Intermolecular Exchange and Stabilization of Recombinant Human αA- and αB-Crystallin*

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Lens α-crystallin subunits αA and αB are differentially expressed and have a 3-to-1 ratio in most mammalian lenses by intermolecular exchange. The biological significance of this composition and the mechanism of exchange are not clear. Preparations of human recombinant αA- and αB-crystallins provide a good system in which to study this phenomenon. Both recombinant αA- and αB-crystallins are folded and aggregated to the size of the native α-crystallin. During incubation together, they undergo an intermolecular exchange as shown by native isoelectric focusing. Circular dichroism measurements indicate that the protein with a 3-to-1 ratio of αA and αB-crystallins has the same secondary structure but somewhat different tertiary structures after exchange: the near-UV CD increases after exchange. The resulting hybrid aggregate is more stable than the individual homogeneous aggregates: at 62 °C, αB-crystallin is more susceptible to aggregation and displays a greater light scattering than αA-crystallin. This heat-induced aggregation of αB-crystallin, however, was suppressed by intermolecular exchange with αA-crystallin. These phenomena are also observed by fast performance liquid chromatography gel filtration patterns. The protein structure of αB-crystallin is stabilized by intermolecular exchange with αA-crystallin.

α-Crystallin is an oligomer protein of two subunits, αA and αB, in a ratio of 3 to 1 for most mammalian lenses. The αA- and αB-crystallins are differentially expressed in the lenses of humans and most other mammalian species; αB-crystallin appears earlier than αA-crystallin and is dominant in epithelial cells, but the αA-to-αB ratio increases with differentiation (1–4). The significance of the differential expression is not clear. Moreover, how the two subunits interchange after expression and the significance of the 3-to-1 ratio are also not well established.

The rather high concentration of α-crystallin in the lens and its extremely high thermostability (5) may be crucial in the protection against aggregation of β- and γ-crystallins. But how the thermostability is achieved is not fully understood. One possibility is through an intermolecular exchange that results in a dynamic quaternary structure. The observation that α-crystallin is the major crystallin in the lens HMW2 aggregates and water-insoluble fraction (6) may arise from some mechanisms that cause the loss of this dynamic quaternary structure.

In vitro study of α-crystallin has been handicapped by the fact that α-crystallin and its two subunits, especially isolated from human lenses, are already extensively modified and are difficult to obtain in their pure, native state. Previously, the two subunits were isolated from α-crystallin under denatured conditions and were unable to refold to the native state. The use of recombinant DNA technology to clone human α-crystallin has largely solved these problems (7–10). We have recently prepared both recombinant human αA- and αB-crystallins and performed a comparative study between them (10). The recombinant αA- and αB-crystallins exist in solution as homo-oligomers of molecular mass ~600 kDa (10). In this report, we present evidence of the interoligomeric exchange of the αA- and αB-crystallins and the stabilization of protein structure by subunit interchange. The biological significance of the 3-to-1 ratio of αA and αB subunits in vivo is discussed.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human αA- and αB-crystallins were prepared from a cDNA library of human lens epithelial cells, kindly provided by Dr. Toshimichi Shinohara. The procedures for cloning, expression, and protein purification have been described previously (10). Proteins in 0.05 M phosphate buffer, pH 7.4, were used in all measurements unless otherwise stated. Protein concentrations were determined by calculated extinction coefficients based on a protein amino acid sequence as described by Mach et al. (11). The amino acid sequences for human lens αA- and αB-subunits were published previously (12, 13). The Pierce bicinchoninic acid (BCA) assay was used to determine protein concentrations in the previous report (10). Protein concentrations determined by extinction coefficient are considerably greater than those determined by BCA assay.

Human infant lenses were obtained as surgical discards from patients with retinopathy of prematurity (Dr. Tatsuio Hiresoe, Massachusetts Eye and Ear Infirmary). α-Crystallin was isolated by gel filtration in FPLC (14).

Intermolecular Exchange Probed by IEF—Exchange between αA and αB was studied with a fixed ratio (1:1) (concentration of each, 1 mg/ml) at various incubation times and with various ratios at a fixed incubation time at 37 °C. IEF was performed on precast IsoGel-agarose IEF gels (pH range, 3–10; FMC BioProducts, Rockland, ME) under native conditions. Gels were stained with Coomassie Brilliant Blue R-250.

CD Measurements—Protein secondary and tertiary structural changes were investigated by far- and near-UV CD measurements in an Aviv circular dichroism spectrometer (model 60 DS) (15, 16). The reported CD spectra are the average of five scans, smoothed by polynomial curve fitting. The fit was checked with a statistical test so that the original data were not oversmoothed. The CD data were expressed as molar ellipticity in degrees cm² dmol⁻¹.

