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Deamidation of α-A Crystallin from Nuclei of Cataractous and Normal Human Lenses

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Purpose: To quantitate the extent of deamidation of asparagine-101, glutamine-50, and glutamine-6 of α-A crystallin in the nucleus from human cataractous and normal lenses.
Methods: Reverse phase chromatography was used to prepare α-A crystallin from total proteins of the nucleus from cataractous and age-matched normal human lenses. Synthetic peptides were made corresponding to the expected amidated and deamidated tryptic fragments containing asparagine-101, glutamine-50, and glutamine-6. The peptides were used to identify and quantitate amidated and deamidated forms of tryptic fragments from α-A crystallin eluting from a reverse phase column.
Results: Significant amounts of deamidation of asparagine-101 and glutamine-50, but not glutamine-6, were present in α-A crystallin from nuclear sections of both cataractous and age-matched normal lenses. Quantitative analysis of tryptic peptides containing these residues indicated no statistical difference in deamidation in cataractous versus normal lenses.
Conclusions: There was no significant difference in the extent of deamidation of asparagine-101, glutamine-50, and glutamine-6 for α-A crystallin, purified from the nucleus of cataractous versus age-matched normal lenses. These results strongly suggest that deamidation of these residues does not play a role in the biogenesis of human nuclear cataract.

It has been known for many years that glutamine and asparagine residues can undergo age-dependent, nonenzymatic deamidation, both in vivo and in vitro [1]. Because of their abundance in most proteins, this covalent change is probably the most prevalent posttranslational modification in aging tissue. During conversion of glutamine or asparagine residues to glutamate or aspartate residues, the introduction of a negative charge may alter the structural properties of the protein, resulting in a greater accessibility to the action of endogenous proteases. The same structural changes may also lead to the formation of high molecular weight protein aggregates of sufficient size, that may directly scatter the incident light seen in a human nuclear cataract [2].

As one of the most abundant proteins of the human lens, α-A crystallin has been implicated in the above mentioned structural changes that may be involved in the opacification process [3,4]. In addition, α-A crystallin is a member of the family of small heat shock proteins that play an important role in the prevention of protein denaturation and eventual aggregation [5]. An increase in nonenzymatic deamidation could therefore result in structural changes that may decrease the molecular chaperone properties of this protein, leading to an acceleration of the denaturation process in vivo.

One of the most common forms of human cataractogenesis involves opacification of the lens nucleus, prevalent in older patients. The age-dependent onset of this type of cataract suggests that the process of lens opacification may be linked to an acceleration of biochemical mechanisms involved in the aging process, such as nonenzymatic deamidation of glutamine and asparagine residues. To assess the possible role of deamidation in α-A crystallin during formation of nuclear cataract, this protein was prepared from the nucleus of nuclear cataracts and age-matched normal lenses, followed by tryptic digestion and quantitation of the tryptic fragments containing representative amidated and deamidated forms of specific asparagine and glutamine residues. The results illustrate the feasibility of using synthetic peptide standards to quantitate the deamidation of specific glutamine/asparagine residues, and demonstrate that deamidation of asparagine-101, glutamine-50, and glutamine-6 of α-A crystallin does not play an important role in development of opacification in the nucleus of the human lens.

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METHODS

Lenses: Cataractous lenses, containing opacifications of grade 3-4 [6] were removed by intracapsular extraction. Normal lenses showing no visual opacities were obtained from the National Disease Research Interchange. Informed consent was obtained in all cases, and this research was approved by the institutional human experimentation committee. The lenses were stored at -75 °C until use. The thawed lenses were decapsulated, and the cortical fiber cells were carefully removed by dissection under a microscope, leaving the nucleus, which represented approximately 90% of the total lens mass.

The nucleus was dissolved anaerobically in 2.0 ml solution containing 7 M guanidine hydrochloride, 0.3 M Tris, 10 mM EDTA, pH 8.6, then reduced and carboxymethylated with dithiothreitol and iodoacetate, as previously described [7]. After extensive dialysis against distilled water, protein was determined according to Bradford [8], using bovine serum albumin as standard.

