alpha-Crystallin is known to exhibit chaperone-like activity. We have studied its chaperone-like activity toward the refolding of beta-crystallin upon refolding of this protein from its unfolded state in guanidinium chloride. The chaperone-like activity of alpha-crystallin is less pronounced below 30 °C and is enhanced above this temperature. The plot of percentage protection as a function of temperature shows two transitions; one at 30 °C and another at around 55 °C. We have performed steady state fluorescence, fluorescence polarization, fluorescence quenching, circular dichroism, sedimentation analysis, and gel filtration chromatography to probe the temperature-induced structural changes of alpha-crystallin. Our results show that at above 50 °C, alpha-crystallin undergoes a transition to a multimeric molten globule-like state. Above 30 °C, a minor but detectable perturbation in its tertiary structure occurs that might lead to the observed exposure of its hydrophobic surfaces. These results support our earlier hypothesis that alpha-crystallin prevents the aggregation of other proteins by providing appropriately placed hydrophobic surfaces; a structural alteration induced by temperature forms a part of the general mechanism of chaperone function, because they are required to function more effectively at non-permissible temperatures.

alpha-Crystallin, a multimeric protein composed of both acidic (alpha) and basic (beta) subunits with the molecular mass of approximately 20 kDa, is a major protein of the eye lens. It is also expressed in other tissues such as heart, kidney, brain, muscles, etc. (1–4) and under certain diseased conditions (5–8). It shares both structural and sequence homology with small heat shock proteins and behaves in several ways like small heat shock proteins (9–12). Its expression can be induced by thermal (9) or hypertonic (13) stress. It was believed to play only a structural role in the formation of the transparent and highly refractive tissue of the eye lens. However, the discovery of its presence in non-lenticular tissues and its homology with small heat shock proteins suggests that it might have other functional properties.

alpha-Crystallin is shown to prevent the heat-induced aggregation of other crystallins and enzymes like a molecular chaperone (14). The chaperone-like property of this crystallin has been investigated by several workers in order to gain insight into both the mechanism of this property and its relevance to the lens transparency (15–30). alpha-Crystallin from the old human lenses (28) and from the senile-induced cataract lenses of an animal model (29) are found to exhibit decreased chaperone-like activity. The chaperone-like activity of alpha-crystallin might be important in the formation and maintenance of the lens transparency, and the loss of transparency can be attributed to the loss of function of alpha-crystallin (17).

Earlier we investigated the chaperone-like activity of alpha-crystallin toward the photo-induced aggregation of crystallin (17), aggregation of insulin (18), and on the refolding of beta- and gamma-crystallins (19). We observed that alpha-crystallin can prevent photo-aggregation of crystallin, and this chaperone-like activity of alpha-crystallin is enhanced severalfold at temperatures above 30 °C. This enhancement parallels the exposure of its hydrophobic surfaces as a function of temperature, probed using hydrophobic fluorescent probes such as pyrene (17) and 8-anilinonaphthalene-1-sulfonate (18). We therefore concluded that alpha-crystallin prevents the aggregation of other proteins by providing properly placed hydrophobic surfaces; a structural transition above 30 °C might therefore be important for its chaperone-like property (17).

Maiti et al. (31) studied the secondary structure of alpha-crystallin by far UV-circular dichroism and reported that alpha-crystallin is heat stable and does not denature at temperatures up to 100 °C. Surewicz and Olesen (32) observe that alpha-crystallin undergoes a major thermotropic transition with a midpoint at 60–62 °C, which appears to correspond to the transition observed by Walsh et al. (33) and our own observation (18). The details of the structural alterations in terms of secondary, tertiary, and quaternary structures of alpha-crystallin as a function of temperature, however, are not yet understood.

The commonly used model system, thermal aggregation of beta-crystallin does not allow variable temperature studies. Hence, we have used refolding-induced aggregation of beta-crystallin and also investigated the structural aspects of alpha-crystallin in the context of its chaperone-like activity.

EXPERIMENTAL PROCEDURES

Materials—Sephacryl S-300 (high resolution), Bio-Gel A-1.5 m, and Bio-Gel A-5 m were purchased from Pharmacia Biotech Inc. GdmCl was procured from Serva. All other chemicals used in this study were of analytical grade. alpha- and beta-crystallins were isolated and purified as described in our earlier studies (15–20).

