The identification of a reaction site of glutathione mixed-disulphide formation on γ S-crystallin in human lens

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The glutathionylation of human lens proteins was examined by Western-blot analysis with an anti-GSH antibody and scanning. Several different glutathionylated proteins were observed, and a 47 kDa band was of particular interest. This band did not appear after SDS/PAGE under reducing conditions, suggesting that it was a glutathionylated fraction. The 47 kDa band was found principally in the outer part of the lens, the cortex, but not in the lens nucleus where older proteins are present. The 47 kDa component

was composed of β B1-, β B2- and γ S-crystallin, with the γ S-crystallin having glutathione bound at Cys-82 and at Cys-22, Cys-24 or Cys-26. We conclude that when glutathione becomes bound to γ S-crystallin, it causes it to bind in turn to the β -crystallin polypeptides to form a dimer.

Key words: conformation, crystallin, glutathione, glutathionylation, lens, mass spectrometry (MS), mixed disulphide.

INTRODUCTION

The lens is an avascular cellular tissue in the middle of the eye. Its function is to focus light on to the retina and, hence, it should have a high refractive index and a high degree of transparency [1]. In the fully formed lens, there is a single layer of epithelial cells inside the anterior capsule, which divide and migrate to the equator. In the equator, they elongate to form fibre cells. This places new cells around pre-existing cells but without cell loss. The lens nucleus contains cells that have been present since birth. During elongation, the cell nuclei and other organelles are lost, ensuring minimal absorption or scattering of light. However, the absence of DNA and RNA indicates that the fibre cells are not capable of renewing their proteins or replacing damaged proteins; thus these cells are dependent on the crystallin proteins synthesized before organelle loss [2].

Crystallins, which are the structural proteins of the lens, make up 90% of the total protein. α -Crystallin is a heteromultimer composed of two different subunits, α A and α B, each having a molecular mass of approx. 20 kDa [2]. β -Crystallins form a multi-gene family of basic and acidic polypeptides of 23–35 kDa and combine to form 40–200 kDa dimers, trimers, tetramers and higher oligomers with high polydispersity. γ -Crystallins have a mass of 20 kDa and do not usually form oligomers.

GSH is a tripeptide with a free thiol group. The glutathione concentration is approx. 6 mM in the lenses of young human and decreases with age and cataract [3]. GSH is essential for maintaining the thiol groups of proteins, including enzymes, preventing disulphide cross-linking, which can disrupt their function. GSH maintains tissue levels of ascorbate and α -tocopherol and protects cells against oxidative damage, free radical damage, xenobiotics and other types of stress. Glutathione protects NaK-ATPase and thus helps to maintain cation levels in the lens [4].

All these processes produce GSSG, which can be recycled to the reduced form (GSH) via glutathione reductase using NADPH, which is produced by the pentose phosphate pathway. If this system is overloaded and GSH is oxidized more rapidly than it can be reduced, GSSG would accumulate and form GSH–protein

adducts with the thiol groups of native crystallins (PSSG, protein-S-S-glutathione), which may destabilize and unfold the crystallins.

Decrease in glutathione was one of the earliest changes identified in diabetic rat lens before opacification [5]. Subsequently, a decrease in protein thiol was found to occur. In the early stages of diabetic cataract, a small increase in glutathione mixed disulphide (PSSG) was seen, which decreased later [6]. Formation of mixed disulphides can be induced in incubated rat and monkey lenses by peroxide [6], probably as a result of protein thiol reacting with GSSG, produced from the oxidation of GSH by H_2O_2 [7].

During cataractogenesis, the lens proteins unfold, exposing thiols that were buried [8]. These reactive thiols can form mixed disulphides with glutathione and cysteine [3]. As the severity of the cataract increases, the total protein thiol decreases and the total protein disulphide increases [9,10]. These changes occur mostly in the nucleus, where the oldest proteins are present. Reaction of native crystallins with GSSG causes a conformational change [11]; therefore GSSG resulting from GSH oxidation would cause the crystallins to unfold, rendering their thiol groups even more susceptible to reaction with GSSG [12]. As cataract formation proceeds, crystallins unfold, thus exposing thiol groups; hence, there is an opportunity for further PSSG formation, which would destabilize crystallins further. As PSSG formation seems to play a role in cataract formation, there has been interest in how and where GSH forms disulphide bonds with the various crystallins.

