Specific Glutamine and Asparagine Residues of γ-S Crystallin Are Resistant to in Vivo Deamidation*

Received for publication, April 3, 2000, and in revised form, May 26, 2000
Published, JBC Papers in Press, June 7, 2000, DOI 10.1074/jbc.M002809200

Larry Takemoto‡ and Daniel Boyle

From the Division of Biology, Ackert Hall, Kansas State University, Manhattan, Kansas 66506

It has been hypothesized that resistance to nonenzymatic deamidation of asparagine and glutamine residues may be an important determinant of protein stability in vivo. As a test of this hypothesis, we analyzed the central region of old human lenses, which contain proteins such as γ-S crystallin that were synthesized during the fetal-embryonic periods of development. Total protein from the fetal-embryonic region of old human lenses was digested with trypsin, followed by resolution of tryptic fragments containing amidated and deamidated forms using high pressure liquid chromatography-reverse phase chromatography together with synthetic peptide standards and mass spectral analysis. The results demonstrate no detectable deamidation of glutamine 92, glutamine 96, asparagine 143, and glutamine 170 from γ-S crystallin from old human lenses, consistent with the hypothesis that very long-lived proteins can contain asparagine and glutamine residues that are extremely resistant to in vivo deamidation.

Previously studies have shown that α-S crystallin, purified from total proteins of the human lens, contains some glutamine residues that are resistant to in vivo deamidation (2). Because the β and γ crystallins belong to a different family of lens proteins, and because they comprise the majority of proteins present in the dry weight material of the lens, it is important to verify whether asparagine and glutamine residues in this class of lens proteins are also relatively resistant to in vivo deamidation.

Unfortunately, it is impossible to purify any of the β and γ crystallins from the aged human lens because they undergo a series of post-translational reactions that results in extensive heterogeneity in their molecular weights and charge properties. In addition, in the aged human lens, a significant percentage of β and γ crystallins are water insoluble, making it even more difficult to quantitatively assess the degree of deamidation of asparagine and glutamine residues of specific crystallins. To circumvent these difficulties, we first used trypsin to digest total lens proteins from the fetal-embryonic region of aged human lenses and followed this by using synthetic peptides corresponding to selected amidated and deamidated tryptic peptides of γ-S crystallin. These synthetic peptides have been used as markers, to develop the optimal acetonitrile gradients for separation of amidated and deamidated peptides. Because of the large number of tryptic peptides generated from treatment of lens proteins, when necessary we have also used sequential gradients containing different ion-pairing agents to resolve the amidated and deamidated species. As an indication of possible deamidation reactions occurring in the β and γ crystallins during aging, we have chosen to resolve possible amidated and deamidated species of glutamine 92, glutamine 96, asparagine 143, and glutamine 170 from γ-S crystallin. The results of our analyses demonstrate no detectable deamidation of these residues during the entire lifetime of lenses from aged human donors, consistent with the hypothesis that very long-lived proteins contain at least some glutamine and asparagine residues that are resistant to the deamidation process.

**EXPERIMENTAL PROCEDURES**

Normal human lenses ages 57, 60, and 66 years were obtained from the National Disease Research Interchange, Philadelphia, PA. The whole lenses were stored at −75 °C. After thawing the lens at room temperature, the fetal-embryonic region of each lens was obtained by dissection (3). This region of the lens was dissolved completely in 7 M guanidine hydrochloride, 10 mM EDTA, and 0.5 M Tris-hydrochloride, pH 8.6, and was then reduced by carboxymethylation and dialyzed against distilled water as described previously (2). Protein was determined as described previously (4) using bovine serum albumin as standard. The lyophilized protein (3.0 mg) was digested with 25 g of sequencing grade trypsin (Roche Molecular Biochemicals) for 16–20 h at 37 °C in 0.30 ml of buffer containing 0.01% (w/v) sodium azide and 0.1 M Tris-hydrochloride, pH 7.4.

