THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 52, Issue of December 29, pp. 41004–41010, 2000
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Printed in U.S.A.

Packing-induced Conformational and Functional Changes in the Subunits of α-Crystallin*

Received for publication, August 23, 2000, and in revised form, September 18, 2000
Published, JBC Papers in Press, October 9, 2000, DOI 10.1074/jbc.M007686200

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The heteroaggregate α-crystallin and homoaggregates of its subunits, αA- and αB-crystallins, function like molecular chaperones and prevent the aggregation of several proteins. Although modulation of the chaperone-like activity of α-crystallin by both temperature and chaotropic agents has been demonstrated *in vitro*, the mechanism(s) of its regulation *in vivo* have not been elucidated. The subunits of α-crystallin exchange freely, resulting in its dynamic and variable quaternary structure. Mixed aggregates of the α-crystallins and other mammalian small heat shock proteins (sHSPs) have also been observed *in vivo*. We have investigated the time-dependent structural and functional changes during the course of heteroaggregate formation by the exchange of subunits between homoaggregates of αA- and αB-crystallins. Native isoelectric focusing was used to follow the time course of subunit exchange. Circular dichroism revealed large tertiary structural alterations in the subunits upon subunit exchange and packing into heteroaggregates, indicating specific homologous and heterologous interactions between the subunits. Subunit exchange also resulted in quaternary structural changes as demonstrated by gel filtration chromatography. Interestingly, we found time-dependent changes in chaperone-like activity against the dithiothreitol-induced aggregation of insulin, which correlated with subunit exchange and the resulting tertiary and quaternary structural changes. Heteroaggregates of varying subunit composition, as observed during eye lens epithelial cell differentiation, generated by subunit exchange displayed differential chaperone-like activity. It was possible to alter chaperone-like activity of preexisting oligomeric sHSPs by alteration of subunit composition by subunit exchange. Our results demonstrate that subunit exchange and the resulting structural and functional changes observed could constitute a mechanism of regulation of chaperone-like activity of α-crystallin (and possibly other mammalian sHSPs) *in vivo*.

α-Crystallin, the major lens protein of the mammalian lens, is a member of the family of ubiquitous small heat shock proteins (1–4). Like most other small heat shock proteins, α-crystallin exists as a high molecular mass complex of a large number of subunits and prevents the aggregation of a number of proteins like a molecular chaperone (5). In most mammals, the two types of subunits of α-crystallin, αA- and αB-crystallins, are present at a molar ratio of 3:1 (6). They are encoded by evolutionarily related genes and have >50% sequence identity (7). αA- and αB-crystallins form homoaggregates of high molecular mass and display chaperone-like activity, albeit to different extents (8, 9). The demonstration of their independent, non-lenticular expression in tissues such as the heart, thymus, lung, kidney, retina, and brain (10–13) and overexpression of αB-crystallin under stress (14–19) and in disease conditions (20–25) suggests individual roles for αA- and αB-crystallins. In the lens, αA- and αB-crystallins are expressed differentially, with αB-crystallin being expressed earlier in the lens epithelial cells; the ratio of αA- to αB-crystallin increases during differentiation to fiber cells (26–30). The significance of this differential expression is not clearly understood. It is not clear as to how the two subunits interchange after expression, leading to the formation of the heteroaggregate. The arrangement of αA- and αB-crystallin subunits in the α-crystallin heteroaggregate is also speculative.

α-Crystallin is normally isolated as an oligomeric complex with an average of 40 subunits and a molecular mass of 8×10^5 Da (6), although its size distribution can vary from 3×10^5 to 1.5×10^6 Da, depending on the age of the tissue from which it was isolated (31–36) and isolation conditions such as temperature, pH, and ionic strength (37–40). Cryoelectron microscopy studies of human recombinant αB-crystallin showed that it has a variable quaternary structure (41). The quaternary structure of α-crystallin is also dynamic. Calf lens α-crystallin that is isolated into five subpopulations with distinct molecular masses returns to its original distribution upon mixing (42), indicating a dynamic equilibrium between α-crystallin oligomers. Homoaggregates of αA- and αB-crystallins are known to exchange subunits among themselves and with the α-crystallin heteroaggregate (43–46). Exchange of subunits between the native and phosphorylated forms of α-crystallin has also been detected (43). Subunits of HSP27 [1] exchange freely with homoaggregates of αA-crystallin (47). These findings suggest that the subunits of α-crystallin are freely exchangeable, indicating a dynamic structure for α-crystallin and therefore its polydisperse character. Moreover, the demonstration of mixed aggregates of αB-crystallin and HSP27 in human heart and *in vitro* (48–51) suggests a dynamic quaternary structure for most mammalian small HSPs. The relevance of subunit exchange with regard to the functionality of small HSPs has not been investigated.

Earlier studies from our laboratory have shown that the chaperone-like activity of α-crystallin is temperature-dependent (52–54). α-Crystallin shows negligible protection against DTT-induced insulin aggregation or UV light-induced γ-crys-

* This work was supported in part by the Department of Biotechnology, Government of India. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by a student fellowship from the University Grants Commission.

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[1] The abbreviations used are: HSP, heat shock protein; DTT, dithiothreitol.

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tallin aggregation at temperatures below 30 °C, but offers significant protection above 30 °C. The enhanced chaperone-like activity at elevated temperatures is due to a temperature-induced structural perturbation that leads to the concomitant exposure of appropriately placed hydrophobic surface(s), which sequester aggregation-prone proteins (52). Interestingly, the exchange of subunits between α-crystallin molecules is also temperature-dependent (43, 44). The subunits of α-crystallin do not exchange at 4 °C, but do so at 37 °C. This result indicates that the structurally perturbed form of α-crystallin, which displays higher chaperone-like activity, exchanges subunits. Earlier studies on the subunit exchange of α-crystallin have investigated the dependence of subunit exchange on experimental parameters such as temperature, pH, salt concentration, and the presence of calcium ions (43–46). The functional significance of subunit exchange is, however, not known.

