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Biochemistry

α-Crystallin can function as a molecular chaperone

(aggregation/heat shock/renaturation/eye lens proteins)

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ABSTRACT **The α-crystallins (αA and αB) are major lens structural proteins of the vertebrate eye that are related to the small heat shock protein family. In addition, crystallins (especially αB) are found in many cells and organs outside the lens, and αB is overexpressed in several neurological disorders and in cell lines under stress conditions. Here I show that α-crystallin can function as a molecular chaperone. Stoichiometric amounts of αA and αB suppress thermally induced aggregation of various enzymes. In particular, α-crystallin is very efficient in suppressing the thermally induced aggregation of β- and γ-crystallins, the two other major mammalian structural lens proteins. α-Crystallin was also effective in preventing aggregation and in refolding guanidine hydrochloride-denatured γ-crystallin, as judged by circular dichroism spectroscopy. My results thus indicate that α-crystallin refracts light and protects proteins from aggregation in the transparent eye lens and that in nonlens cells α-crystallin may have other functions in addition to its capacity to suppress aggregation of proteins.**

α-Crystallin is one of the major lens structural proteins in the vertebrate eye. In mammalian lenses, α-crystallin can reach levels of ≈50% of the total lens structural protein mass (1). There are two genes in the α-crystallin family, one for αA-crystallin and one for αB-crystallin. Studies on the structure of the α gene in various animals prove it to be highly conserved (1, 2). In humans, the α-crystallin gene, which is located on chromosome 21, encodes a polypeptide of 173 residues. The αB gene, which is located on chromosome 11, encodes a 175-residue polypeptide. There is ≈58% sequence homology between αA and αB. αA- and αB-crystallins always occur as heterogeneous high molecular weight aggregates. The range of molecular weights observed can vary from >1,200,000 to 300,000 with an average molecular weight of ≈800,000 in many species. Ingolia and Craig (3) made the seminal discovery that the small heat shock proteins in *Drosophila* are related to mammalian α-crystallin. More recently, another protein, the major egg antigen from *Schistosoma mansoni*, was found to have homologies to heat shock proteins and α-crystallins (4). The findings suggest that α-crystallin originated from the small heat shock protein family (5). Until about 1985, it was generally accepted that the α-crystallins were lens-specific. However, since that time, evidence has accumulated that α-crystallins exist in many nonlenticular tissues (6, 7). The occurrence of αB has been demonstrated in a multitude of cells and tissues including heart, lung, spinal cord, skin, muscle, brain, kidney, and retina (8–10). Interestingly, αB-crystallin has been associated with several neurological disorders. It accumulates in Rosenthal fibers in Alexander disease (10), in Lewy bodies in diffuse Lewy body disease (11), and in reactive glia in Creutzfeldt–Jakob disease (12). αB-Crystallin gene expression has been observed in scrapie-infected hamster brain cells (13), in NIH 3T3 mouse fibroblasts expressing Ha-*ras* and v-*mos* oncogenes (14), and in fibroblasts from patients with Werner syndrome (15). Recent results that αB-crystallin can be induced by heat stress (16) or hypertonic stress (17) are consistent with αB-crystallin being a member of the small heat shock protein family. Since only αB-crystallin was found in nonlenticular tissues and cells, it was commonly accepted that αA-crystallin is lens-specific. However, recent evidence shows that αA is present in spleen and thymus (18). α-Crystallin, therefore, can no longer be considered lens-specific.

A major unanswered question is "What is the function of α-crystallin?" In the eye lens, the vast amount of α-crystallin makes it clear that its functional role is to be part of the refractive element mass that is needed to produce the necessary refractive index. Does it have another nonrefractive function in the lens? What is the function of α-crystallin in other tissues? The normal distribution of α-crystallin in many cell types, its occurrence in neurological diseases, and its increased expression under stress conditions suggest that it must play an important role in cell function.

In this report, I present data showing that α-crystallin functions as a molecular chaperone.

MATERIALS AND METHODS

Enzymes. All enzymes were obtained from Sigma.

