Preferential Interaction of Alpha Crystallin With Denatured Forms of Gamma Crystallin

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Purpose. To characterize the possible interaction of alpha crystallin with partially denatured forms of gamma crystallin.

Methods. Gamma crystallin was denatured in the presence of guanidine hydrochloride, then dialyzed in the presence or absence of alpha crystallin. The high-molecular-weight complex formed in the presence of alpha was characterized by gel filtration chromatography, electron microscopy, and quantitative Western blot analysis.

Results. Relative to native alpha or reconstituted aggregates of purified alpha, the higher molecular weight complex possessed a greater mean diameter and contained increased amounts of gamma crystallin.

Conclusions. Alpha crystallin preferentially interacts with partially denatured forms of a lens protein, consistent with its putative role as a functional molecular chaperone in the intact lens.

MATERIALS AND METHODS

Newborn bovine lenses were obtained from Antech, Inc. (Tyler, TX), and were stored at −75°C until use. In the authors' opinion, methods for obtaining tissue adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Alpha and gamma crystallins were purified from the water-soluble fraction of the lens cortex as described previously, using a TSK G3000SW column (Perkin-Elmer, Norwalk, CT). The amount of protein in each fraction was determined according to the BCA method (Pierce Chemical Co., Rockford, IL), using bovine serum albumin as standard. After dialysis and lyophilization, the gamma crystallin fraction was reduced and carbox-
yamidated (RCA) in the presence of 7 M guanidine hydrochloride, followed by dialysis and lyophilization. To determine the possible interaction of alpha with different forms of gamma crystallin, 1.80 mg alpha was mixed with 0.36 mg native or RCA gamma in 0.50 ml solution containing 6.0 M guanidine hydrochloride, 10 mM Tris-HCl, pH 7.4. After incubation at 22°C for 15 minutes, the solution was dialyzed for 30 to 36 hours at 4°C against a solution containing 10 mM Tris-HCl, pH 7.4, with several changes of the buffer, then dialyzed for 4 to 6 hours against TSK buffer (0.06 M sodium phosphate, 0.1 M sodium sulfate, pH 7.0). The dialysate was centrifuged at 10,000g for 5 minutes, and 50% of the supernatant was injected into a Biosep S4000 gel permeation column (300 mm X 7.8 mm, Phenomenex, Torrance, CA). Proteins were resolved at a flow rate of 0.5 ml/min, using TSK buffer.

The major peaks were collected, and 5% of the material was precipitated in acetone to remove salts, then dissolved in sample buffer and analyzed by Western blot analysis using 15% (v/v) polyacrylamide gel electrophoresis. For the purposes of quantitation, known amounts of alpha crystallins, gamma crystallins, or RCA gamma crystallins were resolved at the same time. The blots were probed with rabbit polyclonal antisera made against human alpha and bovine gamma crystallins, which were furnished by Dr. S. Zigler, National Eye Institute. After binding of radioiodinated protein A, protein bands of the resulting autoradiograph were quantitated using scanning densitometry.

Approximately 10% of the remaining material from the S-4000 column was applied to Formvar- and carbon-coated copper grids, stained with uranyl acetate, exposed to osmium tetroxide vapor, and visualized by electron microscopy as previously described. (11) Statistical analysis of aggregate diameters was carried out according to the Mann-Whitney test, using the Number Cruncher Statistical Package (J.L. Hintze, Kaysville, UT).

RESULTS

Figure 1 shows the profiles of various mixtures of alpha and gamma crystallins, after their dialysis and resolution using a Biosep S4000 gel permeation column. Figure 1A shows the elution times for a mixture of alpha and gamma crystallins that was not subjected to prior denaturation with guanidine hydrochloride. The 15.6-minute and 21.6-minute elution times represent the peaks for native alpha and native gamma crystallins, respectively. Guanidine hydrochloride treatment and dialysis of alpha alone (Fig. 1B), followed by S-4000 chromatography results in a peak eluting at a similar time (15.4 minutes).

Denaturation of the binding protein by guanidine hydrochloride, followed by dialysis or dilution to renatur-
turation. In Figure 1C, alpha crystallins and unmodified gamma crystallins were incubated in the presence of 6.0 M guanidine hydrochloride, followed by dialysis and S-4000 column chromatography. Compared with the 15.6-minute peak for native alpha in Figure 1A, guanidine hydrochloride treatment results in an earlier eluting peak (13.5 minutes), termed the high-molecular-weight aggregate (HMWA) peak. Also present is a smaller peak eluting at 15.5 minutes, which probably represents uncomplexed alpha, and is termed the native alpha peak.

