Published in final edited form as:

Biochem J. ; 474(14): 2475–2487. doi:10.1042/BCJ20170268.

Hotspots of Age-Related Protein Degradation. The Importance of Neighbouring Residues for the Formation of Non-Disulfide Crosslinks derived from Cysteine

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Abstract

Over time, the long-lived proteins that are present throughout the human body deteriorate. Typically, they become racemised, truncated and covalently crosslinked. One reaction responsible for age-related protein crosslinking in the lens was elucidated recently and shown to involve spontaneous formation of dehydroalanine (DHA) intermediates from phosphoserine.

Cys residues are another potential source of DHA, and evidence was found for this in a number of lens crystallins. In the human lens, some sites were more prone to forming non-disulfide covalent crosslinks than others. Foremost among them was Cys 5 in β A4 crystallin. The reason for this enhanced reactivity was investigated using peptides.

Oxidation of Cys to cystine was a pre-requisite for DHA formation and DHA production was accelerated markedly by the presence of a Lys, one residue separated from Cys 5. Modelling and direct investigation of the N-terminal sequence of β A4 crystallin, as well as a variety of homologous peptides, showed that the epsilon amino group of Lys can promote DHA production by nucleophilic attack on the alpha proton of cystine. Once a DHA residue was generated, it could form inter-molecular crosslinks with Lys and Cys. In the lens, the most abundant crosslink involved Cys 5 of β A4 crystallin attached via a thioether bond to glutathione. These findings illustrate the potential of Cys and disulfide bonds to act as precursors for irreversible covalent crosslinks and the role of nearby amino acids in creating “hotspots” for the spontaneous processes responsible for protein degradation in aged tissues.

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Keywords

Human aging; protein modification; crosslinking

Introduction

The human body contains many proteins that are long-lived (LLPs)[1] As a result of the longevity of these proteins, numerous non-enzymatic posttranslational modifications (PTMs) can occur including racemization[2], deamidation[3, 4], oxidation, glycation[5], truncation, and covalent crosslinking[6]. The human lens is a long-lived tissue with no turnover of proteins in the differentiated lens fiber cells[7] with new cells layered on top of existing cells as we age. As a consequence, proteins in the center of the lens are old as the individual, this enables a snapshot of aging by sectioning of the lens from the cortex (youngest) to the nucleus (oldest). This makes the lens an ideal tissue to use for the study of non-enzymatic aging processes that occur to LLPs throughout the human body.

In age-related nuclear cataract lenses, a significant amount of protein remains insoluble even after the lenses have been extracted with 8M urea. A large amount of this can be solubilized by the addition of a reducing agent [8]. The extraction data alone, suggested the formation of both disulfide and non-disulfide covalent protein–protein crosslinks [9, 10] and this was supported by chromatographic data which revealed that the extent of non-disulfide crosslinking increased as the cataract worsened [8, 11]. It is thought that protein aggregation, crosslinking, and insolubilization processes contribute to the development of age-related cataract [9]. Despite the importance of this covalent crosslinking, little was known of its molecular basis until very recently.

Several mechanisms for crosslinking had been proposed based on the identification of novel compounds from lens digests, for example, advanced glycation end products [12], ϵ -(γ -glutamyl)lysine[13], lanthionine (LAN), histidinoalanine (HAL), and lysinoalanine (LAL) [14–16] as well as compounds derived from 3-hydroxykynurenine[17]; however, the identification of the crystallins involved, and the exact sites of crosslinking has remained unknown until recently[6].

LAN, HAL, and LAL are crosslinks formed via a dehydroalanine (DHA) intermediate. DHA is commonly found in proteins of foodstuffs that have been treated with heat or alkali [18], and under these conditions, DHA is formed from a hydroxide ion-induced β -elimination reaction. This process also occurs in long-lived proteins under physiological conditions as evidenced by the detection of DHA-derived compounds in tissues such as the human lens [10] and teeth [19] [20]. Amino acids that can be converted to DHA include Cys [21], Ser, and phosphoSer [18, 20]. Once DHA is produced, it is reactive and potentially becomes subject to nucleophilic attack by Cys, His, and Lys. If such residues react with DHA, protein–protein crosslinks can be formed [15, 20, 22].

