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Age-related changes of alpha-crystallin aggregate in human lens

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Summary. Lens alpha-crystallin, composed of two subunits alpha A- and alpha B-crystallin, forms large aggregates in the lens of the eye. The present study investigated the aggregate of human lens alpha-crystallin from elderly and young donors. Recombinant alpha A- and alpha B-crystallins in molar ratios of alpha A to alpha B at 1:1, corresponding to the aged sample, were also studied in detail. We found by ultra-centrifugation analysis that the alpha-crystallin aggregate from elderly donors was large and heterogeneous with an average sedimentation coefficient of 30 S and a range of 20–60 S at 37 °C. This was higher compared to the young samples that had an average sedimentation coefficient of 17 S. The sedimentation coefficients of recombinant alpha A- and alpha B-crystallins were approximately 12 S and 15 S, respectively. Even when recombinant alpha-crystallins were mixed in molar ratios equivalent to those found in vivo, similar S values as the native aged alpha-crystallin aggregates were not obtained.

Changes in the self-association of alpha-crystallin aggregate were correlated to changes in chaperone activity. Alpha-crystallin from young donors, and recombinant alpha A- and alpha B-crystallin and their mixtures showed chaperone activity, which was markedly lost in samples from the aged alpha-crystallin aggregates.

Keywords: Aging – Aggregation – Analytical ultracentrifugation – Lens alpha-crystallin

Introduction

The mammalian lens is composed of three major structural proteins, alpha-, beta-, and gamma-crystallins. These structural proteins are present in high concentrations and have defined interactions that contribute to the transparency of the lens. They provide the refractive index needed to focus light onto the retina. With aging, the crystallins undergo various modifications with subsequent aggregation and insolubilization. These processes contribute to the development of age-related lens opacity. Since the turnover of crystallins in the lens is very low, their ability to maintain their native conformation is crucial.

Alpha-crystallin is a large molecule with a molecular weight of approximately 800 kDa and is comprised of two kinds of polypeptides, alpha A- and alpha B-crystallin (Groenen et al., 1994). Recent papers showed that the ratio of alpha A- to alpha B-crystallin in the fetal lens obtained from humans is about 2:1, and this ratio decreases to about 3:2 and 1:1 in the water-soluble fraction of a lens obtained from a 54/55 year-old (Ma et al., 1998) and 80 year-old (Swamy and Abraham, 1991), respectively. Since the alpha A- and alpha B-crystallin monomers are approximately 20 kDa, the aggregate molecules contain approximately 40–50 subunits. The molecular weight of the alpha-crystallin aggregates increase to more than 1000 kDa during aging. Eventually, the aggregates become insolubilized. The mechanisms of aggregation and insolubilization of alpha-crystallin are not known, but may be related to various post-translational modifications such as deamidation (Voorter et al., 1988; Miesbauer et al., 1994; Takemoto and Boyle, 2000), racemization and isomerization (Masters et al., 1977; Fujii et al., 1994a, b), truncation (Takemoto and Emmons, 1991; Emmons and Takemoto, 1992), phosphorylation (Miesbauer et al., 1994; Takemoto, 1996c), oxidation (Takemoto, 1996b; Finley et al., 1998a, b), glycation (Argirova and Breipohl, 2002), and disulfide bond formation (Takemoto, 1996a; Cherian-Shaw et al., 1999). In our previous studies, we reported that specific aspartic acid (Asp) residues in alpha A- and

alpha B-crystallin were highly inverted from L-isomer to D-isomer and isomerized from the normal alpha-linkage to the beta-linkage of the peptide-bond (Fujii et al., 1994a, b). The truncation of alpha-crystallin subunits, the increase of intramolecular disulfide bonding, and advanced glycation may also perturb the normal close-packing structure of proteins. Therefore, it is predicted that the nature of aged alpha-crystallin aggregate must be different from that of the young alpha-crystallin aggregate. This is because post-translational modifications most likely leads to the distortion in the higher order structure of alpha-crystallin. The previous studies have not completely characterized the property of human lens alpha-crystallin aggregate from the elderly donors. In the present study, we characterized the human alpha-crystallin aggregate from both elderly and young donors and compared the aggregates to recombinant alpha A- and alpha B-crystallins, using ultra-centrifugation analysis.

