Chapter 4

THE TRIFLUOROACETIC ACID-INDUCED MOLTEN GLOBULE-LIKE STATES OF α-CRYSTALLIN AND ITS SUBUNITS
4.1. Introduction

Globular proteins are believed to undergo unfolding co-operatively with an equilibrium between the completely folded native state and the unfolded state. The relative population of these two states in equilibrium depends on conditions such as denaturant concentration, pH and temperature. Such "all or none" transitions have been observed for small proteins (Privalov, 1979) and protein domains (Privalov, 1982). However, several globular proteins have been reported to unfold through partially folded or unfolded intermediate states. The most well characterised partially folded or unfolded intermediate state(s) is the so called molten-globule state(s) (Ohgushi and Wada, 1983). These are observed either as equilibrium intermediate states in unfolding pathway or as kinetic intermediate states in the refolding pathway, exhibiting native-like or substantial amount of secondary structure with little or no tertiary structure and highly solvent exposed hydrophobic surfaces (Ptitsyn, 1992). Such intermediate states of several proteins have been studied in urea (Holzman et al., 1986), guanidinium chloride (Herold and Kirshner, 1990) and temperature-induced unfolding (Poklar et al., 1997), in extremely low (Fink et al., 1990) or high pHs (Goto and Fink, 1989), in presence of trichloroacetic acid, trifluoroacetic acid and their salts (Goto et al., 1990) and in the presence of trifluoro ethanol (Buck et al., 1993). Most of the earlier studies are concerned with the molten-globule states of monomeric proteins. However, only few studies are available on the molten-globule states of multimeric proteins.

Earlier studies from our laboratory on the temperature-induced structural changes of α-crystallin found that structurally perturbed α-crystallin exhibits enhanced chaperone-like property (Raman and Rao, 1994, 1997). Under these conditions, α-crystallin exists as a multimeric molten-globule (Raman and Rao, 1997). A similar multimeric unfolding intermediate of α-crystallin has been observed in 1.5 M GdmCl which has altered tertiary structure and prevents the aggregation of β-crystallin much better than the native state (Das and Liang, 1997). In our attempts to address the molecular basis for the requirement of two gene products, αA- and αB-crystallin for α-crystallin, their possible modulatory role and their differential activity and stability, we have investigated the trifluoroacetic
acid-induced unfolding of homo- and hetero-aggregates of αA- and αB-crystallins. αA- and αB-Crystallins lose native tertiary structure, but retain significant secondary structure in the presence of 0.1% TFA. However αA- and αB-crystallin display a differential behaviour with regard to the aggregation status of the unfolding intermediates. While αA-crystallin forms a multimeric molten globule state, αB-crystallin exhibits a monomeric molten globule State, in the presence of trifluoroacetic acid. The oligomeric status of the molten globule-like state of the α-crystallin heteroaggregate is strongly dependent on the salt concentration. Unlike αA-crystallin, at very low salt concentrations, αB-crystallin exists as a random coil in the presence of TFA. Our results also indicate the higher susceptibility of αB-crystallin to pH dependent structural change. These results should also prove useful in understanding of the partially folded/unfolded states of multimeric proteins in general.

4.2. MATERIALS AND METHODS

Trifluoroacetic acid (TFA) and 8-anilinonaphthalene-1-sulfonic acid (ANS) were purchased from Aldrich Chemical Company, USA. Superose 6 FPLC column was purchased from Pharmacia, USA.

4.2.1. Preparation of Hetero- and Homo- aggregates of αA- and αB-crystallin

We have isolated α-crystallin at 4°C, from the cortex of calf eye lens, as described earlier in chapter 2. This calf eye lens α-crystallin is a heteroaggregate of αA- and αB- crystallins at a mole ratio of 3:1 (Delcour and Papaconstantinou, 1974). This preparation was used in our entire study as the heteroaggregate (referred to as α-crystallin).

The individual αA- and αB- crystallins were separated and purified from α-crystallin (isolated as mentioned above) by reverse phase HPLC on a C4 column using a gradient of water and acetonitrile containing 0.1% TFA. Homoaggregates of αA- and αB-crystallins were prepared as described in chapter 2. To unfold the proteins in the presence of TFA, the proteins were equilibrated for three hours in 10 mM sodium
phosphate buffer containing 0.1% TFA (v/v) and the specified amount of NaCl for all experiments. The pH of such a solution is around 2.5.

