



# Characterization of $\alpha$ A-crystallin from high molecular weight aggregates in the normal human lens

Noriko Fujii,<sup>1</sup> Minori Awakura,<sup>1</sup> Larry Takemoto,<sup>2</sup> Mitsushi Inomata,<sup>3</sup> Takumi Takata,<sup>1</sup> Norihiko Fujii,<sup>1</sup> Takeshi Saito<sup>1</sup>

<sup>1</sup>Research Reactor Institute, Kyoto University, Osaka, Japan; <sup>2</sup>Division of Biology, Kansas State University, Manhattan, KS;

<sup>3</sup>Biomembrane Research Group, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan

**Purpose:** Lens  $\alpha$ -crystallins, composed of two subunits of  $\alpha$ A- and  $\alpha$ B-crystallin, form low molecular weight (LMW) water soluble aggregates with an average molecular mass of approximately 800 kDa. In the intact lens, some of the  $\alpha$ -crystallins are associated with even larger high-molecular-weight (HMW) aggregates which are thought to be precursors of components found in the water insoluble fraction. Although the mechanism of HMW aggregation and insolubilization are not known, the process is considered to be related to cataract formation. The purpose of the present study is to compare  $\alpha$ A-crystallins from HMW and LMW forms in order to help understand the mechanism of insolubilization.

**Methods:** HMW and LMW  $\alpha$ A-crystallins were isolated from lenses of 50 year old and 2 year old human donors and compared with respect to chaperone activity and fluorescence. We also analyzed isomerization and racemization of Asp-58 and Asp-151 residues in  $\alpha$ A-crystallin from HMW and LMW fractions.

**Results:** The chaperone activity of HMW  $\alpha$ A-crystallin was lower than that of LMW  $\alpha$ A-crystallin. Fluorescence spectra indicated that HMW  $\alpha$ A-crystallin was more hydrophobic than that of LMW. Isomerization of normal  $\alpha$ -linkage to abnormal  $\beta$ -linkage at both Asp-58 and Asp-151 residues markedly increased in HMW  $\alpha$ A-crystallin compared with that of LMW  $\alpha$ A-crystallin. The D/L ratio of  $\beta$ -Asp-58 of either HMW or LMW forms were higher than 1.0, showing that inversion occurred in this site. In addition, the D/L ratio of the Asp-151 residue from HMW  $\alpha$ A-crystallin was significantly lower than that of the LMW form.

**Conclusions:** These results were qualitatively the same as those previously found in  $\alpha$ A-crystallin from total proteins of cataractous versus normal lenses, suggesting that changes in the native structure of  $\alpha$ A-crystallin associated with the HMW fraction of normal lenses reflect the same changes that occur to a greater degree in total proteins of human cataractous lenses.

$\alpha$ -Crystallin, a major protein of the ocular lens, is thought to play a role in maintaining lens transparency. In its native state,  $\alpha$ -crystallin is a large, water-soluble low molecular weight aggregate (LMW) with a molecular weight of approximately 800 kDa comprised of two kinds of polypeptides,  $\alpha$ A- and  $\alpha$ B-crystallins, containing 173 and 175 amino acid residues, respectively. Since the  $\alpha$ A and  $\alpha$ B monomers are approximately 20 kDa, the aggregate molecules contain approximately 40 subunits. As aggregation of  $\alpha$ -crystallin progresses, it becomes part of high molecular weight (HMW) aggregates (more than 1,000 kDa) and insoluble proteins. In aged and cataractous lenses, a large amount of HMW, aggregated  $\alpha$ -crystallin is observed. The mechanisms of aggregation and insolubilization of  $\alpha$ -crystallin are not known, but may be related to post-translational modifications such as deamidation [1-3], racemization and isomerization [4-13], truncation [2,9,14-16], phosphorylation [2,17], oxidation [2,18-20], an increase in intramolecular disulfide bonding [2,21], and glycation [22]. Racemization and isomerization of amino acids in proteins can cause major changes in their structure, since

different side chain orientations can induce an abnormal peptide backbone. Truncation of  $\alpha$ -crystallin subunits, an increase of intramolecular disulfide bonding, and advanced glycation end products (AGEs) also may perturb the normal close-packing structure of crystallins. Therefore, these post-translational modifications can induce a partial unfolding of  $\alpha$ -crystallin aggregates, resulting in a reduction of chaperone-like activity, followed by the eventual formation of cataracts. Previous studies indicated that oxidation and isomerization of  $\alpha$ A-crystallin decrease its chaperone activity [23,24]. In the present study we characterized the LMW and HMW forms of  $\alpha$ A-crystallin in order to better understand the effects of posttranslational modifications such as truncation, isomerization, and racemization of select amino acids upon the aggregation behavior and chaperone activity of the molecule.

## METHODS

*Purification of HMW and LMW  $\alpha$ A-crystallin from normal lenses of human donors:* Normal human lenses from 50 year old and approximately 2 year old donors (3-4 in each group) were obtained from the National Disease Research Interchange (Philadelphia, PA). HMW and LMW forms of  $\alpha$ -A crystallin were prepared as previously described [3]. The lenses were homogenized in phosphate buffer (0.06 M sodium phosphate, 0.1 M sodium sulfate, pH 7.0) at 4 °C. The homogenate was

Correspondence to: Noriko Fujii, Ph.D., Research Reactor Institute, Kyoto University, Kumatori, Sennan, Osaka 590-0494, Japan; Phone: +81-724-51-2496; FAX: +81-724-51-2630; email: [nfujii@hl.rii.kyoto-u.ac.jp](mailto:nfujii@hl.rii.kyoto-u.ac.jp)

centrifuged at 16,000x g for 15 min at 4 °C, and the supernatant was applied to a Biosep-SEC-S 4000 gel filtration column (7.8 mm x 300 mm, Phenomenex, Torrance, CA). Fractions corresponding to the HMW and LMW forms of  $\alpha$ -A crystallin were collected, followed by reverse phase chromatography of each fraction to purify  $\alpha$ -A crystallin [3]. The  $\beta$ L-crystallin fraction from human lenses was prepared using a Sephacryl S 300 HR gel-filtration column (26 mm x 600 mm, Pharmacia, Tokyo, Japan), which was equilibrated with 0.1 M  $\text{Na}_2\text{SO}_4$ , 0.1 M  $\text{KH}_2\text{PO}_4$  phosphate buffer (pH 7.5). Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, CA) with bovine serum albumin as a standard.