Materials and methods

Isolation of water soluble alpha-crystallins from aged and young human lenses

Human (80-year range) lens samples were a gift from Dr. Sato (Science University of Tokyo). Young lenses were excised from eyes obtained from two day old donors from the Lions Eye Bank of Oregon, USA. 80-year range lens samples were homogenized in phosphate buffer (0.06 M sodium phosphate, 0.1 M sodium sulfate, 1 mM phenylmethylsulfonyl fluoride pH 7.0) at 4 °C. The lens homogenates were centrifuged at 16,000 × g for 30 min at 4 °C, and the supernatant was fractionated by size exclusion chromatography on a TSK 4000 SW column which had been equilibrated with the same buffer. Fractions containing alpha-crystallin were collected. The alpha-crystallin from human lenses of 0-year range was also purified by size exclusion chromatography in a similar buffer as previously described (Lampi et al., 1998). The purity of the proteins was checked with SDS-PAGE using 15% polyacrylamide gels in accordance with the method of Laemmli (Laemmli, 1970).

Expression of human recombinant alpha A- and alpha B-crystallin

A DNA fragment containing human alpha A-crystallin was obtained by PCR amplification from first-stranded cDNAs of human fetal brain (Clontech, CA) with a sense primer of 5′-CCATGGACGTGACCA TCCAG-3′ and an antisense primer of 5′-GGCTGCTATCTAAAG GAGT-3′. The sense primer was designed to include the *Nco*I site where the underlined nucleotide sequence shows the restriction site. The PCR reaction was carried out for 40 cycles of denaturation (94 °C, 30 s), annealing (60 °C, 30 s), and extension (72 °C, 60 s). The PCR product was gel-purified and subcloned by pBluescript TA vector (Strategene, CA). The *Nco*I-*Bam*HI digestion product of the DNA fragment of alpha A-crystallin was ligated into the *Nco*I-*Bam*HI site of the T7 expression vector pET-3d (Novagen, WI). The resulting construct was transformed into the *Escherichia coli* strain BL21 (DE3) pLysS (Novagen). Transformed *E. coli* cells were grown at 37 °C in LB medium containing 50 μg/ml ampicillin and 30 μg/ml chloramphenicol, and cultured at 37 °C until an A_{600} of 1.0 was reached. Isopropyl thio-beta-D-galactoside was added at a final concentration of 0.3 mM and the cells were further cultured for 5 h at 37 °C.

E. coli cells were harvested by centrifugation at 2000 *g* for 10 min, resuspended in 20 mM Tris/HCl (pH 8.0) containing 30 mM NaCl, 1 mM EDTA, 1 mM PMSF. The cell extract was obtained from ten minutes ultra sonication and centrifuged at 20,000 *g* for 45 min to prepare the supernatant.

Purification of human recombinant alpha A- and alpha B-crystallin

The supernatant of expressed *E. coli* cell suspension was separated by Q Sepharose XL (26 mm × 200 mm, Amersham Bioscience) equilibrated with 20 mM Tris/HCl buffer (pH 8.0) containing 1 mM EDTA. Alpha A- and alpha B-crystallins were eluted with linear gradients between 0 and 1 M NaCl, (flow rate 5 ml/min). The fractions eluting between 250–350 mM NaCl were recovered. The alpha A-crystallin fraction was applied to a Superose 6 column (16 mm × 600 mm; Amersham Bioscience), while the alpha B-crystallin fraction was applied to an equilibrated Sephacryl S-300 column (26 mm × 600 mm, Amersham Bioscience). Both columns were equilibrated with 20 mM Tris/HCl buffer (pH 8.0) containing 150 mM NaCl and 1 mM EDTA. The same buffer was used as the eluent with a flow rate of 1.5 ml/min. Alpha A-crystallin was eluted with 160–190 ml of eluent while the alpha B-crystallin was eluted with 120–150 ml of buffer. Alpha A- and alpha B-crystallin fraction were further purified with a Uno Q-6 column (Bio Rad laboratory) equilibrated with 20 mM Tris/HCl buffer (pH 8.0) containing 1 mM EDTA. Alpha A- and alpha B-crystallins were eluted with a linear gradient between 0 and 1 M NaCl, (flow rate 5 ml/min) and the fractions eluting between 270–350 mM NaCl were recovered.