Approximately 2–10% of the resulting solution was resolved on a 4.6 × 250-mm C18 column (Vydac, Hesperia, CA) using various linear gradients of acetonitrile in 0.1% (v/v) trifluoroacetic acid (see figure legends for specific gradients). Synthetic peptide standards were used.
to select gradients for the optimal resolution of peptides. Regions of the elution profile corresponding to selected amidated or deamidated tryptic peptides of γ-S crystallin were collected followed by lyophilization and further resolution using the same column with various linear gradients of acetonitrile in 0.1% (v/v) heptfluorobutyric acid (see figure legends for specific gradients). Synthetic peptides corresponding to amidated and deamidated tryptic peptides were synthesized by Research Genetics (Huntville, AL) using Fmoc (N-(9-fluorenyl)methoxy-carbonyl) chemistry. When necessary, peptide preparations were further purified using reverse phase chromatography. Characterization of peptides by mass spectrometry and Edman degradation was performed by the Biotechnology Core Facility of Kansas State University.

RESULTS

Table I lists synthetic peptide standards corresponding to expected tryptic fragments containing amidated or deamidated forms of glutamine 92, glutamine 96, asparagine 143, and glutamine 170. These synthetic peptides were used as markers to develop the optimal acetonitrile gradient, for the reverse phase resolution of these peptides from the tryptic digest of total proteins from the fetal-embryonic region of the aged human lens.

Table I Synthetic peptide standards

| γ-S residues | Sequence             |
|-------------|----------------------|
| 84–94 (amidated) | AVHLPSGGQYK           |
| 84–94 (deamidated) | AVHLPSGGFYK           |
| 95–100 (amidated) | IQIFEK               |
| 95–100 (deamidated) | IEIFEK               |
| 131–145 (amidated) | VLEGWIFYELPNYR       |
| 131–145 (deamidated) | VLEGWIFYELPDYR       |
| 158–173 (amidated) | KPIDNGASSPAVQSFPR    |
| 158–173 (deamidated) | KPIDNGASSPAVESFR     |

Fig. 1 shows the elution of tryptic peptides from the fetal-embryonic region of a 60-year-old human lens. A linear gradient of 30–40% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid was used over a period of 60 min, because it was found that these conditions clearly separated the amidated and deamidated forms of the γ-S sequence 131–145 (see solid and split arrows in Fig. 1). A peak eluting at 28.5 min coincided with the expected elution time of the amidated peptide, but no peak was found with the expected time of the deamidated form of this same sequence. Mass spectral analysis of the 28.5 min peak showed that it comprised a single component of M, 1898.7, which within experimental error was the same as the calculated value of 1898.2 for the amidated form of sequence 131–145. The results of Fig. 1 therefore demonstrate that no detectable deamidation of asparagine 143 in sequence 131–145 has occurred during the approximately 60 years since the protein was synthesized.

In a similar manner, synthetic peptides of γ-S sequence 158–173 were used to develop the optimal acetonitrile gradient for the separation of the amidated and deamidated forms of this sequence. Fig. 2 shows a major peak eluting at 19.5 min (solid arrow) that coincides with the elution time of the amidated form of the peptide. In addition, there were two minor peptides that eluted at the approximate time of the deamidated form (split arrow). To unambiguously determine whether any of these minor peaks corresponded to the deamidated form, the elution interval designated by the horizontal bar was collected, followed by resolution using a different acetonitrile gradient containing 0.1% (v/v) heptfluorobutyric acid instead of trifluoroacetic acid as ion-pairing agent. Previous results have shown these different ion-pairing agents can interact differently with peptides (5), making it possible to resolve a complex mixture of peptides on a reverse phase column by using sequential gradients containing either trifluoroacetic acid or heptfluorobutyric acid. Fig. 3 shows that the amidated form of sequence 158–173 was present as a peak eluting at 29.5 min, with the absence of any peaks eluting at the elution time of the deamidated form of this sequence. Mass spectral analysis of the 29.5 min peak demonstrated that it comprised a single component of M, 1730.1, which within experimental error is the same as the calculated M of 1729.9 for the amidated sequence.

