Methylation and carbamylation of human γ-crystallins

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Abstract

Accessible sulfhydryls of cysteine residues are likely sites of reaction in long-lived proteins such as human lens crystallins. Disulfide bonding between cysteines is a major contributor to intermolecular cross-linking and aggregation of crystallins. A recently reported modification of γS-crystallins, S-methylation of cysteine residues, can prevent disulfide formation. The aim of this study was to determine whether cysteines in γC-, γD-, and γB-crystallins are also S-methylated. Our data show that all the γ-crystallins are S-methylated, but only at specific cysteines. In γD-crystallin, methylation is exclusively at Cys 110, whereas in γC- and γB-crystallins, the principal methylation site is Cys 22 with minor methylation at Cys 79. γD-crystallin is the most heavily methylated γ-crystallin. γD-Crystallins from adult lenses are 37%–70% methylated, whereas γC and γB are ~12% methylated. The specificity of γ-crystallin methylation and its occurrence in young clear lenses supports the idea that inhibition of disulfide bonding by S-methylation may play a protective role against cataract. Another modification, not reported previously, is carbamylation of the N termini of γB-, γC-, γD-crystallins. N-terminal carbamylation is likely a developmentally related modification that does not negatively impact crystallin function.

Keywords: Human lens crystallins; cataract; cysteine S-methylation; N-terminal carbamylation; N-terminal acetylation

The primary function of the eye lens is to focus images on the retina. This function is possible because of the unique composition of the lens, where closely packed proteins account for ~38% of the wet mass. More than 90% of the lens proteins are structural proteins, called crystallins. The crystallins are organized into three main classes, α-, β-, and γ-crystallins, on the basis of the size of assemblies they form and their sequence homologies. The γ-crystallins, monomers with molecular masses of ~21 kD, constitute ~30% of the proteins in the nuclei of adult human lenses. Four γ-crystallin genes are expressed in humans: γS, γB, γC, and γD (Meakin et al. 1985; Heon et al. 1999). Several types of hereditary cataracts have been linked to mutations in human γ-crystallin genes (Heon et al. 1999; Stephan et al. 1999; Kmoch et al. 2000; Ren et al. 2000; Santhiya et al. 2002). A single base alteration in the γD-crystallin gene resulting in cysteine rather than arginine at residue 14 is associated with a type of hereditary, juvenile-onset punctate cataract (Stephan et al. 1999). This mutation in γD-crystallin does not cause conformational changes or destabilize the protein, but the presence of the additional exposed cysteine creates an opportunity for disulfide cross-linked oligomerization of the mutated protein that leads to cataracts (Pande et al. 2000). γ-Crystallins, which are rich in cysteine residues, are the most abundant disulfide-bonded crystallins present in the high molecular weight aggregates typical of senile nuclear cataracts (Takemoto and Azari 1977; Kodama and Takemoto 1988; Hejtmancik 1998; Lapko et al. 2002a). In addition, cysteine residues of lens crystallins are susceptible to other post-translational modifications that can lead to nondisulfide cross-linking (Garner et al. 2000).

Recent evidence of methylation at two specific cysteines of γS-crystallins suggests a possible protective mechanism against cataracts (Lapko et al. 2002b). Methylation of these exposed cysteine residues prevents their participation in the intermolecular disulfide bonding that leads to protein cross-linking, aggregation, and eventually cataract. To determine whether other members of the γ-crystallin family also have

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this post-translational modification, γC-, γB-, and γD-crystallins were isolated and analyzed by mass spectrometry. S-methylation was found among all γ-crystallins. In addition, carbamylation of the N termini of γC-, γD-, and γB-crystallins was established as a major post-translational modification during lens aging.

Results

This study identified several major post-translational modifications of human γ-crystallins and the age-related changes in these modifications. Quantitation of the post-translational modifications is approximate because the mass spectral responses of the modified and unmodified proteins and/or peptides may not be equivalent. The responses may also be affected by signal suppression due to coeluting components. In addition, there are undoubtedly some selective losses during the chromatographic isolations. A 19-year-old lens was chosen to illustrate these data (Figs. 1–4, below) because it has the major modifications of an adult lens (Datiles et al. 1992; Lampi et al. 1998; Ma et al. 1998; Lapko et al. 2002b), but fewer of the minor modifications that make isolation of proteins from older lenses more difficult and complicate the mass spectra. In addition, as much as 95% of the nuclear proteins from a 19-year-old lens are soluble, allowing characterization of the crystallins using soluble extracts. In this report, the term modification refers to in vivo post-translational reactions, whereas the term derivatization refers to in vitro reactions used in the analysis.

Post-translational modifications of γC-crystallin

The mass spectrum of soluble γC-crystallins from the nucleus of a 19-year-old lens (Fig. 1A) shows a peak at 20,747 Da corresponding to full-length γC-crystallin with a free N terminus and a second peak at 20,790 Da, suggesting carbamylation (+43 Da) or acetylation (+42 Da). The mass spectrum of these proteins after derivatization with 4-vinylpyridine showed, in addition to the two major components, two additional minor components, 91 Da lower than the major peaks at 21,496 and 21,539 Da (Fig. 1B). This decrease of 91 Da is indicative of S-methylation of cysteine residues and is present because methylation (+14 Da) prevents 4-vinylpyridine derivatization, which would add 105 Da. The intensity of the peaks indicates that S-methylation of these nuclear γC-crystallins is only about 6%.

To identify the major site(s) of modification and distinguish between carbamylation and acetylation, peptides from γC-crystallins digested with trypsin, chymotrypsin, or Asp-N proteases were analyzed. The accuracy in determining peptide masses (±0.2 Da) allows a 43 Da increase due to carbamylation to be distinguished from a 42 Da increase due to acetylation. Both reactions can occur at amino groups in the side chains of lysines or at free N termini of proteins.

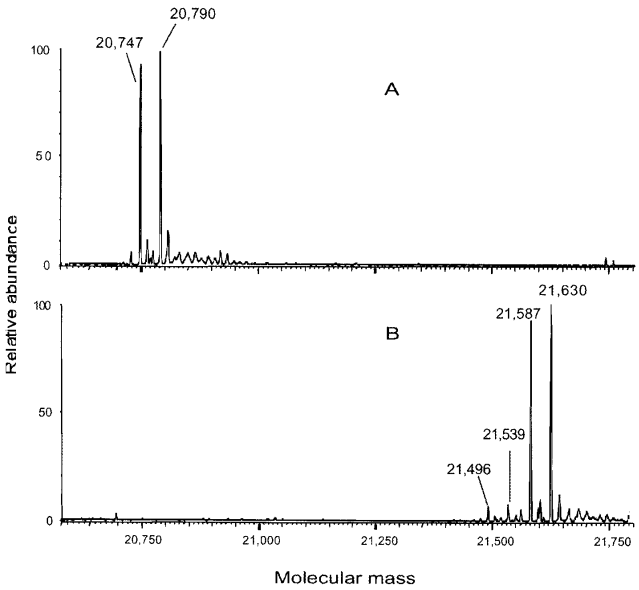

Figure 1. Reconstructed ESI mass spectra of γC-crystallin isolated from the nucleus of a 19-year-old lens. (*A*) Underivatized. The mass at 20,747 Da corresponds to unmodified γC-crystallin, whereas the mass at 20,790 Da indicates carbamylation or acetylation. (*B*) Derivatized with 4-vinylpyridine. The masses at 21,496 and 21,539 Da are 91 Da lower than the major peaks, indicating the presence of a methylated cysteine.

