Age-dependent deamidation of glutamine residues in human γS crystallin: Deamidation and unstructured regions

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Abstract: Human aging is associated with the deterioration of long-lived proteins. Gradual cumulative modifications to the life-long proteins of the lens may ultimately be responsible for the pronounced alterations to the optical and physical properties that characterize lenses from older people. γS crystallin, a major human lens protein, is known to undergo several age-dependent changes. Using proteomic techniques, a site of deamidation involving glutamine 92 has been characterized and its time course established. The proportion of deamidation increased from birth to teen-age years and then plateaud. Deamidation at this site increased again in the eighth decade of life. There was no significant difference in the extent of deamidation between cataract and age-matched normal lenses. Gln92 is located in the linker region between the two domains, and the introduction of a negative charge at this site may alter the interaction between the two regions of the protein. Gln170, which is located in another unstructured part of γS crystallin, showed a similar deamidation profile to that of Gln92. As the other Gln residues in β-sheet regions of γS crystallin appear to remain as amides, modification of Gln92 and Gln170 thus conforms to a pattern whereby deamidation is localized to the unstructured regions of long-lived proteins.

Keywords: deamidation; glutamine; age-related cataract; human lens; racemization

Introduction

One aspect of human aging involves the degradation of long-lived proteins. This takes place at a number of sites and organs in the body.[1–6] Because of the presence of multiple cell types, cell complexity, low protein copy numbers, and the insolubility of long-lived proteins at some sites, it is neither straightforward to characterize the major sites of modification nor to obtain data on the time course of each modification.

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These limitations are obviated in the human lens as the major structural proteins, the crystallins, are abundant and consist of fewer than 10 major polypeptides. There is also no turnover of proteins after they are packaged into a single type of cell: fiber cells.[7] In addition, because lens proteins belong to more than one family, it may be possible to derive lessons on age-related polypeptide post-translational modification (PTM) that apply more widely in the body.

Maintenance of the structure of one crystallin, γS crystallin, appears to be crucial for long-term transparency of the lens, as two conservative point mutations (Gly-Val)[8] and (Val-Met)[9] have been shown to be responsible for human hereditary cataract. Deamidation of γ-crystallins can significantly affect protein structure,[10] and therefore, it is important to characterize the sites and rates of significant

