Racemization of Two Proteins over Our Lifespan: Deamidation of Asparagine 76 in γS Crystallin Is Greater in Cataract than in Normal Lenses across the Age Range

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Purpose. Long-lived proteins are widespread in man, yet little is known about the processes that affect their function over time, or their role in age-related diseases.

Methods. Racemization of two proteins from normal and cataract human lenses were compared with age using tryptic digestion and LC/mass spectrometry. Asp 151 in αA crystallin and Asn 76 in γS crystallin were studied.

Results. Age-dependent profiles for the two proteins from normal lenses were different. In neither protein did the modifications increase linearly with age. For αA crystallin, racemization occurred most rapidly during the first 15 years of life, with approximately half of L-Asp 151 converted to D-isoAsp, L-isoAsp, and D-Asp in a ratio of 3:1:0.5. Values then changed little. By contrast, racemization of Asn 76 in γS crystallin was slow until age 15, with isoAsp accounting for only 5%. Values remained relatively constant until age 40 when a linear increase (1%/year) took place. When cataract lenses were compared with age-matched normal lenses, there were marked differences in the time courses of the two crystallins. For αA crystallin, there was no significant difference in Asp 151 racemization between cataract and normal lenses. By contrast, in γS crystallin the degree of conversion of Asn 76 to isoAsp in cataract lenses was approximately double that of normals at every age.

Conclusions. Modification of Asn and Asp over time may contribute to denaturation of proteins in the human lens. An accelerated rate of deamidation/racemization at selected sites in proteins, such as γS crystallin, may contribute to cataract formation. (*Invest Ophthalmol Vis Sci.* 2012;53:3554–3561) DOI:10.1167/iovs.11-9085

Small changes to proteins can have dramatic consequences for tissues, and can be sufficient to impair their function.[1,2] This is clearly illustrated by the human lens where a number of mutants have been described that give rise to cataract. Most are point mutations in crystallins.[3–8] While these genetic defects are responsible for only a tiny fraction of human cataract, they highlight the fact that a single amino acid substitution in just one crystallin can be sufficient to lead to lens opacification.

An important question is whether similar changes to crystallins over time could result in age-related cataract, which is responsible for the vast majority of human cataract. If so, what are these changes? Deamidation is one such modification, and it has been studied by several groups.[9–12] A survey of deamidation of every crystallin in aged human lenses was published recently.[13] It revealed large differences in the extent of deamidation between individual crystallins, as well as demonstrating that some Asn and Gln sites in a particular crystallin were subject to extensive deamidation, whereas other Asn and Gln residues in the same protein were unchanged.

Both Asp and Asn residues can undergo racemization via an intramolecular condensation reaction that leads to succinimide formation.[14] Four possible products are formed by ring opening of the succinimide: D-Asp, D-isoAsp, L-isoAsp, and L-Asp. In many tissues an enzyme, protein-L-isoaspartyl methyltransferase (PIMT),[15] is present that can partially ameliorate the damaging effect of these modifications by methylating some of the products and directing the equilibrium reaction back to the succimide.[16] PIMT appears however to be only of limited effectiveness in mitigating such deleterious protein modifications since it is inactive on a major product, D-isoAsp.[15,17,18] In addition, there is no known way of reforming the amide side chain of Asn, so that succinimde formation from Asn inevitably leads to the introduction of a negative charge at that site. For Asn, a variation of the intramolecular condensation reaction can lead to cleavage of the peptide bond.[14,19]

Although long-lived proteins are present at many sites in the human body,[20–27] little is known about the major posttranslational modifications that affect their structure and properties over time. Such processes, if significant, may contribute to a loss of organ/tissue function and ultimately disease. Racemization is a quantitatively significant modification of crystallins[28,29] that increases with age, such that in 60- to 70-year-old human lenses, each polypeptide contains, on average, between two and three D-amino acids.[30] Of particular significance, cataract lenses contained higher levels of D-Asp and D-Ser than age-matched normal lenses.[30]

In the current investigation, the authors studied age-dependent changes in one crystallin representative from each of the two major lens protein groups, that is, the α and β/γ crystallin families. One, an Asp residue in αA crystallin (Asp 151), has been shown to be significantly racemised in old human lenses.[31] The other is an Asn residue in γS crystallin (Asn 76) and was chosen because it was found to be almost twice as deamidated in 60- to 70-year-old cataract lenses as in normal controls.[13] Particular attention was directed at deter-

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mining the time course of deamidation and of discovering the major products formed at each site.

