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In vivo acetylation identified at lysine 70 of human lens αA-crystallin

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Abstract

Posttranslational modification of protein lysyl residues that change the net charge of the molecule may alter the protein conformation. Such modifications are of particular significance among lens proteins, because conformational changes are associated with the development of cataract. A previously unidentified acetylated form of αA-crystallin has been isolated from the water-soluble portion of human lenses. The αA-crystallins were fractionated by anion exchange HPLC into seven peaks, each containing more than one form of αA-crystallin. The previously reported deamidated and phosphorylated forms were identified by their molecular masses, determined by electrospray ionization mass spectrometry. In addition to these modifications, approximately 5% of αA-crystallin had a modification that decreased the charge by one and increased the molecular mass by 42 u. This modification, identified as acetylation, was located uniquely at Lys 70. Like any modification that alters the surface charge, acetylation may affect protein conformation and intermolecular interactions, thereby altering the solubility or chaperone properties of αA-crystallin. Acetylation of lysine 70 is potentially significant since it is located in a region that has been implicated in the chaperone activity of αA-crystallin.

Keywords: acetylation; ion exchange; lens crystallins; mass spectrometry

Proper close-packing of the proteins in the eye lens is required for transparency. The α-crystallins, which comprise approximately 30% of the lens proteins, contribute to proper close packing through both their function as structural proteins and their chaperone-like activity in maintaining the solubility of other lens crystallins (Horwitz, 1992; Muchowski et al., 1997). Post-translational modifications that affect either of these functions may contribute to cataractogenesis. Because lysyl residues have a positive charge under physiological conditions, they are likely participants in ionic interactions required for close-packing. Modifications that alter the charge of lysyl groups may be expected to alter the close packing of crystallins in the lens. Modified lysyl residues and their effects on aggregation properties have been observed primarily with lens crystallins incubated in vitro with the modifying agents. Several in vivo modifications at lysyl resides have been implicated in the development of cataract, yet these modifications have been reported to be present at fewer than 1% of the lysines (Dunn et al., 1989; Dyer et al., 1991; Nagaraj et al., 1991) and the exact locations of the modifications have not been specified. We report an in vivo modification of human αA-crystallin, located only at Lys 70. This modification, which removes one positive charge of αA-crystallin and increases the molecular mass by 42 u, is present in approximately 5% of αA-crystallin molecules.

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Results

The water-soluble αA-crystallins of each lens were separated into seven peaks by anion exchange chromatography at pH 6.2, each containing more than one species. Figure 1 is a representative chromatogram showing the results for a 66 year old lens. Because of the limited amount of modified protein in any one lens, five lenses were used to obtain complete characterization of the modifications. The number of peaks and relative intensities of the anion exchange chromatograms for the different lenses changed little with the age of the lens. Identifications of the modified αA-crystallins were based on the molecular masses of the proteins in each fraction, determined by electrospray ionization mass spectrometry (ESIMS). The first peak to elute by anion exchange chromatography, which is off-scale in Figure 1, is primarily αA-crystallin (M_r 19,951), unmodified except for the N-terminal acetylation common to all α-crystallins, and a minor component of αA-crystallin missing the C-terminal serine (M_r 19,864) (Miesbauer et al., 1994). The ESI mass spectrum of the proteins in the first peak is shown in Figure 2A. The later elution times of the subsequent peaks indicate that the proteins in these peaks have an increased negative charge. The major component of the second peak (data not shown) also had a molecular mass in agreement with the mass calculated from the sequence of αA-crystallin. This protein was tentatively identified as a deamidated form of αA-

