CHAPTER III

TEMPERATURE-INDUCED STRUCTURAL ALTERATIONS IN α-CRYSTALLIN
3.1. INTRODUCTION

As described in the previous chapter, α-crystallin exhibits chaperone-like activity in preventing the aggregation of other proteins in a temperature-dependent manner. A transition was observed in the chaperone-like activity of α-crystallin - its activity increases several fold at temperatures above 30°C. This implicates a possible structural transition of α-crystallin which is associated with its activity. In order to understand the structural basis for the temperature-dependent enhancement of the chaperone-like activity of α-crystallin, we have carried out circular dichroism, fluorescence, analytical ultracentrifugation and gel filtration studies at different temperatures.

3.2. MATERIALS AND METHODS

Sephacryl S-300 (high resolution) was purchased from Pharmacia, USA. Pyrene and 8-anilinonaphthalene-1-sulfonic acid (ANS) were purchased from Aldrich Chemical Company, USA. Acrylamide was procured from Serva. All other chemicals used in this study were of analytical grade.

3.2.1. Probing the hydrophobic surfaces of α-crystallin with pyrene

α-Crystallin solution (0.3 mg/ml) in 10 mM phosphate buffer, pH 7.2, containing 100 mM NaCl was equilibrated at the required temperature for 5min. Then 20μl (containing 1μMoles) of methanolic solution of pyrene was added to 1ml of α-crystallin solution and the resulting suspension was stirred for 30min (the solubilization was found to reach completion
within 15 minutes). The mixture was centrifuged at 5000 x g and the optical density of the supernatant was measured at 338 nm against blank solution containing the protein, in a Hitachi-330 UV-Vis. spectrophotometer. Fluorescence spectra were recorded in a Hitachi F4000 fluorescence spectrophotometer at the excitation wavelength of 338 nm with the excitation and emission band passes of 3 nm.

3.2.2. Probing the hydrophobic surfaces of α-crystallin with ANS

α-Crystallin (0.3mg/ml) in 10mM phosphate buffer of pH 7.4 containing 100mM NaCl was incubated with 20μM sodium 8-anilino-1-naphthalene sulfonic acid (ANS) at 20°C for 2 hrs. This sample was equilibrated at the required temperature in the sample holder of Hitachi F4000 fluorescence spectrophotometer using Julabo thermostated water bath for 5 min. The actual temperature of the sample was monitored as mentioned above. Fluorescence spectra were recorded with the excitation wavelength of 365nm. The excitation and emission band passes were 3nm and 1.5nm respectively.

3.2.3. Fluorescence of α-crystallin

Fluorescence measurements were performed using a Hitachi F4010 fluorescence spectrophotometer. For fluorescence studies, a 0.2mg/ml sample of α-crystallin in 30mM sodium phosphate buffer (pH 7.4) containing 100mM NaCl was used. Fluorescence studies 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 in side the cuvette was monitored using the Physitemp type-T
microthermocouple thermometer.

3.2.3.1. *Intrinsic fluorescence*

Fluorescence spectra were recorded with the excitation wavelength set at 295nm to preferentially excite tryptophan residues. The excitation and emission band passes were set at 5 and 1.5nm respectively. All spectra were recorded in corrected spectrum mode.

3.2.3.2. *Fluorescence polarization*

Fluorescence polarization measurements were performed using Hitachi fluorescence polarization accessory. The sample was excited at 295nm and the emission intensity was monitored at 340nm. Polarization values were calculated using the following equation.

\[
P = \frac{(I_{VV} - G I_{VH})}{(I_{VV} + G I_{VH})}
\]

The polarization value, \( P = \frac{(I_{VV} - G I_{VH})}{(I_{VV} + G I_{VH})} \) where \( I_{VV} \) and \( I_{VH} \) are the measured fluorescence intensities with the excitation polarizer vertically oriented and the emission polarizer vertically and horizontally oriented, respectively. \( G \) is a correction factor and is equal to \( I_{HV}/I_{HH} \).

3.2.3.3. *Quenching of intrinsic tryptophan fluorescence with acrylamide*

Fluorescence quenching experiments were performed using the neutral quencher, acrylamide. The sample was excited at 295nm. The emission intensity at 340nm was measured with successive additions of 7\( \mu \)l of 7M acrylamide to 1.2ml of the 0.2mg/ml \( \alpha \)-crystallin in 30mM sodium phosphate buffer (pH 7.4) containing 100mM 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.