4.2.2. Circular Dichroism Studies

Near- and far-UV Circular dichroism spectra of the protein (1mg/ml) in 10 mM sodium phosphate buffer (pH 7.4) containing 130 mM NaCl (unless mentioned otherwise) in the absence and in the presence of 0.1% TFA were recorded using a JASCO J-715 spectropolarimeter. The path lengths of the sample cells were 1 cm and 0.1 cm for the near- and far-UV CD respectively.

4.2.3. Fluorescence Studies

4.2.3.1. Intrinsic tryptophan fluorescence

The tryptophan fluorescence spectra of the protein (0.1 mg/ml) in 10 mM sodium phosphate buffer (pH 7.4) containing 130 mM NaCl in the absence and in the presence of 0.1% TFA were recorded using a Hitachi F-4000 fluorescence spectrophotometer with the excitation wavelength set at 295 nm in the correct spectrum mode. The excitation and emission bandpasses were set at 5 and 3 nm respectively.

4.2.3.2. Fluorescence quenching studies

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 addition of 7 μl of 7 M acrylamide to 1.2 ml of 0.2 mg/ml α-crystallin in 10 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl in the presence and in the absence of 0.1% TFA. The fluorescence intensities were corrected for the dilution and inner filter effect.

4.2.3.3. Fluorescence of ANS Bound to α-crystallins

Fluorescence spectra of αA-, αB-, and α-crystallins (0.1 mg/ml) in 10 mM sodium phosphate buffer (pH 7.4) containing 130 mM NaCl and 100 μM ANS either in the absence or in the presence of 0.1% TFA were recorded using a Hitachi F-4000 fluorescence spectrophotometer in correct spectrum mode. The excitation wavelength
was set at 365 nm. The excitation and emission band passes were set at 5 nm and 3 nm respectively.

In another experiment, increasing amounts of TFA (up to 0.1%) was added to αA-, αB-, and α-crystallin in 20 mM citrate-phosphate buffer and the resultant pH measured after each addition. A similar titration was performed with each of the samples containing 100 μM ANS and the fluorescence intensity at 485 nm measured. The excitation wavelength was set at 365 nm. The fluorescence intensity was plotted against the pH of the medium upon each addition of TFA.

4.2.3.4. Time resolved fluorescence

We have investigated the fluorescence of α-crystallin-bound ANS by time-resolved fluorescence measurement using a LS-100 luminescence spectrophotometer from Photon Technology International, Canada, operated in the time-correlated single photon counting mode. Photon counts (5000) were collected at the peak channel. Lamp profiles were measured at the excitation wavelength of 381 nm using Ludox as the scatterer. The fluorescence intensity decay was measured at 490 nm. The sample and the scatterer were alternated after every 10% of acquisition. The stored data in a multichannel analyser were transferred to an IBM PC for analysis. Intensity decay curves were fitted as a sum of exponential terms: \( F(t) = \sum \alpha_i \exp(-t/\tau_i) \), where \( \alpha_i \) is a pre-exponential factor representing the fractional contribution to the decay of the component with a lifetime \( \tau_i \). These decay parameters were obtained using non-linear least-squares iterative fitting program based on Marquardt algorithm (Bevington, 1969). The best fitting of the decay curve was evaluated by the low \( \chi^2 \) value, the weighted residuals and the auto-correlation function of the weighted residuals. A fit was considered good when plots of the weighted residuals and the auto-correlation function showed random deviation about zero with a \( \chi^2 \) value close to one.

4.2.4. Gel filtration chromatography

Gel filtration chromatography of αA-, αB-, and α-crystallin (0.1 mg/ml) in 10 mM sodium phosphate buffer (pH 7.4) containing 130 mM NaCl in the absence and in the presence of 0.1% TFA was performed on a Superose 6 FPLC column. The elution of the protein samples was performed at a flow rate of 0.3 ml/min and monitored at 280
nm. In other experiments the concentration of NaCl was as mentioned.

4.3. Results and discussions

As mentioned, earlier studies from our laboratory have showed the existence of a multimeric molten globule-like state of $\alpha$-crystallin above 60°C (Raman and Rao, 1997). This state of the protein has enhanced chaperone-like activity in preventing the aggregation of other proteins. A partially unfolded state of $\alpha$-crystallin in the presence of 0.8-1.0 M guanidinium chloride also has been shown to exhibit enhanced chaperone-like activity (Das and Liang, 1997). Our studies on the temperature dependence of the chaperone-like activities of homo- and heteroaggregates of $\alpha$A- and $\alpha$B-crystallin showed $\alpha$B-crystallin to have a higher hydrophobicity compared to $\alpha$A-crystallin. However, while separating them by reverse phase chromatography in the presence of TFA, $\alpha$B-crystallin elutes before $\alpha$A-crystallin.