crystallin, since deamidation is a modification that retards elution on anion exchange, but causes only a one mass unit increase in molecular mass, an increase too small to be differentiated in the ESIMS spectrum of a 20 kDa protein. The third peak of Figure 1, the shoulder marked by the arrow, contained a protein with a molecular mass 42 u higher (M_r 19,993) than unmodified αA-crystallin (Fig. 2B). A 42 u increase in molecular mass is consistent with acetylation. In addition, this peak contained minor components with molecular masses probably due to deamidated αA-crystallin (M_r 19,951), deamidated αA minus serine (M_r 19,864) and αA minus serine plus 42 u (M_r 19,906). The deamidated αA-crystallins may be due to overlap with the second peak. The principal components of the fourth peak of the ion exchange were phosphorylated forms of αA-crystallin (Fig. 2C), which have two additional negative charges and an increase of 80 u in molecular mass. The peak at M_r 20,029 is αA-crystallin with one phosphorylation; the peak at M_r 19,943 can be attributed to αA minus the C-terminal serine with one phosphorylation. The molecular masses of the proteins in the fifth through seventh peaks could be assigned to combinations of phosphorylations, deamidations, and the +42 u modification of αA-crystallin, which were consistent with the fifth peak containing proteins with two more negative charges than unmodified αA-crystallin, and the sixth and seventh peaks containing proteins with three and four more negative charges, respectively. The ESI mass spectrum of the seventh peak, which is barely discernible as a distinct peak in the ion exchange chromatogram, is shown in Figure 2D. Possible identifications of the proteins, consistent with four additional negative charges and within ±3 u of calculated masses, are given in Table 1. In addition to acetylation, these modifications include one or two deamidations and one or two phosphorylations, as well as loss of the C-terminal serine. Except for the acetylation, which is being reported here for the first time, these modifications have been previously observed (Miesbauer et al., 1994; Lund et al., 1996). In those investigations, the phosphorylated serines and deamidated asparagines and glutamines were located by observation of peptides with appropriately altered molecular weights in digests of αA-crystallin. The deamidations are also consistent with the fact that αA-crystallins from adult lenses show little change in molecular weight, but an increase in acidity compared with juvenile lenses (Lampi et al., 1998; Ma et al., 1998).

Because the mass accuracy of ESIMS is approximately 0.01% (±2 Da for a protein of 20,000 Da), both acetylation (+42 u) and carbamylation (+43 u) are consistent with the increase in molecular mass. To ascertain whether the +42 modification could possibly be due to carbamylation that might have occurred while the protein was in the 6 M urea of the buffer used for the ion exchange separation, the α-crystallin fraction from reversed phase HPLC was analyzed by ESIMS without the ion exchange procedure. This sample also showed the presence of a protein with a molecular mass 42 u higher than unmodified αA-crystallin even though it had not been exposed to urea, demonstrating that the increase was not due to carbamylation during the isolation.

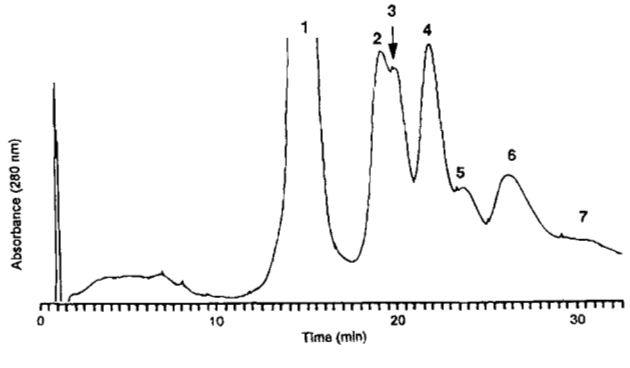

Fig. 1. Chromatogram of the anion exchange separation of αA-crystallins at pH 6.2. The peak due to unmodified αA-crystallins is off-scale to illustrate the presence of other forms of αA. The peak marked with an arrow contains αA-crystallin modified at Lys 70.

