The damaging effect of UV-C irradiation on lens α-crystallin

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Purpose: To evaluate the effect of UV-C irradiation on the structural properties of α-crystallin and its chaperone activity.

Methods: α- and β-L-crystallins were isolated from bovine lenses using gel chromatography. The purified α-crystallin was subjected to UV-C irradiation (254 nm; 1, 2, 5, 10, 20, 50 J/cm²). We measured the tryptophan fluorescence, circular dichroism (CD) spectroscopy in the far UV, and the chaperone activity of both irradiated and non-irradiated α-crystallin.

Results: The tryptophan fluorescence of α-crystallin decreased, whereas the N-formylkynurenine fluorescence increased markedly with increasing doses of UV-C irradiation. Both the oxidation of Met and the racemization of Asp of α-crystallin increased at a dose of 1-2 J/cm² and then gradually decreased. The CD spectrum showed that the secondary structure of α-crystallin altered with increasing radiation dose, and almost all of the β-sheet structure was lost at doses above 50 J/cm². The chaperone activity of α-crystallin irradiated with doses under 5 J/cm² remained intact. However, it was reduced to only 40% after irradiation at 10 J/cm².

Conclusions: Our study suggests that photo-oxidation of tryptophan residues in α-crystallin may be one of the events that affects the three-dimensional packing array and chaperone activity of this lens protein.

α-Crystallin, a major protein of the lens, is thought to play a role in maintaining lens transparency. In its native state, α-crystallin is a large, water soluble aggregate with an average molecular mass of approximately 800 kDa. α-Crystallin comprises two polypeptides, αA- and αB-crystallin, which contain 173 and 175 amino acid residues, respectively. The amino acid sequences of αA- and αB-crystallin share approximately 55% sequence identity [1,2]. Previous studies have demonstrated that α-crystallin belongs to the family of small heat-shock proteins [3] and functions as a molecular chaperone [4]. This chaperone-like activity may be related to the reported hydrophobic binding of partially denatured proteins to α-crystallin [5-8]. α-Crystallin undergoes various posttranslational modifications such as oxidation [9,10], racemization [11,12], deamidation [13-15], and truncation [14,16-18] that may alter its secondary or higher order structure and may decrease its chaperone properties.

Ultraviolet (UV) irradiation is believed to be one of the stress factors in cataract formation during natural aging. A previous study has shown that exposure of α-crystallin to UV-B induces the photodegradation of tryptophan residues in α-crystallin and causes a loss of chaperone activity [19].

Furthermore, it has been reported that gamma irradiation induces the oxidation of tryptophan (Trp) and methionine (Met) residues [20,21], and the isomerization of aspartic acid (Asp) residues [21] in αA-crystallin, which are correlated with the loss of the chaperone-like activity. However, these modifications occur only at a high level of gamma radiation (0.5-5 kGy level). UV-C irradiation does not generate directly hydroxyl radicals in water and its energy is less than that of gamma irradiation. Nevertheless, the present study shows that UV-C irradiation induces the oxidation of Trp and Met residues, racemization of the Asp residue and the loss of secondary structure in α-crystallin, as well as showing that the modified protein has reduced chaperone-like activity.

METHODS

Purification of α-crystallin and βL-crystallin: Bovine (approximately 5 years old) lenses were homogenized in 50 mM NaCl and 1 mM EDTA/50 mM Tris-HCl buffer (pH 7.4). The proteins were fractionated into water soluble and water insoluble fractions by centrifugation at 15,000x g for 40 min at 4 °C. The water soluble fraction was loaded onto a Sephacryl S300 HR gel filtration column (26x600 mm; Pharmacia, Tokyo, Japan), equilibrated with 50 mM Tris HCl buffer (pH 7.8), and the α-crystallin and βL-crystallin fractions were collected.

UV-C irradiation of α-crystallin: α-Crystallin (1 mg/ml, 6 ml) was irradiated with different doses (0.2, 0.5, 1, 2, 5,10, 20, and 50 J/cm²) of UV-C irradiation at 254 nm using a germicidal UV-C lamp (GL-10; Matsushita Electric Industrial Co., Ltd., Osaka, Japan) at a dose rate of 3.3 microW/cm². According to the manufacturer, these lamps emit predominantly monochromatic light of 253.7 nm.