GSSG can form mixed disulphides with all the crystallins, especially with β L- and γ -crystallins, which have a high thiol content [7]. After the addition of GSH, protein thiols were restored. Different crystallin–GSH mixed disulphides have been prepared *in vitro*. Bovine γ B-crystallins formed mixed disulphides by reacting with GSH or GSSG, and one, two or three glutathione molecules became attached [13]. Since GSH is polar, addition of GSH to crystallins changes their surface charge, adding one positive and two negative charges. Thus addition of GSH causes conformational changes in crystallins. Recently, a small proportion of γ B-crystallins from bovine lenses was shown by MS to have GSH adducts at Cys-15, Cys-18, Cys-22 and Cys-41.

Abbreviations used: MALDI–TOF, matrix-assisted laser-desorption ionization–time-of-flight; PSSG, protein-S-S-glutathione (mixed disulphide).

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γ B-crystallin from H_2O_2 -treated lenses had an additional adduct at Cys-109 [14]. Anything which increases the oxidation of GSH will contribute to opacification; conversely, anything which helps to maintain or boost the GSH levels in the lens will help to decrease opacification. The aim of the present study was to identify reaction sites for GSSG on lens proteins.

MATERIALS AND METHODS

The Bristol Eye Bank and Oxford Eye Hospital provided the clear human lenses. Oxford Eye Hospital also provided cataract nuclei from routine extracapsular cataract extractions. Dr R. M. Broekhuysen (University of Nijmegen, Nijmegen, The Netherlands) provided cataract lenses of grades I–IV on the Pirie Cataract Scale [15]. Lenses were dissected out, frozen and stored at -20°C until use. For SDS/PAGE and Western-blot analyses, materials and equipment were obtained from Bio-Rad Laboratories. The primary antibody anti-GSH was from Virogen (Watertown, MA, U.S.A.) and the secondary antibody and detection materials were from Promega (Madison, WI, U.S.A.). Other chemicals were obtained from Sigma.

Sample preparations

Frozen lenses were thawed, decapsulated and then separated into the cortex and nuclear fractions by gentle rubbing between the thumb and the index finger (with gloves). Once separated, the fractions were frozen to -80°C and freeze-dried. A solution of 5 mg/ml lens proteins was dissolved in SDS-gel-loading buffer and homogenized. The samples (1.5 ml) were transferred to Eppendorf tubes and placed in a boiling-water bath for 5 min.

SDS/PAGE

SDS/PAGE (12.5 % gel) was performed as described in [16] under reducing and non-reducing conditions. Samples (10 μl) were loaded on to the 4 % polyacrylamide SDS-containing stacking gel. Protein bands were visualized by Coomassie Blue staining (1.25 %) and then destained in methanol/acetic acid/water (5:1:4, by vol.) for 2 h.

Western-blot analyses

Samples were run on SDS/polyacrylamide gel as above using prestained molecular-mass standards. The gels were blotted on to PVDF and blocked with milk powder. The membrane was incubated with the primary antibody anti-GSH for 1 h (1:1000 dilution) and then rinsed for 5 min in TBS/T [0.5 ml of Tween 20/100 ml of TBS (2.42 g of Trizma base and 8 g of NaCl made up to 1000 ml and adjusted to pH 7.6)], after which the membrane was incubated with the secondary antibody anti-mouse IgG (1:7500 dilution) for 1 h. The membrane was then rinsed three times in TBS-T and then stained in Western Blue-stabilized substrate with alkaline phosphatase till immunodetected bands developed. Preliminary experiments optimized the concentrations of primary and secondary antibodies.