Materials and Methods

Extraction and Tryptic Digestion of Lens Proteins

Normal human lenses were obtained from the Sydney Eye Bank, with ethical approval from the University of Sydney and fetal lenses from the Endocrinology Department, Prince of Wales Hospital, Randwick, NSW, Australia. Cataractous lenses were obtained from the K.T. Seth Eye Hospital, Rajkot, Gujarat, India. Proteins were extracted using a previous method.[30] Two groups of normal lenses were examined: one in which total protein from the lens nucleus was digested and another in which insoluble proteins were studied. Insoluble protein was obtained following extraction of lenses in 7M guanidine HCl, 100 mM Tris, pH 8, then overnight dialysis against four changes of MilliQ water (Millipore, Bedford, MA) at 4°C. The samples were centrifuged for 15 minutes at 16,000*g*. The pellets were lyophilized and denoted as insoluble protein. Another set of normal lenses underwent the same procedure described above; however, the content of the dialysis bags was freeze-dried without centrifugation. This was termed total protein.

Proteins (2 mg/mL) from individual lens nuclei were homogenized in 7 M guanidine hydrochloride (Gdn-HCl), 100 mM Tris, pH 7.0, and centrifuged (16,000*g*, 10 minutes). A 20-fold molar excess of dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, MO) was added and incubated for an hour at 37°C. The solution was adjusted to pH 8.0 with NaOH and a 40-fold molar excess, relative to Cys, of iodoacetamide (IAA) (Sigma-Aldrich) was added and the solution was incubated for 30 minutes at 37°C in the dark. 200 μg of alkylated protein was removed and diluted into 100 mM Tris-buffer, pH 8.0 (1:20). The reduced and alkylated proteins were digested with 1:100 (enzyme:substrate) of trypsin (Promega, Madison, WI) and incubated at 37°C for 16 hours. Following digestion, approximately 40 μg of peptides from each sample were desalted and concentrated using a Zip tip (0.6 μL C18 resin) (Millipore). Peptides were eluted using acetonitrile (ACN):formic acid (FA):H_2O (70:0.5:29.5, vol/vol) and freeze dried. The lyophilized peptides were resuspended in FA:heptafluorobutyric acid (HFBA):H_2O (1:0.05:98.85, vol/vol).

Peptide Standards

IQTGLDATHAER, which corresponds to tryptic peptide 146–157 of human αA crystallin, was synthesized by Peptide 2.0 (Chantilly, VA) with L-aspartic acid, L-isoaspartic acid, D-aspartic acid, or D-isoaspartic acid at position 151. WMGLNDR, which corresponds to tryptic peptide 72–78 of γS crystallin, was purchased from Peptide 2.0. The L-aspartic acid, L-isoaspartic acid, D-aspartic acid, and D-isoaspartic forms of WMGLDDRLSSCR were from Peptide 2.0 and the corresponding forms of WMGLDDR obtained by tryptic digestion. Each peptide was dissolved with FA:HFBA:H_2O (1:0.05:98.85, vol/vol) and analyzed by LC–MS/MS. Optimized LC conditions were then used to run human lens digests. Further confirmation of the elution positions for γS crystallin peptides was carried out by spiking in 100 fmol of the standard peptides into the digests.

Liquid Chromatography–Mass Spectrometry (LC–MS)

Tandem mass spectra were acquired using a quadrupole time-of-flight (QTOF) Ultima mass spectrometer with a nanospray source (Waters/Micromass, Manchester, UK). Digested peptides were separated by 1D LC using a NanoCapLC autosampler system (Waters, Milford, MA) and the system was comprised of three pumps (pump A, B, and C), an autosampler, a sample cooler, and a switch valve. A partial loop injection method was used; 2 μL of the sample was picked up with a flush volume of 4 μL. After 20 minutes of sample loading onto the analytical column, with a flow rate of ~350 nL/min (mobile phase A from pump A (H_2O:FA (99.9:0.1, vol/vol), the 10-port switch valve (Valco, Houston, TX) was automatically switched into line with buffers to bypass the injection valve and loop. Peptides were eluted with acetonitrile in mobile phase B (from pump B, H_2O:ACN:FA (19.9:80:0.1, vol/vol). The separation of the peptides was performed on a fritless analytical Magic C18 reversed phase column with 3 μm packing material (Michrom Bioresources, Auburn, CA) particle size 200 Å; 75 μm inner diameter; 360 μm outer diameter and ~20 cm long, manufactured according to Gatlin.[32] Two different gradients were optimized for human αA and γS crystallin, and peptides were eluted with a flow rate of ~200 nL/min over 80 minutes. For αA crystallin a linear gradient of 10% to 17% mobile phase B (vol/vol) was used, while that for γS crystallin was a linear gradient of 19% (vol/vol) to 28% (vol/vol) mobile phase B. The analytical column was connected via a fused silica capillary (~20 cm; inner diameter = 25 μm, outer diameter = 360 μm) to a low volume tee (Upchurch Scientific, Silsden, UK) where a high voltage (2400 V) was applied to the capillary and the column tip positioned ~0.5 cm from the Z-spray inlet of a QTOF tandem mass spectrometer. The mass spectrometer was operated in positive ion mode, with a source temperature of 80° C and a cone voltage of 50 V. The QTOF was operated in data-dependent acquisition mode (DDA), with an acquisition of TOF MS survey scan (m/z 350–1700, 1 second) and the two largest multiply charged ions (counts > 30) were sequentially selected by Q1 for MS/MS analysis. Argon was used as collision gas, and optimum collision energy would be chosen based on its charge state and mass. Tandem mass spectra were accumulated for up to 2 seconds (m/z 50–2000). Individual fragment spectra obtained for each of the precursors were processed using MassLynx software version 4.0 SP4 (Waters/Micromass) to obtain centroid MS/MS data and the corresponding peak lists in the format of pkl files.