Fig. 4 shows the resolution of tryptic peptides from the same lens region using an acetonitrile gradient of 10–25% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid. The conditions would be expected to include the elution times of the amidated and
The results of Figs. 1–6 therefore demonstrate that for the fetal-embryonic region of a 60-year-old lens, there is no detectable deamidation of asparagine 143, glutamine 170, glutamine 92, and glutamine 96 from γ-S crystallin. In an identical manner, the fetal-embryonic regions of two other lenses, age 57 and 66 years, were also shown to contain the same γ-S sequences in only the amidated form (results not shown). Taken together, these results demonstrate the extraordinary stability of some of the asparagine and glutamine residues of γ-S crystallin toward in vivo deamidation during aging of the normal human lens.

**DISCUSSION**

Because nonenzymatic deamidation of asparagine and glutamine residues is thought to be an important factor in the turnover of proteins, it is possible that resistance to deamidation, in vivo, is a characteristic of long-lived proteins. Previous reports have characterized the deamidation rate of proteins with half-lives as short as 3–4 h and as long as 3–4 months (1). Rapidly growing cells contain many enzymes and structural proteins that deamidate rapidly (1), an observation consistent with the importance of deamidation in protein turnover. In contrast, very little information is available on proteins that are extremely stable in vivo. Resistance to deamidation of such proteins might support the hypothesis that deamidation rates play a role in their relative rates of turnover in vivo.

The central, fetal-embryonic region of the aged human lens is perhaps the best tissue to use in studying the relationship between nonenzymatic deamidation and protein stability of long-lived proteins. This region comprises so-called fiber cells that are lacking intracellular organelles. Although preliminary studies suggest a possible intercellular exchange of proteins within the central region of the lens (6), it is generally believed that proteins within this region were synthesized early in the lifetime of the individual during the fetal-embryonic period of lens development. The current study has taken advantage of the availability of old human lenses, in which the central fetal-embryonic region contains a relatively small number of proteins for which complete sequences have already been deter-
Deamidation Resistance of Glu and Asp Residues

It is hypothesized that because the crystallins exhibit remarkable stability over the lifetime of the individual, they should contain asparagine and glutamine residues that are relatively resistant to in vivo deamidation. By using synthetic peptides corresponding to expected amidated and deamidated tryptic fragments, it is now possible to determine precisely the optimal high pressure liquid chromatography gradient to separate the two forms from themselves and from other contaminating peptides. A key part of this methodology involves the sequential use of two different ion-pairing agents, trifluoroacetic acid and heptafluorobutyric acid, in the acetonitrile gradients. Previous studies have demonstrated that these two reagents differentially interact with peptides (5), making it possible to resolve and quantitate the presence of specific sequences among the hundreds of peptides present in a tryptic digest of total lens. In addition, it is anticipated that this experimental approach can be used to quantitate deamidation of specific asparagine and glutamine residues in any aged tissue, where it is expected that many peptide fragments will be generated after tryptic digestion.

Previous studies using α-A crystallin purified from total proteins from the same region of the aged human lens have suggested that some, but not all, selected glutamine residues were also resistant to age-related deamidation (2). Because α-A crystallin has a sequence and probable structure completely different from the β/γ crystallins, the present study has investigated the possibility that a member of the β/γ family of crystallins might also contain glutamine and/or asparagine residues resistant to deamidation. The β and γ crystallins of the human lens have related sequences and similar structures, together making up the bulk of proteins from the lens.

The results of the current study demonstrate that one of the major members of the β/γ crystallin family, γ-S crystallin, contains some glutamine and asparagine residues that are extremely resistant to in vivo deamidation, consistent with the hypothesis that resistance to age-dependent deamidation is a property common to long-lived proteins. Specific tryptic peptides were chosen that contained only a single glutamine or asparagine residue. This experimental approach minimized the number of synthetic peptide standards necessary for development of the optimal acetonitrile gradients. With the appropriate synthetic peptides, however, this same methodology can also be used to identify and quantitate deamidation in tryptic fragments containing multiple glutamine and/or asparagine residues.