**Chaperone activity assay for heat-induced aggregation of  $\beta$ L-crystallin:** A chaperone activity assay was used to measure the ability of  $\alpha$ -crystallin to protect  $\beta$ L-crystallin against heat-induced aggregation.  $\beta$ L-Crystallin (200  $\mu\text{g}$ ) in 0.1 M  $\text{KH}_2\text{PO}_4$  buffer (pH 7.5) with or without 20 or 40  $\mu\text{g}$  of  $\alpha$ A-crystallin was incubated at 60 °C. The final volume of each reaction mixture was 0.7 ml [ $\beta$ L-crystallin/ $\alpha$ A-crystallin=10/1 or 5/1 (w/w)]. Aggregation of  $\beta$ L-crystallin was monitored by absorption due to light scattering at a wavelength of 360 nm. The chaperone activities of  $\alpha$ -A crystallin from HMW and LMW fractions from old donors (50 year old) and the

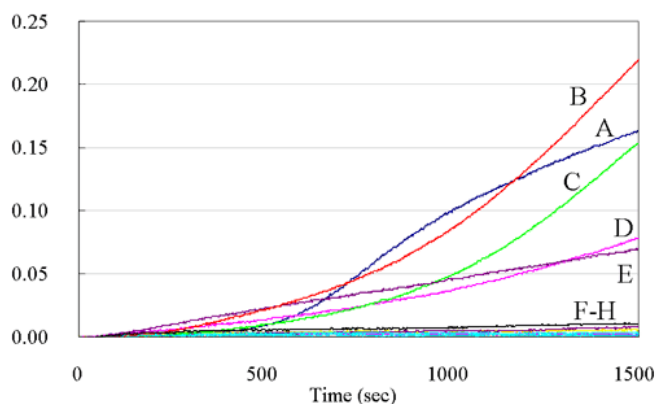


Figure 1. Chaperone activity of HMW and LMW  $\alpha$ -crystallins. Chaperone activity of  $\alpha$ -crystallin from the HMW and LMW fractions from 50 year old lenses and from the LMW fraction from 2 year old lenses. This assay was used to measure the ability of  $\alpha$ -crystallin (20 or 40  $\mu\text{g}/700 \mu\text{l}$ ) to protect  $\beta$ L-crystallin (200  $\mu\text{g}/700 \mu\text{l}$ ) against heat-induced aggregation at 60 °C. **A:**  $\beta$ L-Crystallin in the absence of  $\alpha$ A-crystallin; **B:**  $\beta$ L-Crystallin in the presence of HMW  $\alpha$ A-crystallin from 50 year old tissue ( $\beta$ L/ $\alpha$ A= 5/1 (w/w)); **C:**  $\beta$ L-Crystallin in the presence of HMW  $\alpha$ A-crystallin from 50 year old tissue ( $\beta$ L/ $\alpha$ A= 10/1 (w/w)); **D:**  $\beta$ L-Crystallin in the presence of LMW  $\alpha$ -crystallin from 50 year old tissue ( $\beta$ L/ $\alpha$ A= 5/1 (w/w)); **E:**  $\beta$ L-Crystallin in the presence of LMW  $\alpha$ -crystallin from 50 year old tissue ( $\beta$ L/ $\alpha$ A= 10/1 (w/w)) **F:**  $\beta$ L-Crystallin in the presence of LMW  $\alpha$ -crystallin from 2 year old tissue ( $\beta$ L/ $\alpha$ A= 10/1 (w/w)); **G:** HMW  $\alpha$ -crystallin from 50 year old tissue (40  $\mu\text{g}/700 \mu\text{l}$ ) in the absence of  $\beta$ L-crystallin; **H:** LMW  $\alpha$ -crystallin from 50 year old tissue (40  $\mu\text{g}/700 \mu\text{l}$ ) in the absence of  $\beta$ L-crystallin; **I:** LMW  $\alpha$ -crystallin from 2 year old tissue (40  $\mu\text{g}/700 \mu\text{l}$ ) in the absence of  $\beta$ L-crystallin.

LMW fraction from a young donor (2 year old) were compared.

**Fluorescence measurement:** Fluorescence was measured with a F-4500 Hitachi fluorescence spectrophotometer (Hitachi, Tokyo, Japan). Bis-ANS (4,4'-dianilino-1,1'-binaphthalene-5,5'-disulfonic acid, Molecular Probes, Junction City, OR) was used to probe the change in hydrophobicity, with an excitation wavelength at 395 nm and emission scanning between 420 and 550 nm [25]. Aliquots (20  $\mu\text{l}$ ) of bis-ANS ( $4.5 \times 10^{-4}$  M) were added to 3 ml of protein sample (0.1 mg/ml) and allowed to incubate at room temperature for 15 min before measurement.

**Preparation of antibodies of anti-1-154 degraded  $\alpha$ A-crystallin:** Polyclonal antibodies against the native forms of  $\alpha$ A- and  $\alpha$ B-crystallins were produced as previously described [26]. An antibody against the truncated form of bovine  $\alpha$ A-crystallin (1-154) was produced in rabbits using a synthetic antigen peptide corresponding to residues 149-154 (GVDAGH) with a cysteine residue added to the N-terminus as a site for conjugation to carrier protein. Affinity purification of antibodies was carried out using antigenic peptides immobilized on epoxy-activated Sepharose 6B (Amersham Pharmacia Biotech, Buckinghamshire, England).