To identify and locate the modification responsible for the 42 u increase in mass, the proteins in fraction 3 (Fig. 1) were digested with endoproteinase Asp-N, the resulting peptides isolated, and the molecular masses of the peptides determined by on-line HPLC/ESIMS. The chromatogram in Figure 3A shows the total ion current monitored by the mass spectrometer as the peptides eluted from the HPLC column. Mass spectra of the fractions marked by arrows B and C are shown in the lower traces (Fig. 3B,C). The peptide with MH^+ 882 corresponds to peptide 69–75 (DKFVIFL) of αA-crystallin; MH^+ 924 is exactly 42 u higher, suggesting that it is peptide 69–75 with the +42 modification. Because mass accuracy is ±0.3 u for peptide analysis, the modification could be attributed, unequivocally, to acetylation (+42 u) and not carbamylation (+43 u). Peptides with molecular masses corresponding to all other regions of αA-crystallin were present in the Asp-N digest. The +42 modification was found only at Lys 70; no other modification consistent with a +42 u change was observed. These assignments were confirmed by MS/MS analysis, shown in Figure 4. The upper trace is the MS/MS spectrum of the peptide with MH^+ 882 and the lower trace is the MS/MS spectrum of MH^+ 924. The masses of the fragments formed from cleavage along the backbone of peptide 69–75 with the charge remaining at the N-terminus (the b-series (Roepstorff & Fohlman, 1984)) correspond to the masses found in the upper spectrum. The masses in the lower spectrum indicate the presence of an additional 42 u on the second residue from the N-terminus, Lys-70. These data confirmed that the observed modification is acetylation of Lys 70 of αA-crystallin.

Further confirmation of the acetylation of Lys 70 was obtained from a tryptic digest of αA-crystallin with the +42 modification. Because modification at Lys 70 may prevent trypsin cleavage at Arg 68 and Lys 70, observation of a peptide with MH^+ 1,623 (Fig. 5) is consistent with peptide 66–78 from αA-crystallin with a +42 u modification at Lys 70. The major peak in the spectrum is due to the peptide with two protons attached (MH_2^{2+} 812). In this tryptic digest, the expected peptides corresponding to all other regions of αA-crystallin were present (Fig. 6). Pepsin digestion of the peptide with MH_2^{2+} 812 yielded peptides with molecular masses consistent with its identification as residues 66–78, acetylated at Lys 70. These peptides are indicated with a P in Figure 6.

Discussion

The importance of lysyl residues to the proper close packing of the lens crystallins has been illustrated by the many in vitro studies in which conformational changes and aggregation of lens crystallins occurred during incubation with a lysine modifying reagent (Harding & Rixon, 1980; Beswick & Harding, 1984, 1987; Bensch et al., 1985; Crompton et al., 1985; Huby & Harding, 1988; Ortwerth et al., 1988, 1992; Ajiboye & Harding, 1989; Swamy & Abraham, 1989; Raza & Harding, 1991; Prabhakaram & Ortwerth, 1992;