Preparation and Digestion of α-A Crystallin: The total dialyzed material containing both soluble and precipitated proteins was lyophilized, and approximately 0.5 mg of total lens protein was dissolved completely in 0.3 ml of 7 M guanidine hydrochloride, then resolved, using a wide pore C_{18} column (4.6 mm x 250 mm, Rainin Instrument Co., Walnut Creek,

CA), with a linear gradient of 30 - 80% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid, and flow rate of 1.0 ml/min. As previously described [7], the last peak eluting from the column was taken as α-A crystallin. The purification procedure was repeated three more times, and the pooled α-A crystallin peaks were lyophilized, then incubated 16-20 hours in 0.2 ml of solution containing 0.1 M Tris (pH 7.4), 0.01% (w/v) sodium azide, and 12.5 μg sequencing grade trypsin (final concentration 62.5 μg/ml) from bovine pancreas (Boehringer Mannheim Corp., Indianapolis, IN).

Quantitation of Amidated and Deamidated Peptides: Approximately 10% of the resulting solution was resolved using either a C_8column (4.6 mm x 250 mm; Phenomenex, Torrance, CA) or a C_{18} column (4.6 mm x 250 mm; Vydac, Hesperia, CA), using various linear gradients of acetonitrile in 0.1% (v/v) trifluoroacetic acid. Synthetic peptides, corresponding to the expected amidated and deamidated tryptic peptides of α-A crystallin, were synthesized using F-MOC chemistry, by Research Genetics (Huntsville, AL). Purity and correct sequence of each peptide were verified by mass spectral analysis and/or N-terminal sequencing, by either Research Genetics, or by the Biotechnology Core Facility of Kansas State University. Synthetic peptides were used to develop the best acetonitrile gradient for resolution of amidated and deamidated forms of the tryptic peptide. Identities of the eluted peptides from tryptic digests were confirmed by Edman degradation and/or mass spectral analysis. The synthetic peptides were also used as standards for quantitation. Known amounts of each peptide were resolved using reverse phase chromatography, and the areas of the peaks were used to construct a standard curve for quantitation of the same peptides from tryptic digests.

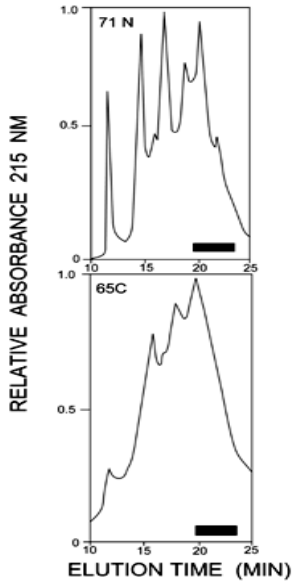

Figure 1. Preparation of α-A crystallin. Resolution of total proteins from nuclear sections of a 71 year old normal lens (top; 71N) and 65 year old cataractous lens (bottom; 65C) with opacification of the nucleus. See Methods for details of lens dissection, protein dissolution, and reduced carboxymethylation. Approximately 0.5 mg of protein was resolved on a C_{18} wide pore column (4.6 mm x 250 mm, Rainin Instruments), using a linear gradient of 30 - 80% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid, over a period of 30 min, at a flow rate of 1.0 ml/min. Horizontal bars designate the α-A crystallin peaks collected for further analysis.

RESULTS

Figure 1 shows reverse phase elution profiles for the resolution of total lens proteins from the nucleus of a 71 year old normal lens (top) and 65 year old cataractous lens (bottom). In both cases, the last peak was collected and digested with sequencing grade trypsin. Previous results demonstrated that all detectable α-A crystallin eluted in this peak (results not shown).

