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Glutathiolation Enhances the Degradation of γC-crystallin in Lens and Reticulocyte Lysates, Partially via the Ubiquitin–Proteasome Pathway

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Abstract

PURPOSE—*S*-glutathiolated proteins are formed in the lens during aging and cataractogenesis. The objective of this work was to explore the role of the ubiquitin–proteasome pathway in eliminating *S*-glutathiolated γC-crystallin.

METHODS—Recombinant human γC-crystallin was mixed with various concentrations of glutathione (GSH) and diamide at 25°C for 1 hour. The extent of glutathiolation of the γC-crystallin was determined by mass spectrometry. Native and *S*-glutathiolated γC-crystallins were labeled with ^{125}I, and proteolytic degradation was determined using both lens fiber lysate and reticulocyte lysate as sources of ubiquitinating and proteolytic enzymes. Far UV circular dichroism, tryptophan fluorescence intensity, and binding to the hydrophobic fluorescence probe 4,4′-dianilino-1,1′-binaphthalene-5,5′-disulfonic acid (Bis-ANS), were used to characterize the native and glutathiolated γC-crystallins.

RESULTS—On average, two and five of the eight cysteines in γC-crystallin were glutathiolated when molar ratios of γC-crystallin-GSH-diamide were 1:2:5 and 1:10:25, respectively. Native γC-crystallin was resistant to degradation in both lens fiber lysate and reticulocyte lysate. However, glutathiolated γC-crystallin showed a significant increase in proteolytic degradation in both lens fiber and reticulocyte lysates. Proteolysis was stimulated by addition of adenosine triphosphate (ATP) and Ubc4 and was substantially inhibited by the proteasome inhibitor MG132 and a dominant negative form of ubiquitin, indicating that at least part of the proteolysis was mediated by the ubiquitin-proteasome pathway. Spectroscopic analyses of glutathiolated γC-crystallin revealed conformational changes and partial unfolding, which may provide a signal for the ubiquitin-dependent degradation.

CONCLUSIONS—The present data demonstrate that oxidative modification by glutathiolation can render lens proteins more susceptible to degradation by the ubiquitin–proteasome pathway. Together with previous results, these data support the concept that the ubiquitin–proteasome pathway serves as a general protein quality-control mechanism.

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Lens fiber cells contain a very high concentration of proteins in the cytoplasm. This gives the lens a high refractive index and minimizes light scatter at the membrane–cytoplasm interface. The structural lens proteins, namely α-, β- and γ-crystallins, are subjected to extensive posttranslational modifications during aging, such as phosphorylation, deamidation, glycation, truncation, and the formation of disulfide bonds.[1–5] The latter is caused by oxidation of protein thiol groups and can result in intramolecular and/or intermolecular cross-links via protein–protein disulfides, or disulfide formation between the cysteinyl residues of lens proteins and other low molecular weight thiols in the lens—that is, glutathione (GSH) and cysteine.[6,7] The lens has a very high concentration of GSH—2 to 4 mM in 19- to 21-year-old human lenses—and the level of free cysteine is approximately 1% to 3% of that of free GSH.[6] Whereas free cysteine in the lens is pretty evenly distributed, there is a decreasing gradient of GSH from the lens epithelium to the nucleus.[6] Of the mixed disulfides, protein-*S*-*S*-glutathione (PSSG) predominates over protein-*S*-*S*-cysteine (PSSC) and protein-*S*-*S*-γ-glutamyl cysteine (PSSGC).[7,8] Protein-thiol mixed disulfides are known to accumulate both in old human lenses[7] and in human cataractous lenses.[9–11] In addition, increased protein-thiol mixed disulfides are associated with the development of cataract induced by various reagents, such as naphthalene,[12] ultraviolet radiation,[13,14] and hyperbaric oxygen.[15] Therefore, accumulation of protein-thiol mixed disulfides in the lens may be associated with cataract development.

Of the three major classes of crystallins, γ-crystallins have a high cysteine content and are susceptible to glutathiolation. In human lenses, mRNA for five different γ-crystallins—γS-, γA-, γB-, γC- and γD-crystallin—have been identified, but only γS-, γC- and γD-crystallins are abundantly expressed.[3] Detailed analysis of γS-, γC-, and γD-crystallins from human lenses showed that disulfide bonding, *S*-methylation and deamidation are the major posttranslational modifications of these crystallins.[16,17] Early work by Kodama and Takemoto,[18] investigating disulfide-linked crystallins associated with fiber cell membranes in human cataractous lenses, showed that γ-crystallin was the predominant protein involved in this interaction. Glutathiolated γS-crystallin has been found in human lenses[19] and formation of glutathiolated γB-crystallin was demonstrated after intact bovine lenses were exposed to hydrogen peroxide.[20] Although γC-crystallin contains the highest content of cysteine among the three predominant γ-crystallins in the lens, glutathiolation of γC-crystallin has not been reported. Based on the high homology in sequence and similarity in structure among various γ-crystallins, it is reasonable to assume that γC-crystallin is also glutathiolated in vivo upon oxidative stress. However, if glutathiolated γC-crystallin were more susceptible to proteolysis and were being rapidly degraded,[21] the chance of detecting glutathiolated γC-crystallin would decrease. Consistent with this speculation, levels of γC-crystallin in the nucleus of human lenses decrease with aging, whereas levels of γS- and γD-crystallins in the lens increase.[17]

