CHAPTER IV

RAPID REFOLDING STUDIES ON CRYSSTALLINS
4.1. INTRODUCTION

Several proteins need the assistance of molecular chaperones to fold correctly to their native state. Whether chaperones themselves need such assistance is not clear. Few studies have attempted to answer this question. Chemically synthesised GroES not only folds and assembles properly, but also shows complete activity in refolding of rubisco in the presence of GroEL (Mascagni et al., 1991). Urea unfolded GroES regains its native assembly upon removing the denaturant (Mascagni et al., 1991). GroEL, dissociated to its monomeric form by urea reassembles to its native polymeric form in the presence of Mg-ATP and the presence of native GroEL enhances such reassembly (Lissin et al., 1990). A more recent study shows that GroEL refolds and assembles to its native dodecameric structure in the presence of salts (Ybarra and Horowitz, 1995). In order to find out whether α-crystallin spontaneously refolds to its native state without any external assistance, we have performed rapid refolding of the protein from its denatured state in urea (Raman et al., 1995b). In addition, we have also carried out rapid refolding studies on the other lenticular crystallins such as the multimeric β- and the monomeric γ- crystallins.

4.2. MATERIALS AND METHODS

4.2.1. Unfolding and rapid refolding of crystallins

Crystallins were dissolved in 20mM phosphate buffer, pH 7.4, containing 8 M urea and equilibrated at room temperature for 5 hrs. Rapid refolding was achieved by diluting 50μl of this sample into 950μl of 10mM phosphate buffer (pH 7.4) free of denaturant. The refolding buffer also contained 100mM NaCl. The solution was vortexed for 30 sec and
equilibrated at room temperature for 1 hr. Turbidity of the solutions was measured by monitoring the optical density at 500nm in a Hitachi-330 spectrophotometer. The samples were centrifuged at 5000 g for 15 min. and the optical density at 280nm of the supernatant was measured. In another experiment, 50μl of the sample in denaturant (8M urea) containing 0.3mg of β- or γ-crystallin was added to 950μl of refolding buffer containing the required amount of α-crystallin. Rapid refolding of β- or γ-crystallin together with α-crystallin was performed by adding 50μl of the denatured protein sample containing 0.3 mg of β- or γ-crystallin and varying amounts of α-crystallin, to 950 μl of the refolding buffer. The absorption coefficients (for 1mg/ml) of 0.83, 2.3, 2.1 and 2.1 for α- (Thomson and Augusteyn, 1983), βH, βL- and γ-crystallins (Siezen, 1984) respectively were used for determination of their concentrations. The percentage recovery of the target protein was calculated from the OD280 assuming that recovery of α-crystallin is 100%. This assumption was verified in a separate experiment where only α-crystallin was refolded as mentioned earlier.

4.2.2. Fluorescence spectroscopy

Fluorescence measurements were made on a Hitachi F4000 fluorescence spectrophotometer. Synchronous scanning fluorescence spectra were recorded as described by Rao (1991). The excitation and emission monochromators were set at 250 nm and 290 nm respectively to get Δλ of 40 nm and both the monochromators were scanned simultaneously. Red edge excitation shift study (Rao et al., 1989; Rao and Rao, 1994) was performed by taking fluorescence spectra of the native and refolded α-crystallin with excitation wavelengths of 280, 295, 300, 305 nm. The excitation and emission band passes were set at 3
and 1.5 nm respectively.

4.2.3. *Circular dichroism measurements*

Near and far UV CD spectra of the native and refolded α-crystallins were recorded on a Jasco J-20 or Jasco J-715 spectropolarimeter. Alpha-crystallin refolded at 1mg/ml was used for CD studies. Spectra were recorded using 0.05cm and 2cm path length cells for far UV and near UV regions respectively.

4.2.4. *Sedimentation velocity measurements*

Sedimentation velocity measurements were carried out on native and refolded α-crystallin solutions (4mg/ml) using a Spinco E analytical ultracentrifuge. The detection system was based on Schlieren optics. Runs were performed at 68000 rev./min at an average temperature of 25±0.5°C. S_{20,w} values were obtained by applying appropriate corrections for temperature and solvent viscosity.