Refolding of beta-Crystallin—beta-Crystallin was unfolded in 50 mM Tris-HCl buffer (pH 8.3) containing 6.5 M GdmCl, and the sample was incubated at 25 °C for approximately 16 h. Refolding of beta-crystallin was performed at the final protein concentration of 0.133 mg/ml by 50-fold dilution of the stock of unfolded protein into the refolding buffer. The refolding buffer was 30 mM sodium phosphate (pH 7.4) containing 100 mM NaCl. The refolding experiment was performed at different temperatures by preequilibrating the refolding buffer with and without alpha-crystallin (0.65 mg/ml) at the required temperature for 5 min. The

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‡ To whom correspondence should be addressed. Tel.: 91-40-7172-241; Fax: 91-40-7171-195; E-mail: mohan@ccmb.globemail.com.

1 The abbreviations used are: GdmCl, guanidinium chloride; CD, circular dichroism.
sample was kept at the same temperature for 5 min before the measurement of the turbidity of the sample. The turbidity was measured as optical density at 500 nm.

**Fluorescence Studies**—Fluorescence measurements were performed using a Hitachi F45010 fluorescence spectrophotometer. For fluorescence studies, a 0.2 mg/ml sample of α-crystallin in 30 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl was used. Fluorescence measurements at different temperatures were performed by placing the samples in the cuvette holder, which was thermostated at the required temperatures using a Julabo circulating water bath. The actual temperature inside the cuvette was monitored using the Physitemp type-T microthermocouple thermometer. Fluorescence spectra were recorded with the excitation wavelength set at 295 nm. The excitation and emission band passes were set at 5 and 1.5 nm, respectively. All spectra were recorded in corrected spectrum mode.

Fluorescence polarization measurements were performed using a Hitachi fluorescence polarization accessory. The sample was excited at 295 nm, and the emission intensity was monitored at 340 nm. Polarization values were calculated as \( P = (I_{VV} - G I_{VH})(I_{VV} + G I_{VH}) \) where \( I_{VV} \) and \( I_{VH} \) are the measured fluorescence intensities with the excitation and emission polarizers oriented vertically or the excitation and the emission polarizers oriented vertically and horizontally, respectively. \( G \) is a correction factor and is equal to \( I_{VV}/I_{VH} \).

Fluorescence quenching experiments were performed using the neutral quencher, acrylamide. The sample was excited at 295 nm. The emission intensity at 340 nm was measured with successive additions of 7 µl of 7 M acrylamide to 1.2 ml of 0.2 mg/ml α-crystallin in 30 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl. The total volume of the acrylamide added was not more than 9% of the sample volume. The fluorescence intensities were corrected for the dilution.

**Circular Dichroism Measurement**—Circular dichroism (CD) spectra were recorded using a Jasco J-715 spectropolarimeter. CD spectra of α-crystallin at different temperatures were recorded using water-jacketed cuvettes. The required temperatures were maintained using a Julabo circulating water bath with the actual temperature inside the cuvette measured using the Physitemp type T microthermocouple thermometer. A 1 mg/ml sample of α-crystallin in 30 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl was used. Far UV-CD spectra and near UV-CD spectra were recorded using 0.01-cm and 1-cm path length cuvettes, respectively. The spectra shown in the present study were the cumulative average of at least four repeat scans.

**Gel Filtration Experiments**—Gel filtration chromatography of α-crystallin was performed at different temperatures using a Sephacryl S-300 (high resolution) water-jacketed column (0.9 × 70 cm) equilibrated at the required temperature using the Julabo water circulating water bath. 0.3 ml of 2.1 mg/ml α-crystallin solution was loaded onto the column equilibrated with 30 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl and eluted with the same buffer. Fractions (0.5 ml) were collected, and absorbance at 280 nm was measured. Elution profile of blue dextran (molecular mass, 2000 kDa) on the column was measured. Appropriate corrections for temperature and solvent viscosity.

**RESULTS AND DISCUSSION**

α-Crystallin is known to prevent aggregation of proteins like a molecular chaperone. The molecular mechanism of this chaperone-like activity of α-crystallin is not yet completely understood. We have investigated the aggregation of β₂-crystallin upon refolding from its GdmCl-unfolded state and the effect of α-crystallin on this aggregation. Fig. 1A shows the aggregation of β₂-crystallin upon refolding at 0.133 mg/ml from its unfolded state (in 6.5 M GdmCl) by 50-fold dilution into the refolding buffer at various temperatures. As seen from the figure, the extent of aggregation of β₂-crystallin gradually increases with refolding temperature in the absence of α-crystallin. The aggregation of β₂-crystallin is relatively less in the presence of α-crystallin, but the aggregation profile with refolding temperature is parallel to that containing no α-crystallin until 30 °C.

Interestingly, above 30 °C, the extent of aggregation is remarkably reduced in the presence of α-crystallin. Fig. 1B shows the percentage protection that the α-crystallin offers to β₂-crystallin as a function of refolding temperature. It is evident from the figure that the chaperone-like activity of α-crystallin is enhanced above 30 °C, with two distinct transitions, one at 30 °C and the another above 55 °C.