It has been proposed that during aging of the lens, proteins are damaged by UV radiation and various oxidative species, and diminished ability to degrade those damaged proteins may cause their aggregation, cross-linking, precipitation, and subsequent cataract formation.[22–26] The ubiquitin-proteasome pathway (UPP) is one of the dominant proteolytic systems that are responsible for nonlysosomal protein degradation in eukaryotic cells. The UPP is known to prefer oxidized proteins to their native forms as substrates[27,28] and is therefore considered an important protein quality control mechanism. Proteins destined for degradation by the UPP are tagged with ubiquitin for subsequent recognition and degradation by the 26S proteasome. Several steps of the UPP machinery require adenosine triphosphate (ATP) and, therefore, ATP dependency is a hallmark of UPP-mediated proteolysis. Our previous studies demonstrated that mature lens fibers, including fibers in the lens nucleus, have a fully functional UPP[29,30] and that the UPP activity increases in response to mild oxidative stress.[31]

We have shown that oxidized crystallins, including α-, β- and γ-crystallins, are degraded at a faster rate than native crystallins, and that native γ-crystallin is resistant to proteolysis.[28,32]

The objective of this study was to test the hypothesis that glutathiolated γ-crystallin is preferentially degraded by the UPP. γC-crystallin was chosen for this study because it is one of the major γ-crystallins in human lens and it contains the highest content of cysteine and is therefore a potential target of glutathiolation. Incubation with GSSG in vitro can cause glutathiolation of γ-crystallins. However, only a fraction (20%) of the protein can be glutathiolated by incubation with GSSG at a 1:15 molar ratio at 37°C for 3 hours. It is not feasible to compare the susceptibility of glutathiolated proteins using such a mixture. To investigate the susceptibility of glutathiolated crystallins to proteolysis, we needed to obtain a high proportion of glutathiolated crystallins. To facilitate this study, we applied an efficient method of glutathiolation using GSH and diamide, a thiol-specific reagent.[33,34] This method glutathiolates γC-crystallin in a dose-dependent manner. The present data show that glutathiolated γC-crystallin is preferentially degraded, at least in part, by the UPP. The data also indicate that glutathiolation-associated conformational changes may be the signals of increased susceptibility to UPP-mediated proteolysis.

MATERIALS AND METHODS

Trizma (Tris-base), dithiothreitol (DTT), creatine phosphate, creatine phosphokinase, ATP, 2-deoxyglucose, Coomassie blue R-250, and chloramine T were purchased from Sigma-Aldrich (St. Louis, MO). Acrylamide, *N,N′*-methylene-bis-acrylamide, *N,N,N′,N′*-tetra-methylenediamine, sodium dodecyl sulfate (SDS), glycine, and protein molecular mass standards were obtained from Bio-Rad (Hercules, CA), and magnesium chloride was from Fisher Scientific (Fairlawn, NJ). ^{125}I-labeled protein A was purchased from Perkin Elmer (Boston, MA). MG132 was from Calbiochem (La Jolla, CA).

Bovine eyes were purchased from a local abattoir and the lens cortex was homogenized with 50 mM Tris-HCl containing 1 mM DTT (pH 7.6). After centrifugation at 100,000*g* for 10 minutes, the supernatant was used as the source of the UPP for degradation assays. Rabbit reticulocytes, constituting 90% of the red blood cells were purchased from Green Hectares Company (Oregon, WI), and lysate was prepared as previously described.[32]

Recombinant γC-crystallin

Details regarding construction of the gene for recombinant human γC-crystallin, its expression and purification of the expressed protein have been described previously.[35,36] The identity of the protein was confirmed by Western blot analysis, and the purity was established by SDS-PAGE. Protein concentrations were determined by using a protein assay reagent (Coomassie Plus; Pierce, Rockford, IL), with bovine serum albumin as the standard.

Glutathiolation

To produce glutathiolated γC-crystallin, recombinant human γC-crystallin was incubated with GSH and diamide at molar ratios of 1:2:5 or 1:10:25, respectively (γC-crystallin-GSH-diamide). The incubation was performed at 25°C for 1 hour in Tris-HCl buffer (50 mM, pH 8.0), after which nonbound GSH and diamide were removed by gel filtration (PD-10 Sephadex columns; GE Healthcare, Piscataway, NJ). Pilot experiments indicated that similar extents of glutathiolation can be achieved within a broad range of protein concentrations, as long as the ratios of γC-crystallin-GSH-diamide remain the same. Because most of the biophysical characterizations and subsequent proteolysis cannot be performed in highly concentrated protein solutions, we used 0.5 to 1.0 mg/mL of γC-crystallin in the glutathiolation reaction. The extent of glutathiolation of γC-crystallin was determined by SDS-PAGE run on 15% separating gels under nonreducing conditions (without β-mercaptoethanol in the Laemmli and running buffers) as well as by reversed phase HPLC coupled with an inline ESI-ion-trap mass spectrometer (Esquire-LC; Bruker Daltonik GmbH, Leipzig, Germany).