The membranes were scanned at 500 dpi in Photoshop 5.5 and then transferred to LabWorks image acquisition and analysis software package for quantification by densitometry. The band(s) of interest were identified and the IOD (integrated optical density) was calculated. The density of a band represented the amount of anti-GSH bound. The density of each band was determined relative to the total density of that lane. This procedure was repeated three times and the means and S.D. were calculated.

Figure 1 SDS/PAGE of the whole human lens under reducing (R) and non-reducing (N) conditions

Greater smearing was observed under non-reducing conditions. α A-, α B- and γ -crystallins were identified. The high-molecular-mass protein has not entered into the gel and the amount increases with age of the lens. However, the total high-molecular-mass protein is the same for each sample under both reducing and non-reducing conditions. MMs, molecular masses.

Identification of the unknown SDS/PAGE band by MS

SDS/polyacrylamide gels were stained with GelCode Blue Stain Reagent (Pierce). The bands of interest were cut out and digested overnight with trypsin (Promega) in the ratio 1:30 in 50 mM ammonium bicarbonate. A 0.5 μl aliquot of the tryptic peptides was mixed with 0.5 μl of α -cyano-4-hydroxycinnamic acid matrix and loaded on to a MALDI-TOF (matrix-assisted laser-desorption ionization–time-of-flight) target plate before placing in the Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems, Warrington, Cheshire, U.K.). The computer program Protein Prospector MS-Fit was used to analyse the mass map for each of the proteins analysed.

RESULTS

Mixed disulphides in human lenses were identified and quantified by assessing the binding of an antibody directed to glutathione (anti-GSH) by Western-blot analysis after separation by SDS/PAGE. All SDS/PAGE analyses were performed under non-reducing conditions unless stated otherwise. Previous experiments using MS have shown glutathione adducts in the α -crystallin fraction of human lenses and human lenses from renal-failure patients [17,18].

SDS/PAGE of reduced and non-reduced samples is shown in Figure 1, with the crystallins prominent between 20 and 29 kDa. On the Western blot, the band of 47 kDa is very sharp; therefore it could represent a major species for GSH binding (Figure 2). It is more defined in the non-reduced gel. α - and β -crystallins are also more defined in the non-reducing gel, implying that they have glutathione attached.

The identity of the band of 47 kDa (band 3) is unknown; none of the major crystallin subunits appears at 47 kDa. This region is where cytoskeletal proteins and enzyme polypeptides might be found. The density of the band generally decreases with age (Figure 2).

To investigate the decrease in mixed disulphides in cataract lens, clear and cataract lens samples were compared. Samples were run on SDS/polyacrylamide gel as before and then transferred to Western blots. A precise band is seen at 47 kDa in Figure 2. However, the band appears to be absent from cataract lens samples. Results of densitometry (Figure 3) show that cataract has

Figure 2 Western-blot analysis of the gel shown in Figure 1 using anti-GSH antibody

Proteins that produced stronger bands under non-reducing conditions are indicated. Band 1, 22 kDa, tentatively assigned to a subunit of α -crystallin; band 2, 25 kDa, tentatively assigned to a β -crystallin subunit; band 3, 47 kDa, unknown and requires further investigation. The 47 kDa band was only present under non-reducing conditions; band 4, high-molecular-mass insoluble protein. MMs, molecular masses.

Figure 3 Densitometry to investigate the effect of age and cataract on an approx. 47 kDa band

Comparison of normal (N) and cataract (C) lens samples, with age (in years) given under each bar. * $P < 0.01$ by Student's t test. For the older group (approx. 86 years old), there is a significant decrease in the density of this band in cataract lens compared with normal lens. This decrease is not significant for the younger age group (approx. 68 years old). Younger groups were not studied due to the lack of availability of young, normal lenses.

significantly less amount of 47 kDa protein with bound GSH. The amount of protein-bound glutathione on this component decreased with cataract formation.

