

α-Crystallin Can Act As a Chaperone Under Conditions of Oxidative Stress

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Purpose. Previous studies have shown that α-crystallin, a major lens protein, acts as a chaperone preventing the thermal denaturation of other lens crystallins. However, there has not been an examination of the α-crystallin chaperone ability with respect to the types of insult thought to cause human cataract. Therefore, an examination of the chaperone potential of α-crystallin under conditions of oxidative stress was undertaken.

Methods. Oxidation of α, beta low (βL), and γ-crystallins was performed with an ascorbate FeCl₃–EDTA–H₂O₂ system. Thermal denaturation was carried out by heating preparations at 62°C or 72°C. After protein denaturation, 360 nm scatter was measured. Protein-complex formation was measured with a TSK gel G4000 SW 600 × 7.5 mm exclusion column.

Results. This study indicates that: (1) α-crystallin markedly reduces the 360-nm light scatter of γ-crystallin caused by oxidation at 37°C. (2) α-crystallin appears to protect the γ-crystallin thiol groups from extensive oxidation. (3) Oxidation of α-crystallin causes only a small change in its ability to prevent heat-induced scattering of either γ- or βL-crystallin. (4) Oxidation of both α- and γ-crystallin does not significantly affect the ability of α-crystallin to inhibit 360-nm light scattering of γ-crystallin at 72°C. (5) Oxidation of βL-crystallin decreases its susceptibility to thermally induced scattering, but, conversely, oxidation of γ-crystallin increases such susceptibility. (6) Oxidation of βL-crystallin at 37°C produces only a slight increase in light scatter, in contrast to observations obtained with γ-crystallin. (7) α-crystallin provides long-term protection against thermally induced scatter of βL-crystallin but not of γ-crystallin. High-performance liquid chromatography (HPLC) analysis suggests that the α–γ-crystallin complex gradually becomes insoluble at 72°C, in contrast to the α–βL-crystallin complex. Differing from thermal insult, α-crystallin causes a marked decrease in γ-crystallin light scattering under long-term oxidation. (8) The α–γ-crystallin complex that results from oxidation represents a weak interaction because it cannot be isolated with procedures used to obtain the thermally induced complex. (9) This work confirms a previous study demonstrating that each α monomer (α₉) contains a binding site for a partially denatured crystallin.

Conclusions. The overall results indicate that α-crystallin can act as a chaperone under conditions of oxidative stress, decreasing the light scatter and thiol oxidation of other crystallins. Because oxidative stress is thought to be present under normal physiological conditions, it is probable that α-crystallin contributes to the mechanisms that maintain the lens in a transparent state. Invest Ophthalmol Vis Sci. 1995;36:311–321.

It is now well established that α-crystallin can act as a chaperone protecting other proteins from thermal insult. The possibility that α-crystallin may be a chaperone was suggested by the sequence homology between the α monomers and the small heat shock proteins. However, it was not until Horwitz demonstrated that α-crystallin can suppress the heat-induced aggregation of β- or γ-crystallins that it became appar-
ent that α-crystallin is a chaperone. This work was quickly confirmed by other laboratories.

α-crystallin is a complex macromolecule composed of two polypeptides, αA and αB, that have considerable sequence homology and are held together noncovalently to form giant aggregates ranging from 700 to 1000 kd. The isolated α monomers, α\textsubscript{m}, will aggregate independently and they also have been shown to act as chaperones. The quaternary structure of the macromolecule has not been determined, and different models have been proposed, including globular and micelle structures and, most recently, a rhombic dodecahedron model. In the latter models, every monomer has sites exposed on the surface of the macromolecule.

There is substantial evidence that oxidative stress is an initiating or early event in the development of maturity onset cataract. It has been found that patients with cataract have elevated levels of H\textsubscript{2}O\textsubscript{2}; the observed concentrations of H\textsubscript{2}O\textsubscript{2} are capable of causing cataract in organ culture; there is extensive oxidative damage to lens proteins, lipids, and DNA in patients with cataract; and the damage to cellular constituents observed when lenses in culture are subjected to oxidative stress is similar to that reported with human cataractous material.

Although it has been reported that α-crystallin is able, under certain conditions, to refold urea or guanidine-denatured protein, there have been no studies on whether α can act as a chaperone under conditions that may cause cataract. Because of the importance of oxidative stress in inducing cataract, it was of interest to determine whether the α-crystallin chaperone activity is effective under conditions of oxidation. In the presence of marked oxidative stress, α-crystallin can minimize protein aggregation and light scattering and can decrease the degree of thiol oxidation of other crystallins.

**MATERIALS AND METHODS**

All materials used were of the highest quality available and were obtained from major vendors such as Sigma (St. Louis, MO) and Bio-Rad (Hercules, CA).