Proteolytic Degradation Assay and Statistical Analysis

γC-crystallin was labeled with ^{125}I by the chloramine T method.[22] Free ^{125}I and small peptides were removed by centrifugation with microconcentrators(Centricon 10; Amicon, Beverly, MA). Degradation of γC-crystallin was assayed essentially as described by Huang et al.[23] but using bovine lens fiber supernatant or rabbit reticulocyte lysate as the source of ubiquitinating and proteolytic enzymes. Briefly, the proteolysis reaction mixture, in a final volume of 25 μL, contained 30 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 1 mM DTT, and 15 μL lens fiber lysate (150 mg/mL protein), or reticulocyte lysate (300 mg/mL protein). For determination of ATP- and Ubc4-dependent proteolysis, 2 mM ATP, 10 mM creatine phosphate, 6 μg of creatine phosphokinase and 0.4 μg of recombinant Ubc4 were included in the assay. The latter was expressed and purified essentially as described by Wing and Jain.[37] Pilot experiments suggested that there is sufficient free ubiquitin in lens and reticulocyte lysates; therefore, no exogenous ubiquitin was added in these assays. Degradation was initiated by addition of 4 to 10×10^4 cpm of ^{125}I-labeled γC-crystallin, and the reaction mixtures were incubated at 37°C for 90 minutes. The reaction was terminated by addition of 200 μL ice-cold 10 mg/mL bovine serum albumin, immediately followed by 50 μL of 100% TCA (yielding a final concentration of 18.2% TCA), after which the samples were left on ice for 10 minutes. The extent of degradation was determined as the amount of TCA-soluble ^{125}I-labeled fragments of γC-crystallin. The total TCA-insoluble count at time 0 was defined as 100%. The degradation observed with the addition of ATP and Ubc4 is referred to as total degradation, whereas the difference between total degradation and degradation without addition of ATP and Ubc4 is denoted as ATP-/Ubc4-stimulated degradation. All experiments were performed in triplicate and typically repeated twice. For statistical analysis, data from several experiments were pooled and Student's *t*-test with the Bonferroni correction for multiple comparisons was used. $P < 0.025$ (due to the Bonferroni correction) was considered statistically significant.

To determine whether the degradation measured in this assay was mediated by the proteasome, reticulocyte lysate was preincubated with the proteasome inhibitor MG132 for 30 minutes before the start of the reaction, yielding a final concentration of 24 μM in the assay. To determine ubiquitin-dependent degradation, the proteolytic assay was also performed with the addition of 2 μg of a dominant negative form of ubiquitin, 4-hydroxynonenal-modified ubiquitin (HNE-Ub), which specifically inhibits ubiquitin-dependent proteolysis (Shang et al., unpublished data, 2005). This results in ~1:1 ratio of endogenous wild-type ubiquitin and added modified ubiquitin. HNE-modified ubiquitin was prepared by incubating purified ubiquitin with HNE at a molar ratio of 1:3 for 2 hours at 37°C. HNE-modified ubiquitin was separated from unmodified ubiquitin and free HNE by reversed phase HPLC. HNE-modification was verified by mass spectrometry analysis. Corresponding control samples received wild-type ubiquitin.

Spectroscopic Measurements

Fluorescence was measured with a spectrofluorometer (model RF-5301PC; Shimadzu, Columbia, MD). Tryptophan (Trp) fluorescence emission was scanned with an excitation wavelength of 295 nm. For determination of hydrophobicity, the extrinsic probe 4,4′-dianilino-1,1′-binaphtalene-5,5′-disulfonic acid (Bis-ANS; $\varepsilon = 23 \times 10^3\ cm^{-1} \cdot M^{-1}$ at 395 nm; Invitrogen, Eugene OR) was used.[38] Far UV circular dichroism (CD) spectra were obtained with a spectrometer (model 60 DS; Aviv, Lakewood, NJ). Five scans were recorded, averaged, and expressed in molar ellipticity, with units defined as $deg \cdot cm^2 \cdot dmol^{-1}$, using a polynomial-fitting program. To depict the secondary structure motifs of γC-crystallin we used the software Prosec.[39] All spectroscopic measurements were repeated at least twice with similar results. The experiments were performed at 25°C with native and glutathiolated γC-crystallin in 50 mM Tris-HCl buffer (pH 7.6).