As part of an ongoing study, the crosslinking of proteins in the human lens is being investigated using mass spectrometry. Early data revealed that Cys 5 in β A4 crystallin was highly modified, so the enhanced reactivity of this site was investigated using a combination

of computational and *in-vitro* peptide studies. Understanding the molecular basis for age-related protein crosslinking is important since it is a characteristic feature associated with aging in several long-lived postmitotic cells, for example, neurons, retinal pigment epithelial cells, cardiac myocytes, and skeletal muscle fibers [23–25].

Experimental

Human lens sample preparation and mass spectrometry analysis

Human lenses were obtained from NDRI (Philadelphia, PA). Lenses were sectioned equatorially to a thickness of 30 μm in a cryostat (LEICA CM 3050S) at -21°C . Equatorial sections were picked up on a piece of parafilm and dried at room temperature. Different regions of the lens were separated by punching through the parafilm using AcuPunch trephines (Acuderm Inc, Ft. Lauderdale, FL). The inner nucleus region was obtained by punching the middle of the section using a 4.5 mm diameter AcuPunch. Further punching the remaining section with a 6 mm diameter punch gave the outer nucleus region. The remaining tissue section was collected as cortex. Proteins on parafilm rings, typically 8–9 sections pooled, were collected by five sequential washes using 100 μL of 25 mM Tris, 150 mM NaCl, 5 mM EDTA, pH 7.4. The samples were passed through a 25 g needle 5 times and centrifuged at 20,000 g for 30 min. The supernatant was collected as the water soluble fraction (WSF). The pellets were vortexed in 100 μL of 8 M urea in 25 mM Tris, 150 mM NaCl, 5 mM EDTA, pH 7.4 and centrifuged at 20,000g for 30 min. The pellets were further extracted with 8 M urea once and the supernatants from both extractions were pooled as the urea soluble fraction (USF). Typical protein yields were for the WSF were: 2.4mg (cortex), 1.9mg (outer nucleus), and 2.9mg (inner nucleus) and for the USF were: 250 μg (cortex), 330 μg (outer nucleus) and 420 μg (inner nucleus). Proteins in WSF and USF were reduced by adding DTT to a final concentration of 10 mM. The samples were incubated at 56°C for one hour. Iodoacetamide was then added to a final concentration of 55 mM and the samples were incubated in the dark at room temperature for 45 min. Proteins in WSF and USF were precipitated by a method of chloroform/methanol precipitation and digested by trypsin in 10% ACN with enzyme to protein ratio of 1:100. The digestion was done overnight at 37°C . Tryptic peptides corresponding to 0.5 μg of total protein were separated on a one-dimensional fused silica capillary column (200 mm x 100 μm) packed with Phenomenex Jupiter resin (3 μm mean particle size, 300 $\text{\AA}$ pore size) coupled with an Easy-nLC system (Thermo Scientific). An 86-minute gradient was performed, consisting of the following: 0–60 min, 2–45% B; 60–70 min, 45–95% B; 70–73 min, 95% B; 73–74 min, 95–2%B, 74–86 min, 2%B (column equilibration). The eluate was directly infused into a Q Exactive instrument (Thermo Scientific, San Jose, CA) equipped with a nanoelectrospray ionization source. The data-dependent acquisition method consisted of MS1 acquisition ($R=70,000$), using an MS AGC target value of $1\text{e}6$, followed by up to 15 MS/MS scans ($R=17,500$) of the most abundant ions detected in the preceding MS scan. The MS2 AGC target value was set to $2\text{e}5$ ions, with a maximum ion time of 200 ms, and a 5% under fill ratio, and intensity threshold of $5\text{e}4$. HCD collision energy was set to 27, dynamic exclusion was set to 5s, and peptide match and isotope exclusion were enabled.

