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The Formation of Oxidatively Induced High-Molecular-Weight Aggregate of α-/γ-Crystallins

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α-/γ-Crystallin interactions under oxidation with ascorbate–$FeCl_3$–EDTA–H_2O_2 followed by dialysis have been studied. A high-molecular-weight aggregate (HMWA) composed of α- and γ-crystallin was observed for the mixture of the dialyzed α-crystallin and the oxidized γ-crystallin through gel-filtration chromatography. This illustrates an interaction between α-crystallin and partially denatured γ-crystallin induced by oxidation. No HMWA formation was observed under the condition without dialysis and/or with the addition of catalase to the oxidized γ-crystallin prior to the addition of α-crystallin. More HMWA was formed by oxidized γ-crystallin followed by the addition of α-crystallin than by simultaneous oxidation of both α- and γ-crystallins. Conformational changes of α-crystallin during oxidation analyzed by circular dichroism spectra showed that oxidized α-crystallin can gradually be restored to an ordered structure through dialysis. The overall results imply that structural changes of both α- and γ-crystallins and dialysis are required to form HMWA. The observation of this oxidatively induced chaperone/substrate complex suggests that an efficient chaperone-like protective action against oxidative insults may exist *in vivo*.

The mammalian lens has an inordinately high protein content, of which three major lens proteins, α-, β-, and γ-crystallin, have long been recognized (1). Lens crystallins are remarkable in that they are organized in a very sophisticated manner to maintain lens transparency, and have little or no protein turnover. γ-Crystallin is a group of six homologous 21 kDa monomer proteins. α-Crystallin is a polymeric protein resulting from two closely related polypeptides, αA- and αB-crystallin, each with a molecular weight of about 20 kDa (2). Being a protein of an average mass around 800 kDa, the quaternary structure of α-crystallin remains a mystery, though many models have been proposed to meet the various experimental and theoretical data (3–8). In 1982, Ingolia and Craig found that α-crystallin shares 40% sequence homology with *Drosophila* small heat shock protein (9). α-Crystallin was known as a lens structural protein before Horwitz, in 1992, showed it capable of functioning as a molecular chaperone against thermal aggregation (10). More recently, α-crystallin, especially αB-crystallin, has been identified in other tissues throughout the body (11, 12), suggesting a putative role as a functionally important chaperone.

To date, a number of papers have shown α-crystallin acting as a molecular chaperone under conditions of heat (thermal) insult (13–15). Among these, Das *et al.* reported that α-crystallin, under heat stress, undergoes an irreversible conformational transition, with a marked increase in surface hydrophobicity, correlating well with the increase of its chaperone-like activity (14). Wang and Spector reported a stable complex of α-crystallin with γ-crystallin or β_L-crystallin when both were incubated at 70°C for various periods (16). Lee *et al.* showed that short-term preincubation of α-crystallin with its substrates at 60°C for 20 min. resulted in the formation of stable complexes between α-crystallin and its substrates, and the chaperone activity is greatly enhanced under these conditions (17). Further, a few studies have shown that α-crystallin's chaperone-like activity protects against oxidative stress and ultraviolet irradiation (18, 19). However, in terms of maintaining lens transparency and protecting the lens from cataracts, it is necessary to consider the physiological conditions of eye. It is, in fact, physiologically impossible that the high temperatures employed in the above studies exist *in vivo,* and thus the chaperone behavior of α-crystallin arising from thermal stress is of debatable significance in terms of protecting lens transparency and preventing cataract formation.

Risks that lead to cataract development include UV-irradiation, oxidative insults, aging and physiological disorders such as diabetes (20, 21). Ramachandra reported a significant amount of H_2O_2 (30 μM) in the

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aqueous humor of the normal human eye (22). Thus, study of the chaperone-like activity of α-crystallin induced by oxidation under physiological conditions becomes more significant, yet there have been only a few studies of α-crystallin's chaperone-like protection of its substrates under oxidative stress (16, 18, 23, 24). Nevertheless, it is reasonable to assume that an efficient chaperone-like protective action against oxidative insults indeed exists *in vivo.* Spector *et al.* have shown that α-crystallin can decrease the light scattering and thiol oxidation of other crystallins under condition of various oxidative stress (16). Lee *et al.* have shown that inhibition of oxidation-induced γ-crystallin aggregation is enhanced when both α- and γ-crystallin are incubated simultaneously at 60°C for 20 min (17). However, both groups failed to isolate the α-crystallin/substrate complex under the oxidative conditions employed in their studies. In this study, a high molecular weight aggregate (HMWA) composed of chaperone/substrate (α-crystallin/γ-crystallin) has been isolated in response to conditions of oxidative stress. Successful isolation of this chaperoning complex required both oxidative incubation and dialysis. Characterization of oxidized α-crystallin during dialysis by CD spectroscopy shows oxidized α-crystallin can regain its ordered structure through dialysis.

