Deamidation of Specific Glutamine Residues from Alpha-A Crystallin during Aging of the Human Lens[†]

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ABSTRACT: Although it has been hypothesized that age-dependent deamidation of glutamine and/or asparagine residues may play an important role in the turnover of proteins in vivo, surprisingly little is known concerning the extents of deamidation of biologically important proteins with very long half-lives. Alpha-A crystallin is the most abundant protein of the adult human lens, which contains long-lived proteins in the central fetal-embryonic region that were synthesized before birth of the individual. Peptides, corresponding to tryptic fragments of alpha-A crystallin, were synthesized with either the expected glutamine-6, glutamine-50, and glutamine-147 residues, or deamidated glutamic acid residues at the same positions. These synthetic peptides were used to identify and quantitate the amidated versus deamidated forms of each tryptic fragment of alpha-A crystallin from the fetal-embryonic region of lenses from donors of increasing age up to 64 years old. The results demonstrate that all three glutamine residues are very stable, with glutamine-50 undergoing a maximum of approximately 30% deamidation after 64 years postsynthesis, while glutamine-6 and glutamine-147 undergo no detectable deamidation during the same period of time. Together, these results are consistent with the hypothesis that resistance to age-dependent, nonenzymatic deamidation may be an important prerequisite for the stability of proteins in vivo.

Both glutamine and asparagine residues of biologically important proteins have been shown to undergo nonenzymatic deamidation during the in vivo aging process (*1*). Because of the abundance of these residues, deamidation is considered the most common post-translational modification occurring in living systems. The introduction of a negative charge can result in structural changes, possibly making the protein more susceptible to endogenous proteases. In this regard, it has been hypothesized that nonenzymatic deamidation of glutamine and asparagine residues may act as a "biological clock" to program the turnover of proteins (*2*). Consistent with this hypothesis is the general correlation between increasing half-life of the protein with decreasing mole percent of glutamine plus asparagine (*3*).

Despite the possible importance of the deamidation process in the turnover of proteins, almost nothing is known concerning the extent of deamidation of proteins with very long half-lives. Knowledge of specific glutamine and/or asparagine deamidation half-lives is necessary before any conclusions can be made concerning the effects of deamidation upon protein stability.

Perhaps the best tissue for studying proteins with very long half-lives is the lens of the adult human eye. Fiber cells located in the fetal-embryonic region of the nucleus contain alpha, beta, and gamma crystallins which were synthesized only up to the time of the birth of the individual, resulting in a region of the lens that is comprised exclusively of proteins that coincide with the age of the donor. By using this tissue, a recent report (*4*) has determined that asparagine-101 of alpha-A crystallin has an approximate half-life of 15−20 years in the intact lens, making it the longest half-life of deamidation so far reported for any protein.

However, to determine if very long half-lives of deamidation correlate with the stability of this protein, it is first necessary to determine the half-lives of other glutamine and/or asparagine residues of the same protein, to establish a general trend. In the following report, we have determined the extents of deamidation of glutamine-50, glutamine-6, and glutamine 147 from alpha-A crystallin found in the fetal-embryonic regions of lenses from different age donors. The results indicate that even after approximately 64 years postsynthesis of the protein, all three of these glutamine residues of alpha-A crystallin have either low or undetectable levels of deamidation.

EXPERIMENTAL PROCEDURES

Lenses were obtained from the National Disease Research Interchange, and were stored at −75 °C until use. For the lens of age 41 days, the whole decapsulated lens was used for analysis, since most of the proteins were synthesized before birth. For all other lenses, the fetal-embryonic region was dissected as previously described (*5*). In an adult human lens from a 60-year-old donor, this region constituted approximately 15 vol % of the total lens mass.

The lens material was dissolved completely under anaerobic conditions using 1.0 mL of 7 M guanidine, 0.3 M Tris, 10 mM EDTA, pH 8.6, followed by reduced carboxymethylation using dithiothreitol and iodoacetate (*6*). After extensive dialysis against distilled water, protein concentration was determined according to Bradford (*7*), using bovine serum

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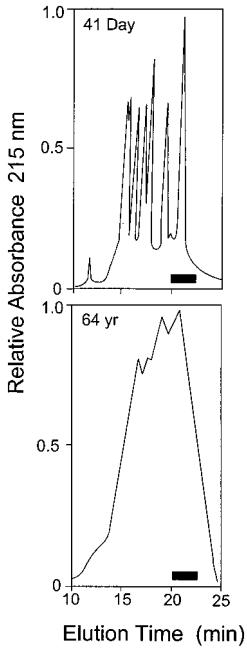