**Protein Isolation**

α, β\textsubscript{IV}, β\textsubscript{L}, and γ-crystallin were prepared from the outer 40% to 50% of calf lenses in most respects as previously described. Calf eyes were obtained from a local abattoir within a few hours of death. The lenses were promptly removed and either were immediately used or were stored at −80°C until needed. The inner section of the lens was excised with a trephine, and the cortex was homogenized in 60 mM sodium phosphate, pH 7, containing 0.1 M NaCl, 2 mM EDTA, and 2 mM diethylenetriaminepentaacetic acid (DTPA). After centrifugation at 27,000g for 15 minutes at 4°C, the supernatant was immediately applied to a Biogel A-1.5 m column (2.6 X 100 cm). The protein was eluted at 4°C with the above buffer except that the NaCl was removed. Only the pooled peak fractions were taken for further study. These fractions were dialyzed against distilled water under argon and then lyophilized. Purity was checked by SDS-PAGE and amino acid composition, and concentrations were also initially determined by amino acid analyses using a Beckman (Fullerton, CA) 6300 amino acid analyzer. The protein characteristics were found to agree with earlier reports.

**Determination of Protein Light Scatter**

Light scattering at 360 nm was used to measure denaturation and aggregation. Measurements were made with a Beckman DU-70 spectrophotometer equipped with a six-cell holder and temperature controlled with a Lauda K2-RD circulator. Temperature in the cuvettes was monitored by insertion of a thermometer in one of the six cells in the sample chamber. Cells were covered with Teflon caps to prevent evaporation. During 30-minute experiments, the total volume was 0.50 ml, and scattering was recorded automatically every 30 seconds. In longer experiments, 1-ml volumes were used. Scattering was recorded automatically every 5 minutes during the first 2 hours and manually every 2 hours for the remainder of the experiment. After 2 hours, the samples were briefly sonicated before measurement. The average time to reach 37°C was approximately 1 minute; for higher temperatures, it was approximately 3 minutes.

**HPLC Analyses**

A Waters 840 HPLC system with a M-45 solvent delivery system, a model 441 detector and a 350 computer was used in conjunction with a TSK gel G4000 SW 600 X 7.5 mm exclusion column. The column was calibrated with blue dextran (2000 kd), thyroglobulin (660 kd), and γ globulin (160 kd). All analyses were performed at ambient temperatures. The mobile phase was 50 mM phosphate, pH 6.8, 50 mM NaCl. An isocratic flow rate of 1 ml/minute was used. Protein elution was followed at 214 nm and 280 nm.

**Oxidation of Protein**

The oxidizing conditions varied with the preparation because the crystallins differed in the extent to which they are susceptible to oxidation. With all conditions, H\textsubscript{2}O\textsubscript{2} and hydroxyl radical, and probably O\textsubscript{2}⁻, were available as oxidants.
α-Crystallin Chaperone Activity Under Oxidative Stress

7.4, 0.25 mM sodium ascorbate, 0.05 mM FeCl₃, 0.15 mM EDTA, and 0.20 mM H₂O₂. At the end of the incubation, 10 μl of 1.9 U/μl catalase was added. After a few minutes, the preparation was cooled to 4°C until used.

γ<sub>ox</sub>, β<sub>ox</sub>, and α<sub>ox</sub> 2 mg of protein in 2 ml containing 150 mM phosphate buffer, pH 7.4, 0.50 mM sodium ascorbate, 0.10 mM FeCl₃, 0.30 mM EDTA, and 0.50 mM H₂O₂ was incubated at 20°C for 48 hours. The reaction was stopped with 15 μl of 1.9 U/μl of catalase; after a few minutes, it was chilled to 4°C until use.

β<sub>ox</sub>, α<sub>ox</sub> 2 mg of protein in 2 ml containing 150 mM phosphate buffer, pH 7.4, 0.50 mM sodium ascorbate, 0.10 mM FeCl₃, 0.30 mM EDTA, and 2 mM H₂O₂ was incubated at 20°C for 48 hours. The reaction was stopped with 30 μl of 1.9 U/μl of catalase.

Thiol Determinations
Thiol was measured by a modification of the 5,5’-dithiobis(2-nitrobenzoic acid) DTNB colorimetric method. Aliquots of 100 μl were added to 400 μl containing 100 mM Tris, pH 8.2, 2 mM EDTA, 2 mM DETAPAK, and 6 M guanidine. 35 μl of 10 mM DTNB in methanol was added, and, after 20 minutes, the samples were read at 412 nm against a DTNB buffer blank. Standard curves were prepared with freshly prepared γ-glutamylcysteinylglycine held at pH 3 until use.

