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Degradation of C-terminal Truncated αA-crystallins by the Ubiquitin–Proteasome Pathway

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Abstract

PURPOSE—Calpain-mediated C-terminal cleavage of αA-crystallins occurs during aging and cataractogenesis. The objective of the present study was to explore the role of the ubiquitin-proteasome pathway (UPP) in degrading C-terminal truncated αA-crystallins.

METHODS—Recombinant wild-type (wt) αA-crystallin and C-terminal truncated αA_{1-168}-, αA_{1-163}-, and αA_{1-162}-crystallins were expressed in *Escherichia coli* and purified to homogeneity. The wt and truncated αA-crystallins were labeled with ^{125}I, and proteolytic degradation was determined using both lens fiber lysate and reticulocyte lysate as sources of ubiquitinating and proteolytic enzymes. Far UV circular dichroism, tryptophan fluorescence intensity, and binding to the hydrophobic fluorescence probe Bis-ANS were used to characterize the wt and truncated αA-crystallins. Oligomer sizes of these crystallins were determined by multiangle light-scattering.

RESULTS—Whereas wt αA-crystallin was degraded moderately in both lens fiber and reticulocyte lysates, αA_{1-168}-crystallin was resistant to degradation. The susceptibility of αA_{1-163}-crystallin to degradation was comparable to that of wt αA-crystallin. However, αA_{1-162}-crystallin was much more susceptible than wt αA-crystallin to degradation in both lens fiber and reticulocyte lysates. The degradation of both wt and C-terminal truncated αA_{1-162}-crystallins requires adenosine triphosphate (ATP) and was stimulated by addition of a ubiquitin-conjugating enzyme, Ubc4. The degradation was substantially inhibited by the proteasome inhibitor MG132 and a dominant negative mutant of ubiquitin, K6W-Ub, indicating that at least part of the proteolysis was mediated by the UPP. Spectroscopic analyses of wt and C-terminal truncated αA-crystallins revealed that C-terminal truncation of αA-crystallin resulted in only subtle changes in secondary structures. However, C-terminal truncations resulted in significant changes in surface hydrophobicity and thermal stability. Thus, these conformational changes may reveal or mask the signals for the ubiquitin-dependent degradation.

CONCLUSIONS—The present data demonstrate that C-terminal cleavage of αA-crystallin not only alters its conformation and thermal stability, but also its susceptibility to degradation by the UPP. The rapid degradation of αA_{1-162} by the UPP may prevent its accumulation in the lens.

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Lens fiber cells contain high concentrations of proteins in the cytoplasm, which gives the lens a high refractive index and minimizes light-scattering at the membrane-cytoplasm interface. α-Crystallins are the major proteins of the mammalian lens that constitute approximately 35% of its dry weight and exist as large heterogeneous oligomers composed of two types of subunits, αA-crystallin and αB-crystallin, in a molar ratio of 3 to 1 in most mammalian lenses.[1] The two subunits have molecular masses of ~20 kDa with approximately 57% amino-acid sequence homology. In humans, αA-crystallin contains 173 amino acids and αB-crystallin contains 175.[2,3] Both subunits are also expressed in other tissues.[1,4] In addition to being a major structural protein, α-crystallins have a chaperone-like activity that suppresses protein aggregation or promotes the refolding of unfolded proteins. Thus, α-crystallins play important roles in maintaining the transparency of the lens.[1,5–7]

Protein aggregation and precipitation is one of the causes of lens opacification. Studies indicate that calpain-mediated cleavage of lens crystallins plays an important role in the aggregation and precipitation of lens proteins.[8–11] This unregulated cleavage of crystallins may result in the precipitation of β-crystallins or cytoskeletal proteins[12–14] or reduction of chaperone activity of α-crystallins.[15] Calpains belong to a superfamily of structurally related, calcium-activated cysteine proteases.[16–18] Some calpains are ubiquitously expressed, whereas others are tissue specific.[17,19–21] Studies of various cataract animal models have suggested that Lp82 and calpain-2 may be the major calpains involved in murine cataractogenesis.[10,11,22–28]

Both α- and β-crystallins, but not γ-crystallins, are susceptible to calpain-mediated cleavage.[29] Whereas α-crystallins are cleaved by calpains at the C terminus,[30] β-crystallins are cleaved closer to the N terminus.[31] C-terminal cleavage of αA-crystallin by calpains can occur at several sites. The major cleaved products of αA-crystallin in the lens include αA_{1-151}, αA_{1-156}, αA_{1-163}, and αA_{1-168}.[30] αA_{1-162} can be generated by incubating αA-crystallin with m-calpain in vitro,[30] but αA_{1-162} is barely detectable in normal lenses.[30,32] However, αA_{1-162} is readily detected in diabetic cataractous lenses.[32] The accumulation of truncated αA-crystallins in cataractous lenses could result from an increase in production and/or a decrease in degradation of the truncated products by other proteases.

The ubiquitin-proteasome pathway (UPP) is one of the proteolytic systems that selectively degrade modified or damaged proteins. We have demonstrated in previous studies that lens cells (both lens epithelial cells and lens fiber cells) have a fully functional UPP[33–38] and that the UPP preferentially degrades damaged or modified proteins, including oxidized, glutathiolated and thermally denatured proteins.[37,39–42] To investigate the role of the UPP in degradation of C-terminal cleaved αA-crystallins, we compared the susceptibility of wt and the C-terminal truncated αA-crystallins to UPP-mediated degradation.

The data presented indicate that C-terminal truncation of αA-crystallin significantly altered its susceptibility to UPP-mediated degradation. Whereas the susceptibility of αA_{1-163} to UPP-mediated degradation was similar to that of wild-type (wt) αA-crystallin, αA_{1-168} was less susceptible and the αA_{1-162} more susceptible than wt αA-crystallin to UPP-mediated degradation. The rapid degradation of αA_{1-162} by the UPP may explain why αA_{1-162}-crystallin is barely detectable in the normal lenses. Because the αA_{1-162}-crystallin is less thermally stable and prone to aggregation, the timely degradation of the αA_{1-162}-crystallin may prevent its accumulation and aggregation in the lens.