We showed earlier that the chaperone-like activity of α-crystallin toward the photo-aggregation of γ-crystallin (17) and dithiothreitol-induced aggregation of insulin (18) is more pronounced at temperatures above 30 °C. Differential scanning calorimetric studies on α-crystallin by Walsh et al. (33) showed two endothermic transitions, a relatively minor one at 35–45 °C and another major transition at around 60 °C. On probing the hydrophobic surfaces of α-crystallin using the hydrophobic fluororescent probe, pyrene, we showed that the hydrophobic surfaces of α-crystallin are exposed above 30 °C, with two distinct transitions, one at ~30 °C and the another at ~55 °C (17). We obtained similar results with another probe, 8-anilinonaphthalene-1-sulfonate (18). Subsequently, Das and Surewics (21) also reported similar temperature-induced exposure of hydrophobic surfaces of α-crystallin using bis-8-anilinonaphthalene-1-sulfonate. Smith et al. (30) used hydrogen-deuterium exchange of amide proton to study the conformational aspects of α-crystallin as a function of temperature. Their observation supports our hypothesis that α-crystallin prevents the aggregation of non-native structures of target proteins by providing appropriately placed hydrophobic surfaces (17) and extended it further by suggesting the regions of α-crystallin that may become exposed with temperature (30).

They observed that the hydrophobic regions around the residues 32–37 and 72–75 of α₂ and 28–34 of α₁ become solvent-exposed above 30 °C. Our earlier studies (15–18) as well as the
present study (Fig. 1) clearly show a temperature-dependent chaperone-like activity of α-crystallin. It is, therefore, important to study in detail the temperature-induced conformational changes of α-crystallin in the context of its chaperone-like activity.

Fig. 2A shows the far UV-CD spectra of α-crystallin at different temperatures. Fig. 2B shows the change in the mean residual ellipticity at 205 and 222 nm as a function of temperature. It is evident from the figure that the ellipticity value at 205 or 222 nm is not significantly altered at temperatures <50 °C. Above 50 °C, the ellipticity at 205 nm increases sharply, whereas the ellipticity at 222 nm increases only marginally. This result is consistent with the earlier report of Maiti et al. (31) and Surewicz and Olesen (32). However, it is to be noted that the far UV-CD spectra of α-crystallin above 50 °C do not indicate a random coil structure but indicate an alteration in the native structure.

As mentioned earlier, most of the earlier studies have been focused on the secondary structure of α-crystallin at different temperatures. However, the tertiary structure of α-crystallin as a function of temperature, to the best of our knowledge, has not been studied. Since higher order structures appear to have a major role in the chaperone-like activity of α-crystallin, we studied the tertiary structure of α-crystallin and its alteration with temperature using fluorescence and near UV-circular dichroism. We observed that the fluorescence emission maximum of α-crystallin is shifted from 335 nm at 20 °C to 338 nm at 62 °C. This indicates that the tryptophan residues of α-crystallin are relatively more exposed to the solvent at 62 °C. It does not indicate complete exposure of tryptophan residues as in the cases of complete unfolding of proteins. We also studied the variation of the microenvironment of tryptophan residues of α-crystallin by monitoring the accessibility of tryptophan residues to the neutral fluorescence quencher, acrylamide. Fig. 3A shows the Stern-Volmer plot of the quenching of the intrinsic fluorescence of α-crystallin when excited at 295 nm as a function of temperature. Fig. 3B shows the variation of \( F_0/F \) value (which is indicative of the extent of quenching) at the quencher concentration of 0.45 M as a function of temperature. This value starts increasing gradually above 30 °C and more sharply above 50 °C. This indicates that the tryptophan residues of α-crystallin are becoming more accessible to the quencher at temperatures above 30 °C. On the other hand, hen egg white lysozyme, a protein that is known to be stable up to temperatures as high as 75 °C (34), does not show such an increase in the accessibility of its tryptophan residues to the quencher with temperature as shown in Fig. 3B, suggesting that the quenching behavior seen in the case of α-crystallin is due to specific structural perturbation. Fig. 4 shows the variation of polarization of the intrinsic fluorescence of α-crystallin at 340 nm as a function of temperature. As seen from the figure, the polarization value decreases more significantly above 30 °C. This suggests that the restriction in the mobility of the tryptophans of α-crystallin due to the microenvironment (side chain packing) is significantly reduced at higher temperatures.