In this study, we have investigated the subunit exchange between αA- and αB-crystallin homoaggregates and studied the consequent time-dependent structural and functional changes. Our results indicate that the packing of subunits in α-crystallin involves specific interactions between αA- and αB-crystallins that alter the structure of the individual subunits. We found that packing-induced structural changes in the subunits affect the chaperone-like activity of the proteins. We also demonstrate *in vitro* that altering the composition of heteroaggregates by the incorporation of αB-crystallin subunits by subunit exchange can modulate chaperone-like activity. We feel that this is of significance, as it is known that αB-crystallin expression increases under a variety of stress conditions. αB-Crystallin expressed under stress conditions can incorporate into preexisting oligomeric small HSPs to increase/modulate its chaperone-like activity. Although the modulation of the chaperone-like activity of α-crystallin *in vitro* by temperature (52–54) and chaotropic agents (55, 56) is known, its regulation *in vivo* remains largely unexplored. Our results demonstrate that the rearrangement of subunits in α-crystallin, due to its dynamic quaternary structure, may provide a possible mechanism to regulate its chaperone-like activity *in vivo*.

EXPERIMENTAL PROCEDURES

Isolation of α-Crystallin—Calf lens α-crystallin was isolated and purified as described earlier (57). The fractions corresponding to α-crystallin were pooled and concentrated at 4 °C using an Amicon ultrafiltration unit with M_r 30,000 cutoff. The concentrated solution of α-crystallin was stored in Tris-HCl buffer at 4 °C. α-Crystallin was used as the heteroaggregate of αA-and αB-crystallins. The ratio of αA- to αB-crystallin was ~3:1 (w/w) in this heteroaggregate as determined by urea-polyacrylamide gel electrophoresis.

Separation of αA- and αB-Crystallins—The subunits of α-crystallin, αA- and αB-crystallins, were separated and purified on a C4 reverse-phase column using a water/acetonitrile gradient containing 0.08% trifluoroacetic acid. The peaks corresponding to αA- and αB-crystallins were lyophilized and stored at −20 °C. Homoaggregates of αA- and αB-crystallins were generated as described earlier (9).

Subunit Exchange between Homoaggregates—Homoaggregates of αA- and αB-crystallins, filtered separately through 0.45-μm Millipore filters, were mixed at various ratios (5:1, 3:1, 1:1, 1:3, and 1:5 (w/w) αA/αB-crystallin) to a final concentration of 1 mg/ml in 10 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl. The exchange of subunits was initiated by incubation at 37 °C and stopped by cooling to 4 °C. To monitor the rate of subunit exchange, the homoaggregates were mixed at a ratio of 3:1 (w/w) αA/αB-crystallin and incubated at 37 °C. Samples were taken at various intervals of time (20, 40, and 60 min and 1.5, 2, 2.5, 3, 4, 6, 8, 12, and 24 h) and transferred to 4 °C to stop further exchange of subunits. As a control, a sample was kept at 4 °C for 24 h without exposure to a temperature of 37 °C. This was done to check if any residual exchange occurred at 4 °C. These samples were used for CD, gel filtration, and protection assays to measure chaperone-like activity. The extent of exchange was monitored by native isoelectric focusing in the pH range of 3–10. Incubation at 37 °C for 24 h was sufficient to ensure complete exchange.

Isoelectric Focusing of Homo- and Heteroaggregates—Homoaggregates and heteroaggregates resulting from subunit exchange experiments were analyzed on acrylamide gels (3% T, 3% C) containing 13% (v/v) glycerol and an ampholyte mixture of the following composition: 1.81% (v/v) Pharmalytes 6–8, 2.26% (v/v) Pharmalytes 3.5–10, and 1.81% (v/v) Pharmalytes 2.5–5. Gels (10 × 20 cm with a thickness of 1 mm) were cast between GelBond film (supported on a glass plate) and a glass plate treated with dimethyldichlorosilane. Flat bed electrophoresis was carried out in an Amersham Pharmacia Biotech electrophoresis unit using strips soaked in 1 M phosphoric acid and 1 M sodium hydroxide and an Amersham Pharmacia Biotech constant power supply unit. Limit settings were 2500 V and 70 mA. Prefocusing was carried out for 45 min at a 30-watt constant power supply. Subsequently, the samples were focused for 30 min at 15 watts and then for 120 min at 30 watts. A Julabo thermostatted water bath was used to maintain a constant temperature of the gel bed at 4 °C. The pH gradients were determined by simultaneously running pI markers (Amersham Pharmacia Biotech). These were further confirmed by cutting the gel to 0.5-cm pieces and extracting the ampholytes with 2 ml of water for 48 h. The pH of the resulting solution was measured. After the gels were fixed for 1 h in an aqueous solution of 30% (v/v) ethanol and 11.5% (w/v) trichloroacetic acid and rinsed with 2% (v/v) acetic acid, they were stained for 45 min in a solution of 1.15% (w/v) Coomassie Blue dye (BDH, page blue 83) in 25% (v/v) ethanol and 8% (v/v) acetic acid until a clear background was obtained.

