Age-Dependent Deamidation of Lifelong Proteins in the Human Lens

Peter G. Hains and Roger J. W. Truscott

PURPOSE. Deamidation is a common posttranslational modification in human lens crystallins and may be a key factor in the age-related denaturation of such lifelong proteins. The aim of this study was to identify the sites of deamidation in older lenses.

METHODS. High-performance liquid chromatography/mass spectrometry of tryptic digests was used to identify sites of deamidation in the major human lens crystallins. Older normal and age-matched cataractous lenses were compared with fetal lenses.

RESULTS. Approximately equal numbers of glutamine and asparagine residues were deamidated in older lenses; however, the extent of deamidation of Asn was three times greater than that of Gln (Asn, $22.6\% \pm 3.6\%$; Gln, $6.6\% \pm 1.3\%$). Individual crystallins differed markedly in their extent of deamidation, and deamidated residues were typically localized within discrete regions of the polypeptides. A large percentage (42%) of the sites of deamidation were characterized by the presence of a basic amino acid one residue removed from the original Gln or Asn. At nine such sites, the extent of Asn deamidation averaged 50% in aged lenses. There were few differences in deamidation between crystallins of aged normal and nuclear cataractous lenses.

CONCLUSIONS. Equal numbers of Asn and Gln residues are deamidated in crystallins from aged normal and cataractous lenses. Deamidation of Asn/Gln in lifelong proteins, such as those in the lens, may be governed to a significant degree by base-catalyzed processes. (*Invest Ophthalmol Vis Sci.* 2010;51:3107–3114) DOI:10.1167/iovs.09-4308

The human lens is an ideal tissue for the study of protein aging because crystallins are abundant and are present for a lifetime.¹ Numerous posttranslational modifications to these polypeptides have been documented; the most abundant is deamidation.^{2,3} Deamidation of Asn and Gln residues has been described for many proteins,^{4,5} and it has been postulated that the introduction of a negative charge at a site that was formerly neutral may represent a “molecular clock” that could regulate protein turnover.⁶ To examine this, research has been undertaken with the aim of understanding the factors responsible for promoting deamidation of side chain amides.⁷ An examination

of hundreds of peptides in which the residues flanking Asn and Gln were varied revealed that Gly, His, Glu, Ser, and Tyr facilitated deamidation. The mechanism of deamidation can involve a cyclic succinimide intermediate that, in the case of Asn, also leads to the formation of L-aspartyl, L-isoaspartyl, D-aspartyl, and D-isoaspartyl residues.⁸ Deamidation of crystallins has been documented,^{9–12} and several sites of Asp racemization in older lens crystallins have also been elucidated.¹³ Primary sequence is not the only factor determining the loss of ammonia from the amide residues of proteins. Secondary, tertiary, and quaternary structures significantly affect the rate of deamidation, and other factors may play a part within a biological environment. The extent of deamidation at particular sites in proteins can be predicted using computational means once crystal structures are available.⁶

Given that the hydrolysis of amides is time dependent, it could be predicted that proteins that are synthesized before birth and do not turnover may have few Asn/Gln residues adjacent to amino acids known to promote deamidation. The overall prevalence of Asn/Gln in crystallins is not significantly different from the average of all proteins.⁶ Deamidation of crystallins in older persons is detectable and may influence the properties of the human lens, as documented by David et al.,² who showed that insoluble aggregates, which form progressively over time from soluble crystallins, have a higher degree of deamidation. It is not surprising that substantial deamidation of Gln and Asn would lead to protein unfolding. With the use of recombinant approaches, it has been demonstrated that the replacement of just one Asn by Asp in BB1-crystallin leads to measurable changes in physical properties, especially in the propensity of the protein to form aggregates.¹⁴ Deamidation of glutamines in β A3,¹⁵ β B1-crystallin,¹⁶ and γ D-crystallin¹⁷ diminishes the stability of the proteins. Maintenance of the structural integrity of crystallins is becoming increasingly recognized as vital for the proper functioning of the lens and, therefore, vision.^{18–20}