Previous studies [7] have also shown that it is possible to resolve the amidated versus deamidated forms of asparagine-101, present in the tryptic peptide HNER/HDER, corresponding to residues 100-103 of α-A crystallin from the aging human lens. Figure 2 shows the resolution of a tryptic digest of α-A crystallin from the nuclear region of a 71 year old normal lens (top) and 65 year old cataractous lens with nuclear opacity (middle). The amidated HNER form is clearly resolved from the deamidated HDER form, as shown by resolution of the HNER and HDER synthetic peptides (Figure 2, bottom). The results of Figure 2 also show similar degrees deamidation of α-A crystallin from cataractous versus normal lens. As

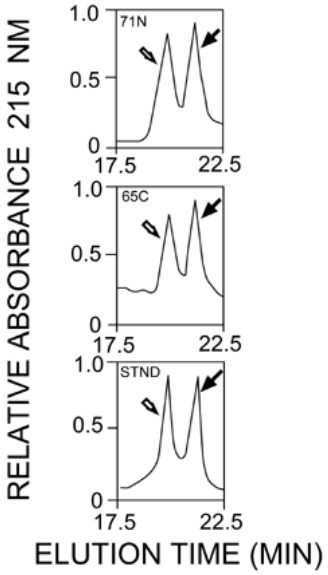

Figure 2. Quantitation of asparagine-101 deamidation. Resolution of amidated and deamidated forms of tryptic peptides corresponding to sequence 101-103 of α-A crystallin. The tryptic digest was resolved on a C_8 reverse phase column (4.6 mm x 250 mm, Phenomenex) using a linear gradient of 0 - 10% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid over 27 min, followed by a linear gradient of 10 - 60% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid, over a period of 5 min. Only the part of the elution profile showing peptides of interest is shown. Top, digest from 71 yr old normal lens (71N); middle, digest from 65 yr old cataract lens (65C) with opacification of the nucleus; bottom, synthetic peptide standards (STND). Open arrows designate elution of amidated peptide HNER, closed arrows designate elution of deamidated peptide HDER.

shown in Table 1, identical analyses were conducted using four other normal lenses and four other cataractous lenses with nuclear opacification. Within experimental error, the nuclear region from both types of lenses contained the same percent deamidation of asparagine-101.

Using synthetic peptides as markers and standards for quantitation, previous studies have also shown that it is possible to quantitate the extent of glutamine-50 deamidation in the tryptic fragment QLSFR of α-A crystallin during aging of the normal human lens [9]. Figure 3 shows the same reverse phase column conditions as previously described [9] for the tryptic digests of α-A crystallin from the nucleus of 71 year old normal lens (top) and 65 year old cataractous lens with nuclear opacity (middle). Based upon resolution of the QLSFR and ELSFR standards (Figure 3, bottom), the amidated and deamidated forms of the peptide can be clearly resolved and quantitated. The deamidation of glutamine-50 for five normal lenses and five cataractous lenses is shown in Table 1. Within experimental error, α-A crystallin from the nuclear region of both normal and cataractous lenses contained the same amounts of deamidation, demonstrating no changes in the conversion of glutamine-50 to glutamate-50 during lens opacification.

Figure 4 shows the resolution of tryptic digests containing the sequence 1-11 from α-A crystallin. This sequence contains the N-terminal methionine, which is acetylated and is partially oxidized to methionine sulfoxide in the aging human lens [10]. It also contains a glutamine at residue 6, which can potentially be deamidated. For use as markers and standards, four peptides were synthesized corresponding to the acetylated N-terminus without methionine oxidation and without deamidation (AcMDVTIQHPWFK), the acetylated N-terminus without methionine oxidation and with deamidation (AcMDVTIEHPWFK), the acetylated N-terminus with methionine oxidation and without deamidation (AcM[0]DVTIQHPWFK), and the acetylated N-terminus with methionine oxidation and with deamidatiion (AcM[0]DVTIEHPWFK).