Light-scattering Measurement—Conformational stability was determined by treatment with heat (17). Aggregation induced by heat was measured by light scattering in a Shimadzu fluorometer (model RF-5301PC). Briefly, a protein solution of αA- or αB-crystallin or mixtures of various ratios were incubated at 37 °C for 36 h. Light scattering was measured at 62 °C maintained by a Brinkmann Lauda circulator (model RCS-6). Preliminary measurements at temperatures lower and...
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RESULTS

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IEF Study—IEF shows that there was a time-dependent exchange between αA- and αB-crystallin subunits at 37 °C (Fig. 1A). The exchange was faster at 37 °C than at 25 °C (data not shown). The original αA and αB bands became blurred with time, and eventually only one band was observed; a steady-state was reached after incubation for 30 h. The focusing pattern did not change upon further incubation. It should be noted that IEF of α-crystallin in the native state showed a broad band possibly because of microheterogeneity in the charge or structure. Similar IEF patterns were observed by van den Oetelaar et al. (18) in an exchange study using calf lens αA- and αB-crystallins.

The intermolecular exchange occurred with any ratio of the two subunits. Fig. 1B shows the focusing pattern of mixtures with various αA- and αB-crystallin ratios after incubation for 80 h at 37 °C. The newly formed hybrid aggregates displayed one band with characteristic pl values that are the number average of the pl values of the initial two components.

CD Study—CD of far- and near-UV regions was measured for the 3-to-1 ratio samples of αA- and αB-crystallins before and after exchange. The exchange was induced by incubation at 37 °C for more than 30 h, which IEF indicated a complete exchange. The lack of change in far-UV CD (data not shown) but an increase of near-UV CD (Fig. 2) indicates that intermolecular exchange causes no gross structural change. An increase of near-UV CD usually indicates that the protein structure has become more compact. The controls and αA- or αB-crystallin alone did not show a CD increase. Near-UV CD of α-crystallin isolated from human infant lenses was included in Fig. 2 for comparison; it does not overlap completely with that of 3αA-1αB-crystallin. Apparently, either in vivo and in vitro intermolecular exchanges are different or some modifications had already occurred in the human lens sample.

Thermal Aggregation of αA- and αB-Crystallins

Light-scattering Study—Fig. 3A shows the scattering intensity at 62 °C for αA- and αB-crystallin and for mixtures of various ratios of αA to αB with a fixed concentration of αB-crystallin. The samples were incubated at 37 °C for 30 h to ensure a complete exchange between αA and αB subunits. αB-Crystallin alone displayed an increasing intensity with time, but the intensity of αA-crystallin alone increased very little, indicating that αB-crystallin was more susceptible to aggregation. The addition of αA-crystallin to αB-crystallin suppressed aggregation, and the extent of suppression increased with increasing amounts of αA-crystallin until a 3-to-1 ratio was reached. Further increase in αA-crystallin decreased the suppression; the sample of 3-to-1 ratio was the most stable aggregate. Fig. 3B is a summary of the scattering measurements with the y axis as maximal change of slope in the light-scattering curves. These results may indicate that αA-crystallin stabilizes αB-crystallin from heat-induced aggregation. The aggregation shown in Fig. 3A may not be in a final state of equilibrium; the scattering intensity may continue to increase if further incubation is undertaken. Our purpose was
to compare the initial responses by these samples, from which we may infer their thermostability.

**CD Study**—Tertiary structural change was monitored by near-UV CD; Fig. 4 shows that CD intensity decreased and became negative in the whole near-UV region for the 3αA-1βB hybrid protein at a high temperature (62 °C). Upon cooling to room temperature, the original intensity was not restored, and the CD spectrum was almost the same as that at 62 °C. The irreversible change was also observed previously in calf α-crystallin samples (17). The decreased near-UV CD may reflect the loosening of protein structure by partial unfolding. Similar decreased CD was observed for homo-protein tallin samples (17). The decreased near-UV CD may reflect the loosening of protein structure by partial unfolding. Similar decreased CD was observed for homo-protein tallin samples (17).

**FPLC Gel Filtration**—To determine whether increased scattering intensity of α-crystallin at 62 °C is really due to aggregation, FPLC gel filtration was performed. The samples were preincubated at 62 °C for 20–60 min and then cooled to room temperature before being applied to the column. Preliminary results of αA and αB of the present study and our previous study of calf lens α-crystallin (17) indicate that the extent of FPLC change is dependent on incubation temperature and time. The data we present here are fixed in both parameters (preincubation at 62 °C and for 20 min) so a comparison can be made. Fig. 5 shows that the peak position for αA-crystallin shifted very little after heating, but the peak position for αB-crystallin shifted to a higher molecular weight, and some HMW aggregates were eluted at void volume. The peak shift for the 3αA-1βB mixture was slightly greater than that of αA-crystallin but less than that of αB-crystallin, and no HMW aggregate was observed at void volume.

