiological UVR dose being administered here (51, 54). It is thus reasonable to
expect a different photo-damage pathway(s) to be encountered under the experimental conditions used here.

Interestingly, adding tryptophan’s 42, 130, and 156 back (by examining triple W:F mutants with a single Trp remaining) slightly increased photo-aggregation rates relative to NoTrp HyD-Crys, with W156 increasing the least and W130 the most, while adding W68 back slightly decreased the rate of photo-aggregation. It would seem, overall, that having one tryptophan of the four is more deleterious for HyD-Crys photo-aggregation vulnerability than having none at all. The photo-protective effect may require more than one tryptophan to be present, and there may be differences in the photo-damage vulnerability and photo-protection contributed by the different tryptophans.

The two triple W:F mutant HγD-Crys constructs with only a moderately fluorescent tryptophan remaining (W42 or W130) photo-aggregated more rapidly than those with only a quenched tryptophan remaining (W68 or W156). This seems consistent with the analysis of Chen et al., and suggests that the presence of a stronger fluorophore, and thus perhaps a longer-lived photochemically active species, conveys stronger photo-aggregation propensity than a weak fluorophore, which is photochemically active for a far shorter duration (55). Future experiments will address the effects of combinations of W:F mutations on photo-aggregation.

An alternative explanation for the behavior of these multiple mutant HyD-Crys constructs is that, by modifying the hydrophobic core, the mutants proteins are destabilized or have taken on a non-native conformation relative to WT.
Assuming the mechanism of the observed photo-aggregation involves an unfolding or partial unfolding step, these folding or structural changes would be responsible for the apparent photo-aggregation rate change. However, previous experiments have characterized the multiple W:F mutant HyγD-Crys constructs and found they adopted stable, WT-like structures (40). CD thermal denaturation experiments showed that the mutants are, indeed, somewhat destabilized compared to WT (Table 1) but still unfold about 40°C above room temperature. Together, these suggest the changes in photo-aggregation behavior result from phenomenon other than destabilization.

Another explanation for the differences in photo-aggregation between WT and W:F mutants could be intra-protein cross-linking. If the conserved tryptophan residues become photo-excited and cross-linked to other sites within an individual HyγD-Crys, such a reaction could compete with inter-protein cross-linking and thus slow the observed aggregation with the formation of photo-products with near identical molecular weights to monomeric HyγD-Crys. Inserting W:F mutations would then be removing a competing chemical reaction pathway, not disrupting an energy transfer mechanism. However, this scenario is unlikely given the current data. Significant tryptophan-based intra-protein cross-links would cause a change in the absorbance spectra of the samples, which was not detected. Intra-molecular cross-links, while not creating a significant difference in molecular weight between uncross-linked and cross-linked proteins, would also create small differences in their mobility in an SDS-PAGE gel, which were not seen.
From the current study, tryptophan is unlikely to be the site of photo-
damage, raising questions about which sites in HγD-Crys play key roles in UVR-
induced photo-aggregation. Excited state energy transfer occurs from tyrosine to
tryptophan (44), and the current results suggests one or a set of HγD-Crys’
fourteen tyrosines could be an important site of absorption and/or photo-damage.
Besides tryptophan, a number of amino acids, including cysteine, tyrosine, and
histidine, can participate in excited state radical chemistry that could lead to
photochemical covalent cross-linking (56).

It seems likely that the mechanism of the observed photo-aggregation
involves absorption at a non-tryptophan aromatic site, the generation of a free
radical on a residue of HγD-Crys, followed by several steps of covalent cross-
linking to other HγD-Crys subunits. The absorption and reaction steps of this
mechanism could proceed through an array of sites on the protein, or be very
specific and involve a small number of residues. Preliminary analysis using mass
spectrometry has not found evidence for a single dominant covalent cross-link,
suggesting that cross-links are occurring at a diverse set of sites across HγD-
Crys. The initial results have, however, identified significant oxidation of C18 in
irradiated samples, consistent with previous reports on lens protein oxidation (57,
58).

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SUPPLEMENTARY MATERIALS

Figures S1, S2, and S3 can be found at DOI: xxxx-xxxxxx.s1.

REFERENCES


Table 1. Circular Dichroism Thermal Unfolding Data for HγD-Crys Mutant Constructs

<table>
<thead>
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<th>Construct</th>
<th>Melting Temperature (°C)</th>
<th>Standard Deviation (°C)</th>
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<tr>
<td>NoTrp</td>
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<td>0.11</td>
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FIGURE CAPTIONS

Figure 1. (a) X-ray crystallography structure of HγD-Crys (38) (PDB ID: 1HK0) displayed in ribbon form, with its 4 conserved Trp residues (W42 purple, W68 blue, W130 green, W156 red) highlighted in space filling form. (b) Graph of WT HγD-Crys Trp fluorescence emission spectra upon 295 nm excitation in the native (solid line) and GuHCl unfolded (dashed line) states.

Figure 2. Changes in solution turbidity measured at OD_{600} (solid triangles, left axis) and changes in soluble protein concentration measured using the BCA assay on samples (open squares, right axis) as a function of UVR exposure time.
Samples contained 0.25 mg/ml of WT HγD-Crys in sample buffer, and were incubated at 25 °C.