4.2.5. *Gel filtration experiments*

Gel filtration experiments were performed using a Bio Gel A-1.5m column (0.8 x 96 cm) equilibrated with Tris-HCl buffer, pH 7.2, containing 100mM NaCl, 1mM EDTA and 0.02% sodium azide with a flow rate of 15ml/hr. Fractions (0.9 ml) were collected and the optical density at 280nm was monitored.
4.3. RESULTS AND DISCUSSION

We have studied the refolding properties of α-, β- and γ- crystallins using the rapid refolding method (Raman et al., 1995b). This is one of the widely used methods (Saxena and Wetlauffer, 1970; Tandon and Horowitz, 1989; Kumar et al., 1994) to study the refolding properties of a protein where the protein from its unfolded state in denaturants such as urea or GdmCl is rapidly transferred to condition(s) which favour its folding as opposed to the slow process of removing the denaturant by dialysis. Crystallins were subjected to refolding at different concentrations by 20-fold dilution of the denatured stock in 8M urea into the refolding buffer. Figure 4.1A shows the turbidity profiles of crystallins as a function of the amount of protein taken for the refolding. Interestingly, α-crystallin remains clear upon rapid refolding in the concentration range studied, while βH, βL and γ- crystallins aggregate and yield turbid solutions. Turbidity increases with concentration. Refolding of α-crystallin even at a concentration of 13mg/ml yields a clear solution. The amount of protein recovered in the soluble form, after removing the precipitate by centrifugation, is shown in figure 4.1B. As evident from the figure, α-crystallin is almost completely recovered in the soluble form while the other crystallins kinetically partition to aggregate in a concentration-dependent manner. The recovery of γ-crystallin appears to be comparatively lower. This may be either because γ-crystallin is more prone to aggregation or due to other factors such as preferential adsorption to the surface of the reaction tube.

4.3.1. Characterization of refolded α-crystallin

Since the refolding of α-crystallin yields a clear solution, we performed circular
Figure 4.1. Rapid refolding of \( \alpha\)-\((-\circ-), \beta_H\)-\((-\bullet-), \beta_L\)-\((-\Delta-), and \gamma\)-\((-\blacktriangle-\text{c}rystallins. (A) Turbidity profile upon refolding at different concentrations of crystallins. Turbidity was measured as the optical density at 500nm. (B) Variation of recovery of the crystallins in soluble form as a function of concentration. Percentage recovery of proteins is with respect to the amount taken for refolding. Concentrations of the crystallins are those obtained after dilution into refolding buffer.
dichroism (CD) and fluorescence spectroscopy to determine whether the refolded α-crystallin attains its native conformation. Figure 4.2A shows the far UV CD spectra of native and refolded α-crystallin. These spectra overlap within experimental error, suggesting that it regains almost all its secondary structure. Comparison of near UV CD spectra of native and refolded α-crystallin (figure 4.2B) suggests that tertiary structure is also regained.

The fluorescence emission maxima of native and refolded α-crystallins, upon excitation at 295 nm, do not differ significantly; they are 336 nm and 336.6 nm respectively. The synchronous spectrum of a mixture of fluorophores or fluorophore in different local microenvironment is likely to be more informative than the normal emission or excitation spectrum (Lloyd and Evett, 1977). Synchronous scan might be expected to provide a signature and reflect minor structural perturbations. An earlier study on α-, β-, and γ-crystallins using synchronous fluorescence scanning suggests that the synchronous scan spectra of these crystallins are distinguishable and appear to provide specific signature (Rao (1991)). The synchronous scan spectra of the native and refolded α-crystallin superimpose on each other as shown in figure 4.3, indicating that the microenvironments of the aromatic amino acids are indistinguishable in both the cases.