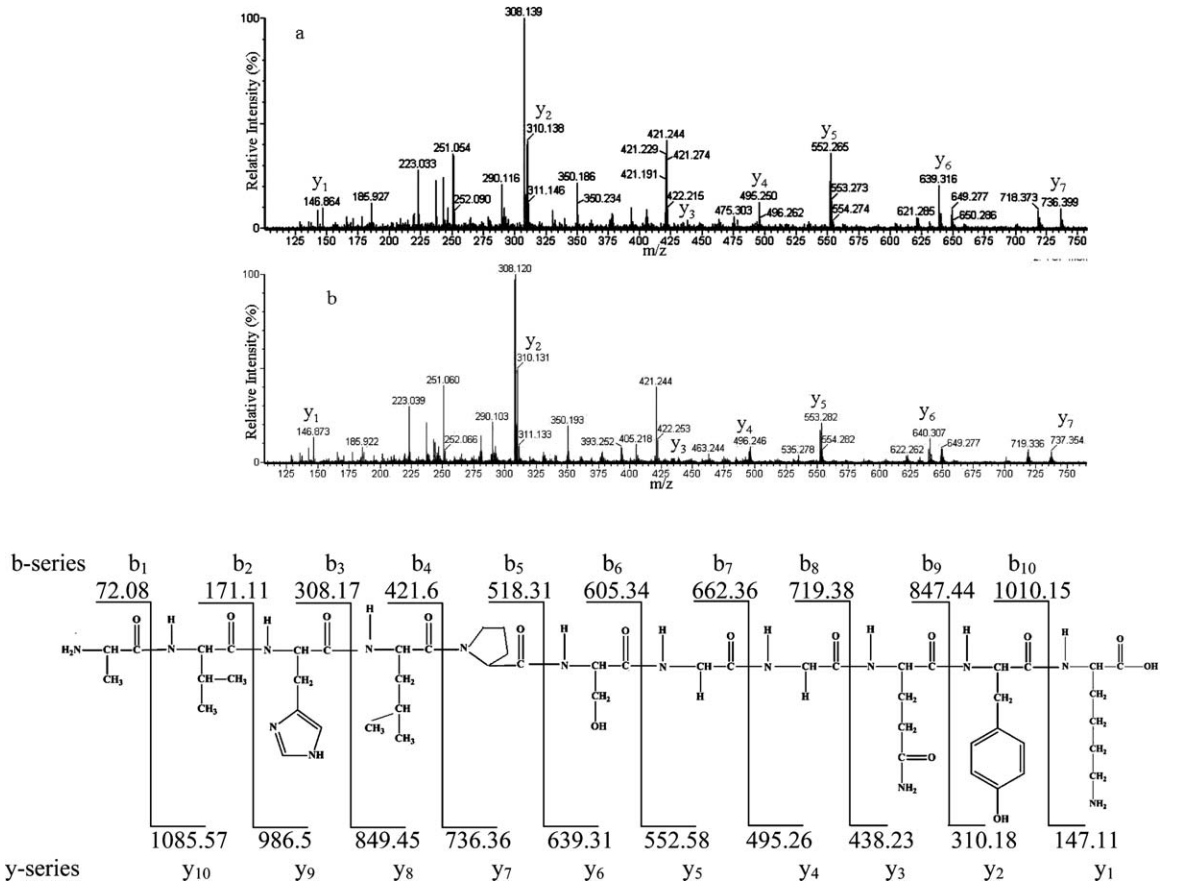

Figure 1. MS/MS spectra of (a) AVHLPSGGQYK ($m/z = 386.2$) and (b) the corresponding deamidated form of the peptide ($m/z = 386.5$). Spectra were obtained from separated HPLC peaks of a tryptic digest from an 88-year-old human lens. (c) The theoretical *y*- and *b*-ion fragments of AVHLPSGGQYK from γS crystallin. Masses were generated using the molecular weight calculator (http://ncrr.pnl.gov/software/).

deamidation in the human lens. Unfortunately, there is controversy in the literature concerning the sites and extent of deamidation in γS crystallin. With regard to Gln92, a residue located in the crucial linker region between the two domains, some authors have reported no deamidation,[11,12] whereas others have found significant deamidation[13,14] in older lenses.

In an effort to obtain information on the predominant types, and time courses, of modifications to proteins that turnover slowly, and are therefore submerged in physiological solution for decades, we have undertaken a program of research to determine crystallin modifications from human lenses of different ages.

In this study, we characterized a site of glutamine (Gln) deamidation in γS crystallin and monitored its rate of conversion to Glu92 as a function of time and compared this with deamidation of Gln170 and Glns in β-sheet regions of the protein.

Results

With age, both Gln and asparagine (Asn) residues can undergo deamidation.[13–19] When a peptide or protein is deamidated, its molecular weight increases by 0.98 Da at each site.[20] In this project, we first analyzed a tryptic peptide, AVHLPSGGQYK, derived from γS crystallin that appeared on the basis of LC/MS investigation, to show age-dependent deamidation. As the triply charged molecular ion for the original Gln-containing tryptic peptide has a mass of 386.2, the deamidated form should have a mass of 386.5. These two versions had different LC retention times, with the Gln92-containing version eluting first (~48 min), followed by the deamidated form (50–51.5 min).

To confirm the sequences of the two peptides, tandem mass spectrometry (MS/MS) was carried out, as there should be an increase in mass for some of the fragment ions if the peptide is indeed deamidated.[21] Two observations were made from the MS/MS spectra (Fig. 1). First, the experimental masses for the *y*-ions for the Gln-containing version AVHLPSGGQYK matched closely the theoretical values (Fig. 1). In contrast, there was an increase in mass (1 Da) for y_5 (553.282), y_6 (640.307), and y_7 (737.354) fragments for the deamidated peptide AVHLPSGGEYK. Second, some *b*-ion fragments for the deamidated version also had a mass of 1 Da higher than the amidated form e.g. b_8 (719.336),

