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Modifications of human βA1/βA3-crystallins include S-methylation, glutathiolation, and truncation

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Abstract

Disulfide bonding of lens crystallins contributes to the aggregation and insolubilization of these proteins that leads to cataract. A high concentration of reduced glutathione is believed to be key in preventing oxidation of crystallin sulfhydryls to form disulfide bonds. This protective role is decreased in aged lenses because of lower glutathione levels, especially in the nucleus. We recently found that human γ-crystallins undergo S-methylation at exposed cysteine residues, a reaction that may prevent disulfide bonding. We report here that βA1/A3-crystallins are also methylated at specific cysteine residues and are the most heavily methylated of the human lens crystallins. Among the methylated sites, Cys 64, Cys 99, and Cys 167 of βA1-crystallin, methylation at Cys 99 is highest. Cys 64 and Cys 99 are also glutathiolated, even in a newborn lens. These post-translational modifications of the exposed cysteines may be important for maintaining the crystallin structure required for lens transparency. Previously unreported N-terminal truncations were also found.

Keywords: human lens crystallins; cataract; in vivo protein modification; S-methylation; glutathiolation

A high concentration of proteins in the ocular lens provides the refractive index required for focusing light on the retina. Major structural proteins, called crystallins, which have molecular masses of 20–28 kDa, account for >90% of the lens proteins. Based on sequence homology and the size of aggregates isolated under physiological conditions, human crystallins are organized into three classes, α-, β- and γ-crystallins. Because lens crystallins undergo little or no turnover, there is opportunity for numerous post-translational modifications as the lens ages (David et al. 1996; Lampi et al. 1998; Ma et al. 1998; Takemoto and Boyle 1998; Slingsby and Clout 1999; Hanson et al. 2000). Some modifications, identified in young clear lenses, may reflect normal development and maturation of the lens, while others, associated with aged lenses, could negatively impact crystallin conformation, aggregation state, or solubility, resulting in increased light scattering and eventual loss of lens transparency.

The free sulfhydryls of cysteine residues are among the most reactive functional groups in proteins. Cysteine residues in lens crystallins are susceptible to oxidation forming mixed disulfides with low molecular weight thiols such as glutathione, cysteine, and γ-glutamylcysteine (S-thiolation) (Dickerson and Lou 1993), and protein–protein disulfide bonds (Spector and Roy 1978; Lapko et al. 2002a). Although the physiological role of mixed disulfides is not well understood (Lou and Dickerson 1992; Kamei 1993; Feng et al. 2000), there is a correlation between these modifications and the color and opalescence of the lens nuclei (Lou et al. 1999). Also, the association of protein–protein disulfide bonds with crystallin aggregation and insolubilization suggests that intermolecular disulfide bonding has a role in cataractogenesis (Kodama and Takemoto 1988; Kodama et al. 1988; Stephan et al. 1999; Pande et al. 2000; Lapko et al. 2002a).

In a normal lens, the reduced form of glutathione (GSH) is the major factor in maintaining protein sulfhydryl groups in the reduced form (Lou and Dickerson 1992; Cotgreave and Gerdes 1998; Klatt and Lamas 2000). This role is achieved by direct scavenging of reactive oxygen species (Coan et al. 1992) as well as by producing reducing equivalents for enzymes involved in maintenance of redox equilibria (Hayes and McLellan 1999; Klatt and Lamas 2000).

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Although lens GSH concentration is normally ~5 mM (Dickerson and Lou 1997), GSH synthesis is slower in aged lenses (Rathbun and Murray 1991) and lower GSH levels have been reported in the lens nuclei of most forms of cataract (Lou et al. 1999; Bova et al. 2001). Recent data showing methylated cysteines in human lens crystallins (Lapko et al. 2002b, 2003a; Searle et al. 2004) suggest that other reactions may also protect reactive sulfhydryls. The high levels of cysteine methylation in γS- and γD-crystallins isolated from young clear lenses indicate that S-methylation does not negatively affect the functions of these proteins and, perhaps, may be beneficial in preventing disulfide bonding.

In the present paper, we describe previously unreported modifications of βA1/A3-crystallins including methylation and glutathiolation of cysteines. These modifications were detected using the same methodology that permitted identification of methylation as a major modification of human lens γS-crystallins (Lapko et al. 2002b). Key to identification of cysteine modifications is comparison of protein mass spectra of nuclear and cortical crystallins from human lenses of different ages, both prior to and after derivatization with cysteine-specific reagents such as 4-vinylpyridine and iodoacetamide. βA1- and βA3-crystallins are products of the same HuβA1/A3 gene containing two in-frame initiation codons (Hogg et al. 1986). Protein synthesis initiated from these two codons generates the two proteins, which are identical except that βA3-crystallin has 18 more amino acids at the N terminus than βA1-crystallin has. The N-terminal methionine of βA3-crystallin is acetylated. The N-terminal residue of βA1-crystallin is an acetylated alanine corresponding to residue 19 of βA3 (Lampi et al. 1997). Both βA1- and βA3-crystallins are truncated even in newborn lenses, with a loss of four and 22 residues from their N termini, respectively, yielding identical truncated forms (Lampi et al. 1998; Ma et al. 1998). These forms will be designated here as Δ4 with the residues numbered according to their positions in βA1-crystallin. Further truncations, which occur during lens maturation, will also be designated as Δ followed by the number of residues missing from the N terminus of βA1-crystallin. Among the β-crystallins, substantial levels of methylated cysteine were found only in βA1/A3-crystallins. Similar to γ-crystallins, βA1/A3-crystallins are methylated at specific cysteine residues. βA1/A3-Crystallins are also glutathiolated even in young lenses. Both methylation and glutathiolation may be important in maintaining crystallin structure.