Tandem mass spectra were analyzed using a suite of custom-developed bioinformatics tools. All MS/MS spectra were converted to mzML files by Scansifter, a tool under development at Vanderbilt University Medical Center and searched on a 2,500 node Linux cluster supercomputer using a custom version of the TagRecon algorithm [26]. Trypsin specificity was used with a maximum one missed cleavage site. The data were searched with a static modification of carbamidomethylation of cysteine and variable modifications of oxidation of methionine and GSH modification on Ser, Thr, Cys and Lys. All of the GSH modified peptides TagRecon: high-throughput mutation identification through sequence tagging tide measurements reported in this paper require below 5 ppm mass accuracy and manual verification of their tandem mass spectra.

Peptides studies

Ac-TLQCTK (corresponding to the N-terminal tryptic peptide of β A4 crystallin) and related peptides Ac-TLACAK, Ac-TLACTK, Ac-TLACAR, Ac-TLACAH, Ac-TLACKA, Ac-TLKCAA, Ac-TKACAA, Ac-TLACAK-(Ac) and Ac-ACGF were synthesized by GLS Biochem (Shanghai, China) and dissolved in 100mM phosphate pH 7.0 (1mg/mL). Each peptide was incubated at 37°C or 60°C and aliquots removed at regular intervals. 50 μ g of each aliquot was injected onto a C18 RP HPLC column (Jupiter 5 μ C18 300Å, Phenomenex) and separated on a Shimadzu HPLC (SPD- 10A) and monitored at 216nm and 241nm. An 65-minute gradient was performed, consisting of the following: 0–30 min, 2–20%B; 30–40 min, 30–80% B; 40–45 min, 80%B (solvent A 0.1%,TFA 2% CH₃CN, Solvent B 0.1% TFA, 100% CH₃CN).

Computer modeling of peptides

Disulfide-linked dimers of AcTLQCTK₂, AcTLACAK₂ and AcTLACKA₂ were modeled manually in COOT [27]. Two different starting configurations were used for each dimer, and each of the six resulting systems were simulated for 50 ns. All MD trajectories were calculated by NAMD [28] using the CHARMM36 all-atom force field [29–31]. Structures were embedded in cubic water boxes and overlapping solvent molecules were deleted. Sodium and chloride ions were added (target salt concentration 100 mM) such that the systems had zero net charge. All simulations were run as isothermal–isobaric ensemble (temperature 310 K; pressure 101.325 kPa) with periodic boundary conditions. Temperature was controlled using Langevin dynamics (damping constant 5 ps⁻¹). Pressure control used the Nosé-Hoover Langevin piston (period 100 fs; decay rate 50 fs). A multiple time-step approach was used with 1, 2, and 4 fs for bonded, non-bonded and long-range electrostatic calculations respectively. The Particle-mesh Ewald with a grid resolution of 1 Å was used to calculate long-range electrostatic forces. van der Waals' interactions were smoothly scaled to zero between 10 and 12 Å. All systems in this study were subjected to energy minimization (10,000 steps) prior to equilibration by MD. Frames were saved for analysis every 10,000 steps (10 ps). VMD [32] was used for all trajectory analysis.

Results

Sites of cysteine breakdown in the human lens

In human nuclear cataract lenses, crystallins become crosslinked by both disulfide and non-disulfide bonds and the extent of non-disulfide crosslinking increases as the cataract worsens [6, 8, 11]. Until recently, little was known about the reactions responsible for this process.

In the current study, sites of non-disulfide crosslinking in lens proteins were investigated by mass spectrometry. In a previous publication, DHA arising from the breakdown of phosphoSer residues, lead to the formation of both crystallin-crystallin and crystallin-GSH crosslinks. Since GSH is abundant in the lens [33] (~2–20mM) it was hypothesized that if breakdown of protein Cys leading to the formation of DHA occurred it would produce mostly GSH adducts. In agreement with this, several GSH-modified Cys sites in lens crystallins were identified and are displayed in Table 1. Cys 5 in β A4 crystallin was found to be the most highly modified (see Figure 1) with ~50% of this residue existing as a thioether crosslink by age 20. This was approximately 200-fold higher than other Cys residues at a similar age. The reasons for the high degree of Cys 5 modification were investigated further.