**SDS-PAGE and western blot analysis:** The HMW and LMW  $\alpha$ -crystallin fractions from 50 year old and LMW  $\alpha$ -crystallin from 2 year old samples were electrophoresed by the method of Laemmli [27] using 15% SDS-polyacrylamide gels. The proteins in the gels were stained with Coomassie brilliant blue and/or transferred onto PVDF membranes and the blotted proteins were incubated with the first antibody (anti-1-154 degraded  $\alpha$ A-crystallin antibody) followed by incubation with peroxidase-conjugated anti-rabbit IgG as the second antibody. The antigen polypeptides were visualized using diaminobenzidine as substrate.

**Enzymatic digestion and isolation of peptides:** All preparations of  $\alpha$ -crystallins were digested with trypsin (Wako,

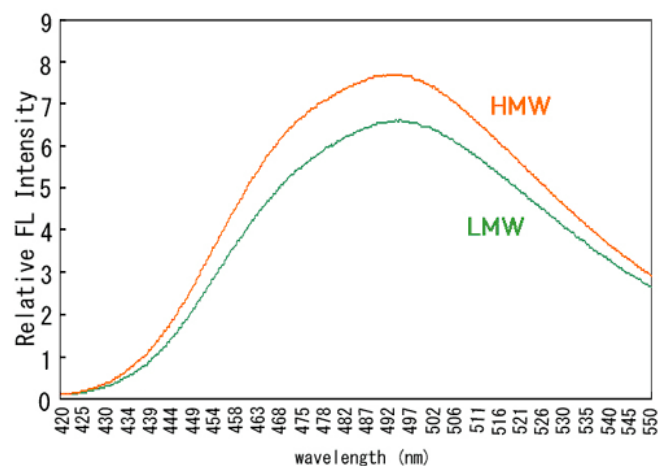


Figure 2. Bis-Ans fluorescence spectra. Bis-Ans fluorescence spectra of LMW and HMW  $\alpha$ -crystallin from lenses of 50 year old donors. Excitation wavelength was 395 nm and emission scanning was between 420 and 550 nm. Bis-Ans concentration was 15  $\mu\text{M}$ .

Osaka, Japan) for 20 h at 37 °C in buffer containing 0.1 M Tris-HCl buffer, 20 mM CaCl<sub>2</sub>, pH 7.6 at an enzyme-to-substrate ratio of 1/50 (mol/mol). The resulting tryptic peptides (T) were separated by RP-HPLC (LC-10A, Shimadzu, Kyoto, Japan) using a C18 column (TSK gel-ODS -80 TM, 4.6 x 250 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 using 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, Kompact 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,  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA, 10 mg) was dissolved in 1 ml of solution containing two parts of 0.1% (v/v) trifluoroacetic acid in water to one part of acetonitrile. The sample peptide (0.5 ml) was added to an equal volume (0.5 ml) of the matrix solution on the plate, and then dried. Each sample was present at a level of a few picomoles per spot.

**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-butylloxycarbonyl-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, 3.9 x 300 mm, Waters, 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 (Applied Biosystems 477A/120A, Foster City, CA).

## RESULTS

**Chaperone activity:** Since chaperone activity has been proposed to be a major function of  $\alpha$ -crystallin, we measured  $\alpha$ -A crystallin's chaperone activity through its ability to protect another protein from heat-induced aggregation. As shown in Figure 1, aggregation of  $\beta$ L-crystallin increased at 60 °C in the absence of  $\alpha$ -A crystallin, but this aggregation was completely suppressed by the presence of  $\alpha$ -A crystallin from the LMW fraction of lenses from 2 year old sources. However, protection from aggregation decreased markedly using  $\alpha$ A-crystallin from the LMW fraction of older lenses (50 year old sources), with a further decrease in activity using  $\alpha$ A-crystallin from the HMW fraction of the same lens [ $\beta$ L/ $\alpha$ A=10/1 (w/w)]. The chaperone activity of HMW  $\alpha$ A-crystallin decreased dramatically in the case of  $\beta$ L/ $\alpha$ A=5/1 (w/w). Since HMW  $\alpha$ A-crystallin did not aggregate at the same concentration without  $\beta$ L-crystallin, the aggregation is due to the mixture of  $\beta$ L-crystallin and HMW  $\alpha$ A-crystallin.

**Fluorescence:** Bis-ANS is an anionic hydrophobic fluorescent probe that binds to the polar interface, and has been used in the study of protein hydrophobicity [28]. Figure 2 shows that the fluorescence intensity of the HMW form of  $\alpha$ A

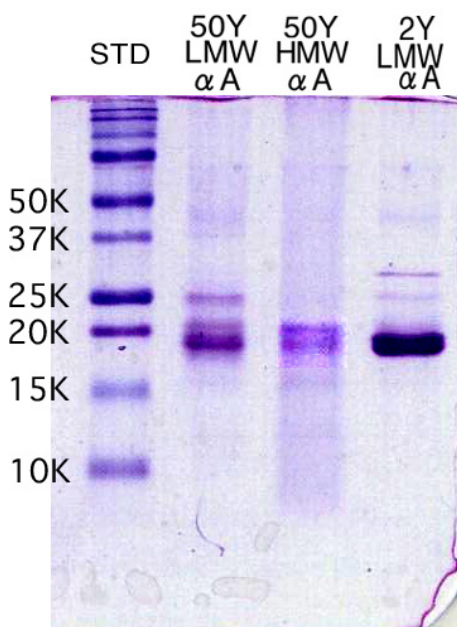


Figure 3. SDS-PAGE analysis. SDS-PAGE analysis of  $\alpha$ A-crystallin from the HMW and LMW fractions from lenses of 50 year old donors and from the LMW fraction from 2 year old donors.