3.2.4. Circular dichroism studies on α-crystallin

Circular dichroism spectra of α-crystallin at different temperatures was recorded using water jacketed cuvette. The required temperatures were maintained using a Julabo circulating water bath. The actual temperature inside the cuvette was measured using the Physitemp type T microthermocouple thermometer. A sample of 1mg/ml α-crystallin in 30mM sodium phosphate buffer (pH 7.4) containing 100mM NaCl was used. Far and Near UV-CD spectra were recorded using 0.01cm and 1cm pathlength cuvettes respectively. The spectra shown in the present study were the cumulative average of four repeated scans.

3.2.5. Gel filtration of α-crystallin at different temperatures

Gel filtration chromatography of α-crystallin was performed at different temperatures using a Sephacryl S-300 (high resolution) water jacketed column (0.9 x 80 cm) equilibrated at the required temperature using the Julabo circulating water bath. 0.3ml of 2.1mg/ml α-crystallin solution was loaded on the column equilibrated with 30mM sodium phosphate buffer (pH 7.4) containing 100mM NaCl and eluted with the same buffer. Fractions (0.5ml) were collected and absorbance at 280nm was measured. The elution profile of blue dextran (mol. mass 2000 kDa) on the column was obtained to measure the void volume at different temperatures.
3.2.6. **Sedimentation analysis**

Sedimentation velocity measurements were done on 4mg/ml native and temperature modified α-crystallin solutions using a Spinco E analytical ultracentrifuge. The detection system was based on Schlieren optics. Runs were performed at 60,000 rpm at an average temperature of $25 \pm 0.5^\circ$C. $S_{20,w}$ values were obtained by applying appropriate corrections for temperature and solvent viscosity.

3.3. **RESULTS AND DISCUSSION**

Since α-crystallin exhibits a temperature-dependent chaperone-like activity in preventing the aggregation of other proteins, it is necessary to understand the mechanism involved in such processes in terms of the temperature-induced structural changes of α-crystallin. We have attempted to investigate structural features of α-crystallin at different temperatures in the context of its chaperone-like activity (Raman and Rao, 1994 and 1997).

3.3.1. **Exposure of hydrophobic surfaces of α-crystallin with temperature**

As described in the previous chapter, γ-crystallin partially unfolds and exposes its hydrophobic surfaces upon irradiation with UV-light and α-crystallin prevents this photo-induced aggregation of γ-crystallin by forming a stable complex. This implies that the major force involved in the interaction of the chaperone and the target protein may be hydrophobic in nature.
Since we have observed that the chaperone-like activity of α-crystallin in preventing photo-aggregation of γ-crystallin depends on temperature, we have investigated the accessibility of the hydrophobic surface of α-crystallin as a function of temperature by solubilizing the hydrophobic fluorophore, pyrene. Pyrene is almost insoluble in water. α-Crystallin (0.3 mg/ml) increases the solubility of the apolar molecule, pyrene, to about 6-7 fold at room temperature when compared to the solubility in buffer alone. The free energy of transfer ($\Delta G_{tr}$) of pyrene from buffer to the α-crystallin solution is calculated to be -1055 cal/mol at 291 °K (see table 3.1), indicating an energetically favorable interaction. The solubility, as measured by the absorbance at 338 nm, increases remarkably after 30 °C as shown in figure 3.1.A which parallels the temperature-dependent protective ability of α-crystallin (chapter II). The solubility does not change significantly between 30-50 °C. There is a remarkable increase in the solubility above 50 °C. This second transition appears to correspond to the second transition observed in differential scanning calorimetric studies by Chakrabarti and co-workers (Walsh et al., 1991). The $\Delta G_{tr}$ of pyrene from buffer to α-crystallin solution at 312 °K and at 336 °K are calculated to be -1427 cal/mol and -2070 cal/mol respectively (table 3.1). Thus, the accessibility of the hydrophobic surface of α-crystallin, as reflected in the enhanced solubility of the hydrophobic molecule, increases with temperature.

