In an earlier report we showed that incubation of α-crystallin with oxidized glutathione results in significant loss of its chaperone-like activity. In the present study, we determined the effect of protein-glutathione mixed disulfides (PSSG), formed at Cys-131 in bovine αA-crystallin, and Cys-131 and Cys-142 in human αA-crystallin, on the function of α-crystallin as a molecular chaperone. After incubation of calf and young human αA-crystallin fractions with oxidized glutathione, levels of PSSG were determined by peroxamic acid oxidation of the mixed disulfides followed by reversed-phase high pressure liquid chromatography separation of phenylisothiocyanate-derivatized glutathione sulfonic acid. Levels of PSSG increased from 0.01 to 0.14 nmol/nmol (20 kDa) in bovine αA-crystallin and from 0.022 to 0.25 nmol/nmol in human αA-crystallin. The presence of glutathione adducts at Cys-131 and Cys-142 were confirmed by mass spectral analysis. The chaperone-like activity was determined by the heat denaturation assay using β-L-crystallin as the target protein. To examine the reversibility of the effect of mixed disulfides on chaperone activity, studies were done before and after reduction with the glutathione reductase system. Increased levels of PSSG resulted in lower chaperone activities. Treatment with the glutathione reductase system led to 80% reduction in PSSG levels with a concomitant recovery of the chaperone activity. These results suggest that cysteine(s) in the αA-crystallin subunit play an important role in the function of α-crystallin as a molecular chaperone.

α-Crystallin is one of the major eye lens proteins, representing about 35% of the total protein in the lens. It comprises two homologous subunits, αA and αB, with a molecular mass of 20 kDa each. Native α-crystallin exists as an oligomer with a molecular mass in the 360–800 kDa range. Historically, it was thought to be lens-specific, playing a structural role in maintaining transparency and thus facilitating light transmission to the retina. The idea that α-crystallin is purely lens-specific, playing only a structural role in the lens is no longer tenable. α-Crystallin, especially the αB subunit, is known to be widely expressed in other tissues, such as heart, kidney, placenta, lungs, and skeletal muscle (1–5). αA-Crystallin is found in the thymus and spleen (4, 6). αB-Crystallin accumulates in certain pathological conditions in the central nervous system, such as in Alexander’s disease, a degenerative neurological disorder (2), multiple sclerosis (7), and Lewy body disease (8). The natural occurrence of α-crystallin in various cell types and its increased expression in neurological diseases and other stress conditions like heat stress (9) and hypertonic stress (10) suggest an important functional role besides a structural role.

Ingolia and Craig discovered sequence similarities between small heat shock proteins (hsp) of Drosophila and α-crystallins (11). Based on this sequence homology both αA- and αB-crystallins are considered members of the hsp family. αB-Crystallin and hsp 27 can be induced in cell lines under stress conditions such as heat shock, oxidative stress, and exposure to transition metals (12, 13). One of the functions of a heat shock protein is to act as molecular chaperone, binding to partially denatured proteins preventing further denaturation and/or facilitating refolding of proteins to their native state. α-Crystallin has been shown to have such a chaperone-like function and is known to form a stable complex with denatured or partially unfolded proteins, preventing further aggregation (14–17). During aging and cataractogenesis of the lens, both subunits of α-crystallin undergo extensive post-translational modifications such as deamidation (18, 19), isomerization, racemization (20), oxidation (21), intramolecular disulfide bond formation (22), mixed disulfide formation (23, 24), and glycation (25). The ocular lens is under constant oxidative stress. In most types of cataracts, the common factor appears to be depletion of glutathione (GSH1), presumably through the formation of oxidized glutathione (GS(GG), which in turn forms protein-glutathione mixed disulfides (PSSG) (23, 24, 26, 27). Human lenses exposed to oxidative stress have elevated levels of PSSG (27), a precursor of protein disulfides. PSSG and protein-cysteine mixed disulfides (PSSC) are two major forms of protein-bound thiols in human lenses (24, 27).