Reconstitution of alpha-crystallin heteroaggregates

The heteroaggregate composed of recombinant alpha A- and alpha B-crystallin was reconstituted based on the procedures described in a previous paper (Gupta and Srivastava, 2004). The purified recombinant alpha A- and alpha B-crystallin were mixed at 1:1 ratio and then an equivalent volume of 8 M Gdn HCl (final concentration 4 M GdnHCl) was added. This was followed by incubation for 6 h at 4 °C. After which, a dialysis against 50 mM Tris-HCl, pH 7.9, at 5 °C for 48 h with four changes of the buffer for the renaturation was performed.

Analytical ultracentrifugation

Sedimentation analysis was carried out at 37 °C using a Beckman Coulter Optima XL-I analytical ultracentrifuge. The rotor was equipped with 12 mm 2-channel center pieces and quarts windows. Concentration profiles of the sample were monitored by a UV scanner at 280 nm. The sedimentation velocity experiments were performed with 0.8 mg/ml of solution at a rotor speed of 10,000–20,000 rpm. The calculated sedimentation coefficient was converted to the standard condition (20 °C in water) to obtain $s_{20,w}$.

Fluorescence spectra

In order to observe changes in protein hydrophobicity, the sample was assayed with bis-ANS (4,4′-dianilino-1,1′-binaphthalene 5,5′-disulfonic acid, Molecular probes, Junction City, OR), and the resulting fluorescence spectra were recorded with an excitation wavelength of 395 nm and emission scanning wavelength between 420 nm and 550 nm using a F-4500 Hitachi spectrofluorometer (Hitachi, Tokyo, Japan) (Musci et al., 1985). The assay was performed according to a previously described procedure (Liang and Akhtar, 2000). In brief, aliquots (20 μl) of bis-ANS (4.5×10^{-4} M) were added to 3 ml of each protein sample and allowed to stand at room temperature for 15 min before measurement.

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. One mg of beta L-crystallin in 50 mM Tris HCl buffer (pH 7.8) with or without 0.1 mg of alpha-crystallin was incubated at 60 °C. The final volume of each reaction mixture was 0.7 ml. Aggregation of beta L-crystallin was monitored by absorption due to light scattering at a wavelength of 360 nm.

Results

Isolation of alpha-crystallin fractions from human lenses

The alpha-, beta-, and gamma-crystallin fractions were separated by size exclusion chromatography using a TSK G-4000SW HPLC column. Figure 1 shows a typical HPLC profile with absorbance at 280 nm. The size of alpha-crystallin obtained from the elderly donors is greater than that of alpha-crystallin from young donors. Figure 2 shows the SDS-PAGE of alpha-crystallins from aged and young donors. The monomeric alpha A- and alpha B-crystallin (molecular weights of 20 kDa) bands from elderly donors were not distinct and the quantity of cross-linked products increased (Fig. 2, lane 2).

Isolation of recombinant alpha A- and alpha B-crystallin

The size exclusion chromatography of recombinant alpha A- and alpha B-crystallin is shown in Fig. 3. The results showed that both recombinant alpha A- and alpha B-crystallin indeed formed high aggregates that corresponded in size with the aggregates from the 0 year old human lenses as shown in Fig. 1. This indicated that recombinant alpha A- and alpha B-crystallins folded and aggregated in *E.coli*

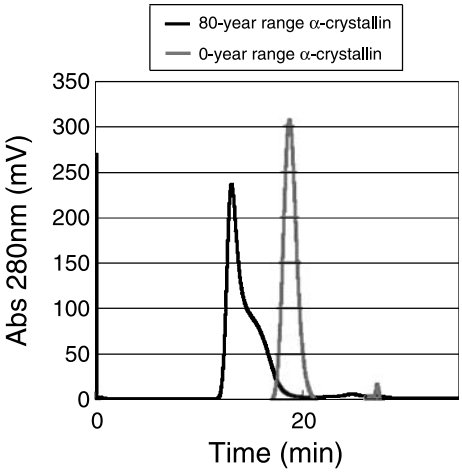

Fig. 1. Size exclusion HPLC of alpha-crystallin samples from lenses of 80-year range and 0-year range human donors using a TSK-4000 SW column. The mobile phase contained 0.06 M sodium phosphate and 0.1 M sodium sulfate (pH 7.0). The flow rate was 1 ml/min. The absorbance was recorded at 280 nm