A similar profile is observed when the hydrophobicity of the microenvironment of the solubilized pyrene molecule in α-crystallin is monitored as a function of temperature. The intensity ratio of the vibronic emission peak 3 to the vibronic emission peak 1 ($I_3/I_1$, also called as Ham ratio) of the fluorescence spectrum of pyrene is widely used as a reporter of the apolarity around the probe (Nakajima, 1976 a&b). In water, the ratio is around 0.5 and in
Table 3.1

Free energies of transfer of pyrene from buffer to α-crystallin

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Temperature, °K</th>
<th>$\Delta G_{tr}$ (cal.mol$^{-1}$)</th>
</tr>
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<tbody>
<tr>
<td>1.</td>
<td>291</td>
<td>-1055</td>
</tr>
<tr>
<td>2.</td>
<td>312</td>
<td>-1427</td>
</tr>
<tr>
<td>3.</td>
<td>328</td>
<td>-1905</td>
</tr>
<tr>
<td>4.</td>
<td>336</td>
<td>-2070</td>
</tr>
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* All measurements were made with a 0.3 mg/ml solution of α-crystallin in 10 mM phosphate buffer of pH 7.2 containing 100 mM NaCl. Concentrations of pyrene were measured using absorbance values at 338 nm. $\Delta G_{tr}$ were calculated from the equation, $\Delta G_{tr} = -RT \ln (C_a/C_b)$; where $C_b$ and $C_a$ are solubilities in buffer and in the α-crystallin solution.
Figure 3.1. Variation of accessibility of hydrophobic surfaces of α-crystallin with temperature. (A) Solubility of pyrene in α-crystallin with temperature. (B) The ratio of the vibronic emission peak 3 to peak 1 of fluorescence spectrum of pyrene solubilized in α-crystallin with temperature. (inset) Fluorescence spectra of pyrene in water (---) (I3/I1 = 0.54) and in methanol (---) (I3/I1 = 0.72): showing the vibronic emission peaks. If = normalized fluorescence intensity.
a hydrophobic environment the ratio approaches 1 or even more. It is evident from figure 3.1.B that α-cry stallin offers a surface of considerable hydrophobicity at 20 °C, as the ratio is about 0.79. The ratio increases with the solubilization temperature with distinct change in slope around 30 °C and 50 °C.

Figure 3.2 shows the change in the emission maximum of ANS bound to α-cry stallin with temperature. It is to be noted that this study was performed by monitoring the fluorescence of ANS bound to α-cry stallin at the respective temperature unlike in the case of pyrene where the spectra were recorded after cooling the sample to room temperature. As seen from the figure, the emission maximum is red shifted gradually above 30°C (and more sharply above 50°C), indicating that the ANS bound sites of α-cry stallin are getting exposed to water.

The protection ability of α-cry stallin against photo-aggregation of γ-cry stallin or DTT induced aggregation of insulin or aggregation of β₅-cry stallin upon its refolding parallels these trends obtained using pyrene and ANS, suggesting the involvement of hydrophobic interactions between α-cry stallin and the target proteins.

Hydrophobic interactions play a significant role in intra- and inter-protein interactions including the recognition between chaperones and partially unfolded proteins. GroEL binds to the "molten globule" like state (with increased hydrophobicity) of dihydrofolate reductase and rhodanese (Martin et al., 1991). Some proteins and enzymes in the partially opened state expose the hydrophobic surface which leads to non-specific hydrophobic interactions and aggregation in the absence of chaperones. Thus appropriately
Figure 3.2. Fluorescence of α-crystallin bound ANS as a function of temperature.
placed hydrophobic surfaces appear to be important in chaperone activity.

3.3.2. Secondary structure of α-crystallin

α-Crystallin has been shown to have mainly a β-sheet structure and almost no α-helices as monitored by far-UV CD spectrum (Li and Spector, 1974; Liang and Chakrabarti, 1982; Siezen and Argos, 1983). Also, Fourier-transform infrared spectroscopic analysis indicated the presence of 40-50% β-sheet and 5-10% α-helical structure (Lamba et al., 1993). The temperature-induced alterations of the secondary structure of α-crystallin remain controversial. Maiti et al. (1988) have studied the secondary structure of α-crystallin by far UV-circular dichroism and reported that α-crystallin is heat stable and does not denature at temperatures up to 100 °C. Surewicz and Olesen (1995) have studied Fourier-transformed infrared spectroscopy of α-crystallin and reported that α-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. (1991) and our own observation (Raman and Rao 1994). In order to understand the temperature effect on the secondary structure of α-crystallin, we have recorded circular dichroism spectra of α-crystallin at different temperatures. Figure 3.3.A shows the far UV-CD spectra of α-crystallin at different temperatures. Figure 3.3.B shows the change in the mean residual ellipticity at 205 nm and 222 nm as a function of temperature. It is evident from the figure that the ellipticity value at 205 nm or 222 nm is not significantly altered below 50 °C. Above 50°C, the ellipticity at 205 nm increases sharply while the ellipticity at 222 nm increases only marginally. This result is consistent with the earlier report of Maiti et al. (1988) and Surewicz and Olesen (1995). However, it is to be noted that the far UV-CD spectra of α-crystallin above 50 °C do not indicate unfolding of
Figure 3.3. (A) Far UV-CD spectra of α-crystallin at different temperatures; (---) 20°C; (-------) 37°C; (.........) 47°C; (-.-.-.) 58°C and (-.-.-.-.) 62°C. (B) The mean residue weight ellipticity of α-crystallin at (○) 205nm and (●) 222nm at different temperatures.
the protein to a random coil structure but indicate an alteration in the native structure.