RESULTS

Glutathiolation of γC-crystallin in the Presence of GSH and Diamide

SDS-PAGE without reducing agent showed a 21-kDa band for recombinant γC-crystallin (Fig. 1). Incubation of γC-crystallin with GSH in the presence of diamide yielded proteins with higher apparent molecular weight in the absence of β-mercaptoethanol (Fig. 1). In contrast, the modified proteins migrated similarly to the unmodified in the presence of β-mercaptoethanol (Fig. 1), suggesting that the slower migrating bands in the absence of β-mercaptoethanol were mixed disulfides. Mass spectrometry analysis revealed that the molecular mass of the native γC-crystallin used in this study was 20,748, and 20,930 (an adduct of γC-crystallin and AEBSF, a protease inhibitor used during purification of the recombinant γC-crystallin; Fig. 2A). Incubation of γC-crystallin with GSH using a molar ratio of 1:2 resulted in a dominant form (45%) of γC-crystallin with a molecular weight of 21,664, corresponding to three GSHs per γC-crystallin molecule (Fig. 2B). In addition, there were relatively small fractions of unmodified γC-crystallin (14%) or γC-crystallin modified by one (18%) or two (22%) GSHs (Fig. 2B). The weighted average was two GSHs per γC-crystallin. When γC-crystallin was incubated with GSH at the ratio of 1:10 in the presence of diamide, the masses of γC-crystallin became more heterogeneous, ranging in molecular weight from 21,664 to 22,947 (Fig. 2C). Approximately 35% was modified with three GSHs, 8% with five GSHs, and 56% with six GSHs. The weighted average was five GSHs per γC-crystallin.

Enhanced Proteolytic Degradation of Glutathiolated γC-crystallin

Proteolytic degradation of native γC-crystallin was undetectable in the lens fiber lysate without ATP supplementation and low (0.78%) when ATP was supplemented (Fig. 3A). Glutathiolation of γC-crystallin resulted in a significant increase in the susceptibility to proteolysis (Fig. 3A). Addition of ATP further increased the degradation of glutathiolated γC-crystallin. The data suggest that glutathiolated γC-crystallin is more susceptible than native γC-crystallin to proteolysis in lens fiber lysate and that at least 42% of the degradation of glutathiolated γC-crystallin was ATP-dependent.

As we demonstrated previously, the data suggest that the mature lens fibers have a functional ubiquitin-dependent proteolytic system. However, the large quantity of endogenous crystallins, including glutathiolated crystallins, in lens fiber lysate could compete with ^{125}I-labeled glutathiolated γC-crystallin, resulting in an apparent low degradation rate. To assess further whether glutathiolated γC-crystallin is indeed degraded by the UPP, we determined the degradation of glutathiolated γC-crystallin by using another source of the UPP. Reticulocytes, like lens fibers, are terminally differentiated and denucleated cells, and they retain an active UPP. As in lens fiber lysate, native γC-crystallin was resistant to degradation in reticulocyte lysate (Fig. 3B). In comparison, glutathiolation resulted in a dramatic increase in the rate of degradation of γC-crystallin, and this degradation was ratio dependent with regard to total degradation. With the addition of ATP and Ubc4, the degradation rates of γC-crystallin with an average of two and five GSHs were 16- and 30-fold higher, respectively, than the degradation of unmodified γC-crystallin under the same conditions (Fig. 3B). The increased susceptibility to proteolysis is not due to treatment with diamide, because treatment with diamide alone at a 1:25 molar ratio only marginally enhanced the degradation of γC-crystallin (data not shown). Ubc4 is a ubiquitin conjugating enzyme that shows selectivity to abnormal proteins. The amount of Ubc4 is limited in the reticulocyte lysate, and therefore addition of exogenous Ubc4 enhanced our ability to detect the ubiquitin-dependent degradation. Addition of Ubc4 enhanced ATP- and ubiquitin-dependent degradation. However, addition of Ubc4 had no effect on ATP-independent degradation (Shang F, unpublished data, 2005). Addition of ATP and Ubc4 to the assay increased proteolysis by 120% and 41% for the γC-crystallin modified by an average of two and five GSHs, respectively (Fig 3B). The ATP-independent

degradation of glutathiolated γC-crystallin was proportional to the extent of modification. For example, γC-crystallin modified by an average of five GSHs was degraded three times faster than the γC-crystallin that was modified by an average of two GSHs. However, the net ATP/Ubc4-dependent degradation of γC-crystallins modified by an average of two and five GSHs were similar (Fig. 3C).

To corroborate that the UPP is involved in the degradation of glutathiolated γC-crystallin, we tested the effects of the proteasome inhibitor MG132 and a dominant negative form of ubiquitin, HNE-ubiquitin, on degradation of γC-crystallin in reticulocyte lysate. The data demonstrate that approximately 40% of the ATP-/Ubc4-stimulated degradation (the enhancement over the degradation without added ATP) of γC-crystallin with two GSHs was inhibited by the proteasome inhibitor MG132 (Fig. 4A) and approximately 60% was inhibited by HNE-ubiquitin (Fig. 4B). The inhibition rates were similar for γC-crystallin modified by five GSHs (not shown). These data demonstrate that at least part of the ATP-/Ubc4-dependent proteolytic activity was mediated by the UPP. Because it is impossible to inhibit the proteasome completely with MG132, and because HNE-Ub also fails to abrogate UPP-dependent proteolysis completely, the present data represent minimal estimates of the contribution of the UPP to the overall proteolysis.