Chaperone activity assay for heat induced aggregation of βL-crystallin: A chaperone activity assay was used to measure the ability of α-crystallin to protect βL-crystallin against heat induced aggregation. One mg of βL-crystallin in 50 mM Tris HCl buffer (pH 7.8) with or without 0.1 mg of α-crystallin was incubated at 60 °C. The final volume of each reaction mixture was 0.7 ml. Aggregation of βL-crystallin was monitored by absorption due to light scattering at a wavelength of 360 nm. The chaperone activity of gamma irradiated and non-irradiated α-crystallin was compared.

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Circular dichroism: Circular dichroism (CD) spectra in the far UV and near UV regions were obtained using a spectropolarimeter (Jasco J-720, Tokyo, Japan). A cylindrical quartz cell with a 0.2 mm path length was used.

Fluorescence: Fluorescence was measured with an F-4500 Hitachi spectrofluorometer (Hitachi, Tokyo, Japan). Trp and N-formylkynurenine fluorescence spectra were obtained with excitation wavelengths of 295 nm and 330 nm, respectively. In order to observe changes in protein hydrophobicity, the sample was assayed with 4,4'-dianilino-1,1'-binaphthalene 5,5'-disulfonic acid (bis-ANS; Molecular probes, Junction City, OR), and the resulting fluorescence spectra were recorded with an excitation wavelength of 395 nm and emission scanning between 420 nm and 550 nm [22]. The assay was performed according to a previously described procedure [23]. In brief, aliquots (20 µl) of bis-ANS (4.5x10^-4 M) were added to 3 ml of each protein sample and allowed to stand at room temperature for 15 min before measurement.

Enzymatic digestion and isolation of peptides: All preparations of α-crystallin were digested with trypsin (Wako, Osaka, Japan) for 20 h at 37 °C in 0.1 M Tris-HCl buffer (20 mM CaCl₂, pH 7.6) at an enzyme to substrate ratio of 1:50 (mol:mol). The resulting tryptic (T) peptides were separated by RP-HPLC (LC-10A; Shimadzu, Kyoto, Japan) using a C₁₈ column (TSK gel-ODS-80TM, 4.6x250 mm; Tosoh, Tokyo, Japan) with a linear gradient of 0-40% acetonitrile plus 0.1% trifluoroacetic acid at a flow rate of 0.8 ml/min. Absorbance was monitored at 215 nm, and the peptides were collected by a fraction collector (FRC10; Shimadzu, Kyoto, Japan).

Matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOFMS): All spectra were obtained using a matrix assisted laser desorption/ionization time of flight mass spectrometer (MALDI-TOFMS, Compact MALDI IV; Shimadzu Kyoto, Japan). The MALDI-TOFMS spectrometer was operated with a 337 nm nitrogen laser and an ion acceleration voltage of 20 kV. Data were collected in reflection mode as signals of positive ions. For the matrix, α-cyano-4-hydroxycinnamic acid (CHCA, 10 mg) was dissolved in 1 ml of solution containing a 2 to 1 ratio of 0.1% trifluoroacetic acid in water to acetonitrile. The sample peptide (0.5 µl) was added to an equal volume (0.5 µl) of the matrix solution on the plate and then dried. Each spot contained a few picomoles of sample peptide.

Determination of D/L ratio of amino acids: Peptide samples were lyophilized in tubes and hydrolyzed with gas-phase 6 N HCl for 7 h at 108 °C. After hydrolysis, the samples were derivatized with o-phthalaldehyde (OPA) and N-tert-butylxycarbonyl-L-cysteine (Boc-L-Cys) to form diastereoisomers. The D/L ratio of amino acids was determined by RP-HPLC with a C₁₈ column (Nova-Pak ODS, 3.9x300 mm; Waters, Tokyo, Japan) using fluorescence detection (wavelengths: excitation, 344 nm; emission, 433 nm). The samples were eluted at 30 °C with a linear gradient of 5-47% acetonitrile plus 3% tetrahydrofuran in 0.1 M acetate buffer (pH 6.0) over 120 min at a flow rate of 0.8 ml/min.