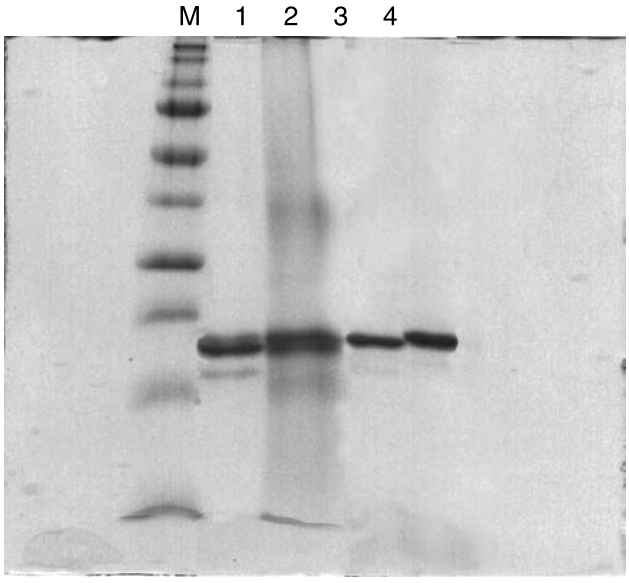

Fig. 2. Identification of alpha-crystallin samples by SDS-PAGE using a 15% poly acrylamide gel. *1* Alpha-crystallin from donors in 0-year range; *2* alpha-crystallin from donors in 80-year range; *3* recombinant alpha A-crystallin; *4* recombinant alpha B-crystallin. All bands were stained with Coomassie Brilliant Blue

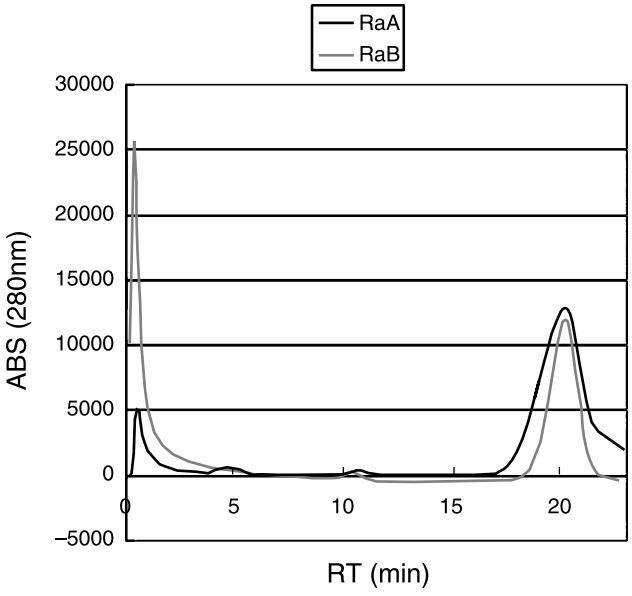

Fig. 3. Size exclusion HPLC of recombinant alpha A- and alpha B-crystallin samples on a TSK-4000 SW column. The mobile phase contained 0.06 M sodium phosphate and 0.1 M sodium sulfate (pH 7.0). The flow rate was 1 ml/min. The absorbance was recorded at 280 nm

even without post-translational modifications such as N-terminal acetylation. As shown in Fig. 2, the recombinant alpha A- and alpha B-crystallin were clearly purified (lanes 3, 4). The bands were identified by Western blotting using the anti-alpha A or alpha B-crystallin antibody, respectively (data not shown).