Model studies of synthetic peptides

To investigate the basis for the high level of GSH modification of Cys 5 in β A4 crystallin, model studies were undertaken using Ac-TLQCTK, which corresponds to the N-terminal tryptic peptide of β A4 crystallin. For consistency, all synthetic peptide numbering in the following sections will follow the convention of the canonical sequence of β A4 crystallin in which the N-terminal Met has been removed. Therefore a Cys residue at position 4 in a peptide corresponds to Cys 5 in β A4 crystallin.

Ac-TLQCTK was initially incubated at 37°C in phosphate buffer pH 7.0 and monitored for the generation of DHA by HPLC. These results are shown in figures 2 and 3. Ac-TLQCTK readily oxidized forming a disulfide linked dimer, such that after 7 days little, or no, free Cys remained (Figure 3a). Concurrently as the disulfide increased, a peak eluting just prior to the monomer was observed. This was confirmed by MS/MS analysis to be DHA derivative (Figure 4). This suggested that the formation of DHA resulted from initial formation, then breakdown of the disulfide and this is consistent with literature data [34, 35]. To confirm this, the disulfide linked dimer was purified by HPLC and re-incubated. There was no significant difference in the rate of DHA formation starting with either the purified disulfide or from oxidized Ac-TLQCTK in the original incubation (data not shown). If Ac-TLQCTK was incubated with DTT to prevent disulfide bond formation, no DHA formation was observed. This suggests that the formation of DHA from Cys depends on prior oxidation to the disulfide and this is in agreement with the requirement for formation of a good leaving group.

Incubation of Ac-TLQCTK also led to the generation of a number of new HPLC peaks (Figure 2). These components were analyzed by mass spectrometry and were found to represent covalently crosslinked peptides formed between DHA and either Cys or Lys residues. These assignments were based on MS/MS fragmentation data (Figure 5). The structure of the lysine intermolecular crosslinked dimer was confirmed by purifying Ac-

TLQ(DHA)TK and incubating it separately. Over time, a new HPLC peak appeared at 16.3 min, which had the same MS/MS spectrum as that of the peak labelled in Figure 2. As shown in Figure 3b, thioether formation occurred quickly after DHA formation, and then plateaued as the content of free sulfhydryl groups declined. The lysinoalanine crosslink formed at a slower rate and was only detected between two DHA modified peptides. A diagram illustrating the structures of these crosslinks is shown in Figure 6.

In order to study why the formation of DHA from Ac-TLQCTK occurred so rapidly, several homologous peptides were incubated (See Table 2). Replacement of Gln 4 (Ac-TLACTK) or Thr 6 (Ac-TLACAK) with Ala did not appreciably alter the rate of DHA formation. However, substitution of Lys 7 by an Ala residue (Ac-TLACAA), reduced DHA formation approximately 6 fold, with the rate of DHA formation becoming similar to that observed for Ac-ACGF. To confirm that the ϵ -amino group of Lys was promoting DHA formation, the lysine residue was acetylated (Ac-TLACAK-(ϵ Ac)). This markedly reduced DHA formation. These data are consistent with the epsilon amino group of the C-terminal Lys acting as a nucleophile to promote the β elimination of Cys from the disulfide and thus the formation of DHA. (See Figure 7).

If indeed such an intramolecular reaction were occurring, it should be constrained by conformational factors. Consistent with this proposition, placing a Lys next to Cys (Ac-TLACKA) resulted in 3-fold reduction in the formation of DHA compared to Ac-TLQCTK. In this case modelling showed that the ϵ -amino group of Lys is less likely to be able to attack the α -carbon of Cys.

If the side chain of Lys is involved in promoting DHA formation in these peptides, one prediction would be that placing a Lys on the N-terminal side of Cys one residue removed (e.g. Ac-TKACAA) would yield a similar rate of DHA formation as Ac-TLQCTK, where the Lys is located on the C-terminal side. This was indeed found to be the case (Table 2). These data indicate that for maximal promotion of DHA, Lys needed to be one residue separated from the Cys residue.