Preparation of Lens Crystallin Fractions. Bovine α-, β_H-, β_L-, γ_S-, and γ-crystallin fractions were prepared as follows. Briefly, freshly excised bovine eyes were obtained from a local slaughterhouse. The lenses were removed and separated into nucleus and cortex. The cortical material was homogenized in 20 mM Tris·HCl (pH 7.6) containing 0.1 M NaCl and 1 mM NaN_3. A water-soluble fraction was obtained by centrifuging the homogenate in a Beckman model J2-21 at 27,000 × *g* for 20 min at 4°C. The supernatant containing the soluble crystallins was then fractionated by gel filtration in a Sephacryl S-200 (Pharmacia) high-resolution column (2.5 cm × 100 cm). The fraction containing α-crystallin was eluted in the void volume. Distinct peaks corresponding to the β_H, β_L, γ_S, and γ fractions were collected separately. Further purification of α- and γ-crystallin fractions was achieved by rechromatographing these fractions on a Pharmacia FPLC system using a Superose 6 HR 10/30 prepacked column. The buffer used was 50 mM sodium phosphate, pH 7.4/0.1 M NaCl. Densitometric analysis of the α-crystallin fraction after SDS/polyacrylamide gel electrophoresis showed it to be composed of αA and αB at a ratio of 3:1. αA and αB were separated and purified by using isoelectric focusing in the presence of urea. A Bio-Rad Rotofor preparative isoelectric focusing cell was used.

Aggregation Measurements. The aggregation of the various proteins upon heat denaturation was determined by measuring the apparent absorption due to scattering at 360 nm in a Beckman DU 70 spectrophotometer equipped with a six-cell-holder accessory and a Peltier temperature controller accessory. The temperature of the samples in the cells was measured by inserting a small bead thermocouple inside one of the six cells in the holder. Cells used were Teflon-

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stoppered, black-walled, and 2 mm wide with a 10-mm pathlength. In each experiment, the total volume in the cell was 400 μl. The scattering in each cell was recorded automatically every 20 sec. Proteins and buffers were mixed in the cell at room temperature. The average time for the mixture in the cell to reach the predetermined temperature of the cell holder was ≈5 min.

Denaturation/Renaturation Studies of γ-Crystallin With and Without α-Crystallin. γ-Crystallins, containing the native mixture of γ_I–γ_{IV} that was obtained from the S-200 column, were denatured by adding 3.5 mg of protein (in 0.1 ml of Tris buffer) to 0.7 ml of 6 M guanidine hydrochloride. The mixture was incubated at room temperature for 1 hr and then dialyzed against 1 liter of the Tris buffer containing 1 mM dithiothreitol at 4°C for 8 hr or overnight, with several changes of buffer. The dialyzed solution was then centrifuged at 18,000 × *g* for 20 min at 4°C, and the supernatant was used for further experiments. To study the effects of α-crystallin on renaturation, the same amount of γ-crystallin was first denatured with guanidine hydrochloride (final concentration, 5.25 M). After incubation, 55 mg of α-crystallin in 0.84 ml of Tris buffer was added to the denatured γ-crystallins and the solution was dialyzed and processed as described above. α- and γ-crystallins were separated by chromatography on an HR-6 column with the FPLC system, as described above.

RESULTS

Recently, the molecular chaperone GroEL from *Escherichia coli* was shown to facilitate the refolding of citrate synthase by suppressing aggregation (19). Subsequently, GroEL was shown to be very efficient in suppressing the thermally induced aggregation of α-glucosidase (20). I therefore decided to use α-glucosidase in my preliminary experiments. Titration of α-crystallin with a solution of α-glucosidase heated to 48°C showed that α-crystallin was as effective as GroEL in the suppression of aggregation (data not shown). The kinetic behavior of the system was essentially similar to that published for the α-glucosidase–GroEL system (see figure 2C in ref. 20). As was observed for GroEL, substoichiometric amounts of α-crystallin slowed down the rate of aggregation but did not suppress it, whereas a stoichiometric amount or a small molar excess was sufficient to suppress aggregation.

To determine whether the protective effects of α-crystallin could be extended to other enzymes, eight additional enzymes were tested. Typical results are shown below for two of the enzymes tested.

Thermal Aggregation of Alcohol Dehydrogenase. Solutions of horse liver alcohol dehydrogenase started to aggregate at temperatures >39°C. The kinetics of aggregation at 48°C is shown in Fig. 1. Within 5 min, the scattering started to increase rapidly (Fig. 1, curve 1). The addition of α-crystallin significantly reduced the aggregation (Fig. 1, curves 2–4). By using a native molecular weight of 80,000 for alcohol dehydrogenase and a molecular weight of ≈360,000 for α-crystallin at 48°C (21), an alcohol dehydrogenase to α-crystallin molar ratio of 15:1 significantly reduced the aggregation rate (Fig. 1, curve 2). At an alcohol dehydrogenase to α-crystallin molar ratio of 5:1 the aggregation was almost completely suppressed (Fig. 1, curve 3). At an alcohol dehydrogenase to α-crystallin molar ratio of ≈1:1, there was no evidence of any aggregation (Fig. 1, curve 4). The addition of bovine serum albumin, ovalbumin, or β- or γ-crystallin at the same or even higher concentrations failed to protect alcohol dehydrogenase from aggregation (data not shown).