Results

Analysis of mass spectra of undigested proteins

Post-translational modifications of βA1/A3-crystallins were determined by mass spectral analysis of βA1/A3-crystallins isolated by size exclusion chromatography (Fig. 1A) followed by reversed-phase HPLC of the β-crystallins (Fig. 1B). Only the water-soluble proteins were examined in this study, but for lenses up to 19 yr old, the water-soluble portion includes at least 93% of the crystallins. Therefore, these spectra include nearly all the βA1/A3-crystallins.

In an 11-d-old lens, intact βA1- and βA3-crystallins are the major forms; yet nearly a fourth of the proteins have lost four (from βA1) or 22 (from βA3) residues from the N terminus (Table 1). Additional truncations at the N termini along with other post-translational modifications make interpretation of mass spectra of βA1/A3-crystallins from older lenses rather difficult. However, comparative analysis of proteins isolated from lenses of different ages facilitates identification of the mass spectral peaks. As described in the Materials and Methods section, the nuclear crystallins were obtained by boring through the center of the lens and then trimming the ends of the cylinder. These ends combined with the remainder of the lens are referred to as the cortex.

Masses for nontruncated βA1/A3-crystallins were easily detected among the crystallins from the cortex of an 11-yr-old lens (Fig. 2A, peaks at 23,102 Da and 25,192 Da) and in 19-yr-old lenses (spectra not shown). The major peak in this spectrum (Fig. 2A) at 22,646 Da corresponds

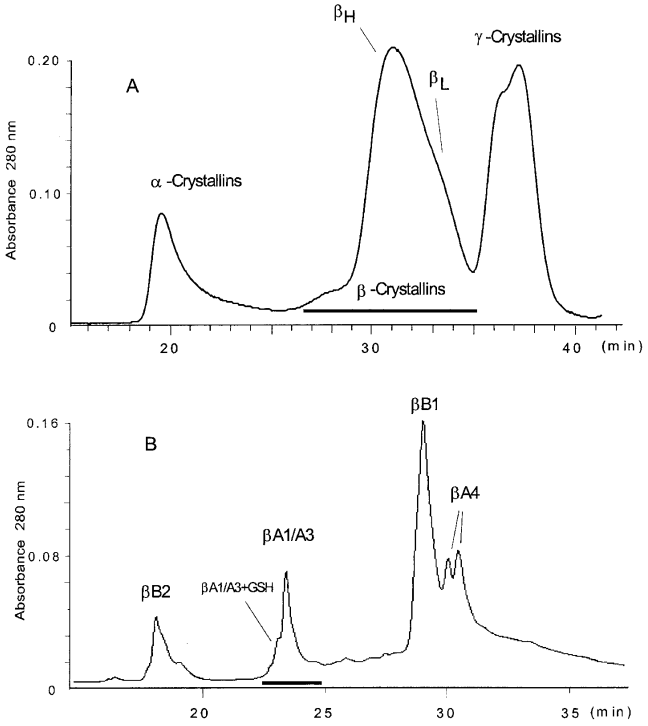

Figure 1. (*A*) Size exclusion chromatography of soluble lens crystallins from an 11-yr-old lens. (*B*) Reversed-phase HPLC of the β-crystallins indicated by the bar in *A*. The βA1/A3-crystallin fraction analyzed by MS is indicated by a bar.

Table 1. *Percentages of βA3/A1-crystallins and their major truncated forms in human lenses of different ages*

	Inner nucleus			Remainder of lens		
βA3/A1 forms	11 d	11 yr	19 yr	11 d	11 yr	19 yr
βA3	50			55	10 ± 1	6 ± 2
βA1	27			30	10 ± 2	6 ± 1
Δ4βA1	23	46 ± 3	27 ± 3	15	52 ± 2	50 ± 4
Δ7βA1		23 ± 2	23 ± 2		13 ± 3	15 ± 2
Δ8βA1		30 ± 1	50 ± 4		14 ± 1	22 ± 3

Percentages were estimated from the relative intensities of protein mass spectral peaks. The data are from the following lenses: one 11-d-old, two 11-yr-old, and four 19-yr-old lenses.

to the previously described Δ4 truncated protein. Peaks at 22,351 Da and 22,294 Da are due to further truncation of the proteins with losses of seven and eight residues from the N terminus of βA1-crystallin (or 25 and 26 residues of βA3). Small peaks with mass increases of 14 Da at 22,660 Da and 22,308 Da (Fig. 2A) indicate possible methylation.

The mass spectrum of the βA1/A3-crystallins isolated from the nucleus of the same lens (Fig. 2B) shows an increase in truncated forms Δ7 and Δ8 (peaks at 22,352 Da and 22,294 Da) as well as small amounts of proteins missing nine and 11 residues. The truncation sites are illustrated in Figure 3. The peaks with an additional 14 Da are more prominent among the nuclear proteins (Fig. 2B) than the cortical proteins (Fig. 2A), and further methylation is indicated by additional peaks at +28 Da and +42 Da.

Mass spectra of 4-vinylpyridine-derivatized nuclear proteins provided conclusive evidence of S-methylation (Fig. 2C). Well resolved peaks with molecular masses 91 Da less than the expected masses of the derivatized forms of βA1/A3-crystallins indicate methylation of cysteine residue(s). Dimethylated species were also easily identified among 4-vinylpyridine-derivatized proteins (Fig. 2C). Identification of trimethylated proteins was more problematic because the mass of underivatized proteins with three methylations differs by only one mass unit from the carbamylated species. Derivatization separated the masses of the trimethylated proteins and carbamylated proteins, but the mass spectral peaks for the trimethylated forms were then barely detectable.

The presence of a small peak at 22,951 Da (Fig. 2A,B) with a molecular mass 305 Da higher than the major Δ4 truncated form, and its absence after reduction of the proteins suggests the presence of a glutathione adduct. These glutathiolated species, which have similar abundances in the nuclei and cortex (Fig. 2A,B), are the major component of samples collected from the front shoulder of the βA1/A3-crystallin HPLC fraction (Fig. 1B).