Molecular modeling

To further understand the experimental data, molecular modelling of Ac-TLQCTK was undertaken. For all simulations, the number of frames where a lysine- $N\zeta$ atom approached within 4 Å of a Cys -C α H (intra-chain) were counted. This was based on the reasoning that this distance is approximately the maximum that would allow base-mediated elimination of Cys disulfide to DHA to occur. In the case of Ac-TLQCTK₂, and Ac-TLACAK 2.29%, and 1.69% of frames were found where this distance criterion was met, respectively. For AcTLACKA₂, the corresponding figure is 0.41%. Figure 8 shows examples of conformations where the minimum distance criterion is satisfied

Effect of Arg and His on DHA formation

We investigated the possibility that the side chains of His and Arg could also act as nucleophiles to promote the β elimination of cystine. Two peptides, Ac-TLACAH and Ac-TLACAR were compared to Ac-TLACAK. DHA formation with the Arg peptide (Ac-

TLACAR) took place ~ 3-fold slower than that of Lys (Ac-TLACAK) (Table 2), though this was higher than Ala at the same position. Replacement of Lys by His resulted in a rate of DHA formation approximately 60% that of Lys (Ac-TLACAK) (Table 2). Therefore, both Arg and His can also act to promote DHA formation, with His being the more effective of the two basic amino acid residues.

Protein data base search

Using knowledge that Lys at either the N+2 or N-2 position can promote DHA formation from Cys residues, the human Uniprot database (02/15/17) was searched for the frequency of these N+/-2 amino acid sequences. 201,093 matches were found. Since flexibility is known to be a pre-requisite for such modification to occur, the database of known intrinsically unstructured proteins [36] was also searched. A list of 101 candidate proteins was obtained, which is displayed in Supplementary Table 1. Significantly, a number of these proteins are known also to be long-lived [1], including collagen and nuclear pore complex proteins.

Discussion

This paper describes a mechanism by which a non-reducible protein crosslink can form from a Cys residue as a result of tissue aging. In addition, it provides an explanation for the high reactivity of one particular Cys in a protein within the human lens and the products that are formed. It was estimated that by age 20 more than 50% of Cys 5 in β A4 crystallin had been modified by a thioether crosslink with glutathione.

Using a synthetic peptide corresponding to the N-terminal tryptic peptide of β A4 crystallin (Ac-TLQCTK) together with various homologues, the rate of DHA formation was examined. Three factors were found to be important. Oxidation to the disulfide appeared to be a prerequisite for DHA formation. The presence of a free Lys epsilon amino group in the peptide chain accelerated DHA production and furthermore, the exact position of the Lys residue relative to the Cys was important. If the Lys was immediately adjacent to the Cys, only limited promotion of DHA was observed. Maximal formation of DHA was detected if the Lys residue was one residue separated from the Cys; as it is in β A4 crystallin. Introduction of Lys separated from Cys by one amino acid on the C-terminal side also led to an accelerated formation of DHA. From these data we propose a mechanism involving attack of the epsilon group of Lys on the alpha carbon of the newly formed cystine residue leading to beta elimination. Computational modelling supported this mechanism. This nucleophilic attack could occur intramolecularly as described, or could possibly involve another amino group, either as part of a disulfide-linked peptide or within a nearby protein.

Whilst it was evident that beta elimination at a cystine site could be promoted by the presence of Lys, other basic amino acids, Arg and His were also investigated. Arg had little impact on the rate of DHA formation which is consistent with the guanidinium group being a poor nucleophile. However, replacement of Lys by His accelerated DHA formation to a level approximately 60% of that observed with Lys (Table 2). In a previous study the presence of His one residue removed, had a significant effect on the extent of deamidation of

Asn in lens crystallins [37] suggesting that the influence of nearby basic residues on the spontaneous processes associated with aging may be a more general phenomenon.

Another factor not specifically investigated here, is the importance of conformational flexibility in allowing the protein modifications to take place. Other spontaneous age-related PTMs, such as deamidation, are confined to unstructured regions of proteins [38, 39]. Other β A crystallins investigated by NMR contain a flexible N-terminal “tail”[40], so it is likely that Cys 5 in β A4 crystallin is present in a similar unstructured region in the lens. Using mass spectrometry, a number of protein-protein crosslinks were also found in adult human lenses. As expected, some of these also involved Cys 5 of β A4 crystallin and these will be documented in a separate publication.