The cataract lenses used in this experiment had no cortex, since they were from extracapsular surgery (Oxford Lenses, Oxford Eye Hospital). This could mean that the 47 kDa mixed disulphide is predominantly a cortical protein and, hence, it is not seen in cataract simply because the cortex is not present. To assess this situation, other cataract lenses from intracapsular cataract extraction in the Netherlands were obtained that had intact cortex.

There was a higher level of smearing with increased severity of the cataract from grade I to III; by grade IV, there is probably so much protein in the insoluble fraction that there appears to be less glutathionylated protein on Western blots (result not shown). There was a decrease in anti-GSH binding of the α -crystallin fractions with cataract. A precise band appeared at 47 kDa, as seen in Figure 2. However, the band appears to stain less in the nuclear region compared with the cortical region of cataracts. This means that the mixed disulphide is present in cataract lenses, or is simply absent or greatly modified in the nuclear portion so that the

Figure 4 Densitometry to investigate the effect of cataract on a 47 kDa protein band with bound GSH

The cataract grades I–IV are as defined by Pirie [15]. Nu, nucleus; Co, cortex. Scale bars represent S.D. * $P < 0.01$ by Student's t test.

Figure 5 Western-blot analysis using anti-GSH antibody

There is decreased staining of the α -crystallin band with cataract. MM, molecular mass. Age (in years) and type (N, normal; C, cataract) of lens samples is given beneath the gel.

antibody does not recognize it. Densitometry confirmed that the density of the 47 kDa band was, in every case, lower in the nuclear portion compared with the cortical portion (Figure 4). The density decreased gradually in both the nuclear and cortical preparations with severity of the cataract.

There was a decrease in anti-GSH binding of the α -crystallin fractions with cataract (Figure 5). A precise band shows up at 47 kDa as shown in Figure 2, principally in the cortical region of cataracts. This verifies the suggestion that the 47 kDa band is predominantly a cortical protein; indeed, this may not be due to a difference between normal and cataract lenses but a difference between the nucleus and cortex of the lens. Densitometry confirmed that the density of the approx. 47 kDa band is found predominantly in the lens cortex and that the density decreases with cataract (results not shown).

Identification of the 47 kDa protein band by MS

The 47 kDa band was cut from a non-reducing gel of a clear 24-year-old lens and then digested with trypsin. The digested peptide fragments were analysed by MS. The resulting mass 'fingerprint' spectrum is shown in Figure 6. The results are summarized in Table 1, showing the candidates β B1-, β B2-, γ C- and γ S-crystallins (Figure 7). When GSH, with a molecular mass of 0.307 kDa, forms a disulphide bond with a cysteine residue, two hydrogen atoms are eliminated, resulting in an increase in protein mass of 305 Da. Therefore, for each fragment, 305 Da was added on to the peptide mass and 610 or 915 Da was added if multiple cysteine residues were present. The resulting masses, representing possible GSH adducts of cysteine, were sought. Bovine γ B-crystallin has been shown to react with GSH to form mixed disulphides with

Figure 6 MALDI-TOF mass spectrum of the tryptic digest from the 47 kDa band isolated from human lens on SDS/12% polyacrylamide gel

Table 1 Proteins identified by MS, which are candidates for forming GSH-protein mixed disulphides

Protein	No. of matched masses	Coverage of protein (%)
β B1-crystallin	13/55	59
β B2-crystallin	9/55	50
γ C-crystallin	7/55	40
γ S-crystallin	6/55	41

one, two or three glutathione molecules attached to the protein [13].

β B1 and β B2 were both present in the 47 kDa component. Masses (m/z) of the peptides derived from β B1 and identified by MS are listed in Table 2 with their sequences and positions in the overall sequence. These peptides accounted for 59% of the total sequence of β B1-crystallin (Table 1), and for four or five of the major peaks in the mass spectrum (Figure 6). The masses of β B2 peptides identified by MS are listed in Table 3 with their sequences and positions. They accounted for 50% of the total sequence of β B2 (Table 1) and for at least two major peaks of the mass spectrum.