4.3.1. Circular dichroism of $\alpha$-crystallins in presence of TFA

In the present study, we set out to investigate the unfolding of both the hetero- and homoaggregates of $\alpha$A- and $\alpha$B-crystallins in the presence of low concentrations of trifluoroacetic acid. Figure 4.1 shows the far-UV CD (figure 4.1A) and near-UV CD (figure 4.1B) spectra of $\alpha$-crystallin in the absence and in the presence of 0.1% TFA. It is evident from Figure 4.1B that $\alpha$-Crystallin loses almost all the chiral structure in the near-UV region in the presence of TFA. The circular dichroism observed in the far-UV CD region (figure 4.1A), indicative of secondary structure content is not lost. It is interesting to note that the chirality in the far-UV CD region indeed increases in the presence of TFA. Such an increase in chirality in partially unfolded structures is not uncommon, while the enhancement is generally more pronounced in the acid-induced unfolding studies. For example, in human carbonic anhydrase B the negative molar ellipticity at 210 nm is nearly four times greater in the acid molten globule state than in the native state (Jagannadham and Balasubramanian, 1985). This does not necessarily indicate a change in secondary structure, as, far-UV CD spectra can be influenced by aromatic side chains. These side chains can contribute to the CD spectra not only in the
Figure 4.1 Far- (A) and Near- (B) UV circular dichroism spectra of α-crystallin in the absence (-----) and in presence of 0.1% TFA (........).
near-UV region but also in the far-UV CD region (Sears and Baychok, 1973; Manning and Woody, 1989). Since the aromatic groups can have both positive and negative bands in the far-UV region (Brahms and Brahms, 1980; Manning and Woody, 1989), the far-UV CD spectra of the molten globule states can be either more or less pronounced than those of the native state. Earlier studies (Maiti et al., 1988; Surewicz and olesen, 1995; Raman and Rao, 1997) on the circular dichroism of α-crystallin at higher temperatures also showed an enhanced chirality in the far-UV region. Thus, our results suggest that α-crystallin retains substantial amount of secondary structure in the presence of TFA. Since the temperature dependence of chaperone-like activity, hydrophobicity, and molecular stabilities of the two subunits of α-crystallin are quite different, we have studied the effect of TFA on the individual subunits. Figure 4.2 and 4.3 compare the effect of TFA on the near and far-UV CD spectra of the individual homoaggregates. In the presence of TFA both the subunits lose considerable tertiary structure as monitored by near-UV CD (figure 4.2). The changes are more pronounced for αB-crystallin as compared to αA-crystallin. The signals contributed by phenylalanine at 258 and 265 nm, though reduced in intensity, are not lost completely. The signals between 270-290 nm are diminished considerably for αS-crystallin, similar to that observed as a function of temperature in chapter 2. The far-UV CD spectra of the individual homoaggregates of αA- and αB-crystallin figure 4.3, show similar changes as the heteroaggregate, in the presence of TFA. The extent of increase in the signal is higher for αB-crystallin as compared to αA-crystallin. Upon increase in temperature α-crystallin shows a similar enhancement in the CD signal as seen in chapter 2.