Conformational Changes with Glutathiolation of γC-crystallin

Previous investigations suggest that oxidative modifications to proteins that result in altered proteolytic susceptibility, may involve conformational changes. To determine whether this occurs with glutathiolation, we monitored the changes in Trp fluorescence, hydrophobicity, and CD spectra. Trp fluorescence displayed an emission maximum at 325 to 331 nm for the native and the glutathiolated γC-crystallins (Fig. 5A), which is consistent with previous data on γC-crystallin.[40] Glutathiolation of γC-crystallin decreased the emission intensity by 22% to 36% (γC-crystallin modified by 2 GSH) and 50% to 56% (γC-crystallin modified by 5 GSH), respectively. The decrease in Trp fluorescence upon glutathiolation may reflect changes in the tertiary structure of GSH-modified γC-crystallin. Using the extrinsic probe Bis-ANS, GSH-modified γC-crystallin exhibited a pronounced, typically threefold, increase in intensity (Fig. 5B), indicating that glutathiolation of γC-crystallin results in the exposure of hydrophobic residues of this protein.

The far UV CD showed a trough at 215 to 218 nm, characteristic of β-pleated sheet conformation (Fig. 6) and glutathiolation resulted in decreased β-sheet content and increased content of random coil, as evidenced by a wavelength shift of the trough. The content of α-helix, β-sheet, β-turn, and random coil, as calculated by the program ProSec,[39] was 10%, 52%, 19%, and 20% for native γC-crystallin and 3%, 49%, 16%, and 32% for the γC-crystallin modified by five GSHs.

DISCUSSION

Mature lens fibers are synthetically quiescent. The crystallins produced by the differentiating lens epithelium and immature lens fibers must function for the entire lifetime of the organism. During aging, the crystallins undergo extensive posttranslational modifications, some of which appear to be pathologic, leading to aggregation and cataract formation. Oxidative modifications of lens proteins, including formation of protein-thiol mixed disulfides appear to be cataractogenic,[7,11] and it has been demonstrated that GSH is the major sulfhydryl compound in these protein-thiol mixed disulfides.[41] Prior work has demonstrated that oxidatively modified proteins are substrates for the UPP,[23,24,28,32] and we asked if glutathiolation might be one of the oxidative modifications that provides a signal for selective degradation by the UPP.

In the present study, we used γC-crystallin as the model substrate to test whether glutathiolated lens proteins are degraded by the UPP. γC-crystallin was chosen as a model substrate, because it has the highest thiol content among crystallins—eight cysteines per γC-crystallin molecule[42]—and it is one of the most abundant γ-crystallins in human lenses.[1,3] Glutathiolated γB- and γS-crystallin have been identified in human lenses and bovine lenses after oxidative stress.[19,20] However, there is no report regarding the glutathiolation of γC-crystallin in vivo. Given the high content of cysteine in γC-crystallin and its structural similarity to other γ-crystallins, we expected that γC-crystallin would be susceptible to glutathiolation. Consistent with our prediction, the present data show that γC-crystallin was readily glutathiolated in the presence of GSH and diamide. Consistent with previous studies that have shown that native γ-crystallin is a poor substrate for the proteasome,[32,43,44] native γC-crystallin was resistant to degradation in both lens fiber lysate and reticulocyte lysate (Fig. 3). In contrast, glutathiolated γC-crystallin was degraded in both lens fiber lysate and reticulocyte lysate. At least 40% to 50% of the degradation of glutathiolated γC-crystallin in lens fiber lysate and reticulocyte lysate was ATP-dependent, indicating the involvement of the UPP in degradation of glutathiolated crystallin. These data are consistent with our hypothesis that the UPP is a general protein quality-control mechanism, which selectively degrades damaged or abnormal proteins. The selective degradation of the glutathiolated proteins by the UPP may explain the age-and cataract-related loss of γ-crystallins.[3,17,45–47] It should be noted that although the total degradation rates of γC-crystallin increased with increasing extent of glutathiolation, the ATP- and Ubc4-dependent degradation γC-crystallin did not correlate with the extent of glutathiolation. Based on these data, we speculate that a moderate glutathiolation is sufficient to trigger the degradation of γC-crystallin by the UPP. Extensive glutathiolation may cause overall conformational changes that may render the γC-crystallin to degradation by other less specific proteases, such as trypsin-like protease, calpain or the 20S proteasome, which degrade proteins in an ATP-independent manner.

Whereas native γC-crystallin is quite resistant to proteolysis,[32] glutathiolated γC-crystallins are readily degraded by the UPP. It appears that glutathiolation provides a signal for UPP-mediated degradation. Previous studies have indicated that exposure of hydrophobic patches of a protein can serve as a signal for the UPP.[48,49] Consistent with that idea, glutathiolation induced partial unfolding of γC-crystallin, as indicated by increased content of random coil, increased hydrophobicity, and decreased Trp fluorescence (Fig. 5, Fig. 6). Therefore, it is plausible that the conformational changes associated with glutathiolation may provide the basis for the UPP to discriminate glutathiolated γC-crystallin from native γC-crystallin.