The shift in the fluorescence emission wavelength upon excitation with the wavelength of red edge of the absorption spectrum of a fluorophore is termed as red edge excitation shift (REES). This effect is usually observed for polar fluorophores in motionally restricted media (Demchenko, 1982; Lakowicz and Keating-Nakamoto, 1984; Macgregor and Weber, 1986; Demchenko, 1988; Rao et al., 1989; Chattopadhyay and Mukherjee, 1993; Rao and Rao, 1994). Single-tryptophan proteins (Demchenko, 1988), crystallins in intact eye lens (Rao, et al., 1989) and in solutions (Rao and Rao, 1994) have been studied using this...
Figure 4.2. Circular dichroism of native (-----), refolding intermediate (with in 6 min. of initiation of refolding) (............) and refolded (at 1 mg/ml)(-------) α-crystallin. (A) Far UV CD spectra. (B) Near UV CD spectra.
Figure 4.3. Synchronous fluorescence spectra of native (——) and refolded (-----) α-crystallin. Δλ = set at 40nm. Both excitation and emission band passes were set at 3nm. $I_f$ = normalised fluorescence intensity (arbitrary units); $\lambda$ = excitation wavelength (nm).
technique. The magnitude of the shift in the observed emission maximum can be correlated to the restriction that the microenvironment imposes on the fluorophore. Both the native and the refolded α-crystallin show a 6 nm REES (336 to 342 nm) upon shifting the excitation wavelength from 295 nm to 305 nm. This indicates that the microenvironments around the tryptophans in the native and the refolded α-crystallin (or at least its influence on the mobility of the tryptophans) are identical.

To find out the state of the quaternary structure of the refolded protein, we have performed gel filtration chromatography and sedimentation velocity measurement. Analysis of the elution profiles (figure 4.4) of the native and refolded α-crystallin suggests that the refolded α-crystallin might form a marginally smaller multimer compared to the native form. The sedimentation coefficient ($S_{20,w}$ value) of the native α-crystallin, determined to be 17-18S, is in good agreement with the reported value for the αc form (Siezen et al., 1980). The sedimentation pattern of the refolded α-crystallin shows a single peak (figure 4.5) with a $S_{20,w}$ value of 16 suggesting that the size of the refolded α-crystallin is marginally smaller than the native α-crystallin. Packing alterations, if any, without significant shape and size changes might not be detected in the sedimentation studies.

These results indicate that α-crystallin regains most of its three dimensional structure upon rapid refolding and it does not need any external assistance. RNase A which is known to refold completely to its active form at low concentrations (<0.1mg/ml), misfolds and aggregates upon refolding at concentrations higher than 0.1 mg/ml (Kumar et al., 1994). It is interesting to note that α-crystallin refolds to its native state even at concentration as high as 13 mg/ml.
Figure 4.4. Elution profile of native (-o-) and refolded (-•-) \(\alpha\)-crystallin on a Bio Gel A-1.5m column. The amount of protein loaded was 1mg in both the cases. The elution positions of a, blue dextran (2000 kDa); b, thyroglobulin (670 kDa) and c, lysozyme (14.4 kDa) are indicated by arrows.
Figure 4.5. Sedimentation pattern of native (A) and refolded (B) α-crystallin.
4.3.2. Refolding of β- and γ-crystallin in the presence of α-crystallin

As described in chapter II, γ-crystallin undergoes photo-aggregation and α-crystallin prevents this aggregation by forming a complex in a temperature-dependent manner. Alpha-crystallin is also known to prevent the thermal aggregation of β- and γ-crystallins (Horwitz, 1992). We find that rapid refolding of β- and γ-crystallins also leads to aggregation and turbidity (figure 4.1). We have, therefore, studied the effect of α-crystallin on this aggregation of β- and γ-crystallins. The recovery of these proteins in the soluble form increases to some extent when α-crystallin is present in the refolding buffer. The recovery of these proteins with increasing concentration of α-crystallin is shown in figure 4.6. The increase in the α-crystallin concentration marginally enhances the recovery of β- and γ-crystallin as shown in the figure. Even a ten fold excess α-crystallin does not improve the recovery of γ-crystallin to more than 25-30%.

It is important to note that these experiments were performed at room temperature (25°C). As described in the earlier chapters, the chaperone-like activity of α-crystallin is enhanced above 30°C due to structural perturbations. Temperature-dependent chaperone-like activity of α-crystallin is also observed on the refolding of β-crystallin (see chapter II, figure 2.10).