MATERIALS AND METHODS

Construction of C-terminal Truncated αA-crystallins

To mimic C-terminal cleavage by calpains, recombinant wt and C-terminal truncated αA_{1-168}, αA_{1-163}, and αA_{1-162}-crystallins were expressed and purified to homogeneity. To construct these truncated αA-crystallin, human αA-crystallin cDNA in the pAED4 vector[43] was used as the template and 5′-ACTCCATGGACGTGACCATCCAG-3′ was used as the forward primer for the PCR-based cloning. The reverse primers for αA_{1-168}, αA_{1-163}, and αA_{1-162} were 5′ - ACAGGATCCTTAGGTGGGCTTC-3′, 5′-AATGGATCCTTACCGCGACACG-3′ and 5′- CATATGTTACGACACGGGGATGG-3′, respectively. PCR conditions were as follows: predenaturing at 94°C for 2 minutes, followed by 35 cycles of denaturing at 94°C for 15 seconds, annealing at 63°C for 40 seconds, and extension at 72°C for 40 seconds. PCR products were analyzed on a 1% agarose gel and purified (QIAquick Gel Extraction Kit; Qiagen, Chatsworth, CA). Purified PCR products of wt and truncated αA-crystallins were digested with *Nco*I and inserted into *Nco*I and *Bam*HI (the *Bam*HI site was blunted by a Klenow fragment of DNA polymerase I) sites of the pET15b vector, to generate pET15b-αA, pET15b-αA_{1-168}, pET15b-αA_{1-163}, and pET15b-αA_{1-162} plasmids. The sequences of these plasmids were verified by DNA sequencing.

Expression and Purification of Recombinant wt and Truncated αA-crystallins

The expression plasmids pET15b-αA, pET15b-αA_{1-168}, pET15b-αA_{1-163}, and pET15b-αA_{1-162} were transformed into competent *E. coli* BL21 (DE3) cells. Growth, induction, and purification of the recombinant proteins were performed essentially as described elsewhere.[43,44] Briefly, the expression of αA-crystallin was induced by adding 1 mM IPTG (isopropyl-β-d-thiogalactopyranoside) to the culture medium and incubating for 4 hours. The bacteria were collected by centrifugation at 3000g for 10 minutes. The bacterial pellets were lysed in the lysis buffer (50 mM Tris-HCl, 1 mM EDTA [pH 7.6]) by sonication, and the cell lysates were centrifuged at 30,000*g* for 20 minutes. Whereas >90% of wt αA and αA_{1-168} were in the supernatant, >50% of αA_{1-163} and αA_{1-162} were in the pellets. To retrieve αA_{1-163} and αA_{1-162}, we subjected the pellets to extraction with the lysis buffer containing 0.5% Tween-20 with a brief sonication. The supernatant of Tween-20 extraction was combined with the water-soluble fraction for purification of the recombinant proteins. The supernatants were first fractionated with a DE52 column. Both wt- and C-terminal truncated αA-crystallins were eluted in the fractions containing 100 to 200 mM NaCl. After concentration with a centrifugal filter device (Millipore, Bedford, MA), the αA-containing fractions were further purified with a Sephacryl S-300 size-exclusion column, with 50 mM Tris-HCl buffer containing 150 mM NaCl (pH. 7.6) as the mobile phase. The concentrations of purified proteins were determined by measuring absorption at 280 nm, with the absorbance coefficients of A 0.1% at 0.742, 0.741, 0.764, and 0.771 for wt αA, αA_{1-168}, αA_{1-163}, and αA_{1-162}, respectively, calculated based on the amount of aromatic amino acids.[45] The purity of wt and truncated proteins was analyzed on 15% SDS-polyacrylamide gels under reducing conditions and stained with Coomassie blue (Brilliant Blue R250; Sigma-Aldrich, St. Louis, MO). To determine the oligomer states of wt and truncated αA-crystallins, we analyzed the αA-crystallin solutions by size-exclusion chromatography with an inline light-scattering, absorbance, and refractive index detectors.[1] A Sepharose column (6HR 10/30; GE Healthcare, Piscataway, NJ) was connected in line with a UV detector (UV-900; GE Healthcare), a multiangle laser light-scattering detector (Dawn-EOS; Wyatt Technology Corp., Santa Barbara, CA), and a refractive index detector (Optilab-DSP; Wyatt Technology Corp.). Samples were loaded onto the column at a concentration of 1 mg/mL and eluted with 50 mM sodium phosphate buffer containing 100 mM NaCl (pH 7.0).

Study of Conformational Changes

Circular dichroism (CD) spectra were obtained with a circular dichroism spectrometer (Aviv Circular Dichroism Spectrometer model 60 DS; Aviv Associates, Lakewood, NJ). Proteins in 50 mM Tris-HCl buffer (pH 7.6; 0.1 mg/mL) were used. For each CD measurement, five scans were recorded, averaged, and followed by a polynomial fitting program. The CD was expressed in units of deg/cm²/dmol. Fluorescence was measured with a Shimadzu spectrofluorometer (model RF-5301PC; Shimadzu Instruments, Columbia, MD). Tryptophan fluorescence spectra were determined using an excitation wavelength of 295 nm. Bis-ANS fluorescence emission spectra were scanned between 460 and 560 nm with an excitation wavelength of 395 nm. Aliquots of 50 μL of Bis-ANS (5.5×10^{-5} M stock solution) were added to 1 mL of αA-crystallin solution (0.1 mg/mL in 50 mM Tris-HCl buffer; pH 7.6) until saturation was reached. The samples were then incubated for 10 minutes at room temperature before the fluorescence spectra were determined.

Thermal Stability Measurements

Thermal stability was studied by time-dependent changes in light-scattering at 400 nm using a fluorescence spectrophotometer (excitation and emission wavelengths were set at 400 nm) when wt or truncated αA-crystallins (in 50 mM sodium phosphate buffer; pH 7.6) were incubated at 65°C. The protein melting temperature (Tm) was determined with a VP-capillary differential scanning calorimeter (MicroCal, Northampton, MA). The protein concentration was 1 mg/mL in 50 mM Tris-HCl buffer (pH 7.6). The starting temperature was 10°C, and the final temperature was 110°C. The scanning rate is 100°C/hour. Commercial software (Origin; MicroCal) was used to calculate the Tm of the proteins.

Preparation of Lens Fiber Lysate and Reticulocyte Lysate

Fresh calf eyes (within 6 hours of death) were purchased from a local meat-packing company, and the outer layers of lens cortex was homogenized with 50 mM Tris-HCl buffer containing 1 mM dithiothreitol (DTT; pH 7.6). After centrifugation at 100,000g for 10 minutes, the supernatant was used as the source of the UPP for degradation assays. Rabbit reticulocytes were purchased from Green Hectares Company (Oregon, WI). After three washes with PBS, the packed recticulocytes were lysed with an equal volume of 10 mM Tris-HCl containing 1 mM DTT (pH 7.6) as previously described.[39] After centrifugation at 50,000*g* for 60 minutes, the supernatant was used as the source of the UPP for degradation assays.