4.3.2. Fluorescent properties of α-crystallins in TFA

4.3.2.1. Intrinsic tryptophan fluorescence

To investigate the TFA induced changes in the tertiary structure of the proteins, we have recorded fluorescence spectra (Figure 4.4) of the various samples. Figure 4.4 (A) and (B) show the intrinsic tryptophan fluorescence spectra of αA- and αB-crystallin respectively, in the absence and presence of 0.1% TFA. The tryptophan emission maximum provides an indication of whether the tryptophan residues (Trp-9 in αA-crystallin and Trp-9 and Trp-60 in αB-crystallin) are buried or exposed. Since all these
Figure 4.2 Near-UV circular dichroism spectra of (A) αA-crystallin and (B) αB-crystallin in the absence (-----) and presence (.........) of 0.1% TFA
Figure 4.3 Far-UV circular dichroism spectra of (A) αA-crystallin and (B) αB-crystallin in the absence (——) and presence (---) of 0.1% TFA
Figure 4.4 (A) Intrinsic tryptophan fluorescence spectra of (A) αA-crystallin and (B) αB-crystallin in the native (——) and the partially unfolded state in 0.1% TFA (—-).
residues are in the N-terminal domain (residues 1-63), which is hydrophobic and believed to participate in high molecular weight aggregation, they should be relatively buried. αA- and αB-crystallin homoaggregates display emission maxima at 336 and 339 nm respectively, which are close to that for calf lens α-crystallin. In the presence of 0.1% TFA, the respective emission maxima red shift to 344 nm and 347 nm with a reduction in their fluorescence intensities. The red shift in the emission maximum indicates exposure of the tryptophan residues of the protein to the polar water. Since the emission maximum of completely exposed tryptophan varies from 352-354 nm, our result show that αA-, and αB-crystallins are not yet completely unfolded, and retains some structure in the presence of TFA. The tryptophans of αB-crystallin are more accessible to polar water than that in αA-crystallin, in the presence of 0.1% TFA. We have also studied the quenching of the tryptophan fluorescence of αA-, and αB-crystallins in the absence and presence of TFA by acrylamide. Stern-Volmer plots (Figure 4.5) clearly demonstrate the increased access of protein side chains to acrylamide in the presence of 0.1% TFA. Under native conditions, acrylamide accesses the tryptophans in αB-crystallin more easily than the lone tryptophan in αA-crystallin. Table 1 tabulates the fractional accessibility and the bimolecular quenching constants as derived from the modified stern-volmer plot. All these results show that α-crystallin heteroaggregates as well as αA- and αB-crystallin homoaggregates exhibit substantial amount of secondary structure and lack tertiary structure in the presence of 0.1% TFA. The pH of 10 mM sodium phosphate (pH 7.4) containing 0.1% (v/v) TFA was found to be close to 2.5. Under such low pH, several proteins exhibit partially unfolded states known as an "A-state" formed either in the presence or the absence of salt.

4.3.2.2. ANS as an extrinsic fluorescent probe

The "A-state" of α-lactalbumin is well characterised to be a molten-globule state (Dolgikh et al., 1981). Such partially unfolded states of proteins often exposes their hydrophobic surfaces which are buried in their native state (Baldwin, 1993; Ptitsyn, 1995; Fink, 1995; Das and Liang, 1997). Stevens and Augustyn studied pH-induced unfolding of α-crystallin in glycine-HCl and reported that the partially unfolded state of the protein exhibits reduced binding of the hydrophobic fluorescent probe, 6-(p-toluidino)-2-naphthalenesulfonic acid, indicating lack of exposure of hydrophobic surfaces (Stevens
Figure 4.5 Stern-Volmer plot of Tryptophan fluorescence quenching of the native (filled symbols) and the partially unfolded state in 0.1% TFA (open symbols) of αA-crystallin (circles) and αB-crystallin (triangles) by acrylamide. F₀ and F are the fluorescence intensities in the absence and presence of the quencher respectively.
Table 4.1

Fractional accessibility and Stern-Volmer quenching constant of the accessible fraction

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<th>Fa</th>
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<tr>
<td>αA</td>
<td>1</td>
<td>1.35</td>
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<tr>
<td>αA + 0.1% TFA</td>
<td>1</td>
<td>1.9512</td>
</tr>
<tr>
<td>αB</td>
<td>1</td>
<td>1.456</td>
</tr>
<tr>
<td>αB+0.1% TFA</td>
<td>1</td>
<td>2.415</td>
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and Augusteyn, 1993). This led them to conclude that the molecule had undergone acid-expansion rather than a conversion to a molten globule state. Considering the molten globule-like nature of the partially unfolded states of α-crystallin and its subunits one would expect increased exposure of hydrophobic surfaces. In order to find out whether the partially unfolded states of α-crystallin heteroaggregate and αA- and αB-crystallin homoaggregates detected by us in the presence of TFA, expose hydrophobic surfaces, we studied the binding of the hydrophobic fluorescent probe ANS to the proteins.