RESULTS
Crystallins Vary in Susceptibility to Thermal Insult
Before considering the effect of oxidation on the chaperone activity of α-crystallin, it was necessary to define the crystallin response to thermal insult. Crystallins vary significantly in their response to heat-induced insult. As shown in Figure 1, α-crystallin (α) does not heat denature as measured by light scatter at temperatures as high as 72°C. In contrast, β-L-crystallin (β<sub>L</sub>) is particularly sensitive to heat-induced denaturation, demonstrating significant 360 nm scatter at 62°C and increased scatter at 72°C. The γ-crystallins (γ) show a sensitivity to heat-induced aggregation between α- and β-L-crystallin with little denaturation at 62°C but a level of aggregation approaching β<sub>L</sub>-crystallin at 72°C. However, even at 72°C, the rate of aggregate formation with γ-crystallin is definitively slower than with β<sub>L</sub>-crystallin.

Characteristics of α-Crystallin Chaperone Activity During Long-Term Thermal Insult
It has been shown that α-crystallin probably interacts with other crystallins with a 1:1 binding of the α monomers (α<sub>m</sub>) of approximately 20 kd to the monomeric units of the other crystallins. Thus, the degree of protection against thermal insult is determined by the relative amount of α<sub>m</sub> present with respect to the interacting unit of the other crystallin. These conclusions have been based on relatively short-term experiments of approximately 30 minutes.

Because investigation of the effect of oxidation on the chaperone ability of α-crystallin required, in some experiments, insult periods longer than 30 minutes, the effect of long-term exposure to high temperature was examined. As shown in Figure 2, most of the 360-nm scatter was produced during the first hour of exposure at 72°C with either β<sub>L</sub>- or γ-crystallin alone. After 10 hours of thermal insult, only 15% of the β<sub>L</sub>- or γ-crystallin does not aggregate (Table 1). With β<sub>L</sub>-crystallin, as expected, almost all the heat-induced aggregation was eliminated, with concentrations of α-crystallin equal to those of β<sub>L</sub>-crystallin. Furthermore, the α-crystallin chaperone activity did not appear to decay during the 10-hour incubation period.

Surprisingly, the results with γ-crystallin were markedly different. Although, as expected, molar monomer levels of α-crystallin equivalent to γ-crystallin prevented significant 360-nm scattering during a 1-hour exposure to 72°C, with longer periods of exposure, considerable light scattering was observed. The rate at which the temperature-induced aggregation occurred was dependent on the mole ratio of γ-crystallin to α<sub>m</sub>. In the absence of α-crystallin, maximum scattering was approached within 1 hour. With increasing levels of α-crystallin, scattering was diminished. Even with an excess of α-crystallin (3 α<sub>m</sub>:2 γ-crystallin), significant scattering developed after a few hours. At
FIGURE 2. Long-term protection of βL- (A) and γ-crystallin (B) from heat-induced aggregation at 72°C with varying amounts of α-crystallin in 1 ml. The scattering was recorded automatically for the first 2 hours and manually for the remaining time. After 2 hours, the samples were sonicated before the scattering measurement.

10 hours, the scattering was approximately the same with α-γ-crystallin ratios of either 1 or 1.5. This scattering is only somewhat less than that found with γ-crystallin alone. Under the same conditions, α-crystallin alone shows insignificant scatter after 10 hours.

To confirm that the scattering data are indicative of the formation of large aggregates, selected samples similar to those used in Figure 2 were filtered through a 0.2-μm Gelman acrodisc filter, and the absorption of the resultant filtrate was measured at 280 nm. As shown in Table 1, no significant change in α-crystallin absorption was observed during the 10-hour exposure to 72°C. βL-crystallin absorption at 280 nm dropped precipitously at 2 hours and slightly more at 10 hours, leaving approximately 15% of the original A280. However, when 0.2 mg βL-crystallin and 0.2 mg α-crystallin are mixed, little change in absorption is found during the 10-hour period. The A280max observed equals approximately the sum of the individual protein preparations. With γ-crystallin alone, most of the protein has been removed by filtration at 2 hours. When 0.2 mg γ-crystallin is mixed with 0.3 mg α-crystallin, the A280max at zero time approximately reflects the sum of the individual A280max observations, and little change is observed at 2 hours. However at 10 hours, approximately 50% of the A280max is removed from the solution.

It is difficult to interpret these data. Either the α-γ-crystallin complex is gradually insolubilized or there is a slow dissociation of the complex, leading to insolubilization of γ-crystallin with the α-crystallin remaining in solution. Based on the A280max data, either approximately 50% of the complex has been insolubilized or about 10% of the γ-crystallin remains in solution with the α-crystallin. In either case, a clear instability of the complex is observed.