Proteolytic Degradation Assay and Statistical Analysis

Both wt and C-terminal truncated αA-crystallins were labeled with ^{125}I by the chloramine T method.[39] Free ^{125}I and small peptides were removed by Sephadex G25 desalting columns, and labeled proteins were concentrated with microconcentrators (Centricon 10; Amicon, Beverly, MA). The specific activity of ^{125}I-labeled proteins ranged from 0.2 to 0.5 μCi/μg. An approximately equal amount of substrate was used for the degradation assay (~100 ng/assay). Degradation of the wt and truncated αA-crystallins was determined as described by Huang et al.,[46] but we used bovine lens fiber lysate or rabbit reticulocyte lysate as sources of ubiquitinating and proteolytic enzymes. Briefly, the proteolysis reaction mixture, in a final volume of 25 μL, contained 50 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 1 mM DTT, and 15 μL lens fiber lysate (150mg/mL protein), or reticulocyte lysate (300 mg/mL protein). For determination of adenosine triphosphate (ATP)- and Ubc4-dependent proteolysis, 2 mM ATP, 10 mM creatine phosphate, 6 μg creatine phosphokinase, and 0.4 μg recombinant Ubc4 was included in the assay. The latter was expressed and purified essentially as described by Wing and Jain.[47] Pilot experiments suggested that there is sufficient free ubiquitin in lens fiber lysate and reticulocyte lysate; therefore, no exogenous ubiquitin was added in these assays. Degradation was initiated by addition of 4 to 10×10^4 cpm of ^{125}I-labeled αA-crystallins, and

the reaction mixtures were incubated at 37°C for 2 hours. The reactions were terminated by addition of 200 μL of ice-cold 10 mg/mL bovine serum albumin, immediately followed by 50 μL of 100% TCA (yielding a final concentration of 18.2% TCA), after which the samples were left on ice for 10 minutes. The extent of degradation was determined as the amount of TCA-soluble ^{125}I-labeled fragments of αA-crystallin. The total TCA-insoluble count at time 0 was defined as 100%.

In pilot experiments, the percentage of degradation was proportional to the incubation time for the first 2 hours. The percentage of degradation during the 2-hour incubation reflected the susceptibility to degradation. Since the substrate was not saturating and the degradation was expressed as a percentage of the labeled substrate that was degraded during the 2 hours of incubation, the susceptibility to degradation is not affected by variation in the molar amount of the labeled substrates, at least under these experimental conditions.

The degradation observed with the addition of ATP and Ubc4 is referred to as total degradation, whereas the difference between total degradation and degradation without addition of ATP and Ubc4 is denoted as ATP-stimulated degradation. All experiments were performed in triplicate and repeated two to four times. For statistical analysis, data from several experiments were pooled and analyzed using Student's *t*-test.

To determine whether the degradation measured in this assay was proteasome-dependent, MG132, a proteasome inhibitor, was added to the system at a final concentration of 24 μM. To determine ubiquitin-dependent degradation, the proteolytic assay was also performed with addition of 2 μg of a dominant negative mutant ubiquitin (K6W-Ub), which specifically inhibits ubiquitin-dependent proteolysis.[48] This results in ~1:1 ratio of endogenous wt ubiquitin and added K6W-Ub.

RESULTS

Expression and Purification of wt and C-terminal Truncated αA-crystallins

The wt and truncated αA-crystallins were expressed in *E. coli* BL21 (DE3) and expression of recombinant proteins was monitored by SDS-PAGE. The wt and αA_{1-168}-crystallins were mainly found in the water-soluble fraction, whereas >50% of αA_{1-163} and αA_{1-162}-crystallins were found in the water-insoluble fraction. The wt and αA_{1-168}-crystallin were purified from the water-soluble fraction to near homogeneity (~95%). The αA_{1-163} and αA_{1-162}-crystallins were first retrieved from the water-insoluble fraction with a 50 mM Tris-HCl buffer containing 0.5% Tween-20 (pH. 7.6), and the proteins were then purified to near homogeneity (~95%) using the same procedures as used for the purification of wt protein. As shown in Figure 1, the purified proteins migrated predominantly as a single ~20-kDa band on SDS-PAGE. The differences in migration due to C-terminal truncations were clearly detectable. These differences in migration may reflect not only the changes in molecular sizes of these truncated proteins, but also the changes in charges of the truncated proteins.

Degradation of C-terminal Truncated αA-crystallins by the UPP

As shown in Figure 2A, a small percentage of wt and truncated αA-crystallins were degraded in the lens fiber lysate, and the degradations of all these αA-crystallins were totally ATP-dependent. The degradation was not detectable without ATP supplementation (data not shown). When ATP was supplied, wt αA-crystallin was degraded 0.91% ± 0.43% (Fig. 2A). Under the same conditions, the degradation of αA_{1-168}, αA_{1-163}, and αA_{1-162}-crystallins was 0.32% ± 0.68%, 1.341% ± 0.32%, and 3.74% ± 1.87%, respectively. When the proteasome was inhibited with MG132, the degradation of αA_{1-162}-crystallin was reduced to 1.43 ± 1.13 (Fig. 2A), indicating the involvement of the UPP in the degradation of this truncated protein. However,

inhibition of the proteasome had little effect on the degradation of wt αA, αA_{1-168}, and αA_{1-163} in the lens fiber lysate (Fig. 2A). These data suggest that C-terminal truncated αA_{1-162}-crystallin, but not αA_{1-168}, or αA_{1-163}, is selectively degraded by the UPP in lens fiber lysate.

As we have demonstrated, mature lens fiber cells have a functional UPP, and α-crystallins are natural substrates of the UPP.[38,46] The large quantity of endogenous α-crystallins in the lens fiber lysate could compete with ^{125}I-labeled αA-crystallins, resulting in decreased degradation of the labeled substrates. To further demonstrate the effects of C-terminal truncation of αA-crystallin on its degradation by the UPP, we determined the degradation of wt and C-terminal truncated αA-crystallins in reticulocyte lysates. Like lens fiber cells, reticulocytes are terminally differentiated and denucleated cells. As we previously demonstrated,[46] wt αA-crystallin was degraded in reticulocyte lysate in an ATP-dependent manner (Fig. 2B). Similar to that observed in lens fiber lysate, C-terminal truncated αA-crystallins had different susceptibilities to degradation in reticulocyte lysates. The percentages of degradation of wt αA, αA_{1-168}, αA_{1-163}, and αA_{1-162}-crystallins in reticulocyte lysate were 3.74 ± 0.82, 1.26 ± 0.38, 3.8 ± 0.68 and 12.1 ± 1.49, respectively (Fig. 2B). These data indicate that whereas αA_{1-163} was degraded similarly to that of wt αA-crystallin, αA_{1-168} was less susceptible, and αA_{1-162} was more susceptible than wt αA-crystallin to degradation. When proteasome inhibitor was added, 80% to 95% of degradations of wt and C-terminal truncated αA-crystallins were inhibited (Fig. 2B), suggesting the involvement of the UPP in degradation of these proteins.