Amino acid sequence analysis: Amino acid sequences were determined by Edman degradation on a pulsed-liquid protein sequencer, equipped with an on-line phenylthiohydantoin (PTH) amino acid analyzer (477A/120A; Applied Biosystems, Foster City, CA).

Figure 1. Molecular chaperone-like activity of normal and UV-C irradiated α-crystallin. The chaperone-like activity was assayed by measuring the heat induced aggregation of βL-crystallin at 60 °C.
RESULTS

Chaperone activity: A chaperone-like role has been proposed to be the main function of α-crystallin. Here, we measured the chaperone activity of α-crystallin by determining its ability to protect another protein from heat induced aggregation. As shown in Figure 1, aggregation of β-L-crystallin increased at 60 °C in the absence of α-crystallin, but this aggregation was completely suppressed by the presence of native non-irradiated α-crystallin. The chaperone activity of α-crystallin irradiated under 5 J/cm^2 of UV-C remained constant. However, this activity was reduced to 40% after irradiation at 10 J/cm^2 and decreased with increasing doses of UV-C irradiation.

CD measurements: To determine whether UV-C irradiated α-crystallin retains its native secondary structure, we analyzed the structure of irradiated and non-irradiated α-crystallin by circular dichroism (CD) spectroscopy in the far UV (Figure 2). At doses of under 10 J/cm^2, there were no significant differences between the spectra of irradiated and non-irradiated α-crystallin, which showed a typical β-sheet conformation. However, the secondary structure was altered with increasing doses of UV-C irradiation and the β-sheet structure was almost completely lost above 50 J/cm^2.

Fluorescence: α-Crystallin has three Trp residues (Trp_9 in αA-crystallin and Trp_9 and Trp_60 in αB-crystallin). These residues contribute to the Trp fluorescence observed at 330 nm. Following UV-C irradiation, Trp is well known to oxidize to N-formylkynurenine (NFK), which has a fluorescence maxima at 440 nm. Figure 3 shows the change in relative intensities of the Trp and NFK fluorescence after UV-C irradiation of α-crystallin. The intensity of the Trp fluorescence reduced with increasing doses of UV-C irradiation, whereas that of NFK rose with increasing doses of UV-C irradiation.

Bis-ANS is an anionic hydrophobic fluorescent probe that binds to a polar interface. Figure 4 shows the bis-ANS fluorescence spectra of normal and UV-C irradiated α-crystallin. The bis-ANS fluorescence intensity of α-crystallin decreased with increasing doses of UV-C irradiation.
with increasing doses of UV-C irradiation and decreased markedly with doses greater than 10 J/cm². Thus, UV-C irradiation leads to a decrease in the exposure of hydrophobic surfaces of \(\alpha\)-crystallin.

**Oxidation of T1 peptides of \(\alpha\)-A-crystallin:** In order to determine whether individual amino acid residues were modified by UV-C irradiation, samples of normal and irradiated \(\alpha\)-crystallin were digested with trypsin. The resulting peptides were fractionated by RP-HPLC and then identified by protein sequencing and time of flight (TOF)-MS spectra. As shown in Figure 5, in addition to the T1 peptide (Ac-MDIAIQHPWFK: 1427 Da) of \(\alpha\)-A-crystallin, the oxidized T1 peptide (1443 Da), with a mass of 16 Da more than the native T1 peptide, was observed. A previous study has shown that the 1443-Da peptide that elutes 10 min earlier than the native T1 is singly oxidized at Met₁ of the T1 peptide (Ac-M(O)DIAIQHPWFK: 1443 Da) of \(\alpha\)-A-crystallin [21]. After UV-C irradiation, the relative abundance of Met₁-oxidized T1 peptide in \(\alpha\)-crystallin increased, reaching an abundance five times higher than that of native T1 peptide at a UV-C dose of 5 J/cm², and then decreased with higher doses of radiation (Table 1). This decrease is due to the appearance of more oxidative species, such as doubly oxidized T1 peptide.