A major lens protein, α -crystallin, is a chaperone and binds other crystallins as they denature, forming high-molecular weight aggregates.²¹ Because there is no protein turnover in the center of the lens, after age 40 all the α -crystallin that was present at birth has been used in forming such aggregates. At this point, the stiffness of the lens increases dramatically,²² a phenomenon implicated in presbyopia—the inability to focus on near objects—that affects everyone once they reach age 45 to 50. Age-related nuclear cataract, a major cause of blindness, typically becomes noticeable a decade or more after presbyopia and is characterized by an even greater degree of protein modification such that in advanced cases, half the protein present in the lens center becomes colored, cross-linked, and insoluble in 8 M urea.²³ It is unknown which factors are chiefly responsible for the progressive age-dependent denaturation of the crystallins in lenses. Heat is one possibility,²⁴ and deamidation, if it were substantial, would be another that could lead to extensive unfolding.

From the Save Sight Institute, University of Sydney, Sydney, NSW, Australia.

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Corresponding author: Roger J. W. Truscott, Save Sight Institute, 8 Macquarie Street, Sydney, NSW, 2001, Australia; rjw@eye.usyd.edu.au.

In this study we characterized the sites of deamidation in all the major crystallins in the human lens using tryptic digestion coupled with mass spectrometry. Analysis of the data revealed a novel sequence determinant implicated in protein deamidation. Because other tissues in the body^{25,26} that contain proteins are present for all our lives and organs with likely candidate polypeptides^{27–29} that remain to be investigated, this study also represents a step toward understanding the processes involved in age-dependent denaturation of these lifelong proteins and the consequences of this for human aging.

METHODS

Lens Preparation

The research followed the tenets of the Declaration of Helsinki. Normal human lenses were obtained from the Sydney Eye Bank, with ethics approval from the University of Sydney (ethics approval number 7292). Cataractous lenses were obtained from K.T. Seth Eye Hospital, Rajkot, Gujarat, India. Two sets of fetal lenses, each containing two lenses (set 1, 2 lenses, 17 weeks' gestation; set 2, 1 lens, 17.5 weeks' gestation plus 1 lens, 19 weeks' gestation), three normal (59, 65, 80 years) lenses, and three nuclear cataractous lenses (60, 65, 80 years), graded as type III–IV according to the Pirie classification system were used. Because of their size, complete fetal lenses were used, whereas the nucleus was separated from the cortex for each of the adult lenses by coring through the visual axis with a 5-mm diameter trephine and removing 0.5 mm from each end of the core. Lens cores (nuclei) were analyzed in this study, and regions could be compared directly with fetal samples because the lens grows throughout life by the addition of cells to the lens formed in utero.

Lens samples (50 mg/mL) were homogenized with 7 M guanidine-HCl, 100 mM Tris, pH 7.0, and centrifuged for 10 minutes at 16,000g. Protein (10 mg) was then reduced and alkylated as outlined.³⁰ Alkylated protein (1.8 mg) was diluted 1 in 8 with 100 mM Tris-HCl, pH 8.0 containing 19 μ g trypsin (Promega, Madison, WI). The vial was layered with argon and incubated at 37°C overnight. After digestion, approximately 200 μ g protein from each sample was desalted and concentrated (PerfectPure C18 tips; Eppendorf, Hamburg, Germany). Before two-dimensional (2D) LC-MS/MS analysis was performed, all samples were analyzed by MALDI-TOF MS to ensure that digestion was complete.