The results above indicate that αA_{1-162}, but not αA_{1-168} and αA_{1-163}, is more susceptible than wt αA-crystallin to UPP-mediated degradation. To determine further the involvement of ubiquitin and ubiquitination in the selective degradation of αA_{1-162}, we assessed the effect of a dominant negative mutant ubiquitin (K6W-ubiquitin).[48] As shown in Figure 3A, addition of K6W-ubiquitin not only reduced the degradation of wt and αA_{1-162}-crystallins, but it also diminished the difference in degradation between wt and αA_{1-162}-crystallins (Fig. 3A). These data further demonstrated that αA_{1-162}-crystallin was preferentially degraded in a ubiquitin-dependent manner. To further corroborate the requirement of ubiquitination in degradation of wt and αA_{1-162}-crystallins, we determined the effects of a ubiquitin-conjugating enzyme, Ubc4, on degradation of wt and αA_{1-162}-crystallins. Previous work demonstrated that Ubc4 plays a role in degradation of abnormal proteins and that the level of Ubc4 is limiting in reticulocytes.[41] The difference in degradation between wt and αA_{1-162}-crystallins was marginal when Ubc4 was not added (Fig. 3B). Addition of Ubc4 only slightly increased the degradation of wt αA-crystallin. In contrast, addition of Ubc4 significantly increased the degradation of αA_{1-162}-crystallin (Fig. 3B). The data further confirm that the UPP plays a role in the selective degradation of C-terminal truncated αA_{1-162}-crystallin.

Effects of C-terminal Truncation on the Secondary and Tertiary Structures of αA-crystallin

Conformational changes are often recognized by the UPP as signals of degradation.[41,42] To investigate the molecular mechanisms that underlie the altered susceptibility of C-terminal truncated αA-crystallins to UPP-mediated degradation, we characterized the secondary, tertiary and/or quaternary structures of wt and C-terminal truncated αA-crystallins by comparing their CD spectra, tryptophan fluorescence, and surface hydrophobicity.

The far UV CD spectrum reflects the secondary structure of a protein. Figure 4A shows that C-terminal truncations of αA-crystallin slightly altered the CD spectra and that different truncations had different effects on the CD spectrum. The effects of C-terminal truncation on contents of α-helix, β-sheet, β-turn, and random coil are summarized in Table 1. In general, C-terminal truncation did not significantly alter the secondary structures. C-terminal truncated αA-crystallins had a moderate increase in contents of β-sheet and a decrease in contents of β-turn. The C-terminal truncations had no significant effect on the contents of α-helix and random

coil (Table 1). For accurate determination of the secondary structure of a protein, the CD spectrum should be scanned from 260 to 180 nm.[50] For comparative purposes, the far UV CD spectrum in this study was scanned from 260 to 200 nm. This may affect the accuracy of the estimated contents of α-helix, β-sheet, β-turn, and random coil of these proteins, but it is sufficient for evaluating the conformational changes of the truncated proteins.

Tryptophan fluorescence provides information on the gross positioning of tryptophan residues, which usually reflects the tertiary or quaternary structures of a protein. Protein conformational changes are often accompanied by altered intensity and/or a shift of the wavelength of fluorescence emission maxima.[51] We found that C-terminal truncations altered the intensity of tryptophan fluorescence and the wavelength of fluorescence emission maxima (Fig. 4B). Whereas αA_{1-162} and αA_{1-163} showed a 20% to 40% increase, αA_{1-168} showed a decrease in tryptophan fluorescence intensity.

Conformational changes are often associated with changes in the surface hydrophobicity. Bis-ANS (a hydrophobic probe) is nonfluorescent in aqueous solution and becomes fluorescent when it binds to the hydrophobic residues on the surface of a molecule. As shown in Figure 4C, αA_{1-162}-crystallin exhibited an increase in Bis-ANS fluorescence intensity, indicating an increase in hydrophobicity. In contrast, αA_{1-163}- and αA_{1-168}-crystallins showed a decrease in Bis-ANS fluorescence, indicating a decrease in hydrophobicity.

Effect of C-terminal Truncation on the Thermal Stability of αA-crystallin

As a member of the small heat shock protein family, αA-crystallin is thermally stable and is capable of preventing heat-induced aggregation and precipitation of other proteins.[5,6] To characterize further the structural changes associated with C-terminal truncated αA-crystallins, we compared the thermal stability of wt and truncated αA-crystallins. As shown in Figure 5, αA_{1-168}-crystallin was more thermally stable than was wt αA-crystallin. Incubation of wt αA-crystallin at 65°C resulted in a slight increase in light-scattering, but incubating αA_{1-168}-crystallin under the same condition did not cause detectable changes in light-scattering. αA_{1-163}-crystallin was also more stable than wt αA-crystallin, but not as stable as αA_{1-168}-crystallin. However, αA_{1-162}-crystallin was most susceptible to heat-induced aggregation. When incubated at 65°C, αA_{1-162}-crystallin began to aggregate as early as 5 minutes. The altered thermal stabilities of the C-terminal truncated αA-crystallins were confirmed by differential scanning calorimetry analysis. The Tm of wt αA-crystallin was 59.65 ± 0.14°C. The Tm of αA_{1-168}-, αA_{1-163}-, and αA_{1-162}-crystallins were 64.06 ± 0.09°C, 60.44 ± 0.06°C, and 57.65 ± 0.16°C, respectively. These data indicate that the C-terminus of αA-crystallin plays a role in determining thermal stability of αA-crystallin.