**Racemization of the Asp₁₅₁ residue in \(\alpha\)-A-crystallin:** We have reported previously that Asp₁₅₁ in \(\alpha\)-A-crystallin is stereochemically labile to allow the conversion of L-Asp to D-Asp during aging [24] and UV-B irradiation [25]. Here, we measured the ratio of Asp₁₅₁ racemization in normal and irradiated \(\alpha\)-crystallin. First, we identified the Asp₁₅₁ containing peptides, namely, the T₁₈ peptide (IPSGVD₁₅₁AGHSER) of non-irradiated and irradiated \(\alpha\)-crystallin by sequence analysis and mass analysis. Subsequently, the T₁₈ peptide was hydrolyzed and derivatized with OPA and Boc-L-Cys to form diastereoisomers as described in the Methods. The D/L ratio of amino acids was determined by RP-HPLC. As shown in Table 1, the D/L ratio of Asp₁₅₁ doubled with a UV-C irradiation dose of 1 J/cm² and then decreased gradually with increasing doses of radiation.

**DISCUSSION**

The present study focused on the effects of UV-C irradiation on \(\alpha\)-crystallin in comparison to the effects of gamma ray irradiation, which we reported in our previous paper. Because gamma irradiation is well known to dissociate H₂O and to result in OH or H radicals, we predicted that \(\alpha\)-crystallin would be influenced by these radicals and that various modifications including changes in the higher order structure of the protein would be observed. We found, however, that modifications of the lens protein in vitro occurred only at high doses of gamma radiation (0.5-5 kGy level), a level that we are never exposed to in daily life. By contrast, substantial damage, such as various modifications of the primary, secondary, and quaternary structure of \(\alpha\)-crystallin were observed following UV-C irradiation at a low dose (1-50 J/cm²), corresponding to levels that living organisms commonly encounter.

This damage may be caused by the presence of Trp residues in the protein. Trp residues in lens protein absorb UV rays, which cause photodamage to the protein [26]. It is thought
that the protein damage is mediated by the excitation and subsequent photooxidation of Trp to N-formylkynurenine (NFK), which then acts as an efficient photosensitizer [27]. It has been proposed that the excited triplet state of NFK reacts either with ground-state molecular oxygen to form singlet oxygen ($^{1}O_{2}$) or with protein amino acids to generate free radicals. Accordingly, it has been demonstrated that $^{1}O_{2}$, superoxide anion ($O_{2}^{-}$) and hydrogen peroxide (H$_{2}$O$_{2}$) can all cause photodamage to lens crystallin [28].

The fluorescence of α-crystallin, excited at 290 nm, is dominated by emission from Trp$_{9}$ in αA- and αB-crystallin and from Trp$_{60}$ in αB-crystallin. The destruction of Trp fluorescence of α-crystallin upon exposure to UV irradiation has been reported by many researchers [29,30]. The current study has shown that the Trp residues in α-crystallin markedly decreased at low dose of UV-C irradiation. A decrease in Trp fluorescence of about 30% was observed at 1 J/cm$^2$, subsequently 70% of Trp fluorescence was lost at 5 J/cm$^2$ irradiation, and finally Trp fluorescence was completely lost after irradiation at 20 J/cm$^2$ (Figure 3). The effect of UV-C irradiation on the loss of Trp was greater than the effect of gamma radiation on bovine α-crystallin. A loss of about 30% of Trp was observed after radiolysis with 50 krad (500 Gy) of gamma irradiation [20]. In parallel with the loss of Trp fluorescence, the corresponding NFK fluorescence increased markedly in a dose dependent manner (Figure 3). NFK can act as a UV photosensitizer and can generate reactive oxygen species. The oxidation of Met$_{1}$ may be caused by these reactive oxygen species.

The β-sheet structure of α-crystallin was retained after UV-C irradiation at doses lower than 10 J/cm$^2$. However, this secondary structure was lost with doses of UV-C irradiation higher than 20 J/cm$^2$ (Figure 2). This effect of UV-C on the secondary structure of α-crystallin is quite different from the effect of gamma irradiation on α-crystallin reported previously. α-Crystallin retained its secondary structure even after a dose of gamma irradiation as large as 4 kGy [21]. Thus, UV-C has a much greater effect than gamma irradiation on the secondary structure of α-crystallin. Gamma irradiation damages the protein by generating various radicals via water molecules, whereas the damage by UV-C may cause excitation of Trp/NFK, which in turn would cross-link the protein and subsequently cause loss of its secondary structure.