Data Analysis

The triply charged ions for each version of the γS and αA crystallin-derived tryptic peptides listed above were extracted, and the intensities from the extracted ion chromatogram (XIC) determined using MassLynx software. Peak areas of specific peptides were calculated using a mean smoothing method (Number of Smooths: 2, window size (scans): ± 3). The MS/MS spectrum of each peptide was matched to the XIC, ensuring the peak area used corresponded to that of the matched peptide and not an isobaric peptide. For αA crystallin, all forms of the peptide (L-Asp, L-isoAsp, D- isoAsp, and D-Asp) were summed and modification for each one was expressed as a percentage of the total peak area. For γS crystallin, deamidation was calculated by combining the areas of the two DD forms, that is, WMGLDDR, and expressing it at a percentage of the total WMGLDDR + WMGLNDR peak area (see Supplementary Fig. 1, http://www.iovs.org/lookup/

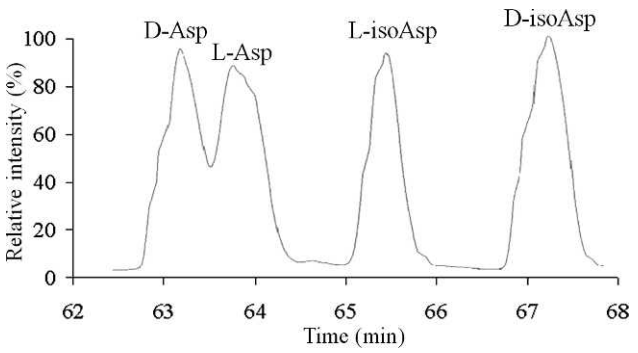

Figure 1. Representative HPLC trace showing the separation of the four forms of the αA crystallin tryptic peptide (146-157) IQTGLDATHAER. Peptides were synthesized containing D-Asp, D-isoAsp, L-Asp, or L-isoAsp at position 151.

suppl/doi:10.1167/iovs.11-9085/-/DCSupplemental). The amount of isoAsp is expressed as the percentage of two DD peak areas of this tryptic peptide. Simple linear regression analysis and Mann–Whitney *U* tests were performed using a previous method.[30]

Results

One objective of the current study was to chart the time course of racemization at two sites in human proteins as a function of age. An Asp residue in αA crystallin (Asp 151) was selected, as it was known from previous work to be subject to modification.[31,33] The rate of change in this Asp was compared with that of an Asn residue in γS crystallin (Asn 76), since this site was found to undergo significant deamidation with age, and importantly, to be more highly modified in cataract than in age-matched normal lenses.[13] Lenses across the age range were examined and the extent of modification at both sites was compared.

αA Crystallin—Normal Lenses

The four forms of the αA-crystallin tryptic peptide (146–157) IQTGLDATHAER, containing D-Asp, D-isoAsp, L-isoAsp, or L-Asp at position 151, were synthesized and a solvent gradient was developed that resolved them by HPLC (Fig. 1). This permitted relative quantitation of the four forms in lens digests as a function of age. The appearance of the three modified forms of Asp as a function of age in total protein from the lens nucleus is shown in Figure 2. The shapes of the curves for the two D-forms were almost identical (Figs. 2a, 2b). There was an increase in the proportion of both D-forms until the mid-teens, after which there was no appreciable increase. D-isoAsp was the most abundant, representing 40% of the Asp by age 20, whereas D-Asp accounted for approximately 12%.

By contrast, L-isoAsp (Fig. 2c) increased more slowly, reaching a similar level to that of D-Asp (~12%) by age 50. When the amounts of D-Asp, D-isoAsp, and L-isoAsp were combined, they accounted for two-thirds of the total Asp 151 by age 50.