Figure 4 (bottom) shows that all four of these peptides could be resolved as previously described [9] by a linear gradient of 23.0 - 30.0% (v/v) acetonitrile in 0.1 (v/v) triflouroacetic acid. Figure 4 also shows the resolution of tryptic peptides from a digest of α-A crystallin from the nucleus of a 71 year old normal lens (top) and 65 year old cataractous lens (middle). For both types of lenses, the major forms of the sequence 1-11 are the unoxidized, amidated sequence (large open arrows) and the oxidized, amidated sequence (small open arrows). Not detectable are the unoxidized, deamidated sequence (large solid arrows) and the oxidized, deamidated sequence (small solid arrows), demonstrating no significant deamidation of glutamine-6, either in normal or cataractous lenses. Table 1 shows that identical results were obtained after analysis of α-A crystallin from the nuclear region of four other normal and four other cataractous lenses.

Table 1. Deamidation of Asn-101 & Gln-50

Donor Age (yr)	Deamidation Asn-101*	Deamidation Gln-50*	Deamidation Gln-6
Normal lens:			
59	52.6 ± 1.3	16.7 ± 1.3	Not Detectable
61	51.8 ± 0.8	15.1 ± 2.5	Not Detectable
64	54.1 ± 0.7	14.2 ± 2.9	Not Detectable
66	54.7 ± 2.9	14.2 ± 3.3	Not Detectable
71	53.8 ± 2.5	18.9 ± 6.9	Not Detectable
AVERAGE ± S.E.	53.4 ± 1.2	15.8 ± 2.0	
Cataractous lens:			
59	57.0 ± 2.5	21.6 ± 3.5	Not Detectable
60	46.1 ± 4.5	14.8 ± 1.7	Not Detectable
60	49.2 ± 5.3	14.8 ± 1.3	Not Detectable
65	56.1 ± 2.2	15.7 ± 3.0	Not Detectable
70	52.5 ± 3.2	17.1 ± 1.0	Not Detectable
AVERAGE ± S.E.	52.2 ± 4.6	16.8 ± 2.8	

*Percent deamidation as described in Methods, average of three determinations ± standard error.

DISCUSSION

As one of the most abundant proteins of the adult lens, post-translational changes in the sequence of α-A crystallin may result in structural changes that play a key role in the development of age-dependent nuclear opacities. It has been hypothesized that structural changes in lens proteins such as α-A crystallin can result in the formation of high molecular weight aggregates large enough to scatter incident light [2]. Structural changes can also alter the short-range order of protein-protein interactions necessary for the maintenance of the transparent properties of the lens [11]. Finally, structural changes can alter the molecular chaperone properties of the α-A molecule [5], resulting in an altered ability to inhibit the denaturation and eventual aggregation of lens proteins.

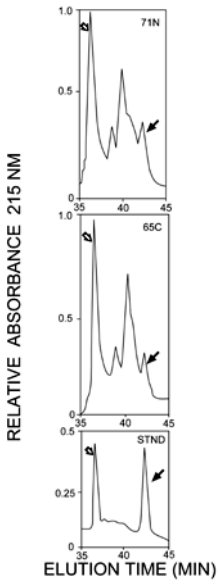

Figure 3. Quantitation of glutamine-50 deamidation. Resolution of amidated and deamidated forms of tryptic peptides corresponding to sequence 50-54 of α-A crystallin. The tryptic digest was resolved on a C_{18} reverse phase column (4.6 mm x 250 mm, Vydac) using a linear gradient of 12 - 17% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid over a period of 60 min, followed by linear gradient of 17 - 60% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid over a period of 5 min. Only the part of the elution profile showing peptides of interest is shown. Top, digest from 71 yr old normal lens (71N); middle, digest from 65 yr old cataractous lens (65C) with opacification of the nucleus; bottom, synthetic peptide standards (STND). Open arrows designate elution of amidated peptide QLSFR, closed arrows designate elution of deamidated peptide ELSFR.

All of these changes may at least in part be the result of posttranslational modifications such as oxidation, truncation, and deamidation that have been known to occur in the α-A crystallin molecule during the process of normal aging in the human lens [12]. Based upon these findings, a primary objective of current studies is to determine if any of the above mentioned modifications are accelerated or inhibited during the opacification process. This is especially relevant for the process of nonenzymatic deamidation, which is probably the most commonly found posttranslational modification in aging lens tissue. Introduction of a negative charge following the deamidation process may lead to structural alterations of the α-A crystallin molecule, possibly resulting in formation of high molecular weight aggregates, a change in short-range order involving α-A crystallin, and/or a change in the ability of the α-A molecule to prevent denaturation and aggregation in vivo.