Sites of major post-translational modifications of βA1/A3-crystallins

Truncations of βA1/A3-crystallins, evident in the mass spectra of undigested proteins (Fig. 2A,B), were confirmed by MS/MS analysis of isolated truncated N-terminal tryptic peptides (residues 5–14, 8–14, and 9–14 of βA1-crystallin) and Asp-N peptides 10–18 and 12–18. Three of five truncations were adjacent to proline residues (Fig. 3). Methylation of cysteine residues was evaluated from MALDI MS spectra of tryptic digests of the proteins after derivatization with 4-vinylpyridine. Three of the five cysteine-containing peptides of βA1/A3-crystallins from the nucleus of an 11-yr-old lens had the signature of methylation: additional peaks with a mass 91 Da lower than the expected masses of the derivatized peptides. This 91-Da decrease due to derivatization with 4-vinylpyridine (Friedman 2001) helped identify methylated proteins and the methylated sites in tryptic peptides. Peptides that MALDI indicated might be methylated were further analyzed by LC/MS/MS, confirming methylation at Cys 64 (Fig. 4), Cys 99, and Cys 167 (Table 2).

LC MS/MS analysis of peptides from 19-yr-old lenses also showed two tryptic peptides with a mass of 1517.8 Da, βA1-crystallin peptide 92–104 with methylation at Cys 99 and peptide 7–18 of γS-crystallin. Even when contamination by γS-crystallin was small, the strong MS response of γS peptide 7–18 interfered with direct estimation of methylation at βA1 Cys 99. Therefore, separation of βA1 92–104 and γS 7–18 by online LC/MS was important for estimation of the extent of methylation at Cys 99. Iodoacetamide-derivatized peptides were chosen for estimation of abundance by LC/ESI MS/MS analysis because methylated and iodoacetamide-derivatized peptides have similar ionization efficiencies and charged state distributions. Peptides derivatized with 4-vinylpyridine are less satisfactory for quantitation because they are not stable during MS/MS analysis, yielding complicated MS/MS spectra, and because their charged state distributions differ from the methylated peptides.

MALDI mass spectra of tryptic peptides from βA1/A3-crystallins isolated from an 11-d-old lens indicated the presence of GSH adducts in peptides 47–72 and 92–107, each containing one cysteine residue (Cys 64 and Cys 99, respectively). Analysis of these peptides by LC/MS/MS confirmed the assignments (spectra not shown).

The peptides in the tryptic, Asp-N, and chymotryptic digests were searched for evidence of several previously reported modifications in βA1/A3-crystallins including phosphorylation at Ser 142 and Thr 109, methylation at Arg 119, and acetylation of Lys 104, Lys 107, and Lys 113 (MacCoss et al. 2002). Even with specific ion monitoring, peptides with these modifications were not detected, suggesting that if they are present, the abundances are very low. Carbamylation of the free N termini of truncated βA1/A3-crystallins was found in 11- and 19-yr-old lenses.