Thermal Aggregation of Other Enzymes and Crystallins. Other enzymes treated similarly were phosphoglucose isomerase, glutathione *S*-transferase, enolase, aldolase, lactate dehydrogenase, citrate synthase, and carbonic anhydrase. The kinetics for heat denaturation and aggregation for all enzymes were similar to that shown in Figs. 1 and 2, and for all enzymes, the addition of stoichiometric amounts of α-crystallin resulted in the suppression of thermal aggregation (data not shown).

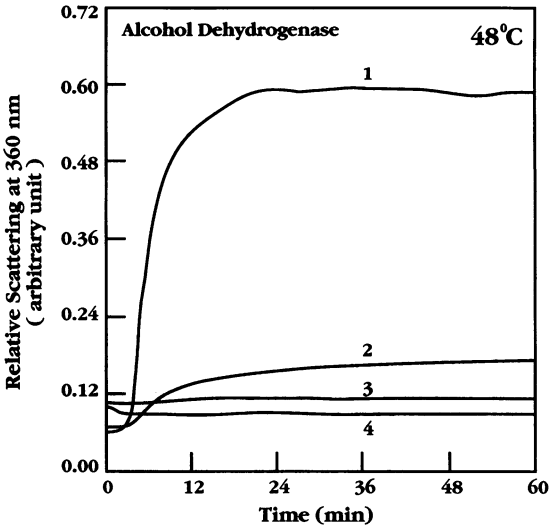

FIG. 1. Aggregation of alcohol dehydrogenase at 48°C in the absence and presence of α-crystallin. In each experiment, 0.13 mg of alcohol dehydrogenase (horse liver) in 50 mM sodium phosphate (pH 7) was used with the following additions: Curves 1, none; 2, plus 0.04 mg of α-crystallin; 3, plus 0.12 mg of α-crystallin; 4, plus 0.55 mg of α-crystallin. The final volume of each reaction mixture was 0.4 ml and the pathlength was 10 mm.

Since α-crystallins were effective in the suppression of thermal aggregation of the various enzymes, I tested the effects of α-crystallin on the other major lens structural proteins (the β-crystallins and the γ-crystallins) and on a native mixture of soluble bovine crystallins. α-, β-, and γ-crystallins are known to be relatively thermally stable (21–29). Thus, higher temperatures were used for the induction of aggregation.

The Effects of α-Crystallin on the Thermally Induced Aggregation of β-Crystallin. When a β_L fraction of the lens crystallin was heated to 55°C, aggregation occurred as shown in Fig. 3, curve 1. As with the enzymes, addition of α-crys-

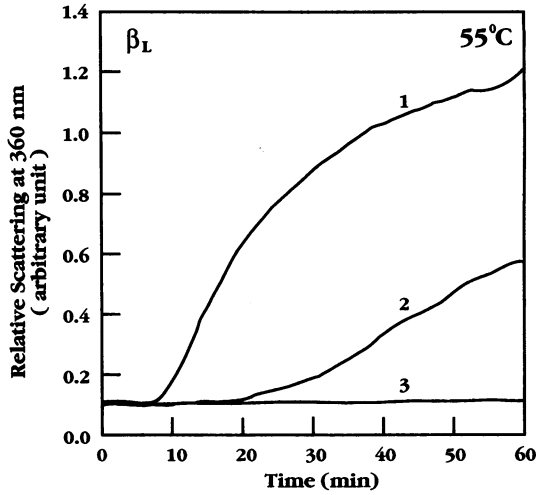

FIG. 2. Aggregation of β_L-crystallin at 55°C in absence and presence of α-crystallin. In each experiment, 0.26 mg of β_L in 50 mM sodium phosphate (pH 7) was used with the following additions: Curves: 1, none; 2, plus 0.013 mg of α-crystallin; 3, plus 0.13 mg of α-crystallin. Other conditions are as in Fig. 1.