FIGURE 1: Preparation of fractions enriched in alpha-A crystallin. See Materials and Methods for details of lens solubilization, reduced alkylation, and resolution using reverse-phase chromatography. The solid horizontal bars designate the alpha-A crystallin fractions taken for further analysis.

albumin as standard, and 0.5 mg of protein was resolved using a C_{18} reverse-phase column (4.6 mm × 250 mm; Rainin Instrument Co., Woburn, MA), using a linear gradient of 30%−80% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid, at a flow rate of 1.0 mL/min over 30.0 min. The last peak eluting from the column was collected as alpha-A crystallin (*6*).

The alpha-A crystallin peaks from four identical runs were pooled, lyophilized, and digested for 16−20 h at 37 °C using 0.2 mL of total solution containing 0.1 M Tris, pH 7.4, 0.01% (w/v) sodium azide, and 12.5 μg of sequencing grade trypsin from bovine pancreas (Boehringer Mannheim, Indianapolis, IN). Approximately 10% of the digest was resolved on a C_{18} reverse-phase column (4.6 mm × 250 mm; Vydac, Hesperia, CA), using various linear gradients of acetonitrile−water in 0.1% (v/v) trifluoroacetic acid.

Synthetic peptides corresponding to expected amidated and deamidated tryptic fragments of alpha-A crystallin from human lens were synthesized by Research Genetics (Huntsville, AL), using FMOC chemistry. Purity and identity of the synthetic peptides were verified by mass spectral analysis and/or N-terminal sequencing at Research Genetics or at the Biotechnology Core Facility of Kansas State University.

RESULTS

Figure 1 shows typical elution profiles of protein from the total lens of a 41-day-old donor (top), and from the fetal-embryonic region of a 64-year-old donor (bottom). As previously determined, the last peak eluting from the reverse-phase column was alpha-A crystallin (*6*). This peak was collected, lyophilized, and digested with sequencing grade trypsin.

To identify and quantitate the degree of deamidation of specific glutamine residues of alpha-A crystallin, synthetic peptides were made that corresponded to the expected tryptic fragments containing either the amidated or deamidated form of glutamine/glutamic acid. These peptides were chosen because they contained only a single glutamine residue that could be deamidated. In addition, they were short enough to be easily synthesized in high yield and purity.

Table 1: Identity of Synthetic Peptides

name	sequence	identity
peptide 1	AcMDVTIQHPWFK	alpha-A 1−11 (amidated)
peptide 2	AcMDVTIEHPWFK	alpha-A 1−11 (deamidated)
peptide 3	AcM[O]DVTIQHPWFK	alpha-A 1−11 (oxidized, amidated)
peptide 4	AcM[O]DVTIEHPWFK	alpha-A 1−11 (oxidized, deamidated)
peptide 5	QSLFR	alpha-A 50−54 (amidated
peptide 6	ESLFR	alpha-A 50−54 (deamidated)
peptide 7	IQTGLDATHAER	alpha-A 146−157 (amidated)
peptide 8	IETGLDATHAER	alpha-A 146−157 (deamidated)

Table 1 lists the synthetic peptides corresponding to expected tryptic peptides of alpha-A sequences 1−11, 50−54, and 146−157, with and without deamidation of the single glutamine residue. Peptides 1 and 2 correspond to amidated and deamidated forms of residues 1−11, with acetylation of the N-terminal methionine (*8*). In addition, since this same methionine has also been reported to be partially oxidized to methionine sulfoxide (*8*), peptides 3 and 4 correspond to amidated and deamidated forms of the sequence after oxidation of methionine. Peptides 5 and 6 represent amidated and deamidated forms of residues 50−54, and peptides 7 and 8 correspond to amidated and deamidated forms of residues 146−157. Each of the synthetic peptides was used to determine the best acetonitrile gradient to clearly separate the amidated versus deamidated forms of the expected tryptic fragments. In addition, the synthetic peptides were used to construct a standard curve, so that the moles of amidated and deamidated species could be used to compute the percent deamidation.