HPLC Analyses Indicate That the α-γ-Crystallin Complex Becomes Insoluble in a Time-Dependent Manner During Thermal Insult

To examine this process more critically, HPLC experiments using TSK 4000 columns were undertaken with a ratio of 1.5:1 α-γ-crystallin (Fig. 3A). Under conditions similar to those shown in Table 1 and Figure 2, after incubation at 72°C, the mixture of α- and γ-crystallin was filtered and then passed through the HPLC column that previously had been calibrated to determine where standard proteins of different sizes would elute. Elution of the proteins was monitored at 214 nm. At zero time, the expected peaks were obtained (Fig. 3A). Peak 2 had an elution volume similar to that obtained with α-crystallin and eluted as a 800 ± 50 kd component. Peak 3 corresponded to the elution position of γ-crystallin, but a molecular weight could not be assigned because of nonlinearity and poor resolution in the low molecular weight range. At zero time, the ratio of the relative areas of the HPLC peaks (peak 2/peak 3 = 1.30 ± 0.10/0.84 ± 0.10) corresponded to the ratio of the initial concentration of the proteins (1.55). After 0.5 hour of incubation, the α-crystallin peak almost disappeared and a new larger peak, peak 1, appeared (relative area = 1.90 ± 0.10) with a molecular size of approximately 1.1 X 10^6. The γ-crystallin peak was now much reduced, representing about 1/3 of the original peak (relative area 0.28 ± 0.02). The area of the new peak equaled the sum of the α-crystallin peak and 1/3 of the original γ-crystallin area. Thus, the total areas of the original α- and γ-crystallin peaks were distributed in the new peak and the remaining γ-crystallin peak. As anticipated from the Fig-

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ure 2B scatter data, little change was observed at 2 hours in either area or elution position of either peak 1 or peak 3, but at 10 hours, there was a large decrease in peak 1 to about 37% of its original size (relative area, 0.72 ± 0.05). No additional peak appeared, and the γ-crystallin peak did not change significantly.

The data clearly support the conclusion that an α-γ-crystallin complex was present at 0.5 hours, with γ-crystallin/αm = 0.44 increasing the molecular weight of the native α-crystallin aggregate theoretically to 1.15 × 10⁶ d, approximately the value found experimentally. The change in molecular weight was also approximately what would be expected from the observed increase in the 214-nm absorption found in peak 1. The results suggested that although there was probably a distribution in the size of the α-γ-crystallin complex, the relatively sharp peak indicated that most α-crystallin aggregates interacted with γ-crystallin to form complexes with approximately the same level of saturation of α monomers. The decrease in the peak 1 area at 10 hours indicated that much of the α-γ-crystallin complex aggregated, contributing to the 360-nm scatter observed in Figure 2B, and it was sufficiently large to be removed by the 0.2-μm filter as shown in Table 1. This interpretation is supported by no reappearance of the α-crystallin HPLC peak (Fig. 3A). The results are in reasonable agreement with the observations shown in Table 1. The loss of the α-γ-crystallin complex A₂₈₀nm absorption, thus, was the result of insolubilization of the complex at 72°C. The remaining A₂₈₀nm material resulted from the remaining soluble α-γ-crystallin complex and the γ-crystallin that remained in solution. It had been shown that some γ-crystallin does not become heat denatured.⁴

In contrast to the α-γ-crystallin experiments, the TSK 4000 HPLC results with βL-crystallin showed the appearance of a new, higher molecular weight peak of approximately 1.1 × 10⁶, which appeared after 0.5 hours of thermal insult but did not decrease in area with time (Fig. 3B). As with the γ-crystallin peak, a small βL-crystallin peak remained and apparently was resistant to thermal insult at 72°C (23% of the original βL-crystallin peak area). The size and quantity of the α-βL-crystallin complex (relative area at 10 hours was 96% of areas of starting components) agreed with the values determined from the filtering experiments (A₂₈₀ nm at 10 hours was 94% of initial A₂₈₀ nm).

### Oxidation Has Only a Small Effect on α-Crystallin Chaperone Ability

To obtain varied levels of oxidation, the crystallins were subjected to different oxidizing conditions. In all cases, the same constituents were used for the oxidation, Fe³⁺, EDTA, ascorbate, and H₂O₂. However, concentrations and the time of oxidation were varied. As shown in Table 2, with a higher concentration of H₂O₂ and longer incubation times, it was possible to reduce α-crystallin thiol levels by approximately 70% (αox₂; βL-crystallin thiol levels by approximately 87% (βox₂), and γ-crystallin thiol levels by approximately 44% (γox₂). Under less forceful conditions and with a shorter exposure to oxidative stress, less thiol was oxidized (ox-1).