Assay for Chaperone-like Activity—Insulin at a concentration of 0.2 mg/ml (in 10 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl) in the presence or absence of a 0.1 mg/ml concentration of the various reconstituted proteins was equilibrated at the required temperature for 10 min with constant stirring in the cuvette using a Julabo thermostatted water bath. The actual temperature in the cuvette was monitored with a Physitemp Microthermocouple thermometer system. The reduction of insulin was initiated by the addition of 30 μl of 1 M DTT to 1.5 ml of sample, and the extent of aggregation was monitored by measuring the scattering at 90° with the excitation and emission monochromators in a Hitachi-4000 fluorescence spectrometer set at 465 nm and excitation and emission band passes at 1.5 nm. Results are expressed as percentage protection. Percentage protection is calculated as follows: $(I_t - I_{t+\alpha})/I_t$, where I_t is the intensity of scattered light for target protein insulin and $I_{t+\alpha}$ is the intensity of scattered light in the presence of homoaggregates or heteroaggregates of αA- and αB-crystallins.

Circular Dichroism Measurements—The CD measurements were carried out in 10 mM phosphate buffer using a Jasco J-715 spectropolarimeter. αA- and αB-crystallin concentrations were 1.0 mg/ml in 10 mM sodium phosphate and 100 mM NaCl (pH 7.4). Water-jacketed 1- and 0.01-cm path length cells were used for recording near- and far-UV CD spectra, respectively. αA- and αB-crystallins at a ratio of 3:1 (w/w) at a final concentration of 1 mg/ml were used for the temperature-dependent subunit exchange and the associated CD measurements. Spectra of the mixture of homoaggregates (3:1 αA/αB-crystallin) were recorded prior to and after subunit exchange (by incubating at 37 °C). Sample temperature was maintained at 15 °C in water-jacketed sample cells of 1 and 0.01 cm using a Julabo thermostatted water bath. In another experiment, subunit exchange was carried out in the water-jacketed sample cell by maintaining the sample temperature at 37 °C. Spectra were acquired at 15-min intervals initially and later at 0.5- and 1-h intervals. The shutter was kept closed during incubation intervals to prevent possible photodamage to the protein. The sample temperature was monitored using a Physitemp Microthermocouple thermometer.

RESULTS AND DISCUSSION

αB-Crystallin and, to a much lesser extent, αA-crystallin are found as homoaggregates in a variety of tissues, including the brain, spleen, and heart (10–13). In the lens, however, the two proteins are found to exist only as a heteroaggregate, α-crystallin, although there has been a report showing that αB-crystallin may exist in minute amounts as an independent homoaggregate in the lens (59). α-Crystallin from the mammalian lens is a heteroaggregate of αA- and αB-crystallins present at a molar ratio of 3:1. The significance of this ratio, if any, is not clear. Sun and Liang (45) found that αA-crystallin at a ratio of 3:1 αA/αB-crystallin is most efficient in prevention of thermal aggregation of αB-crystallin. The composition of the α-crystallin heteroaggregate varies with age and during the differen-

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tiation of the lens epithelial cells to fiber cells (26–30) and between various species (60–63). It is known that homoaggregates of αA- and αB-crystallins can exchange subunits among themselves as well as with the α-crystallin heteroaggregate (43–46). This observation led to the proposal of a "dynamic quaternary structure" model of α-crystallin that can freely exchange subunits (43). Moreover, it is possible to produce heteroaggregates of varying subunit composition by co-refolding αA- and αB-crystallins in various ratios (64, 65). The presence of two types of subunits in the α-crystallin heteroaggregate and the dynamic and variable nature of this aggregate raise questions about the packing of subunits in it. Are the interactions between the subunits nonspecific, dictated mainly by hydrophobic interactions, or are there any specific and preferential interactions? Are the subunits packaged in the heteroaggregate more like rigid spheres, or do the subunits show a degree of structural flexibility during packaging in the heteroaggregate? These questions gain relevance in the light of the observations that α-crystallin as well as both homoaggregates of its individual subunits differ in their stability and the efficiency of their chaperone-like function (9). The exchange of subunits occurs efficiently only at temperatures close to 37 °C, but not below 15 °C (43, 44). We have used this temperature dependence of subunit exchange to control the extent of subunit exchange between homoaggregates of αA- and αB-crystallins by allowing exchange at 37 °C and stopping it at 4 °C. We hypothesized earlier that αB-crystallin, due to the higher chaperone-like activity and propensity for structural change, may modulate the chaperone-like activity of α-crystallin either directly or by inducing packing changes in the heteroaggregate (9). Structural perturbation of the individual subunits in the heteroaggregate may translate to quaternary structural alterations, which could modulate the chaperone-like activity of α-crystallin. Conversely, packing alterations of the subunits may reflect in tertiary structural changes within the subunits. We have tried to address these questions by studying the time-dependent structural and functional changes upon heteroaggregate formation by subunit exchange between homoaggregates.

If there are no specific interactions between the subunits in the α-crystallin heteroaggregate, then the near-UV CD spectrum of the heteroaggregate should be an algebraic sum of those of the αA- and αB-crystallin homoaggregates. In other words, the spectrum synthesized by the algebraic summation of the spectra of αA- and αB-crystallin homoaggregates would indicate if homologous interactions (αA-αA, αB-αB), as seen in homoaggregates, are equivalent to heterologous interactions (αA-αB), as seen in the α-crystallin heteroaggregate. Fig. 1 shows the near-UV CD spectra of αA- and αB-crystallin homoaggregates and the α-crystallin heteroaggregate. Also shown are the near-UV CD spectra of a mixture of αA- and αB-crystallin homoaggregates at a ratio of 3:1 (w/w) immediately upon mixing and after allowing for subunit exchange by incubation at 37 °C for 24 h. The near-UV CD spectra of αA- and αB-crystallin homoaggregates show differences mainly in the 270–290 nm region, where tryptophan and tyrosine contribute to the observed signal (Fig. 1*A*). The near-UV CD spectrum of a mixture of αA- and αB-crystallin homoaggregates (3:1, w/w) does not overlap with that of the α-crystallin heteroaggregate (Fig. 1*B*). This non-additive nature suggests alterations in the subunit structure upon incorporation into the heteroaggregate. The spectrum of the mixture (3:1 (w/w) αA/αB-crystallin) bears a closer resemblance to that of the αA-crystallin homoaggregate (Fig. 1*A*). This was expected due to the predominance of αA-crystallin in the mixture. At this point in time, the proteins exist as homoaggregates, with little or no subunit exchange