Peptide 1–6 of γC-crystallin was found both with and without addition of 43 Da. Tandem mass spectrometric (MS/MS) analysis of the unmodified (Fig. 2A) and the modified (Fig. 2B) peptides confirmed that the modification was carbamylation of the N terminus. A search for other sites of carbamylation or acetylation gave evidence that the N terminus was also acetylated. The acetylated peptide eluted after the carbamylated one during on-line HPLC/MS analysis. The total ion current for the carbamylated N-terminal peptide was 8–10 times higher than for the same peptide with acetylation. These data indicated that the mass spectral peak ~43 Da higher than γC was primarily due to N-terminal carbamylation.

Tryptic digests of γC-crystallins derivatized with 4-vinylpyridine were used to locate S-methylated cysteines. With this derivatization, all of the cysteine-containing peptides gave strong signals on both electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) mass spectrometry. Peptides containing S-methylated cysteines were recognized readily from the 91 Da decrease in mass. Analysis by MS/MS demonstrated that a peptide with a mass of 2034.9 Da had the same sequence as peptide 15–31 (2125.9 Da), but with methylation at Cys 22. In addition, minor methylation was detected at Cys 79. The methylation at Cys 22 was two to three times more abundant than at Cys 79 (data not shown). Further examination of peptides from γC-crystallin yielded no additional sites of methylation.

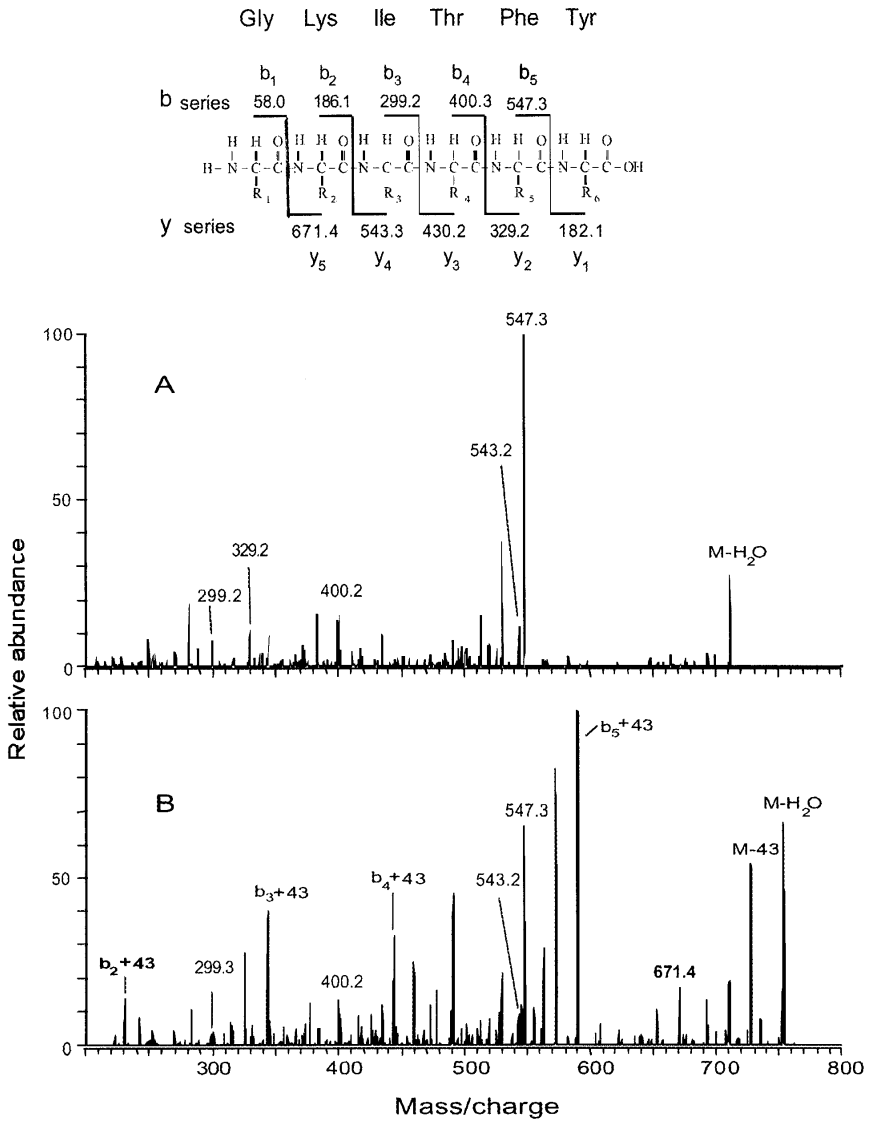

Figure 2. MS/MS spectra of the N-terminal chymotryptic peptide 1–6 of γC-crystallin with (*A*) a free N terminus (m/z 728.4) and (*B*) with a carbamylated N terminus (m/z 771.4). The b- and y-fragments at m/z 229.3 and m/z 671.4 are critical for identification of the carbamylation site. The presence of b_2 +43 ion and y_5 without modification, as well as absence of a peak at m/z 714.4, which would have indicated carbamylation at Lys 2, show that the N-terminal Gly is carbamylated. The presence of some b-series ions without the modification is consistent with previous observations that the carbamyl group is unstable in MS/MS analysis (Lapko et al. 2001; Van Driessche et al. 2002). The amino acid sequence and expected b- and y-fragments for peptide 1–6 are given at the *top*.

Post-translational modifications of γD-crystallin

In contrast to the simple mass spectra of γC- and γS-crystallins, the mass spectrum of γD-crystallins from the nucleus of a 19-year-old lens had eight major components ranging from 20,521 to 20,665 Da (Fig. 3A, Table 1). The complicated spectrum is due to combinations of three major post-translational modifications, that is, truncation of the C-terminal serine, carbamylation of the N terminus, and S-methylation of cysteines. Peaks 1–4 are due to full-length γD-crystallin (M_r 20,607) and its methylated and/or carbamylated forms, whereas peaks 5–8 are truncated γD-crystallin (M_r 20,520) and its methylated and/or carbamylated species. Molecular masses and identities of these forms and their derivatives are given in Table 1.