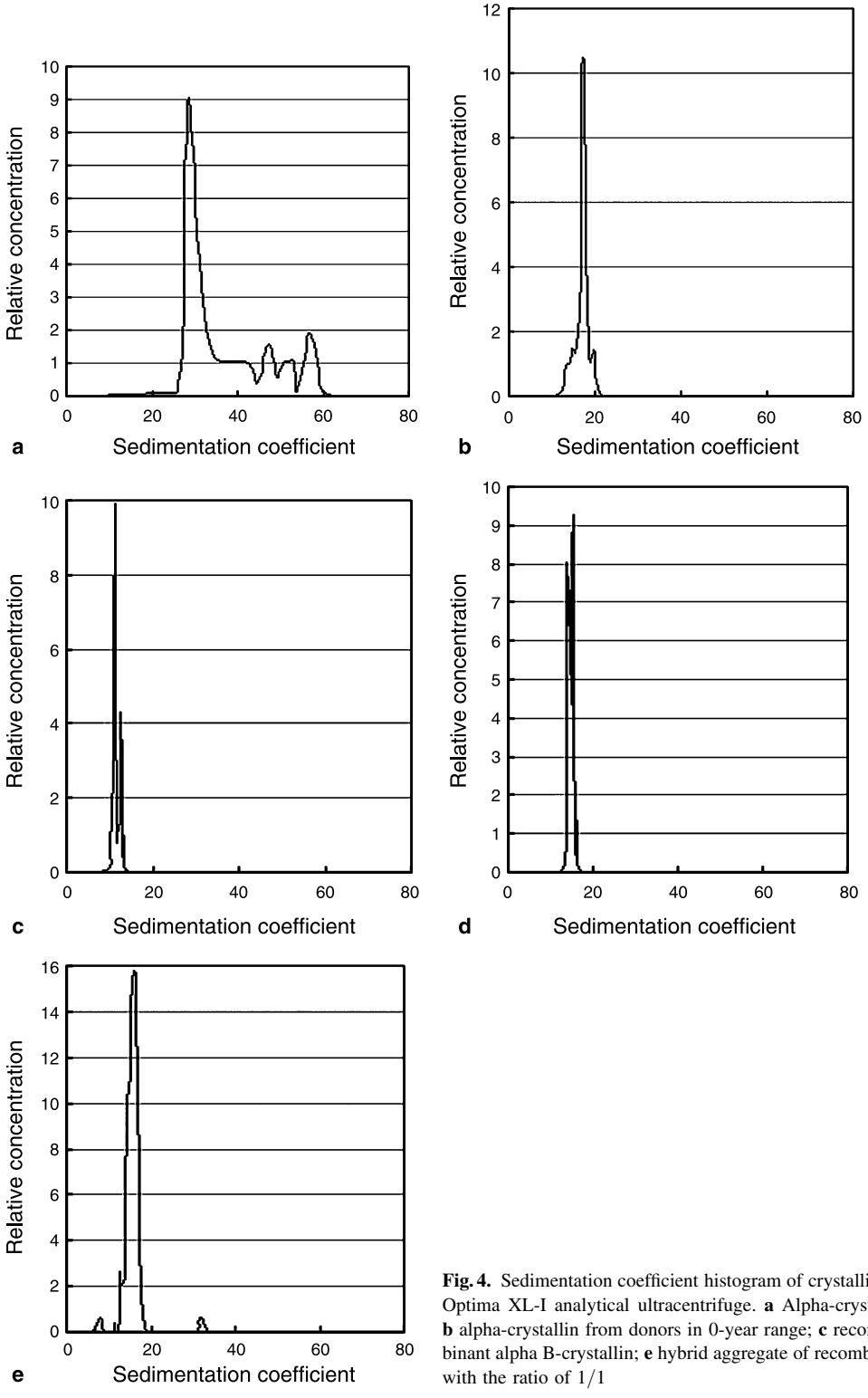

Fig. 4. Sedimentation coefficient histogram of crystallins at 37 °C using a Beckman Coulter Optima XL-I analytical ultracentrifuge. **a** Alpha-crystallin from donors in 80-year range; **b** alpha-crystallin from donors in 0-year range; **c** recombinant alpha A-crystallin; **d** recombinant alpha B-crystallin; **e** hybrid aggregate of recombinant alpha A- and alpha B-crystallin with the ratio of 1/1