2D LC-MS/MS

Dried peptides were dissolved in 0.5% (vol/vol) heptafluorobutyric acid and 1% (vol/vol) formic acid (FA) and were separated by nano-LC using an HPLC (Ultimate 3000; Dionex, Amsterdam, Netherlands) and autosampler system (Dionex). Peptides (5 μ L) were loaded onto a microcolumn (0.76 mm $\times$ ~15 mm; SCX; Dionex) containing HPLC media (Poros S10; Applied Biosystems, Foster City, CA) and eluted sequentially using 5, 10, 15, 20, 25, 30, 40, 50, 100, 250, 500, and 1000 mM ammonium acetate (20 μ L). The initial unbound fraction and each of the salt steps were concentrated and desalted on a microprecolumn (500 μ m $\times$ 2 mm; C18; Michrom Bioresources, Auburn, CA) with 98% (vol/vol) water, 2% (vol/vol) acetonitrile, and 0.1% (vol/vol) FA (buffer A) at 20 μ L/min. After a 4-minute wash, the precolumn was switched (Valco 10 port valve; Dionex) into line with a fritless nanocolumn (75 μ m $\times$ ~10 cm) containing C18 media (5 μ m, 200 Å Magic; Michrom Bioresources). Peptides were eluted using a linear gradient of H₂O/CH₃CN (98:2, 0.1% [vol/vol] FA) to H₂O/CH₃CN (55:45, 0.1% [vol/vol] FA) at 350 nL/min over 30 minutes. High voltage (1800 V) was applied to a low-volume tee (Upchurch Scientific, Oak Harbor, WA), and the column tip was positioned approximately 0.5 cm from the heated capillary (T = 200°C) of a mass spectrometer (LTQ FT Ultra; Thermo Electron, Bremen, Germany). Positive ions were generated by electrospray, and the mass spectrometer (LTQ FT Ultra; Thermo Electron) was operated in data-dependent acquisition mode. Each sample was analyzed by LC/MS once.

A survey scan (m/z 350–1750) was acquired in the Fourier transform ion cyclotron resonance cell (resolution = 100,000 at m/z 400, with an initial accumulation target value of 1,000,000 ions in the linear ion trap). As many as seven of the most abundant doubly or triply charged ions (>2000 counts) were sequentially isolated and fragmented within the linear ion trap using collision-induced dissociation, with an activation $q = 0.25$ and an activation time of 30 ms at a target value of 30,000 ions. The m/z ions selected for MS/MS were dynamically excluded for 60 seconds. All data were acquired in profile mode and searched against the SWISS-PROT 54.3 database (17,400 human sequences; made available by Swiss Institute of Bioinformatics) with a range of posttranslational modifications (PTMs) using MASCOT (version 2.2.03; Matrix Science, London, UK), with the enzyme specificity set to semi-trypsin. The numbers of MS/MS spectra searched were as follows: fetal, 12,585 and 14,262; normal 16,186, 10,011, and 16,156; cataract, 12,120, 14,378, and 17,761. The following PTMs were listed as variable modifications in addition to deamidation: acetylation (N-term), Arg55, a newly noted unknown modification to Arg³¹; carboxymethyl, *N*-ethylmaleimide, methylation (C); oxidation (H, W, M); peptide tolerance, 8 ppm; fragment tolerance, 0.6 Da; one missed cleavage allowed. MASCOT results were processed with the Trans-Proteomic Pipeline (version 3.2.0); a global false-discovery rate of $P < 0.05$ was chosen using PeptideProphet statistics once the MASCOT search was fully processed by this algorithm.

Label-Free Quantitation

Peptides were quantitated using *SuperHirn* version 0.05 beta.³² *SuperHirn* automatically aligns LC-MS total ion currents and matches the peptides across a number of samples using MS/MS information in addition to retention time, peptide mass, and charge state. Only peptides with a PeptideProphet score >0.9 were used for this analysis, giving an error level of $P < 0.025$. Although *SuperHirn* shares MS/MS information across aligned features, it may still be possible to obtain quantitative data for a modification from a same sample at the MS level even though a PTM might not have been identified in one sample at the MS/MS level. *SuperHirn* was used to compare the RP-HPLC profile of all 13 SCX fractions from each of the normal and cataractous lens samples. Default processing parameters were used for data processing. An alignment tree was built, followed by LC-MS alignment and intensity normalization. This resulted in 13 files containing quantitative information for numerous peptides identified by MS/MS. A Perl script was written to take each file output from *SuperHirn* and then sort and combine the data for any given peptide. The output was a single-tab delimited file containing the summed intensities for each peptide, with a separate column for each lens sample, in each SCX fraction in which it was found.

Statistical Analysis

Quantitative data are presented as relative abundance of all forms of the peptide containing a deamidation compared with all other versions of that peptide—that is, the intensity values for all forms of a detected peptide were summed, and the intensity value of the deamidated form was divided by that of the deamidated plus nondeamidated value. Because the data were obtained without the use of labeled internal standards, the extent of deamidation values shown in the figures are semiquantitative. Data were analyzed statistically (Prism, version 4; GraphPad, San Diego, CA) using two-way ANOVA with Bonferroni posttest. Significance was taken as $P < 0.05$. In most cases, three values each from normal and cataractous lenses were used for analysis. In some cases only two values were available for adult lenses; these are noted in the figures.