The present data confirm earlier spectroscopic analyses regarding α- and γ-crystallin, showing that formation of mixed disulfides leads to partial unfolding.[50] The conformational change of glutathiolated γC-crystallin is not surprising, since formation of mixed disulfides adds a negative charge to each neutral cysteine involved. Conformational instability and aggregation has been demonstrated in α- and γB-crystallin mixed disulfides,[21] as well as in a mutant form of γC-crystallin which exhibited the same type of conformational changes as the glutathiolated γC-crystallin in this study.[40] A possible scenario in vivo is that glutathiolation of crystallins leads to initial partial unfolding, thereby increasing the susceptibility to various covalent modifications such as oxidation, glycation, and methylation, resulting in subsequent aggregation, precipitation, and cataract formation. Therefore, timely degradation of glutathiolated crystallins could prevent the subsequent modification, aggregation, and precipitation of glutathiolated proteins.

Lens fibers contain high levels of GSH. It is generally believed that transient *S*-glutathiolation in response to oxidative stress may serve as a protective mechanism that prevents protein thiols from irreversible oxidation. In most cases, glutathiolated proteins are rapidly deglutathiolated by thioltransferase or other GSH S-transferases.[6] Although the rapid degradation of

glutathiolated proteins by the UPP may result in the premature destruction of reversibly modified proteins, the reversible inactivation of the UPP by glutathiolation[51,52] may provide a checkpoint to prevent the premature destruction of cellular proteins. The activities of the ubiquitin-activating enzyme (E1) and the ubiquitin-conjugating enzyme (E2) were reduced when the GSSG-GSH ratio was increased upon exposure to H_2O_2, and there is also evidence of glutathiolation of E1 and E2 enzymes. In addition, ubiquitin-dependent proteolysis was found to be regulated by the GSSG-GSH ratio.[53] When the redox-status was restored upon recovery from stress, the UPP regained its activity.[53] Therefore, we speculate that the UPP and thioltransferase may work coordinately to eliminate glutathiolated proteins. It is plausible that only the proteins that are not rapidly deglutathiolated by thioltransferase are degraded by the UPP.

Together with previous reports, this work further demonstrates that the UPP is a general protein quality mechanism in the lens, which selectively degrades damaged proteins, including glutathiolated proteins. We previously showed that, in addition to epithelial cells, mature fiber cells, including the fibers in the nucleus, retained a functional UPP.[29,30] However, UPP activity decreases during maturation and aging of lens fibers. The UPP in nuclear fibers of lenses from old rats could not respond to mild oxidative stress as in lenses from younger rats.[25,31] In addition, large protein aggregates can also inhibit the proteasome.[54] We speculate that failure of timely degradation of damaged proteins, including glutathiolated proteins, could lead to the formation of protein aggregates, which in turn inhibits the UPP and further compromises the ability to degrade damaged proteins, thereby creating a vicious cycle that would eventually lead to cataract.[24] Therefore, maintaining or restoring the activity of the UPP in the lens is a reasonable approach for preventing cataract, particularly nuclear cataract.

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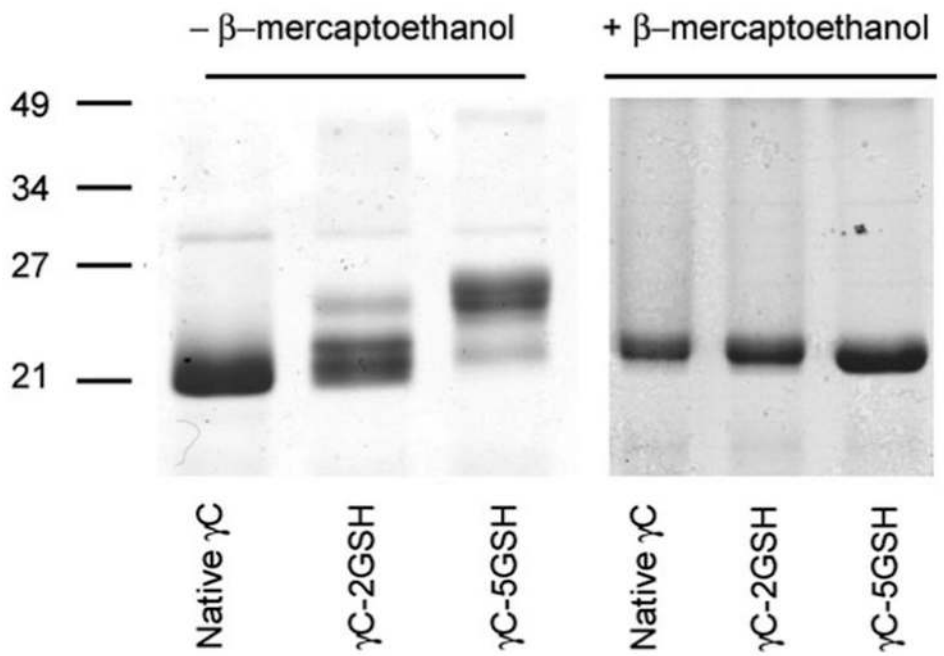

FIGURE 1.