Recently, mass spectral analysis has been used to approximate the deamidation of various glutamine and asparagine residues of γ-S crystallin from whole human lens (7). The results suggested that glutamine 92 and glutamine 170 undergo significant deamidation during the aging process. The reasons for the discrepancy with the present results are not known, but it may be related to differences in the methods of quantitation. In addition, the previous study (7) characterized the water-soluble fraction of whole lenses, whereas the present study quantitated possible deamidation of total proteins from the fetal-embryonic region of old lenses. A priori, one would expect increased deamidation in total proteins from the oldest part of the lens, although it is possible that increased deamidation may allow for a greater proportion of γ-S crystallin to be found in the water-soluble fraction. Further studies, involving quantitation of the same protein fraction by the two different methodologies, are necessary to obtain a more accurate determination of the extents of deamidation of these two residues.

Although the reasons for the exceptional stability of glutamine 92, glutamine 96, asparagine 143, and glutamine 170 of γ-S crystallin toward age-related deamidation are not well understood, such information would be important in understanding the molecular basis of selective deamidation during the aging process. Analysis of the extent of deamidation of peptides and proteins has established that secondary protein structure (8), as well as the primary sequence near the asparagine residue (9), may determine the liability toward deamidation. γ-S protein from aged human lens should provide an ideal system to study the effects of protein structure upon deamidation, because much is known about the three-dimensional structure of this polypeptide (10). Molecular modeling of this protein has suggested that glutamine 92, glutamine 96, asparagine 143, and glutamine 170 are all solvent-exposed. Resistance to age-dependent deamidation for such residues may be particularly important because they may be involved in the short-range order of crystallins that has been hypothesized to be necessary for lens transparency (11).

In addition to the primary sequence, it is also possible that different environments of cells containing long-lived versus short-lived proteins may play an important role in the stability of proteins in vivo. According to this theory, the same polypeptide sequence could have different half-lives depending upon cell-specific factors that may affect the rates of turnover. An excellent test of this hypothesis would be to determine the half-lives and rates of deamidation of crystallins in lens versus non-lens tissue. With the methodology described in this report, it should be possible to quantitate the deamidation rates of all major crystallin species in different tissues, to obtain unique insights into protein structural parameters and/or cellular environments that may result in the differential resistance of specific glutamine and asparagine residues toward in vivo deamidation.

Acknowledgments—The authors thank P. Buhr for technical assistance and K. Wyatt for the production of figures.

REFERENCES
1. Robinson, A. B., and Rubi, C. J. (1974) Curr. Top. Cell. Regul. 8, 248–295
2. Takemoto, L., and Boyle, D. (1998) Biochemistry 37, 13681–13685
3. Takemoto, L., and Boyle, D. (1998) Exp. Eye Res. 67, 119–120
4. Bradford, M. (1976) Anal. Biochem. 72, 248–254
5. Guo, D., Mant, C. T., and Hodges, R. S. (1987) J. Chromatogr. 386, 205–222
6. Shestopalov, V. I., and Bassnett, S. (2000) J. Cell. Sci. 113, 1913–1921
7. Hanson, S. R., Smith, D. L., and Smith, J. (1998) Exp. Eye Res. 67, 301–312
8. Xie, M., and Schowen, R. L. (1999) J. Pharm. Sci. 88, 8–13
9. Tyler-Cross, R., and Schirch, V. (1991) J. Biol. Chem. 266, 22549–22556
10. Zarina, S., Slingsby, C., Jaenicke, R., Zaidi, Z. H., Driessen, H., and Srinivasan, N. (1994) Protein Sci. 3, 1840–1846
11. Delaye, M., and Tardeau, A. (1983) Nature 302, 415–472

3 C. Slingsby, personal communication.
Specific Glutamine and Asparagine Residues of γ-S Crystallin Are Resistant to in Vivo Deamidation
Larry Takemoto and Daniel Boyle

J. Biol. Chem. 2000, 275:26109-26112.
doi: 10.1074/jbc.M002809200 originally published online June 7, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M002809200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 11 references, 2 of which can be accessed free at
http://www.jbc.org/content/275/34/26109.full.html#ref-list-1