The fluorescence of the probe, ANS is sensitive to the polarity of the microenvironment of the probe bound surfaces. Upon binding to the apolar surfaces or in a less polar environment, its fluorescence intensity is enhanced and the emission maximum is shifted to lower wavelengths. It is used to probe hydrophobic surfaces in proteins (Cardamone and Puri, 1992) and to detect intermediates in unfolding or refolding pathways of proteins (Semisotnov et al., 1991). Figure 4.6 shows the fluorescence spectra of ANS bound to αA- and αB-crystallin in the absence and in the presence of 0.1% TFA. It is evident from the figure that the partially unfolded state of the protein in TFA binds ANS several fold higher compared to the native protein. The fluorescence emission maxima are red shifted to around 495 nm in the presence of TFA indicating solvent exposure of the hydrophobic, dye binding sites. Figure 4.7 shows the relative fluorescence intensity of the protein-bound ANS as a function of pH upon addition of increasing amounts of TFA. α-Crystallin exhibits a transition below pH 5.5. The enhancement of ANS binding upon lowering the pH occurs at a higher pH and more co-operatively for αB-crystallin compared to αA-crystallin. The TFA-induced changes in the α-crystallin molecule is found to be reversible as the fluorescence spectrum of the protein-bound ANS upon removing the TFA by dialysis is almost similar to that of the native α-crystallin bound ANS (inset figure 4.7). This result shows that the partially unfolded state of α-crystallin in TFA, which exhibits higher solvent exposed hydrophobic surfaces compared to the native protein, can be refolded to a state wherein its hydrophobicity is comparable to the native protein.

We have further probed the hydrophobic surfaces of α-crystallin in its native and its partially unfolded states by time resolved fluorescence of ANS bound to the protein. Table II shows the analysis of the time-resolved fluorescence decay of the protein bound ANS in its native state and in its partially unfolded state in 0.1% TFA. The fluorescence lifetime of ANS increases as the polarity of the environment decreases (Analysis of the
Figure 4.6 (A) Fluorescence spectra of ANS bound to (A) αA-crystallin and (B) αB-crystallin in their native (-- - --), the partially unfolded states (-----) in 0.1% TFA.
Figure 4.7 Fluorescence intensity of ANS bound to α-crystallin (-●-), αA-crystallin (-○-) and αB-crystallin (-△-) as a function of the pH of the medium, upon addition of increasing quantity (0-0.1%) of TFA. (Inset) fluorescence spectrum of ANS bound to native (——), partially unfolded in 0.1% TFA (- - -), and refolded (-----) α-crystallin. $I_r$ is normalised fluorescence intensity.
fluorescence decay of ANS in methanol and butane-1-ol shows the existence of a single emitting species with fluorescence lifetimes of around 6 ns and 10 ns respectively). Figure 4.8 shows the representative fluorescence decay profile of ANS bound to \(\alpha\)-crystallin. The decay profile can be best fitted with three exponentials. The data indicate the presence of three sets of decay components with the life times of 0.875 ns, 11.671 ns and 21.622 ns with the corresponding pre-exponential factors of 0.551, 0.26 and 0.189. The long life time component (21.6 ns with the pre-exponential factor of 0.189) indicates the presence of a highly hydrophobic surface on the native \(\alpha\)-crystallin. The probe bound to the partially unfolded state of \(\alpha\)-crystallin in the presence of TFA exhibits bi-exponential fluorescence decay with the life times of 7.341 ns and 15.916 ns with the corresponding pre-exponential factors of 0.511 and 0.489. This result indicates that the minor component of the highly hydrophobic pocket (s) (represented by the 21.622 ns component) present in the native state is not available and new hydrophobic surfaces become available in the partially unfolded state. As mentioned earlier these hydrophobic surfaces of the partially unfolded state of the protein binds the probe several fold higher resulting in the enhanced fluorescence intensity (figure 4.8).

The molten globule states of proteins are characterised by significant loss of tertiary structure, but the presence of substantial amount of secondary structure. The packaging of the side chains in the protein becomes flexible and the hydrophobic interior becomes more solvent accessible. Moreover, this state is more compact compared to the fully denatured extended state, though relatively expanded compared to the native state. One could speculate that a structure, in which helices and/or \(\beta\)-strands are formed with some degree of stability and which is under the considerable packaging constraint of being condensed and approximately spherical, will have to be near to the native topology. Under these circumstances, precise intra- and inter-subunit steric and spatial interactions may also be affected. Hydrophobic interactions between proteins, being non-specific in nature, however, may be retained. Since \(\alpha\)-crystallin and its subunits are multimeric proteins, it was of interest to investigate the effect of TFA on their quaternary structures.

