Comparison of ultraviolet induced photo-kinetics for lens-derived and recombinant β-crystallins

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Purpose: The photobiology of purified recombinant crystallins has not been studied. Here we examine photo-induced aggregation of purified recombinant mouse βA3-crystallin (rβA3) and compare it with that of βL-crystallins isolated from bovine lenses.

Methods: rβA3-Crystallin was expressed in baculovirus-infected Sf9 cells and purified by ion-exchange and gel-filtration chromatography. Protein solutions (pH 7.4) were irradiated at room temperature using a 308 nm excimer laser and light scattering was registered by attenuation of an unabsoled beam of red light (670 nm).

Results: Irradiation of bovine α-crystallin, βL-crystallin, rβA3-crystallin and γB-crystallin resulted in formation of insoluble aggregates with subsequent light scattering. Different slopes and threshold energies were observed for light scattering by each of these species. Sensitivity to ultraviolet irradiation induced light scattering as determined from threshold energies varied, with γ-crystallins showing the greatest sensitivity, the βL- and rβA3-crystallins showing an intermediate sensitivity and α-crystallins much less sensitive. Low doses (100 J/cm²) resulted in irreversible formation of water soluble oligomers but no insoluble aggregates as indicated by changes in light transmission. The photo-behavior of rβA3 was similar to mixed βL-crystallin and different from that of α- and γ-crystallins.

Conclusions: Ultraviolet induced sensitivity of purified recombinant crystallins reflects that of mixed crystallin populations and should provide an indication of the pathogenicity of specific crystallin sequence changes associated with lens aging and hereditary cataract.

Both animal and human studies demonstrate the importance of the lens crystallins in establishing and maintaining lens transparency [1]. Structural modifications of crystallins in human senile cataract mimic many ultraviolet (UV) induced changes of crystallins in solution. X-ray diffraction analysis demonstrated a complete loss of native structural organization of γ- and β-crystallins in senile nuclear cataract, and similar changes result from UV irradiation of bovine crystallins solutions [2].

Major classes of ubiquitous crystallins include the α-crystallins, which are molecular chaperones related to small heat shock proteins [3], and the βγ-crystallins. The βγ-crystallins share a common 2-domain structure [4-6]. Each domain comprises 2 “Greek key” motif folds forming a core structure of two twisted β-pleated sheets. The β-crystallins also have amino and carboxy terminal extensions or “arms” [7]. Under the conditions at which they are usually studied, β-crystallins associate into higher order complexes while the γ-crystallins exists in solution as monomers [8,9].

Cataracts are a significant cause of vision loss. Hereditary cataracts can be caused by mutant crystallins having altered stability, solubility, or interactions, and this could be exacerbated upon prolonged exposure of the lens to ultraviolet light. Even normal crystallins may photo-oligomerize and precipitate from solution, resulting in lens opacity [2]. It was shown recently that a splice mutation of βA3-crystallin is associated with autosomal dominant zonular cataract formation [10,11], and changes in α-[12-15], βB2- [16,17], and γC- and γD-crystallins [18-21] are associated with congenital cataracts.

β-Crystallins are more resistant to photo-aggregation than γ-crystallins [22]. Although the core domain structures of γ- and β-crystallins are very similar, γ-crystallins have only rudimentary terminal extensions but the β-crystallins have both, amino- and carboxy-terminal extensions [7]. This suggests that while the role of β-crystallin terminal extensions in the photo-aggregation process is unknown, their photo-behavior might be explained by differences in the structure of terminal extensions. While the susceptibility of βγ-crystallins to UV radiation have been well characterized [22-25], recombinant crystallins have not previously been studied. Here we compare the photo-kinetics of UV induced light scattering by mouse rβA3-crystallin with that of bovine α-, βL- and γ-crystallins. The photo-behavior of rβA3-crystallin is similar to mixed βL-crystallin and different from that of α- or γ-crystallin. This suggests that the UV induced light scattering kinetics of normal or modified recombinant crystallins can be used to model pathological properties of crystallins upon UV exposure in the lens.