Sedimentation analysis

In order to characterize the alpha-crystallin aggregates obtained from aged and young human lenses and recombinant alpha A- and alpha-B-crystallins, sedimentation coefficients were calculated using the second moment method and Van Holde-Weischet method. This yielded the sedimentation coefficient of the samples under the standard condition ($s_{20,w}$). Figure 4a shows the distribution of sedimentation coefficients of the alpha-crystallin from aged human donors. The sedimentation coefficient ($s_{20,w}$) of the alpha-crystallin from the 80 year range was determined to be mostly 30 S, but S values were widespread from 22 S to 60 S. On the other hand, the sedimentation coefficient ($s_{20,w}$) of the alpha-crystallin from the 0 year range was less heterogenous with an S value calculated to be 17 S (Fig. 4b). Figures 4c and d show the S values of recombinant alpha A- and alpha B-crystallin, respectively. Both recombinant crystallins are homogenous aggregates compared with that of the native alpha-crystallin from elderly donors. Recombinant alpha A-crystallin had sedimentation coefficients, 11–13 S, and it was less than that of recombinant alpha B-crystallin, which had a coefficient of 15 S. Figure 4e shows the reconstituted heteroaggregates composed of the recombinant alpha A-crystallin and alpha B-crystallin at the ratio of 1/1. The 16 S was different from the value by the simple addition of the 11–13 S for alpha A and 15 S for alpha B. The 16 S of the heteroaggregate is approximately the same as that of the 0 year-range of human lens crystallin. The hybrid ratio of alpha A to alpha B-crystallin in human lens decreases during aging (Ma et al., 1998). The molar ratio of 1/1 for alpha A- to alpha B- is the expected value for the 80-year human lenses (Swamy and Abraham, 1991). However, the S value of the recombinant heteroaggregate at the molar ratio of 1:1 was smaller than that

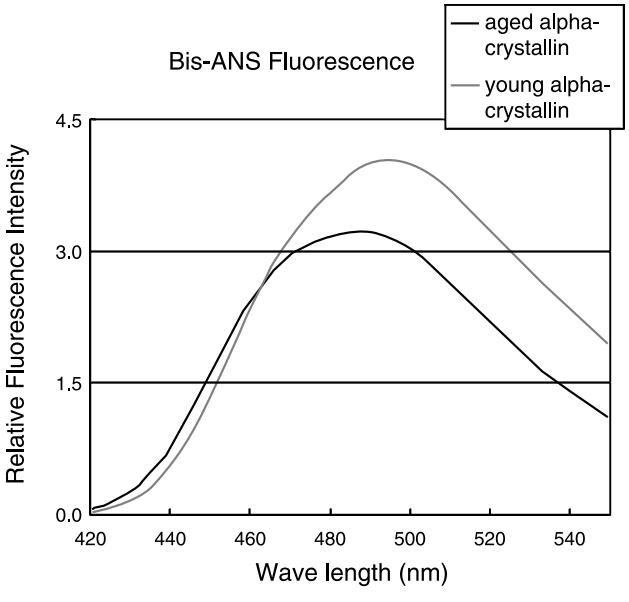

Fig. 5. Bis-ANS fluorescence spectra of aged and young alpha-crystallin. The excitation wavelength was 395 nm. The protein concentration was 0.1 mg/ml in 50 mM Tris/HCl buffer (pH 7.8)