Horwitz first reported the molecular chaperone-like function of α-crystallin (14). The chaperone function of α-crystallin has been shown to decline with aging in human lenses (28). Therefore, determining the factors that lead to its dysfunction as a chaperone is important in understanding the age-related changes causing opacification of the lens. The central focus of this communication is the formation of mixed disulfide between α-crystallin and oxidized glutathione, and the effect of mixed disulfides on chaperone activity of α-crystallin. Preliminary studies have shown that incubation of bovine α-crystallin with GSSG leads to a significant decrease in the chaperone-like activity (28). However, it is not certain whether protein-glutathione mixed disulfides are the true cause of this decrease. This

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The abbreviations used are: GSH, glutathione; PSSG, protein-glutathione mixed disulfides; GSSG, oxidized glutathione, PSSC, protein-cysteine mixed disulfides; GR, glutathione reductase; HPLC, high pressure liquid chromatography; PITC, phenylisothiocyanate.
investigation shows that incubation with GSSG causes mixed disulfides to form at Cys-131 of bovine α-crystallin and at both Cys-131 and Cys-142 of human α-crystallin. Additionally, we show that the chaperone activity can be recovered by reducing the mixed disulfides with glutathione reductase (GR).

EXPERIMENTAL PROCEDURES

Freshly collected calf lenses were obtained from a local abattoir. Human lenses from the Eye Bank of the Medical College of Georgia were pooled from 12–20-year-olds. Calf and human lenses were homogenized in 50 mM Tris, 50 mM NaHSO₄, 20 mM EDTA, pH 7.4. After centrifugation at 10,000 × g for 1 h at 4 °C, the supernatant water-soluble fractions were collected. About 30 mg of the soluble protein was resolved by preparative Sephacryl S-300-HR chromatography on a 100 × 1.5-cm column. The purity of calf and human crystallin fractions used in this study was confirmed by SDS-polyacrylamide gel electrophoresis (29).

In Vitro Modification of α-Crystallin—The α-crystallin fraction from calf lenses or 12–20-year-old human lenses (5 mg/ml) in 50 mM Tris buffer, 20 mM EDTA, pH 7.4 containing 0.02% NaN₃, was sterile filtered through 0.2 μm filters (Gelman Sciences) and incubated for 24 h with 25 mM GSSG. After incubation, analysis of proteins by SDS-polyacrylamide gel electrophoresis did not reveal any degradation during incubation. To reduce the PSSG formed during the GSSG incubations, half of the incubation mixtures from both bovine and human α₁-crystallins were further incubated for 90 min with 10 units/ml of GR and 2 mM NADPH. Part of the old (60–70 years) human α₁-crystallin (10 mg/ml) was also subjected to treatment with GR and NADPH.

Determination of Protein-Glutathione Mixed Disulfides—The procedure of Kumari et al. (30) with minor modifications was used to determine protein-glutathione mixed disulfides. About 10 mg of the calf or human α₁-crystallin were precipitated in 10% trichloroacetic acid, washed thoroughly in 5% trichloroacetic acid, methanol/ether, 1:1 and oxidized with performic acid, which releases glutathione as glutathione sulfonic acid. The sulfonic acid was derivatized by PITC. The phenylthiocarbamyl derivative was separated by HPLC on a phenomenex C-18 RP-HPLC column, and absorbance was monitored at 254 nm. The α₁-crystallin was further purified by preparative gel filtration (Bio-Rad, Hercules, CA) on a Sephacryl S-300 column.

Levels of Protein(α-Crystallin)-Glutathione Mixed Disulfides—Glutathione mixed disulfides were determined by reversed-phase HPLC separation of glutathione sulfonic acid after oxidation with performic acid (Fig. 1). The α₁-crystallin from calf and young human lenses, before and after GSSG treatment, was used for these analyses; the results are summarized in Table I. Calf α₁ fraction contained a very low level (0.01 nmol/nmol of 20-kDa monomer) of PSSG. After GSSG treatment, the level increased 14-fold to 0.142 nmol/nmol. Treatment of young human α₁-crystallin with 25 mM GSSG increased the PSSG content to 0.25 nmol/nmol of protein. Since about one-third of calf (or young human) α₁-crystallin is αβ-crystallin (32) containing no cysteine, the level of α₁-crystallin modification was approximately 0.21 nmol/nmol in calf α₁-crystallin and 0.38 nmol/nmol in human α₁-crystallin. Subsequent treatment with GR almost completely reduced the in vitro formed PSSG.