RESULTS

The overall aim of this investigation was to determine the extent and location of deamidation in crystallins of older normal lenses. These data were compared with those obtained

from analyses of age-matched cataractous lenses and of fetal lenses. The aim was to identify which sites are quantitatively of most importance for age-related protein denaturation and to discover whether there were significant differences in cataractous lenses that could imply deamidation plays a significant role in the development of age-related cataract. To avoid any issues of fractionation of modified individual crystallins that may occur during purification and could affect the data on the extent of deamidation, whole lens samples were solubilized and examined directly.

The human lens is composed of nine major crystallins: α A, α B, β A1/A3, β A4, β B1, β B2, γ S, γ C, and γ D.³³ These nine abundant polypeptides contain a total of 150 Asn and Gln residues. Of these, we obtained MS/MS data for 134 sites in crystallins isolated from lenses in the sixth to eighth decades. Of the total we detected, 55 were quantified and 24 were deamidated to a small degree, though those levels were too small for accurate quantification. In 55 sites no deamidated version of the peptide was found, and 16 possible deamidation sites were not observed. In this study we define “not deamidated” as residues in which <1% of the tryptic peptide containing Asp or Glu in place of the parent Asn or Gln residues was detected. The high resolving power and the rapid scan speed of the Fourier transform mass spectrometer allows ready differentiation of the 1-Da mass difference between Asn/Gln-containing tryptic peptides and their corresponding deami-

dated counterparts. We compared the central (nuclear) regions of older human lenses with fetal lenses. Because the lens grows throughout life by the addition of cells to a preexisting core, the data obtained from the fetal lenses serves as a control for age-dependent deamidation.

Similar numbers of Gln and Asn residues were found to be deamidated in the crystallins from older lenses; however, the extent of deamidation of the Asn sites was higher, in agreement with previous studies with peptides^{34,35} and proteins.⁴ In human lenses from the sixth to eighth decades, 29 Gln were deamidated compared with 26 Asn residues, and the extent of the deamidation for sites at which this occurred averaged $22.6\% \pm 3.6\%$ for Asn and $6.6\% \pm 1.3\%$ for Gln. Plots of the extent of deamidation at each site for the nine major human crystallins are shown in Figure 1. The average extent of deamidation for the age-related nuclear cataract (ARNC) and the corresponding age-matched normal lenses at each site is depicted. β B3-crystallin, a less abundant lens protein, is not included because the sequence coverage was <60%.

Several features are apparent. First, the number of deamidated residues varies considerably between the various crystallins. In β B2- and γ C-crystallin, only one residue was found to be deamidated in the aged normal lenses. By contrast, nine sites of deamidation were found in β B1-, eight in β A4-, five in γ S-, and six in β A1/A3-crystallin. Second, the extent of deamidation varied substantially. Some crystallins from older lenses con-

FIGURE 1. Extent of deamidation in the nine most abundant crystallins of the human lens. The percentage of deamidation for each residue is shown for aged normal and age-related nuclear cataractous lenses. (a) α A-crystallin. (b) α B-crystallin. (c) β A1/A3-crystallin. (d) β A4-crystallin. (e) β B1-crystallin. (f) β B2-crystallin. (g) γ C-crystallin. (h) γ D-crystallin. (i) γ S-crystallin. Results are presented as mean $\pm$ SEM. Unless otherwise noted on the figure, $n = 3$; x-axes, sites of modification; y-axes, percentages of deamidation. Solid bars: normal. Hatched bars: cataract. (*) Deamidation not detected. (§) Residue shown to be deamidated but no quantitative data available. (†) Residue not detected. Sites at which deamidation of cataract and normal lenses was significantly different are shown ($P < 0.05$).

tained sites that were almost totally deamidated. These included β A1/A3-, β A4-, γ S-, and γ D-crystallins. By contrast, in β B2-crystallins, the most highly deamidated residue in older normal lenses was Gln146, and the extent of deamidation was approximately 3%. In α A-crystallin, the most deamidated sites were Asn123 and Gln126, and the extent of deamidation was approximately 10%. Third, the sites of deamidation were often localized to discrete regions. For example, in α A-crystallin, deamidation was found only in the putative C-terminal domain. In β A1/A3-crystallin, the most substantial deamidation in the protein was found at three sites contained within a region spanning residues 103 to 133. In γ C-crystallin, the only site of deamidation was localized to the peptide bridging the two domains. In most cases, the most highly deamidated sites were those of Asn residues. These features are discussed in greater detail for each crystallin.