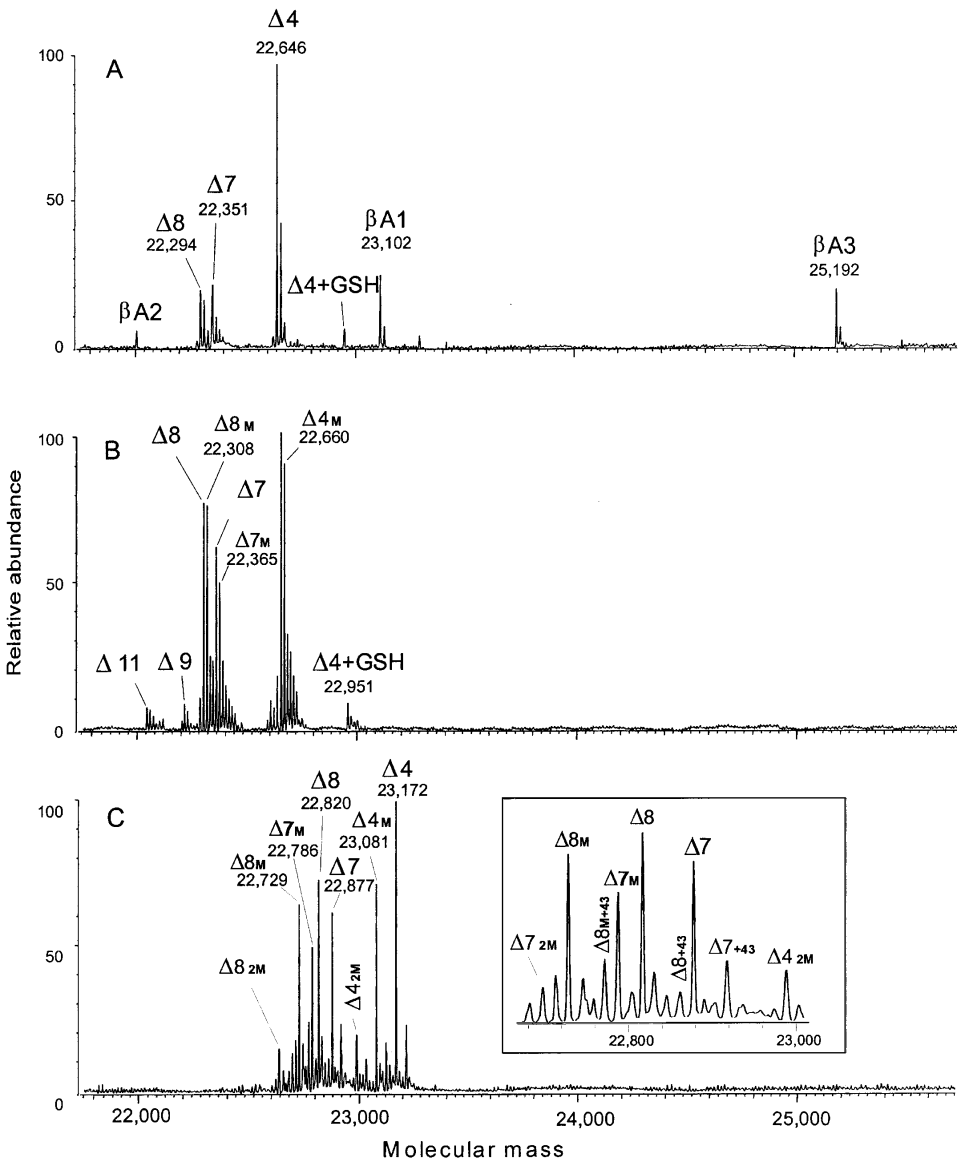

Figure 2. Reconstructed ESI mass spectra of βA1/A3-crystallins isolated from an 11-yr-old lens: (*A*) from the cortex, (*B*) from the nucleus of the lens. Peaks corresponding to truncated βA1/A3-crystallins with the loss of four, seven, eight, nine, and 11 N-terminal residues of βA1 are marked as Δ4, Δ7, Δ8, Δ9, and Δ11, respectively. (M) Monomethylated species; (Δ4+GSH) the glutathione adduct of βA1 missing four residues from the N terminus. (*C*) The nuclear crystallins from the 11-yr-old lens shown in *B* after reduction, derivatization with 4-vinylpyridine, and separation by reversed-phase HPLC. Masses of the Δ4, Δ7, and Δ8 species (at 23,172, 22,877, and 22,820 Da) indicate derivatization of five cysteine residues in each protein. The monomethylated species had molecular masses decreased by 91 Da (peaks at 23,081 for Δ4m, 22,786 for Δ7m, and 22,729 Da for Δ8m). A dimethylated species is designated as "2M." (*C*,*inset*) An expanded region for 22,860–23,000 Da. Peaks marked as "+43" are the carbamylated forms. The experimentally observed masses of the proteins were within 2 Da of values calculated from the sequences.

Changes in abundances of the major post-translational modifications

Both truncation and methylation of βA1/A3-crystallins increase with age and are higher in the nucleus than in the cortex. Intact βA1/A3-crystallins are found in the cortex of lenses as old as 70 yr (data not included in this study), but only truncated proteins are detected in the nuclei of 11-yr-old (Fig. 2B) and older lenses. Comparison of spectra for nuclear and cortical βA1/A3-crystallins from 11-yr-old and 19-yr-old lenses shows more truncation among nuclear than cortical proteins from both ages.

Both nuclear and cortical proteins from 19-yr-old lenses are more methylated than those from 11-yr-old lenses (Table 2). In the nuclei of 11-yr-old lenses, the abundance of methylated Δ4 and Δ8 βA1-crystallins is similar to the

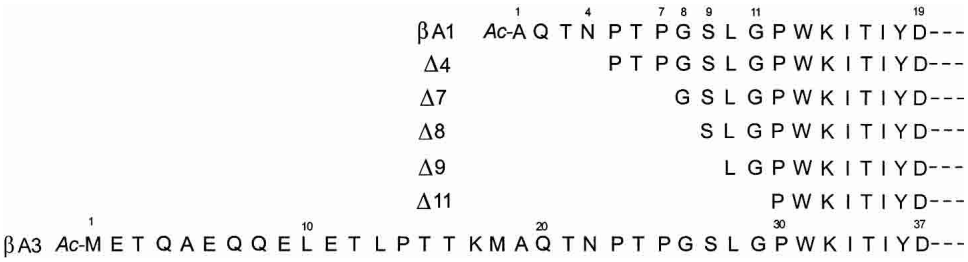

Figure 3. N-Terminal amino acid sequences of βA1/A3-crystallins and their major in vivo degradation products. (*Ac-*) *N*-Acetyl group.

nonmethylated forms (Fig. 2B), while in the nuclei of the 19-yr-old lenses, methylated Δ8 βA1-crystallin is the predominant form. Nonmethylated Δ4 is the most abundant form in the cortex of lenses from both ages (Table 1). Total methylation of nuclear βA1/A3-crystallins is almost twice the methylation of cortical βA1/A3-crystallins from 11- and 19-yr-old lenses (Table 2).

These trends of increased methylation with age and differences between the cortex and nucleus are illustrated by the Δ4 form. Methylation of this form is nearly 30% in the cortex of 11-yr-old lenses (Fig. 2A), ~35% in the cortex of 19-yr-old lenses (data not shown), 47% in the nuclei of 11-yr-old lenses (Table 2), and >75% in the nuclei of 19-yr-old lenses (data not shown).

Our data showed no changes in the specificity of methylation with aging. For example, Cys 99 was the major methylation site in βA1/A3-crystallins isolated from both 11- and 19-yr-old lenses (Table 2). Approximately 10% of the truncated forms in the nucleus of an 11-yr-old lens are carbamylated at the N terminus (Fig. 2C).

Contrary to S-methylation and truncation, glutathiolation is similar among βA1/A3-crystallins isolated from lenses of different ages. Even in an 11-d-old lens, ~15% of βA1/A3-crystallins are glutathiolated. Derivatization with iodoacetamide without reduction of disulfide bonds was used to analyze the GSH-containing fractions from 11-d-old and 19-yr-old lenses. With this approach, much better recovery of disulfide-bonded peptides was obtained than when nonderivatized proteins were digested. Both in 11-d-old and 19-yr-old lenses, Cys 64 and Cys 99 were the major sites of glutathiolation. The possibility of glutathiolation at Cys 167 was suggested by the presence of a very minor peak at 2682.0 Da corresponding to tryptic peptide 160–178 plus glutathione (spectra not shown). This peptide without glutathione produced a strong response in MALDI mass spectra.