Figure 2 shows typical elution profiles for the quantitation of glutamine-50 deamidation from the tryptic digest of alpha-A crystallin from a 41-day-old lens (top) versus the fetal embryonic section of a 64-year-old lens (bottom). At 41 days, only the amidated peptide 5 form is present (solid arrow), with no detectable amounts of the deamidated peptide 6 form (open arrow). At 64 years, the amidated peptide 5 form is still present (solid arrow), with the additional presence of the deamidated peptide 6 form (open arrow). The peak areas of known amounts of peptide 5 and peptide 6 were used to construct a standard curve, to compute the percentage deamidation defined as the moles of ESLFR per moles of QLSFR plus ELSFR, multiplied by 100.

Figure 3 shows the results of this quantitation. No detectable deamidation is present in a whole lens of age 41 days or in the fetal-embryonic region of lenses 10 years and 26 years of age. After this time, deamidation of glutamine-50 slowly increases with age, to a maximum of approximately 30% at 64 years of age.

A similar approach was used to determine the degree of deamidation of glutamine-6, present in the N-terminal tryptic peptide 1−11. Since the N-terminal methionine may be in either the oxidized methionine sulfoxide or unoxidized form (*8*), peptides 1−4 were used as markers to identify the elution times of the amidated and deamidated forms of glutamine. Figure 4 shows that the unoxidized, amidated form of

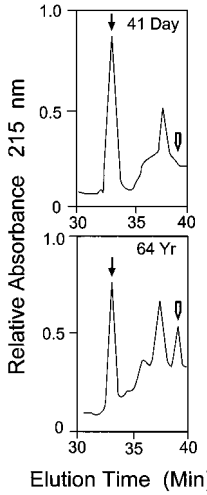

FIGURE 2: Resolution of peptides corresponding to amidated and deamidated forms of glutamine-50 from tryptic digests of alpha-A crystallin from the whole lens of a 41-day-old donor (top) and fetal-embryonic region of a lens from a 64-year-old donor (bottom). Peptides were resolved using a C_{18} reverse-phase column, with a linear gradient of 13%−16% (v/v) acetonitrile in water in 0.1% (v/v) trifluoroacetic acid, over a period of 60.0 min and flow rate of 1.0 mL/min. Only the elution profile containing peptides of interest is shown. Solid arrows designate the elution time of the amidated form of alpha-A crystallin sequence 50−54 (peptide 5), and open arrows designate the elution time of the deamidated form of alpha-A crystallin sequence 50−54 (peptide 6).

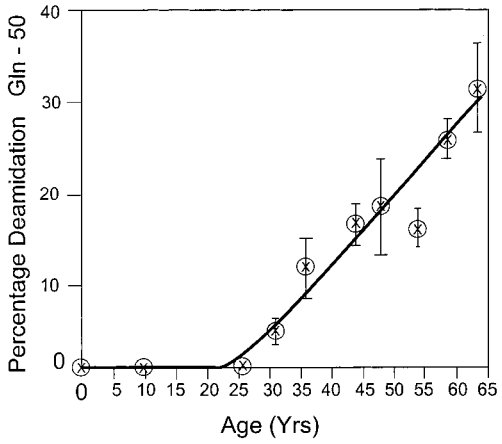

FIGURE 3: Deamidation of glutamine-50 during aging of the human lens. See Material and Methods for details of quantitation. The *x*-axis designates the age of the lens taken for analysis. Each value is the average of three determinations ± standard error.

sequence 1−11 (peptide 1, large solid arrow) is the predominant species present in a tryptic digest of alpha-A crystallin from a 41-day-old lens (top) and from a 64-year-old lens (bottom). Present in the 64-year-old sample, but lacking in the 41-day-old sample, is a small amount of the oxidized, amidated form of sequence 1−11 (peptide 2, small solid arrow at approximately 16 min). Figure 4 also shows that neither the unoxidized nor the oxidized form of the deamidated sequence 1−11 (peptides 2 and 4, small and large open arrows) was present in a tryptic digest of alpha-A crystallin from the 41-day-old lens (top) or the fetal-embryonic section of the 64-year-old lens (bottom). Identical analysis of fetal-embryonic sections from lenses of intermediate age resulted in the same conclusion (results not shown). Together, the results suggest that glutamine-6 from alpha-A crystallin is very stable, with little or no deamidation, even after 64 years of aging.