3.3.3. **Tertiary structure of α-crystallin**

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. We have, therefore, studied the tertiary structure of α-crystallin and its alteration with temperature using fluorescence and near UV-circular dichroism. We have observed that the fluorescence emission maximum of α-crystallin is shifted from 335±1nm at 20 °C to 338±1nm at 62 °C. This indicates that the tryptophan residues of α-crystallin are relatively, albeit marginally, more exposed to the solvent at 62 °C. It does not indicate complete exposure of tryptophan residues as in the case of complete unfolding of proteins. We have 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. Figure 3.4.A shows the Stern-Volmer plot of the quenching of the intrinsic fluorescence of α-crystallin, when excited at 295nm, as a function of temperature. Figure 3.4B shows the variation of Fo/F value (which is indicative of the extent of quenching) at a fixed quencher concentration of 0.354M 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. Hen egg white lysozyme, a protein which is known to be stable up to temperatures as high as 75°C (Shih et al., 1995), on the other hand, does not show such an increase in the accessibility of its tryptophan residues to the quencher with temperature as shown in Figure 3.4B. This
Figure 3.4. (A) Stern-Volmer plot of tryptophan fluorescence quenching of $\alpha$-crystallin at (o) 20°C, (●) 30°C, (Δ) 37°C, (▲) 46°C and at (□) 58°C by acrylamide. (B) The extent of quenching ($F_0/F$; where $F_0$ and $F$ are fluorescence intensity at 340nm in the absence and in the presence of the quencher respectively. The excitation wavelength was 295nm) of the fluorescence of (o) $\alpha$-crystallin and (●) lysozyme at different temperatures. The extent of quenching was determined at the quencher concentrations of 0.354M and 0.275M for $\alpha$-crystallin and lysozyme respectively.
suggests that the quenching behaviour seen in the case of \( \alpha \)-crystallin is due to a specific structural perturbation.

Fluorescence polarization measurements reveal the mobility of a fluorophore and the effect of its microenvironment on its mobility. Upon excitation with plane polarized light, the emitted light from fluorophores is also polarized. This polarization is a result of the photoselection of fluorophores according to their orientation relative to the direction of the polarized excitation. However, the emission is depolarized if the mobility of the fluorophore is unrestricted: Rotational diffusion of fluorophores is a common cause of depolarization. In proteins, the tertiary structure (side chain packing) is the major factor which reduces the mobility of the fluorophore, tryptophan. Thus, the extent of fluorescence polarization is a measure of the restriction that the microenvironment imposes on the mobility of the tryptophan residues of proteins. Figure 3.5 shows the variation of polarization value of the intrinsic fluorescence of \( \alpha \)-crystallin at 340nm 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 \( \alpha \)-crystallin due to the microenvironment (side chain packing) is significantly reduced at higher temperature.

Figure 3.6A shows the near UV-CD spectra of \( \alpha \)-crystallin at various temperatures. The variation of the mean residual weight ellipticity at 272.5nm with temperature is shown in Figure 3.6B. 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, \( \alpha \)-crystallin lacks any rigid tertiary structural packing. This result is consistent with the observed decrease in fluorescence polarization and the increased accessibility of the tryptophan residues of \( \alpha \)-
Figure 3.5. Polarization of the tryptophan fluorescence of α-crystallin at various temperatures.
Figure 3.6. (A) Near UV-CD spectra of α-crystallin at different temperatures: (-----) 20°C; (-------) 37°C; (.........) 47°C; (-.-.-.-) 58°C and (-.-.-.-.) 62°C. (B) The mean residue weight ellipticity at 272nm of α-crystallin at different temperatures.
crystallin to the quencher, acrylamide at higher temperatures. Thus, all our fluorescence and near UV-CD studies show that \( \alpha \)-crystallin loses its tertiary structure with temperature and the loss is more pronounced at temperatures above 50°C.