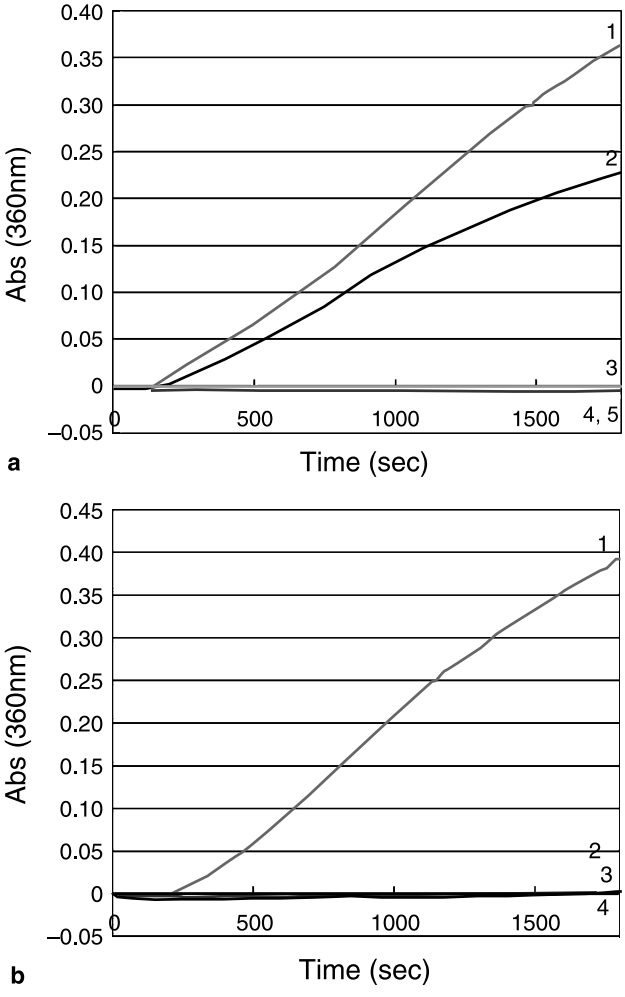

Fig. 6. Molecular chaperone-like activity of alpha-crystallin samples. Assay was used to measure the ability of alpha-crystallin (0.1 mg/700 µl) to protect beta L-crystallin (1.5 µg/700 µl) against heat-induced aggregation at 60 EC. **a** Alpha-crystallin from donors in 80-year range, and 0-year range. *1* Beta L-crystallin in the absence of alpha-crystallin; *2* Beta L-crystallin in the presence of alpha crystallin of 80-year range (beta L/alpha = 15/1 (w/w)); *3* Beta L-crystallin in the presence of alpha-crystallin of 0-year range (beta L/alpha = 15/1 (w/w)); *4* Alpha-crystallin of 80-year range in the absence of beta L-crystallin; *5* Alpha-crystallin of 0-year range in the absence of beta L-crystallin. **b** Recombinant alpha A- and alpha B-crystallin. *1* Beta L-crystallin in the absence of alpha-crystallin; *2* Beta L-crystallin in the presence of recombinant alpha A-crystallin (beta L-crystallin/recombinant alpha A = 15/1(w/w)); *3* Beta L-crystallin in the presence of recombinant alpha B-crystallin beta L-crystallin/recombinant alpha B = 15/1(w/w)); *4* recombinant alpha A- and alpha B-crystallin

of the aged alpha-crystallin. Furthermore, the S distribution of the heteroaggretate was not different from that of the elderly samples (Fig. 4e).

Fluorescence

Bis-ANS is an anionic hydrophobic fluorescent probe that binds to a polar interface. Figure 5 shows the bis-ANS fluorescence spectra of aged and young alpha crystallins. The bis-ANS fluorescence intensity of alpha-crystallin decreased with aging, showing that aging leads to a decrease in the exposure of the hydrophobic surfaces of alpha-crystallin.

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 Fig. 6a, aggregation of beta L-crystallin increased at 60 °C in the absence of alpha-crystallin. However, this aggregation was completely suppressed by the presence of alpha-crystallin from the lenses of 0-year range. On the other hand, protection from aggregation decreased markedly using alpha-crystallin from 80-year range. Figure 6b shows the chaperone activity of recombinant alpha A- and alpha B-crystallins. The recombinant crystallins have stable chaperone activity and there are no differences between the activity of alpha A- and alpha B-crystallin in this assay.

Discussion

It is well known that alpha-crystallins form very high molecular weight aggregates during aging, and that the aggregates may be related to cataract formation in the human eye lens. Although the self-association of alpha-crystallin has not yet been established, the aggregated proteins may be precursors of components that eventually become insoluble. The Asp 58 and 151 in alpha A-crystallin and Asp 36 and 62 residues in alpha B-crystallin from 80 year-old donors were highly inverted from L-isomer to D-isomer and isomerized from the normal alpha-linkage to the beta-linkage of the peptide-bond (Fujii et al., 1994a, b). These uncommon isomers of Asp residues in protein may be one of the triggers of abnormal aggregation. Almost all studies concerning the size of these aggregates have been limited to size-exclusion chromatography, except for earlier reports for bovine alpha-crystallin (Siezen and Berger, 1978; Thomson and Augusteyn, 1983; Chiou et al., 1989). Therefore, in the present study we have characterized the aggregate forms of native alpha-crystallin from human aged and young donors, and recombinant alpha A- and alpha B-crystallins, together with their heteroaggregates. This study clearly demonstrated that the size and the distribution of the aggregate of aged alpha-crystallin differed remarkably from that of young alpha-crystallin. As shown in Fig. 1, the size-exclusion choromatography indicated that the aged alpha-crystallin aggregates were greater than young alpha-crystallin aggregates. However, the difference of the size distribution of the aggregates of the crystallins between old and young donors is not known. In this report, information of size distribution of the aggregates was determined from the more rigorous analysis of sedimentation velocity. The sedimentation coefficient of alpha-crystallin from young donor lenses was 17 S (Fig. 4b). In contrast, the sedimentation coefficient of aged human alpha-crystallin was greater, around 30 S, and was very polydispersed ranging from 20–60 S (Fig. 4a). The increase in S value indicates not only an increase of size but also a change of shape, from sphere to an elongated shape of aggregate. Since, previous studies determined aggregate sizes from size-exclusion chromatography and SDS-PAGE, this is the first report of the S distribution of aggregate. The increase in aggregate size, size distribution and shape change upon aging may lead to altered function of the alpha-crystallin. The hydrophobic surface charge of the aged alpha-crystallin was markedly decreased (Fig. 5). We have also shown a decrease in the hydrophobic surface charge of alpha-crystallin due to UV-C damage (Fujii et al., 2004). The heterogeneity and large aggregate size and shape of alpha-crystallin along with the reduction in surface hydrophobicity of aged alpha-crystallin may explain the lost of chaperone activity that was observed (Fig. 6a).