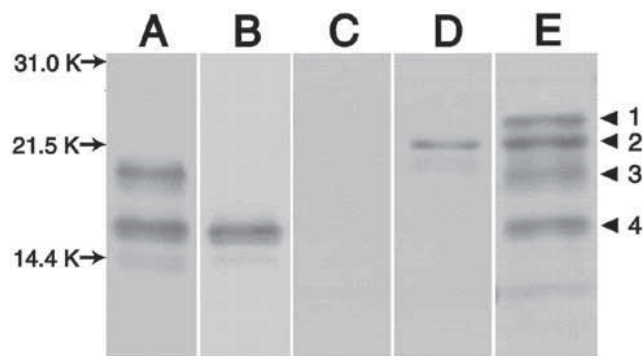


Figure 4. Characterization of antibodies. Characterization of antibody specific to the truncated form of  $\alpha$ A-crystallin (1-154). The  $\alpha$ -crystallin fraction prepared from bovine lenses was analyzed by western blotting. The blots were stained with the anti-native form of  $\alpha$ A-crystallin antibody (lane A), with the anti-truncated form of  $\alpha$ A-crystallin antibody (lane B), with the anti-truncated form of  $\alpha$ A-crystallin antibody in the presence of 500  $\mu$ g/ml antigenic peptide (CGVDAGH, lane C), with the anti-native form of  $\alpha$ B-crystallin antibody (lane D). Lane E is the Coomassie Brilliant Blue staining pattern of the PVDF membrane. Protein bands: 1, unknown; 2, native form of  $\alpha$ B-crystallin (1-175); 3, native form of  $\alpha$ A-crystallin (1-173); 4, truncated form of  $\alpha$ A-crystallin (1-154).

-crystallin is higher than that of the LMW form, indicating that the HMW aggregated form of  $\alpha$ A-crystallin was more hydrophobic than the LMW form of  $\alpha$ A-crystallin, which could possibly result from partial unfolding of the molecule.

**SDS-PAGE analysis:** Figure 3 shows SDS-PAGE of HMW and LMW  $\alpha$ A-crystallin from the 50 year old sample and the 2 year old sample of human lenses. The monomeric  $\alpha$ A-crystallin (molecular weight of 20 kDa) band from HMW the 50 year old sample was not distinct, which may be caused by an increase in crosslinked products and other post-translational modifications.

**Characterization of antibody against the truncated form of  $\alpha$ A-crystallin:** To evaluate the specificity of the antibody against the truncated form of 1-154  $\alpha$ A-crystallin, the  $\alpha$ -crystallin fraction prepared from bovine lenses was analyzed by western blotting (Figure 4). The antibody against the native form of  $\alpha$ A-crystallin reacts with both the intact approximately 20-kDa and truncated forms (Figure 4). In contrast, the anti-truncated form of  $\alpha$ A-crystallin antibody recognizes only the truncated form and does not react with its intact 20 kDa form (Figure 5). Immunostaining with the antibody in the presence of antigenic peptide results in disappearance of the immunoreactive band (Figure 4, lane C). These results indicate that the antibody possesses strict specificity towards the C-terminal sequence of the truncated form of  $\alpha$ A-crystallin (1-154). There are several possible reasons why the anti-truncated  $\alpha$ A-crystallin (1-154) antibody does not cross-react at all with the antigenic peptide sequence (GVDAGH) present in the native  $\alpha$ A-crystallin molecule. First, the corresponding portion in native form may not be exposed to the outside of the molecule, there-

fore it does not fit the antigen binding domain of the antibody; or the newly formed amino group on the C-terminal histidine of truncated  $\alpha$ A-crystallin is an essential part of the epitope for the antibody.

**Degradation of  $\alpha$ A-crystallin peptide from HMW and LMW fractions:** Figure 5 shows western blotting of HMW and LMW  $\alpha$ A-crystallin using anti-1-154 degraded  $\alpha$ A-crystallin antibody. Since the antibody specifically cross reacted with degraded  $\alpha$ A-crystallin 1-154 but not with full-length  $\alpha$ A-crystallin 1-173, the result indicated an increased degradation of the HMW  $\alpha$ A-crystallin compared with that of the LMW  $\alpha$ A-crystallin.

**Isomerization and racemization of the Asp-151 and Asp-58 residue in HMW and LMW  $\alpha$ A-crystallin:** We have reported previously that Asp-151 [7-12] and Asp-58 [13] in  $\alpha$ A-crystallin is stereochemically labile to allow the conversion of L-Asp to D-Asp or isomerization of  $\alpha$ -Asp to  $\beta$ -Asp during aging. Here, we measured the ratio of Asp-151 and Asp-58 isomerization and racemization in HMW  $\alpha$ A-crystallin to that in LMW  $\alpha$ A-crystallin from 50 year old donor lenses. First, we identified the Asp-151-containing peptides, namely, T18 peptides (IQTGLD151ATHAER) of HMW and LMW  $\alpha$ A-crystallin by sequence and mass analysis.

The T18 peptide was separated into two peaks by RP-HPLC owing to the difference between  $\alpha$ - and  $\beta$ -linkages of the Asp-151 residue (Figure 6). We designated the  $\beta$ -Asp-151-containing T18 peptide T18- $\beta$ , and the  $\alpha$ -Asp-151 containing peptide T18- $\alpha$ . Although the molecular masses of T18- $\beta$  and

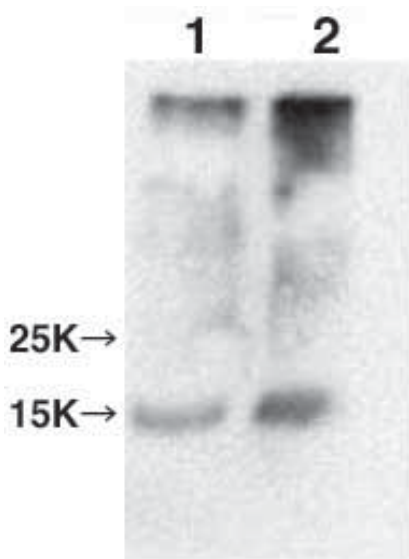


Figure 5. Western blot analysis. Detection of 1-154 degraded  $\alpha$ A-crystallin in HMW and LMW fraction by western blot analysis. Western blot analysis was performed with a rabbit anti-1-154 degraded  $\alpha$ A-crystallin antibody. Lane 1 is LMW  $\alpha$ A-crystallin and lane 2 is the HMW  $\alpha$ A-crystallin from lenses of 50 year range.