MATERIALS AND METHODS

Preparation of rat lens crystallins. Female Sprague–Dawley rats at the age of 3-4 weeks old were sacrificed by asphyxiation in a dry ice chamber. The lenses were homogenized in a pH 7.4, 50 mM Tris buffer containing 0.1% NaN_3 and then centrifuged at 17,000 *g* for 60 minutes to separate the water soluble and water nonsoluble portions. The supernatant was then applied to a Sephacryl S-300 column (600 × 26 mm) for gel permeation. The column was calibrated with high and low molecular standards purchased from Pharmacia. The isolated crystallins were pooled and dialyzed against water (4 × 1500 ml) at 4°C for 24 h, and then stored at −70°C after lyophilization. Low molecular weight α-crystallin (α_L-crystallin) and γ-crystallin, a native mixture of γ_I-γ_{IV}, were used in this study. Protein concentration was estimated by Bio-Rad protein assay of the dye-binding method.

Oxidation of rat lens crystallins. Oxidation of α-crystallin and γ-crystallin were performed with an ascorbate–$FeCl_3$–EDTA–H_2O_2 system. The oxidizing conditions were according to our previous study (18). Briefly, 1 mg of γ-crystallin was incubated for 1, 5, 10, 15, 20, 23, and 25 h at ambient temperature in 3.6 ml of pH 7.4, 50 mM Tris–HCl buffer containing 1 mM ascorbate, 0.2 mM $FeCl_3$, 0.6 mM EDTA, and 0.5 mM H_2O_2. At the end of incubation, 0.6 ml of α-crystallin (1.8 mg/ml) was added, then was dialyzed against the same buffer (4 × 1000 ml) overnight. After dialysis, this mixture was centrifuged at 9000 rpm × 30 min to remove precipitate, if any, then, was applied to a Sephacryl S-300 column for gel filtration.

Isoelectric focusing and SDS–PAGE analyses. To analyze the composites of the HMWA fraction collected from Sephacryl S-300 column chromatography, isoelectric focusing (IEF) electrophoretical analysis was performed. SDS–PAGE electrophoresis was also used to examine oxidized α- and γ-crystallins. Both electrophoreses experiments were performed by using Pharmacia LKB PhastSystem. IEF was performed on a precast IEF gel (PhastGel IEF 5-8 purchased from Pharmacia) under native conditions and SDS–PAGE electrophoresis was performed on a Gradient 8-25 electrophoresis gel purchased from Pharmacia. The gels were visualized by staining with Coomassie blue.

Circular dichroism measurement of oxidized α-crystallin. Circular dichroism (CD) spectra of α-Crystallin under the oxidative system of ascorbate (1 mM)–$FeCl_3$ (0.2 mM)–EDTA (0.6 mM)–H_2O_2 (0.5 mM) for 5 h with or without dialysis were obtained by using a JASCO J-720 spectropolarometer at room temperature. The CD spectra were the average of 3 scans. Far- and near-ultraviolet (UV) CD spectra of α-crystallin were recorded at wavelengths of 200–240 nm and of 250–340 nm, respectively. A cell pathlength of 0.1 cm and a protein concentration of 0.2 mg/ml were used for far-UV CD measurement, while for near-UV CD measurement a cell pathlength of 1 cm and a protein concentration of 1 mg/ml were used. Spectra were expressed in terms of molar ellipticity (deg · cm^2)$dmol^{-1}$. All spectra were corrected for the baseline obtained with the buffer.