A combination of approaches was used to identify each γD-crystallin. These approaches included comparison of the spectra of proteins from the 19-year-old lens with proteins isolated from 11-day-old and 2-year-old lenses, and derivatization of the cysteines with different reagents. As shown in Table 2, the γD-crystallins in an 11-day-old lens are primarily unmodified and not truncated. There are two minor

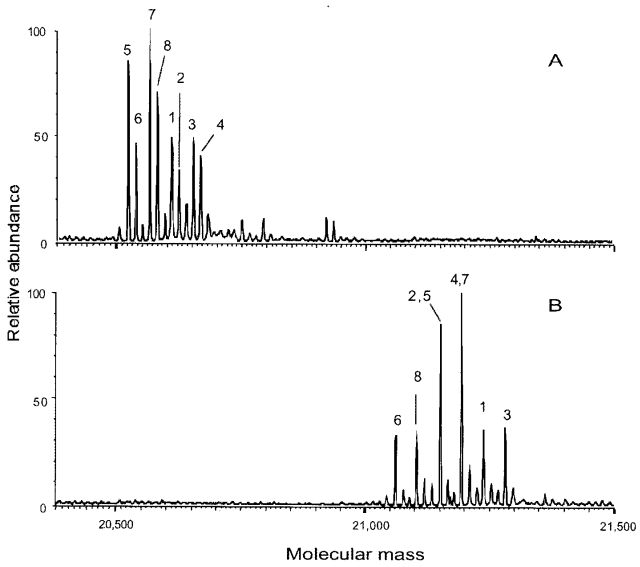

Figure 3. Reconstructed ESI mass spectra of γD-crystallins isolated from the nucleus of a 19 year-old lens. (*A*) Underivatized. (*B*) Derivatized with 4-vinylpyridine. The masses and identities of each numbered peak are given in Table 1.

species, one methylated and one carbamylated. In the 2-year-old lens, the abundance of these minor species is higher, and in addition, truncated γD-crystallins are present. Once it was established that γD-crystallins have these three modifications in 11-day-old and 2-year-old lenses, the masses of γD-crystallins from older lenses could be explained by combinations of methylation, carbamylation, and truncation.

Derivatization with 4-vinylpyridine was also helpful in identifying modifications in γD-crystallins because it alters both the masses and the elution pattern of the proteins in reversed-phase HPLC, allowing better isolation of some γD-crystallin species. Underivatized γ-crystallins elute in the order, γS-, γD-, γB-, and γC-crystallin, whereas after derivatization with 4-vinylpyridine, the order changes to γC-, γS-, γB-, and γD-crystallin. Derivatization with 4-vinylpyridine also permitted the S-methylated (+14 Da) forms to be distinguished from possible oxidized (+16 Da) or N-methylated products because after derivatization, the masses of S-methylated species are 91 Da lower than the corresponding nonmethylated species, whereas oxidized and N-methylated forms are, respectively, 16 and 14 Da higher.

Because all of the tryptic peptides of 4-vinylpyridine derivatized γD-crystallins produced strong peaks in MALDI MS, the sites of methylation and the extent of methylation were determined by use of tryptic peptides. In addition, peptide 107–112 from Asp-N digestion of iodoacetamide-derivatized γD was used to determine the methylation status of Cys 108 and Cys 110. A selected ion plot for methylated peptide 107–112 (m/z 787.2, data not shown) showed only one peak and the MS/MS spectrum of this peak demonstrated that Cys 110 is the only methylated residue in this peptide (Fig. 4).

In addition to S-methylation, N-terminal carbamylation, N-terminal acetylation, and truncation of the C-terminal serine were found in the peptides from γD-crystallin. Both carbamylation and acetylation of the N-terminal Gly residue of γD-crystallin were present, with the peaks due to carbamylation approximately eight times larger than those due to acetylation. Truncation of the C-terminal Ser was confirmed by MS/MS analyses of tryptic peptides 168–172 and 169–172 (data not shown). The various masses of γD-crystallins (Fig. 3; Table 1) could thus be attributed to combinations of methylation at Cys 110, carbamylation (or acetylation) of the N terminus, and truncation of the C-terminal serine.

Table 1. *Experimentally determined masses (Da) of the major human* γD*-crystallins*

Peak number (Fig. 3)	In vivo modification	Underivatized	4-Vinylpyridine-derivatized	Iodoacetamide-derivatized
1	None	20,608	21,238	20,950
2	S-Methylation	20,622	21,147	20,907
3	Carbamylation	20,651	21,281	20,993
4	S-Methylation and carbamylation	20,665	21,190	20,950
5	Truncation	20,521	21,151	20,863
6	Truncation and S-methylation	20,535	21,060	20,820
7	Truncation and carbamylation	20,564	21,194	20,906
8	Truncation, S-methylation and carbamylation	20,578	21,103	20,863

Table 2. *Estimated abundance (%) of soluble γD-crystallins from human lenses of different ages*

	Inner nucleus						Remainder of lens				
Modification	11 days	2 years[a]	11 years	19 years	50 years	72 years	11 days	11 years	19 years	50 years	72 years
None	89	65	26	11	5		89	38	21	20	17
Methylation	4	6	8	6	5		4	6	6	6	6
Carbamylation	7	16	13	11	4	7	7	13	13	10	8
Methylation and carbamylation			5	9	6	6		4	6	4	4
Truncation		12	26	20	12	7		21	19	15	8
Truncation and methylation			6	11	20	19		4	7	11	15
Truncation and carbamylation			13	22	19	20		10	18	15	14
Truncation and methylation and carbamylation			4	12	26	40		3	9	19	28
Total methylation	4	6	23	37	59	70	4	17	29	41	53
Total carbamylation	7	16	34	49	55	68	7	30	46	50	57
Total truncation		12	49	64	77	86		39	54	60	65

[a] The inner nucleus of the 2-year-old lens was not analyzed separately.
Proteins present at less than 3% are not listed.
The value for carbamylation includes minor N-terminal acetylation.

γB-crystallin and its major modifications

Although molecular biology data indicate that there are at least six closely related human γ-crystallins (Wistow and Piatigorsky 1988; Graw 1997), only three γ-crystallins—γS, γC, and γD—are abundant. In a recent study analyzing peptides from digests of the total proteins of a 4-year-old cataractous lens, peptides derived from γB-crystallin were detected (MacCoss et al. 2002). In the present study, the presence of a protein with a molecular mass of 20,776 Da (Fig. 5A) corresponding to the sequence of γB-crystallin, is supporting evidence that γB is expressed. This protein, which accounted for ~4% of the total γ-crystallins, eluted in reversed-phase HPLC as a small peak between γD- and γC-crystallins with some overlap with both. Identification of the peptides from digestion of this protein with trypsin and Asp-N proteases confirmed the published sequence (den Dunnen et al. 1985).