3.3.4. Quaternary structure of \( \alpha \)-crystallin

As mentioned above, \( \alpha \)-crystallin undergoes gradual tertiary structural alterations up to 50°C and loses almost all its tertiary structure above this temperature. However, the secondary structure of \( \alpha \)-crystallin does not seem to be altered significantly up to approximately 50°C but is altered above this temperature. Since \( \alpha \)-crystallin is a multimeric protein, it is possible that its quaternary structure is also perturbed with temperature. The quaternary structure of \( \alpha \)-crystallin, however, is not completely understood. There are several models proposed for the quaternary structure of \( \alpha \)-crystallin - a three-layered structure (Siezen et al., 1980; Tardieu et al., 1986), a micelle-like structure (Augusteyn and Koretz, 1987), a combination of the micellar and three-layer model (Walsh et al., 1991), a rhombic dodecahedron (Wistow, 1993) and a pore like structure (Carver et al., 1994). However, none of these models explains all the properties of \( \alpha \)-crystallin. In the absence of X-ray crystallographic data, the quaternary structure of \( \alpha \)-crystallin remains speculative and is a matter of controversy. \( \alpha \)-Crystallin is isolated as spherical molecules with diameter of about 15nm and molecular mass of 800-900 kDa \((S_{20,w} \sim 17-18S, \text{also called as } \alpha_c\text{-crystallin})\) (Siezen et al., 1980). Thomson and Augusteyn (1983) reported that when the isolation is performed at 37°C, \( \alpha \)-crystallin exhibits a molecular weight of approximately 360kDa \((S_{20,w} \sim 12S, \text{called as } \alpha_m\text{-crystallin})\). van den Oetelaar et al., (1985), however, reported that at physiological ionic strength, pH and temperature, \( \alpha \)-crystallin is
still isolated as a large aggregate of molecular mass of 800kDa. The aggregation state of α-crystallin and hence the sedimentation coefficient and molecular mass vary with the experimental conditions such as ionic strengths, temperature and pH (Siezen et al., 1980; Tardieu et al., 1986). We have performed gel filtration chromatography of α-crystallin at different temperatures on a Sephacryl S-300 (high resolution) column provided with a jacket for water circulation. The protein is allowed to pass through the column which is equilibrated at the required temperatures. The column matrix used withstands high temperatures and is autoclavable (Pharmacia catalogue). The void volume of the column was measured at different temperatures using the elution profile of blue dextran. α-Crystallin elutes at 37°C as a marginally smaller particle compared to that at 22°C as shown in figure 3.7. At 62°C, the elution position of the major portion of α-crystallin with respect to the elution position of blue dextran (at 62°C) is almost the same as at 22°C. However, the elution profile shows more heterogeneous populations of α-crystallin at 62°C (figure 3.7). 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 have also performed sedimentation analysis on α-crystallin samples pre-incubated at different temperatures. The sedimentation coefficient ($S_{20,w}$) of α-crystallin in our experimental conditions is found to be about 18S. It is reduced to 16.2S and 14.7S upon incubating at 37°C for 1hr and 20hr 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 20hrs is also not changed appreciably (data not shown). Siezen et al. (1980) found
Figure 3.7. Elution profile of α-crystallin at (o) 22°C, (●) 37°C and at (△) 63°C on a Sephacryl S-300 (High Resolution) column. The elution positions of blue dextran (Mol. wt 2000 kDa) at (a) 63°C; (b) 22 or 37°C, (c) thyroglobulin (669 kDa), (d) ferritin (440 kDa), (e) catalase (232 kDa) and (f) γ-crystallin (20 kDa) are also indicated. Refer section 3.2 for details.
that the temperature (37°C)-modified α-crystallin is indistinguishable from the native protein by electron microscopy (in shape and size of the particle) but has a lower sedimentation coefficient. They have suggested a structural expansion for the temperature-modified protein. Whether the temperature-induced change in the molecular weight of the protein is important in the chaperone-like activity of α-crystallin is not clear. The conversion of the native α-crystallin (18S) to the smaller particles (16-14S) appears to be a slow process. Siezen et al. (1980) have observed that the sedimentation coefficient is lowered appreciably upon incubating α-crystallin at 37°C only after 5hrs. Tardieu et al. (1986) have reported that the hydrodynamic radius of α-crystallin decreases significantly by incubating at 37°C for a day. The elution of α-crystallin in our gel filtration column is complete within 2hrs of application of the sample to the column. It is also important to note that the assay for the chaperone-like property of α-crystallin performed by us and other workers takes much less time. For example, the chaperone-like activity of α-crystallin towards the aggregation of βI-crystallin (upon refolding) is performed within 10min (Chapter II). During this time period, no appreciable change in the sedimentation coefficient is expected. However, during this time period, there is an observable change in the tertiary structure of α-crystallin (figure 3.6). Thus, it appears that the tertiary structural alterations may be important for increased chaperone-like activity of α-crystallin. However, we cannot 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, which might also result in the observed enhancement in the chaperone-like activity of α-crystallin.