RESULTS AND DISCUSSION

Formation of HMWA with the addition of α-crystallin to oxidized γ-crystallin. Gel-filtration chromatographic data regarding chaperone interaction between normal α-crystallin and oxidized γ-crystallin is shown in Fig. 1, from which it is found that α-crystallin interacts with oxidized γ-crystallin, not with native γ-crystallin, to form HMWA. This result reveals that α-crystallin is capable of protecting oxidatively insulted γ-crystallin from aggregation by forming a HMWA. Interestingly, it is found that the amount of HMWA increases as the oxidation time for γ-crystallin increases. It reaches maximum after 20-h oxidation, then decreases. The amount of γ-crystallin also decreases as the oxidation time increases without the concomitant increase of HMWA for oxidation over 20 h. These results suggest that in order to interact with α-crystallin, γ-crystallin needs to undergo a certain degree of structural alternation, under or beyond which may result in no interaction with α-crystallin or precipitation of γ-crystallin. To examine whether this HMWA resulted from the aggregation of α-crystallin or γ-crystallin alone or was a α/γ-crystallin complex, HMWA fraction was further analyzed by IEF electrophoresis. Figure 2 illustrates the results of IEF electrophoresis, in which lanes 1, 2, 3, and 4 are the characteristics of the HMWA fractions (peak 3 of Fig. 1), native γ-crystallin, native α-crystallin and pI marker, respectively. Lane 1 contains two components corresponding respectively to α-crystallin and γ-crystallin, indicating that the HMWA is composed of both α-crystallin and γ-crystallin. It has been reported that α-crystallin interacts with denatured, nonaggregated crystallin to form HMWA under conditions of thermal incubation, or of urea, or of guanidine hydrochloride incubation, to form HMWA (17, 18). However, no paper has shown a chaperone/substrate complex under oxidative stress. Since oxidation is presumed a major factor during the development of cataracts, the observation of the formation of water-soluble HMWA under oxidative insults is significant evidence demonstrating that the chaperone function of α-crystallin contributes at least partly to lens transparency.

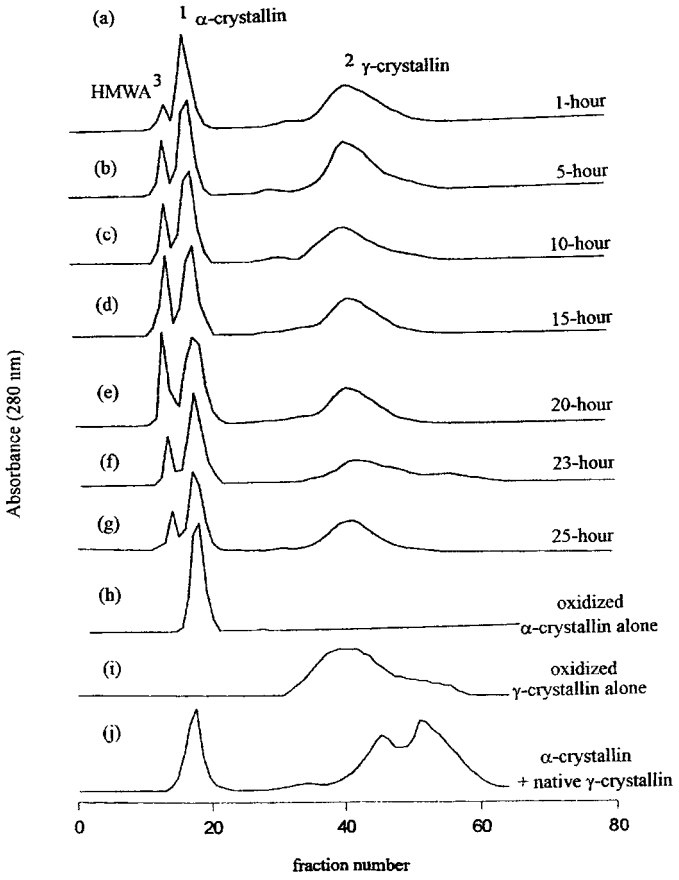

FIG. 1. The interaction of α-crystallin with oxidized γ-crystallin. Gel-filtration analyses of dialyzed α-crystallin/oxidized γ-crystallin samples. (a–g) Elution profile of dialyzed mixtures, γ-crystallin oxidized for 1, 5, 10, 15, 20, 23, 25 h, respectively. (h) 15-h-oxidation α-crystallin for comparison. (i) 15-h-oxidation γ-crystallin for comparison. (j) mixture of native γ- and α-crystallins for comparison.