Effect of C-terminal Truncation on the Oligomerization of αA-crystallin

As other small heat shock proteins, αA-crystallin normally forms oligomers of 700 to 800 kDa in its native state. Other studies have shown that signals for ubiquitin-dependent degradation could be masked by the formation of a native oligomer.[52,53] To investigate whether altered susceptibility of the C-terminal truncated αA-crystallins to UPP-mediated degradation is related to changes in their oligomerization, we determined the oligomeric states of the wt and C-terminal truncated αA-crystallins by multiangle light-scattering analysis. Indeed, C-terminal truncation significantly altered the oligomeric states of αA-crystallin. The sizes of wt αA-crystallin ranged from 300 to 1000 kDa and size at the peak was 700 kDa (Fig. 6A). The sizes of αA_{1-168}-crystallin ranged from 300 to 900 kDa, and the size at the peak was 580 kDa (Fig. 6A). In addition, a small fraction (<10%) of αA_{1-168}-crystallin had a size of 90 kDa (data not shown). The sizes of αA_{1-162}-crystallin were more heterogeneous, ranged from 400 to 1300 kDa, and the size at the peak was 500 kDa (Fig. 6A). Surprisingly, the sizes of αA_{1-163}-crystallin were more heterogeneous and much larger than that of wt αA-crystallin. The majority

of αA_{1-163}-crystallin was eluted in the void volume of the Sepharose 6HR 10/30 column. The size of the αA_{1-163}-crystallin eluted in the void volume ranged from 8,000 to 70,000 kDa and the peak size was ~21,400 kDa, as detected by multiangle light-scattering (Fig. 6B). Even in the fraction that was resolved by the column, the sizes αA_{1-163}-crystallin were much bigger than that of wt αA-crystallin, ranged from 800 to 3000 kDa. These data suggest that C-terminal truncation alters the oligomerization of αA-crystallin and that αA_{1-163}-crystallin tends to form large and heterogeneous aggregates.

DISCUSSION

Accumulation and precipitation of damaged proteins in the lens is associated with cataract formation. In addition, activation of calpains by elevated calcium concentrations in the lens is known to contribute to protein precipitation and cataractogenesis in several model systems.[10,11,22,25,28,54,55] Calpains do not degrade proteins to amino acids. Instead, they cleave proteins into large fragments.[17] Calpain-cleaved proteins either lose solubility[14,27,31] or lose function.[15,54,56] Therefore, accumulation of calpain-cleaved proteins in the lens may be cytotoxic and cataractogenic.

Levels of calpain-cleaved proteins are determined by the rates of production and the rates of clearance by other proteases. In the lens, various proteases, such as amino-peptidases,[57–59] trypsin-like proteases,[60,61] and the UPP[34,35,37,38,62–66] may play a role in the clearance of calpain-cleaved proteins. Among these proteases, the UPP has been demonstrated to be involved in degradation of modified or damaged proteins, including oxidized, denatured, glutathiolated, and mutant proteins.[35,37,41,42,46,62,67,68] Therefore, we hypothesized that the UPP also plays a role in degradation of calpain-cleaved proteins. To test this hypothesis, recombinant C-terminal truncated αA-crystallins were used as model substrates for the UPP in lens fiber and reticulocyte lysates. We found that αA_{1-162} was more susceptible than wt αA-crystallin to degradation by the UPP in both lens fiber and reticulocyte lysates. Whereas αA_{1-163} was degraded similar to wt αA, αA_{1-168} was less susceptible to UPP-mediated degradation.

Consistent with the long lives of lens proteins, only a small fraction of the labeled substrates were degraded during the 2 hours of incubation with lens fiber lysate. Whereas 0.91% ± 0.43% of wt αA-crystallin was degraded, 3.74% ± 1.87% of αA_{1-162}-crystallin was degraded during this period. Although the difference in susceptibility of wt and C-terminal truncated αA-crystallins to degradation is not dramatic in a biochemical sense, this difference could have physiological significance. For example, 1% degradation of the substrate during the 2 hours of incubation means the protein has a half-life of 100 hours. If the degradation increases to 4%, it indicates that the half-life of the protein decreases to 25 hours. Because the chronic accumulation and precipitation of damaged proteins is associated with cataract, a three- to four-fold change in the half-live of a protein could make a significant difference in composition, structure, and optical functions of the lens over a lifetime.

αA_{1-162} is the major product of m-calpain-cleaved αA-crystallin, and it has been detected in diabetic cataractous lenses, but not in normal lens.[32] The rapid degradation of αA_{1-162} by the UPP may be a reason for the absence of αA_{1-162} in normal lenses. The accumulation of αA_{1-162} in diabetic cataract lenses could be caused by enhanced m-calpain activity and/or by reduced UPP activity, or both. Since αA_{1-162} is less thermally stable, the rapid degradation by the UPP prevents it from accumulation and aggregation under stress conditions.

The data also indicate that not all C-terminal truncated αA-crystallins are selectively degraded by the UPP. For example, αA_{1-168} is less susceptible than wt αA-crystallin to UPP-mediated degradation, and αA_{1-163} is degraded similarly to wt αA-crystallin. The relative resistance to

UPP-mediated degradation of these two C-terminal truncated αA-crystallins is consistent with their presence in normal lenses.[30,32] It appears that some of the calpain-cleaved αA-crystallins, such as αA_{1-168}, have to be further cleaved by another protease or peptidases, to be recognized and degraded by the UPP. Consistent with this hypothesis, many of the cleaved fragments of lens proteins, particularly β-crystallins, do not match the calpain-cleavage sites,[69] which indicates that calpain-cleaved products are readily trimmed by other proteases or peptidases.

The different percentages of substrates degraded under these conditions may reflect the different proportion of degradation-prone conformers among the comparison groups. As indicated by the multiangle light-scattering analysis (Fig. 6), the molecular masses of the oligomers of these αA-crystallins are not uniform, indicating heterogeneity of conformation in each of these αA-crystallins. These data indicate that, whereas αA_{1-162} tends to adapt a degradation-prone conformer, αA_{1-168} tends to adapt a degradation-resistant one.

In an attempt to identify the signals for UPP-mediated degradation, we compared the secondary and tertiary structures of wt and different C-terminal truncated αA-crystallins. C-terminal truncations of αA-crystallin resulted in only subtle changes in the CD spectrum, indicating that the C-terminal truncation did not significantly alter the secondary structures. C-terminal truncation also altered tryptophan fluorescence and surface hydrophobicity. Consistent with conformational changes, C-terminal truncation changed oligomeric states and thermal stabilities of αA-crystallin. Whereas αA_{1-168} and αA_{1-162} formed slightly smaller sizes of oligomers, αA_{1-163} tended to form large aggregates. Among these biochemical-biophysical changes, surface hydrophobicity and thermal stabilities of these truncated αA-crystallins correlated with the susceptibilities to UPP-mediated degradation, which indicates that the thermal stability and surface hydrophobicity of a protein, but not the changes in CD spectrum, tryptophan fluorescence or oligomeric states, can predict the susceptibility to UPP-mediated degradation. However, we cannot rule out the possibility that the subtle changes in secondary, tertiary and/or quaternary structures may contribute to the changes in surface hydrophobicity and thermal stabilities of the truncated crystallins.