γS Crystallin—Normal Lenses

The four forms of the tryptic peptide, WMGLDDR, containing D-Asp, D-isoAsp, L-isoAsp, or L-Asp at position 76 were synthesized. These correspond to the four products expected from succinimide-mediated deamidation of γS crystallin 72–78 (WMGLNDR). The ND form (i.e., the peptide prior to deamidation) had a different retention time from the DD versions (Supplementary Fig. 1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-9085/-/DCSupplemental). In this case, the four DD forms could not be resolved by HPLC. The two isoAsp forms co-eluted, as did D-Asp and L-Asp versions. The dependence of deamidation on age was calculated by summing the areas of the two DD forms of the peptide and expressing this as a percentage of the DD+ND forms (Fig. 3a). Deamidation was observed to increase up to the mid-teens, then stabilize before increasing again after age 40.

It was also of interest to determine the identities of the Asp forms produced by deamidation. This is illustrated in Figure 3b, which shows the time course of D-isoAsp + L-isoAsp formation. In this graph, isoAsp is expressed as a percentage of the total DD forms. By age 15, only ~5% of Asn 76 could be accounted for by the formation of isoAsp, and this percentage changed little until age 40. After that time, the rate of formation of the isoAsp forms increased significantly, such that by the ninth decade D-isoAsp and L-isoAsp comprised almost half of the total. Such a pattern is consistent with Asp being formed initially and then being converted to isoAsp over time.

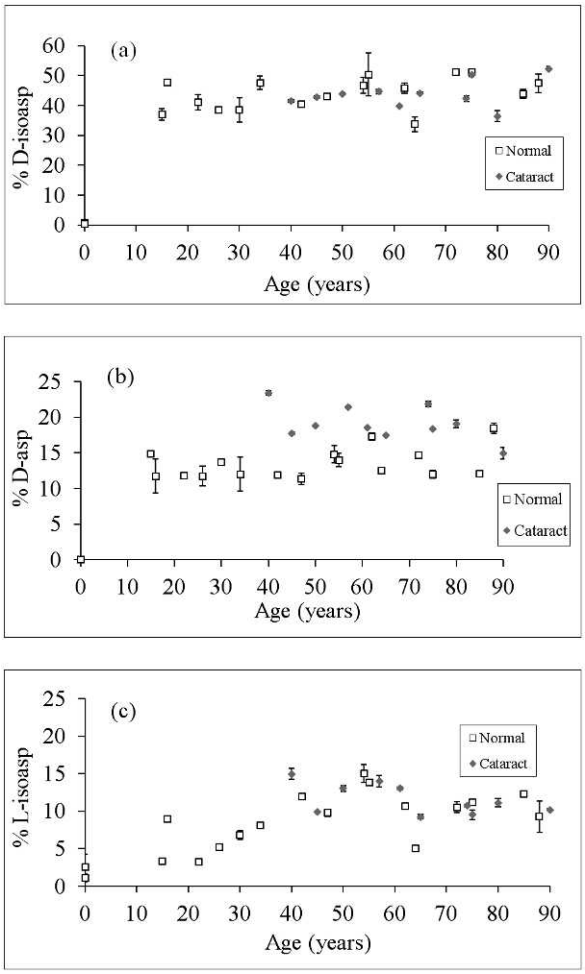

Figure 2. Racemization of Asp151 in αA crystallin (total protein). (Symbols: □ Normal; ◆ Cataract) (**a**) The percentage of D-isoaspartic acid as a function of age, in normal and cataract lens proteins. (Normal: $y = 0.0994x + 38.9$, $R^2 = 0.0813$, two-sided $P = 0.425$; Cataract: $y = 0.102x + 37.4$, $R^2 = 0.125$, two-sided $P = 0.317$). Mann–Whitney *U* test, two-sided $P = 0.363$. (**b**) The percentage of D-aspartic acid as a function of age, in normal and cataract lens proteins. (Normal: $y = 0.0592x + 10.1$, $R^2 = 0.142$, two-sided $P = 0.283$; Cataract: $y = -0.0767x + 24.04$, $R^2 = 0.252$, two-sided $P = 0.139$). Mann–Whitney *U* test, two-sided $P = 0.0002$. (**c**) The percentage of L-isoaspartic acid as a function of age, in normal and cataract lens proteins. (Normal: $y = -0.0375x + 13.3$, $R^2 = 0.0454$, two-sided $P = 0.555$; Cataract: $y = -0.0721x + 16.2$, $R^2 = 0.326$, two-sided $P = 0.0845$). Mann–Whitney *U* test, two-sided $P = 0.809$.

αA Crystallin—Cataract and Normal Lenses

A group of cataract lenses was treated in the same manner as the normal lenses and the results for tryptic digestion and LC/MS analysis of the total lens protein are shown in Figure 2. Age-related cataract lenses are available only after age ~40.