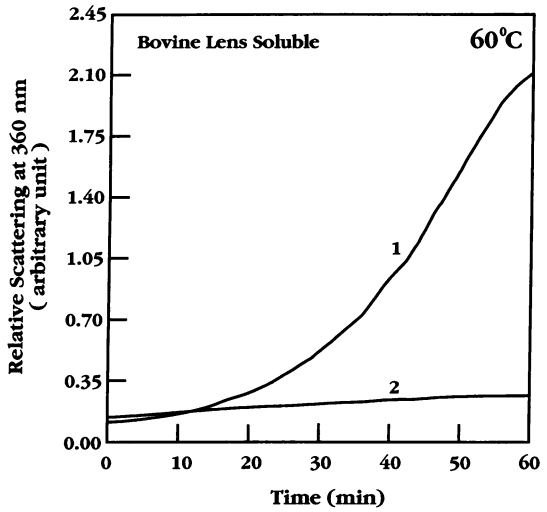

FIG. 3. Aggregation of lens total soluble fraction at 60°C after the removal of α-crystallin. Curves: 1, 1.75 mg of total lens soluble protein obtained after ultracentrifugation at 500,000 × g for 30 min in a Beckman TL-100 to selectively remove most of the α-crystallin; 2, 2 mg of native total bovine soluble fraction not centrifuged. All other conditions the same as in Fig. 1.

tallin suppressed the aggregation process. If the average native molecular weight of β_L was ≈60,000 and of α-crystallin was 360,000, then a β_H- to α-crystallin molar ratio of 120:1 produced a significant delay the aggregation process and lowered the rate of aggregate formation. With a β_L- to α-crystallin molar ratio of 12:1, aggregation was completely suppressed (Fig. 2, curve 3).

α-Crystallin obtained from whole bovine lens or from many other mammalian lenses is a ≈3:1 mixture of αA and αB. To determine whether αA and αB separately are effective in the suppression of thermal aggregation, purified αA and αB were prepared. Both αA and αB were effective in suppressing the thermal aggregation of β_L (data not shown).

The Effects of α-Crystallin in the Suppression of Thermal Aggregation of Unfractionated Total Lens Soluble Proteins. To test whether α-crystallin acts as a molecular chaperone on a native mixture of lens proteins, experiments were conducted on a total soluble protein fraction of a bovine lens homogenate consisting of α, β_H, β_L, γ_S, and γ fractions and all other low molecular weight soluble components that are native to the lens. When such a mixture was heated to 60°C, relatively little aggregation and scattering occurred (Fig. 3, curve 2). However, the selective removal of α-crystallin by ultracentrifugation without dilution or fractionation of the sample caused the remaining β- and γ-crystallins to aggregate (Fig. 3, curve 1). Adding back α-crystallin to a freshly ultracentrifuged supernatant fraction reversed the effect and suppressed the aggregation at 60°C (data not shown).

Thermal Aggregation and Denaturation/Renaturation of γ-Crystallin in Guanidine Hydrochloride. When solutions of γ-crystallins (containing essentially γ_I–γ_{IV}) were heated to 66°C, the kinetics of aggregation were similar to that obtained for β_L (see Fig. 2). α-Crystallin was as efficient in suppression of the γ-crystallin heat aggregation as it was with the β_L-crystallin (data not shown).

The possibility that α-crystallin can assist in the renaturation of denatured proteins was tested with guanidine hydrochloride-denatured γ-crystallin. At >5 M guanidine hydrochloride, γ-crystallin denatures irreversibly (22). When an aliquot of γ-crystallin was incubated at room temperature for 1 hr in 5.25 M guanidine hydrochloride, after dialysis, >80% of the total protein precipitated and was irreversibly denatured. The process of precipitation began within 10–20 min with the commencement of the dialysis. If, however, α-crystallin was added to the denatured γ-crystallin after exhaustive dialysis, ≈95% of the total protein remained in solution. To determine whether the refolded γ-crystallin assumed its native conformation, the γ-crystallin was separated from the α-crystallin and subjected to circular dichroism (CD) spectroscopy. Fig. 4 shows the far- and near-UV CD spectra of native and renatured γ-crystallin. No significant differences were observed between the native (curve 1) and renatured (curve 2) γ-crystallin spectra.