The correlation between thermal stability and susceptibility to UPP-mediated degradation indicate that the residues that are recognized by the UPP also play a role in maintaining the thermal stability of a protein. The positive correlation between thermal vulnerability and susceptibility to degradation may be a mechanism for the selective degradation of proteins that are in unstable conformations. This mechanism may play an essential role in preventing the accumulation and aggregation of thermal labile proteins. Hence, this protein quality-control mechanism may be essential for maintaining the transparency of the lens.

The susceptibilities of the wt and C-terminal truncated αA-crystallins were determined in this study by using purified proteins. In the lens, C-terminal truncated αA-crystallins may form complexes with wt αA-crystallin, αB-crystallin, or other small heat shock proteins. It remains to be determined whether the C-terminal truncated αA-crystallins in complexes with wt α-crystallins are degraded in the same manner as in the isolated form. We will determine the effect of wt α-crystallins on degradation of C-terminal truncated αA-crystallins in future studies.

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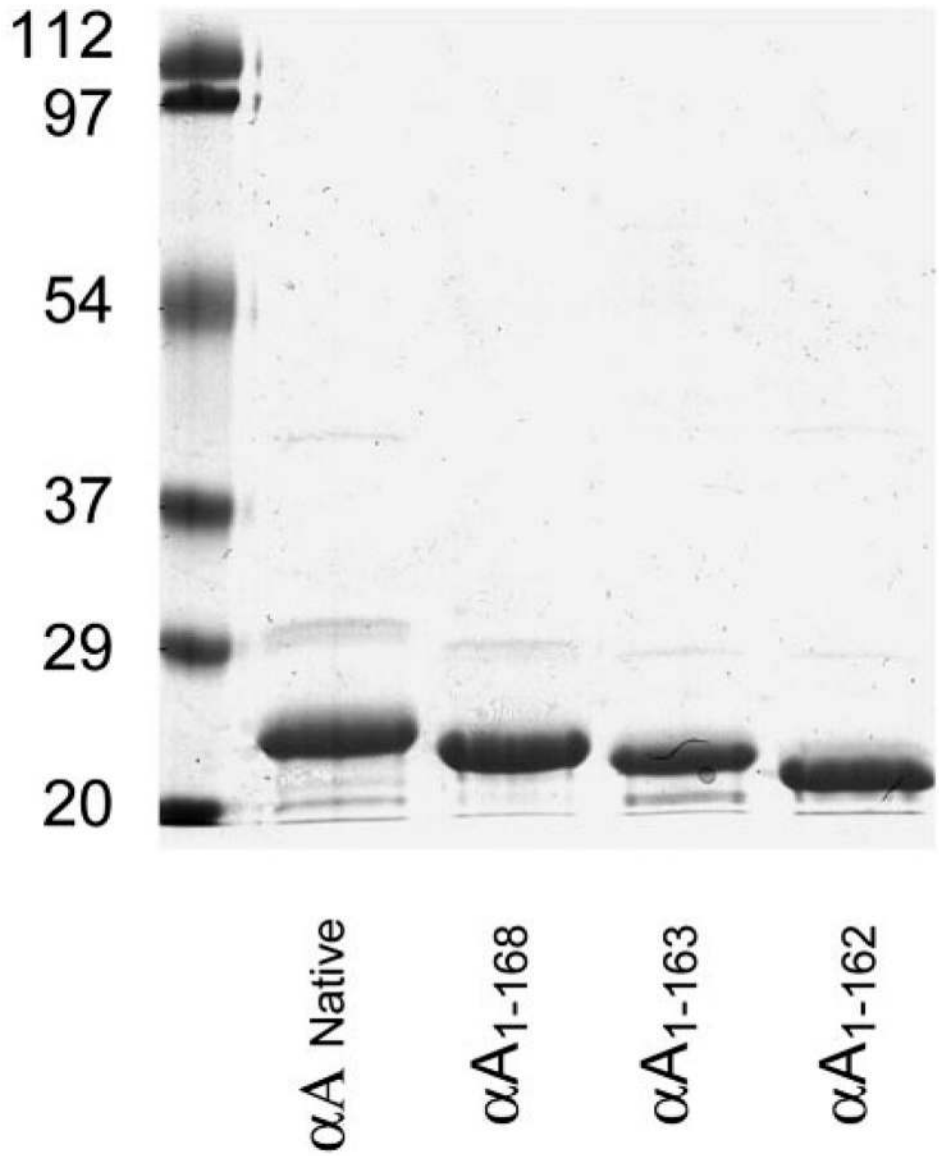

FIGURE 1.

SDS-PAGE of wt and three truncated forms of αA-crystallin. *Lane 1*: low-molecular-weight protein standards, the molecular weights were labeled as kDa; *lane 2*: purified wt αA-crystallin; *lane 3*: purified truncated αA_{1-168}-crystallin; *lane 4*: purified truncated αA_{1-163}-crystallin; *lane 5*: purified truncated αA_{1-162}-crystallin. Twenty micrograms of each purified protein was loaded and the gel was stained with Coomassie blue R-250.