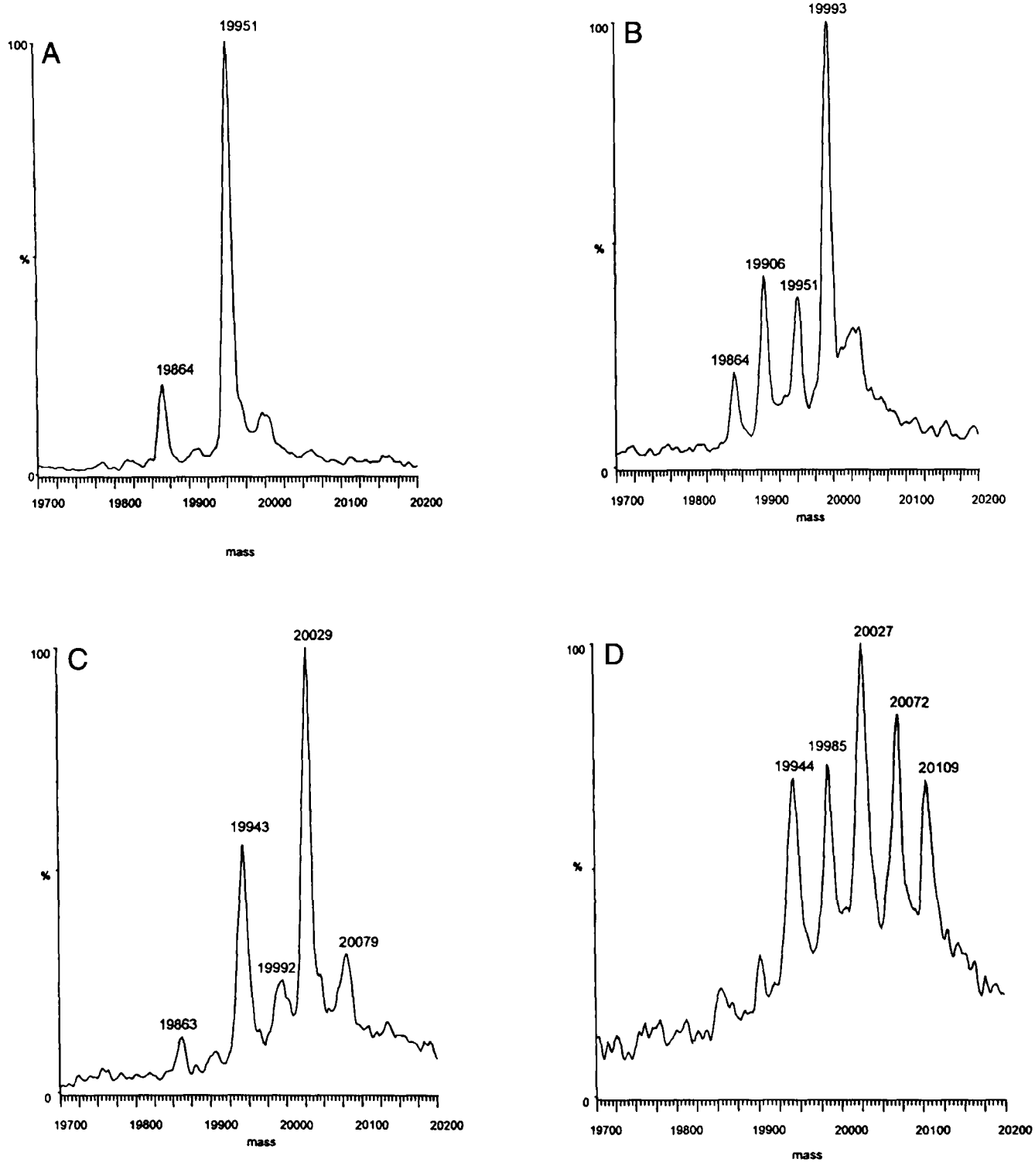

Fig. 2. Reconstructed mass spectra showing the proteins present in the peaks marked in Figure 1. **A:** The major component of peak 1 is unmodified αA-crystallin (calculated M_r 19,952). A minor component missing the C-terminal serine (calculated M_r 19,865) is also evident. **B:** The proteins in peak 3 were identified as αA-crystallins with one less positive charge. The masses were consistent with identification as αA acetylated at Lys 70 (calculated M_r 19,994), αA deamidated (calculated M_r 19,953), αA deamidated and minus the C-terminal Ser (calculated M_r 19,866), and αA minus Ser 173 and acetylated at Lys 70 (calculated M_r 19,907). **C:** The major components of peak 4 are αA with one phosphorylation (calculated M_r 20,032) and αA minus Ser 173 plus phosphorylation (calculated M_r 19,945). **D:** The elution time of peak 7 suggests that these αA-crystallins have four additional negative charges. Masses in the spectrum, assigned to modified forms of αA-crystallin consistent with four additional negative charges, are given in Table 1.

Table 1. *Assignment of molecular masses in the ESIMS analysis of proteins in peak 7 of the cation exchange HPLC of αA-crystallins*[a]

Experimental M_r	Calculated M_r	Probable modifications: Acetylated Lys 70	Minus C-terminal Ser	Plus phosphate	Deamidation
19,944	19,947		Yes	One	Two
19,985	19,988	Yes	Yes	One	One
20,027	20,025		Yes	Two	
20,072	20,075	Yes		One	One
20,109	20,112			Two	

[a]The HPLC chromatogram is Figure 1; the ESI mass spectrum is Figure 2D.