Figure 3. Parameters of UVR-induced protein aggregation of WT HγD-Crys observed using OD₆₀₀: (a) Irradiation of varying concentrations of WT HγD-Crys: 2 mg/ml (solid squares), 1.25 mg/ml (open diamonds), 0.75 mg/ml (solid triangles), 0.5 mg/ml (X’s), 0.38 mg/ml (solid circles), 0.15 mg/ml (dashes). (b) UVR-induced aggregation of 0.25 mg/ml WT HγD-Crys samples using varying doses of UVR as measured via radiometer: 2 mW/cm² (X’s), 1.5 mW/cm² (solid triangles), 1 mW/cm² (open diamonds), 0.5 mW/cm² (solid squares). (c) UVR exposure of a 1 mg/ml WT HγD-Crys sample in the absence or presence of atmospheric oxygen. Protein and buffer samples prepared anaerobically were irradiated, then opened to the atmosphere at 60 minutes (denoted by arrow). (d) Exposure of 1 mg/ml WT HγD-Crys samples to decreasing ranges of the UV lamp’s emission spectrum using glass filters blocking all light shorter than a wavelength cutoff: no filter (open diamonds), 280 nm filter (solid squares), 295 nm filter (solid triangles), 305 nm filter (dashes), 320 nm filter (X’s).

Figure 4. Transmission electron micrograph of uranyl-acetate stained UVR-induced aggregate from a 1 mg/ml WT HγD-Crys sample at 30 minutes irradiation at 2 mW/cm². The inset is a negative control micrograph of unirradiated WT HγD-Crys.
Figure 5. (a) Scanned image of a krypton-stained gel from SDS-PAGE of UVR-induced aggregate samples from a 1 mg/ml WT HγD-Crys sample taken at a series of UVR exposure times: 0 min (lane 1), 25 min (lane 2), 45 min (lane 3), 70 min (lane 4), 100 min (lane 5), 120 min (lane 6). Marked sites: large protein aggregates unable to enter gel (i), high molecular weight species (ii), HγD-Crys dimer-sized band (iii), monomeric HγD-Crys band (iv), lower molecular weight degradation products (v). (b) Graphs of quantification of band density for the monomeric approx. 20 kDa band (left axis) and the dimeric approx. 40 kDa band (right axis) from the gel image in (a).

Figure 6. (a) Turbidity development at OD$_{600}$ of 1 mg/ml WT HγD-Crys sample (black triangles and solid line, right axis) and dimer gel band density quantification from SDS-PAGE of the same WT HγD-Crys sample (open squares and dotted line, left axis) versus UVR exposure time. Curves were generated using polynomial fits. (b) The same data is presented from (a), examining just the earliest time points from 0 to 10 minutes of UVR exposure.

Figure 7. (a) Absorbance spectra of WT HγD-Crys at increasing UVR exposure times, taken from 1 mg/ml samples diluted to 0.1 mg/ml into GuHCl to minimize aggregate light scattering: 0 min (black), 9 min (red), 18 min (blue), 27 min (green), 36 min (orange). (b) Graph of the change in solution turbidity at 600 nm (solid squares) and absorbance at 280 nm (open triangles) over UVR exposure time of the same WT HγD-Crys samples exposed to UVR as in (a).
Figure 8. (a) Comparison of UVR-induced aggregation of W:F mutant constructs of $H\gamma D$-Crys by monitoring $OD_{600}$ of 0.5 mg/ml protein solutions in sample buffer over irradiation time. (b) Comparison of the concentration dependences of UVR-induced aggregation of W:F mutant $H\gamma D$-Crys constructs by analyzing the apparent rate of aggregation (steepest linear slope of $OD_{600}$ curves) versus protein concentration. W42-only $H\gamma D$-Crys (purple triangles), W68-only $H\gamma D$-Crys (blue hatches), W130-only $H\gamma D$-Crys (green squares), W156-only $H\gamma D$-Crys (red diamonds), WT $H\gamma D$-Crys (black circles), NoTrp $H\gamma D$-Crys (black dashes).

Figure S1. (a) Changes in solution turbidity measuring $OD_{600}$ (red triangles), $OD_{350}$ (green diamonds), and $OD_{280}$ (blue squares) over UVR exposure time. Samples contained 0.25 mg/ml WT $H\gamma D$-Crys in sample buffer. (b) Lag time to turbidity development versus concentrations of WT $H\gamma D$-Crys observed via $OD_{600}$ (red triangles), $OD_{350}$ (green diamonds), and $OD_{280}$ (blue squares). Turbidity data were fit to sigmoidal curves and the time to increase to 0.05 au above baseline $OD$ at time 0 was calculated as the lag time.

Figure S2. Changes in solution turbidity measuring $OD_{600}$ of samples at pH 4 (blue diamonds), pH 5 (red squares), pH 6 (green triangles), pH 7 (purple X's), pH 8 (turquoise hatches), and pH 8.8 (orange circles) over UVR exposure time. Samples contained 0.25 mg/ml WT $H\gamma D$-Crys in sample buffer. Solutions at pH 4 and 5 were produced by adding concentrated HCl to sample buffer beforehand.
Solutions pH's were determined using a Thermo Orion PerpHect LogR Meter (model 330) with 8156BNUWP probe.

Figure S3. Changes in solution turbidity of 0.25 mg/ml WT H7D-Crys samples, measuring OD$_{600}$, of samples at 4°C (blue diamonds), 16°C (red squares), 25°C (green triangles), and 37°C (purple X’s) over UVR exposure time.