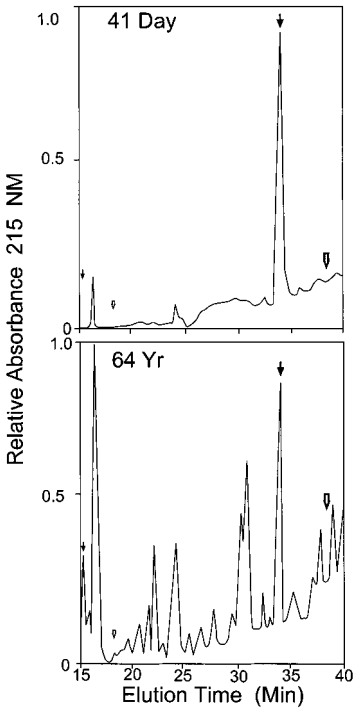

FIGURE 4: Resolution of peptides corresponding to amidated and deamidated forms of glutamine-6 from tryptic digests of alpha-A crystallin from the whole lens of a 41-day-old donor (top) and fetal-embryonic region of a lens from a 64-year-old donor (bottom). Peptides were resolved using a C_{18} reverse-phase column, with a linear gradient of 23%−30% (v/v) acetonitrile in water in 0.1% (v/v) trifluoroacetic acid, over a period of 60.0 min and flow rate of 1.0 mL/min. Only the elution profile containing peptides of interest is shown. Small solid arrows designate the elution time of the oxidized and amidated form of alpha-A crystallin sequence 1−11 (peptide 3), large solid arrows designate the elution time of the unoxidized and amidated form of alpha-A crystallin sequence 1−11 (peptide 1), small open arrows designate the elution time of the oxidized and deamidated form of alpha-A crystallin sequence 1−11 (peptide 4), and large open arrows designate the elution time of the unoxidized and deamidated form of alpha-A crystallin sequence 1−11 (peptide 2).

In an identical manner, peptides 7 and 8 were used to determine the degree of deamidation of glutamine-147 in the alpha-A crystallin sequence 146−157. Figure 5 shows the elution profile of the tryptic digest of alpha-A crystallin from total 41-day-old lens (top) and the fetal embryonic section of a 48-year-old lens (bottom). In fetal-embryonic sections greater than approximately 48 years of age, tryptic digests lacked both the amidated and deamidated forms of sequence 146−157 (peptides 7 and 8). The absence of both forms of the peptide is probably due to the known age-dependent cleavage from the C-terminal region of alpha-A crystallin during the aging process (*8*). As shown in Figure 5, the digests from the 41-day-old lens and 48-year-old lens both contained the amidated sequence (peptide 7, solid arrows), but no detectable deamidated sequence (peptide 8, open arrows). Analysis of tryptic digests of alpha-A crystallin preparations from the fetal embryonic section of lenses of intermediate age between 41 days and 48 years also demonstrated the absence of any detectable deamidated form of the sequence 146−157 (results not shown). The digest from the fetal-embryonic region of the 48-year-old lens did contain a major peptide that eluted at approximately 26 min that is not present in the digest from the 41-day-old

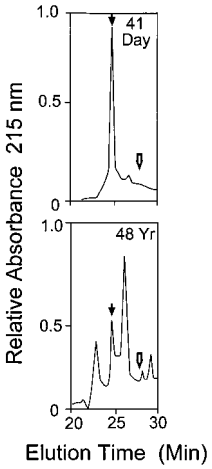

FIGURE 5: Resolution of peptides corresponding to amidated and deamidated forms of glutamine-147 from tryptic digests of alpha-A crystallin from the whole lens of a 41-day-old donor (top) and the fetal-embryonic region of a lens from a 48-year-old donor (bottom). Peptides were resolved using a C_{18} reverse-phase column, with a linear gradient of 12%−17% (v/v) acetonitrile in water in 0.1% trifluoroacetic acid, over a period of 60.0 min and flow rate of 1.0 mL/min. Only the elution profile containing peptides of interest is shown. Solid arrows designate the elution time of the amidated form of alpha-A sequence 146−157 (peptide 7), and open arrows designate the elution time of the deamidated form of alpha-A crystallin sequence 146−157 (peptide 8).

lens. Sequence and mass spectral analysis of this peptide demonstrated that it was not from alpha-A crystallin, but rather corresponded to the tryptic peptide 143−152 from gamma-4 crystallin, which was also present in the alpha-A preparation.