In Figure 1D, the gamma preparation was first reduced and carboxamidated before dialysis, to ensure that complete renaturation was blocked. Under these conditions, the HMWA eluting at 13.6 minutes was also present when the dialysate was resolved by S-4000 chromatography.

Figure 1E shows that guanidine hydrochloride treatment of gamma alone, followed by dialysis, does not result in the HMA peak. The absence of a detectable peak represents the probable precipitation of gamma. Identical results were obtained for RCA gamma (results not shown). The lack of a gamma or HMWA peak in Figure 1E reflects the absence of alpha, which binds to gamma and prevents precipitation during the dialysis renaturation procedure.

Together, the results shown in Figure 1 suggest that after denaturation and partial renaturation in the presence of alpha, some of the gamma may bind to alpha crystallins, preventing its precipitation, and producing an alpha–gamma aggregate of higher molecular weight.

To verify the presence of these higher molecular weight aggregates, electron microscopy was used to compare their size with aggregates from native alpha. Negative staining (Fig. 2) shows the presence of particles that could be measured and quantitated as shown in the histogram in Figure 3. Relative to native alpha or reconstituted alpha alone (Fig. 3, A and B), guanidine hydrochloride treatment and dialysis of alpha in the presence of gamma (Fig. 3C) or RCA gamma (Fig. 3D) produced aggregates with a greater range of size, resulting in a larger mean value (12.01 ± 3.66 in Fig. 3C; 11.98 ± 3.51 in Fig. 3D) as compared with 9.53 ± 2.31 in Figure 3A and 10.6 ± 3.07 in Figure 3B. Statistical analysis using the Mann-Whitney test demonstrated that the populations of aggregates shown in Figure 3C and Figure 3D were significantly larger than those shown in Figure 3B (P = 0.009).

To characterize the protein composition of the HMWA plus native alpha peaks versus the peaks from native or reconstituted alpha, the peaks from S-4000 chromatography were collected, followed by determination of alpha and gamma crystallin by using antisera to gamma and alpha crystallin. Figure 4 shows the results of Western blot analysis, which was used to quantitate the amounts of alpha and gamma crystallin as shown in Table 1. The HMWA plus native alpha peaks from Figures 1C and 1D contain significant amounts of gamma crystallin (326 ± 24 μg and 328 ± 12 μg, respectively), when compared with the amounts of gamma found in the major alpha peak obtained from reconstituted alpha alone (3.4 ± 0.3 μg) or from native alpha incubated with native gamma (20.4 ± 3.0 μg). The amount of gamma crystallins present in the HMWA plus native alpha peaks accounts for approximately 90% of the material added to the original guanidine-hydrochloride–treated mixture, demonstrating that almost all the gamma has remained in solution by being complexed with alpha.

**DISCUSSION**

Recent studies using an in vitro assay have demonstrated that the alpha crystallins are able to protect other proteins from heat-induced denaturation and aggregation. Recent studies using an in vitro assay have demonstrated that the alpha crystallins are able to protect other proteins from heat-induced denaturation and aggregation. This observation suggests that in the intact lens, one of the major functions of alpha crystallins is to protect lens polypeptides against the extensive amounts of denaturation that could eventually result in cataractogenesis. Based on studies of molecular chaperones from other cell types, the mechanism of this protection must involve direct interaction of alpha with the partially denatured lens protein.

To test this hypothesis, we characterized the binding of alpha crystallin to native versus denatured forms of the gamma crystallin. We dissolved both alpha crystallin and gamma crystallin in 6.0 M guanidine hydrochloride, then renatured them by dialysis. The results in Figures 1 to 3 clearly show that guanidine hydrochloride treatment and subsequent dialysis of a mixture of alpha and gamma crystallin results in the production of larger aggregates than those obtained after identical treatment of alpha alone.