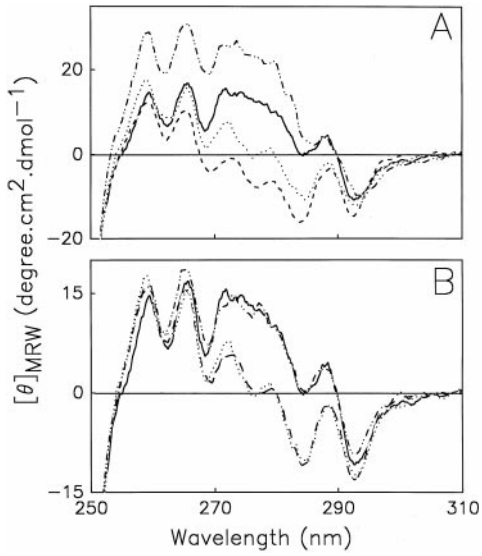

FIG. 1. *A*, comparison of near-UV CD spectra of the α-crystallins. ——, α-crystallin; – – –, αA-crystallin; –··–, αB-crystallin; ····, synthesized spectrum for αA- and αB-crystallin homoaggregates mixed at a 3:1 (w/w) ratio. *B*, tertiary structural changes upon subunit exchange between homoaggregates monitored by near-UV CD. – – – and –··–, αA- and αB-crystallin homoaggregates mixed at a 3:1 (w/w) ratio before and after subunit exchange, respectively. The synthesized spectra for αA- and αB-crystallin homoaggregates mixed at a 3:1 (w/w) ratio (····) and the α-crystallin heteroaggregate (——) are shown for direct comparison. *MRW*, mean residue weight.

having taken place. A spectrum synthesized by the algebraic addition of the individual homoaggregate spectra ((3 × αA-crystallin spectrum + αB-crystallin spectrum)/4) overlaps with that of the mixture of homoaggregates (Fig. 1*B*), but not with that of α-crystallin (Fig. 1*A*). Incubating the mixture at 37 °C allows for subunit exchange and generates heteroaggregates. The near-UV CD spectrum of the mixture following subunit exchange for 24 h is almost identical to that of the native α-crystallin heteroaggregate (Fig. 1*B*). The main difference in the near-UV CD spectra of the homoaggregates before and after subunit exchange is in the 270–290 nm region. The near-UV CD spectra of the 3:1 (w/w) mixture of αA- and αB-crystallins did not show any change when incubated at 4 °C for 24 h (data not shown). In an earlier study, we investigated the temperature-dependent changes in the structure and function of αA- and αB-crystallin homoaggregates (9). We found that unlike αA-crystallin and contrary to the well known temperature stability of the α-crystallin heteroaggregate (57, 58), αB-crystallin loses considerable tertiary structure at 45 °C. Interestingly, the maximum CD signal change observed was also in the 270–290 nm region. To monitor the changes in tertiary structure as a function of subunit exchange, we recorded the near-UV CD spectra of the homoaggregates as a function of time of exchange at 37 °C. Fig. 2 plots the change in ellipticity at 284 and 265 nm as a function of time as the subunit exchange proceeded. The change was much higher at 284 nm than at 265 nm. This is because, at 265 nm, the ellipticity value of the α-crystallin heteroaggregate is not different from the algebraic sum (3:1) of those of the homoaggregates. The observed change in ellipticity at 284 nm upon subunit exchange saturated by 2 h.

The effect of subunit exchange on the secondary structure of the subunits was monitored by far-UV CD (Fig. 3). The far-UV CD spectra of αA- and αB-crystallin homoaggregates show only minor differences from the spectrum of the α-crystallin heteroaggregate (9). The spectrum of the 3:1 (w/w) mixture of αA- and αB-crystallin homoaggregates is almost similar to that of

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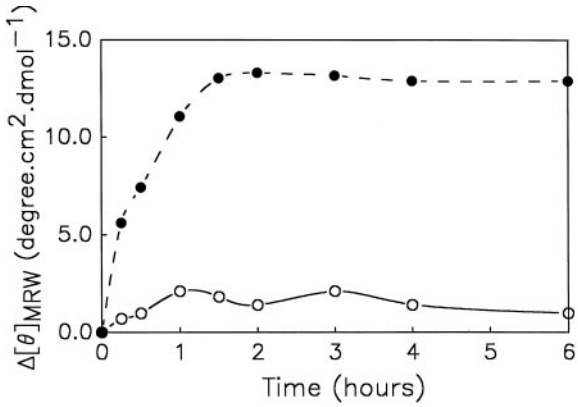

FIG. 2. **Change in ellipticity at 284 nm (●) and 265 nm (○) as a function of time as subunit exchange proceeds.** *MRW*, mean residue weight.