METHODS

rβA3-Crystallin was expressed in a baculovirus expression system and purified by combination of ion-exchange and gel-
filtration chromatography as described previously [26]. Briefly, Sf9 cells were infected with recombinant AcMNPV virus containing the rβA3-crystallin coding sequence. Infected Sf9 cells were harvested after 72 h, washed with phosphate buffer saline (PBS) and stored as a pellet at -70 °C. The frozen cells were lysed by freeze-thawing in lysis buffer consisting of 50 mM tris(hydroxymethyl)aminoethane (Tris) HCl, pH 8.5, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT) and the thiol protease inhibitor E-64 (Roche Diagnostics, Indianapolis, IN) at 2.8 mM, and rβA3-crystallin was purified by ion-exchange chromatography on a DE-52 column followed by gel-filtration chromatography on a Superdex 75 column. The identity of rβA3-crystallin was verified by sodium dodecyl sulfate (SDS)-PAGE and Western blot analysis. Stock solutions of commercial (Sigma-Aldrich, St. Louis, MO) bovine α-, βL-, and γ-crystallins of 0.25-1.0 mg/ml concentration were prepared in 0.1 M phosphate buffer at pH 7.4. Protein concentration before and after irradiation was measured by absorption spectroscopy at 280 nm using a Beckman UV DU-62 spectrophotometer.

Laser irradiation and light scattering were performed as follows. The samples were brought to room temperature in 1X PBS more than 1 h prior to beginning UV irradiation experiments. All samples were irradiated at an ambient temperature of 22-24 °C. Sample temperatures did not increase by more than 1-2 °C in the course of irradiation. The apparatus constructed to detect increases in light scattering attenuation with UV dose is shown schematically in Figure 1. The protein samples were irradiated using an excimer laser (Lambda-Physik EMG-201-MSC; average power <0.5 W, 308 nm, XeCl, 50 pulses/sec, pulse width 20 nsec). The full laser intensity at 0.5 W average power was decreased by using the approximately 5% reflection from each surface of an uncoated quartz beam splitter. Under these conditions, the peak power delivered to the sample during the laser pulse is less than 500 mW. The light scattering attenuation studies were conducted by

Figure 1. Schematic of apparatus A. Apparatus A allows simultaneous excimer laser irradiation and transmittance measurement. Excimer laser irradiation is performed at a wavelength of 308 nm and transmittance (light scattering attenuation) was continuously monitored at a wavelength of 670 nm.

Figure 2. UV induced light scattering of βL-crystallin. A: Two subsequent runs of UV induced light scattering of two aliquot samples of βL-crystallin solution on apparatus A. Measurements are performed at protein concentration of 1 mg/ml. B: Superdex 75 10/30 gel-filtration chromatography of samples obtained for non-irradiated sample (upper profile) and after the simultaneous irradiation of βL-crystallin in two cuvettes at 100 J/cm² (low dose, middle and bottom profiles). Void and column volumes were 7 and 24 ml respectively. Fraction size was 1 ml.
passing an unabsorbed beam of red light (Thorlabs 1011 series diode laser; \(\lambda_{\text{max}}=670\) nm, bandwidth about 5 nm, full width at half maximum, \(<3\) mW CW) through the cuvette containing the protein solutions, perpendicular to the excimer laser path. This method, which monitors the increasing loss of light due to scattering (light scattering attenuation), is distinct from the more common light scattering measurement in which scattered photons normal to the incident probe beam are observed. The red light was detected using a monochromator and photomultiplier (PMT) combination to reject ambient light and the scattered photolysis beam. It was necessary to attenuate the diode laser beam using neutral density filters to move the initial flux to a linear region of the PMT’s response curve. The rectangular beam from the probe laser passes through more than 50% of the total solution volume, all of which is uniformly irradiated by the excimer laser. The output of the PMT is then integrated over 1 s using a digital voltmeter and the resultant measurement is transmitted to the data collection computer using an IEEE-488 interface. The computer logs and archives the raw intensity data, which are then processed, to yield plots of optical density (OD) vs. dose. The sensitivity of the system was sufficient to detect changes in the solution’s optical density on the order of O.D. 0.02. The change in optical density is presumably due to light scattering by large, insoluble aggregates of the proteins. This instrument, located at the National Institute of Standards and Technology, is referred to as instrument A.