Modifications of γB were determined from peptides produced by enzymatic digestion after HPLC separation of the 4-vinylpyridine-derivatized proteins. The mass spectrum of γB isolated from a 19-year-old lens suggested the presence of carbamylation and minor methylation (Fig. 5B). MS/MS analysis of an Asp-N peptide at m/z 900.4 showed it had the sequence of γB peptide 1–7 with carbamylation at the N terminus. Other modifications of γB included minor methylations at Cys 22 and Cys 79, as well as minor amounts of N-terminal acetylation, similar to the post-translational modifications of γC-crystallin.

In vitro methylation of human γ-crystallins

The possibility that the enzyme betaine-homocysteine methyltransferase (BHMT) could methylate γ-crystallins was tested using BHMT isolated from monkey lenses and recombinant human BHMT. For these experiments, total γ-crystallins from an 11-day-old human lens were the substrate, and both betaine and dimethylacetothetin were tested as donors of methyl groups (Garrow 1996). The initial level of methylation of γS- and γD-crystallins in the 11-day-old lens sample was ~5%. No increase in methylation was detected, even after 8 h of incubation.

Age-related changes in abundances of major species γ-crystallins

The inner nuclei and the remaining portions of lenses were analyzed separately to determine age-related changes in the modifications of γC- and γD-crystallins. For γC-crystallin, carbamylation increased with age to 50%–64% in adult lenses (Table 3). In lenses of 11 years and older, N-terminal carbamylation was 8–10 times more abundant than acetylation, but in the 11-day-old lens, carbamylation and acetylation were both about 2%–3%. Methylation of γC-crystallins increased to 12% in adult lenses. In all lenses, modifications were similar for γC-crystallins from the inner nucleus and the remainder of the lens.

Despite their complicated mass spectra, the age-related changes in the post-translational modifications of γD-crys-

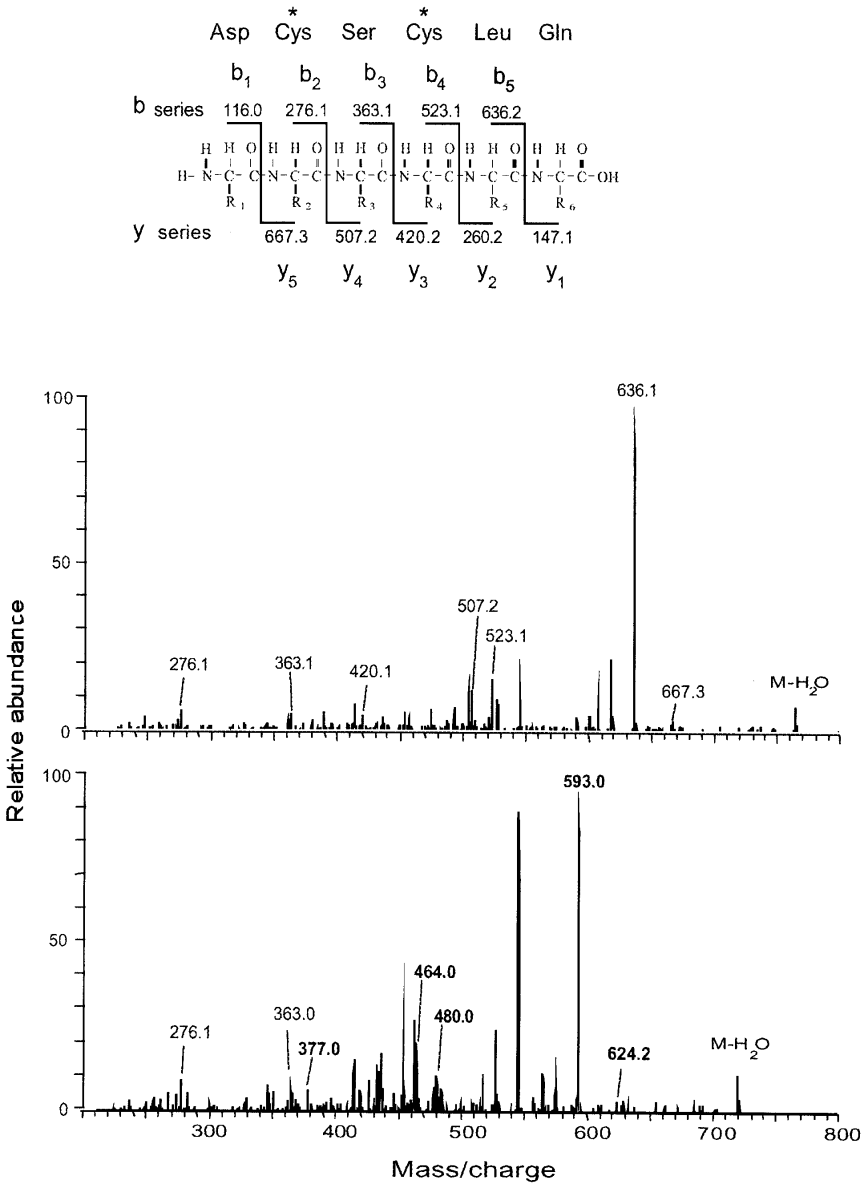

Figure 4. MS/MS spectra of peptides corresponding to 107–112 from an Asp-N digest of iodoacetamide-derivatized γD-crystallin isolated from the nucleus of a 19-year-old lens containing (*A*) no methylated cysteines (m/z 782.3) and (*B*) one methylated Cys residue (m/z 739.3). Because Cys 110 was methylated (+14 Da) and could not be derivatized by iodoacetamide (+57 Da), all fragments (b_4, b_5, y_3, y_4, y_5) that included Cys 110 showed a net loss of 43 Da. The expected y- and b-fragments for the peptide with both cysteines derivatized are given at the *top*.

tallins from lenses, ages 11 days to 19 years, could be estimated from the abundances of the MS peaks of the undigested proteins. Because mass spectra of the 50- and 72-year-old lenses were substantially more complicated, the extent of modification was estimated from mass spectra of peptides. The levels of carbamylation of γD- and γC-crystallins were similar (Tables 2 and 3), with slightly higher carbamylation among γD-crystallins from the inner nuclei. Methylation of γD-crystallins was 4–6 times higher than γC, again with slightly higher methylation in the inner nuclei than in the remaining portion of the lens. The specificity of methylation did not vary with the age of the lens. Probably, the most prominent difference between γD-crystallins from inner nucleus and the remainder of the lens was the higher level of nonmodified full-length γD-crystallin in the outer portions of the 52- and 70-year-old lenses compared with the inner nuclei. Also, in the 52- and 70-year-old lenses, carbamylation and methylation of truncated γD is several times higher than carbamylation and methylation of the full-length protein (Table 2).