Results of Electrospray Ionization Mass Spectrometry of Human α₁-Crystallin—The molecular weights of the α₁-crystallins were determined using an on-line reversed-phase microbore column (5-cm × 1.0-mm inner diameter Vydac C-4, 300 A) attached to an electrospray ionization mass spectrometer (Micromass Platform II Quadrupole, Manchester, UK). The sample, 20 μl of the incubation mixture, was injected into the column at a flow rate of 50 μl/min. A post-column splitter directed 5 μl/min to the mass spectrometer and 45 μl/min to a UV monitor and fraction collector. A linear gradient of 35–60% acetonitrile in water, with 0.1% trifluoroacetic acid, over 25 min separated the α₁- and αβ-crystallins. MassLynx software was used to calculate the molecular weights of the proteins from the multiple charged peaks of the electrospray ionization mass spectrum.

Molecular Chaperone Assay—Chaperone-like activity of α₁-crystallin was determined by performing heat denaturation studies according to Horwitz (14). The ability of the unmodified or modified human or bovine α-crystallin preparation to protect calf β₁-crystallin (used as the target protein) from heat-induced denaturation and aggregation was assessed as follows: 40 μg of α₁-crystallin fraction was added to 400 μg of β₁-crystallin into a 1.5-ml cuvette and made up to a final volume of 1 ml with 50 mM phosphate buffer, pH 7.0. The cuvette was placed in a temperature-regulated cell holder attached to a Shimadzu model UV 160 spectrophotometer. Light scattering due to protein denaturation and aggregation was monitored (scanned) at 360 nm absorbance over a time period of 3000 s at 55 °C or 1800 s at 58 °C.

Other Methods—SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (29) using the Bio-Rad Mini-Protein II system. 10 μg of the samples were loaded onto a reducing gel and run at 200 volts. Protein concentrations were determined according to the method of Bradford (31).

FIG. 1. Reversed-phase HPLC elution profile of PITC-derivatized glutathione sulfonic acid released from α₁-crystallin bound glutathione mixed disulfides. α₁-crystallin fraction was incubated (5 mg/ml) for 24 h with or without 25 mM oxidized glutathione (GSSG) in Tris buffer. After dialysis, GR (10 units/ml) and NADPH (2 mM) were incubated with half of α₁ + GSSG for a further 1.5 h. Aliquots containing 10 mg of protein from each incubation were precipitated with trichloroacetic acid, oxidized by performic acid, derivatized with PITC, separated by C-18 RP-HPLC column, and absorbance was monitored at 254 nm. A, α₁-crystallin alone; B, α₁ + GSSG; C, α₁ + GSSG + GR; D, PITC-derivatized standards; glutathione sulfonic acid and cysteic acid.
indicates relative abundance of the proteins. However, such comparisons between two mass spectra are not valid. The mass spectra (Fig. 2, A and B) show that all the peaks with masses of 20,032 and below belong to α-A-crystallin without GSH adducts. The remaining six major peaks are generated entirely due to in vitro thiolation with GSSG. There is about 50% modification of α-A-crystallin by mixed disulfide formation (Fig. 2B).