Riley & Harding, 1993, 1995; Swamy et al., 1993; Abraham et al., 1994). Even though acetylation alters the charge of the lysyl residue in the same way as the other modifications and would be expected to have a similar effect on lens opacity, acetylation of lens proteins was observed to be anti-cataractogenic (Crompton et al., 1985; Rao et al., 1985; Rao & Cotlier, 1988; Harding et al., 1989; Blakytny & Harding, 1992). This apparent contradiction was resolved in a subsequent study showing that cysteinyl residues are also acetylated, and that the anti-cataract effect of acetylation is more likely due to irreversible acetylation of cysteinyl residues, preventing disulfide bonding, than to acetylation of lysine (Qin et al., 1993). Had it been possible to acetylate the lysyl residues without modifying the cysteinyl residues, investigators might have observed the same deleterious effect on opacity as other lysyl modifications. Removal of the positive charge at the lysyl residue would be expected to interfere with ionic interactions required for proper packing, thus contributing to conformational changes. Harding has proposed that such conformational changes may facilitate formation of disulfide bonds, leading to aggregates that are responsible for opacity (Harding, 1972, 1973).

A new perspective on the importance of acetylation in molecular mechanisms was recently proposed in an investigation that showed dramatically increased stimulation of the tumor suppressor p53 by acetylation of lysyl residues in the C-terminus (Gu & Roeder, 1997). This study indicated that protein acetylation is a fundamental regulatory mechanism in transcription, raising the possibility

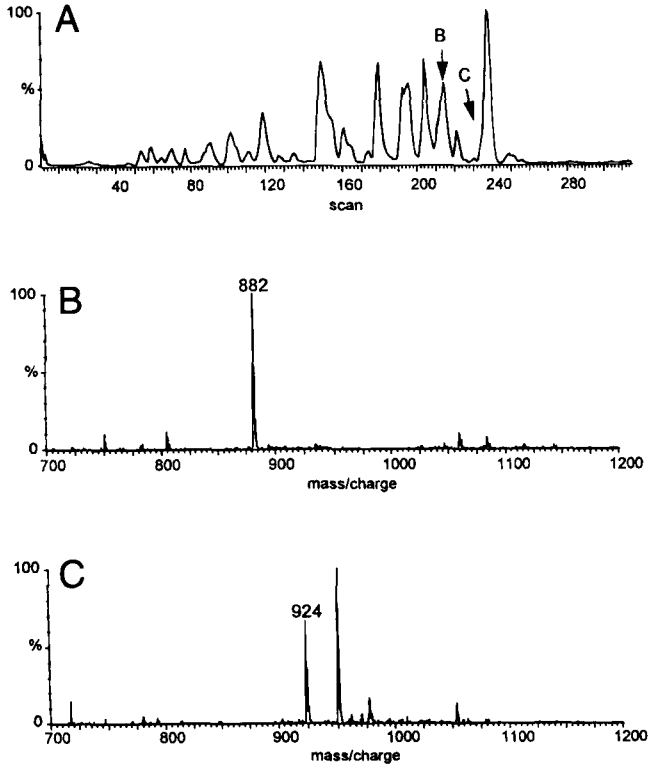

Fig. 3. Mass spectrometric analysis of an Asp-N digest of modified αA-crystallin from the peaked marked with an arrow in Figure 1. **A:** The total ion current produced as the digest is separated by reversed phase HPLC on-line to the mass spectrometer. **B:** Mass spectrum of the peptide present in Peak B of the total ion current. The mass at MH^+ 882 corresponds to peptide 69–75 (DKFVIFL) of αA-crystallin. **C:** Mass spectrum of the peptides present in the shoulder marked by arrow C. The mass at MH^+ 924 corresponds to peptide 69–75 (DKFVIFL) plus an additional 42 u. The peak at 951 can be attributed to the doubly charged ion for peptide 35–50.