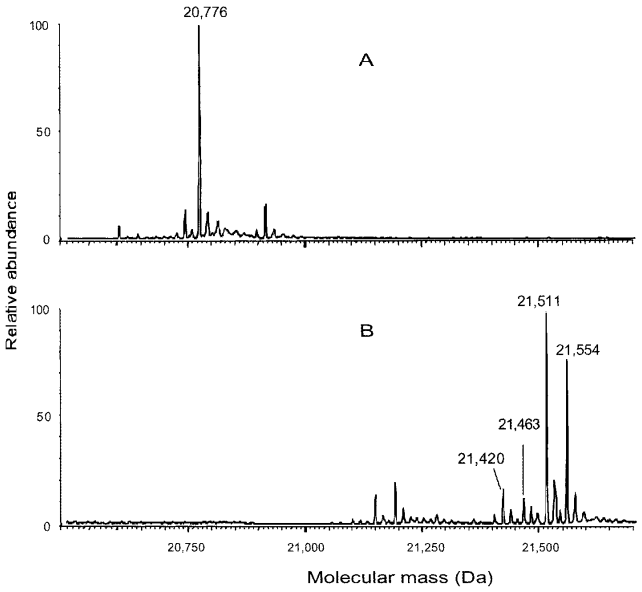

Figure 5. (*A*) Reconstructed ESI mass spectrum of γB-crystallin isolated from an 11-day-old lens. The mass at 20,776 Da corresponds to unmodified γB-crystallin. Small peaks due to γS, γC, and γD are also evident. (*B*) Mass spectrum of γB-crystallin from the nucleus of a 19-year-old lens after derivatization with 4-vinylpyridine and isolation by reversed-phase HPLC. The mass at 21,511 Da corresponds to γB-crystallin with all seven Cys residues derivatized. The mass at 21,554 Da is the carbamylated protein. The peaks 91 Da lower than the major peaks at 21,420 and 21,463 Da indicate the presence of minor S-methylation. The small peaks at 21,150 and 21,194 Da are γD-crystallins.

Discussion

Although γ-crystallins account for ~30% of the soluble protein of adult lenses, the post-translational modifications of these crystallins are not yet completely characterized. Previous publications have reported truncations, disulfide bonding, methionine oxidation, and deamidation of γ-crystallins (Abbasi et al. 1998; Hanson et al. 1998, 2000; Lapko et al. 2002a). In addition, phosphorylation and oxidation of tyrosines and methylation of lysines were reported in a 4-year-old cataractous lens (MacCoss et al. 2002). Recently, methylation of specific cysteine residues was identified as one of the most abundant post-translational modifications in clear human lenses (Lapko et al. 2002b). We now report that S-methylation is a major post-translational modification of all of the γ-crystallins. In addition, we found N-carbamylation of γC-, γD-, and γB-crystallins. Identification of these post-translational modifications in γ-crystallins was facilitated by instrumental developments that have improved the resolution of protein mass spectra.

S-methylation of cysteine residues in crystallins is of particular interest because methylated cysteines are not capable of forming the disulfide bonds that lead to high molecular weight aggregates typical of cataract. Protein methylation, which has been implicated previously in numerous functions such as signal transduction, regulation of gene activity, and protein sorting (Armitage 1999; Davie and Dent 2002; Kouzarides 2002), typically occurs at side chains of lysine, arginine, glutamic, and aspartic acids, and at some N- and C-terminal residues (Park and Paik 1990). Beside our report of S-methylation of γS-crystallin (Lapko et al. 2002b), the only other reports of in vivo methylation of cysteine are S-methylation of hemoglobin following exposure to methylating agents (Bailey et al. 1981; Ferranti et al. 1996), self-methylation of methyltransferases involved in methylation of cytosine in DNA (Szilak et al. 1994), and repair of DNA damage caused by alkylating agents (Olsson and Lindahl 1980). Only about 0.02% of human hemoglobin is S-methylated (Tornqvist et al. 1988), whereas about 50% of γD-crystallin in older lenses is methylated.

Methylation in γ-crystallins is highly specific. γD-, γS-, γC-, and γB-Crystallins have six, seven, eight, and seven cysteine residues, respectively. Among these cysteines, only Cys 26 of γS-crystallin and Cys 110 of γD-crystallin are heavily methylated. Comparison of the sites of S-methylation and the amino acid sequences (Fig. 6) suggests certain patterns in the methylation of γ-crystallins. Two major sites of methylation, Cys 26 of γS-crystallin and Cys 110 of γD-crystallin have the sequence: Asp-Cys-X-Cys, in which X = Asp in γS or Ser in γD. Both sites are within acidic clusters. The cysteine residue corresponding to Cys 26 of γS-crystallin is conserved in both γC- and γB-crystallins (Fig. 6), but the level of methylation at this Cys residue in γC- and γB-crystallins is much lower than in γS-crystallin.

Surface exposure of amino acid residues is an important factor in the reactivity of some functional groups of proteins. The extent of cysteine methylation in γ-crystallins also appears to be influenced by accessibility. Both Cys 26 of γS- and Cys 110 of γD-crystallin are among the most exposed cysteines. γC-crystallin has several cysteine residues exposed to solvent, however, the total methylation of the protein is low. Minor methylations were found at Cys 22 (accessibility of 44 Å) and Cys 79 (36 Å), but the most exposed Cys residue of γC-crystallin, Cys 153 (52 Å), did not have detectable methylation. These data indicate that accessibility alone is not sufficient for high methylation of cysteines. In this study, methylation at sites other than cysteine residues was not detected. The methylation at Lys 6 of γS-crystallin, reported recently by MacCoss et al. (2002), was not seen, indicating that this methylation, if present, is extremely low.