Discussion

The exceptional longevity of the lens proteins is unique. In the nucleus, the crystallins are as old as the organism itself. This longevity raises a question of how the structure of lens crystallins is preserved during aging. Many modifications associated with aging are deleterious, leading to protein insolubility and cataract (Kamei et al. 1997; Shih et al. 2001; Takemoto 2001; Lapko et al. 2002a; Srivastava and Srivastava 2003), while others occur early in life in clear lenses with no apparent damaging effects (Datiles et al. 1992; Miesbauer et al. 1994; Lampi et al. 1998; Ma et al. 1998). This study extends our knowledge about the major post-translational modifications in young human lenses. Among proteins in general, methylation at cysteine residues is not prevalent. The trace amounts of S-methylcysteine reported in a number of proteins (Tornqvist et al. 1988) suggest that this modification has little physiological significance in these proteins. This study demonstrates that cysteine methylation is an abundant post-translational modification of human βA1/A3-crystallins as it is in γS- and γD-crystallins (Lapko et al. 2002b, 2003a) and that methylation occurs even in young lenses.

Exposed cysteines are among the most reactive residues in proteins. They are extremely sensitive to oxidation. Accessible cysteines can also form mixed disulfides (with low molecular weight thiols) and both intra- and intermolecular disulfide bonds. Such cross-linking of crystallin subunits is a physiologically abnormal process leading to aggregation and precipitation of the proteins and development of cataract (Kodama et al. 1988; Stephan et al. 1999; Pande et al. 2000). In vitro studies have demonstrated formation of protein disulfide cross-links and lens opacification under conditions of oxidative stress such as hyperbaric oxygen (Giblin et al. 1988, 1995) and exposure to hydrogen peroxide (Brigelius et al. 1983; Hanson et al. 1999). Modification of cysteines by methylation or glutathiolation could be beneficial by inhibiting formation of protein–protein disulfide bonds.

Identification of a mechanism explaining in vivo methylation of crystallins is a challenging task. The major sites of S-methylation in human γD- and γS-crystallins share certain structural similarities such as nearby cysteine residues (Fig. 5). The lack of homology both among βA1/A3-crystallin sequences containing methylated cysteines and

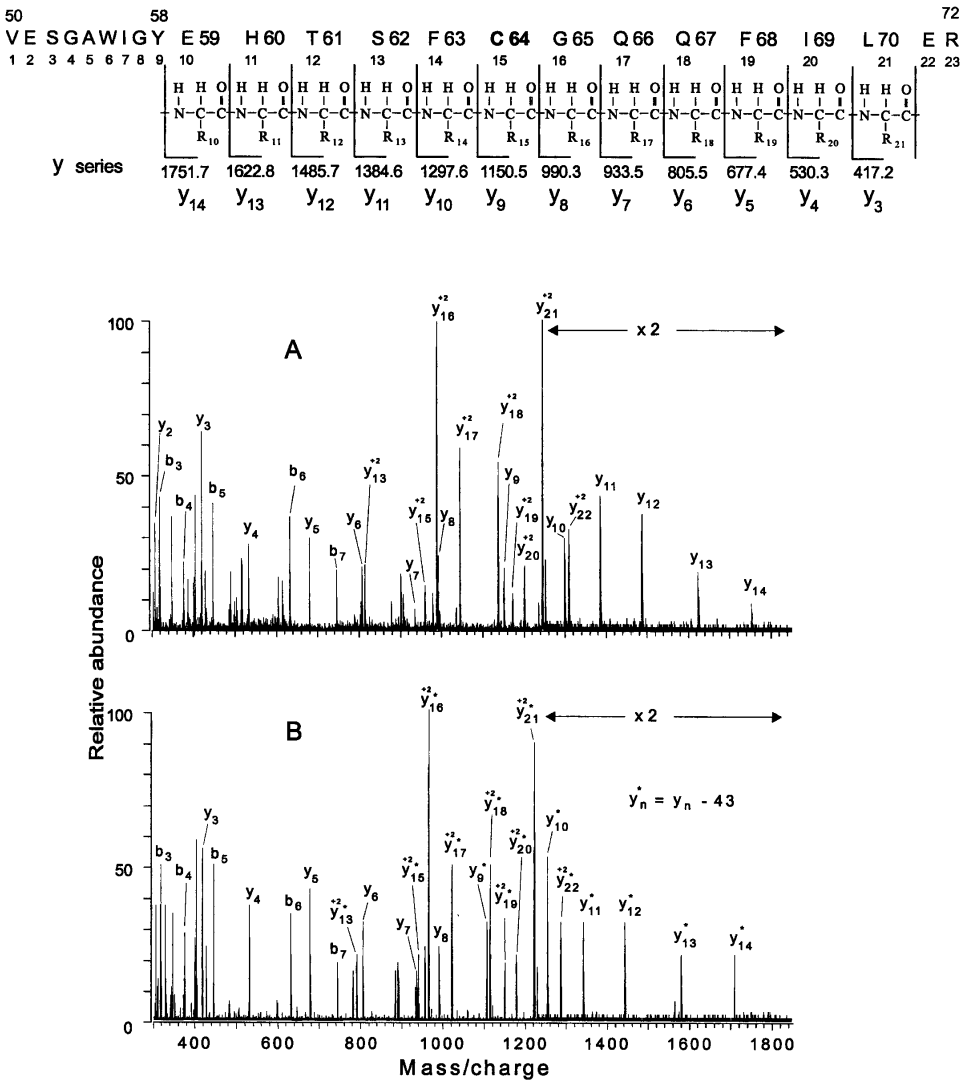

Figure 4. MS/MS spectra (Q-T of Ultima) of tryptic peptide 50–72 of human βA1-crystallin with (*A*) iodoacetamide-derivatized Cys 64 (m/z 905.7^{+3}) and with (*B*) methylated Cys 64 (m/z 891.4^{+3}). Both spectra contain a complete series of y-fragments confirming the expected sequence given at the *top*. The m/z values of y-fragments from both sides of Cys 64 (derivatized with iodoacetamide) are given below the sequence. Note that y-fragments y_9 and higher in spectrum B have m/z values 43 Da lower than m/z values of the corresponding y-fragments of the iodoacetamide-derivatized peptide in spectrum A, confirming methylation at Cys 64.