Fig. 5A shows the near UV-CD spectra of α-crystallin at various temperatures. The variation of the mean residual mass ellipticity at 272.5 nm with temperature is shown in Fig. 5B. The ellipticity value at 272.5 nm decreases gradually till about 50 °C and falls steeply to about zero by 62 °C. This result shows that at 62 °C, α-crystallin lacks any rigid tertiary structural packing. This result is consistent with the observed decrease in fluorescence polarization and the increased accessibility of tryptophan residues of α-crystallin to the quencher at higher temperatures. Thus, all our fluorescence and near UV-CD studies show that α-crystallin loses its tertiary structure with temperature, and the loss is more pronounced at temperatures above 50 °C.

As mentioned above, α-crystallin undergoes gradual tertiary structural alterations up to 50 °C and loses almost all its ter-
and pH (35, 36). It is, therefore, important to study the quaternary structure above this temperature. However, the secondary structure of α-crystallin does not seem to be altered significantly up to approximately 50 °C but is altered above this temperature. Since α-crystallin is a multimeric protein, it would be important to find out whether the quaternary structure is perturbed with temperature. The quaternary structure of α-crystallin, however, is not completely understood. There have been several models proposed for the quaternary structure of α-crystallin, a three-layered structure (35, 36), a micelle-like structure (37), a combination of the micellar and three-layer model (33), a rhombic dodecahedron (38), and a porcine-like structure (37), a combination of the micellar and three-layer model (33), a rhombic dodecahedron (38), and a porcine-like structure (39). In the absence of x-ray crystallographic data, the quaternary structure of α-crystallin remains speculative and is a matter of controversy.

The aggregation state of α-crystallin and hence the sedimentation coefficient and molecular mass also vary with experimental conditions such as ionic strengths, temperature, and pH (35, 36). It is, therefore, important to study the quaternary structure of α-crystallin (the aggregation state, size, etc.) with temperature under our present experimental conditions in the context of its chaperone-like activity. We have performed gel filtration chromatography of α-crystallin at different temperatures on a Sepharose S-300 (high resolution) column provided with a jacket for water circulation. The protein was allowed to pass through the column, which was equilibrated at the required temperatures. The column matrix used withstands high temperatures and is autoclavable (Pharmacia). The void volume of the column was measured at different temperature using the elution profile of blue dextran. It is evident from Fig. 6 that α-crystallin elutes at 37 °C as a marginally smaller particle compared with that at 22 °C. At 62 °C, the elution position of the major portion of α-crystallin with respect to the elution position of blue dextran is not very different from its position at 22 °C. However, the elution profile shows more heterogeneous populations of α-crystallin at 62 °C (Fig. 6). It is important to note that the rearrangement in the relative occupancies of the subunits with respect to one another or perturbations in the domain packing within the individual subunits with temperature, if any, may not be detected in the chromatographic profiles.

We also performed sedimentation analysis on α-crystallin samples pre-incubated at different temperatures. The sedimentation coefficient (s20,W) of α-crystallin in our experimental conditions is found to be about 18 S. It is reduced to 16.2 and 14.7 S upon incubating at 37 °C for 1 and 20 h, respectively. It is to be noted that our gel filtration experiments show only marginal change in the elution profile of α-crystallin at 37 °C. We have also found that the elution profile of α-crystallin sample, which is pre-incubated at 37 °C for 20 h, is also not changed appreciably (data not shown). Siezen et al. (36) showed by electron microscopy that the mean diameter of the temperature (37 °C)-modified α-crystallin is the same, within experimental error, as the native protein. The electron micrographs also essentially reveal the same shape. On the other hand, the size distributions of the two proteins differ significantly (compare Fig. 5A with Fig. 5E in Ref. 36), assuming that the sample preparation for electron microscopy did not affect the distribution. These investigators also showed that temperature-modified α-crystallin has a lower sedimentation coefficient, suggesting a structural difference as well as a difference in measured molecular mass. Whether the temperature-induced change in the molecular mass of the protein has any role in the chaperone-like activity of α-crystallin is not clear. The conversion of the native α-crystallin (18 S) to the smaller particle(s) appears to be a slow process. Siezen et al. (36) observe that the sedimentation coefficient is lowered appreciably upon incubating α-crystallin at 37 °C only after 5 h. Tardieu et al. report the
hydrodynamic radius of α-crystallin changes significantly by incubating at 37 °C for a day. The elution of α-crystallin in our gel filtration column is complete within 2 h of application of the sample to the column. It is also important to note that the assay procedure for the chaperone-like property of α-crystallin followed by us and other workers takes much less time. For example, the chaperone-like activity of α-crystallin toward the aggregation of β2-crystallin (upon refolding) in the present study is performed for 10 min. During this time period, no appreciable change in the sedimentation coefficient is expected. However, during this time period, there is a significant alteration in the tertiary structure of α-crystallin (Fig. 5). Thus, it appears that the tertiary structural alterations precede the quaternary structural alterations (in terms of molecular mass) of α-crystallin with temperature. We cannot, however, rule out the possibility of quaternary structural alterations in terms of the relative arrangement of subunits and/or packing of domains within the subunits themselves.