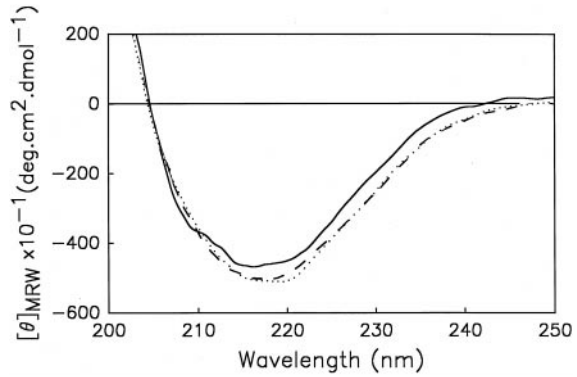

FIG. 3. **Secondary structural changes upon subunit exchange as monitored by far-UV CD.** Shown are the CD spectra of a 3:1 (w/w) αA/αB-crystallin mixture of homoaggregates before exchange (——), after 4 h of exchange (– – –), and 24 h of exchange (····). *MRW*, mean residue weight; *deg*, degrees.

α-crystallin (data not shown). Upon subunit exchange by incubating the mixture at 37 °C, no significant changes were observed. Taken together, these results indicate that a change from homologous subunit packing (as in homoaggregates) to heterologous subunit packing (as in α-crystallin) alters the tertiary, but not the secondary, structures of αA- and αB-crystallins. The secondary structure of α-crystallin is exceedingly stable against perturbation by temperature (57, 58) and, to a lesser extent, by chaotropic agents (56). The primary β-pleated secondary structure of HSP25 is stable up to a pH of 4.5 and at a neutral pH up to a temperature of 60 °C (66). The tertiary structure of the α-crystallins is more labile and is lost at 60 °C (9) and in 0.8 M guanidinium chloride (56). Using cryoelectron microscopy, Haley *et al*. (41) showed that the outer surfaces of αB-crystallin homoaggregates have an extremely variable and dynamic structure. The interactions involved in quaternary structure formation must be predominantly on the surface of the proteins. Increasing the temperature would increase the dynamics of the flexible elements on the surface. This could be a reason why the subunits of α-crystallin freely exchange at or above 37 °C, but not at lower temperatures. Here, it is interesting to note that electron microscopy studies of two-dimensional crystals of GroEL showed that it has unusual plasticity (67). Perhaps such a high degree of flexibility is required for molecular chaperones to have a low specificity, enabling them to bind a variety of protein folding intermediates. This could constitute a general mechanism by which a few chaperones could interact with the folding intermediates of a large number of proteins.

To ascertain if the time course of subunit exchange correlates

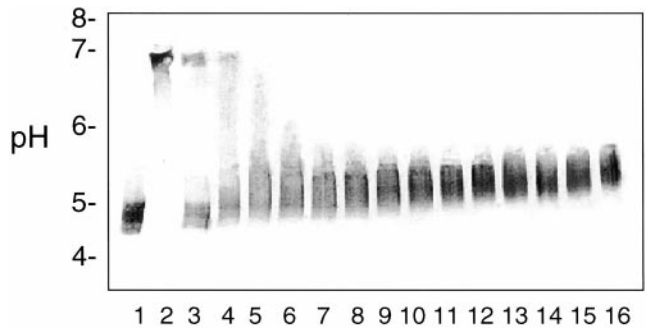

FIG. 4. **Native isoelectric focusing of heteroaggregates generated upon subunit exchange between homoaggregates of αA- and αB-crystallins.** *Lanes 1* and *2*, αA-crystallin and αB-crystallin respectively; *lanes 3–16*, isoelectric focusing of a mixture of homoaggregates (3:1 (w/w) αA/αB-crystallin) incubated at 37 °C for various intervals of time: 0, 15, 30, and 45 min and 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 15, and 24 h, respectively.

with the time course of the tertiary structural changes observed upon mixing homoaggregates, we used the difference in the pI values of the αA- and αB-crystallin homoaggregates as a tool to follow the course of subunit exchange. Fig. 4 shows the time course of heteroaggregate formation upon mixing αA- and αB-crystallin subunits at a ratio of 3:1 (w/w) as monitored by native isoelectric focusing. Homoaggregates of αA-crystallin focused to a pI range of 4.5–5.2, whereas those of αB-crystallin focused to a pI range of 6.4–6.9. The broad pI ranges of the homoaggregates of αA- and αB-crystallins focused in the absence of urea indicate that the homoaggregates have charge microheterogeneity. Earlier studies on the native isoelectric focusing of α-crystallin and homoaggregates of its subunits also revealed a high extent of charge microheterogeneity (43). This may be caused by heterogeneity in the structure of the aggregates with respect to number, folding, or packing of subunits. Previous Monte Carlo simulation of the charge frequency distribution demonstrated that a random combination of subunits could explain the observed charge microheterogeneity of α-crystallin (68). Isoelectric focusing of αA- and αB-crystallin subunits in the presence of 7 M urea does not show such charge heterogeneity (69). It is interesting to note that the pI values observed for αA- and αB-crystallin homoaggregates are lower by ~0.8–1.1 and 0.4–0.7 units, respectively, as compared with their corresponding subunits in 7 M urea. This is indicative of the masking of positive charges, probably due to the folding or aggregation of the subunits. Upon incubation of a mixture of αA- and αB-crystallin homoaggregates at 37 °C, the bands in the acidic region (αA-crystallin homoaggregates) became more basic, whereas those in the basic region (αB-crystallin homoaggregates) moved to a more acidic pI (Fig. 4). At a ratio of 3:1 (w/w) αA/αB-crystallin, the pI of the resulting heteroaggregate is not very different from that αA-crystallin. The formation of heteroaggregates could, however, be followed by monitoring the shift of the αB-crystallin homoaggregates. As the incubation proceeded, more protein focused to regions of an intermediate pI. Most of the change was completed in 2 h, and only minor alterations in the focusing pattern were observed when the incubation was extended to 24 h. A similar experiment with exchange of subunits between homoaggregates at a ratio of 1:1 (w/w) showed a similar trend (data not shown). From these experiments, we were able to conclude that subunit exchange is concomitant with the observed changes in tertiary structure upon mixing of homoaggregates.

