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

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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, αA and αB, 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 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 βB1-, βB2- and γS-crystallin, with the γS-crystallin having glutathione bound at Cys-82 and at Cys-22, Cys-24 or Cys-26. We conclude that when glutathione becomes bound to γS-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.
γ-B-crystallin from H₂O₂-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. Broekhuyse (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.

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. How- ever, the band appears to be absent from cataract lens samples. Results of densitometry (Figure 3) show that cataract has
A glutathione site on human lens γS-crystallin

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.

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 (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 βB1-, βB2-, γC- and γS-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 γB-crystallin has been shown to react with GSH to form mixed disulphides with...
one, two or three glutathione molecules attached to the protein [13].

βB1 and βB2 were both present in the 47 kDa component. Masses (m/z) of the peptides derived from βB1 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 βB1-crystallin (Table 1), and for four or five of the major peaks in the mass spectrum (Figure 6). The masses of βB2 peptides identified by MS are listed in Table 3 with their sequences and positions. They accounted for 50% of the total sequence of βB2 (Table 1) and for at least two major peaks of the mass spectrum.

There was evidence for GSH adducts on γS-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.

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>
<thead>
<tr>
<th>Protein</th>
<th>No. of matched masses</th>
<th>Coverage of protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>βB1-crystallin</td>
<td>13/55</td>
<td>59</td>
</tr>
<tr>
<td>βB2-crystallin</td>
<td>9/55</td>
<td>50</td>
</tr>
<tr>
<td>γC-crystallin</td>
<td>7/55</td>
<td>40</td>
</tr>
<tr>
<td>γS-crystallin</td>
<td>6/55</td>
<td>41</td>
</tr>
</tbody>
</table>

**Table 1** Proteins identified by MS, which are candidates for forming GSH–protein mixed disulphides
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Table 4 Peptides identified with a possible GSH adduct

<table>
<thead>
<tr>
<th>Peptide position</th>
<th>Peptide sequence</th>
<th>Peptide mass (kDa)</th>
<th>Peptide mass, with GSH adduct (+ 305.3 Da)</th>
<th>Mass matched with MS</th>
<th>Difference between 4th and 5th columns</th>
</tr>
</thead>
<tbody>
<tr>
<td>20–35</td>
<td>YDCCCDCAFDHTYLSR</td>
<td>1926.7091</td>
<td>2231.0091</td>
<td>2231.0889</td>
<td>0.0798</td>
</tr>
<tr>
<td>72–83</td>
<td>WMGLEDLSSC</td>
<td>1437.6725</td>
<td>1741.9725</td>
<td>1742.8556</td>
<td>0.8831</td>
</tr>
</tbody>
</table>

Figure 7 Amino acid sequences for the crystallin proteins

Beta crystallin B1

SQAKASAS TVAVNFDGY KKCGAFPGY SPDKYTLAP TVPHTSAK AELPFONRYL
YVIFLENGQ KAAFLGSCS NLADCGFGK RSITVSAGPW VAFQGNSRGM EMYLKEHEY
PFHNYIGSEY RSSWGNSPRP LEEAOQERK SLFEGANPMK WTITFQDRA PSLAVYGFSD
PVGYKVSSG TVWGVVGYQG RGYVTELVE DFRHNQDQA FQPDQSLSR LRPDKWLELEG
PPFVLAFFE P K

Beta crystallin B2

AEGHQGQGR PQSLNPKIII PQEFGQGRS HELGICQPL KETOVRKAGS VLQAGFWVG
YEQNQEQEQ PYTEGNSKR MGGWHTSRR DOLSLIPQVQGSHKLFYIENMWFGQRK
MEIIODGYS FRANQYERK DSVGQVQGTV VQGYPQGVR LGYLLHSGD KNSSSSGAP
PQSGVREIR DMHQNQCAF GSH

Gamma crystallin S

SGITEQFYS KEQHDQGHRH CQDCCDADQY TYLERCNSTK VEGSNVAYE RPPYAGVMYI
LPGQYKYG HFQHSDNKLV SRASVHELDG PQKYTGQEPQ GQFSGQWYRT TEPDPQIMQ
PFIHNEHCK VLQGWIFYRE LPRFGRQYLG LDKKSTQPKQ DWSGAPQAV SFPIRV

Gamma crystallin C

SKYETTIAF QRSTINET QZQFIPQYHE KSIRMVEGBS NWNLKQVRHY QGQTYLLEKQ
ETYTOPQHM LCSISINCNQH ITQVSHAR LKREKKNQK MLREHEGDPQ IQDQHIPSEI
RSIHYLQCK VMLENPHYK RYQYLQGRQ EQGQWNGD ARDQSKRLYV QLY

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

<table>
<thead>
<tr>
<th>Peptide position</th>
<th>Sequence</th>
<th>Modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>131–145</td>
<td>VLEGWIFYELPNYGR</td>
<td></td>
</tr>
<tr>
<td>158–173</td>
<td>KPIDWGAASPVQGSR</td>
<td></td>
</tr>
<tr>
<td>72–83</td>
<td>WMGLEDLSSC</td>
<td></td>
</tr>
<tr>
<td>84–94</td>
<td>AHWLPSGQGYK</td>
<td></td>
</tr>
<tr>
<td>148–154</td>
<td>QYLOKX</td>
<td></td>
</tr>
<tr>
<td>72–78</td>
<td>WMGLNRD</td>
<td></td>
</tr>
<tr>
<td>20–35</td>
<td>YDCCCDCAFDHTYLSR</td>
<td>+ GSH</td>
</tr>
<tr>
<td>72–83</td>
<td>WMGLEDLSSC</td>
<td>+ GSH</td>
</tr>
</tbody>
</table>

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 γS-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 H2O2. 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 γS-, γB-, γD-, γC- and γE-crystallin; however, γS-crystallin was not found in the fractions from lenses treated with 30 mM H2O2 [14]. In both cases, γS-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 βB1- and βB2-crystallins, but no GSH modification was observed, suggesting that the 47 kDa protein might be a dimer formed between γS-crystallin and βB1- and/or βB2-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 superfamilly 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 γS. 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 γS-crystallin (formerly known as βS-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 γS-crystallin, and probably formed as a result of glutathionylation of γS-crystallin, is found almost exclusively in the cortex.

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REFERENCES