It is interesting to note the state of α-crystallin at 62 °C. At this temperature, α-crystallin almost completely loses its tertiary structure although possessing substantial amount of secondary structure. This state of α-crystallin is similar to that of the molten globule state of proteins. Molten globule states of proteins, detected on the unfolding or refolding pathways of proteins, are characterized as having substantial amounts of secondary structure although lacking rigid tertiary structure (41–43). In most of the earlier studies, the molten globule state was obtained either as equilibrium or kinetic intermediate on the unfolding or refolding pathways of monomeric proteins. Recently Singh et al. (44) reported an intermediate of the hexameric enzyme, glutamate dehydrogenase, with molten globule-like properties, based on differential scanning calorimetric studies. Our present study shows that α-crystallin, a multimeric protein, undergoes a transition at around 62 °C in which the near UV-CD spectrum indicates loss of all its tertiary structure, but the far UV-CD spectrum shows an increase in the ellipticity. Increase in the ellipticity in the far UV-CD spectra has also been observed for many proteins in their molten globule state (43). Thus, α-crystallin undergoes a transition to a molten globule-like state at 62 °C.

As mentioned earlier, we have shown that the chaperone-like activity of α-crystallin is more pronounced at temperatures above 30 °C (our earlier studies (17, 18) and the present study). On probing the hydrophobic surfaces of α-crystallin using pyrene, we have shown that the hydrophobic surfaces of α-crystallin are exposed above 30 °C with two perceptible transitions, one at −30 °C and another at −50 °C (17). Differential scanning calorimetric studies on α-crystallin by Walsh et al. (33) show two endothermic transitions, a relatively minor one at 35–45 °C and another major transition at around 60 °C. The chaperone-like activity of α-crystallin in preventing the aggregation of other proteins is generally studied at elevated temperatures (60–75 °C). Our results show that at these temperatures α-crystallin exists in a molten globule-like state. Thus, it appears that this state of α-crystallin is also capable of preventing the aggregation of other proteins. However, the transition observed around 30 °C seems to be important and biologically relevant. At this temperature, α-crystallin undergoes a minor change in its tertiary structure accompanying the exposure of its hydrophobic surfaces (17, 18) (due to the exposure of specific regions (30) of its sequence), whereas its secondary structure is relatively unchanged. It is possible that these changes are due to the reorganization of the subunits within the aggregate and/or small perturbation in the packing of domains within the subunits themselves. This state exhibits enhanced chaperone-like activity in preventing the aggregation of other proteins compared with those states below 30 °C. Our results also suggest that the chaperone assay performed at around physiological temperature would provide more meaningful results rather than those performed at elevated (>50 °C) or lower (<30 °C) temperatures.

Whether the temperature-dependent chaperone property of α-crystallin is common to all heat shock proteins and chaperones is not clear. However, recently there have been several reports of differential function of molecular chaperones at different temperatures. Hayer Hartl et al. (45) observe that the ATP hydrolysis is not required in the activation of rhodanese by the GroEL and GroES machinery at 37 °C, whereas it is essential at 20 °C (45). The heat shock protein, hsp90, known to function as molecular chaperone, binds more effectively to unfolded proteins in its thermally modified form (46). Brunscher et al. (47) report a similar temperature-dependent interaction between GroEL and phage P22 tail-spike protein: above 30 °C, folding intermediates bind to GroEL, whereas below 25 °C GroEL has no apparent interaction with the nascent protein. Hansen and Gafni (48) observe that GroEL, in the absence of GroES and ATP, enhances the refolding of glucose-6-phosphate dehydrogenase but fully arrests the process above 30 °C. Whether the properties of these chaperones at different temperatures are due to structural changes in these chaperones, as observed in the case of α-crystallin, is not known. It is possible that structural alterations induced by temperature form a part of the general mechanism of chaperone function, because they are required to function more effectively at nonpermissible temperatures.

Acknowledgments—We thank Dr. T. Ramakrishna for useful discussions and suggestions and N. Promod for help in sedimentation velocity measurements.

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Bakthisaran Raman and Ch. Mohan Rao

J. Biol. Chem. 1997, 272:23559-23564.
doi: 10.1074/jbc.272.38.23559

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