UV irradiation of r\(\alpha\)-A3-crystallin was repeated in the Institute of Applied Physics of The Russian Academy of Sciences. The construction of the apparatus used for this experiment was similar to that presented in Figure 1 with some differences as follows. Protein samples in 1X PBS with 1 mM DTT and 50 \(\mu\)M tris(2-carboxyethyl)-phosphine hydrochloride (TCEP, Pierce, Rockford, IL) were irradiated with an excimer laser (LPX-200 Lambda Physik, 308 nm, XeCl, pulse power density 75 J/cm\(^2\), 2 pulses/sec). A HeNe laser (10 mW, 633 nm) was used to measure light absorption in a direction perpendicular to the excimer laser beam. This instrument is referred to as instrument B. While instruments A and B produced irradiation at the same wavelength, differences in their design and the presence of strong reducing agents during irradiation on apparatus B resulted in parallel but quantitatively different results for the aggregation threshold and slope, although the values obtained were consistent on each machine. Data will be identified in the figures and text as having been obtained with apparatus A or B.

By using a cuvette with a 10 by 2 mm internal cross sectional area, it was possible to make full use of the 1 cm\(^2\) masked excimer laser beam to irradiate the sample and to monitor the light scattering attenuation using 250 \(\mu\)l samples. Protein concentration ranged from 0.5 to 1 mg/ml. At low doses of irradiation (100 J/cm\(^2\)) there is no observed light scattering attenuation, but some visually observed aggregation of protein near the edges of the cuvette undetectable by the transmittance measurement took place. At medium doses, aggregation was observed near the edges of the cuvette, probably due to the restricted geometry and freedom of diffusion in the cuvette. However, the monitoring beam integrated the light scattering attenuation along the full length of the cuvette. At significantly higher doses a visible ‘snow flake’ aggregation of protein was observed.

### RESULTS

Aggregation curves of two aliquots of 1 mg/ml bovine \(\beta\)L-crystallin samples were similar (Figure 2A). Laser light scattering curves obtained for each protein were very close to the two state transition curves described previously in the literature [22,24]. Increases in light scattering represent the transition from soluble protein into insoluble aggregated protein. Although no change in light transmission occurred at energies of 180 J/cm\(^2\) in either sample, after filtration through a

### Table 1. Parameters of UV induced light scattering transition curves for bovine crystallins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Concentration (mg/ml)</th>
<th>Threshold energy (J/cm(^2))</th>
<th>Aggregation rate (O.D. x cm(^2)/J)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha)-crystallin (A)</td>
<td>1.0</td>
<td>1620</td>
<td>0.001</td>
</tr>
<tr>
<td>(\beta)-crystallin (A)</td>
<td>1.0</td>
<td>310</td>
<td>0.003</td>
</tr>
<tr>
<td>(\gamma)-crystallin (A)</td>
<td>1.0</td>
<td>80</td>
<td>0.010</td>
</tr>
<tr>
<td>(\alpha)-crystallin (B)</td>
<td>0.8</td>
<td>210</td>
<td>0.002</td>
</tr>
<tr>
<td>(\beta)-crystallin (A)</td>
<td>0.8</td>
<td>180</td>
<td>0.002</td>
</tr>
<tr>
<td>(\gamma)-crystallin (B)</td>
<td>0.5</td>
<td>70</td>
<td>0.011</td>
</tr>
<tr>
<td>(\alpha)-A3-crystallin (B)</td>
<td>0.7</td>
<td>60</td>
<td>0.013</td>
</tr>
</tbody>
</table>