It might be expected that if Lys were present at a N+2 or N-2 site relative to Cys, in unstructured regions of other proteins, it may also promote β elimination and the formation of DHA. Database searching of human protein sequences revealed that such 3 amino acid triads were abundant, so it is probable that other long-lived proteins in the body might also be prone to the changes described here. For example, a thioether crosslink has been documented in IgG1 that involves Cys 103 which is located in the flexible hinge region together with a N+2 Lys. [41]

Based on our observations, once DHA forms from Cys, there are several possible subsequent reactions that can occur. The major one appears to be reaction with a free Cys sulfhydryl group, leading to the formation of a thioether crosslink. In addition, in the peptide modelling experiments we detected reaction with lysine residues to form an intermolecular lysinoalanine crosslink. Evidence for intramolecular lysinoalanine crosslinking was also obtained, however this needs further validation. Histidinoalanine either did not form, or formed significantly more slowly than the other crosslinks and was below the detection limit of our measurements. It is clear that if free sulfhydryl groups, such as GSH, are present in excess (e.g. in the lens) then the formation of lysinoalanine crosslinks will be greatly reduced. This is in accord with the known reactivity of sulfhydryl and amino groups. In the lens, GSH concentrations are typically high (2–20mM) and it could be hypothesized that, in addition to acting as an antioxidant, GSH has a role in scavenging DHA residues that form and thus preventing potentially harmful protein-protein crosslinks. This would explain the high amount of thioether crosslinked GSH to Cys 5 in β A4 crystallin. The lens data also imply that there is significant disulfide interchange within the normal human lens. In a dynamic system, some Cys 5 of β A4 crystallin becomes oxidized to a disulfide with either GSH or another Cys-containing crystallin. In the vast majority of cases this disulfide is probably re-reduced with GSH, however in some cases, the disulfide undergoes β elimination to form DHA. Once this occurs a crosslink is formed and there is no prospect for reversal. The outcome of DHA formation at Cys 5 in the human lens is summarized in figure 9

In a previous report we characterized protein-protein crosslinks in the human lens that were formed by β -elimination of phosphoSer and phosphoThr residues [6]. Since DHA can also be formed from the spontaneous decomposition of phosphoSer, the relative amount of DHA-formation from phosphoSer in the lens was compared to that from Cys to gauge the relative

impact of crosslinking from each of these amino acids. The relative levels of GSH thioether modification were calculated as described in Table 1. For Ser59 in α B crystallin an average ratio of 0.075 ± 0.0034 was obtained (thioether/unmodified residue) for WSP and from 0.017 ± 0.0070 (thioether/unmodified residue) for USP. For Ser58 in β A3 crystallin (0.01 ± 0.0028 WSP and 0.0077 ± 0.00062 USP). These levels are approximately 15–100 fold lower than the modification at Cys 5 in β A4 crystallin.

The results of this study may also be relevant to the breakdown of proteins in other tissues. For example, in Alzheimer's disease, the microtubule-associated protein Tau, displays increased non-disulfide covalent crosslinking and this crosslinking can also be induced by the incubation of Tau *in vitro* [42]. In Tau, one Cys residue is found in close proximity to a Lys [43] however nothing is known about the structure of the crosslinks. Oxidative stress is also associated with many conditions such as inflammation [44] and this leads to oxidation of protein Cys and disulfide bond formation. On the basis of our data, any tissue undergoing oxidative stress may also display increased DHA formation in the proteins. If true, the formation of thioether crosslinks with GSH or other proteins may be useful as a potential biomarker for oxidative stress within tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Lisa Frank is thanked for developing a program to search the protein databases.

Funding information: Funding for this study was provided by NIH NEI (R01EY024258)

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Figure 1.

Selected ion chromatograms of unmodified β A4 crystallin peptide (top row) and GSH thioether-modified peptide (bottom row) in different lens regions of a 19year-old human lens. A small fraction of the unmodified peptide eluted slightly later has the same mass and fragmentation pattern and is most likely due to racemization of threonine or glutamine residues. The two peaks identified as GSH modified peptide likely correspond to two stereoisomers formed during the nucleophilic attack and are separable by HPLC.

Figure 2.