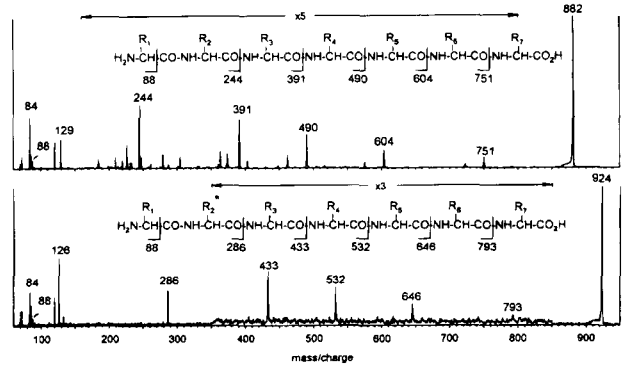

Fig. 4. A: MS/MS spectrum of the peptide in Figure 3B. The masses in the spectrum correspond to the b-series fragments (Roepstorff & Fohlman, 1984) from the sequence DKFVIFL. **B:** MS/MS spectrum of peptide MH^+ 924 in Figure 3C. The masses in the spectrum indicate that this peptide has the same sequence as peptide MH^+ 882 with an additional 42 u at the residue second from the N-terminus (Lys 70).

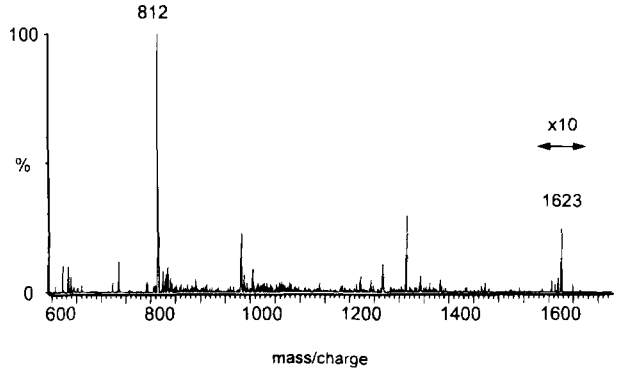

Fig. 5. Electrospray mass spectrum showing the peptide in the tryptic digest of αA-crystallin corresponding to residues 66–78 with an additional 42 u. The calculated mass of the peptide is 1,581. A peak corresponding to the peptide with the additional 42 u, singly protonated, MH^+, at mass/charge 1,623 is shown with tenfold magnification The major peak is the peptide with two protons attached, MH_2^{2+}, at mass/charge 812.

that the observed acetylation of Lys 70 of αA-crystallin may have an as yet undetermined regulatory function.

In vivo evidence of modified lysyl residues has been limited. Age-related accumulation of N^{ϵ}-(carboxymethyl)lysine and fructoselysine, measured by gas chromatography–mass spectrometric analysis of normal human lenses, detected only 8 mmol/ mol of lysine, even in old lenses (Dunn et al., 1989). Similarly, pentosidine detected in human lens proteins was limited to trace concentrations of less than 5 μmol/mol lysine (Dyer et al., 1991). Another product of lysine modification, the fluorophore LM-1, which is purportedly formed by the reaction of ascorbic acid oxidation products with lysine, accounted for less than 2% of the total fluorescence of the water-insoluble fraction of cataractous lenses (Nagaraj & Monnier, 1992). In contrast, acetylation of Lys 70 of αA-crystallin, reported in this publication, has modified approximately 5% of the αA-crystallin molecules, somewhat less than the approximately 20% phosphorylation of αA-crystallin in the adult lens (Miesbauer et al., 1994; Takemoto, 1996), but significantly greater than other reported lysyl modifications. Because α-crystallins are a major component of the water-insoluble portion of the lens

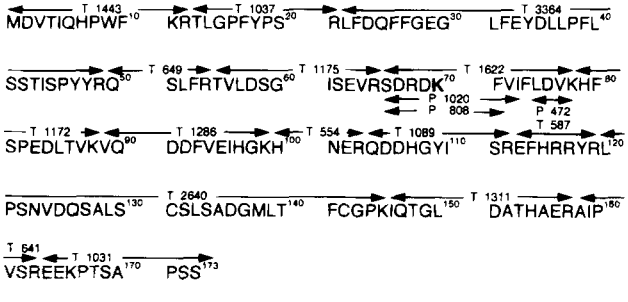

Fig. 6. The sequence of human αA-crystallin. The peptides found in a tryptic digest of αA-crystallin acetylated at Lys 70 are marked above the sequence with T and the molecular mass of the peptide. The mass of 1,622 corresponds to the expected mass of peptide 66–78 plus an additional 42 u. The peptides found in a pepsin digest of the T-1622 peptide are noted below the sequence. Both P-1020 and P-808 are 42 u higher than the masses calculated from the sequence.

that increases with age, it is possible that acetylated αA-crystallins make an even larger contribution to the total lens αA-crystallins than is evident from this examination of the water-soluble portion.