In order to more thoroughly study the alpha-crystallin aggregate, recombinant of alpha A- and alpha B-crystallins were characterized. Since, the individual recombinant alpha A- and alpha B-crystallins eluted at the same retention time as the young alpha-crystallin (Figs. 1 and 3), we predicted that S values of the recombinant alpha-crystallins would be similar to the native alpha-crystallin obtained from young lenses. However, they were not. The S values and the S distribution were smaller than that of native young alpha-crystallin. Their smaller S value may be due to the absence of post translational modifications present in even the very young lenses (Lampi et al., 1998). Furthermore, the homoaggregate of recombinant alpha A-crystallin formed at 11–13 S (Fig. 4a), while the homo-

aggregate of recombinant alpha B-crystallin formed a heavier aggregate at 15 S. These results showed that the size and shape of the recombinant alpha A- and alpha B-crystallin aggregates were entirely different. The tertiary and secondary structure of recombinant alpha A- and alpha B-crystallin also differ (Sun et al., 1997) and this may explain why the S values differed.

Since, the molar ratio of alpha A- and alpha B tends to reach the 1:1 ratio during aging, recombinant alpha-crystallins were mixed in similar ratios. The S value of hetero aggregates composed of a 1:1 molar ratio of alpha A- to alpha B-crystallin was greater than that of the homoaggregate of the individual crystallins (Fig. 4e). This hetero-aggregate with 1:1 ratio is similar to that of the young alpha-crystallin but not the alpha-crystallin from the adult lens. The properties of recombinant alpha A- and alpha B-crystallin aggregate and their hybrid aggregate differed from the native aged alpha-crystallin. It is possible that the alpha-crystallin aggregate from elderly donors had the most age dependent post-translational modifications. Therefore, the self-association mechanism the folding mechanism and packed shape of the aggregates may be different between the native alpha-crystallin and the recombinant alpha crystallins. The post-translational modifications on the primary structure of the protein from aged alpha-crystallin can induce the partial unfolding of alpha-crystallin aggregates, resulting in a reduction of chaperone-like activity, followed by the eventual formation of cataracts. A reason for the loss of chaperone activity of aged alpha-crystallin may cause from the formation of huge aggregate and its heterogeneity.

Trigger of abnormal aggregation of alpha-crystallin is not known but various post-translational modifications may cause the change of the higher order structure of alpha-crystallin. In a previous study, we reported that the Asp 58 and Asp 151 residues inverted and isomerized to form D-beta-Asp residues in the aged alpha A-crystallin (Fujii et al., 1994b). If D-Asp would be formed in alpha A-crystallin that is composed of mainly beta-sheet structure, the higher order structure of the protein would induce the big change because the configuration of the Asp residue would be opposite. In addition to D-Asp formation, beta-linkage of Asp formation may affect a quaternary structure of crystallin because the main chain of the protein would be elongated.

Cherian-Shaw et al. (1999) indicated that intra-molecular disulfide bond between Cys-131 and Cys-142 formed in alpha A-crystallin obtained from lenses of elderly donors may cause the structural disturbance and accounts for the loss of chaperone activity. These various post-translational modifications contribute to the age-dependent loss of the chaperone activity and may induce cataract.

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