α -Crystallins

Each α -crystallin subunit contains few Asn and Gln residues.

α A-Crystallin. In α A-crystallin, the first site of deamidation from the N-terminus was not found until Gln90. Thereafter, all sites were found to be deamidated to some degree, with the lowest extent found at Asn101.

α B-Crystallin. In α B-crystallin, four of the five possible deamidation sites were deamidated with very low levels at Gln108. Gln151, close to the C terminus, was the only residue not deamidated.

β -Crystallins

All the β -crystallins, with the notable exception of β B2-crystallin, were substantially deamidated at several sites.

β A3-Crystallin. β A1 is an abundant, N-terminally truncated form of β A3-crystallin. Deamidation was highly localized. No deamidation was detected in the first 22 residues, containing five possible sites, and indeed little deamidation was detected at any of the Asn or Gln sites in the N-terminal half of the polypeptide until Asn103, with only Asn54 showing appreciable alteration. Asn120, with His Lys as the residues on its C-terminal side, was almost totally deamidated in older lenses, with approximately 20% to 25% deamidation at the flanking, and almost equally spaced, Asn103 and Asn133 residues. These three residues are within, or close to, the connecting peptide, comprising residues 118–123. There seemed to be little evidence of substantial deamidation along the sequence C-terminal from Asn133, which contained 12 possible sites. The coverage in this region, however, was not as high as in other crystallins; four sites were present in peptides that were not detected.

β A4-Crystallin. As was found with β A3-crystallin, deamidation seemed to be localized within one internal region of the protein. Substantial deamidation was detected at all five sites spanning Gln62 to Asn100. As was observed with β A3-crystallin, one site (Asn100) in this zone was almost totally deamidated. Analogous to β A3, this Asn also had two basic amino acids (His Arg) as its C-terminal residues. Outside this 38-amino acid residue region, only Gln111 and Asn113 were deamidated to any significant extent, and these were at low levels (approximately 5% each) with Gln22 at 2%.

β B1-Crystallin. β B1-crystallin contained numerous sites of deamidation, the most highly deamidated of which was Asn124 (approximately 30% deamidated) located within Greek key motif 2. As was found with the other β -crystallins, the region of deamidation was localized, but in β B1-crystallin the region was

TABLE 1. Major Sites of Deamidation in Human Fetal Lenses

Crystallin	Residue	Surrounding Residues	Relative Abundance $\pm$ SEM
α B	N146	LTVNGPR	0.09 $\pm$ 0.01
β A1/A3	N103	SGSNAYH	0.03 $\pm$ 0.02
β A1/A3	N120	CSANHKE	0.16 $\pm$ 0.00
β A4	N100	ACANHRD	0.38 $\pm$ 0.02
β B1	N107	EQSNFRG	0.06 (n = 1)
γ D	Q47	LYEQPNY	0.11 (n = 1)
γ D	N49	EQPNYSG	0.11 (n = 1)
γ D	Q54	SGLQYFL	0.15 $\pm$ 0.16
γ S	N143	ELPNYRG	0.90 $\pm$ 0.10

broader, spanning Asn67 to Gln166. Outside this central zone there was no evidence of significant deamidation.

β B2-Crystallin. β B2-crystallin was markedly different from the other β -crystallins. Deamidation was restricted to a narrow zone, corresponding to the third Greek key motif within the C-terminal domain. Only one site (Gln146), of the 25 possible sites, was deamidated in normal lenses, with the average level of deamidation just 3%. Levels below 1% were detected at Asn115 and Gln154. The unusually low extent of modification of β B2-crystallin is consistent with that of previous studies.³⁶

γ -Crystallins

There are three abundant γ -crystallins in the human lens.³³ Two of these, γ S and γ D, were found to contain one Asn site that was almost totally deamidated in the older lens samples. In both, Asn was flanked C-terminally by Tyr Arg.