For D-isoAsp and L-isoAsp there was no significant difference in the values compared to normal lens proteins (Figs. 2a, 2c). The D-Asp levels were slightly higher for the cataract lenses, and this was statistically significant (Fig. 2b).

A separate group of normal lenses across the age range were examined and compared with another age-matched group of cataract lenses. In this case, only the insoluble proteins were

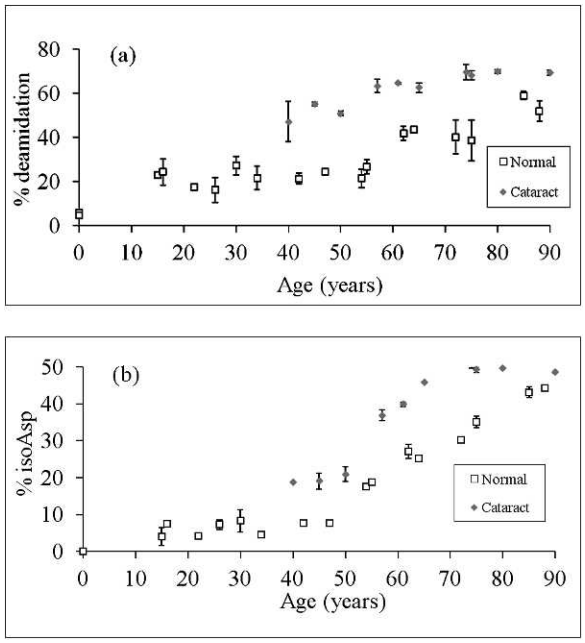

Figure 3. Deamidation of Asn 76 in γS crystallin (total protein). (Symbols: □ Normal; ◆ Cataract) (**a**) The percentage of deamidation as a function of age, in normal and cataract lens proteins. (Normal: $y = 0.769x - 12.6$, $R^2 = 0.8302$, two-sided $P < 0.0001$; Cataract: $y = 0.463x + 32.6$, $R^2 = 0.825$, two-sided $P = 0.0003$). Mann–Whitney U test, two-sided $P = 0.0002$. (**b**) The percentage of (L+D)-isoaspartic acid as a function of age, in normal and cataract lens proteins. (Normal: $y = 0.832x - 27.9$, $R^2 = 0.98$, two-sided $P < 0.0001$; Cataract: $y = 0.757x - 10.3$, $R^2 = 0.837$, two-sided $P = 0.0002$). Mann–Whitney U test, two-sided $P = 0.0375$.

studied in order to see if there were marked differences in the results compared with the total lens proteins. The results for the second cohort of normal lenses were very similar to the first batch of normals. The appearance of the three modified forms of Asp as a function of age is shown in Figure 4. The shapes of the curves for the two D-forms were again very similar (Figs. 4a, 4b). There was an increase in the proportion of both D-forms until the mid-teens, after which there was no appreciable increase. D-isoAsp was the most abundant, representing approximately 35% of the Asp, whereas D-Asp accounted for approximately 12%.

Insoluble proteins from cataract lenses, treated in the same way as normals, were also examined. When compared to the normal lenses there were no statistically significant differences in the graphs (Figs. 4a, 4b).

In the case of L-isoAsp there was considerable variation in the data points, probably reflecting the low absolute values for this modified amino acid. Cataract and normal lenses were not statistically different.

γS Crystallin—Cataract and Normal Lenses

In contrast to the αA crystallin data, in the case of γS crystallin the results from cataract and normal lenses were distinctly different (Figs. 3, 5).

For total protein samples, the extent of deamidation of Asn 76 was consistently higher in cataract lenses than the age-matched normals (Fig. 3a). The values for cataract lenses were almost double those of the normals up to about the age of 70. From age 70 onwards, the slope of the line for cataract lenses appeared to become more horizontal.