The columns list the protein name, protein concentration measured before the sample irradiation, threshold energy determined for each sample in the point where light scattering intensity change start (see Figure 3 and Figure 4), and aggregation rate determined as the slope of the transition curve. Letters in parentheses in column 1 show which apparatus was used for obtaining data for each condition (see Methods section). Direct comparison of threshold and aggregation rates obtained on different apparatus were difficult because of differences in modes of sample irradiation.
0.45 μm filter the protein concentration of the irradiated samples were less than half of the concentration of the sample before irradiation. In contrast to the gel filtration profile seen before irradiation, (Figure 2B, upper profile) the irradiated sample had additional peaks appearing at the expected position of dimers (peak 2) and extending to the column void volume. The two irradiated samples showed similar elution profiles on gel-filtration (Figure 2B, middle and lower profiles). These data clearly demonstrate the formation of soluble βL-crystallin aggregates in samples irradiated at a low dose that showed no increase in light scattering. These ranged from dimers seen as an accentuation of peak 2 in the middle and lower panels of Figure 2B to some larger than 0.45 μm in diameter that were retained during filtration.

The photo behavior of bovine α-, βL-, and γ-crystallins is compared in Figure 3. Light scattering curves were characterized with respect to two parameters; energy threshold for aggregation, obtained by extending the ascending linear part of the transition curve to the x intercept, and aggregation rate, obtained as the slope of the ascending linear part of the curve. These two parameters were extracted from transition curves for bovine α-, βL-, and γ-crystallins shown in Figure 3 and are summarized in Table 1. The threshold energy demonstrates the minimum energy required to initiate detectable light scattering corresponding to the beginning of photo aggregation of the protein sample. At the same concentration (1 mg/ml), the energy threshold of UV induced light scattering decreased for bovine α-, βL-, and γ-crystallins in ratios of 35:4:1. From these data we may conclude that γ-crystallin is more sensitive to photo-aggregation than βL-crystallin, and that α-crystallin is extremely resistant to UV induced light scattering. Light scattering by α-crystallin is negligible up to high doses of UV irradiation.

The second parameter, the aggregation rate, shows the rate of change of the intensity of scattering per energy interval above the threshold value, corresponding to transition of soluble protein into aggregates. Ratios of the aggregation rates were 1:3:10 for α-, βL-, and γ-crystallins respectively (Table 1), demonstrating a high rate of aggregation for γ-crystallin, a low value for the α-crystallin and an intermediate value for the βL-crystallin. This suggests that under a given dose of UV irradiation above the threshold values, γ-crystallin denatured at about 10 times the rate of α-crystallin and βL-crystallin at about 3 times that rate. While aggregation thresholds and rates cannot be directly compared between samples analyzed on different instruments, in each case, aggregation tends to increase with increasing concentration. These data are consistent with previous observations [22].

The UV sensitivities of rβA3 and a non-recombinant bovine βL-crystallin are compared in Figure 4, and the derived thresholds and aggregation rates are shown in Table 1. Threshold energies for rβA3 and bovine βL-crystallins under the same experimental conditions and at the same concentrations are similar (180 and 210 J/cm², respectively). Both proteins also show a similar aggregation rate (0.002 O.D. x cm²/J). Thus the UV induced photo-kinetics of rβA3 and βL-crystallin are similar. This experiment was repeated at different protein concentrations (Figure 5 and Table 1). Once more, a typical biphasic response curve was observed. Threshold energies were 70 and 60 J/cm², and aggregation rates were 0.011 and 0.013 O.D. x cm²/J for βL- and rβA3-crystallins, respectively. This confirmed the similar response curves of βL-crystallin and rβA3 to UV irradiation. In contrast, the bovine α-, βL-, and γ-crystallins show different susceptibility to ultraviolet light induced photo-aggregation, so that combining the data from both experiments indicates that γ-crystallins have the greatest sensitivity, the βL- and rβA3-crystallins have an intermediate sensitivity, and α-crystallins are much less sensitive.