γ S-Crystallin. Four sites of deamidation were detected in the linker region and the C-terminal domain. Asn143 was wholly deamidated. The four sites were characterized by the following C-terminal amino acids: Asp Arg (Asn76), Tyr Arg (Gln 92), Phe His (Gln102), and Tyr Arg (Asn143). In this crystallin were two sites close to the N-terminus (Asn14 and Gln16) in which significantly more deamidation was observed in cataractous lenses than in age-matched normal lenses.

γ C-Crystallin. γ C-Crystallin was one of the least affected proteins and had only one significant site of deamidation (Gln83). This crystallin was also unusual in that Gln rather than Asn was the most highly deamidated residue in normal lenses. Gln83 is contained within the linker region between the two domains. As was found in γ S-crystallin, a residue close to the N-terminus was more highly deamidated in cataractous lenses.

γ D-Crystallin. An eight-residue sequence (Gln47–Gln54) contained 3 of 4 sites of significant deamidation. None of these was flanked by basic residues, but two were flanked by Pro, and all are within the second Greek key motif.³⁷ The most highly deamidated site was Asn137, which is in the same region of the protein, has the same C-terminal sequence (Tyr Arg) as that of γ S-crystallin, and was also highly deamidated.

Fetal Lenses

Incubation of proteins under the conditions used for tryptic digestion can lead to deamidation at susceptible sites. To control for such artifactual deamidation in the adult lenses, the same digestion protocol was used with fetal lens proteins. The results are summarized in Table 1. The data suggest that deamidation at N143 of γ S may be artifactual and that there is likely to be a contribution at some other sites. Deamidation of γ D (Gln47–Gln54) may also be largely an artifact. One other highly deamidated site detected in fetal lenses was N155 in

β B3-crystallin (66%). Deamidation at crystallin sites not listed in Table 1 was not above 2%; most was $<0.5\%$.

Therefore, with the exception of the sites listed, the other deamidation sites identified in the adult lenses (Fig. 1) appeared to be the result of extended exposure to physiological pH and temperature within the eye.

Age-Related Nuclear Cataract and Normal Lenses

In most instances, the deamidation patterns detected in cataractous lenses mirrored those of the normal aged lenses. Because there were three lenses in each group, the cataract findings corroborate the sites of deamidation identified in the normal lenses and indicate that aging, of itself, is the major contributor to deamidation. Some sites (α A, Q147; α B, N78, Q108; β A4, Q64, N82; β B1, N67, N69; γ C, N24; γ D, N160; γ S, N14, N16, N76) showed increased levels of deamidation in ARNC cataractous lenses. Sometimes these residues were located near the ends of polypeptides, as in the N-termini of γ C and γ S-crystallins and the C-terminus of α A-crystallin. Greek key motif 1 within β B1-crystallin contained three residues that were more deamidated than normals. These observations may be consistent with localized unfolding of these polypeptide regions in cataractous lenses. In fewer cases (Fig. 1), deamidation appeared to be greater in normal aged lenses. The reason for this is unclear, but it may simply reflect individual variation or the fact that other posttranslational modifications to the ARNC proteins affected the process of tryptic digestion and analysis.

DISCUSSION

It is becoming apparent that there are several sites in the human body at which proteins may be present for a lifespan. To date these include elastin in the lung,²⁵ crystallins in the lens,¹ dentin in teeth,²⁶ and other, less well-characterized, sites such as collagens in the vitreous humor of the eye²⁸ and cartilage.²⁹ The limited list to date, may well be a reflection of the difficulty in analysis, which requires determination of the ^{14}C content of proteins from donors across the age range relative to atmospheric ^{14}C levels.^{25,27} The implications of the presence of such lifelong polypeptides for human aging and for long-term health are profound because it is clear that over a very long time period, susceptible amino acids will decompose and the proteins may be cleaved and accumulate PTMs.