among regions of βA1/A3- and γ-crystallins with methylated cysteines (Fig. 5) supports an argument against a highly specialized methylase as the methylating agent. However, no methylated residue other than cysteine has been detected. Comparison of the sequences of γ- and βA1/A3-crystallins with the sequence of βB2, whose structure is known (Chirgadze et al. 1991), suggests that the methylated cysteines in γ- and βA1/A3-crystallins all have high solvent-accessibility. Cysteine 34 of βA1-crystallin, which is conserved in all human β-crystallins, is not methylated. The three-dimensional structure of βB2-crystallin shows that this cysteine is buried.

The role of glutathiolation in cataract development is controversial. To date, in vivo glutathiolation has been re-

Table 2. *Percentage methylation in human βA1/A3-crystallins*

Methylated sites	Inner nucleus[a]		Remainder of lens[b]	
	11 yr	19 yr	11 yr	19 yr
Cys 64	12 ± 2	20 ± 2		
Cys 99	25 ± 3	40 ± 5	Total 28 ± 3	Total 38 ± 3
Cys 167	10 ± 3	18 ± 3		

[a] Percentages were estimated from the relative intensities methylated and iodoacetamide-derivatized peptides. The data are from two 11-yr-old and two 19-yr-old lenses.
[b] Percentages were estimated from the relative intensities of protein mass spectral peaks. The data are from two 11-yr-old and four 19-yr-old lenses.

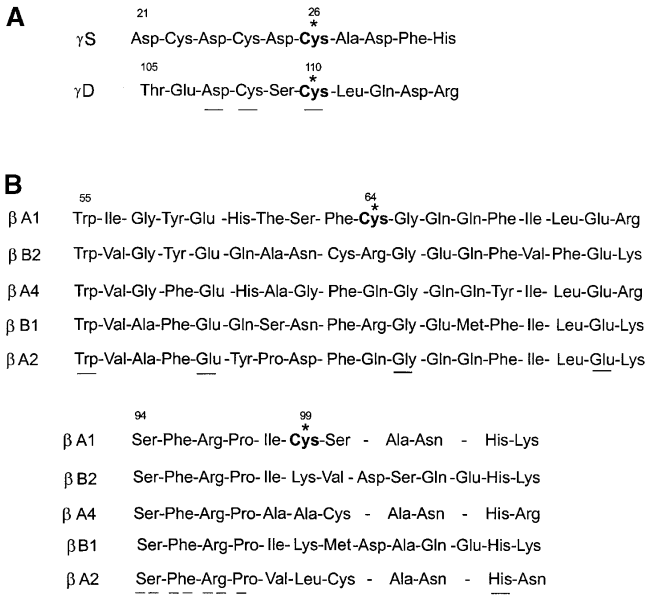

Figure 5. Methylation sites in human γ- and βA1/A3-crystallins. (*A*) Amino acid sequences containing methylation sites in γS- and γD-crystallins. (*B*) Amino acid sequences of homologous regions of human β-crystallins containing the major methylation sites, Cys 64 and Cys 99, of βA1-crystallins. Conserved amino acid residues are underlined.

ported in βA1/A3, βB2, and αA, but not in γ-crystallins (Smith et al. 1995; Feng et al. 2000; Lapko et al. 2003a). Formation of protein disulfides with glutathione (and cysteine) has been proposed as a precursor of protein–protein disulfide cross-links (Lou 2000). This hypothesis is supported by evidence that bovine lenses exposed to 30 mM hydrogen peroxide form glutathiolated γB-crystallins and become opaque (Hanson et al. 1999). Also increased oxidized glutathione and protein-glutathione disulfide bonds have been reported in nuclei of aged lenses (Lou 2000; Bova et al. 2001), perhaps because reduced glutathione is diminished in the nucleus (Lou et al. 1999; Bova et al. 2001). This decrease in reduced GSH may be due to an internal barrier to the movement of small molecules that develops by middle age (Truscott 2003). In contrast, it has also been suggested that glutathiolation is a protective mechanism, preventing irreversible oxidation, disulfide bond formation, and insolubility (Kamei 1993; Cappiello et al. 2000). This hypothesis is supported by evidence that glutathiolated βB2-crystallins are less prone to precipitation (Feng et al. 2000). Our evidence of a relatively high level of glutathiolation of βA1/A3-crystallins from a newborn lens suggests that glutathiolation is not detrimental. Although the role of glutathiolation of βA1/A3-crystallins in young lenses is not clear, this modification results in a species more stable to oxidation than nonglutathiolated proteins, and capable of regenerating the reduced protein. Regeneration of the thiol groups from glutathiolated proteins may occur either chemically or enzymatically (Thomas et al. 1995; Jung and Thomas 1996). In human lenses, thioltransferase and glutathione S-transferase are the major enzymes involved in the dethiolation process (Raghavachari et al. 1999; Lou 2000). A possibility for direct chemical reduction of protein-glutathione disulfide also was shown using intact rabbit lenses (Willis and Schleich 1996). In aged lenses, which have increased levels of oxidized glutathione and a decreased ability to regenerate sulfhydryl groups, accumulation of protein-thiol disulfides may lead to protein–protein disulfide cross-links.