### 4.3.3. Gel filtration chromatography of \(\alpha\)-crystallins in presence of TFA

We have investigated the effect of TFA on the quaternary structure of \(\alpha\)-crystallin
Figure 4.8 Time-resolved fluorescence decay of $\alpha$-crystallin bound ANS. The peak to the left is the decay profile of the scatterer. The peak to the right is the decay profile of the sample. The sample decay curve is fitted to two-exponential function. RESID = weighted residuals. ACORR = autocorrelation function of the weighted residuals. Excitation and emission wavelengths are 381 nm and 490 nm respectively.
and its subunits by gel filtration chromatography. The elution profile of the \( \alpha \)-crystallin heteroaggregate in its native and its partially unfolded state in the presence of 0.1\% TFA and 130 mM NaCl, on a Superose 6B FPLC column is shown in figure 4.9A. In the presence of 0.1\% TFA, \( \alpha \)-crystallin elutes as two peaks: a major peak which elutes as a slightly smaller sized aggregate compared to the native protein and a second relatively minor peak at position corresponding to 20 kDa. As mentioned earlier, the calf eye lens \( \alpha \)-crystallin is made up of \( \alpha A \)- and \( \alpha B \)- subunits in a ratio of approximately 3:1 (Delcour and Papaconstantinou, 1974). Urea-PAGE of the peaks I and II, obtained on eluting \( \alpha \)-crystallin in the presence of TFA, identifies the peaks to be predominantly \( \alpha A \)- and \( \alpha B \)-crystallin respectively (figure 4.9B). \( \alpha A \)-Crystallin which elutes as peak I is a multimer, while \( \alpha B \)-crystallin, peak II, elutes as a monomer. A previous study reported that at low pH (in the presence of glycine-HCl), \( \alpha \)-crystallin dissociates into particles composed of only \( \alpha A \)-crystallin subunits and denatured \( \alpha B \)-crystallin polypeptides (Augusteyn et al., 1988). The protonation of Aspartate-127 in \( \alpha B \)-crystallin was believed to result in the loss of tertiary structure at low pH. Since our work reported in chapters 2 and 3 demonstrate that \( \alpha A \)- and \( \alpha B \)-crystallin subunits behave differently as homo and heteroaggregates, we monitored the affect of TFA on the individual homoaggregates. In the presence of 0.1\% TFA and 130 mM NaCl \( \alpha A \)-crystallin eluted at a position corresponding to the multimeric peak (figure 4.10A) with negligible monomerization, while \( \alpha B \)-crystallin eluted at a position corresponding to the monomeric peak (figure 4.10B). Thus, the minor change in oligomer size of \( \alpha \)-crystallin is due to the loss of \( \alpha B \)-subunits, which appear as the second peak. In many proteins, the extent of unfolding/denaturation under acidic conditions, is dependent on the ionic composition of the medium. To see if ionic composition of the medium may have a role in determining the oligomeric status of the molten globules observed, we performed gel filtration chromatography of \( \alpha \)-crystallin in the presence of 10 mM sodium phosphate containing 0.1\% TFA and 250 mM NaCl (figure 4.11). Under these conditions, the \( \alpha B \)-subunits do not dissociate from the \( \alpha \)-crystallin aggregate. On the contrary, the oligomeric size of the heteroaggregate increases slightly. This is probably because the higher salt concentration leads to greater hydrophobic interactions, while shielding charges.
Figure 4.9 Gel filtration chromatography (A) of the native $\alpha$-crystallin (-----) and $\alpha$-crystallin in the presence of 0.1% TFA and 130 mM NaCl (-------) on a Superose 6B FPLC column. (B) Urea-PAGE of $\alpha$-crystallin and the peaks I and II eluted on Superose 6B column. Lane 1: peak I, lane 2: peak II, lane 3: $\alpha$-crystallin.
Figure 4.10 Gel filtration chromatography of (A) $\alpha$A-crystallin and (B) $\alpha$B-crystallin in their native (---), the partially unfolded states (-----) in 0.1% TFA containing 130 mM NaCl.
Figure 4.11 Gel filtration chromatography of native α-crystallin (——) and α-crystallin in the presence of 0.1% TFA (- - - - -) containing 250 mM NaCl, on a Superose 6B column.
4.3.4. Salt-dependence of the secondary structure of \(\alpha\)-crystallins in presence of TFA