Does oxidation of α-crystallin change its ability to chaperone with respect to thermal insult? As shown in Figure 4, increasing amounts of α-crystallin are increasingly effective in decreasing the 360-nm scatter of γ- and βL-crystallin. The stoichiometry required to prevent aggregation during 30 minutes of heating approaches 1:1 for αm:βL-crystallin and αm:γ-crystallin. These values are approximately the same with oxidized or native species. With βL-crystallin, it appears that 26 μg/ml of αox₁ yields approximately the same results as obtained with 21 μg/ml of native α-crystallin, whereas 26 μg/ml of αox₂ may provide slightly less protection than αox₁. There appears to be an approximately 20% decrease in chaperone effectiveness with αox₂ compared to native α-crystallin. Similar results are obtained in preventing γ-crystallin aggregation. Again, 26 μg/ml of αox₂ yields results similar to 21 μg/ml of native α-crystallin. Thus, it can be concluded that extensive oxidation of α-crystallin does not appreciably affect its ability to act as a chaperone with other crystallins.

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**Table 1. Heat-induced Decrease in Soluble Protein**

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>α</th>
<th>βL</th>
<th>γ</th>
<th>βL + α*</th>
<th>γ + α†</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.180 ± 0.010</td>
<td>0.440 ± 0.020</td>
<td>0.408 ± 0.020</td>
<td>0.610 ± 0.020</td>
<td>0.654 ± 0.020</td>
</tr>
<tr>
<td>2</td>
<td>0.175 ± 0.010</td>
<td>0.090 ± 0.005</td>
<td>0.078 ± 0.005</td>
<td>0.590 ± 0.020</td>
<td>0.635 ± 0.020</td>
</tr>
<tr>
<td>10</td>
<td>0.172 ± 0.010</td>
<td>0.066 ± 0.005</td>
<td>0.060 ± 0.005</td>
<td>0.576 ± 0.020</td>
<td>0.328 ± 0.020</td>
</tr>
</tbody>
</table>

At 72°C, 0.20 mg lens protein (α, βL, or γ) was incubated in 1 ml of 60 mM sodium phosphate, pH 7.0. The incubated samples were filtered with a Gelman Acrodisc 0.2-μm filter. The soluble proteins in the filtrate were measured by the absorbency at 280 nm. Mixtures of crystallins were incubated in the same manner using the amounts of proteins shown below:

* 0.20 mg βL was incubated with 0.20 mg α.
† 0.20 mg γ was incubated with 0.30 mg α.
Oxidation of β-L- and γ-Crystallin Change Their Susceptibility to Thermal Insult

Does oxidation of β-L- or γ-crystallin affect the ability of α-crystallin to act as a chaperone under conditions of thermal insult? In Figure 5A, it is apparent that with increasing oxidation, β-L-crystallin became relatively resistant to heat-induced aggregation as measured by 360-nm scattering. This resulted in less α-crystallin required to prevent β-L-crystallin scattering.

The results obtained after oxidation of γ-crystallin were different. Oxidized γ-crystallin readily aggregated with thermal insult (Fig. 5B). Indeed, γox-i2 is not soluble enough to study under the conditions used in these experiments. At low concentrations of α-crystallin, relative to γ-crystallin, more α-crystallin is needed to achieve the same level of chaperone activity under thermal insult with γox-i1 than with γ. This is probably due to the greater propensity of γox-i1 to aggregate. However, α-crystallin still appears effectively to protect γox-i2 with a molar ratio of αm to γ-crystallin approaching 1:1.

Oxidized α-Crystallin Retains Its Ability to Protect Oxidized γ-Crystallin From Thermal Insult

Although it has been shown that αox acts as a chaperone with native γ-crystallin and that α-crystallin protects γox-i1, it is not apparent from such results that, if both α- and γ-crystallin have been independently oxidized, the αm can protect γox from thermal denaturation. When such an experiment was conducted with αox-i1 and γox-i1, the same degree of protection observed with native α-crystallin was obtained (Fig. 5B). Thus, the prior oxidation of the proteins does not prevent a chaperone-like effect with respect to thermal insult.