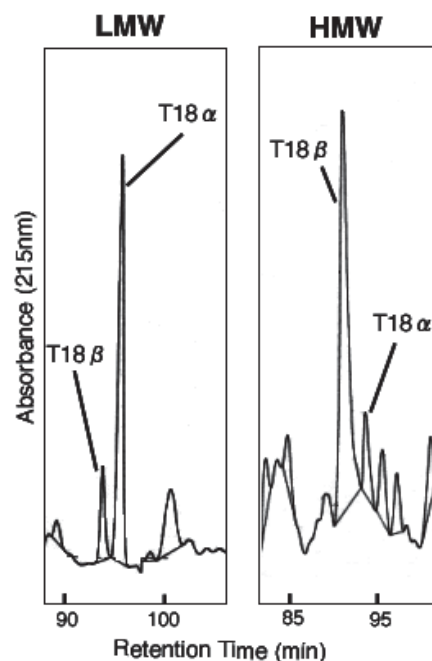


Figure 6. Reversed-phase HPLC of  $\alpha$ A-crystallin. Reversed-phase HPLC chromatogram of T18 peptides of HMW and LMW  $\alpha$ A-crystallins obtained from lenses of 50 year old donors. T18 peptide was separated into two peaks due to the difference between normal  $\alpha$ -linkage and unusual  $\beta$ -linkage. T18 $\beta$  and T18 $\alpha$  represent the T18 peptide containing  $\beta$ -Asp-151 and  $\alpha$ -Asp-151, respectively.

T18- $\alpha$  are both m/z 1,311.6, consistent with the theoretical mass of the peptide, T18- $\beta$  can be distinguished from T18- $\alpha$  by protein sequencing. Sequence analysis of T18- $\alpha$  gave the expected sequence from Ile-146 to Arg-157, whereas T18-beta gave a shortened sequence from Ile-146 to Leu-150 because  $\beta$ -linkages are resistant to Edman degradation. The  $\beta/\alpha$  ratio of Asp-151 of HMW  $\alpha$ A-crystallins was markedly higher than that of LMW  $\alpha$ A-crystallins. Subsequently, we also identified the Asp-58-containing peptides, namely, T6 peptides (TVLD58SGISGVR) of HMW and LMW  $\alpha$ A-crystallin and analyzed the linkage of Asp-58 by a similar methodology. Figure 7 shows the ratio of isomerization of Asp-151 and Asp-58 in  $\alpha$ A-crystallin from HMW and LMW fractions. The  $\beta/\alpha$  ratio of Asp-151 of HMW  $\alpha$ A-crystallin was extremely high, reaching a value of about 3.0, and the ratio of  $\beta/\alpha$  of Asp 58 of  $\alpha$ A-crystallin from HMW is also higher than that of LMW  $\alpha$ A-crystallin. The Mann-Whitney test indicated that the average  $\beta/\alpha$  ratio of Asp-151 and Asp-58 residues in HMW  $\alpha$ A-crystallin was significantly higher than those of LMW  $\alpha$ A-crystallin (for Asp-151:  $p < 0.05$ ; for Asp-58:  $p < 0.05$ ). We analyzed the D/L ratios of the  $\beta$ - and  $\alpha$ -Asp 151 residues from HMW and LMW  $\alpha$ A-crystallins in the 50 year old donors, respectively (Figure 8). Statistical analysis showed that the average D/L ratio of the  $\beta$ -Asp-151 residue in LMW  $\alpha$ A-crystallin was significantly higher than that of HMW  $\alpha$ A-crystallin ( $p < 0.05$ ). In LMW  $\alpha$ A-crystallin, the D/L ratio of  $\beta$ -Asp-151 residue was higher than 1.0, which was consistent with our previous results using normal lenses from elderly human donors [8-12]. Since racemization is defined as a reversible first order reaction, when the D/L ratio reaches 1.0, racemization is in equilibrium. Therefore, the D/L ratios which were higher than 1.0 would not be defined as racemization, but as the stereoinversion of L-Asp to its D-isomer. On the other hand the D/L ratios of  $\alpha$ -Asp-151 residues of LMW and HMW  $\alpha$ A-

crystallin are low (approximately 0.2). There were no significant differences in the D/L ratios for either LMW or HMW  $\alpha$ A-crystallin ( $p = 0.39$ ). In contrast, as shown in Figure 9, the D/L ratio of  $\beta$ -Asp-58 was higher than 1.0 either in HMW or LMW  $\alpha$ A-crystallin, which is also consistent with our previous result [13]. The statistical analysis showed that there were no differences in the D/L ratios of the Asp-58 residue between HMW and LMW  $\alpha$ A-crystallin ( $p = 0.48$ ).

In contrast, the D/L ratios of  $\alpha$ -Asp-58 of LMW  $\alpha$ A-crystallin was significantly higher than that of HMW  $\alpha$ A-crystallin ( $p < 0.05$ ), although the values were low (approximately 0.05-0.3).