SDS-polyacrylamide gel electrophoresis of native and GSH-modified γC-crystallin. Samples were treated with Laemmli buffer, with or without β-mercaptoethanol as indicated, and resolved on 15% separating gels, and the gels were stained with Coomassie Blue. Native γC-crystallin is denoted native γC and glutathiolated γC-crystallins were referred to as γC-2GSH and γC-5GSH, depending on the number of GSH adducts per γC-crystallin molecule, as revealed by subsequent mass spectrometry (Fig. 2). Numbers on the *left* indicate molecular mass of the standards (kDa).

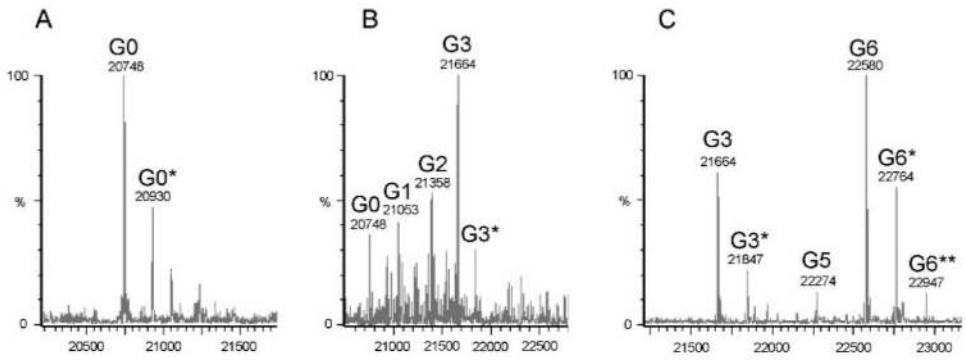

FIGURE 2.

Mass spectrometry characterization of native and glutathiolated γC-crystallin. Native and glutathiolated γC-crystallins were analyzed with LC-MS, and the deconvoluted mass spectra are shown. (**A**) Native γC-crystallin; (**B**) γC-crystallin modified with GSH at a molar ratio of 1:2; (**C**) γC-crystallin modified with GSH at a molar ratio of 1:10. G0 represents unmodified γC-crystallin; G1 represents γC-crystallin modified with 1 glutathione; G2 represents γC-crystallin modified with two glutathiones; G3 represents γC-crystallin modified with three glutathiones; G5 represents γC-crystallin modified with five glutathiones; and G6 represents γC-crystallin modified with six glutathiones. *Adduct of γC-crystallin with single protease inhibitor (+ 182); **adduct of γC-crystallin with two protease inhibitors.

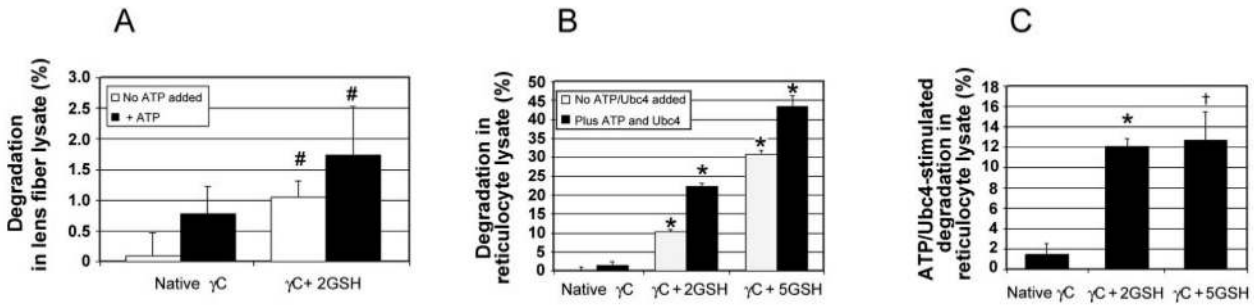

FIGURE 3.

Proteolytic degradation of ^{125}I-labeled native and GSH-modified γC-crystallin. (**A**) Degradation assay performed using bovine lens fiber cell lysate, with or without addition of ATP. (**B**) Degradation assay performed using reticulocyte lysate as the source of UPP-components with (total degradation) and without addition of ATP/Ubc4. (**C**) ATP-/Ubc4-stimulated degradation in reticulocyte lysate. Experiments were performed in triplicate and repeated at least three times with two separate batches of γC-crystallin. Shown are pooled data from all degradation experiments: $n \geq 12$. Mean ± SEM *$P < 0.0005$, †$P < 0.025$, #$P < 0.05$ (compared with native γC-crystallin with/without addition of ATP, respectively).

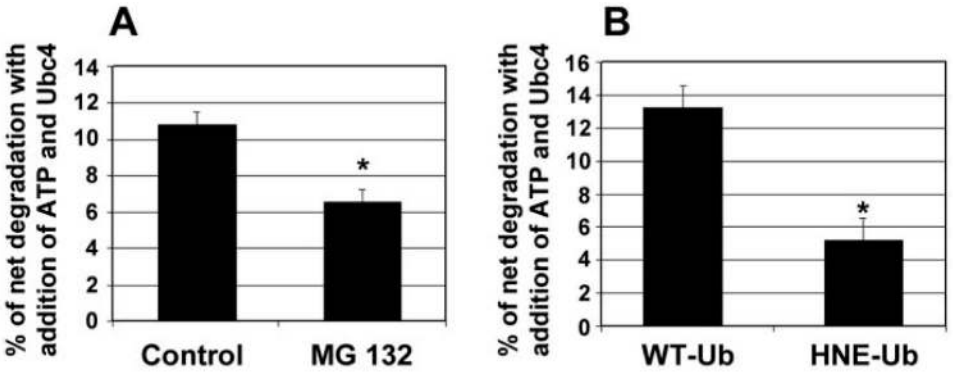

FIGURE 4.