The chaperone activity of α-crystallin irradiated with doses of under 5 J/cm$^2$ was retained. However, it decreased markedly as the radiation dose increased. Only 40% of the native chaperone activity was observed after UV-C irradiation above 10 J/cm$^2$ (Figure 1). This may relate to the decrease in surface hydrophobicity of α-crystallin. Oxidation of the Met$_{1}$ residue of αA-crystallin reached more than 80% at a dose of 2 J/cm$^2$, but then decreased. In addition to amino acid oxidation, racemization of the Asp$_{151}$ residue of αA-crystallin was observed, showing that the D/L ratio of the Asp$_{151}$ residue increased from 0.13 at 0 J/cm$^2$ to 0.22 at 5 J/cm$^2$ and then decreased. We have reported previously that Asp$_{151}$ in αA-crystallin is stereochemically labile to allow the conversion of L-Asp to D-Asp during UV-B irradiation. As shown in column 3, the D/L ratio of Asp$_{151}$ doubled with a UV-C irradiation dose of 1 J/cm$^2$ and then decreased gradually with increasing doses of radiation.

<table>
<thead>
<tr>
<th>Irradiation</th>
<th>(T1+16)/T1</th>
<th>D/L of Asp$_{151}$</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0.27</td>
<td>0.13</td>
</tr>
<tr>
<td>1</td>
<td>0.48</td>
<td>0.27</td>
</tr>
<tr>
<td>2</td>
<td>1.63</td>
<td>0.24</td>
</tr>
<tr>
<td>5</td>
<td>1.4</td>
<td>0.22</td>
</tr>
<tr>
<td>10</td>
<td>1.18</td>
<td>0.2</td>
</tr>
<tr>
<td>20</td>
<td>0.55</td>
<td>0.15</td>
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</tbody>
</table>

This table indicates that after UV-C irradiation, the relative abundance of Met$_{1}$-oxidized T1 peptide in α-crystallin increased, reaching an abundance five times higher than that of native T1 peptide at a UV-C dose of 5 J/cm$^2$. With higher doses of radiation the abundance of peptide decreased. We have reported previously that Asp$_{151}$ in αA-crystallin is stereochemically labile to allow the conversion of L-Asp to D-Asp during UV-B irradiation. As shown in column 3, the D/L ratio of Asp$_{151}$ doubled with a UV-C irradiation dose of 1 J/cm$^2$ and then decreased gradually with increasing doses of radiation.
of αA-crystallin increased to 0.27 at a UV-C dose of 1 J/cm² (Table 1). In previous studies, we have shown that the Asp₁₅₁ residue is stereoechemically labile to allow the racemization of L-Asp to D-Asp in lens αA-crystallin during aging [31]. Although bovine αA-crystallin has 14 Asp and 2 Asn residues, this racemization occurs only in Asp₁₅₁ residues and not in other Asp/Asn residues. We previously investigated the formation of D-Asp at Asp₁₅₁ in αA-crystallin from aged and cataractous human lenses and found that D-Asp might form via a succinimide intermediate. The racemization of Asp depends on the rate of succinimide formation, which in proteins is expected to depend on the residue adjacent to the Asp residue. When the amino acid neighboring the Asp residue has a small side chain, succinimide formation can occur easily because there is no steric hindrance. Because Asp₁₅₁ in αA-crystallin is followed by an alanine residue, a succinimide can be easily formed, and therefore the Asp₁₅₁ residue is labile to enable its racemization to the D-form in αA-crystallin. The increase in racemization of Asp₁₅₁ with increasing UV-C irradiation is similar to the trend observed in aged lenses.

In summary, the present study suggests that the destruction of Trp residues in α-crystallin by UV-C irradiation may be one of the events that triggers the increased oxidation and racemization of amino acid residues in the protein. UV-C irradiation may also cause cross-linking and destroy the secondary structure of the protein. Subsequently, such changes would reduce the chaperone activity of α-crystallin.

α-Crystallin can gradually lose its chaperone activity with aging, probably through the cumulative effects of long-term exposure to UV-C radiation. Further studies on the effects of UV-A and UV-B irradiation on α-crystallin are needed to elucidate in full the effects of amino acid modifications on chaperone activity.

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REFERENCES