## DISCUSSION

Formation of very high molecular weight aggregates of lens proteins is known to occur during aging, and may be relevant to cataract formation in human eye lenses. Although the mechanism of HMW aggregate formation has not yet been established, aggregated proteins may be precursors of components that eventually become insoluble. Therefore in the present study we have characterized the HMW form of  $\alpha$ A-crystallin from normal lenses and compared our results with those obtained from previous studies of  $\alpha$ A-crystallin obtained from total proteins of human cataractous lenses. As shown in Figure 1, the chaperone activity of  $\alpha$ A-crystallin from young (2 year old) lens completely prevented the aggregation of  $\beta$ L-crystallin, while  $\alpha$ A-crystallin from the LMW fraction of older (50 year old) lens was reduced in activity, with further reductions in chaperone activity using  $\alpha$ A-crystallin from the HMW fraction. To form the HMW fraction, we therefore hypothesize that there might occur partial conformational changes, resulting at least in part from increased amounts of degraded polypeptides and/or various post-translational modifications. Since the process of aggregation to form HMW components

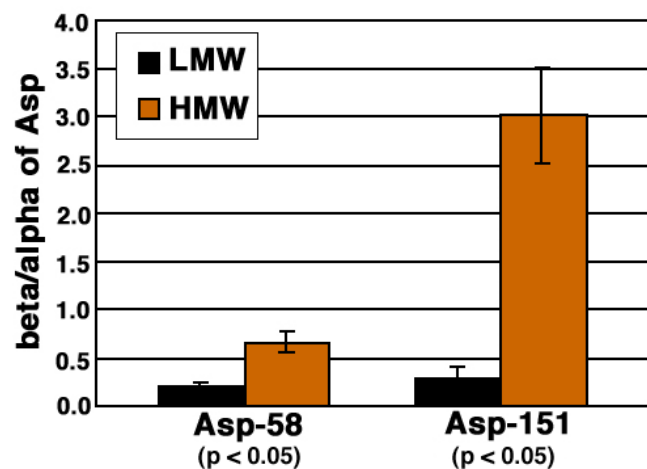


Figure 7.  $\beta/\alpha$  ratio of Asp-151 and Asp-58 from HMW and LMW  $\alpha$ A-crystallins. Ratio of abnormal isomers ( $\beta/\alpha$ ) of Asp-151 and Asp-58 residue of HMW and LMW  $\alpha$ A-crystallins obtained from lenses of 50 year range. The  $\beta/\alpha$  ratio of the Asp-151 was calculated by the ratio of peak area of T18  $\beta/\alpha$  peptides. The  $\beta/\alpha$  ratio of the Asp-58 was calculated by the ratio of peak area of T6  $\beta/\alpha$  peptides.

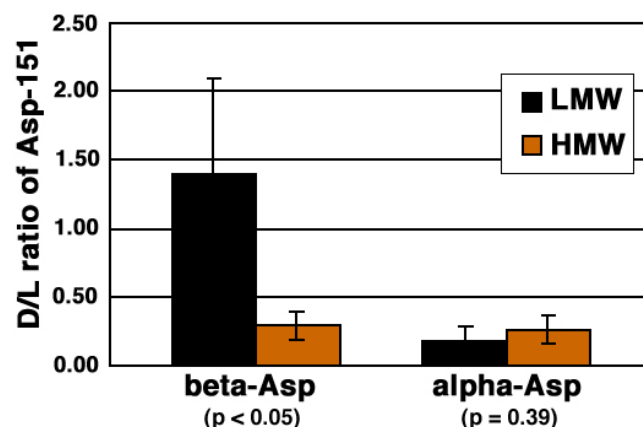


Figure 8. D/L ratio of Asp-151 from HMW and LMW  $\alpha$ A-crystallins. D/L ratio of Asp-151 residue of HMW and LMW  $\alpha$ A-crystallins obtained from lenses of 50 year old tissue. Peptide samples were hydrolyzed and derivatized to form diastereoisomers. The D/L ratio of amino acids was determined by RP-HPLC. See Methods for detail of the quantitation.

most likely results in insolubilization, the HMW form of  $\alpha$ A-crystallin should be more hydrophobic than native  $\alpha$ -crystallin. Figure 2 shows that the hydrophobicity of  $\alpha$ A-crystallin in the HMW aggregates was indeed greater than  $\alpha$ A-crystallin in the LMW fraction. The results strongly suggest that HMW aggregates of  $\alpha$ A-crystallin result from exposure of buried beta-pleated sheets and increased hydrophobic interactions, which is consistent with an earlier report [29].

We have also found a new cleavage site which is a post-translational modification between residues His-154 and Ala-155 in  $\alpha$ A-crystallin from old human lens. Since this cleavage was not found in  $\alpha$ A-crystallin from young lens, the cleavage probably results from aging [9]. Therefore, we prepared a specific antibody against 1-154  $\alpha$ A-crystallin and examined the amounts of the degraded  $\alpha$ A-crystallin in either HMW or LMW fractions by western blotting. There was a significant difference in the amounts of degraded products between HMW and LMW fractions, that is, the amounts of cleaved  $\alpha$ A-crystallin increased markedly in HMW protein (Figure 5). The results indicate that the HMW fraction contains a large amount of degraded  $\alpha$ A-crystallin, which may alter the tertiary structure of the protein.

In addition, we have analyzed the racemization and isomerization of Asp-151 and Asp-58 residues in HMW and LMW fractions of  $\alpha$ A-crystallin. In previous studies we have investigated D- $\beta$ -Asp formation of Asp-151 and Asp-58 in  $\alpha$ A-crystallin obtained from total proteins of aged and cataractous human lenses [8-13,24]. As shown in Figure 10, the D-Asp formation might occur via a succinimide intermediate. L-Succinimide is readily formed by intramolecular cyclization of L- $\alpha$ -Asp. L-Succinimide can be converted to D-succinimide by racemization, and both succinimides can be hydrolyzed to D- $\beta$ -Asp, L- $\beta$ -Asp, D- $\alpha$ -Asp and L- $\alpha$ -Asp residues, respectively. This reaction depends on the rate of succinimide formation, which is expected to depend on the neighboring residue of the Asp residue. When the neighboring amino acid of the Asp residue has a small side chain, succinimide occurs