3.3.5. Molten-globule like state of α-crystallin
It is interesting to note the state of α-crystallin at 62°C. At this temperature, α-crystallin almost completely loses its tertiary structure while 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 characterised as a state having substantial amount of secondary structure while lacking rigid tertiary structure (Ptitsyn, 1992 & 1995; Baldwin, 1993). In most of the earlier studies, the molten-globule state was obtained as either an equilibrium or a kinetic intermediate on the unfolding or refolding pathways of monomeric proteins. Recently, Sing et al. (1996) have 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 (Ptitsyn, 1992). Thus, it appears that α-crystallin may be a second example of molten-globule state observed in a multimeric protein.

3.3.6. Which conformational states of α-crystallin are relevant to its chaperone-like activity in vivo?

As mentioned earlier in chapter I, we have shown that the chaperone-like activity of α-crystallin is more pronounced at temperatures above 30 °C. 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 around 30 °C and
another at around 50 °C (Raman and Rao, 1994). Subsequently, Das and Surewicz (1995a) also reported similar temperature-induced exposure of hydrophobic surfaces of α-crystallin using bis-ANS. Smith et al. (1996) used hydrogen-deuterium exchange of amide proton to study α-crystallin as a function of temperature. Their observation supported our hypothesis that α-crystallin prevents the aggregation of non-native structures of target proteins by providing appropriately placed hydrophobic surfaces (Raman and Rao, 1994) and extended it further by suggesting the regions of α-crystallin that may become exposed with temperature (Smith et al., 1996). They have observed that the hydrophobic regions around the residues 32-37 and 72-75 of αA and 28-34 of αB become solvent exposed above 30°C. Earlier differential scanning calorimetric studies on α-crystallin by Walsh et al. (1991) 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 temperature (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. Around the physiological temperature (37°C) α-crystallin undergoes a minor but detectable change in its tertiary structure accompanying the exposure of its hydrophobic surfaces (Raman and Rao, 1994; Raman et al., 1995) (perhaps due to the exposure of specific regions (Smith et al., 1996) of its sequence) while 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 to 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.

3.3.7. Temperature effect on the chaperone activity of other molecular chaperones

Whether the temperature-dependent chaperone-property of α-crystallin is common to all heat shock proteins and chaperones is not known. However, recently there have been several reports of differential function of molecular chaperones at different temperatures. Hayer Hartl et al., (1996) have observed that ATP hydrolysis is not required in the activation of rhodanese by the GroEL and GroES machinery at 37°C while it is essential at 20°C. The heat shock protein, hsp90, known to function as molecular chaperone, binds more effectively to unfolded proteins in its thermally modified form (Yonehara et al., 1996). Brunschier et al., (1993) have reported a similar temperature-dependent interaction between GroEL and phage P22 tailspike 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 (1994) have observed 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. Leung et al., (1996) have reported a thermal activation of bovine Hsc70 molecular chaperone at physiological temperatures. Whether the properties of these chaperones at different temperatures are due to structural changes in them, 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 non-permissible temperatures.
3.4. CONCLUSIONS

(i) Above 30°C α-crystallin undergoes a minor change in its tertiary structure accompanying the exposure of its hydrophobic surfaces. The secondary structure of α-crystallin is relatively unchanged up to approximately 50°C, but is altered above this temperature. These changes may involve reorganization of the subunits within the aggregate and/or small perturbation in the packing of domains within the subunits themselves.

(ii) At around 62°C α-crystallin undergoes a transition to a molten-globule like state.

(iii) All these results prove the hypothesis stated in Chapter I that α-crystallin prevents the aggregation of non-native structures of proteins by providing appropriately placed hydrophobic surfaces; a transition above 30°C in such a process is indicative of a structural transition involving reorganization of its hydrophobic surfaces which is important in the chaperone-like activity of α-crystallin.

(iv) 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 non-permissible temperatures.