Polydispersity is one of the striking characteristics of the α-crystallin heteroaggregate. The average molecular mass of the α-crystallin heteroaggregate is 800 kDa, whereas those of the homoaggregates of its subunits, αA- and αB-crystallins, are

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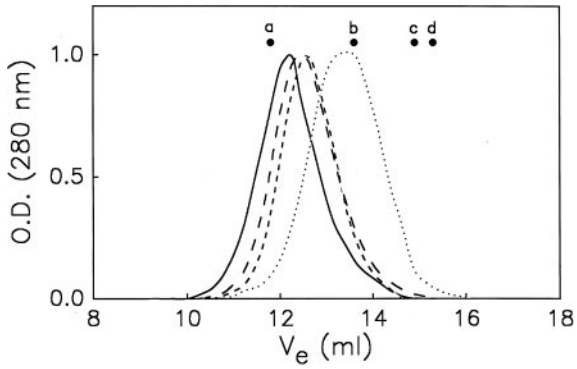

FIG. 5. **Gel filtration chromatography of heteroaggregates generated by subunit exchange.** ····, prior to subunit exchange; - - - - - and – – –, after 4 and 30 h of exchange at 37 °C, respectively; ——, native α-crystallin. The elution positions of thyroglobulin (669 kDa; *point a*), ferritin (440 kDa; *point b*), catalase (232 kDa; *point c*), and aldolase (158 kDa; *point d*) are indicated.

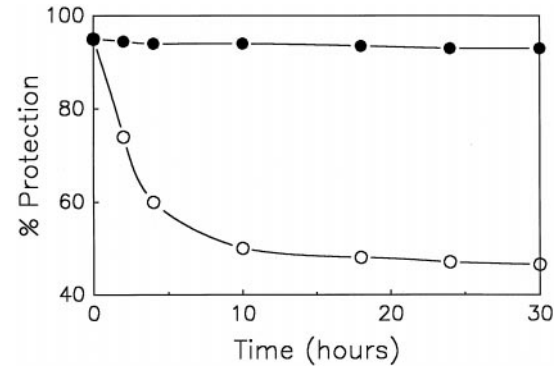

FIG. 6. **Chaperone-like activity against DTT-induced insulin aggregation at 37 °C as a function of time of subunit exchange.** ○, heteroaggregates generated as a result of subunit exchange for various periods of time at 37 °C; ●, control homoaggregates maintained at 4 °C to prevent subunit exchange. The ratio of αA- to αB-crystallin homoaggregates was 3:1 (w/w).

600 and 450 kDa, respectively. The molecular mass of the α-crystallin heteroaggregate is affected by isolation conditions such as pH, ionic strength, and temperature (37–40). We used gel filtration chromatography to study the effect of subunit exchange on oligomer size and polydispersity. Fig. 5 shows the gel filtration profile of a 3:1 (w/w) mixture of homoaggregates (αA/αB-crystallin) on a water-jacketed Superose 6B column run at 4 °C. A mixture of the homoaggregates, injected immediately after mixing, eluted as a broad peak because the ratio of αA- to αB-crystallin was 3:1, and the two peaks did not resolve fully. Incubation of this mixture at 37 °C to facilitate subunit exchange caused a change in elution volumes. With increasing incubation time and the generation of heteroaggregates, the proteins eluted earlier, indicating an increase in oligomer size. Subunit exchange at 37 °C for 4 h increased the size of the heteroaggregate considerably. Upon further incubation, only a minor change in subunit size was observed. The heteroaggregate obtained upon complete exchange after 24 h eluted as a homogeneous peak with a narrower size distribution as compared with a mixture of homoaggregates. Subunit exchange between homoaggregates of αA- and αB-crystallins generates a discreet population of oligomers. The resultant heteroaggregates generated upon subunit exchange are larger than the homoaggregates from which they are generated. A balance of charge and hydrophobic interactions would dictate the size of a soluble aggregate. As the sizes of the aggregates become smaller, the surface/volume ratio of aggregates increases, but the total internal volume (and therefore, burial of hydrophobic surface) decreases. The charges on the surface of the proteins are essential for its solubility, whereas the burial of hydrophobic surfaces is energetically favorable. In the cell cytoplasm, where the pH is close to 7 and ionic strength is ~150 mM, the α-crystallins bear a high effective charge of 50. Their high molecular mass is associated with a high excluded volume fraction equivalent to twice the dry cell volume fraction (70), suggesting an open, non-compact quaternary structure. The large excluded volume fraction and high charge combine to produce the strong repulsive behavior, responsible for lens transparency. Image-restrained analysis of cryoelectron microscopy data of αB-crystallin yielded a similar "open" structure with large voids between subunits (71). The highly charged C-terminal tail of α-crystallin is responsible for the solubility of the heteroaggregate. Siezen *et al.* (34) have shown that successive truncation of residues from the C-terminal tail of αA-crystallin produces successively larger aggregates. The charge on the α-crystallin heteroaggregate is important for the solubility of the α-crystallin-target protein complex. Truncation of the C terminus of α-crystallin decreases its chaperone-like activity (73, 74) and is believed to be one of the post-translational modifications that compromise its molecular chaperone-like function *in vivo*. The homoaggregates of αA- and αB-crystallins differ with respect to their charge and hydrophobicity. Subunit exchange between homoaggregates to form heteroaggregates alters the balance between the charges and hydrophobicity. The alteration of surface charge in the heteroaggregate due to altering subunit composition, as during lens differentiation, could have implications for the functions of α-crystallin in both the maintenance of lens transparency and its chaperone-like activity. The formation of a larger heteroaggregate is probably a compensatory mechanism by which the burial of hydrophobic surfaces in a larger aggregate could lead to its stabilization. This might be a reason why in the lens, where both αA- and αB-crystallins are expressed, they are found to exist together as heteroaggregates of α-crystallin.