DISCUSSION

Although age-dependent, nonenzymatic deamidation is probably the most frequent type of post-translational modification occurring in proteins, very little is known concerning the half-lives of glutamine and/or asparagine deamidation for very stable proteins with long half-lives in vivo. Since there is almost no protein synthesis in the differentiated fiber cells of the lens, proteins from the central fetal-embryonic region of the lens represent macromolecules that were synthesized during fetal development of the organism. By carefully dissecting away the outer cortical and juvenile-adult region of nucleus, it is possible to obtain the fetal-embryonic region from human lenses of different age (*5*).

After solubilization of total proteins from this region under anaerobic conditions, reverse-phase chromatography was used to obtain a fraction highly enriched in alpha-A crystallin. Since the sequence and tryptic cleavage products of this protein are known (*8, 9*), it is now possible to use synthetic peptides to identify and quantitate the fragments that contain either the amidated or deamidated forms of specific glutamine residues. The synthetic peptides can be first used to optimize the gradient and/or type of reverse-phase column, to achieve maximum separation of the amidated versus deamidated tryptic fragments. Mixing of the synthetic peptide standards with the tryptic digest of alpha-A crystallin, followed by resolution of the resulting mixture, can then be used to quickly ascertain the identity of peaks from the tryptic digest that were suspected of being the peptides of interest. Finally, the same synthetic peptides can be used to quantitate the amount of each species, to obtain an estimate of the percent of deamidation occurring in vivo.

The results obtained in the present study suggest that glutamine-6, glutamine-50, and glutamine-147 of alpha-A crystallin undergo very slow, if any, deamidation in the lifetime of the individual. Of these three residues, glutamine-50 shows the most deamidation, starting at approximately 20−25 years postsynthesis, with a maximum degree of deamidation of approximately 30% at 60−65 years postsynthesis. Glutamine-6 and glutamine-147 undergo little, if any, deamidation during the same period.

In the only other study directed toward estimation of glutamine deamidation of alpha-A crystallin in the human lens, Lund et al. (*8*) used mass spectral analysis to approximate deamidation percentages of 50%−60% for glutamine-50, 5%−10% for glutamine-6, and 10%−15% for glutamine-147 in the lens of a 45-year-old donor. These values are difficult to compare with the present results using reverse-phase chromatography, since the study by Lund et al. (*8*) analyzed only the water-insoluble proteins from the total lens of a single donor, while the current study quantitated deamidation of total proteins from the fetal-embryonic region of lenses of different ages. Nonetheless, it is notable that the study by Lund et al. (*8*) and the results presented here both indicate that of the three glutamine residues analyzed, glutamine-50 is the most labile toward age-dependent deamidation, while glutamine-6 and glutamine-147 exhibit relatively lower levels of deamidation. In addition, the results of Lund et al. (*8*) compared with the present results seem quite reasonable, if the assumption is made that the water-insoluble material of the lens is preferentially enriched in deamidated species.

What is apparent from both studies is the remarkably low rate of deamidation of the above-mentioned glutamine residues during aging, especially of glutamine-6 and glutamine-147. Taken together with a previous study showing an in vivo half-life of approximately 15−20 years for asparagine-101 of the same protein (*4*), the results are consistent with the hypothesis that the low rates of deamidation may make significant contributions to the long-term stability of alpha-A crystallin in the aging human lens. On the basis of previous studies that have characterized the in vivo truncation sites of alpha-A crystallin (*8, 10*), almost all of the alpha-A crystallin molecules can exist for decades in the human lens, with the only significant cleavage at the C-terminal region, even though it has been well-documented that active proteases are present in the lens throughout the lifetime of the individual (*11*).

By understanding the molecular mechanisms responsible for the low rates of glutamine deamidation in vivo, it may be possible to better understand the basis for the very low rates of turnover of alpha-A crystallin in the intact human lens. Although poorly understood, previous studies have suggested that the primary sequence, as well as the higher order structure of proteins, may affect the in vitro and in vivo rates of nonenzymatic deamidation (*1*−*3*). Additional studies need to be done, to better understand why some glutamine residues undergo deamidation in a matter of hours (*1*), while other glutamine residues such as glutamine-6, glutamine-50, and glutamine-147 of alpha-A crystallin are not fully deamidated even 64 years after synthesis of the protein. Lens crystallins such as alpha-A crystallin, which

has abundance in the lens, low turnover, and very low rates of glutamine deamidation, would provide an excellent model system to correlate the effects of protein sequence and structure upon resistance to deamidation and subsequent low rates of protein turnover.

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