Although direct nonenzymatic action of a methylating agent in the lens is possible, the high specificity of methylation and the presence of methylated γ-crystallins even in very young lenses suggest that the reaction is enzymatic. The presence of several methyltransferases and methionine adenosyltransferase, a S-adenosylmethionine synthesizing enzyme, in human lenses is consistent with enzymatic methylation in the lens (Geller et al. 1986; Rao et al. 1998;

Table 3. *Estimated abundance (%) of modified human γC-crystallins from human lenses of different ages*

Modification	11 days	2 years[a]	11 years	19 years	43 years	50 years	59 years	72 years	83 years
Carbamylation (inner nucleus)	5	16	38	54	52	54	53	63	61
Carbamylation (remainder)	4	16	35	51	51	52	52	64	62
Methylation (inner nucleus)	n.d.	n.d.	4	6	12	13	12	12	12
Methylation (remainder)	n.d.	n.d.	3	5	11	13	12	11	12

[a] The inner nucleus of the 2-year-old lens was not analyzed separately.
(n.d.) Not detected.
Two independent analyses were performed on each lens extract. The precision was 3% or better.
Data are from the following lenses: one each, 11 days, 2, 11, 59, 72, and 83-year-old lenses; two each, 19, 43, and 50-year-old lenses

Cornish et al. 2002). One of the most abundant proteins in the nucleus of monkey lenses is betaine-homocysteine methyltransferase (BHMT; Rao et al. 1998). This enzyme, which is involved in the biosynthesis of methionine, is also expressed in human lenses (Rao et al. 1998). A novel isozyme, betaine-homocysteine methyl transferase 2 (BHMT2) was identified recently in several human tissues and its expression was found in fetal eyes (Chadwick et al. 2000). Both protein extracts from monkey lenses and recombinant human BHMT were tested for their ability to methylate human γ-crystallins in vitro. No detectable methylation was found, leaving unanswered the question of what enzyme(s) might be involved in the methylation of γ-crystallins. In vivo S-methylation is a very slow reaction developed over decades. Finding conditions that accelerate the reaction in vitro could facilitate detection of S-methylation.

Another abundant post-translational modification identified in this study is carbamylation of the N termini of γB-, γC-, and γD-crystallins. Carbamylation is often associated with the reaction of proteins with isocyanate. Because urea decomposes to isocyanate, carbamylation can occur in urea solutions. The product of the reaction is a protein with $-CONH_2$ (+43 Da) attached to an amino group. The development of cataracts in patients with renal failure was speculated to be the result of increased concentrations of cyanate in the body leading to carbamylation of the lens crystallins (Harding and Crabbe 1984; van Heyningen and Harding 1988), but examination of α-crystallins from uremic patients gave no evidence of carbamylation (Smith et al. 1995). Carbamylation is not necessarily due to the presence of urea or cyanate/isocyanate. Enzymatic carbamylation involving carbamylphosphate as a carbamylating agent is also known. N-carbamylation of aspartate is the first step in the biosynthesis of pyrimidine, and carbamylation of ornithine is involved in the biosynthesis of arginine. The term carbamylation has also been used to describe addition of CO_2 (Lorimer and Miziorko 1980; Golemi et al. 2001). The product of this reaction has a -COOH group (+44 Da) attached. An example of this type of carbamylation is the activation of ribulose-1,5-bisphosphate carboxilase/oxigenase (Rubisco) by covalent binding of carbon dioxide to a specific Lys residue in the active site of the enzyme (Lorimer and Miziorko 1980). Another example is the reversible binding of carbon dioxide to the N-terminal amino group of hemoglobin.

Carbamylation due to addition of $-CONH_2$, as observed in γ-crystallins, occurs most readily at the free N termini of proteins (Martin and Harding 1989) and then at ε-amino groups of lysines. Like the majority of proteins, (Brown 1970; Driessen et al. 1985; Kendall et al. 1990), α-, β-, and γS-crystallins are N-terminally acetylated during translation, but γC-, γD-, and γB-crystallins have free N termini, which are susceptible to carbamylation. The reasons for differences in the status of the N termini of γ-crystallins are not clear. The human γ-crystallins are highly homologous. Even though they have similar molecular masses, γS-crystallin elutes slightly earlier than the other γ-crystallins on size exclusion chromatography, suggesting that the conformation of γS-crystallin is less compact. γS-crystallin has a four-residue insert, TGTK (residues 2–6) not present in the other γ-crystallins. It is possible that the presence of this insert results in conformational differences and affects the status of the N-terminal residue.

Carbamylation of γC- and γD-crystallins, at 45%–60% in adult human lenses is among the more abundant modifications. To our knowledge, the only previously reported in vivo N-terminal carbamylation is in a bacterial high-potential iron-sulfur protein (Van Driessche et al. 2002). The level of carbamylation of this bacterial protein, ~50%, is comparable with the carbamylation of γC- and γD-crystal-

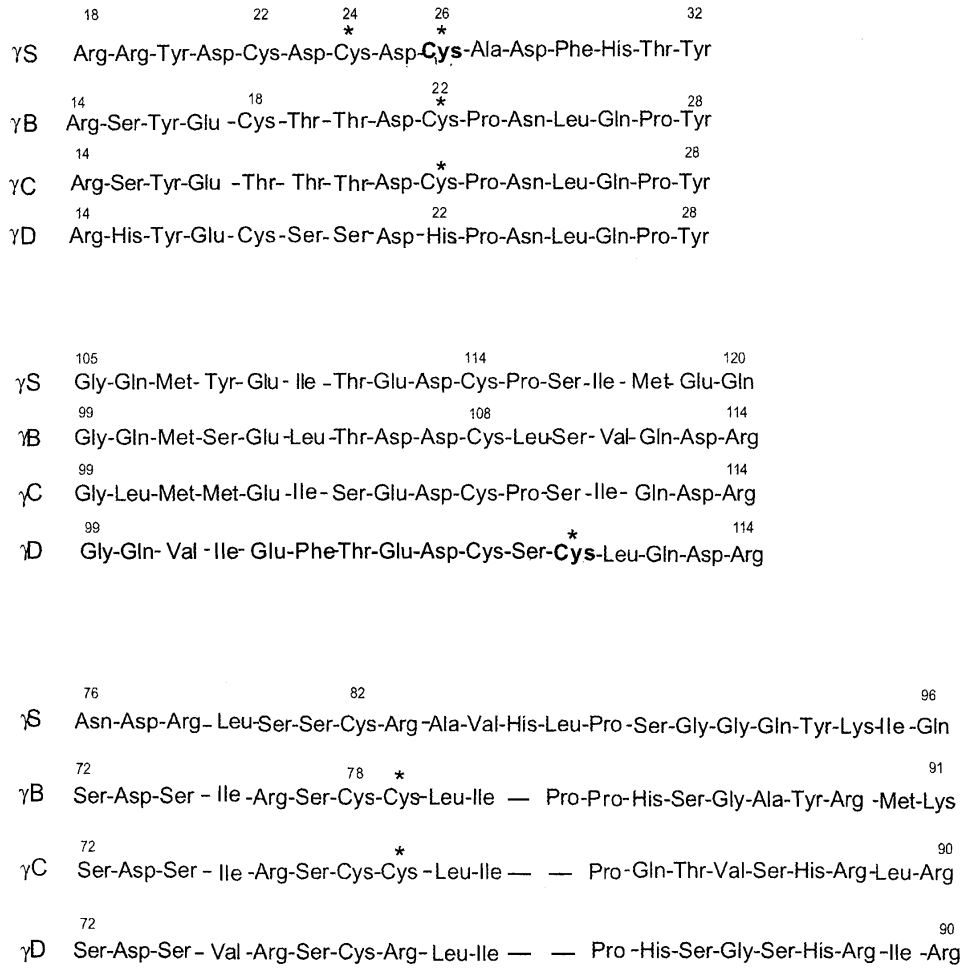

Figure 6. Amino acid sequences of homologous regions of human γ-crystallins containing sites of S-methylation indicated by an asterisk. The major methylation sites, Cys 26 of γS- and Cys 110 of γD-crystallin, are shown in bold.