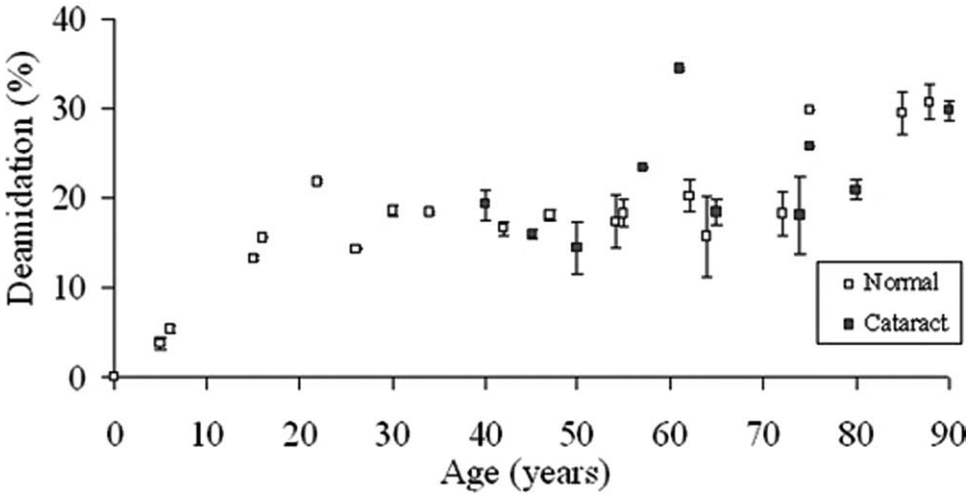

Figure 2. Deamidation of Gln92 in γS crystallin as a function of age. The degree of deamidation of AVHLPSGGQYK is shown for normal and cataract lens proteins. Normal: $y = -026x + 94.1$, $R^2 = 0.725$, two-sided $p < 0.0001$. Cataract: $y = -0.182x + 89.6$, $R^2 = 0.213$, two-sided $p = 0.179$). Mann-Whitney *U*-test, two-sided $p = 0.671$.

which confirmed the site of deamidation at Gln92. Having established by MS/MS that the Gln-containing, and deamidated, versions of the peptide were separated by LC, the proportion of each in lens protein digests could be calculated as a function of age.

The time course of deamidation of Gln92 in γS crystallin, as a function of age, is depicted in Figure 2. There was a linear increase in the proportion of the deamidated form with age, till the mid-teens, after which there was no further increase for the next 50 years. Following that there was a small increase from about age 70 to ~ 30% deamidation (Fig. 2).

The time course of deamidation of another Gln, Gln170, in γS crystallin, as a function of age, is depicted in Figure 3. As can be seen from the crystal structure (Fig. 4), Gln170 is located in another unstructured region of γS crystallin. Up until the age of 60 years, it showed a very similar profile of deamidation with time to that of Gln92. A major difference was in the extent of deamidation, which was approximately an order of magnitude lower than that for Gln92.

A group of cataract lenses was treated in the same manner as the normal lenses, and the results are also presented in Figures 2 and 3. It is important to note that age-related cataract lenses are available only after age ~ 40. There were no significant differences in the deamidation values for either Gln92 or Gln170 when compared with proteins from age-matched normal lenses.

Discussion

Deamidation of Asn and Gln residues takes place with age, in which the side chain amides are converted to aspartic acid and glutamic acid, respectively, and this appears to be a common PTM of

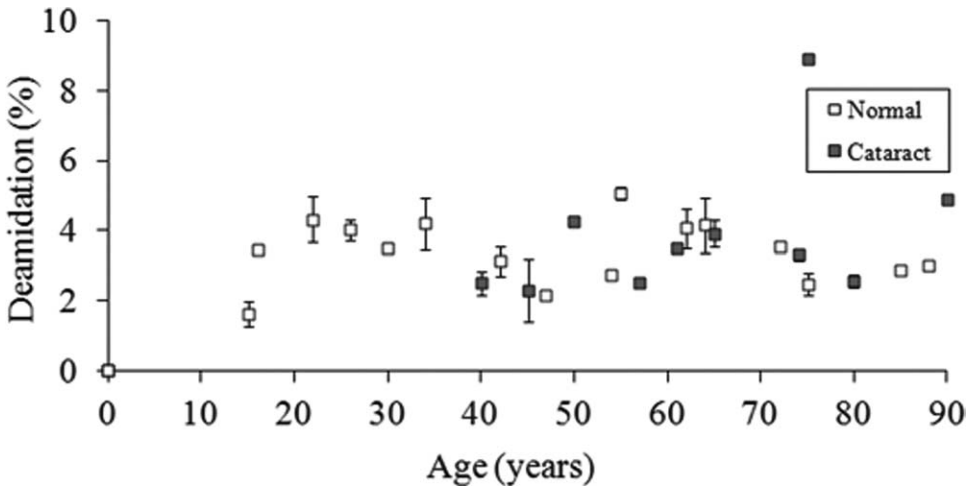

Figure 3. Deamidation of Gln170 in γS crystallin as a function of age. The degree of deamidation of KPIDWGAASPAVQSFR is shown for normal and cataract lens proteins. Normal: $y = -0.22x + 2.07$, $R^2 = 0.189$, two-sided $p = 0.0716$. Cataract: $y = 0.0527x + 0.514$, $R^2 = 0.187$, two-sided $p = 0.212$). Mann-Whitney *U*-test, two-sided $p = 0.853$.