**DISCUSSION**

The intermolecular exchange between αA- and αB-crystallin subunits was demonstrated by IEF in the native state. Similar results have been reported (18), but the αA- and αB-crystallins used were isolated from bovine lenses under denatured conditions, and they were not properly reconstituted (19). Furthermore, the significance of the exchange was not clearly established. Our use of recombinant αA- and αB-crystallins, which are folded in the native form (10), should yield results more relevant to physiological conditions. Although exchange occurs in αA/αB samples with any ratios, a 3-to-1 ratio appears to be most thermostable. Results of heat-induced aggregation indicate that αA-crystallin is thermally more stable than αB-crystallin, probably because of αB’s greater hydrophobicity (10). Hydrophobic interaction makes an important contribution to protein folding at low temperatures, but the hydrophobic entropy decreases as the system becomes enthalpy-driven at high temperatures. Thus, in *vivo* or *in vitro* aggregation between αA- and αB-crystallin (formation of an 600–800-kDa aggregate with a 3-to-1 ratio) may result from a stabilization effect of αA-crystallin on αB-crystallin. The slight shift in the FPLC peak for αA and the 3-to-1 mixture preincubated at 62 °C (Fig. 5) may be due to partial unfolding, which is also apparent in near-UV CD measurements (Fig. 4). This has been reported in the calf lens α-crystallin (17), but the present study indicates that only recombinant αB-crystallin undergoes heat-induced HMW aggregation. The notion of stabilization of αB by αA was recently demonstrated by a study of αA-knockout mice (20). Mice with the targeted disruption of the αA gene developed lens opacification starting from the nucleus and showed dense inclusion bodies that contain mostly αB.

The significance of the intermolecular exchange between αA-
intermolecular exchange of αA- and αB-crystallin subunits. The samples (1 mg/ml) were preincubated at 62 °C for 20 min and cooled to room temperature before application to the Superose-6 column (designated as 62 °C). The controls were not preincubated and were designated as 25 °C. The flow rate is 0.3 ml/min.

The values at the top are the molecular weights of the markers.

and αB-crystallin is its stabilization of the protein structure. But how this stabilization is achieved is difficult to speculate, since we do not know α-crystallin tertiary and quaternary structures; α-crystallin could not be crystallized for x-ray diffraction study. None of the structural models previously proposed, such as the original or modified three-layer model (21, 22), the micelle-like structure (23), or the overlapping two-annulus model (24), can explain the experimental data. It is obvious that the 3-to-1 ratio provides a balance in charge and hydrophobicity that stabilizes the protein structure, but a more definite explanation cannot be attained until the tertiary structure is determined. The near-UV CD clearly indicates that hybrid aggregate has a more compact structure than either αA- or αB-crystallin homo-oligomers. The difference of near-UV CD spectra between in vitro formed 3αA-to-1αB hybrid aggregate and the human infant lens α-crystallin may indicate that they do not have exactly the same tertiary structure, probably either because of different folding and aggregation between in vitro and in vivo conditions or because of modifications in the human lens α-crystallin and lack of modifications in the recombinant α-crystallin. The difference is most pronounced in the 290-nm bands, which originate from tryptophan transition, \( \lambda_{\text{nm}} (15, 25) \). The red shift of the band in the human lens α-crystallin from recombinant α-crystallin (from 292 to 295 nm) indicates that Trp residues in the human lens α-crystallin are more buried than in the recombinant α-crystallin (15, 26). It is difficult to tell what causes this shift, but it is most likely due to a different folded structure (either tertiary or quaternary structure); the shape of near-UV CD spectrum of human lens α-crystallin is very different from that of recombinant α-crystallin.

The protective binding of α-crystallin to the partially unfolded β- and γ-crystallins was reported in in vitro experiments (27, 28). Since no subsequent release of the bound substrate was observed, we should expect plenty of α-β and α-γ complexes in the lens, especially in the aged lens nucleus where modification-related partial unfolding is extensive. Instead of dimers, such as α-β and α-γ, HMW complexes were observed (29). In human lenses, the major component of the soluble HMW and insoluble proteins is α-crystallin; β- and γ-crystallins are the minor components (6). The chaperone complex formation cannot completely explain this phenomenon (19). Therefore, the question arises: why does α-crystallin, whose apparent function is to protect other crystallins from aggregation and denaturation, itself become aggregated and insoluble? Since α-crystallin has been shown to be thermally very stable (5), thermal denaturation and aggregation are unlikely to be responsible for insolubilization. Another possible unfolding mechanism is post-translational modification. Lens crystallins have a long half-life and can accumulate a variety of modifications. Although α-crystallin is thermally more stable than β- or γ-crystallin, it is not more stable in the presence of urea (30, 31); α-crystallin is totally unfolded in 6–7 M urea or 2.5 M guanidine HCl, but 6 M guanidine HCl is required for γ-crystallin to be completely unfolded. Therefore, α-crystallin may be more susceptible to modification-induced structural perturbation than γ-crystallin, which may lead to HMW aggregation and insolubilization. It is possible that when αA-crystallin is extensively modified, it fails to stabilize αB-crystallin. Recently, we have observed an extensive modification in urea-soluble α-crystallin of human lenses; IEF and reverse-phase FPLC showed a greater extent of modification in the αA subunit than in the αB subunit.²

In conclusion, we have demonstrated that lens α-crystallin subunits αA and αB undergo intermolecular exchange, which stabilizes protein conformation. The hybrid aggregate with a 3-to-1 ratio is the most stable combination and may explain why most mammalian lenses contain this ratio of α-crystallin subunits.

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