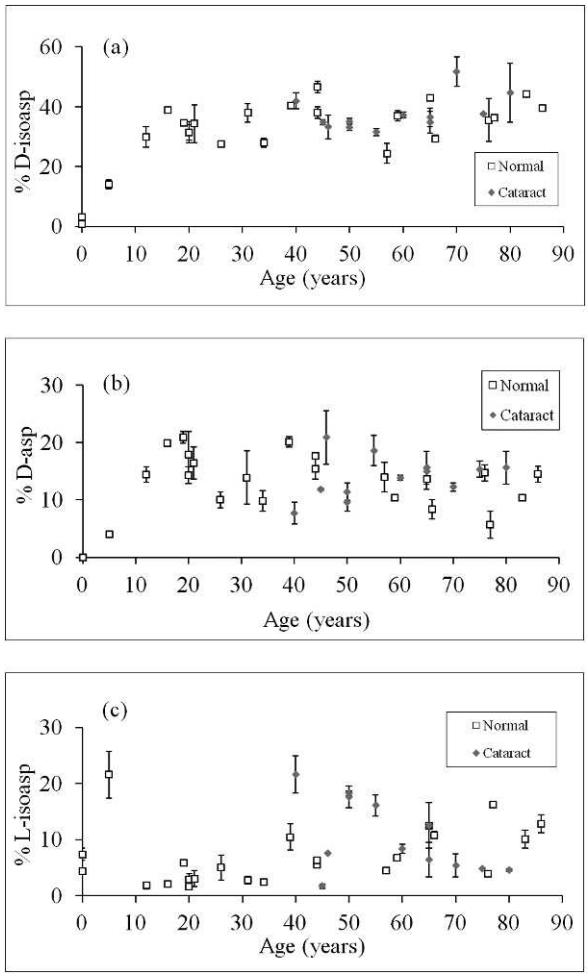

Figure 4. Racemization of Asp151 in αA crystallin (insoluble protein). (Symbols: □ Normal; ◆ Cataract) (**a**) The percentage of D-isoaspartic acid as a function of age, in normal and cataract lens proteins. (Normal: $y = 0.0152x + 36.4$, $R^2 = 0.00114$, two-sided $P = 0.926$; Cataract: $y = 0.211x + 25.4$, $R^2 = 0.218$, two-sided $P = 0.126$). Mann–Whitney U test, two-sided $P = 0.616$. (**b**) The percentage of D-aspartic acid as a function of age, in normal and cataract lens proteins. (Normal: $y = -0.113x + 19.9$, $R^2 = 0.213$, two-sided $P = 0.179$; Cataract: $y = 0.0878x + 8.84$, $R^2 = 0.919$, two-sided $P = 0.338$). Mann–Whitney U test, two-sided $P = 0.354$. (**c**) The percentage of L-isoaspartic acid as a function of age, in normal and cataract lens proteins. (Normal: $y = 0.155x - 1.28$, $R^2 = 0.315$, two-sided $P = 0.0913$; Cataract: $y = -0.275x + 26.5$, $R^2 = 0.289$, two-sided $P = 0.0717$). Mann–Whitney U test, two-sided $P = 0.0931$.

The rate of increase in the percentage of D-isoAsp and L-isoAsp for the cataract and normal lenses from age 40 onwards was also approximately the same (Fig. 3b); however, the absolute values differed and this was reflected in a "statistically significant" difference between the two lines.

When the insoluble proteins from another set of normal and cataract lenses were examined (Fig. 5), once again there was a clear difference in the extent of racemization (Fig. 5a), as well as the percentage of isoAsp (Fig. 5b). As had been observed with αA crystallin, the values mirrored those obtained with the whole lens protein samples.

Since cleavage on the C-terminal side of Asn residues can occur in aged proteins,[19] the relevant peptide (WMGLN) was searched for since this could affect the relative quantification. WMGLN was detected only in one 88-year-old lens, suggesting

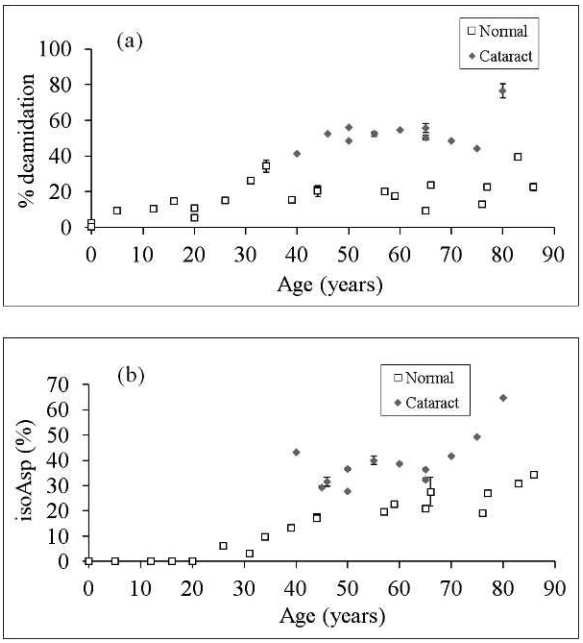

Figure 5. Deamidation of Asn 76 in γS crystallin (insoluble protein). (Symbols: □ Normal; ◆ Cataract) (**a**) Deamidation as a function of age, in normal and cataract lens proteins. (Normal: $y = 0.169x + 9.93$, $R^2 = 0.101$, two-sided $P = 0.371$; Cataract: $y = 0.345x + 32.2$, $R^2 = 0.226$, two-sided $P = 0.139$). Mann–Whitney U test, two-sided $P < 0.0001$. (**b**) The percentage of (L+D)-isoaspartic acid as a function of age, in normal and cataract lens proteins. (Normal: $y = 0.323x + 2.46$, $R^2 = 0.662$, two-sided $P = 0.0042$; Cataract: $y = 0.537x + 8$, $R^2 = 0.453$, two-sided $P = 0.0164$). Mann–Whitney U test, two-sided $P < 0.0001$.

that isomerization/deamidation is a more abundant outcome at this site than peptide bond cleavage.