Among the post-translational modifications of βA1/A3-crystallins that occur during maturation are multiple N-terminal truncations. Loss of four N-terminal residues of βA1- and 18-residues of βA3-crystallin at an early stage of life has been previously established (Lampi et al. 1998; Ma et al. 1998), but this is the first report of further truncations corresponding to loss of seven, eight, nine, and 11 residues of βA1-crystallin. Three of the truncations are adjacent to proline. Truncations C-terminal to proline residues and their functional consequences are well known, being widespread in CD26/DPPIV and homolog proteases (Boonacker and Van Noorden 2003). Also, enzymes that cleave proteins to release proteins with an N-terminal proline have been described (Yoshimoto et al. 1983), but the mechanism(s) for these truncations is not known.

The truncated proteins were abundant among the water-soluble crystallins, suggesting that removal of residues at the N termini of βA1/A3-crystallins has no adverse affect on solubility. Our data showing these truncations in βA1/A3-crystallins from young lenses support the idea that such truncations are more likely functional than harmful (Werten et al. 1999b). It has been suggested that loss of the N-terminal portion of the proteins allows modulation of protein repulsion during lens maturation and maintains the protein concentration gradient required for transparency of the lens (Werten et al. 1999b). In vitro studies of truncated forms of βB1-crystallins also indicate that loss of the N terminus is not as detrimental to its stability as some other post-translational modifications, such as deamidation (Kim et al. 2002).

βA1- and βA3-crystallins are among very few examples of expression of multiple eukaryotic proteins from a single mRNA by a process called leaky ribosomal scanning (Werten et al. 1999a). Occasional skipping of the first start codon results in initiating translation at a second codon and expression of two proteins differing only in the N-terminal region. Analysis of cortical βA1A3-crystallins isolated from lenses of different ages showed very similar relative levels of βA1- and βA3-crystallins throughout life. The N-terminal truncations of these two proteins most likely proceed through the Δ4 form (Fig. 3) resulting in the Δ7, Δ8, Δ9, or Δ11 proteins. Despite the presence of the additional extension of 18 amino acid residues in βA3-crystallins, no truncated intermediates due to

cleavage among the first 18 residues of βA3 were detected.

With the exception of γC-, γD, and γB-crystallins, all the human lens crystallins are cotranslationally acetylated at their N termini. We recently showed that some γC-, γD-, and γB-crystallins in mature human lenses are post-translationally carbamylated. We report here that truncation of portions of the N-terminal regions of βA1/A3-crystallins results in Δ4, Δ7, and Δ8 forms with nonprotected N termini, which may then be N-carbamylated. Due to the relatively short lifetimes of these truncated forms, the level of the carbamylation is lower than carbamylation of γC-, γD and γB-crystallins. Carbamylation of accessible N-termini appears to be a common post-translational modification in lens proteins.

Materials and methods

Isolation of human βA1/A3-crystallins

Seven clear human lenses (one 11-d-old, two 11-yr-old, and four 19-yr-old) were obtained from the National Disease Research Interchange (Philadelphia, PA) or from the Lions Eye Bank (Omaha, NE). Each lens was divided into two parts. The inner nucleus was obtained by cutting a cylindrical sample with a 4-mm cork borer and removing 1.0–1.5 mm from each end of the cylinder. The end pieces were processed with the remainder of the lens. Extraction of crystallins from the two parts of each lens was performed by strong stirring of the tissue in a buffer containing 50 mM 2-[*N*-morpholino]ethane sulfonic acid (MES), 500 mM NaCl, 1 mM EDTA (pH 6.0) under argon at 0°C for 1.5 h. Approximately 1 mL of the buffer was used for each 12 mg of the tissue. The extracts were clarified by centrifugation and fractionated by size exclusion chromatography (Superose 12 HR 10/30 column, Pharmacia Biotech) in the extraction buffer. Because glutathiolated βA1/A3-crystallins have the same elution time as βA2-crystallin on reversed-phase HPLC, a 25 mM sodium citrate buffer containing 100 mM NaCl, 1 mM EDTA (pH 6.2), was used in the size exclusion chromatography isolating glutathiolated βA1/A3-crystallins. With these conditions, the majority of βA1/A3-crystallins eluted in the β_H-fraction, while βA2-crystallins, which form only dimers, eluted in the β_L-fraction (Lapko et al. 2003b). The total β-crystallins or individual β_H and β_L fractions (Ma et al. 1998) were further separated by reversed-phase HPLC using a 20% to 55% gradient of acetonitrile in water with 0.1% trifluoroacetic acid (TFA). The βA1/A3-crystallin fractions were concentrated to dryness, redissolved in 50% acetonitrile-containing 0.3% formic acid, and analyzed by mass spectrometry.

Derivatization of sulfhydryl groups of βA1/A3-crystallins

The βA1/A3-crystallins (20–200 pmol) were dissolved in 300 μL of buffer (250 mM Tris-HCl, 6 M guanidine hydrochloride [GdHCl], 1 mM EDTA, 5 mM dithiothreitol [DTT] at pH 8.5). After incubation for 1 h, the sulfhydryl groups were derivatized by reaction with 15 mM iodoacetamide for 30 min or with 25 mM 4-vinylpyridine (Friedman 2001) for 90 min. The reactions were quenched by adding 30 μL of 2-mercaptoethanol.

When the GSH adducts to βA1/A3-crystallins were to be determined, the crystallins were derivatized with iodoacetamide in a buffer without DTT. A solution of 30 mM iodoacetamide in a 6 M GndHCl buffer (pH 8.5) was added to dry protein samples and allowed to react for 15 min at room temperature. After derivatization, the proteins were desalted by RP HPLC.

Enzymatic digestions of βA1/A3-crystallins

The βA1/A3-crystallins, derivatized with iodoacetamide or 4-vinypyridine, were dissolved in 100 mM ammonium bicarbonate (pH 7.8) and digested at 37°C with modified trypsin (Promega) or chymotrypsin (Sigma) for 8 h or with Asp-N protease (Sigma) for 24 h at an enzyme:protein ratio of 1:50. Digests were freeze-dried and dissolved in 0.1% TFA for online mass spectrometric analysis or in 50% acetonitrile, 0.1% TFA for matrix-assisted laser desorption ionization (MALDI) mass spectrometry. Proteins with GSH-adducts were digested at trypsin:protein ratio of 1:30 for 3 h at 37°C.