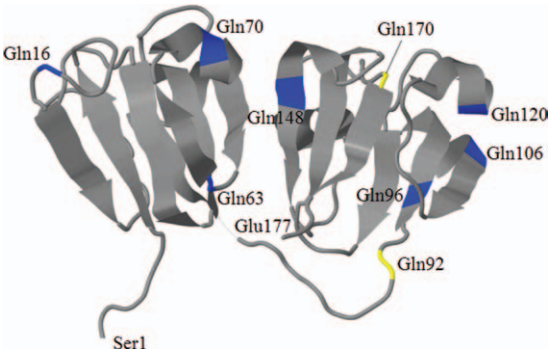

Figure 4. A three-dimensional representative structure of murine γS crystallin obtained from the Protein Data Bank (PDB; http://www.rcsb.org/pdb/home/home.do) showing all possible Gln residues in γS crystallin (blue) with Gln residues 92 and 170 (yellow). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

long-lived proteins.[20,22] In the human lens, deamidation is implicated in protein denaturation, as deamidated residues are found more commonly in insoluble crystallins.[15,23]

One rationale of this study was to resolve a conflict in the literature regarding deamidation of Gln92. Two studies suggested that there was no deamidation of this residue[11,12]; however, we obtained clear mass spectral evidence that there is significant deamidation at this site (Fig. 1). A probable reason for the difference in the two datasets is that the earlier studies that found no deamidation were based solely on HPLC elution times in the absence of mass spectral data.[11,12] Apparently, this method is subject to interference by coeluting species and is not sufficiently sensitive to detect deamidation.

There are a total of nine Gln residues in γS crystallin, yet few[13] appear to undergo significant deamidation. Gln92 is located in the linker region that joins the two domains (Fig. 4). One possible reason for preferential deamidation at Gln92 is that it may be more accessible to solvent and/or is able to adopt a greater range of structures than residues located in the two domains. Conformational flexibility seems to allow a greater degree of PTM. For example, Srivastava and Srivastava[24] have shown that cleavage at the adjacent GG sequence occurs in older lenses and other data (unpublished) shows that the Ser residue on the other side of the GG sequence also undergoes spontaneous cleavage.

Deamidation in proteins is not only affected by tertiary structure but is also influenced by the sequences of amino acid residues adjacent to the Asn or Gln.[20,25,26] When small residues such as glycine and alanine are adjacent to amide-containing side chains, they tend to promote deamidation.[27,28] Therefore, the Gly-Gly sequence in γS crystallin immediately preceding Gln92 should also act to facilitate deamidation.

As deamidation results in the formation of a carboxylate anion, this can alter ionic interactions and protein conformation. Analysis using Jmol software[29] revealed that Gln92 is in close proximity to Glu133 (8.6 Å), Glu112 (10 Å), Asp113 (12.7 Å), and Glu109 (13 Å). Thus, the formation of a Glu residue at position 92 might be expected to cause significant charge–charge repulsion and potentially to lead to a more open, unfolded tertiary structure. This addition of a negative charge could interrupt the short-range interactions of human γS crystallin that have been previously observed.[30] Recent data show that overall deamidation is more prevalent in insoluble proteins than soluble proteins from the same lens,[15] supporting the proposal that deamidation can contribute to denaturation and aggregation.[10,31–33]

In the case of Gln92 and Gln170, the degree of deamidation did not differ significantly between cataract and age-matched normal lenses, which is in agreement with previous data.[13] This may suggest that these particular modifications may contribute to age-related changes to the physical properties of the human lens, but not to cataract formation. On the other hand, other amides in γS crystallin such as Asn14, Asn76, and Gln16 do show distinct differences in the extent of deamidation between cataract and age-matched normal lenses and it could therefore be speculated that these may be causative for cataract.[13] In relation to this point, it should be emphasized that small changes to γ-crystallins, even those that do not cause major changes to the structure of the proteins,[34,35] may be sufficient to induce cataract.