lins. The importance of carbamylation of lens crystallins, its affect on protein conformation and aggregation, and potential to induce cataract have been discussed extensively (Beswick and Harding 1984, 1987; Harding and Crabbe 1984; Crompton et al. 1985; Qin et al. 1992; Smith et al. 1995; Derham and Harding 1999). However, in these studies, the carbamylation was at the ε-amino groups of lysines, which likely affects protein structure very differently from N-terminal carbamylation. The presence of N-carbamylation in γ-crystallins from young lenses suggests that this modification is not detrimental to protein solubility and function.

N-acetylation of γB-, γC-, and γD-crystallins was also detected. In adult lenses, N-acetylation is ~10%–12% of the level of N-terminal carbamylation, but in the 11-day-old lens, acetylation and carbamylation of both γC and γD were comparable (2%–3%). Whether this N-acetylation is due to co- or post-translational acetylation is not known. Usually, N-terminal acetylation of proteins is a cotranslational event, but there are examples of N-terminal acetylation occurring after translation (Dores et al. 1990). N-terminal acetylation is performed by N-terminal acetyltransferases distinct from transferases carrying out internal post-translational acetylation of proteins (Polevoda et al. 1999). Internal post-translational acetylation may have a role in signaling and other cellular functions, similar to that of phosphorylation (Magnaghi-Jaulin et al. 2000; Sterner and Berger 2000; Kouzarides 2002). Despite its wide occurrence, the biological role of N-terminal acetylation remains obscure (Smyth and Zakarian 1980; Tsunasawa and Sakiyama 1984; Tercero et al. 1993).

In addition to S-methylation, N-carbamylation, and N-acetylation of γD-crystallin, we observed the previously reported truncation of the C-terminal Ser residue (Abbasi et al. 1998). This truncation is analogous to truncation of the C-terminal Ser of αA-crystallin (Miesbauer et al. 1994; Takemoto and Gopalarishnan 1994). It has been suggested that truncation of the C terminus of αA-crystallin may contribute to cataractogenesis by affecting the aggregation state and chaperone activity of the protein (Kelley et al. 1993; Takemoto 1999; Thampi et al. 2002). The high level of

truncated γD-crystallins in clear lenses, especially in the nucleus, argues against a similar role for this modification in γD.

In contrast to methylation of γS-crystallin, which is higher in the nucleus than cortex (Lapko et al. 2002b), methylation and carbamylation of γC-crystallins are similar throughout the lens. Although the spatial distribution of modifications may be due to differential localization of enzymes (Rao et al. 1998; Chadwick et al. 2000) or other reactive species, the main factor determining the extent of modifications is probably the age of the protein. Our data on the abundance of γ-crystallins in human lenses (Table 4) are in agreement with previous data that showed no γC- nor γB-crystallin transcripts in 10-year-old lenses (Brakenhoff et al. 1990), but increasing expression of γS-crystallin as the lens ages (Ma et al. 1998). Because the lens grows by adding layers of cells at the periphery, accumulating the oldest proteins in the nucleus and the more recently synthesized in the cortex, cortical γS-crystallins will not have had as much opportunity to become methylated as those in the inner nucleus. In contrast, as there is little biosynthesis of γC-crystallins after birth, all γC-crystallins are of approximately the same age and have similar methylation, regardless of their location in the lens. The abundances of γ-crystallins (Table 4) indicate some redistribution of γC- and other γ-crystallins between the inner nucleus and the remainder of the lens. These data suggest a dynamic organization of the lens allowing diffusion or exchange of proteins from the nucleus.

Materials and methods

Extraction and isolation of crystallins from human lenses

Lenses in this study were from the National Disease Research Interchange or from the Lions Eye Bank. The study included 12 clear lenses from donors, ages 11 days to 83 years old. The donor histories indicated no disorders known to affect lens clarity. The lenses were removed within 12 h of death, shipped on ice, and stored at −80°C until analysis. Each lens was divided into two parts, the inner nucleus (average weight 35 mg) and the remainder of the lens. 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. The two parts of each lens were homogenized and analyzed separately, using ~1 mL of the homogenization buffer (50 mM 2–[N-morpholino]ethane sulfonic acid [MES], 500 mM NaCl, 1 mM EDTA [pH 6.0]) for each 12 mg of the tissue. The tissue was homogenized by strong stirring, under argon, at 0°C for 1.5 h and centrifuged for 30 min at 33,000*g*. The supernatant, which contained the soluble proteins, was used for isolation of soluble γ-crystallins.

Soluble proteins obtained from the inner nuclei and the remaining portions were each fractionated by size exclusion chromatography (Superose 12 HR 10/30 column, Pharmacia Biotech) equilibrated with the homogenization buffer at flow rate of 0.4 mL/min. The total γ-crystallins were collected, and individual γ-crystallins isolated by reversed-phase HPLC (Vydac 4.6 × 150 mm C4 column) equipped with a protein Macrotrap (Michrom BioResources) using a gradient of 20%–55% acetonitrile with 0.1% trifluoroacetic acid (TFA) over 35 min (Hanson et al. 1998). The proteins were concentrated to dryness, redissolved in 50% acetonitrile containing 0.3% formic acid, and analyzed by mass spectrometry.

Determination of abundances of γ-crystallins in human lenses

Protein concentrations in lens extracts and in the γ-crystallin fractions obtained after gel-filtration chromatography were determined using Bradford Reagent (Sigma Chemical Co.). The quantity of individual γ-crystallins (γS-, γC-, γD-, and γB) was estimated from their UV-absorbances using extinction coefficients calculated from their amino acid sequences (Pace et al. 1995). When mass spectra indicated that γ-crystallin fractions were not pure, the crystallins were derivatized with 4-vinylpyridine and separated by reversed-phase HPLC, in which the derivatized crystallins eluted as separate peaks. Each crystallin was quantified by its UV-absorbance.