Wang and Spector reported that it is very difficult to isolate oxidatively induced α-/γ-crystallin complex with the procedure used to obtain the thermally induced complex (16), despite another report showing α-crystallin to have an obvious chaperone function on its substrate via light scattering measurement under oxidative stress (17).

Here we present definite results of formation of HMWA through dialysis of oxidized γ-crystallin with native α-crystallin in an oxidation-reaction-buffer, i.e., dialysis was used as a method of incubation. Oxidation was not terminated before addition of native α-crystallin, and thus oxidants may attack α-crystallin at the beginning of dialysis, altering its structure. To study whether the existence of oxidant in the reaction buffer affects the interaction of α-crystallin with partially denatured γ-crystallin during dialysis, a solution of 50 μl of 28 U/μl catalase was added to the oxidized γ-crystallin before dialysis. After dialysis and followed by the removal of the insoluble aggregate, if any, the mixture was then subjected to the same column for gel filtration. No HMWA formation was observed under this experimental condition (chromatogram is not shown). These results firmly indicate that structural disturbance of α-crystallin is indispensable in order for α-crystallin to display its chaperone ability to form HMWA. On the other hand, it was found that more HMWA was formed by oxidized γ-crystallin followed by addition of α-crystallin to this oxidation-reaction-buffer prior to dialysis than by simultaneous oxidation of γ- and α-crystallins followed by with or without the addition of catalase prior to dialysis (data not shown). This may be due to α-crystallin suffering more severe structural alternation under simultaneous oxidation of both crystallins than under oxidation of γ-crystallin followed by the addition of α-crystallin. These results indicate that α-crystallin needs to be properly oxidized to a partially disordered form in order to form HMWA with oxidized γ-crystallin. Further experiment revealed that if catalase were added to the simultaneously-oxidized α-/γ-crystallin mixture, without dialysis, no HMWA was observed. This reveals that dialysis is a necessary process for α-crystallin to perform its chaperone-like activity. Thus, dialysis is a necessary step, indicating that the oxidation-induced chaperone behavior (mechanism) is different from that of thermally induced chaperone behavior, in which dialysis is not necessary. This also explains why Wang and Spector failed to isolate a α-crystallin/substrate complex under oxidative stress.

Recovery of an ordered structure of oxidized α-crystallin during dialysis. Less HMWA was obtained when α- and γ-crystallins were oxidized simultaneously, suggesting a structural difference for α-crystallin oxidized alone as opposed to α-crystallin oxidized together with γ-crystallin. Thus, α- and γ-crystallins, which had been oxidized simultaneously or respectively for up to 20 h, were analyzed using SDS-Page

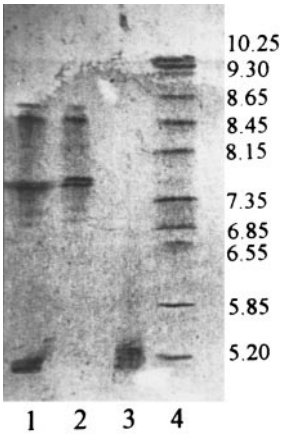

FIG. 2. IEF analysis of fractions from gel-filtration chromatography of Fig. 1. IEF profiles were obtained under native conditions. Lane 1, from peak 3 of Fig. 1; lanes 2 and 3, native γ-crystallin and α-crystallin, respectively; lane 4, pI markers.

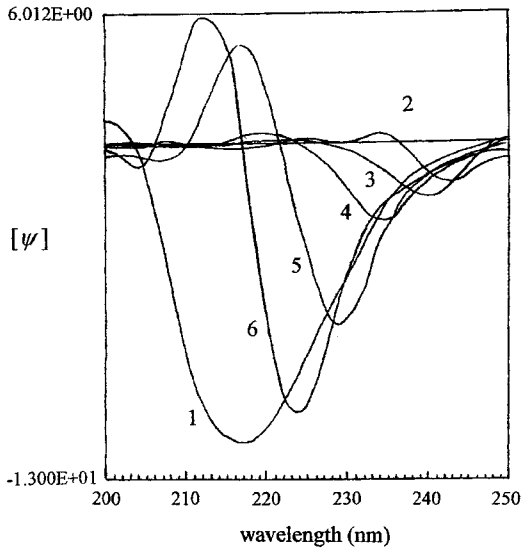

FIG. 3. Far-UV CD spectra of α-crystallin under various conditions. The oxidation system was ascorbate (1 mM)–$FeCl_3$ (0.2 mM)–EDTA (0.6 mM)–H_2O_2 (0.5 mM). Curve 1, native α-crystallin; curve 2, 5-h-oxidation α-crystallin without dialysis; curves 3–6, 5-h-oxidation α-crystallin with 1-, 4-, 8-, and 16-h dialysis, respectively.