The most well-known examples of lifelong proteins are the lens crystallins. There are only a few abundant proteins in this tissue, providing an opportunity to evaluate the extent of changes within all major proteins in one tissue. In this study we focused on deamidation because it has been found in earlier studies to be the most abundant PTM in adult lens proteins. Deamidation of Asn and Gln may play a key role in denaturing crystallins because insoluble proteins that accumulate progressively with age in the human lens^{22,38} have more deamidation than soluble proteins.² This hypothesis is supported by site-directed mutagenesis experiments.¹⁴⁻¹⁶ Deamidation of crystallins from aged lenses has been studied in a number of laboratories,³⁹⁻⁴² but the data are not always in agreement. Some modifications that lead to more acidic α -crystallins were originally ascribed to deamidation, but were later discovered to be due primarily to phosphorylation.⁴³ Other recent mass spectrometric data are more reliable, but to accurately determine sites of deamidation, especially in tryptic peptides with more than one possible site, it is helpful to have access to the high resolving power of FTMS.⁴⁴ This has not been available until recently. As one example, it was reported that increased

deamidation of Asn143 in γ S-crystallin was specific for cataract,⁴⁵ but our data show that in both normal and cataractous lenses, this site is almost totally deamidated. Purification of crystallins could selectively remove modified forms of the proteins and thus bias the results. For this reason, whole lens tissue was examined without fractionation. In general, the sites of deamidation we describe here are in agreement with those shown recently,⁴⁶ but that study did not report quantitative data.

Much work has been performed with the aim of characterizing the features that govern deamidation within proteins, especially the factors responsible for controlling the rate of deamidation at particular sites.^{4,8} Several features are important, including conformation and the identity of the nearest neighbor amino acid.³⁵ The effect of sequence has been studied using model peptides to determine which adjacent residues promote deamidation.

A key finding of the present study was that all nine major structural proteins were deamidated in older lenses but that they were affected very differently, both in terms of the number of residues deamidated and the extent to which Asn and Gln were converted to their corresponding carboxylic acids. For example, β B2-crystallin remained virtually unchanged in the older lenses displaying just one site of deamidation, and the extent in 60-year-old lenses was only 3%. Similarly, γ C-crystallin was characterized by just one site of deamidation. Among the crystallins that displayed the least extent of deamidation were two proteins, β B2-crystallin and α A-crystallin, each of which is thought to assist protein solubility in the lens.^{47,48}

Sites of deamidation in the other crystallins were typically localized within discrete regions of the proteins. The reason for this is unknown but may be related to crystallin packing within the very high protein environment of the lens. Approximately equal numbers of Asn and Gln residues in the crystallins were detected as deamidated, though in model studies Asn is much more labile than Gln.⁶ This finding suggested that factors other than those deduced from peptide data could play a role in deamidation of these very long-lived proteins. In addition, a plot of the expected deamidation derived from peptide incubations,⁶ in contrast to our experimentally derived crystallin data (Fig. 2), showed no clear relationship. There was also no correlation in the extent of deamidation with ASA; hence, solvent exposure alone does not determine whether a residue becomes deamidated. It should be noted that deamidation of Gln, but not Asn, can be catalyzed by enzymes such as transglutaminase,^{49,50} but it is unlikely that this enzyme remains active in the center of older human lenses. Thus, the deamidation of Gln and Asn is likely to be chemically mediated.

There was generally very good agreement between our data and those obtained by Dasari et al.⁴⁶ and Wilmarth et al.² Dasari et al.⁴⁶ did not report the sites we reported for γ S-crystallin. It is important to note, however, that these authors stated they observed only 67% of the known deamidation sites. Wilmarth et al.² reported high levels of deamidation of both N76 and N143 in γ S-crystallin (not reported by Dasari et al.⁴⁶), which is in agreement with our data. A comprehensive table showing the sites of deamidation in lens crystallins is contained in Wilmarth et al.²; and this study also documents the different extents of deamidation for soluble and insoluble proteins. New deamidation sites that we observed are N54, Q164, and Q206 in β A1-crystallin and N100 in β A4-crystallin. When the results of Wilmarth et al.² and our data are viewed together, a picture emerges in which deamidation appears to be important for protein denaturation in the aging lens, but the evidence that deamidation contributes directly to cataract formation is

FIGURE 2. Percentage of deamidation observed for normal (a) and cataractous (b) lenses, plotted against the deamidation rate calculated from published peptide data.⁶ Only lens sites with >1% deamidation are shown.

weaker. It is possible that the few sites we observed to be differentially deamidated in cataractous lenses may be sufficient to cause opacification.