Since subunit exchange between homoaggregates leads to tertiary and quaternary structural changes, it was of relevance to check if these affect the functionality of the heteroaggregate. We studied the chaperone-like activity of the proteins as a function of subunit exchange. Fig. 6 shows the percentage protection offered against DTT-induced insulin aggregation as a function of time allowed for subunit exchange. Interestingly, the protective ability decreased with subunit exchange. It fell to ~50% in ~5–6 h of exchange. Further incubation at 37 °C resulted in only a minor change in chaperone-like activity. The resulting chaperone-like activity observed upon complete subunit exchange is comparable to that of native α-crystallin at 37 °C (9). In contrast, the homoaggregate mixture held at 4 °C showed no change in the protective ability during the same period. The fall in the chaperone-like activity closely followed the time course of subunit exchange and the resulting changes in the tertiary structure of the subunits. It is important to note that the rate of subunit exchange was strongly dependent on the concentration of the interacting homoaggregates. Subunit exchange at a protein concentration of 0.1 mg/ml during the time period of the protection assay was not significant. Moreover, binding of denatured proteins to α-crystallin, as in this protection assay, decreases the rate of subunit exchange considerably (44). Previous studies have shown that changes in α-crystallin oligomer size at elevated temperatures are slow and require many hours to take effect (37, 76), and tertiary structural alterations have been proposed to be responsible for the temperature-dependent enhancement of its chaperone-like activity (58). This result demonstrates that alteration in subunit packing leads to alteration in chaperone-like activity. The

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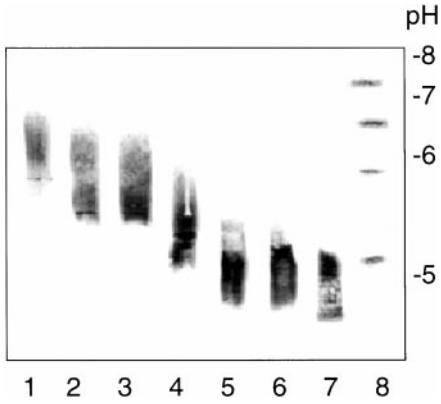

FIG. 7. **Native isoelectric focusing of heteroaggregates of varying composition generated by subunit exchange.** *Lanes 1* and *7*, αB-crystallin and αA-crystallin homoaggregates, respectively; *lanes 2–6*, heteroaggregates of ratios 1:5, 1:3, 1:1, 3:1, and 5:1 (w/w) αA/αB-crystallin, respectively; *lane 8*, native isoelectric focusing markers.

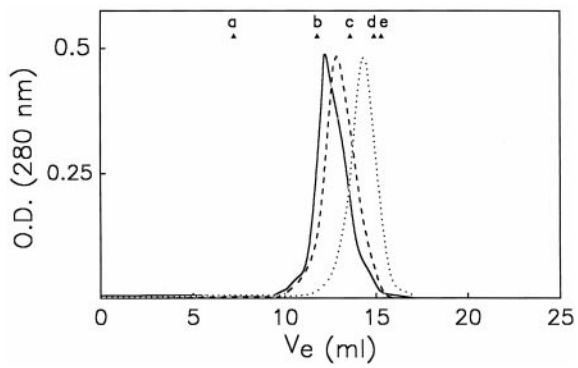

FIG. 8. **Gel filtration of heteroaggregates with subunit compositions of 5:1 (——), 1:1 (– – –), and 1:5 (····) (w/w) αA/αB-crystallin.** The elution positions of blue dextran (2000 kDa; *arrowhead a*), thyroglobulin (669 kDa; *arrowhead b*), ferritin (440 kDa; *arrowhead c*), catalase (232 kDa; *arrowhead d*), and aldolase (158 kDa; *arrowhead e*) are indicated.

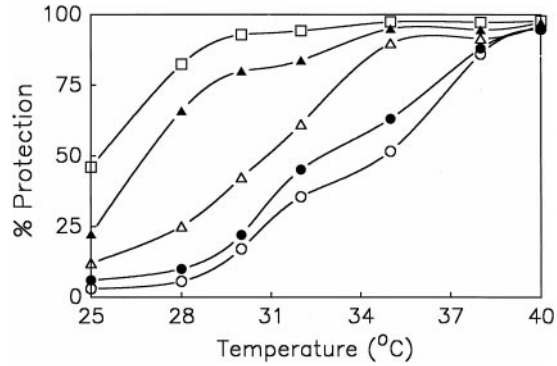

FIG. 9. **Temperature-dependent chaperone-like activity of heteroaggregates with subunit compositions of 5:1 (○), 3:1 (●), 1:1 (△), 1:3 (▲), and 1:5 (□) (w/w) αA/αB-crystallin.**

mechanism of the temperature-induced enhancement of the chaperone-like activity of α-crystallin can be attributable to alteration of subunit packing within the oligomer. Such changes in packaging could alter the relative accessibility of the individual subunits or lead to the generation/exposure of target protein-binding sites.