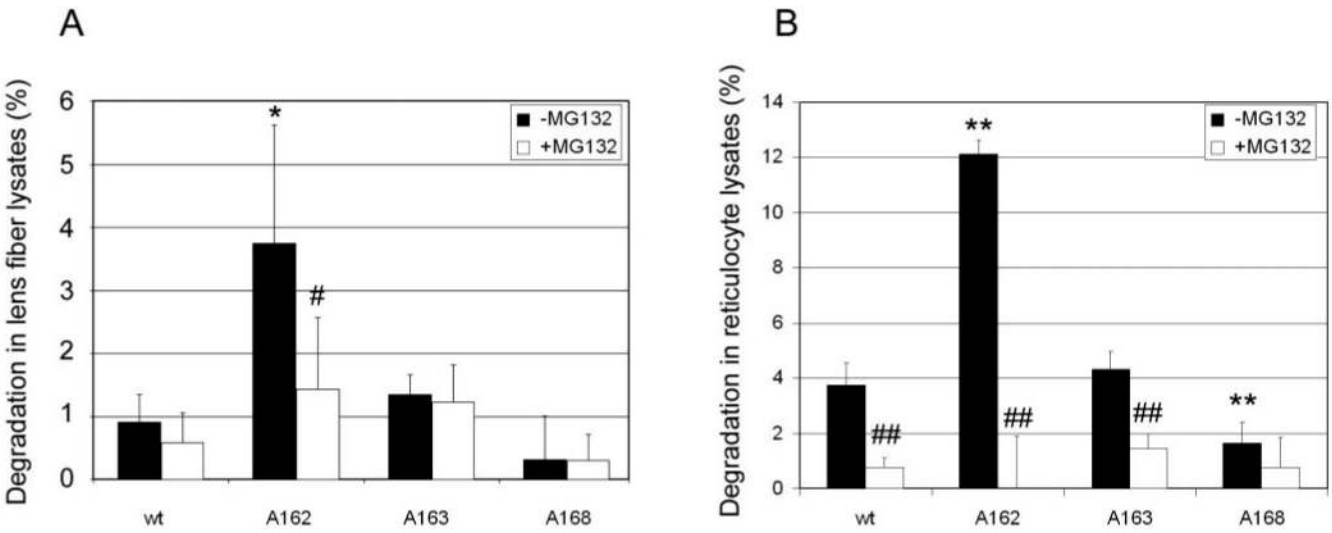

FIGURE 2.

Degradation of wt and C-terminal truncated αA-crystallins. The wt and C-terminal truncated αA-crystallins were labeled with ^{125}I and the ATP-dependent degradation assay was performed using lens fiber cell lysate (**A**) or rabbit reticulocyte lysate (**B**) as the source of UPP components, with or without addition of the proteasome inhibitor MG132. *$P < 0.05$, **$P < 0.001$, compared with the degradation of wt αA-crystallin. #$P < 0.05$, ##$P < 0.001$, in the absence of MG132.

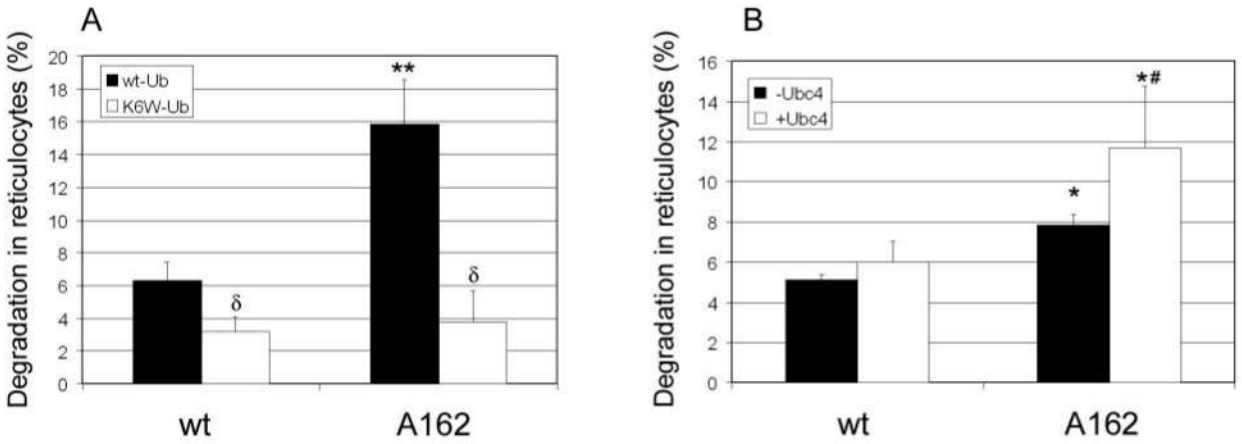

FIGURE 3.

Ubiquitin and Ubc4 are essential for degradation of C-terminal truncated αA_{1-162}-crystallin. (**A**) The wt ubiquitin or dominant-negative mutant K6W-ubiquitin (320 ng/μL; final concentration) was added to the degradation system. (**B**) The degradation was performed in the presence or absence of 20 ng/μL recombinant Ubc4. **P* < 0.05, ***P* < 0.001 compared with the degradation of wt αA-crystallin; δ indicates *P* < 0.01 when compared with the degradation with supplementation of wt ubiquitin; #*P* < 0.05 when compared with the degradation in the absence of Ubc4.

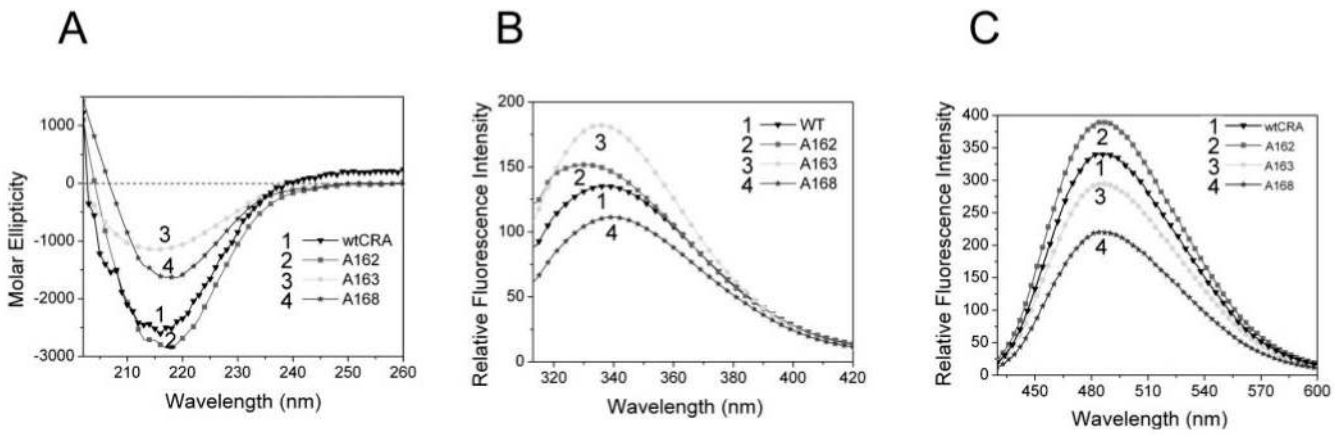

FIGURE 4.

C-terminal truncation induced conformational changes of αA-crystallin. (**A**) Far UV CD spectra. Protein concentration was 0.1 mg/mL in 50 mM Tris-HCl buffer (pH 7.6), and cellpath length was 1 mm. The spectra were an average of five scans smoothed by a polynomial-fitting program. (**B**) Tryptophan fluorescence. Protein concentration was 0.1 mg/mL in 50 mM Tris-HCl buffer (pH7.6). Excitation wavelength was 295 nm. (**C**) Surface hydrophobicity. Bis-ANS was added to protein solutions (0.1 mg/mL in 50 mM Tris-HCl buffer; pH 7.6) to a final concentration of 15 μM. Fluorescence was measured at room temperature, with an excitation wavelength of 395 nm.