Interestingly, another site on γS crystallin also showed a similar increase in age-related deamidation, as well as a difference in cataract compared to normal lenses (Supplementary Fig. 2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-9085/-/DCSupplemental) although the absolute values were lower than for Asn 76. MS/MS analyses confirmed that the site of deamidation was at Asn 14.

Discussion

Age-related deterioration of long-lived proteins can have important consequences at numerous sites in the human body[34–38] and has been proposed to ultimately constrain lifespan.[20,39] Little is known about which processes are quantitatively the most important, or about the rate of change of such processes. The human lens, which contains proteins that do not turnover, provides an opportunity to gather data on the degree and rate of change of specific post-translational modifications.

In this study, two proteins from human lenses were compared. It is known that both Asp and Asn residues in long-lived proteins degrade over time, and that this can involve succinimide intermediates.[14,40,41] The identification of isoAsp products provides evidence that the succinimide pathway is involved rather than, for example, simple hydrolysis of the amide side chain in the case of Asn, or racemization of L-Asp via removal of the alpha proton. In the case of Asn, ring opening of the succinimide results in deamidation with the formation of four Asp isomers.[42] For L-Asp residues, the succinimide intermediate also leads to generation of the four Asp isomers. In this study, two proteins, αA crystallin and γS crystallin, from the same lenses were compared. In neither crystallin was a linear increase in the percentage of the modified amino acid products with age observed.

In the case of αA crystallin, more than half of the L-Asp residues at position 151 had been converted to other structural isomers by age 15. The major product was D-isoAsp. This result indicates strongly the involvement of a succinimide intermediate, as has been found by others for different proteins.[43–47] Interestingly, after early teens, there was no further increase in the amount of D-Asp, D-isoAsp, or L-isoAsp. The reason for this is unclear, but shows that the proportion of isomerized Asp residues should not be used as an indicator of protein age in the absence of other data. It should be noted that our results for Asp 151 in αA crystallin in normal lenses are in broad agreement with a previous study using a more limited age range.[31] In the present study there was also no difference in the percentage of D-Asp, D-isoAsp, or L-isoAsp in αA crystallin from cataract lenses compared with normal lenses.

Comparison of the age-dependent rate of appearance of the three Asp isoforms in αA crystallin was instructive. The shapes of the curves for D-Asp and D-isoAsp were essentially identical, with D-isoAsp being more abundant. This agrees with those found by others for α crystallin[31] and other proteins.[14,48] By contrast, the rate of formation of L-isoAsp was different. This indicates that while the two D-isomers may undergo interconversion in the intact protein, D-Asp, D-isoAsp, and L-isoAsp do not appear to be in equilibrium in the lens. Other factors, for example, the stability of the final products, local folding, or neighboring residues, may determine the ultimate ratio of the individual forms in the modified protein.

In the case of the other crystallin examined, the results were very different. For γS crystallin, all four isomers would not be separated by HPLC, so data for the two isoAsp forms, which co-eluted, are presented together. In γS crystallin, little evidence for succinimide formation in normal lenses was detected before age 40. Indeed, the amount of D-isoAsp + L-isoAsp remained relatively constant at only 5% from age 15 to age 40. After that time, there was a linear increase in the percentage of isoAsp at 1% per year.

There was a difference in the extent of deamidation and the percentage of isoAsp in γS crystallin from cataract lenses when compared with age-matched normal lenses (Figs. 3a, 3b). To illustrate this, the average Asn 76 deamidation in normal lenses for the 40- to 60-year cohort was 23.5±2.58% ($n = 4$) compared to 54.2±6.96% ($n = 4$) for the cataract lenses. This result is in agreement with a previous study, which found a significantly greater extent of deamidation of Asn 76 in γS crystallin from 60- to 80-year-old cataract lenses compared to similarly aged normal lenses.[13] This finding contrasted with the majority of the other Gln and Asn sites in other crystallins where, if deamidation was observed at a particular site, it was essentially the same in cataract and normal lenses. It therefore seems that a greater extent of deamidation at Asn 76 may be a characteristic feature of cataract lenses.