Conversion of Cys to DHA and formation of crosslinked peptides following incubation of Ac-TLQCTK. HPLC trace showing peaks due to Ac- TLQCTK disulfide, Ac-TLQ(DHA)TK and two crosslinked peptides following incubation of Ac-TLQCTK for 28 days at 37° in 0.1M phosphate buffer, pH 7.0. Detection at 216nm. Peaks were analysed by MS/MS (see Fig 5).

Figure 3.

a) Time course showing the loss of Ac-TLQCTK, the formation of the corresponding disulfide linked dimer and the generation of peptide-bound DHA. b) Time course showing the formation of an intermolecular lysinoalanine crosslink and a thioether crosslinked dimer. Incubation conditions as for Figure 3. Each time point is an average of 3 replicates, error bars $\pm$ SD.

Figure 4. Tandem MS/MS of Ac-TLQ(DHA)TK isolated from 37°C incubation. The peptide was formed by the conversion of Cys to DHA; $[MH]^{1+} = 701.38$. The b- and y-ions are labeled, and asterisks indicate the fragment ions that include a DHA residue.

Figure 5. Tandem mass spectra of crosslinked peptides identified from Ac- TLQCTK incubation. a) Thioether crosslinked dimer and b) Lysinoalanine intermolecular crosslinked dimer. For the lysinoalanine crosslinked dimer the tandem mass spectrum of the crosslinked peptides fragments are annotated y and b for the top (DHA-containing) chain and yA and bA for the bottom chain. Ions labeled with an asterisk indicate the fragment ions that include a DHA residue.

Figure 6.
The structures of crosslinked peptides formed from the incubation of Ac-TLQCTK.

Figure 7. DHA formation from cystine can be promoted intramolecularly by the epsilon amino group of a nearby Lys residue. Optimal catalysis occurs if Lys is separated by one residue from the Cys.

Figure 8. Selected snapshots from the simulation of AcTLQCTK₂ (A) and AcTLACKA₂ (B) showing close approach of Lys-N ζ . Each chain of the dimer is shown with carbon atoms green or black. All hydrogen atoms except the Cys- C α H atoms are omitted. The distance between lysine-N ζ and cys-C α H atoms is indicated.

Figure 9.

A proposed scheme illustrating the pathways involved in formation of crosslinked species derived from βA4 crystallin that have been characterized in adult human lenses.

Table 1

Relative abundance of GSH thioether-modified peptides in human lenses

Protein	Site	WSP	USP
		GSH* (Ave $\pm$ SD) n=3	GSH* (Ave $\pm$ SD) n=3
β A4 crystallin	Cys5	1.14 $\pm$ 0.23	0.97 $\pm$ 0.074
γ S crystallin	Cys28	0.0055 $\pm$ 0.0040	0.0088 $\pm$ 0.0014
β B1 crystallin	Cys80	0.00087 $\pm$ 0.00063	0.000037 $\pm$ 0.000052

The relative abundance of GSH-modified peptides at Cys sites in three crystallins as determined by mass spectrometry. The values shown are the ratio of the peak areas of the modified peptide to the peak area of the corresponding unmodified peptide. Average values for three lenses; 19, 21 and 22 year-old are shown. (WSP = Water soluble protein, USP = urea soluble protein).

Table 2

The effect of amino acid sequence on DHA formation from Cys

Peptide	DHA formation* (p mol/hr)	±SD
Ac-TLQCTK	27.9	1.7
Ac-TLACTK	36.2	4.1
Ac-TLACAK	31.2	3.4
Ac-TLQCAK	21.9	2.0
Ac-TKACAA	19.0	0.4
Ac-TLACKA	10.5	1.3
Ac-TLACAH	16.6	0.06
Ac-TLACAR	9.79	1.3
Ac-TLACAA	4.96	0.02
Ac-TLACAK-εAc	2.24	0.49
Ac-ACGF	4.85	2.1

The amount of DHA was determined by RP-HPLC where the DHA peptide was resolved from starting peptide. Peptides were monitored at 216nm and quantification was based on peak area.

* The rate of DHA formation was determined by the slope of line, typically from 48 hr to 148hr. All incubations were performed in triplicate in 100mM phosphate buffer, pH 7.0 at 37°C.