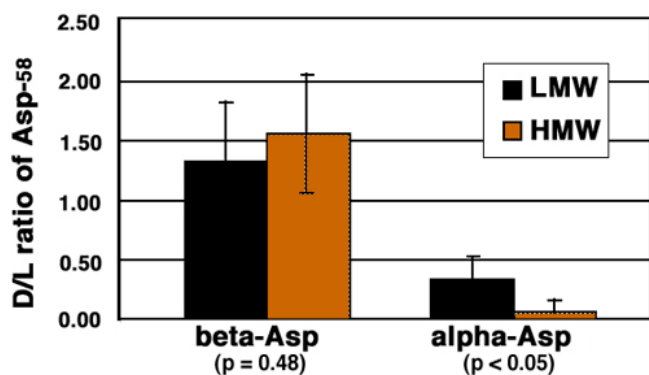


Figure 9. D/L ratio of Asp-58 from HMW and LMW  $\alpha$ A-crystallins. D/L ratio of Asp-58 residue of HMW and LMW  $\alpha$ A-crystallins obtained from lenses of 50 year old tissue. Peptide samples were hydrolyzed and derivatized to form diastereoisomers. The D/L ratio of amino acids was determined by RP-HPLC. See Methods for detail of the quantitation.

easily because there is no steric hindrance. Since Asp-151 and Asp-58 residues in  $\alpha$ A-crystallin are followed by alanine and serine residues, succinimides are easily formed. On the other hand, a previous study indicated that the formation of D-Asp needed the native higher order structure of  $\alpha$ A-crystallin itself, because when the native structure of 80 year old human  $\alpha$ A-crystallin which has a high D/L ratio of Asp-151 (5.0) was unfolded by heating in 6 M urea, the D/L ratio of Asp-151 decreased dramatically to 1.0 [10]. The result suggested that the area surrounding the Asp-151 or Asp-58 residues might have a chiral environment that allows for D-Asp formation in  $\alpha$ A-crystallin.

The native structure of aged  $\alpha$ A-crystallin may be distorted by various post-translational modifications, and the function of D-Asp may be to avoid the unfolding of the structure of  $\alpha$ A-crystallin which is caused by this distortion. In the present study, the D/L ratio of Asp-151 of LMW  $\alpha$ A-crystallin was higher than 1.0, while the value of the HMW form dramatically decreased. The tendency towards a lower D/L ratio was observed in both cataractous lenses[12,13] and  $\gamma$ -

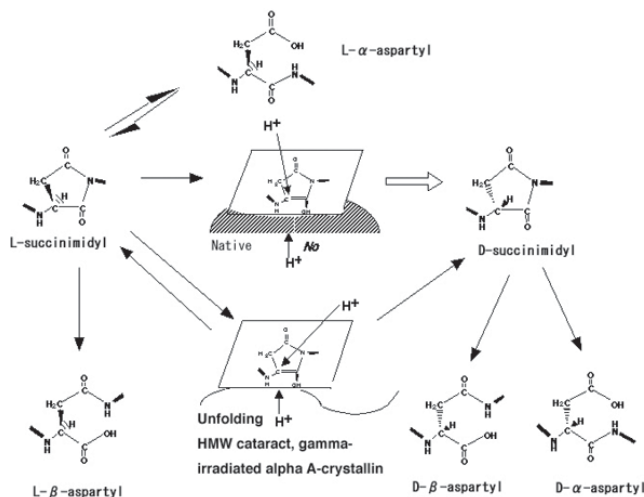


Figure 10. Reaction pathways of inversion and isomerization of Asp residues in protein. Reaction pathways for spontaneous inversion and isomerization of aspartyl residues in protein via a succinimide intermediate. A sterically hindering structure composed of the native higher order structure of  $\alpha$ A-crystallin may be present on the lower side of intermediate [I] (shaded part), resulting in the protonation of intermediate [I] from the upper side of the plane, causing the configuration to be inverted to the D-form [10]. However, when this chiral environment is lost by unfolding of  $\alpha$ A-crystallin, protonation to the intermediate [I] could occur with an equal probability from both sides of the plane, resulting in an increase in L-Asp, and a D/L ratio of 1.0 [10]. In the present study, D- $\beta$ -Asp residues were found at Asp-151 in LMW  $\alpha$ A-crystallin and at Asp-58 in either LMW or HMW, while the Asp-151 of HMW  $\alpha$ A-crystallin showed a low D/L ratio and an increase in  $\beta$ -Asp formation. The results are essentially the same as those found in cataractous and  $\gamma$ -ray irradiated  $\alpha$ A-crystallin, suggesting that D- $\beta$ -Asp formation may help stabilize the higher ordered structure surrounding the Asp residues. The function of the spontaneous stereoinversion may be to relieve the structural distortion within aged  $\alpha$ A-crystallin.

ray irradiated lenses, in a dose dependent manner [24].

The function of D-Asp therefore might be to prevent unfolding of  $\alpha$ A-crystallin, which seems to occur in the formation of cataracts or following irradiation. Taking these results together with our findings here, it seems likely that the area surrounding Asp-151 differs in HMW  $\alpha$ A-crystallin from the area in LMW  $\alpha$ A-crystallin. In contrast, there are no differences in the D/L ratio of  $\beta$ -Asp-58 between LMW and HMW. The higher order structure of surrounding Asp-58 may remain in the native state compared with the region surrounding Asp-151. We also determined the  $\beta/\alpha$  ratios of Asp-151 and Asp-58 in either HMW or LMW  $\alpha$ A-crystallin. The results are consistent with previous results [12,13,24]. As shown in Figure 10, both L- and D-succinimide are easily hydrolyzed. The probability of succinimide opening to  $\beta$ -Asp or  $\alpha$ -Asp is not known, although it favors  $\beta$ -Asp over  $\alpha$ -Asp in small peptides and in human  $\alpha$ A-crystallin. The increase of  $\beta$ -Asp formation in protein should alter the structure and possible function because the peptide backbone goes through the beta-carboxyl group.

In summary, our study suggests that modifications such as an increase in degradation, hydrophobicity, isomerization, and a decrease in racemization may destroy the higher-order structure, including the hydrophobic protein binding site of  $\alpha$ -crystallin and thus reduce its chaperone activity. Further studies are therefore needed to elucidate in full the effects of post-translational modifications on chaperone activity and protein structure.

#### ACKNOWLEDGEMENTS

This work was supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and a grant from the National Institutes of Health.