The subunits of α-crystallin are very prone to form aggregates. In fact, single subunits exist only in concentrated solutions of denaturing agents, and they re-aggregate upon dilution of these agents or after their removal by dialysis. The ratio of subunits is not crucial to form aggregates. Not only dissociated α-crystallin subunits (77–80), but also isolated αA- and αB-crystallin subunits re-associate (64, 81, 82) to homoaggregates. Moreover, it is possible to generate heteroaggregates of varying subunit composition by co-refolding αA- and αB-crystallins mixed at various ratios (64, 65). These results suggest the equivalence of αA- and αB-crystallins in the α-crystallin heteroaggregate.

The α-crystallin in the lens epithelial cells has a subunit composition of 1:3 αA/αB-crystallin; but upon differentiation to fiber cells, the ratio becomes 3:1 αA/αB-crystallin (26–30). The significance of this alteration of the composition of α-crystallin is not clearly understood. Subunit exchange between αA- and αB-crystallin homoaggregates mixed at various ratios also generates heteroaggregates of varying subunit composition (43–45). The composition of the α-crystallin heteroaggregate varies between species (60–63) and during ontogenesis in a single species (72). Kangaroo α-crystallin has a subunit composition of 9:1 (w/w) αA/αB-crystallin, whereas dogfish α-crystallin has a ratio of 1:4. Moreover, αB-crystallin expression is controlled by a heat shock promoter and is known to be overexpressed in a variety of stress (14–19) and diseased (20–25) conditions. In light of the differential structural and functional properties of αA- and αB-crystallins, we investigated the effect of subunit composition on the chaperone-like activity of heteroaggregates. We generated heteroaggregates of varying subunit composition (5:1, 3:1, 1:1, 1:3, and 1:5 (w/w) αA/αB-crystallin) by allowing subunit exchange between homoaggregates, mixed at the corresponding ratios, to proceed for 24 h at 37 °C. Fig. 7 shows the native isoelectric focusing pattern of the resulting heteroaggregates. The native pI of the heteroaggregates is dictated by their composition of acidic and basic subunits. The heteroaggregates also show charge microheterogeneity, such as that observed for the individual homoaggregates. The heteroaggregates with subunit compositions of 5:1 and 1:5 (w/w) αA/αB-crystallin focused at positions comparable to those of the αA- and αB-crystallin homoaggregates, respectively. The change in pI with respect to subunit composition was more significant between the ratios of 3:1 and 1:3 (w/w) αA/αB-crystallin.

We performed gel filtration chromatography to study the effect of subunit composition on the molecular mass of the heteroaggregates (Fig. 8). The molecular mass of the heteroaggregate was dependent on the ratio of αA to αB-crystallin subunits. There was a sudden decrease in the estimated molecular mass of the heteroaggregate when the amount of αB-crystallin subunits predominated in the heteroaggregate. Thomson and Augusteyn (64) reported that, upon co-refolding mixtures of αA- and αB-crystallin subunits at various ratios, a sharp increase in the sedimentation coefficient of the resulting heteroaggregate was observed once αB-crystallin predominated in the heteroaggregate. Interestingly, both studies show an alteration in the molecular mass at the same subunit stoichiometry. The differences may be due to different mechanisms involved in heteroaggregate formation by co-refolding of subunits as compared with by the exchange of prefolded subunits in homoaggregates.

One important difference between the chaperone-like activities of αA- and αB-crystallin homoaggregates is the temperature dependence of their protective abilities (9). At elevated temperatures (~63 °C), the protective abilities of the two proteins are comparable, although at physiological temperatures, αB-crystallin displays higher activity. Even below physiological temperatures, αB-crystallin shows significant protective ability, whereas αA- and α-crystallins do not. The sensitivity of αB-crystallin structure to temperature-induced perturbation may be responsible for these properties. We investigated the effect of altering subunit composition on the temperature-de-

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pendent chaperone-like activity of the heteroaggregate. We used the DTT-induced aggregation of insulin as a model of non-thermal aggregation to study the chaperone-like activity of the heteroaggregates at various temperatures. Fig. 9 shows the abilities of the various heteroaggregates to prevent the DTT-induced aggregation of insulin as a function of temperature. At a protein concentration of 0.1 mg/ml heteroaggregate and 0.2 mg/ml insulin, all the ratios protected at 40 °C. The proteins offered lower protection as the temperature was reduced. The extent of the decrease in protective ability as the temperature was reduced, however, was dependent on the subunit composition of the heteroaggregates. Increasing the content of αB-crystallin in the heteroaggregate increased the effectiveness of the heteroaggregate at lower temperatures. This result demonstrates that incorporation of αB-crystallin into a heteroaggregate could provide a means of increasing the chaperone-like activity of the heteroaggregate. This is of relevance since, as mentioned earlier, αB-crystallin synthesis is induced under stress and disease conditions. Interestingly, HSP27 is also expressed in the eye lens (75).

Our studies demonstrate that packing of subunits in the variable oligomer assembly provides a mechanism for the modulation of chaperone-like activity. The accompanying tertiary structural changes, upon subunit exchange, due to specific interactions between the subunits may be responsible for this modulation. The dynamic nature of the α-crystallin oligomer may provide a mechanism to increase its efficiency by incorporation of αB-crystallin during stress conditions. Due to their homology to α-crystallin and their ability to exchange subunits and to form mixed oligomers, mammalian small HSPs might use a similar form of activity modulation.

Acknowledgment—We thank Ritu Kanwar for useful discussions and suggestions.

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