and the results were compared with that of normal α- and γ-crystallins. It was found that neither truncation nor polymerization of oxidized crystallins was observed, and both normal and oxidized crystallins showed the same SDS–PAGE pattern (data not shown). These results suggest that when α- and γ-crystallins are oxidized simultaneously, the native structure of α-crystallin may be denatured to a degree, rendering it less capable of displaying its chaperone-like activity. Therefore, further characterization of oxidized α-crystallins by circular dichroism spectroscopy was carried out and compared the results with that of native α-crystallin. The results are shown in Figs. 3 and 4. Figure 3 shows far-ultraviolet (UV) CD spectra of α-crystallin under various conditions. Curve 1 is the far-UV CD spectrum of normal α-crystallin, in which the negative band at 218 cm^{-1} is a characteristic of α-crystallin's anti-parallel β-pleated sheet conformation (25). Curves 2, 3, 4, 5, and 6 are far-UV CD spectra of oxidized α-crystallin, dialyzed for 0, 1, 4, 8, and 16 h, respectively. The far-UV CD spectrum reflects protein secondary structure. It is evident that oxidized α-crystallin without dialysis has lost its ordered secondary structure as revealed in curve 2, showing a reduction in ellipticity and peak red-shift. Curves 3 to 5 show that the negative band gradually shifts to the shorter wavelength, from 243 to 224 cm^{-1}, implicating a gradual recovery of an ordered structure. Therefore, the ordered secondary structure was recovered during dialysis, although not completely. Figure 4 shows the near-UV region CD spectra of oxidized and normal α-crystallins with or without dialysis. Curve 1 is the near-UV CD of normal α-crystallin showing three positive bands centered at 256, 264, and 270 cm^{-1}, which is consistent with previous report (26). When oxidized for 5 h without dialysis, α-crystallin showed a significant change in its tertiary structure, as shown in curve 3, indicated by a decreased CD intensity compared to that of native α-crystallin and a loss of its fine structure. Because near-UV CD intensity is indicative of interactions between aromatic amino acid residue side chains such as tyrosine, tryptophan, and phenylalanine, a reduction in CD intensity and loss of fine structure indicate that α-crystallin is not likely to regain its native tertiary form after oxidative insult. The simultaneously oxidized α-crystallin, however, does not seem to be in the state of a randomly unfolded polypeptide, but rather in a partially folded one because, after dialysis for 16 h, the tertiary structure is significantly regained, as shown in curve 2, in which three positive bands at 262, 271, and 279 cm^{-1} are observed. CD study shows that direct oxidation of α-crystallin for 5 h results in a nearly completely denatured structure. Nevertheless, dialysis of oxidized α-crystallin is capable of significant restoration of its secondary and tertiary structures. Thus, this CD study shows that dialysis is a process whereby oxidized α-crystallin gradually regains an ordered structure, with which it interacts with oxidized γ-crystallin to form HMWA.

The polydispersive nature of α-crystallin has been noted in the studies of recombinant α-crystallin (27, 28). It is known the factors that affect the size of