The far UV region (Fig. 4*A*) reflects the secondary structure of γ-crystallin, which is known to be mainly in a β-pleated sheet conformation (30). The near UV spectrum (Fig. 4*B*) reflects mainly the contribution of the aromatic amino acids to the tertiary structure and is very sensitive to various perturbations. The far and near UV spectra of γ-crystallin in ≥5 M guanidine hydrochloride (data not shown in Fig. 4) have been published (22, 24). In the far UV, the 218-nm minimum was totally lost, and a new minimum was

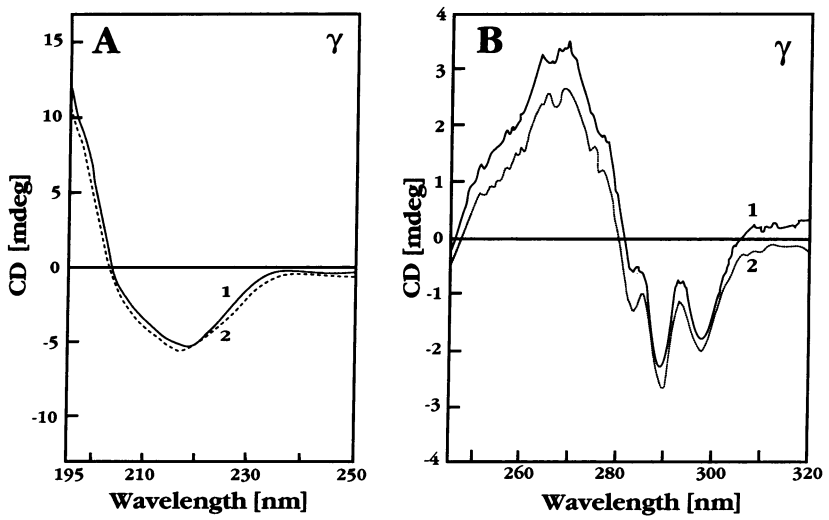

FIG. 4. Far- and near-UV CD spectra of native γ-crystallin fraction and of a γ fraction that was denatured in 5.25 M guanidine hydrochloride and renatured in the presence of α-crystallin. (*A*) Far-UV CD spectra. Curves: 1, native γ fraction; 2, γ fraction after renaturation. The spectra represent averaging of eight scans; pathlength was 0.25 mm. (*B*) Near-UV CD spectra. Curves: 1, native γ fraction; 2, γ fraction after renaturation. The spectra represent averaging of eight scans; pathlength was 10 mm. The absorbance for each sample at 280 nm was 0.6. A Jasco model 600 spectropolarimeter was used. mdeg, millidegree.

observed at 208 nm, suggesting a complete loss of the secondary structure (24). The near UV spectra was also markedly different from that shown in Fig. 4B (22, 24). The reappearance of these CD spectra after dialysis in the presence of α-crystallins provides evidence that the secondary and tertiary structures of the refolded γ-crystallin are similar to those of the native γ-crystallin.

DISCUSSION

Molecular chaperones consist of many classes of proteins that can assist in a multitude of tasks, such as stabilization of native protein conformations, protein folding, mediation and stabilization of correct oligomeric assembly of proteins, translocation of proteins, and protection of proteins from heat denaturation and other stresses (31–34). The present experiments have established that α-crystallin can function as a molecular chaperone. This is consistent with previous studies showing that α-crystallin is related to the small heat shock protein hsp27 (3) and that αB-crystallin is induced by heat (16) and osmotic stress (17). Whereas, in my experiments, α-crystallin by itself was an effective chaperone, it has been shown (35) that the folding process of newly synthesized proteins is a cascade reaction involving three chaperones, DnaK, DnaJ, and GroEL. It remains to be seen whether α-crystallin also acts with other proteins and chaperones in the various cells where it is found.