4.3.3. Co-refolding of β- or γ-crystallin with α-crystallin

As described in the earlier section, the presence of α-crystallin in the refolding buffer
Figure 4.6. Refolding of $\beta_H$ (•), $\beta_L$ (-o-) and $\gamma$ (-Δ-) crystallins in the presence of native $\alpha$-crystallin. The percentage recovery of protein is with respect to the amount of protein taken for refolding which is 0.3mg. The amount of $\alpha$-crystallin in the refolding buffer was varied to get different $[\alpha]/[p]$ (w/w) ratios. $[p] =$ Concentration of $\beta$- or $\gamma$-crystallin.
does not prevent the aggregation of \( \beta \)- or \( \gamma \)-crystallins. Interestingly, when \( \beta \)- or \( \gamma \)-crystallins are denatured together with \( \alpha \)-crystallin and then subjected to refolding, the recovery of \( \beta \)- or \( \gamma \)-crystallins in the solution is increased significantly. Figure 4.7A represents the turbidity profile of solutions obtained by refolding of 0.3mg of \( \beta \)- or \( \gamma \)-crystallin together with increasing amounts of \( \alpha \)-crystallin; the percentage recovery of these proteins is shown in figure 4.8B. Even at a 5 times lesser concentration (w/w), \( \alpha \)-crystallin is able to almost completely prevent the aggregation of \( \beta \)-crystallin. Recovery of \( \gamma \)-crystallin changes from 20% to 60% (figure 4.6 and 4.7B). Thus, when \( \alpha \)-crystallin is refolded together with \( \beta \)- or \( \gamma \)-crystallin, it prevents the aggregation of \( \beta \)- or \( \gamma \)-crystallin very significantly. It would appear that an intermediate state (or assembly) of \( \alpha \)-crystallin formed in its refolding pathway is more effective in preventing the aggregation of the other two crystallins. In order to investigate this transient state, we have studied \( \alpha \)-crystallin refolded from its denatured state in 8M urea within 2-6 min of the initiation of the refolding (figure 4.2). The state of \( \alpha \)-crystallin during this time period is referred to as an "intermediate". The far UV-CD spectrum of this intermediate shows a substantial amount of secondary structure (figure 4.2A). However, the spectral profile is similar to that of \( \alpha \)-crystallin at higher temperatures (e.g., 58°C in figure 3.3). The near UV-CD spectrum of the intermediate, on the other hand, shows a highly reduced tertiary structure (figure 4.2B). All these results suggest that \( \alpha \)-crystallin passes through a kinetic intermediate state on its refolding pathway which is similar to those states of \( \alpha \)-crystallin observed at higher temperatures (see chapter III). This intermediate, possessing more exposed hydrophobic surfaces, interacts with the aggregation prone intermediate states of \( \beta \)- or \( \gamma \)-crystallins and prevents their aggregation upon corefolding with these crystallins. These results show that \( \alpha \)-crystallin is more functional in its structurally perturbed state in preventing the aggregation of other crystallins. Structurally
Figure 4.7. Refolding of $\beta_H$ (-•-), $\beta_L$ (-○-) and $\gamma$ (-Δ-) crystallins together with $\alpha$-crystallin. (A) Turbidity profile upon refolding of mixtures of $\alpha$- and $\beta$- or $\alpha$- and $\gamma$- crystallins at different ratios. Turbidity was measured as optical density at 500nm. (B) Recovery of $\beta$- or $\gamma$- crystallins in soluble form as a function of $[\alpha]/[p]$ ratios. The percentage recovery of protein is with respect to the amount of protein taken for refolding which is 0.3mg. The amount of $\alpha$-crystallin was varied to get different $[\alpha]/[p]$ (w/w) ratios. $[p] = \text{Concentration of} \beta$- or $\gamma$-crystallin.
perturbed state, thus, is important for the chaperone-like activity of α-crystallin.

4.4. CONCLUSIONS

(i) α-Crystallin rapidly refolds and regains most of the secondary, tertiary and quaternary structures. This might be a common property of chaperones and heat shock proteins.

(ii) The other eye lens crystallins such as β- and γ- crystallins aggregate and precipitate out upon such rapid refolding.

(iii) α-Crystallin in the refolding buffer does not prevent the aggregation of β- or γ-crystallin. However, if β- or γ- crystallin is denatured together with α-crystallin and then subjected to the refolding process, α-crystallin almost completely prevents the aggregation of other crystallins.

(iv) A kinetic intermediate state of α-crystallin (2-6min after initiation of refolding) having substantial secondary structure but much reduced tertiary structure is observed. This intermediate of α-crystallin, formed during its refolding, is more effective in preventing the aggregation of β- or γ- crystallins compared to the native α-crystallin.