There was evidence for GSH adducts on γ S-crystallin in peptides 20–35 and 72–83. Peptide 20–35 has three cysteine residues, but there was no evidence for the addition of more than one GSH to this fragment (Table 4). There was no evidence of protein methylation.

Peptide 20–35

The difference calculated between the peptide mass with a GSH adduct (Table 4, column 4) and the fragment mass (column 5) is very small, showing that GSH is attached to one of the three cysteine residues in this part of the protein. No match was found between the peptide mass itself (column 3) and the MS masses. This implies that most of this peptide has GSH attached.

Table 2 Matched peptides of β B1-crystallin identified in the 47 kDa component

Fragment position	Sequence	Mass (Da)
51–60	AAELPPGNRY	1087.5544
61–72	LVVFELENFQGR	1450.7721
73–86	RAEFGGECNSLADR	1611.7235
74–86	AEFGGECNSLADR	1455.6627
93–110	SIIVSAGPWVAFEQSNFR	2008.0245
124–132	WNTWSSSYR	1186.5308
133–143	SDRLMSFRPIK	1365.7277
136–143	LMSFRPIK	991.5750
151–160	ISLFEGANFK	1125.5938
161–182	GNTIEIQGDDAPSLWVYGFSDR	2440.1482
188–202	VSSGTWVGYPGYR	1719.8176
203–214	GYQYLLEPGDFR	1457.7044
215–230	HWNEWGAFQPMQSLR	2014.9320

Table 3 Matched peptides of β B2-crystallin identified

Fragment position	Sequence	Mass (Da)
68–75	GEQVFVEK	983.4816
81–88	WDSWTSRR	1024.4501
89–100	RTDSLRLRPIK	1372.7940
108–119	ILYENPNFTGK	1408.7475
120–139	KMEIIDDVPSFHAHGYQEK	2359.1230
121–139	MEIIDDVPSFHAHGYQEK	2231.0889
145–159	VQSGTWVGYPGYR	1760.8399
172–187	DSSDFGAPHQVQSVR	1726.8152
191–197	DMQWHQR	1000.4431
191–204	DMQWHQRGAFHPSN	1726.8152

Peptide 72–83

The difference calculated between the peptide mass with a GSH adduct (column 4) and the fragment mass (column 5) is within

Table 4 Peptides identified with a possible GSH adduct

Peptide position	Peptide sequence	Peptide mass (kDa)	Peptide mass, with GSH adduct (+ 305.3 Da)	Mass matched with MS	Difference between 4th and 5th columns
20–35	YD CDCDC ADFH T YLSR	1926.7091	2231.0091	2231.0889	0.0798
72–83	WMGLNDR LSSCR	1437.6725	1741.9725	1742.8556	0.8831

Beta crystallin B1

SQAAKASASA TVAVNP^{GP}DT KGKGAPPAGT SPSPGTTLAP TTVPITSACA AELPPGNYRL

VVFELENFQG RRAEFSGECS NLADRGFDRV RSIIVSAGPW VAFEQSNFRG EMFILEKGEY

PRWNTWSSSY RSDRLMSFRP IKMDAQEHKI SLFEGANFKG NTIEIQGDDA PSLWVYGFSD

RVGSVKVSSG TWVGYYPGY RGYQYLLEPG DFRHNEWGA FQPQMQLRR LRDQWHLEG

SFPVLATEPP K

Beta crystallin B2

ASDHTQAGK PQSLNPKIII FEQENFQGHs HELNGPCPNL KETGVEKAGS VLVQAGPWVG

YEQANCRGEQ FVFEKGEYPR WDSWTSSRRT DSLSSLRPIK VDSQEHKIIL YENPNFTGKK

MEIIDDVPS FHAHGYQEKV SSVRVQSGTW VGYQYPGYRG LQYLLEKGDY KDSSDFGAPH

PQVQSVRRIR DMQWHQRGAF HPSN

Gamma crystallin S

SKTGTKITFY EDKNFQGRY **D**C**D**C**D**CA**D**FH **T**YLSR**C**NSIK VEGGTWAVYE RPNFAGYMI

LPQGEYPEYQ **R**W**M**GLNDR**L**S **S**CR**A**VHL**P**SG **G**QY**K**IQIFEK GDFSGQMYET TEDCP**S**IMEQ

FHMREIHSCK **V**LEG**V**WIF**E**Y **L**P**N**YRGR**Q**YL **L**D**K**EY**R**K**P**I **D**W**G**AAS**P**AV**Q** **S**FR**R**IVE