Previous results for γS crystallin had indicated that by the age of 60 years, the degree of deamidation for Gln92 was ~ 20%,[13] which is in agreement with the data in this study and also corresponds with data from just three lenses reported by Hanson *et al.*[14] In this study, we mapped the time course of deamidation and found that the percentage of deamidation increased during childhood and early teens, then remained constant at ~ 20% for five decades (Fig. 2). There was a further increase to ~ 30% in the mid-70s. The reason for this unusual time course is not known. It is possible that one reason for this pattern is that other modifications are taking place at separate regions of the same protein. These may affect protein structure and could therefore influence the extent of deamidation. For example, modifications to γ-crystallins include racemization of Asp/Asn[11,14,36] and truncation.[24] Based on this time course, it does not therefore appear feasible to calculate protein age simply on the basis of the degree of deamidation.

These data illustrate another feature that "not all Glns are created equal." There are nine Gln residues in γS crystallin, and our results clearly show

that Gln92 is deamidated, as is Gln170. Both of these are in unstructured regions of the protein (Fig. 4), as is Gln16, which also appears to be deamidated[13]; however, this was not the focus of this investigation. By contrast, the other Glns are located in β-sheet regions of the protein and are either not deamidated or modified to a degree that was below the level of detection. The one possible exception to this rule is Gln120, which was reported to be deamidated in a previous publication.[13] A Gln in the linker region of γC crystallin is also deamidated with age.[13] In this sense, the Gln data are in accord with Asn deamidation in aged proteins. For example, in γS crystallin, Asn76 in the linker region is the most highly deamidated Asn residue.[37]

Taken together, a hypothesis can be constructed in which deamidation in aged proteins is determined largely by secondary structure, rather than other factors such as nearby residues; although these may influence the degree to which individual Asn and Gln amino acids are deamidated. The importance of secondary structure of proteins in determining deamidation was proposed by Clarke[22] and is supported by data on succinimide hydrolysis in proteins.[38]

As noted above, a picture is emerging from an examination of several proteins as a function of age, both in the lens and outside of the eye. Flexible regions appear to be more prone to age-related modifications such as deamidation and racemization than structured regions.[22,39] In addition, certain amino acid residues, such as Asp, Asn, and Ser, appear to be particularly susceptible to age-related modifications. Understanding the main factors that are responsible for the major PTMs of long-lived proteins may allow us in the future to predict those polypeptides that are most susceptible to age-related PTM and also the regions of these proteins that are most at risk. Such information will assist in characterizing PTMs in proteins such as the nuclear pore proteins that are known to undergo major age-related changes that affect function,[40] but as yet are unexplored in terms of the significant sites of age-related modification.

Conclusion

Structural proteins, such as γS crystallin, are present in the human lens for many decades. Two sites of deamidation, one involving Gln92 in the linker region of γS crystallin and the other Gln170, were characterized. The degree of deamidation for each was found to increase from birth to teen-age years and then to remain constant for four decades. Both sites that deamidated are in unstructured regions of the protein. There was no significant difference in the deamidation of Gln92 or Gln170 between cataract and age-matched normal lenses.

Methods and Materials

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.[41] Total proteins from the lens nucleus were extracted following a previous protocol.[37]

Liquid chromatography mass spectrometry

Tandem mass spectra were acquired using a Waters/Micromass quadruple time-of-flight (QTOF) Ultima mass spectrometer with a nanospray source (Manchester, UK). Tandem mass spectra were acquired using a Waters/Micromass QTOF Ultima mass spectrometer with a nanospray source (Manchester, UK). Peptides were separated by 1D LC using a nanoCap-LC auto-sampler system (Waters, Milford, MA) as described for αA crystallin.[37]

Data analysis

The triply charged ions for the γS crystallin-derived tryptic peptides for both amidated [m/z = 386.2 (Gln92) and 577.3 (Gln170)] and deamidated [m/z = 386.5 (Gln92) and 577.6 (Gln170)] were extracted. Their intensities from the extracted ion chromatogram (XIC) were 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 that the peak areas used corresponded to that of the matched peptide and not to an isobaric peptide. The percentage of deamidation was calculated by using the following formula: [deamidated/(amidated + deamidated)] × 100. A simple linear regression analysis and Mann-Whitney *U*-test were performed using a previous method.[41]

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