α-Crystallin Protects γ-Crystallin From Thiol Oxidation

What happens if there is simultaneous oxidation at 37°C of both α-crystallin and another crystallin? Does the crystallin aggregate? Can α-crystallin protect against oxidation, aggregation, or both? As indicated, it was found that oxidized βL is thermally more stable than native βL and aggregates only to a slight extent, producing little 360-nm light scattering. Therefore, only experiments with γ-crystallin are reported. Under the oxidizing conditions used for this experiment, approximately 20% of the α-crystallin thiol and 50% of the γ-crystallin thiol are oxidized in 17 hours when

### TABLE 2. Thiol Content of Native and Oxidized Crystallins*

<table>
<thead>
<tr>
<th>Crystallin</th>
<th>Native</th>
<th>αox-1</th>
<th>αox-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>0.70 ± 0.05</td>
<td>0.40 ± 0.05</td>
<td>0.20 ± 0.05</td>
</tr>
<tr>
<td>βL</td>
<td>1.50 ± 0.10</td>
<td>0.60 ± 0.05</td>
<td>0.20 ± 0.05</td>
</tr>
<tr>
<td>γ</td>
<td>4.60 ± 0.20</td>
<td>3.80 ± 0.20</td>
<td>2.60 ± 0.20</td>
</tr>
</tbody>
</table>

* nmole SH ± SD 20 μg crystallin.

γox-i1: γ-crystallin (1.0 mg/ml) was oxidized with 0.25 mM sodium ascorbate, 0.05 mM FeCl3, 0.15 mM EDTA, 0.20 mM H2O2, and 150 mM potassium phosphate (pH 7.4) at room temperature for 24 hours. The oxidation was stopped by 10 μl of 1.9 U/μl catalase solution.

γox-i2, βLox-i1, αox-i1: crystallin (1.0 mg/ml) was oxidized with 0.50 mM sodium ascorbate, 0.10 mM FeCl3, 0.30 mM EDTA, 0.50 mM H2O2, and 150 mM potassium phosphate (pH 7.4) at room temperature for 24 hours. The oxidation was stopped by 15 μl of 1.9 U/μl catalase solution.

βLox-i2, αox-i2: crystallin (1.0 mg/ml) was oxidized as above but with 2.0 mM H2O2. 300 μL of 1.9 U/μl catalase was used to stop the reaction.
Each protein is oxidized in the absence of the other (Fig. 6A). With increasing molar abundance of α to γ-crystallin, not only does the level of oxidation decrease, but the loss of thiol is less than would be expected. Based on the observed levels of thiol of the native proteins reported in Table 2 and the loss of thiol observed with α- or γ-crystallin alone, Figure 6A, with α:γ ratios of 1:2, theoretically a 48% loss of thiol would be expected but only 40% was observed; with α:γ of 1:1, 46% is theoretically anticipated but 35% was found; with α:γ of 1.5 to 1, 44% is theoretically expected but 28% was observed (the variation over three experiments gave a standard deviation of ±2%). Thus, it appears that α-crystallin interacts with γ-crystallin early on in the oxidation process and slows the overall rate of oxidation.

It can be argued that the decrease in the thiol oxidation of γ-crystallin in the presence of α-crystallin is due to the additional protein rather than to a protective effect of α-crystallin. Estimation of the level of reactive oxygen species suggested that there was an excess of oxidizing species. To test this assumption more clearly, varying amounts of γ-crystallin alone, α-crystallin alone, or α plus γ-crystallin were oxidized under the identical conditions used in Figure 6. It was found that varying γ-crystallin concentrations from 100 to 250 μg/ml resulted in a thiol loss of 50% ± 2% after 17 hours of oxidation. Varying α-crystallin from 300 to 1000 μg/ml gave thiol losses of 22% ± 2%. Finally, when 100 μg/ml of γ-crystallin was oxidized with 150 μg/ml of α-crystallin and compared with oxidizing 200 μg/ml of γ-crystallin with 300 μg/ml of α-crystallin, 31% and 28% thiol loss was observed, respectively. Thus, in the concentration range used in these experiments, the observed effects are due to α-crystallin protection and not the presence of additional protein. The results suggest that the early
FIGURE 6. Effect of oxidation on thiol loss and relative light scattering of γ-crystallin in the presence of varying concentrations of α-crystallin. (A) Percent thiol loss. (B) Relative 360 nm scattering during 17-hour oxidation. (C) 360 nm scattering during 48-hour oxidation. 200 μg y/ml in the presence of indicated concentrations of α-crystallin were oxidized with 0.25 mM sodium ascorbate, 0.05 mM FeCl₃, 0.15 mM EDTA, 0.20 mM H₂O₂, and 60 mM sodium phosphate, pH 7. EDTA = ethylenediamine-tetraacetic acid.

α,α-γ ox interaction prevents further extensive γ-crystallin oxidation.

One additional experiment was performed to determine whether other proteins might protect γ-crystallin from oxidative stress. The γ globulins do not scatter light under oxidizing conditions; therefore, the presence of γ globulins during the oxidation of γ-crystallin was examined. It was found that the γ globulins at concentrations equal to γ-crystallin offered no protection to γ-crystallin either with respect to aggregation or with respect to thiol oxidation. Similar results with respect to thiol protection were found with β amylase and urease, which have approximately 3.5- to 5-fold more thiol, respectively, than α-crystallin. Such experiments demonstrate that the addition of nonchaperone proteins have no effect on γ-crystallin thiol oxidation or on γ scattering based on γ globulin experiments as well.