Electrospray ionization mass spectrometric analysis of proteins

The βA1/A3-crystallins were dissolved in 50% acetonitrile, 0.3% formic acid, and injected directly into a Q-Tof mass spectrometer (Micromass) using a solvent flow of 5 μL/min. The typical uncertainty in protein mass determinations was 0.005%.

Mass spectrometric analysis of peptides

Peptides produced by enzymatic digestions were analyzed by online capillary reversed-phase HPLC (0.3 × 250 mm, C18-PM; LC-Packings) connected directly to an electrospray ionization ion trap mass spectrometer (Finnigan MAT LCQ). The HPLC gradient was 0%–50% acetonitrile in water, both with 0.01% TFA, over 50 min. The flow rate was 5 μL/min. Peptide elution was monitored at 214 nm. For peptide mapping, the mass spectrometer was routinely operated in the full-scan MS mode with the three most abundant ions of each scan analyzed by MS/MS. A collision energy of 35%–45% was used for the MS/MS analyses. The uncertainty in the peptide mass determinations was ±0.2 Da over the mass range of 100–2000 Da. The zoom mode of operation for a specific mass/charge with a window of ±5 *m/z* was used for detection of peptides containing methylation, glutathiolation, truncation, and carbamylation. Nano-flow reversed-phase HPLC-ESIMS using a Q-Tof Ultima mass spectrometer (Micromass) was also used for peptide mapping when the amount of sample was limited.

The peptides from enzymatic digestions were also analyzed by MALDI MS (Voyager-DE Pro mass spectrometer, Applied Biosystems). Typically, peptides were detected in the reflectron mode of operation. Large peptides were analyzed in the linear mode. α-Cyano-4-hydroxycinnamic acid was the matrix.

Estimation of the abundance of truncated βA1/A3-crystallins

The sites of truncation of βA1/A3-crystallins were confirmed by detecting the corresponding peptides from the N terminus of βA1-crystallins (Ion Trap mass spectrometer; Finnigan MAT LCQ). Carbamylated forms of the N-terminal peptides were identified by

detecting peptides with masses increased by 43 Da. The sequences of the N-terminal peptides were confirmed by MS/MS.

Estimates of the abundances of truncated βA1/A3-crystallins were calculated from the relative intensities of the mass spectral peaks for the proteins derivatized with 4-vinylpyridine as described previously (Lapko et al. 2003a). Calculations of the abundances of the truncated forms as well as other post-translational modifications should be considered only estimates because the mass spectral responses of the modified and unmodified proteins (or peptides) may not be equivalent. In addition, some selective losses could occur during the chromatographic isolations. The proteins included in estimations of truncated βA1/βA3-crystallins were intact βA1/βA3-crystallins, the forms missing four, seven, and eight residues from the N terminus, and the corresponding monomethylated species of each.

Estimation of abundance of S-methylation in βA1/A3-crystallins

Total methylation in nuclear βA1/A3-crystallin was calculated as the sum of estimated abundances of methylations at Cys 64, Cys 99, and Cys 167. The intensities of the peaks in MALDI mass spectra of tryptic digests of the βA1/A3-crystallins βA1/A3-crystallin were used to estimate the extent of methylation at Cys 64 and Cys 167. S-Methylation was recognized as a 91-Da decrease in the mass of a Cys-containing peptide derivatized with 4-vinylpyridine. Methylation at Cys 64 of βA1-crystallin was estimated from relative intensities of peaks for methylated and 4-vinylpyridinylated tryptic peptides of residues 50–72 (peaks at 2672.2 and 2763.2 Da, respectively) as well as the peptides of residues 47–72 (3000.4 and 3091.4 Da). Methylation at Cys 167 was estimated from the tryptic peptides of residues 160–175 or 160–178 of βA1-crystallin. Methylation at Cys 99 was estimated from the relative abundances of peptides from residues 92–107 derived from iodoacetamide-derivatized proteins by monitoring ions at 932.1 and 953.6 *m/z* (doubly charged species) using LC/ESI MS.

Because the abundance of methylation was lower in cortical βA1/A3-crystallins than in the nuclear proteins, the total methylation in cortical βA1/A3-crystallins was estimated from the relative intensities of the mass spectral peaks for the proteins derivatized with 4-vinylpyridine as described previously (Lapko et al. 2003a).

Analysis of glutathiolation of βA1/A3-crystallins

βA1/A3-Crystallins with GSH-adducts (βA1/A3+GSH) were isolated by reversed phase HPLC of the β_H fraction obtained after gel chromatography of β-crystallins from an 11-d-old lens in a 25 mM sodium citrate buffer. Di- and monoglutathiolated βA1/A3-crystallins eluted as separate peaks in front of the βA1/A3-crystallin peak. For older lenses, separation of glutathiolated βA1/A3-crystallins was relatively poor, although the front shoulder of βA1/A3-crystallins peak was enriched in the glutathiolated species.

The sites of glutathiolation in 11-d-old and 19-yr-old lenses were determined by MALDI MS of tryptic digests of the proteins derivatized with iodoacetamide without reduction of disulfide bonds. Tentative assignments of GSH-peptides 47–72 (3289.4 Da), 92–107 (2152.0 Da), and 92–104 (1808 Da) were confirmed by selective ion monitoring with MS/MS analyses of the expected ions. Changes in the abundance of glutathiolation with aging were estimated from mass spectra of nonreduced βA1/A3-crystallins isolated from nuclear and cortical β-crystallins of 11-d-old, 11-yr-old, and 19-yr-old lenses.

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