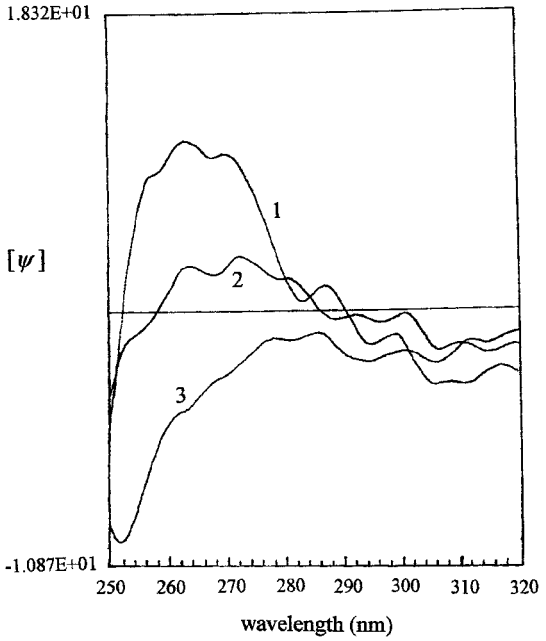

FIG. 4. Near-UV CD spectra of α-crystallin under various conditions. The oxidation system was ascorbate (1 mM)–$FeCl_3$ (0.2 mM)–EDTA (0.6 mM)–H_2O_2 (0.5 mM). Curve 1, native α-crystallin; curve 2, 5-h-oxidation α-crystallin with 16-h dialysis; curve 3, 5-h-oxidation α-crystallin without dialysis.

α-crystallin aggregates include temperature, pH and denaturants. By stepwise denaturation and renaturation of α-crystallin in urea and guanidine hydrochloride, Siezen and Bindel proposed a dissociation/re-association model for the behavior of α-crystallin under perturbation (29). Attempting to study its quaternary structure, the dissociation/re-association process was observed by varying the ambient temperature of α-crystallin, for example, from 25 to 60°C (5). At higher temperatures, from 60 to 80°C, α-crystallin's molecular weight gradually increased (30), which is believed to be due to the re-association of low and high molecular weight α-crystallins through hydrophobic interactions. Therefore, interactions among the subunits of α-crystallin are destroyed and then reconstructed during the process of temperature-induced dissociation/re-association. The ability to prevent undesirable aggregation of soluble denatured γ-crystallin may thus stem from the self-assembling ability of α-crystallin subunits. This study has demonstrated that α-crystallin can, via dialysis, regain ordered structure lost during oxidative insults, and, during dialytic restructuring, α-crystallin may undergo heterogeneous aggregation with oxidatively denatured γ-crystallin. During the HMWA-forming process, the partially denatured α-crystallin quaternary structure must undergo a structural rearrangement partially induced by denatured γ-crystallin, i.e., mutually or co-induced, leading to HMWA formation. It is noteworthy that there should be a prerequisite disturbance to the native quaternary structure of α-crystallin to make α-crystallin capable of chaperone-like complexing, as reflected by native α-crystallin's failure to complex with either native or denatured γ-crystallin. It was found that more HMWA was formed by oxidized γ-crystallin followed by the addition of α-crystallin than by simultaneous oxidation of both α-crystallin and γ-crystallin. This suggests simultaneous oxidation of both crystallins resulted in over-oxidation of α-crystallin, leading to the decrease of its chaperone behavior. This was confirmed when both α- and γ-crystallins were oxidized under ascorbate–$FeCl_3$–EDTA–H_2O_2, with ten times as much concentration of H_2O_2 (i.e., 5 mM), and no HMWA was observed throughout 20-h dialysis. Thus, over-oxidized α-crystallin fails to recover certain ordering and thus fails to form HMWA with oxidized γ-crystallin through dialysis.

Since there is a significant amount of H_2O_2 (30 μM) in the aqueous humor of the normal human eye (22) and we have shown the existence of oxidation-induced chaperone/substrate complex, therefore, it is reasonable to assume that an efficient chaperone-like protective action against oxidative insults indeed exists *in vivo*. It is demonstrated that the partial unfolding of α-crystallin is prerequisite to its chaperone-like ability to form HMWA. The reassembly characteristic of partially denatured disordered α-crystallin has the ability to protect other protein substrates from precipitation. Without dialysis, no oxidation-induced HMWA is detected, which is different than heat-induced complex formation, indicating the oxidation-induced chaperone mechanism is different than the thermally induced. Thus, we propose that the mechanism for the formation of HMWA involves oxidative conformational denaturization of α-crystallin followed by dialysis-mediated renaturization, during which interaction with denatured γ-crystallin occurs. Since dialysis is a necessary step to obtain oxidation-induced chaperone/substrate HMWA and since oxidized α-crystallin can gradually regain an ordered structure through dialysis, it becomes an interesting and important issue to determine the extent of perturbation required to trigger α-crystallin's chaperone-like function.

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