α-Crystallin Provides Long-Term Protection of γ-Crystallin Under Conditions of Oxidative Stress

With the oxidation of γ-crystallin alone, aggregation quickly appears so that within 4 to 5 hours, most of the 360 nm scatter is detected (Fig. 6B). The level of scatter is approximately 50% of that observed with heat aggregation at 72°C. It is interesting that at this point, only 50% of the total thiol oxidation found at 17 hours is observed (Fig. 6A). Unlike short-term, heat-induced denaturation, where 1:1 α:γ ratios reduce the A₃₆₀ nm approximately 10-fold, with oxidation the reduction in scatter is approximately 3-fold. Significant scatter remains. Increasing the α:γ further to 1.5:1 only produces a small additional reduction in scattering. Thus, α-crystallin cannot eliminate light scatter caused by oxidation but can reduce it significantly.

Because the protective effect of α on γ-crystallin remained for a short time with heat-induced aggregation, it was of interest to examine the effect of extended oxidative stress. As shown in Figure 6C, even after 48 hours, there remained approximately 2-fold less scattering in the presence of α-crystallin. Thus, unlike heat-induced insult, the α ox-γ ox complex appears to remain stable and soluble for extended periods although the level of protection decreases (from approximately 3-fold to approximately 2-fold).

After 2 hours of oxidation at 37°C, an attempt was made to examine the α ox-γ ox complex formed with equal starting amounts of α- and γ-crystallin (200 μg). HPLC procedures similar to those described earlier were used. As shown in Table 3, native α-crystallin (peak 1) and γ-crystallin (peak 2) run either separately or together gave similar respective peaks with comparable areas. The α ox material (peak 1) had the same mobility and area as α-crystallin alone. However, the γ-crystallin now was reduced by 12%. Oxidation was also run for 8 hours and then...
DISCUSSION

It has been shown that under conditions of thermal denaturation, α-crystallin acts as a chaperone by interacting only with denatured, nonaggregated crystallins (γ, βL, and βH) and that no interaction occurs with native protein or denatured, aggregated insolubilized components. The interaction with the soluble denatured protein increases in a linear stoichiometric manner until a 1:1 ratio of αm:γ or αm:(βL)m is obtained. If there is more of the denatured protein than of αm present in the macromolecular complex, loss of solubility of the complex occurs. These conclusions are based on short-term studies of heat-denatured species at 65°C and were extended to long-term higher temperature insult in this study. However, because of the instability of the complex formed with oxidized species, it was not possible to isolate this type of complex. Nevertheless, it is likely that the same type of stoichiometry applies to species arising from oxidative stress. In this article, it is definitively shown that α-crystallin can also act as a chaperone when the denaturation is produced by oxidation rather than heat. Because oxidation is a major factor involved in the development of cataract, this is a physiologically relevant insult. Thus, α-crystallin contributes by its chaperone activity in maintaining lens transparency.

The effects of oxidation are not always predictable on the basis of the experiments with thermal insult. With βL after oxidation, the protein becomes less susceptible to heat-induced aggregation. The level of 360 nm scattering produced by extensive oxidation of γ-crystallin is approximately half of what is observed with heat denaturation. The chaperone effect of α- with heat-denatured γ-crystallin decreases with time, but not appreciably with oxidized γ-crystallin. It is probable that the conformation of the protein resulting from thermal denaturation differs from that resulting from oxidatively induced denaturation. The chaperone effect of α-crystallin is dependent on the interaction of the denatured protein with the αm binding site. It can be presumed that specific sites on the denatured protein are required for binding to the α-crystallin. Such sites are unavailable in the native and aggregated heat-denatured protein. In certain situations, these sites may have been modified by oxidation, changing the interaction between the chaperone and the denatured soluble protein. It is interesting that the α-interaction with γ-crystallin decreases the level of oxidation

### TABLE 3. Effect of Oxidation on HPLC Profiles

<table>
<thead>
<tr>
<th>Native</th>
<th>Oxidized</th>
<th>HPLC Peaks</th>
<th>Relative Areas</th>
<th>% γ loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>γ</td>
<td>α</td>
<td>γ</td>
<td>1</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>0.69</td>
<td>0.60</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>0.70</td>
<td>0.60</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>0.72</td>
<td>0.35</td>
<td>42</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>0.72</td>
<td>0.53</td>
<td>12</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>0.68</td>
<td>0.43</td>
<td>28</td>
</tr>
</tbody>
</table>

200 μg of γ and α were oxidized either individually or together with 0.25 mM sodium ascorbate, 0.05 mM FeCl3, 0.15 mM EDTA, 0.2 mM H2O2, and 60 mM sodium phosphate, pH 7.0, at 37°C for 2 hours. The oxidation was stopped by the addition of 15 μl of 1.9 U/μl catalase. The preparations were filtered and then analyzed by HPLC. The elution was followed at 214 nm. All values represent three determinations and have a standard deviation of ±0.01 to 0.02 (see Materials and Methods)

* Represents 8 hours of oxidation.