Involvement of the proteasome and ubiquitin in the degradation of glutathiolated γC-crystallin in reticulocyte lysate. (**A**) MG132 (final concentration 24 μM) or (**B**) HNE-Ub (HNE-ubiquitin, final concentration 10 μM) was added to reticulocyte lysate before the assay was started. Wild-type ubiquitin (WT-Ub) was used as the control. Data shown are the mean ± SEM of results in two independent experiments; each experiment was performed in triplicate. *$P < 0.001$.

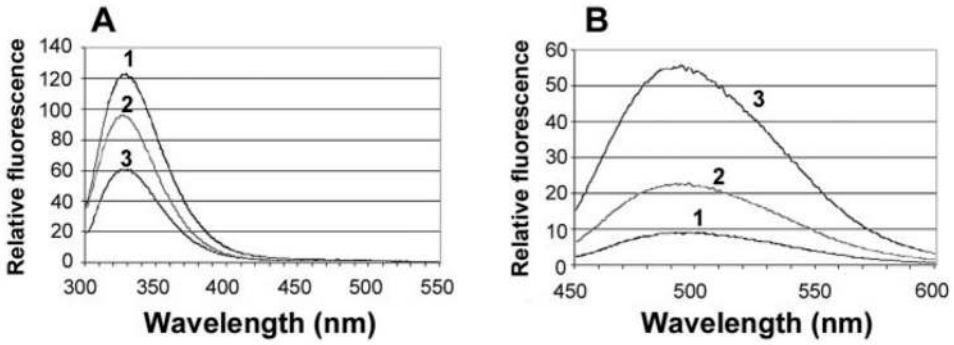

FIGURE 5.

Trp (**A**) and Bis-ANS (**B**) fluorescence spectra of native and GSH-modified γC-crystallin. The numbers refer to native γC-crystallin (1), γC-crystallin modified with an average of two glutathiones (2), and γC-crystallin modified with an average of five glutathiones (3). Protein concentrations of all substrates were 0.15 mg/mL in 50 mM Tris-HCl buffer, pH 7.6.

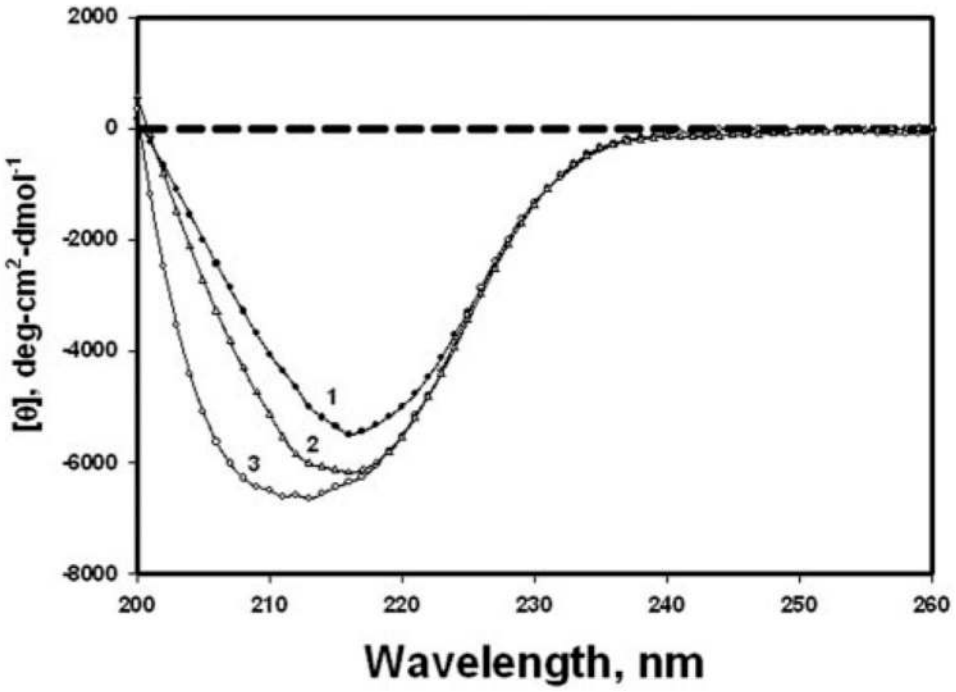

FIGURE 6.

Far UV CD spectra of native and glutathiolated γC-crystallin. Far UV CD spectra for native γC-crystallin (1), γC-crystallin modified with an average of two glutathiones (2), and γC-crystallin modified with an average of five glutathiones (3) were determined. Protein concentrations were 0.2 mg/mL in 50 mM Tris-HCl buffer (pH 7.6). Cell path length was 1 mm.