The reason for this reproducible difference is not known, but it could be hypothesized that a greater degree of deamidation at this site may be causative for cataract. Two lines of evidence support this theory. First, there are two known point mutations of γS crystallin that result in human cataract.[49,50] Both of these are conservative point mutations, and this illustrates clearly that very minor alterations to γS crystallin can be sufficient to cause lens opacification. Second, data obtained from recombinant crystallins show that substitution of just one Asn by an Asp residue, or Gln by Glu, can result in substantial alterations to the properties of the proteins.[12,51–53] Replacement of Asn by an isoAsp would be predicted to lead to even more pronounced changes. These

data also demonstrate that γS crystallin is responsible, in part, for the increased percentage of D-Asp that has been observed in cataract lenses compared with normal lenses.[29,30,54]

While the pronounced increase in deamidation of Asn 76 in cataract lenses compared to normals could be hypothesized to be causative for age-related nuclear cataract, it should be emphasized that increased deamidation at more than one site in γS crystallin may be implicated in denaturation of this protein in the lens. In support of this, examination of the deamidation of Asn 14 (Supplementary Fig. 2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-9085/-/DCSupplemental) revealed a very similar pattern to that of Asn 76. Other sites, for example Gln 92, showed increased deamidation with age but no difference in cataract lenses (data not shown). Gln 170 showed very little deamidation (<5%) across the age range. However, not all potential sites of deamidation can be analyzed using our methodology due, for example, to the length of the tryptic peptides produced, so there may be other deamidation sites that also contribute to denaturation.

With age, the proportion of insoluble protein increases in human lenses.[55,56] In order to see if the Asp modifications were linked to protein insolubility, a separate group of normal and age-related cataract lenses were examined. No major differences were noted between the data sets for total and insoluble proteins suggesting that under the conditions of the study, modification at these sites does not contribute in a major way to protein insolubility. The fact that the data sets for the two different batches of cataract and normal lenses matched closely adds weight to the significance of the findings. One difference between the total and insoluble data sets was that for the total protein, D-Asp in αA crystallin was elevated by ~3% in cataract lenses as compared to the normals of the same age, whereas this pattern was not observed for the insoluble protein data.

Within many cells of the body, an enzyme is present that can ameliorate the time-dependent modification of Asp/Asn residues. Protein isoaspartatemethyltransferase (PIMT)[16] methylates L-isoAsp and D-Asp residues and, via promotion of the succinimide intermediate, can partially reverse such modifications. PIMT is, however, inactive on D-isoAsp, and it is unknown if any methyl transferase acts on modified extracellular proteins, such as elastin or collagen, at other sites in the body. In addition, succinimide formation from Asn results in deamidation, which cannot be reversed and therefore results in the introduction of a negative charge, or alternatively, cleavage of the adjacent peptide bond. It is unlikely that any methyltransferase activity is available in the center of adult lenses, since enzymes appear to be denatured by decades of exposure to body temperature.[57,58]

The regions of both gamma S and αA crystallin that were examined in this study are located in flexible regions of the native proteins.[59–61] Such parts appear to be more susceptible to the post-translational modifications of the type characterized here than Asp/Asn residues located within structured portions.[42,62] From the different appearances of the graphs of αA and γS modification as a function of time (Figs. 2, 3), it appears for these proteins that factors such as unfolding, cleavage, or near neighbor interactions may play a greater role in determining racemization than adjoining sequences of amino acid residues**.** For example, γS crystallin undergoes age-related truncation in the human lens[63] and this cleavage may influence the time course of deamidation of Asn 76, especially if the cleaved portions of γS crystallin are less structured than the intact protein.

There is now a body of literature using recombinant crystallins to show that the introduction of just a single Asp residue in place of an Asn can cause considerable changes to the properties of the proteins.[10,12,52,53] It is likely that the insertion of an isoAsp residue will induce even greater structural alterations since, in this case, an extra carbon is introduced into the peptide backbone in addition to the negative charge. As demonstrated in this study, isoAsp residues represent the major forms at both sites in older lenses.

Conclusions

The presence of different isoforms of Asp strongly indicates the involvement of succinimide intermediates, both in the isomerization of Asp 151 in αA crystallin and the deamidation of Asn 76 in γS crystallin. At neither site was there a linear increase in the percentage of the modified amino acids over the whole age range, and further, the age-dependent profiles for the two proteins were different. This suggests that prediction of the extent of protein deamidation in older tissues, using computer programs based on crystal structures or adjacent amino acid residues, is likely to be fraught with problems.

The finding that there was a consistently greater extent of deamidation of Asn 76 in γS crystallin in all cataract lenses compared with their age-matched controls suggests that this modification, along with others, such as deamidation at Asn 14, could be associated with the development of age-related cataract in man.

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