Deamidation may be at least partially responsible for the previously observed unfolding of α-crystallin molecules during the aging process [13]. This unfolding may not necessarily be deleterious to chaperone function, since thermal denaturation studies have shown that partially unfolded α-crystallin possesses higher chaperone activity in vitro [14,15]. The partially unfolded protein may possess increased exposure to key hydrophobic residues of α-A crystallin that are thought to be involved in chaperone activity [16]. It is also possible that other posttranslational modifications such as oxidation or truncation can influence chaperone function. Based upon in vitro studies [17,18], both these modifications may decrease the ability of α-A crystallin to prevent denaturation of lens proteins.

The present study indicates that for at least asparagine-101, glutamine-50, and glutamine-6, there is no significant increase in deamidation during formation of the nuclear cataract. These residues were chosen because their corresponding amidated and deamidated tryptic peptides were easily synthesized, and because their rates of age-dependent deamidation represented a wide range from essentially no deamidation (glutamine-6 [9]), intermediate deamidation (glutamine-50 [9]), to almost complete deamidation (asparagine-101 [19]) during the lifespan of the human lens. The results suggest that although deamidation may be one of the most common forms of posttranslational modification occurring in α-A crystallin of the aging human lens, there is no acceleration or inhibition of this process for the above mentioned amino acid residues during nuclear opacification.

Nonetheless, this conclusion does not imply that other glutamine/asparagine residues of α-A crystallin or other lens crystallins also do not differ in their rates of deamidation during human cataractogenesis. Previous studies [10] have demonstrated that the extents of deamidation for individual glutamine and asparagine residues differ greatly within the α-A crystallin sequence. In vitro studies have also shown that primary sequence, as well as secondary and tertiary structure, may influence the rate of nonenzymatic deamidation of a protein [1]. Since changes are known to occur in the primary sequence of α-A crystallin [3,20], as well as possibly in the tertiary structure of this protein during cataractogenesis of the human lens, it is possible that some of these changes may influence the extents of deamidation of other glutamine and/or asparagine residues of the α-A crystallin molecule. With the methodology described in the present study, it should be possible to quantitate the degrees of deamidation of other glutamine and asparagine residues of the major crystallins, to verify whether changes in the deamidation rate for any of these residues may correlate with the opacification process in the human lens.

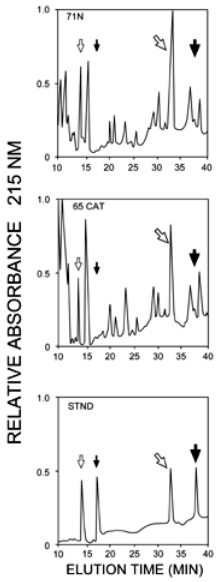

Figure 4. Quantitation of glutamine-6 deamidation. Resolution of amidated and deamidated forms of tryptic peptides corresponding to sequence 1-11 of α-A crystallin. The tryptic digest was resolved on a C_{18} reverse phase column (4.6 mm x 250 mm, Vydac) using a linear gradient of 23 - 30% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid over a period of 60 min, followed by a linear gradient of 30 - 60% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid, over a period of 5 min. Only the part of the elution profile showing peptides of interest is shown. Top, digest from 71 yr old normal lens (71N); middle, digest from 65 yr old cataractous lens (65C) with opacification of the nucleus; bottom, synthetic peptide standards (STND). Large open arrows designate elution of amidated peptide AcMDVTIQHPWFK, small open arrows designate elution of amidated peptide AcM[0]DVTIQHPWFK, large closed arrows designate elution of deamidated peptide AcMDVTIEHPWFK, small closed arrows designate elution of deamidated peptide AcM[0]DVTIEHPWFK.

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