A novel feature discovered in this investigation was the importance of basic amino acids. Forty-two percent of all sites of deamidation had a basic residue one residue removed from the site of deamidation. These data are summarized in Figure 3. The effect was most noticeable when Lys, His, or Arg was on the C-terminal side. On the basis of these data, we predict that the amino groups of such side chains may be able to participate in base-catalyzed hydrolysis of the amide directly or to catalyze the decomposition of the cyclic (e.g., succinimide) intermediates. An effect of Lys or Arg, one residue removed, on promoting the rate of deamidation of Asn has been found using peptides, though this was attributed by the authors⁶ largely to secondary structural effects. The importance of base catalysis in lens proteins may also be inferred indirectly from the fact

that only four Asn residues in human crystallins are located immediately adjacent to a basic residue, and, three of these are His. Of these, we found that two (Asn120, β A3; Asn100, β A4) were totally deamidated in older lenses and that the other two (Asn101, α A; Asn14, γ S) were deamidated to a small extent; however, these basic amino acids are immediately adjacent to negatively charged amino acids, which will affect their reactivity. In addition, there are only three sites at which a basic amino acid is C-terminal to a Gln residue. Interestingly the most highly racemized aspartate residues in another very long-lived polypeptide, human myelin basic protein, also had neighboring Arg residues.⁵¹

It should be noted that lifelong proteins, such as those in the lens, are subjected to numerous PTMs aside from deamidation, and these can influence proteomic investigations. For example, covalent modification of Lys or Arg residues⁵² will impair tryptic digestion. In addition, truncation⁵³ is known to take place in crystallins of older lenses and will affect the methods of analysis and interpretation used in this study. This feature is likely to have contributed to the lack of observation of some residues (Fig. 1). Bearing in mind these factors, it is remarkable that results for the normal and cataractous lenses were so close.

Although the α -crystallins contain few Asn and Gln residues, crystallins in general are not bereft of these amides. On the basis of this study (Fig. 3), we hypothesize that factors that govern deamidation in crystallins, and possibly other such lifelong proteins, may differ subtly from those described for most polypeptides that turn over with a lifetime of days or weeks. Base-catalyzed deamidation may play a greater role in lifelong proteins. In addition, during the course of evolution, it may be that basic residues immediately adjacent to potential sites of deamidation were selected against. Over a period of decades, basic residues that are one residue removed from Gln/Asn may assume greater general importance in determining deamidation in crystallins and possibly in other lifelong proteins. Such inferences must be confirmed by studies on model peptides to determine the exact role of adjacent basic residues.

CONCLUSIONS

All the major structural proteins that are present in the human lens for decades were found to be deamidated, although the extent of deamidation varied considerably. Deamidation involved approximately equal numbers of Asn and Gln residues, and a role for nearby basic residues was inferred from analysis of the peptide sequence data. If this finding is confirmed by model peptide studies, this feature will have to be incorpo-

FIGURE 3. Effect of nearby basic residues on the extent of deamidation. Deamidation sites from all older crystallins were examined for the presence of a basic residue, either next to the site of deamidation ($n \pm 1$) or separated from the deamidation site by one intervening amino acid ($n \pm 2$). $n + 1$ N, a basic residue (R, H, or K) next to, and on the C-terminal side of, an Asn residue; $n + 2$ N, a basic residue one residue removed on the C-terminal side of an Asn residue; $n - 1$ N, a basic residue next to, and on the N-terminal side of, an Asn residue; $n - 2$ N, a basic residue one residue removed on the N-terminal side of an Asn residue. Same notation applies for Q. (*) In crystallins at these sites, the two $n + 1$ sites were also $n + 2$. (§) Residue shown to be deamidated but no quantitative data available. Results for normal, age-related nuclear cataract, and fetal lenses are presented as mean $\pm$ SEM. The number of sites for each category is shown immediately below the bar graph.

rated into computer models that aim to predict sites of deamidation in other old proteins.

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