The reactivity of aspirin with the lysyl residues of proteins implicates chronic aspirin consumption as a possible cause of αA-crystallin acetylation. However, nonenzymatic acetylation by aspirin, which occurs at all lysines (Hasan et al., 1993), is probably not responsible for the observed specific acetylation at only Lys 70. In vitro acetylation of bovine αA-crystallins, effected by incubation of the crystallins in 100 mM aspirin, demonstrated that Lys 166 is the most readily modified and Lys 70 the next most reactive; however, all the lysines reacted (Hasan et al., 1993). Since no evidence for acetylation of Lys 166, or any other lysine other than Lys 70, was found, it seems improbable that the acetylation at Lys 70 is due to a simple nonenzymatic reaction of aspirin with αA-crystallin. Aspirin use among the donors of the lenses used in this study was not documented.

Acetylation at Lys 70 may affect αA-crystallin chaperone function. Das and Surewicz (1995) demonstrated a correlation between α-crystallin chaperone activity and the exposure of hydrophobic regions of α-crystallin. In this study, the hydrophobic regions became exposed, and decreased chaperone activity was observed as the temperature was increased from 38 to 50 °C. These investigators suggested that this correlation implicates the hydrophobic regions of αA-crystallin in chaperone activity. In another study of the effect of temperature on the conformation of αA-crystallin, it was found that a particularly hydrophobic region of αA-crystallin, including peptide 72–75, unfolds at 38 to 50 °C (Smith et al., 1996). If this hydrophobic region contributes to the chaperone activity, acetylation of the nearby Lys 70 could be expected to affect such chaperone function.

Electrophoretic mobilities, including isoelectric focusing and SDS-PAGE, in both one- and two-dimensional gels, are frequently used to isolate and identify various species of the lens crystallins (Datiles et al., 1992; Groenen et al., 1993; Garland et al., 1996; Lampi et al., 1998). In this report, the ESIMS determined molecular masses of the αA-crystallins separated by anion exchange HPLC showed that peaks for each of the variously charged αA-crystallins included more than one protein, demonstrating that pI alone is not sufficient for separation. In addition, many of the modified forms of αA-crystallin have very similar molecular masses. As a consequence, there may be several forms of αA-crystallin with the same charge and a similar molecular mass, migrating to the same position in a two-dimensional gel. The presence of more than one protein at the same position in a two-dimensional gel has previously been demonstrated by analysis of the eluted proteins by mass spectrometry (Lampi et al., 1998). Identification of proteins by molecular masses determined within ±0.01% by ESIMS avoids ambiguity in their assignment. In addition to reporting a previously undetected modification, this report demonstrates the importance of accurate molecular masses for the identification of protein modifications.

Materials and methods

Isolation of αA-crystallin

Normal human lenses from donors 22, 37, 57, 66, and 70 years old were obtained from the National Disease Research Interchange (Philadelphia, Pennsylvania). All lenses were clear; the donors had no diseases known to affect lens opacity. Each lens was analyzed

separately. The lenses were homogenized in 4–5 mL of a buffer (0.5 M NaCl, 0.05 M tris, 0.001 M EDTA, pH 7.4) at 4 °C for 1 h. The supernatant was separated by centrifugation at 15,000 *g* for 1 h, and fractionated into α-, β-, and γ-crystallins by size exclusion chromatography using a 2.5 × 85 cm column (Sephadex G-200). The α-crystallin fraction was separated into αA- and αB-crystallins by reversed-phase HPLC (0.46 × 15 cm Vydac C4 column, Separations Group, Hesperia, California) using a gradient of 0–60% acetonitrile and water, both with 0.1% trifluoroacetic acid at a flow rate of 1 mL/min. The absorbency of the eluate was monitored at 280 nm. The fractions were lyophilized and stored at −20 °C.