#### REFERENCES

1. Voorter CE, de Haard-Hoekman WA, van den Oetelaar PJ, Bloemendal H, de Jong WW. Spontaneous peptide bond cleavage in aging alpha-crystallin through a succinimide intermediate. *J Biol Chem* 1988; 263:19020-3.
2. Miesbauer LR, Zhou X, Yang Z, Yang Z, Sun Y, Smith DL, Smith JB. Post-translational modifications of water-soluble human lens crystallins from young adults. *J Biol Chem* 1994; 269:12494-502.
3. Takemoto L. Increased deamidation of asparagine-101 from alpha-A crystallin in the high molecular weight aggregate of the normal human lens. *Exp Eye Res* 1999; 68:641-5.
4. Masters PM, Bada JL, Zigler JS Jr. Aspartic acid racemization in the human lens during ageing and in cataract formation. *Nature* 1977; 268:71-3.
5. Masters PM, Bada JL, Zigler JS Jr. Aspartic acid racemization in heavy molecular weight crystallins and water insoluble protein from normal human lenses and cataracts. *Proc Natl Acad Sci U S A* 1978; 75:1204-8.
6. Groenen PJ, van den Ijssel PR, Voorter CE, Bloemendal H, de Jong WW. Site-specific racemization in aging alpha A-crystallin. *FEBS Lett* 1990; 269:109-12.
7. Fujii N, Ishibashi Y, Satoh K, Fujino M, Harada K. Simultaneous racemization and isomerization at specific aspartic acid residues in alpha B-crystallin from the aged human lens. *Biochim Biophys Acta* 1994; 1204:157-63.
8. Fujii N, Satoh K, Harada K, Ishibashi Y. Simultaneous stereoinversion and isomerization at specific aspartic acid residues in alpha A-crystallin from human lens. *J Biochem (Tokyo)* 1994; 116:663-9.
9. Fujii N, Momose Y, Yamasaki M, Yamagaki T, Nakanishi H, Uemura T, Takita M, Ishii N. The conformation formed by the domain after alanine-155 induces inversion of aspartic acid-151 in alpha A-crystallin from aged human lenses. *Biochem Biophys Res Commun* 1997; 239:918-23.
10. Fujii N, Harada K, Momose Y, Ishii N, Akaboshi M. D-amino acid formation induced by a chiral field within a human lens protein during aging. *Biochem Biophys Res Commun* 1999; 263:322-6.
11. Fujii N, Takemoto LJ, Momose Y, Matsumoto S, Hiroki K, Akaboshi M. Formation of four isomers at the asp-151 residue of aged human alphaA-crystallin by natural aging. *Biochem Biophys Res Commun* 1999; 265:746-51.
12. Fujii N, Takemoto LJ, Matsumoto S, Hiroki K, Boyle D, Akaboshi M. Comparison of d-aspartic acid contents in alpha A-crystallin from normal and age-matched cataractous human lenses. *Biochem Biophys Res Commun* 2000; 278:408-13.
13. Fujii N, Matsumoto S, Hiroki K, Takemoto L. Inversion and isomerization of Asp-58 residue in human alphaA-crystallin from normal aged lenses and cataractous lenses. *Biochim Biophys Acta* 2001; 1549:179-87.
14. Emmons T, Takemoto L. Age-dependent loss of the C-terminal amino acid from alpha crystallin. *Exp Eye Res* 1992; 55:551-4.
15. Takemoto L, Emmons T. Truncation of alpha A-crystallin from the human lens. *Exp Eye Res* 1991; 53:811-3.
16. Kamei A, Iwase H, Masuda K. Cleavage of amino acid residue(s) from the N-terminal region of alpha A- and alpha B-crystallins in human crystalline lens during aging. *Biochem Biophys Res Commun* 1997; 231:373-8.
17. Takemoto LJ. Differential phosphorylation of alpha-A crystallin in human lens of different age. *Exp Eye Res* 1996; 62:499-504.
18. Takemoto L, Horwitz J, Emmons T. Oxidation of the N-terminal methionine of lens alpha-A crystallin. *Curr Eye Res* 1992; 11:651-5.
19. Finley EL, Dillon J, Crouch RK, Schey KL. Identification of tryptophan oxidation products in bovine alpha-crystallin. *Protein Sci* 1998; 7:2391-7.
20. Finley EL, Dillon J, Crouch RK, Schey KL. Radiolysis-induced oxidation of bovine alpha-crystallin. *Photochem Photobiol* 1998; 68:9-15.
21. Takemoto L. Increase in the intramolecular disulfide bonding of alpha-A crystallin during aging of the human lens. *Exp Eye Res* 1996; 63:585-90.
22. Argirova MD, Breipohl W. Glycated proteins can enhance photooxidative stress in aged and diabetic lenses. *Free Radic Res* 2002; 36:1251-9.
23. Cherian M, Abraham EC. Decreased molecular chaperone property of alpha-crystallins due to posttranslational modifications. *Biochem Biophys Res Commun* 1995; 208:675-9.
24. Fujii N, Hiroki K, Matsumoto S, Masuda K, Inoue M, Tanaka Y, Awakura M, Akaboshi M. Correlation between the loss of the chaperone-like activity and the oxidation, isomerization and racemization of gamma-irradiated alpha-crystallin. *Photochem Photobiol* 2001; 74:477-82.
25. Musci G, Berliner LJ. Probing different conformational states of bovine alpha-lactalbumin: fluorescence studies with 4,4'-bis[1-(phenylamino)-8-naphthalenesulfonate]. *Biochemistry* 1985; 24:3852-6.

26. Inomata M, Nomura K, Takehana M, Saido TC, Kawashima S, Shumiya S. Evidence for the involvement of calpain in cataractogenesis in Shumiya cataract rat (SCR). *Biochim Biophys Acta* 1997; 1362:11-23.
27. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227:680-5.
28. Kato A, Nakai S. Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. *Biochim Biophys Acta* 1980; 624:13-20.
29. Liang JJ, Akhtar NJ. Human lens high-molecular-weight alpha-crystallin aggregates. *Biochem Biophys Res Commun* 2000; 275:354-9.