Gamma crystallin C

GKITFYEDRA FQGRSYETTT DCPNLQPYFS RCNSIRVESG CWMLYERPNY QGQYLLRRG

EYPDYQWWMG LSDSIRSCCL IPQTVSHRLR LYEREDHKGL MMELSEDCPS IQDRFHLSEI

RSLLHVLEGCW VLYELPNYRG RQYLLRPQY RRCQDWGAMD AKAGSLRRVV DLY

Figure 7 Amino acid sequences for the crystallin proteins

Cysteine residues are underlined. In the amino acid sequence of γ S-crystallin, the identified peptides in boldface show 44 % matched sequence coverage of the protein. This confirms that γ S-crystallin is a component of the 47 kDa band.

1 Da. This indicates that GSH was attached to Cys-82 in γ S-crystallin by a disulphide bond. MS analysis identified six unmodified peptides of γ S-crystallin and a further two peptides with GSH attached. The matched peptides are listed in Table 5 with sequence coverage. In total, 44 % of the sequence was covered by the identified peptides (Figure 7). In fact, by this method, γ S-crystallin represents as good a match as β B2. Hence, even though fewer fragments were matched, these fragments were larger, overlapped less and so represented a similar percentage of the total protein sequence.

The 47 kDa band of γ S-crystallin, even with a GSH adduct, must be dimerized either with itself or with another crystallin. γ S-crystallin has a mass of 21 kDa; therefore the dimer + GSH would have a mass of 42 kDa. Probable partners would be β B1

Table 5 Matched peptides of γ S-crystallin found in the 47 kDa component

Peptide position	Sequence	Modification
131–145	VLEGVWIFELPNYRGR	
158–173	KPIDWGAASPAVQSFR	
72–83	WMGLNDR LSSCR	
84–94	AVHLPSGGQYK	
148–154	QYLLDKK	
72–78	WMGLNDR	
20–35	YDCDCDCADFH T YLSR	+ GSH
72–83	WMGLNDR LSSCR	+ GSH

and β B2, also identified in this sample. This would explain their presence in the sample, even though we did not find evidence of their participation in a GSH adduct.

Attachment of GSH to a crystallin via a disulphide bridge would cause a change in surface charge, which would result in a conformational change. If GSH got attached at Cys-22, Cys-24 or Cys-26, then it is feasible that the γ S-crystallin could change conformation, which in turn would lead to dimerization. This is why no match was found in the MS data for the peptide 20–35 without attached GSH, since reaction with GSH induces dimerization of the γ S-crystallin.

DISCUSSION

Glutathione functions to reduce cellular disulphide bonds and is present in very high concentrations in the lens. It acts as an essential antioxidant, vital in maintaining the transparency of this tissue. The size of the GSH pool diminishes as the lens ages, and loss of GSH appears to be influential in the development of cataract. Cataractous lenses show a decrease in the GSH/GSSG ratio compared with clear lenses. The unique development and structure of the lens may explain the need for high levels of GSH.

During cataractogenesis, especially nuclear cataract, the lens proteins unfold and thiols that were buried become reactive [8]. Some of these thiols then react to form both mixed disulphides with glutathione and cysteine and disulphide-cross-linked aggregates in the lens [3,19]. With increasing severity of cataract, there is a decrease in total protein thiol with a concurrent increase in protein disulphide content [8,10].