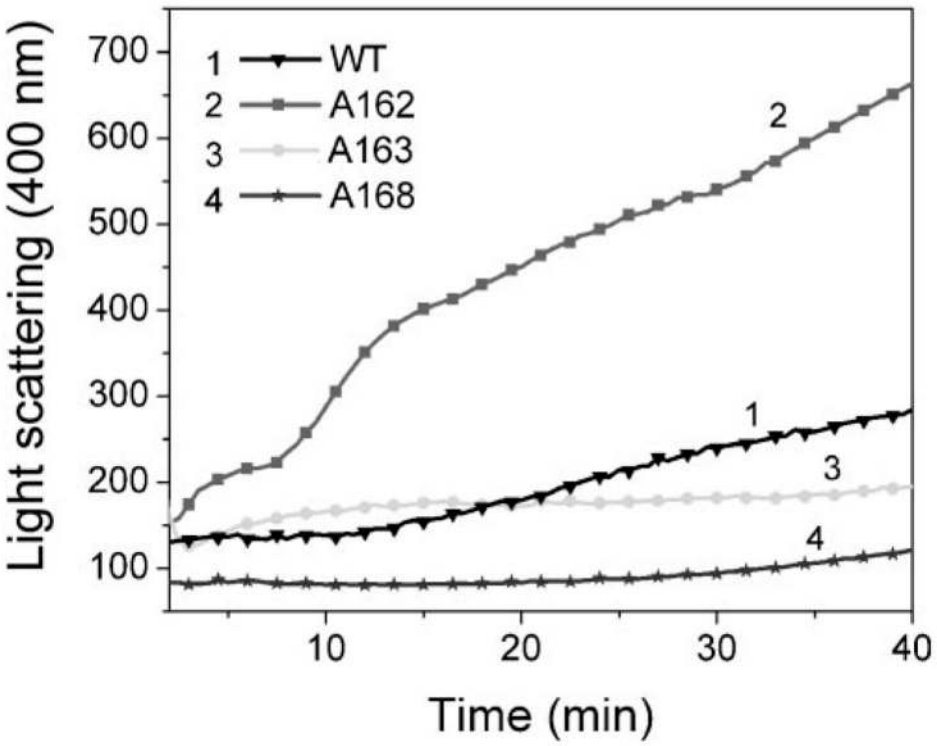

FIGURE 5.
C-terminal truncation alters thermal stability of αA-crystallin. The wt and truncated αA-crystallins in 50 mM sodium phosphate buffer (0.1 mg/mL) were incubated at 65°C and heat-induced light-scattering was detected with a spectrofluorometer. Both the emission and the excitation wavelengths were set at 400 nm.

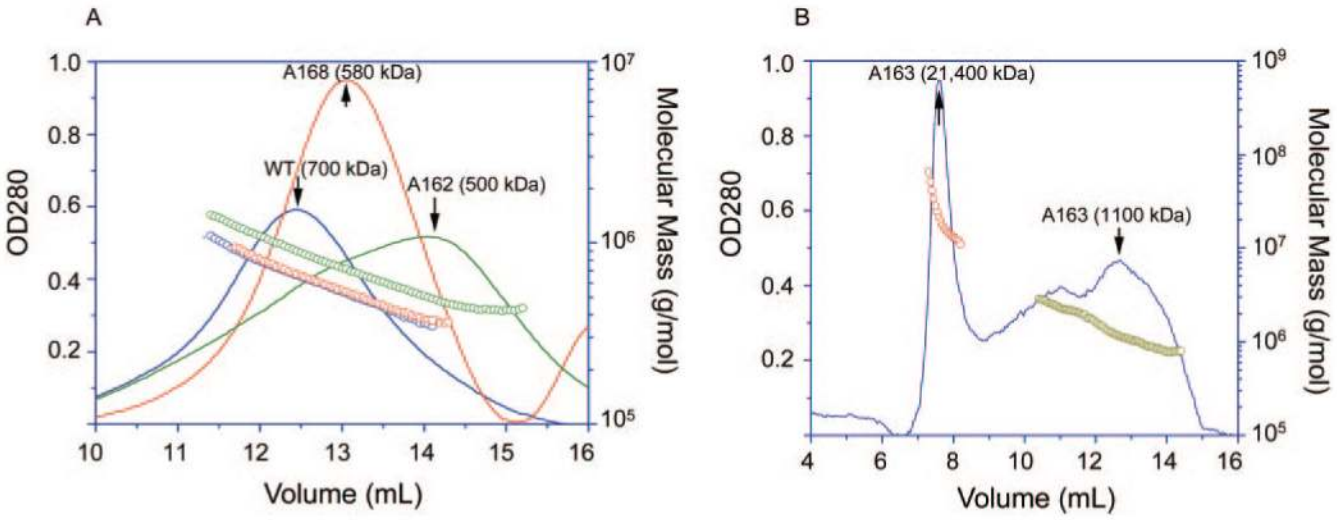

FIGURE 6.

C-terminal truncation alters oligomerization of αA-crystallin. The oligomer sizes of wt and C-terminal truncated αA-crystallins in sodium phosphate buffer containing 100 mM NaCl were determined by size exclusion chromatography coupled with a multiangle light-scattering detector. The *lines* formed by *circles* represented the molecular weight obtained as a function of the elution volumes (left ordinate).

TABLE 1

Effect of C-terminal Truncation on the Secondary Structures of αA-crystallin

	α-Helix	β-Sheet	β-Turn	Random Coil
wt αA	6 ± 0.3	27 ± 0.3	38 ± 0.3	30 ± 0.2
αA_{1-162}	8 ± 0.2	36 ± 1.9	25 ± 1.8	29 ± 1.0
αA_{1-163}	7 ± 0.4	29 ± 0.4	32 ± 0.8	32 ± 0.2
αA_{1-168}	6 ± 1.1	30 ± 2.5	32 ± 2.8	32 ± 1.1

Five far UV CD spectra scans were recorded and averaged and molar ellipticity, with unit defined as deg · cm² · $dmol^{-1}$, was calculated by using a polynomial-fitting program. The contents of α-helix, β-sheet, β-turn and random coil were calculated using PROSEC software.[49] The data are mean ± SD of four individual measurements using two batches of protein preparations.