**Loss of Chaperone-like Function of α-Crystallin by Mixed Disulfide Formation and Restoration by GR**—The target protein for the chaperone assay was calf β-L-crystallin. Characteristically, β-L-crystallin aggregates at elevated temperatures. The addition of α-crystallin either prevented or decreased the heat-induced aggregation of β-L-crystallin, which was measured by light scattering at 360 nm. Short term experiments were done with an α:β ratio of 1:10. The ratio of α to β determines the degree of protection against heat-induced aggregation. After incubation with 25 mM GSSG, α-crystallin showed a diminished ability to prevent the heat-induced aggregation of β-L-crystallin (Fig. 3). Correlation of the decrease in chaperone activity with the formation of mixed disulfides indicated that protein mixed disulfides contributed to the loss of α-crystallin chaperone-like function (see Table I). To confirm that the formation of protein-glutathione mixed disulfides was indeed the cause of this decrease in chaperone function, GR was added in the presence of 2 mM NADPH to reduce the mixed disulfides. The chaperone assay resulted in 40% reversal of chaperone-like activity in the calf α-L-crystallin (Fig. 3). Similar experiments were conducted with αL-crystallin fractions from young human lenses incubated with GSSG. The chaperone assay showed a 54% decrease of the chaperone-like function. Then, with the addition of GR, a 33% recovery of its function was shown (Fig. 4).

**DISCUSSION**

α-Crystallin is one of the most thermostable proteins in the lens. It has been shown to protect against heat-induced denaturation and aggregation of many proteins (14, 15) thus acting as a chaperone by interacting only with denatured nonaggregated crystallins. α-Crystallin differs from true chaperones in that it binds to unfolded proteins in a chaperone-like manner preventing aggregation but does not release its target protein as true chaperones do (33, 34). Nevertheless, this chaperone-like activity seems indispensable in maintaining lens transparency and possibly as a mammalian shock protein in other disorders (6–8). We have attempted to answer whether aging affects this chaperone function and the underlying cause of it in human lenses. Some of the causes of this chaperone malfunction are believed to be posttranslational modifications of α-crystallin by oxidation, glycation, and mixed disulfide formation (28). In this report, we have shown that the formation of glutathione mixed disulfides is a major cause of decreased molecular chaperone-like function of α-crystallin due to aging. This decrease in chaperone function can in turn affect the stability of other crystallins and the state of transparency of the whole lens causing lens opacification.

Lou et al. (23) have reported two or more species of mixed disulfides in the human lens. The two major species of thios bound to proteins are glutathione (PSSG) and cysteine (PSSC). The ratio of PSSG to PSSC in human lenses is 4:1; PSSC in human lenses appears to be concentrated in the nuclear region and the water-insoluble fraction (27). In the present study however, we did not detect PSSC, perhaps because we have used only the α-crystallin from the soluble fraction. Therefore, the focus of the present work is on protein-glutathione mixed disulfides.

Depletion of reduced GSH in the lens is associated with the normal aging process. Depletion of GSH and a concomitant increase of protein-thiol mixed disulfides during aging have been reported earlier (23, 27). In vitro induction of mixed disulfide formation in young human and calf α-crystallin via incubation with GSSG, showed a direct effect of mixed disulfides on chaperone activity (Figs. 3 and 4). Restoration of chaperone activity, as a result of mixed disulfide reduction by GR also supports this conclusion. These results suggest that PSSG formation is one of the major causes of declining chaperone activity.

**TABLE I**

**Levels of protein-glutathione mixed disulfides (PSSG) in various αL-crystallin preparations**

Glutathione mixed disulfides were first converted to glutathione sulfonic acid, derivatized by PITC, separated on a Phenomenex ODS 2 Sphereclone reversed-phase HPLC, and developed isocratically in 50 mM sodium acetate, 0.05% triethylamine (v/v), pH 6.6, and 0.5% acetonitrile (v/v) (see "Experimental Procedures" for the details). Calculations were based on peak areas using the glutathione sulfonic acid standard. Values are mean ± S.D. Number of samples is in parentheses.

| Source of αL-crystallin | Levels of PSSG (20-kDa αL-crystallin) nmol/nmol |
|-------------------------|-----------------------------------------------|
| Calf αL                | 0.010 ± 0.007 (3)                              |
| Calf αL + GSSG         | 0.142 ± 0.053 (4)                              |
| Calf αL + GSSG + GR    | 0.030 ± 0.007 (4)                              |
| Human αL (12–20 years) | 0.021 (2)                                      |
| Human αL (12–20 years) + GSSG | 0.250 (2)                                   |