Most of the results obtained previously [1,3,10,19,20] on thiol content and disulphide accumulation employed biochemical techniques using 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent). In the present study, we have investigated mixed-disulphide formation using antibody binding against glutathione bound to lens proteins. The results show that even young 24-year-old lenses have glutathione bound to α - and β -crystallins. However, there was also a unique well-defined band at approx. 47 kDa, and an increase in GSH antibody binding to the high-molecular-mass protein that did not enter the gel, with age. Binding of a glutathione antibody to the 47 kDa protein decreased with cataract formation and with increasing severity of cataract,

as did α -crystallin binding (Figure 4). Binding of glutathione antibody to the 47 kDa protein was greater in the cortex compared with the nucleus (Figure 4). The 47 kDa band only appears when not reduced, showing that it is a mixed disulphide (Figure 2).

The differences between cortex and nucleus may reflect the unique process of growth of the lens. The epithelial monolayer is located on the inside of the anterior lens capsule. Epithelial cell division leads to the movement of the cells towards the equator, where they elongate into the fibre cells that occupy most of the lens. The continuous elongation of fibre cells places new cells around pre-existing cells but without cell loss, so that cells in the lens nucleus have been present since birth. Only the peripheral cortex still has organelles to maintain its protein-synthetic capabilities. The absence of protein turnover from the nucleus makes long-lived proteins vulnerable to post-translational modifications, leading to unfolding and also exposure of thiol groups. Loss of protein thiol and increase in disulphide-cross-linked aggregates occur preferentially in the nucleus [10,19], where the oldest proteins are to be found. All the major crystallins become oxidized [20]. Although it is probable that conformational changes in crystallins would render their thiol groups more susceptible to reaction with GSSG [12], it has also been shown that reaction of native crystallins with GSSG provokes a conformational change [11].

MS of the 47 kDa band showed that it contained γ S-crystallin with GSH adducts attached to two different cysteine residues: Cys-82 and Cys-22, Cys-24 or Cys-26. These did not correspond to the GSH attachment sites on bovine γ -crystallins found previously [14]. However, the present study has identified the glutathione adducts produced *in vivo* with human lens proteins, whereas Hanson et al. [14] studied adduct formation induced in bovine lenses by incubation with H_2O_2 . Furthermore, they separated the γ -crystallins by HPLC before identifying GSH adducts. After chromatography, the proteins in the control lens peaks were identified according to their molecular mass in the order of elution as γ S-, γ B-, γ D-, γ C- and γ E-crystallin; however, γ S-crystallin was not found in the fractions from lenses treated with 30 mM H_2O_2 [14]. In both cases, γ S-crystallin was lost. If it had dimerized after oxidation, as our results suggest for human lens proteins *in vivo*, then the dimer would not have co-eluted with the γ -crystallins that were taken for HPLC. In the present study, the 47 kDa protein was found to be comprised of β B1- and β B2-crystallins, but no GSH modification was observed, suggesting that the 47 kDa protein might be a dimer formed between γ S-crystallin and β B1- and/or β B2-crystallin. A heterodimer between β - and γ -crystallin is more probable, since there is little evidence for a major γ -crystallin dimerization.

The oligomeric β -crystallins and monomeric γ -crystallins form a superfamily of structural proteins contributing to the refractive properties of the lens. The mammalian β -crystallin family shares approx. 30% sequence identity with γ -crystallins [2,21]. X-ray structures of basic β -crystallin homodimers and several γ -crystallins show similar three-dimensional structures, which reveal the basic quaternary organization of the superfamily as four antiparallel β -strands forming a motif resembling a 'Greek Key' pattern [22]. γ -Crystallins are two-domain monomers of 20 kDa, with the exception of γ S. The compact core of the γ -crystallins is largely hydrophobic, whereas the surface is covered with a large number of interacting charges; these are

probably involved in protein interactions and thereby partly explain the extreme thermostability of the γ -crystallins.

The expression of γ S-crystallin (formerly known as β S-crystallin) ceases after parturition and it is therefore predominantly a cortical protein [23]. This may explain why the 47 kDa component we have identified as containing γ S-crystallin, and probably formed as a result of glutathionylation of γ S-crystallin, is found almost exclusively in the cortex.

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