Isolation of the modified αA-crystallins

The αA-crystallins (~20 nmoles) were dissolved in 1 mL of a buffer (0.01 M bis-tris-HCl, pH 6.2, 6 M urea) and equilibrated at room temperature for 1 h to permit the aggregates to separate into monomers. The proteins were fractionated by anion exchange using a Mono Q HR 5/5 column (Pharmacia, Piscataway, New Jersey) with a 0–30% gradient of the same buffer plus 0.2 M NaCl over 30 min at a flow rate of 1 mL/min. Absorbency of the eluate was monitored at 280 nm. The fractionated proteins were desalted by reversed-phase HPLC on a C4 column (0.46 × 1 cm) and divided with approximately 5% used for molecular mass determinations by electrospray ionization mass spectrometry. The other 95% was lyophilized and stored at −20 °C until further analysis by mass spectrometry after enzymatic digestion.

Electrospray ionization mass spectrometry (ESIMS)

The molecular masses of the proteins were determined with a Micromass Platform II quadrupole mass spectrometer (Micromass, Manchester, UK), calibrated with myoglobin (M_r 16,951.5). The sample was delivered to the analyzer using a 5 μL/min flow of 50:50 acetonitrile:water. The molecular masses of the proteins were determined with an uncertainty of ±2–3 u. The total αA-crystallins before separation by ion exchange as well as each peak from the ion exchange were analyzed by ESIMS.

Enzymatic digestion and analysis of peptides of αA-crystallin

αA-Crystallin (1 nmole), either unmodified or with the +42 u modification, was digested with endoproteinase Asp-N (Boehringer Mannheim, Indianapolis, Indiana) or with trypsin (Sigma Chemical Co., St. Louis, Missouri) at a protein to enzyme ratio of 50:1. Because each lens yielded only about one nanomole of αA-crystallin with the +42 u modification, there was sufficient material for only one digest per lens. For Asp-N digestion, the protein was dissolved in 50 mL of 0.1 M ammonium acetate, pH 8.0 and incubated for 12 h at 37 °C. Endoproteinase Asp-N cleaves N-terminally to aspartic acid and also, with longer incubation times, N-terminally to glutamic acid. For the trypsin digestion, the buffer was 50 μL of 0.1 M tris, pH 7.0, with a 6 h incubation at 37 °C. Trypsin cleaves C-terminally to Lys and Arg, but does not usually cleave at modified residues. The molecular masses of the peptides in the digests were determined by on-line microbore HPLC ESIMS. A post-column splitter directed 5 μL/min to the mass spectrometer and 45 μL/min to a UV detector (ABI model 757, Brownlee Labs Inc., Santa Clara, California), which monitored the peptide elution at 214 nm, followed by a fraction collector. Collected fractions were stored at −20 °C until use in subsequent experiments. The peptide from the tryptic digest, believed to include the modification, was further analyzed after digestion with pepsin (10 μg/mL pepsin in 0.03 M HCl) for 10 min at room temperature. The molecular masses of the resulting peptides were determined by ESIMS.

Tandem mass spectrometry

Tandem mass spectrometry (MS/MS) consists of one mass spectrometer in which the peptide of interest is isolated, a chamber in which fragmentation occurs following collision of the peptide with an inert gas, and a second mass spectrometer in which the masses of the resulting fragments are determined (Biemann, 1990). Peptides fragment along the backbone of the peptide and at some side chains. From the fragmentation pattern produced by collisional activation of the peptide, the identity of the peptide can be determined. The tandem mass spectrometer used in this investigation (Micromass AutoSpec-oaTOF, Manchester, UK) consisted of a conventional magnetic sector instrument as the first mass spectrometer and an orthogonal acceleration time-of-flight analyzer as the second mass spectrometer. Sample ions were desorbed from a matrix consisting of a 1:1 mixture of glycerol/thioglycerol using a 25 keV cesium ion beam and were mass selected by the first mass spectrometer. The fragment masses, produced by collision with xenon gas, were used to confirm the sequence of the peptide and to locate the modified residue.

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