Superdex 75 gel-filtration chromatography of rβA3 samples irradiated with 26, 50 and 170 J/cm² of UV at a wave-
length of 308 nm and then centrifuged at 20,800x g for 30 min at 4 °C is shown in Figure 6. Chromatography of a non-irradiated rβA3 sample, labeled as rβA3 in Figure 6, shows a single major peak at 10.9 ml in phosphate buffered saline buffer with 1 mM DTT and 50 μM TCEP. This peak corresponds to an apparent molecular weight of 29.5 kDa. Chromatography of rβA3 samples irradiated at doses of 26 and 50 J/cm², clearly demonstrate the appearance of an additional peak at elution volume 9.4 ml (57 kDa) and a simultaneous decrease in the major peak. In addition, a smaller peak corresponding to the column void volume can be seen in these samples. A very weak major peak was observed on the chromatogram for the 170 J/cm² UV dose sample without any visible peaks corresponding to higher molecular weight aggregates.

SDS-PAGE and Western blots of the UV irradiated rβA3 samples are shown in Figure 7A and Figure 7B, respectively. Lanes 1 and 2 are non-irradiated samples, with lane 2 being a control for degradation during shipment. They are essentially the same, although there might be a slight increase in dispersed material (smear) visible on lane 2 of the Western blot below the major double band at 27 kDa. The samples irradiated with 26, 50, and 170 J/cm² show progressive loss of this band, until in the 170 J/cm² sample it is barely visible on the SDS-PAGE. This is accompanied in the 26 and 50 J/cm² samples by increased staining below the major band. This is mostly dispersed, but somewhat heavier bands are visible at approximately 18 and 22 kDa. In addition, small molecular weight material not immuno-reactive with anti-βA3 antibodies can be seen at the dye front, peaking in the 50 J/cm² sample and beginning to decrease in the 170 kDa sample. Above the major band there is an additional band at approximately 50 kDa visible only on the Western blot in the 26 and 50 J/cm² samples.

SDS-PAGE and Western blots of insoluble protein pellets dissolved in 4% SDS are shown in Figure 7A and Figure 7B, lanes 7-10. The SDS-PAGE shows very little protein, but does show small peptides at the dye front in the samples irradiated with 26 and 50 J/cm², similar to those seen in the soluble supernatants from these samples. The Western blots of the 26 and 50 J/cm² samples show the rβA3 band at 27 kDa, with smaller immuno-reactive peptides seen diffusely below this major band. Samples irradiated at 50 J/cm² and 170 J/cm² show bands at approximately 50 kDa with a faint band also visible in the unirradiated sample. The 170 J/cm² sample shows loss of the band at 27 kDa, but a general diffuse smear of protein extending from about 17 kDa to over 100 kDa with the appearance of a band at 18 kDa as seen in the soluble fractions.

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Figure 6. Chromatographic profiles of rβA3-crystallin. Chromatographic profiles correspond to the non-irradiated and UV irradiated at doses 26 J/cm², 50 J/cm² and 170 J/cm² rβA3-crystallin, respectively, on apparatus B. Samples of 0.1 ml were loaded to the Superdex 75 10/30HR column at 0.5 ml/min. Protein standards used in gel-filtration chromatography from left to right are bovine serum albumin, ovalbumin, carbonic anhydrase, chymotrypsinogen A, cytochrome c, ribonuclease A and aprotinin, respectively. Void and column volumes are 7.9 ml and 24 ml, respectively.