To check if any secondary structural changes accompany the observed salt-dependent change in oligomeric status of the partially unfolded proteins, we monitored the far-UV CD spectra of \(\alpha\)A- and \(\alpha\)B-crystallins in the presence of TFA, under various salt concentrations (figure 4.12). The far-UV CD spectrum of \(\alpha\)A-crystallin is not significantly affected by salt concentration (figure 4.12A). The secondary structure of \(\alpha\)B-crystallin is however profoundly affected by salt concentration. In a low salt concentration of 10 mM NaCl, \(\alpha\)B-crystallin loses its secondary structure and adopts a random coil conformation. A salt concentration of 50 mM is sufficient to stabilise its structure to a molten globule. Similar observations have been made for the unfolding of beta-lactamase, cytochrome c, and apomyoglobin by HCl (Goto et al., 1990). These proteins undergo extensive unfolding (to a U\(_A\) state) at around pH 2 and conditions of low ionic strength. A further decrease in pH by increasing HCl concentration induces the refolding of the proteins to a structure (the A state) with properties similar to those of molten globule conformations reported for other proteins (Goto et al., 1990). The addition of KCl to the extended conformation unfolded state at pH 2 induces a similar conformational transition, indicating that chloride anion may play a key role in the acid-salt-induced transitions. The conformational transition from random coil to molten globule for \(\alpha\)B-crystallin occurs at a lower salt concentration (10-50 mM NaCl) than that which affects the oligomeric status of the \(\alpha\)-crystallin heteroaggregate (130-250 mM NaCl). Therefore this conformational transition is unlikely to play a role in the dissociation of \(\alpha\)B-crystallin from the \(\alpha\)-crystallin heteroaggregate. This result explains why earlier workers (Augusteyn et al., 1988) observed the dissociation of denatured \(\alpha\)B-crystallin subunits from \(\alpha\)-crystallin. It is probably due to the low ionic strength of the buffers used (0.1 M glycine).

Thus, all our results show that \(\alpha\)-crystallins exhibit highly solvent exposed non-native hydrophobic surfaces in the presence of TFA. Circular dichroism and fluorescence studies show that the protein in the presence of TFA exhibit substantial amount of
Figure 4.12 Far-UV circular dichroism of (A) αA-crystallin and (B) αB-crystallin in 0.1% TFA with varying amounts of salt. 10 mM (——), 50 mM (-----) and 250 mM (······) NaCl.
secondary structure and no tertiary structure. All these properties of the protein are similar to that of molten globule State of proteins. Most of the earlier studies on molten globule state of proteins deals with monomeric proteins. However, only few studies are available on the partially unfolded state of multimeric proteins. Singh et al. (1996), based on calorimetric studies, have shown the existence of a hexameric intermediate with molten globule like properties of the enzyme, bovine-liver glutamate dehydrogenase. An earlier study shows that α-crystallin exhibits a molten-globule-like state at 62°C (Raman and Rao, 1997). Our present study shows that a similar molten globule-like state of the proteins is formed in the presence of TFA. Interestingly, αA-crystallin forms a multimeric-molten globule while αB-crystallin forms a monomeric-molten globule. The pH dependence of the membrane insertion of the pore forming domain of colicin A correlates with its transformation into the molten globule state (van der Goot et al., 1991). In this case the local pH decrease near the negatively charged membrane surface may have an important role in the transformation. It is interesting to note that a significant amount of α-crystallin is found associated with lens membranes (Zhang and Augusteyn, 1993; Cenendella and Chandrashekar, 1993). This association has been shown to be enhanced in vitro under acidic conditions (Cobb and Petrash, 2000). The role of α-crystallin in this context is not understood. One of the poorly understood and controversial aspects of the structure of α-crystallin has been the oligomeric arrangement of the subunits. In vitro translation of αA- and αB-crystallin transcripts have revealed that αB-crystallin folding and oligomerization is promoted by the presence of pre-existing αA-crystallin aggregates (Asselbergs et al., 1978). The author's believe that αA-crystallin may act as a nucleation centre for the subsequent folding of αB-crystallin. The interpretation of in vitro translation experiments are difficult because of low yields, and also the presence of other interfering proteins. Refolding α-crystallin from TFA, in conditions where αB-crystallin is dissociated (130 mM NaCl) would provide a more realistic model, where αA-crystallin may provide an oligomeric nucleation centre for the folding and association of αB-crystallin. As a comparison, refolding from higher salt concentrations would fold the combined heteroaggregate. This report on the molten-globule-like state of α-crystallin is one of very few studies on the partially unfolded states of multimeric proteins.