EDTA = ethylenediaminetetraacetic acid; HPLC = high-pressure liquid chromatography.

was followed by HPLC. Again, no new HPLC peak was found. The αm peak was similar in size and mobility to the initial α-crystallin sample, but a greater loss of γ was observed than with 2 hours of oxidation (28% loss of material). Thus, the results indicate that the αm-γm complex is not sufficiently stable to be detected on HPLC in contrast to the thermally induced α-γ-crystallin complex. The complex appears to dissociate, and the γ-crystallin aggregates.

Some of these results have been confirmed by filtration experiments (Table 4). The native α- and γ-crystallin give typical A280 profiles after filtration with a 0.2-μm filter. Mixing the two crystallins and filtering also gives approximately the expected values. γm produced after 2 hours of oxidation shows approximately the expected loss of A280 after filtration, and the oxidation of α- and γ-crystallin together give a value only 10% lower than a mixture of native α- and γ-crystallin. Thus, the relative quantities of αm and γm detected by HPLC at 214 nm generally agree with the filtration data. The level of γm detected when α-crystallin is present is much higher than in its absence.

### TABLE 4. Effect of Oxidation on Crystallin Solubility

<table>
<thead>
<tr>
<th>Native</th>
<th>Oxidized</th>
<th>A280 nm</th>
<th>% γ loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>0.158 ± 0.005</td>
<td>0.160 ± 0.005</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>0.336 ± 0.010</td>
<td>0.282 ± 0.007</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>0.524 ± 0.010</td>
<td>0.471 ± 0.008</td>
</tr>
</tbody>
</table>

The samples were treated as described in Table 4. Oxidation was carried out for 2 hours. After filtration, the supernatants were analyzed with a Beckman DU-70 spectrophotometer at 280 nm.
of γ-crystallin and the aggregation of the denatured γ-crystallin. The inability of 1:1 ratios of αn:γ-crystallin to protect completely against oxidatively induced aggregation suggests a weakening of the α–γ-crystallin binding with oxidation in comparison to heat-induced insult. That the binding of the α–γ-crystallin complex is weaker in the oxidative situation is further supported by HPLC experiments. Whereas the α–γ-crystallin complex is easily isolated by HPLC fractionation of the products resulting from heat insult, with oxidative stress, such complexes cannot be found. No change in the elution positions of the oxidized α- and γ-crystallin components are observed.

And yet, in spite of a presumed weaker interaction, there is a greater stability of the α–γ-crystallin complex under the oxidative conditions than is found with heat-induced stress based on light scattering results. This may be due to experimental conditions such as the elevated temperatures used for heat insult in comparison to 37°C in the oxidative stress experiments. Of course, it may also be caused by the oxidatively produced modifications.

Although initially there is effective protection against γ-crystallin aggregation when α-crystallin is present during oxidative stress, with time, gradually increasing scatter is observed. Such observations suggest that a similar, but slower, mechanism of aggregation is operative with oxidative stress, as is found with thermal insult. However, the data suggest that it is γ-crystallin that oxidative stress takes out of solution rather than the α–γ-crystallin complex. The chaperone interaction may also facilitate covalent linkage in the complex as a result of further oxidatively induced reactions.

Recently, it was reported that α-crystallin binds denatured γ-crystallin in a central cavity of the α-crystallin aggregate. This conclusion is based on electron microscopic analyses of preparations containing α–γ-crystallin complexes visualized by immunochemical staining with the secondary antibody linked to 5-nm gold particles. It is unlikely that such binding would result in the stoichiometry observed in this and other studies.

Although it is not possible to estimate the volume of the central cavity, it is improbable that more than a few γ-crystallin polypeptides could reside in the α-crystallin macromolecule of 800 kD, which contains approximately 40 monomers. Thus, the two sets of data differ by more than 10-fold. It is improbable that the low binding ratio suggested by the immunochemical experiments could explain a major chaperone role for α-crystallin under physiological conditions unless the chaperone activity caused a return to the native conformation of the bound denatured protein. Observations indicating insolubilization of the complex makes this possibility less likely.

Key Words
hydrogen peroxide, chaperone, thiol oxidation, thermal denaturation, oxidative stress

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α-Crystallin Chaperone Activity Under Oxidative Stress