Figure 7. SDS-PAGE and western blot analysis of rβA3-crystallin. SDS-PAGE (A) and western blot (B) obtained for UV irradiated rβA3-crystallin on apparatus B. Lane 1 is control rβA3; lane 2 is non-irradiated rβA3; lanes 2, 3 and 4 are soluble fractions of rβA3 irradiated at 308 nm with doses 26 J/cm², 50 J/cm² and 170 J/cm², respectively; lanes 6 and 11 show protein standards; lanes 7, 8 and 9 are insoluble fractions of rβA3 irradiated at 308 nm with 26 J/cm², 50 J/cm², 170 J/cm², respectively, and dissolved in 4% SDS; lane 10 is insoluble fraction of non-irradiated rβA3 dissolved in 4% SDS.
An intact double band at 27 kDa is seen in lane 11 of the Western blot, insoluble material from the non-irradiated sample corresponding to lane 2.

DISCUSSION
Overall, these results demonstrate that the crystallins show differential sensitivities to UV-induced light scattering, with the sensitivity of γ-crystallin greater than that of βL-crystallin, which was similar to that of recombinant βA3-crystallin, both of which were much greater than that of α-crystallin. These data are in good agreement with those obtained previously [22]. In addition, the UV sensitivity of βA3 is similar to that of the mixed βL-crystallins. While light scattering reflects the presence of protein aggregates, both the loss of protein from solution on filtration and the sieve chromatography results suggest that protein crosslinking and aggregate formation is present at energy levels well below those which produce light scattering. Thus, as in the lens itself, light scattering is a relatively late result of in vitro exposure of crystallins in solution.

Analysis of irradiated βA3-crystallin by molecular sieve chromatography and SDS-PAGE is consistent with two simultaneous processes occurring in the solutions. The first is protein crosslinking, initially to form dimers and later to form higher molecular weight aggregates that eventually come out of solution and scatter light. This is seen even at the lowest energies studied, and at higher energies begins to be obscured by the second process, photolysis. The data shown in Figure 5, Figure 6, and Figure 7 emphasize this process, because irradiation and transport are carried out in the presence of 50 µM TCEP, a very strong reducing agent that would be expected to minimize the occurrence of disulfide bonds, a major mechanism of crosslinking in photo-oxidized crystallins [27]. In addition, the SDS-PAGE sample buffer had β-mercaptoethanol, which would also tend to reverse disulfide crosslinking. Thus, the aggregation seen in both the chromatography and the gels probably represents a minimum compared to that, which would occur as a result of unopposed photoxidation. It is also likely that centrifugation before chromatography was more efficient at removing aggregates than filtration.

Because the β- and γ-crystallins share a common protein fold in their central domain structures, it might be expected that they would display similar photokinetics in response to ultraviolet irradiation. However, the βL-crystallins appear to be significantly less susceptible to ultraviolet irradiation than the γ-crystallins. While it is tempting to speculate that the presence of terminal extensions on the β-crystallins might in some fashion protect them from non-disulfide crosslinking in response to ultraviolet irradiation, there are a number of other differences between the β- and γ-crystallins that might contribute to this difference. Among these is the tendency of the β-crystallins to form higher order complexes, which would have a complex influence including proximity and decreased effective concentration.

With regards to possible differential susceptibility of the various β-crystallins to form aggregates upon photooxidation, the single amino terminal arm of βA3-crystallin is somewhat longer than that of βB2-crystallin, but certainly shorter than that of βB1-crystallin. Perhaps more relevant are the 8 cysteine residues present in βA3-crystallin of which 4 or 5 appear to be sterically available to form disulfide bonds, the most of any β-crystallin. One way in which these questions are being approached experimentally is through a direct comparison of photokinetics of intact βA3-crystallin with βB2-crystallin and variants of each lacking terminal extensions. In summary, these data suggest that UV-induced light scattering in this in vitro system can be used to assess photo behavior of β-crystallin mutants as well as to clarify mechanisms by which truncation of β-crystallin terminal arms might have on the tendency to undergo light-induced aggregation and photolysis.

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