The molecular mechanisms by which chaperones interact with unfolded proteins are not known. α-Crystallin, which is relatively well-characterized and can be easily purified in relatively large quantities, may be a prime candidate to serve as a model for studying the molecular mechanisms of chaperone functions. Our experiments show that α-crystallin and the *E. coli* GroEL molecular chaperone can similarly suppress thermally induced aggregation of various proteins (20). As with GroEL, the kinetics by which α-crystallin protects from thermal aggregation are dependent upon the incubation temperature employed and the rate of heating, among other parameters. I have not yet analyzed these kinetics but have shown that α-crystallin protects the proteins from thermal aggregation in a saturable manner. In calculating the molar ratios of the various proteins to α-crystallin, I have used a molecular weight value of ≈360,000 for α-crystallin, which is about the lowest value predicted under my experimental conditions (21, 36). Other studies have calculated molecular weight values of >350,000 for α-crystallin at temperatures >45°C (28, 37). My calculations show that, at an approximately stoichiometric concentration of enzymes to α-crystallin, heat aggregation is completely suppressed. This was the case with GroEL and α-glucosidase (20). Under physiological conditions, α-crystallin is always isolated as a heterogeneous multisubunit high molecular weight complex. This raises the following questions: Does the chaperone activity of α-crystallin depend on the size of the complex? Is there a minimum numbers of α-crystallin subunit complexes that can function as a molecular chaperone? Is it possible that, under certain conditions, monomeric α-crystallin polypeptides can act as molecular chaperones? Which domains in the α-crystallin molecule are responsible for the chaperone activity? All these questions await further experimentation.

Interestingly, α-crystallin is more efficient in suppressing the heat-induced aggregation of β- and γ-crystallin than suppressing the aggregation of the enzymes tested. The β-crystallins are multimeric aggregates of M_r 50,000–200,000. By taking a low molecular weight value of ≈60,000, I estimate that, at a β/α molar ratio of 12:1, heat-induced aggregation is completely suppressed. For γ-crystallins, which are monomers of M_r ≈20,000, a γ/α molar ratio of 10:1 also completely suppressed the heat-induced aggregation (data not shown). My preliminary studies show that both αA and αB are similar in their ability to suppress aggregation. Although αA and αB have related amino acid sequences and possess similar structural properties (36, 38), they vary significantly in other properties such as tissue specificity and phosphorylation; in the lens, they exhibit different spatial and temporal distributions. Thus, αA and αB in various cell types may have different chaperone functions, may protect different proteins, or may be maximally active under different conditions.

Although the addition of α-crystallin suppressed aggregation, it did not protect α-glucosidase and alcohol dehydrogenase from heat inactivation (data not shown). This was also the case with GroEL and α-glucosidase, where GroEL by itself did suppress aggregation but did not protect the enzyme from inactivation (20). The denaturation experiments of γ-crystallin provide evidence that α-crystallin assisted in the refolding processes during the removal of the denaturing agent and avoided the irreversible loss due to aggregation that occurs upon dialysis. CD measurements suggest that renatured and native γ-crystallins have similar conformations. However, since γ-crystallin cannot be assayed for any functional or enzymic activity, I cannot state with certainty that the native and refolded proteins have identical conformations and properties.

The choice of α-crystallin as one of the major constituents of the lens protein mass is a striking example of gene sharing (39, 40), whereby one gene encodes a protein with two functions. α-Crystallin is expressed at high levels in the lens where it must contribute to the refractive properties of the lens and, at the same time, can serve as a molecular chaperone, protecting other crystallins and other proteins in the lens from aggregation that will lead to scattering and loss of function (41, 42). Thus, both functions of α-crystallin are needed in the lens. Perhaps other crystallins have functions in addition to their refractive function in the lens. This even raises the possibility that heat shock protein hsp27, a relative of α-crystallin (3), has more than one function in *Drosophila*, inasmuch as *Drosophila* hsp27 appears to have multiple unidentified roles during development (43). It was recently reported that, in human skeletal muscle, the small heat shock protein HSP-28 copurifies with αB-crystallin. This suggests that there might be some specific interactions between HSP-28 and αB-crystallin (44).

α-Crystallin is known to undergo many posttranslational modifications including phosphorylation, acetylation, C-terminal degradation, deamination, and O-liked N-acetylglucosamination (45–52). The effects of all of these modifications on the function of α-crystallin in the lens and other tissues is yet to be determined. Recently, it was shown (53) in *E. coli* that, during heat shock, the chaperone GroEL becomes reversibly phosphorylated. By analogy, the phosphorylation of α-crystallin may also affect its chaperone function (as may its other modifications). In view of my present demonstration that α-crystallin may protect lens proteins from denaturation and light scattering, I propose that the age-related deterioration of α-crystallin may have a central role in the development of cataract. The various age-related posttranslational modifications of α-crystallin may interfere with its chaperone function and lead to the formation of α-crystallin aggregation and aggregates of other proteins.

The chaperone function of α-crystallin should assist in the elucidation of its diverse roles in the many nonlenticular tissue where it is found under normal conditions and in the various neurological disorders and stress conditions where it is overexpressed.

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