**FIG. 2.** Reconstructed electrospray mass spectrum of human α-A-crystallin. Before (A) and after (B) 24 h of incubation with GSSG. The major peaks identified by matching the determined molecular masses within 2 μm of the calculated masses (19) were as follows: αA-Ser, 19,865; αA-crystallin, 19,952; αA + PO4, 20,032; αA + Ser + GSH, 20,170; αA + GSH, 20,257; αA + GSH + PO4, 20,337; αA + Ser + 2 GSH, 20,475; αA + 2 GSH, 20,562; αA + 2 GSH + PO4, 20,642.
activity in aging lenses. Wang and Spector (35) have shown that ο-crystallin can protect oxidation of thiols in γ-crystallin and that 70% oxidation of ο-crystallin thiols with H2O2 decreased only 20% of chaperone activity as compared with the native ο-crystallin. We found a 40% decrease in the chaperone activity in calf ο-crystallin and a 54% decrease in human ο-crystallin after the treatment with GSSG. Calf οA-crystallin has only one cysteine (Cys-131) and the human οA-crystallin contains two cysteines (Cys-131 and Cys-142). Both cysteines in human lenses formed mixed disulfides, as shown by mass spectrometric analysis (Fig. 2). Therefore, human οA-crystallin showed a higher level of modification (Table I) and decrease in chaperone activity than calf οA-crystallin. Takemoto (36) has shown oxidation of Cys-131 and Cys-142 of οA-crystallin forming intramolecular disulfides during human cataract development. Miesbauer et al. (19) showed the presence of disulfides in the water-soluble fraction of ο-crystallin from young lenses. Lund et al. (22) on the other hand showed all cysteines forming disulfide bonds in the water-insoluble fraction of ο-crystallin. Mixed disulfide formation in human ο-crystallin could be the precursor for such disulfide formation. However, the level of ο-crystallin-bound PSSG in the old human lenses is still unknown. Our preliminary data indicated that significant individual variations can be expected because values of 0.997 mol and 0.022 mol of PSSG/mol of 20-kDa protein were observed in two different οA-crystallin preparations from senile lenses (a large number of human lenses are under study). Variations in FSSG levels will not be a surprising observation (24) because of the varying degrees of oxidative stress senile lenses are subjected to. In addition, levels of FSSG will be influenced by the presence of cataracts (24).

The mechanism of the loss in chaperone activity due to mixed disulfide formation is not clear. One explanation is that the modification of the abovementioned cysteines causes conformational changes at the binding site on οA-crystallin. It should be emphasized here that the οB subunit is not expected to be modified, and the conformational changes in the ο-crystallin oligomer are triggered by modification of one or two sites on οA-crystallin. Takemoto and associates (17) have concluded by immunological localization of denatured γ-crystallin binding that the binding site on ο-crystallin is localized in the central region of the oligomeric α-crystallin. However, it is not certain whether the cysteine residues under study are a part of this region, although it is generally believed that the C-terminal domain of οA polypeptide is an integral part of this central region.

One important outcome of this study is the possibility of reversing the age-dependent decline in the ο-crystallin chaperone activity. Young human and calf οA-crystallins that formed mixed disulfides were further incubated with GR in the presence of NADPH to reduce the mixed disulfides. Reduction of the preformed PSSG led to significant improvement in the chaperone activity. This investigation provides insight into the role of protein-mixed disulfides in chaperone function. Controlling the formation of PSSG by activating the redox system may be important in protecting the ο-crystallin, thus delaying or preventing cataractogenesis.