In vitro experiments on methylation of γ-crystallins

The ability of recombinant BHMT and monkey lens crystallins to methylate lens proteins was examined using the total γ-crystallin fraction from a 11-day-old human lens isolated by size-exclusion chromatography. Recombinant human BHMT, with a specific activity ~2000 units/mg (Garrow 1996), was a gift from Dr. T. Garrow. Monkey BHMT was prepared from Rhesus monkey lenses as described by Rao et al. (1998). The reaction mixture included human γ-crystallins (0.8 mg/mL), 5 mM betaine or dimethylacetothetin as the donor of methyl groups, 0.5 or 5 mM DTT, 20 μL BHMT (1 mg/mL), or 20 μL of the β_H fraction of monkey lenses (1 mg/mL), 25 mM NaCl and 50 mM Tris-HCl (pH 7.8). The final

Table 4. *Abundance (mg) of major soluble γ-crystallins in human lenses of different ages*

	Inner nucleus					Remainder of lens				
Lens age	Total protein	Total γ	γS	γD	γC	Total protein	Total γ	γS	γD	γC
11 days	8.10	2.55	0.42	0.57	1.26	5.20	1.51	0.38	0.37	0.62
11 years	8.32	2.71	0.78	0.76	0.80	14.73	3.20	1.32	0.75	0.68
19 years	8.61	2.92	0.90	0.77	0.80	16.24	3.86	1.70	0.75	0.85
50 years	6.02	2.45	1.11	0.65	0.34	26.80	7.10	3.16	1.78	0.75

reaction volume was 110 μL. After incubation for 8 h at 30°C, the γ-crystallins were isolated by reversed-phase HPLC, and the level of methylation was determined by mass spectrometry.

Derivatization of sulfhydryl groups of γ-crystallins

γ-Crystallins (40–200 pmole) were dissolved in 300 μL of a buffer (250 mM Tris-HCl, 6 M guanidine hydrochloride, 1 mM EDTA, 5 mM dithiothreitol at pH 8.5). After incubation for 1 h, the sulfhydryl groups were derivatized by reaction with 15 mM iodoacetamide or iodoacetic acid for 30 min or with 25 mM 4-vinylpyridine for 90 min (Friedman 2001). The reactions were quenched by adding excess dithiothreitol. The derivatized proteins were desalted by reversed-phase HPLC, and analyzed by ESIMS.

Enzymatic digestions of γ-crystallins

γ-Crystallins derivatized with iodoacetamide or 4-vinypyridine were digested with trypsin or chymotrypsin in 100 mM ammonium bicarbonate at 37°C for 8 h or with Asp-N protease for 24 h at an enzyme:protein ratio of 1:50. Asp-N digestion at an enzyme:protein ratio 1:100 for 18 h was used to generate large C-terminal fragments of γD-crystallins. Digests were freeze-dried and dissolved in 0.1% TFA for on-line mass spectrometric analysis or in 50% acetonitrile, 0.1% TFA for MALDI MS.

Analysis of γ-crystallins by ESIMS

Intact γ-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%. Peptides produced by enzymatic digestions were analyzed by on-line capillary reversed-phase HPLC-ESIMS using an ion trap mass spectrometer (Finnigan MAT LCQ). 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 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 specified mass/charge with a window of ±5 m/z was used for detection of carbamylation and acetylation in γ-crystallins and for analysis of methylation in γD-crystallins. Resolution of mass spectra of proteins was enhanced using maximum entropy software (Micromass).

Analysis of peptides by MALDI

Enzymatic digests were also analyzed by MALDI MS (Voyager-DE Pro mass spectrometer, Applied Biosystems). Typically, peptides were detected in the reflectron mode of operation. Large C-terminal fragments of γD-crystallins were analyzed in the linear mode. In all experiments, α-cyano-4-hydroxycinnamic acid was the matrix.

Quantification of major modifications of γ-crystallins

Mass spectral peak intensities of the γC-crystallins derivatized with 4-vinylpyridine were used to calculate the abundance of each modification. In this calculation, the intensity due to each particular modification was divided by the sum of the intensities of the peaks due to all γC-crystallin forms. The calculated values for carbamylation include an ~10% contribution from acetylation due to overlap of the peaks for acetylated and carbamylated γC.

Analyses of protein digests, as well as undigested proteins, were used to estimate the level of γD-crystallin modification. For γD-crystallins from the 50 and 72-year-old lenses, methylation and truncation were estimated from MALDI analysis of the appropriate peptides. Peptide 99–114 in a tryptic digest of 4-vinylpyridine derivatized γD was used to estimate methylation, and Asp-N peptides 113–172 and 113–173 were used to estimate truncation. N-terminal carbamylation was estimated from peaks in the zoom mode of ESIMS for peptides 1–7, unmodified at m/z 694.5 and carbamylated at m/z 737.5.

The relative abundance of the modified forms of γD-crystallin from lenses, 11 days old to 19 years old, were estimated from the size of the mass spectral peaks for each protein. These major forms accounted for ~85% of the total γD-crystallins. The masses of each protein are given in Table 1. The values for the methylated species include both the methylated form (+14 Da) and the oxidized (+16 Da) forms. Corrections calculated from mass spectra of the 4-vinylpyridine derivatives, in which the methylated and oxidized forms were separated, indicated that these forms contributed <3%. Once the abundance of each γD-crystallin form had been estimated, the total methylation was calculated as the sum of the four species that included this modification (methylated γD, methylated and carbamylated γD, methylated and truncated γD, and methylated, truncated, and carbamylated γD). The total level of carbamylation and truncation were calculated similarly.

Estimation of the ratio of N-terminal acetylation to N-terminal carbamylation

The relative abundance of N-terminal carbamylation and acetylation in γC- crystallin was estimated from the ratio of the peaks for peptide 1–6 with carbamylation and acetylation at m/z 771.3 and m/z 770.3, respectively, using the zoom mode of the ion trap mass spectrometer at m/z 770.8 ± 5. Similarly, the ratio of N-carbamylated and N-acetylated γD-crystallins was estimated from the peaks for carbamylated and acetylated peptide 1–6 at m/z 736.8 ± 5.

Accessibility calculations

The surface accessibilities of the cysteine residues in γ-crystallins were calculated with the program GETAREA 1.1 (http://www.scsb.utmb.edu/cgi-gin/get_a_form.tcl) developed at The Sealy Center for Structural Biology, University of Texas Medical Branch, Galveston, TX as described previously (Lapko et al. 2002a). The sequence of each γ-crystallin was superimposed on the X-ray structure of bovine γD-crystallin for these calculations (Chirgadze et al. 1996).

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