The results suggest that formation of the high-molecular-weight aggregates is due to the preferential binding of alpha to denatured forms of gamma crystallin, to produce a supramolecular complex containing both alpha and partially denatured forms of gamma crystallin. This conclusion is supported by the results of Table 1, which demonstrate that the high-molecular-weight aggregates contain much larger amounts of gamma or RCA gamma than do the aggregates of native alpha or alpha reconstituted in the absence of gamma. Using identical conditions to denature and renature gamma crystallin in the presence of alpha, we recently showed that gamma preferentially binds to the central region of the alpha aggregate. Because binding to the central region of the aggregate has been hypothesized to be a common characteristic of molecular chaperones, these results suggest that the alpha–gamma interaction is not due to nonspecific aggregation but rather is the result of a specific interaction between partially denatured forms of gamma and the chaperone-binding site of alpha crystallin.
FIGURE 2. Electron microscopy of aggregate peaks from S-4000 chromatography. See Materials and Methods for details of electron microscopy. (A) Alpha crystallin peak from alpha plus gamma, no guanidine hydrochloride treatment; (B) alpha alone, guanidine hydrochloride treatment plus dialysis; (C) HMWA plus native alpha peaks, alpha plus gamma, guanidine hydrochloride treatment plus dialysis; (D) HMWA plus native alpha peaks, alpha plus RCA gamma, guanidine hydrochloride treatment plus dialysis. The inserted bar in panel (D) represents the distance of 50 nm.

Because the gamma crystallins contain an unusually large number of cysteine and half-cystine residues, reduced alkylation of these amino acids with iodoacetamide would be expected to result in forms of gamma that would not completely renature under any condition. The observation that alpha binds to the same amounts of unalkylated versus RCA gamma after guanidine hydrochloride treatment supports the conclusion that alpha is indeed preferentially recognizing denatured forms of the gamma crystallin structure. This conclusion is consistent with the results of another recent study, which showed that alpha can interact directly with the enzyme carbonic anhydrase after its denaturation by heat.17

It should be realized, however, that the amount of gamma crystallin that binds alpha crystallin after guanidine hydrochloride denaturation and dialysis will depend on several parameters. These include the weight ratio of alpha to gamma, the concentration of the denaturant, and the identity of the protein being denatured. For example, it was previously shown that alpha crystallin facilitated the renaturation of gamma crystallin to a conformation similar to that of native protein.18 Consistent with these earlier findings, we
have found that after guanidine hydrochloride treatment and dialysis, only about 20% to 30% of gamma binds to alpha, whereas the rest is eluted in the uncomplexed form (results not shown).

Previous studies have demonstrated the presence of increased amounts of high-molecular-weight aggregate material in the aging and cataractous lens. Some of this material could be the result of alpha binding to partially denatured forms of the other lens crystallins. Based on the results of this report, we hypothesize that during aging, the large amounts of alpha present in lens fiber cells are necessary for the purpose of binding to and preventing further denaturation of lens proteins. Previous studies of other molecular chaperones have demonstrated that their binding to denatured forms of proteins can be reversed in the presence of adenosine triphosphate. Based on the results of our in vitro binding studies, most, if not all, of the binding between alpha and the denatured protein cannot be reversed with the addition of adenosine triphosphate (results not shown). If a similar situation exists in the intact lens then the relatively large amounts of free alpha found in the water-soluble fraction of young lenses may over the lifetime of the organism be irreversibly complexed with partially denatured proteins produced during the aging process. This pos-

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<thead>
<tr>
<th>Sample</th>
<th>Alpha (ug)</th>
<th>Gamma (ug)</th>
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<tbody>
<tr>
<td>Native alpha + native gamma</td>
<td>1396 ± 46</td>
<td>20.4 ± 3.0</td>
</tr>
<tr>
<td>Guanidine-treated alpha</td>
<td>1446 ± 46</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>Guanidine-treated alpha + gamma</td>
<td>1456 ± 120</td>
<td>326 ± 24</td>
</tr>
<tr>
<td>Guanidine-treated alpha + RCA gamma</td>
<td>1376 ± 114</td>
<td>328 ± 12</td>
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*Average of three separate determinations ± SD. Values normalized to starting amounts of alpha and gamma crystallin.
sibility is consistent with the known, age-dependent decrease of alpha crystallin in the water-soluble fraction of the lens homogenate. As a result of this binding and insolubilization process, the aged lens contains much lower amounts of free alpha, resulting in a diminished ability of the lens to prevent further denaturation of its proteins.

Key Words
alpha crystallin, molecular chaperone interaction

References