REFERENCES

1. Bhat, S. P., and Nagineni, C. N. (1989) Biochem. Biophys. Res. Commun. 158, 319-325
2. Iwaki, T., Kume-Iwaki, A., Liem, R. K. H, and Goldman, J. E. (1989) Cell 57, 71-78
3. Dubin, R. A., Wawrousek, E. F., and Piatigorsky, J. (1989) Mol. Cell. Biol. 9, 1083-1091
4. Kato, K., Shinohara, H., Kurose, N., Goto, S., Inaguma, Y., and Ohshima, K. (1991) Biochem. Biophys. Acta 1086, 173-180
5. Bhat, S. P., Horwitz, J., Srivivasan, A., and Ding, L. (1991) Eur. J. Biochem. 202, 775-781
6. Srivivasan, A. N., Nagineni, C. N., and Bhat, S. P. (1992) J. Biol. Chem. 267, 23337-23341
7. van Noort, J. M., van Sechel, A. C., Bajramovic, J. J., El Ouagmiri, M., Pelman, C. H., Lassmann, H., and Ravid, R. (1995) Nature 375, 788-801
8. Lowe, J., Landen, M., Pike, J., Spendlove, I., McDermott, H., and Mayer, R. J. (1999) Lancet 336, 515-516
9. Klemenz, R., Frohli, E., Steiger, R. H., Schaefer, R., and Aoyama, A. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3652-3656
10. Dasgupta, S., Hohman, T. C., and Carper, D. (1992) Exp. Eye Res. 54, 461-470
11. Ingolia, T. D., and Craig, E. A. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 2360-2364
12. Hickey, E., Brandon, S. E., Potter, R., Stein, G., Stein, J., and Weber, L. A. (1986) Nucleic Acids Res. 14, 4127-4145
13. Arrigo, A. P., Suhaim, J. P., and Welch, W. J. (1988) Mol. Cell. Biol. 8, 5059-5071
14. Horwitz J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10449-10453
15. Horwitz, J., Emmons, T., and Takemoto, L. (1992) Curr. Eye Res. 11, 817-822
16. Rao, P. V., Horwitz, J., and Zigler, J. S., Jr. (1993) Biochem. Biophys. Res. Comm. 190, 786-793
17. Boyle, D., Gopalakrishnan, S., and Takemoto, L. (1993) Biochem. Biophys. Res. Comm. 192, 1147-1154
18. Groenen, P. J. T. A., van Dongen, M. J., Voorter, C. E. M., Bloemendal, H., and de Jong, W. W. (1993) FEBS Lett. 322, 69-72
19. Miesbauer, L. R., Zhou, X., Yang, Z., Yang, Z., Sun, Y., Smith, D. L., and Smith, J. B. (1994) J. Biol. Chem. 269, 12494-12502
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20. Fujii, N., Ishibashi, Y., Satoh, K., Fujino, M., and Harada, K. (1994) Biochim. Biophys. Acta 1204, 157–163
21. Takemoto, L., Horwitz, J., and Emmons, T. (1992) Curr. Eye Res. 11, 651–655
22. Lund, A. L., Smith, J. B., and Smith, D. L. (1996) Exp. Eye Res. 63, 661–672
23. Lou, M. F., Dickerson, J. E., Jr., and Garadi, R. (1990) Exp. Eye Res. 50, 819–826
24. Dickerson, J. E., Jr., and Lou, M. F. (1993) Biochim. Biophys. Acta. 1157, 141–146
25. Swamy, M. S., Abraham, A., and Abraham, E. C. (1992) Exp. Eye Res. 54, 357–345
26. Sippel, T. O. (1966) Invest. Ophthalmol. 5, 568–575
27. Lou, M. F., and Dickerson, J. E., Jr. (1992) Exp. Eye Res. 55, 889–896
28. Cherian, M., and Abraham, E. C. (1995) Biochem. Biophys. Res. Comm. 208, 675–679
29. Laemmli, U. K. (1970) Nature 227, 680–685
30. Kumari, K., Khanna, P., Ansari, N. H., and Srivastava, S. K. (1994) Anal. Biochem. 220, 374–376
31. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
32. Delcour, J., and Papaconstantinou, J. (1974) Biochem. Biophys. Res. Commun. 57, 134–141
33. Roa, P. V., Huang, Q., Horwitz, J., and Zigler, J. S., Jr. (1995) Biochim. Biophys. Acta. 1245, 439–447
34. Carver, J. A., Guerreiro, N., Nicholls, K. A., and Truscott, R. J. (1995) Biochim. Biophys. Acta. 1232, 251–260
35. Wang, K., and Spector, A. (1995) Invest. Ophthalmol. Visual Sci. 36, 311–321
36. Takemoto, L. J. (1996) Biochem. Biophys. Res. Comm. 223, 216–220