Unfolding and refolding of a quinone oxidoreductase: α-crystallin, a molecular chaperone, assists its reactivation

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α-Crystallin, a member of the small heat-shock protein family and present in vertebrate eye lens, is known to prevent the aggregation of other proteins under conditions of stress. However, its role in the reactivation of enzymes from their non-native inactive states has not been clearly demonstrated. We have studied the effect of α-crystallin on the refolding of ζ-crystallin, a quinone oxidoreductase, from its different urea-denatured states. Co-refolding ζ-crystallin from its denatured state in 2.5 M urea with either calf eye lens α-crystallin or recombinant human αB-crystallin could significantly enhance its reactivation yield. αB-crystallin was found to be more efficient than αA-crystallin in chaperoning the refolding of ζ-crystallin. In order to understand the nature of the denatured state(s) of ζ-crystallin that can interact with α-crystallin, we have investigated the unfolding pathway of ζ-crystallin. We find that it unfolds through three distinct intermediates: an altered tetramer, a partially unfolded dimer, which is competent to fold back to its active state, and a partially unfolded monomer. The partially unfolded monomer is inactive, exhibits highly exposed hydrophobic surfaces and has significant secondary structural elements with little or no tertiary structure. This intermediate does not refold into the active state without assistance. α-Crystallin provides the required assistance and improves the reactivation yield several-fold.

Key words: chaperone-like activity, ζ-crystallin, molten globule, reactivation, unfolding.

INTRODUCTION

α-Crystallins, i.e. αA- and αB-crystallins, abundantly present in the eye lens, exhibit sequence similarity to small heat-shock proteins (sHSPs) [1,2]. They are also found in other tissues [3,4]; especially αB-crystallin which is known to be expressed at elevated levels in conditions of stress or disease [5,6]. They are encoded by two different genes [7,8]. Eye lens α-crystallin is a large soluble heteromultimeric protein of approx. 800 kDa composed of both αA- and αB-crystallins. The molar ratios of these subunits in the heteromultimer vary from species to species [9]. Horwitz [10] first reported the ability of the eye lens α-crystallin to protect alcohol dehydrogenase and other crystallins of the lens, such as the β- and γ-crystallins, from thermal aggregation. α-Crystallin also prevents UV-light-induced aggregation of γ-crystallin [11]. Both the polypeptides, either in their homo- or heteromultimeric states, exhibit molecular chaperone-like activity in preventing aggregation of other proteins [12], with αB-crystallin being a more potent chaperone than αA-crystallin [12,13]. Formation of stable complexes of the chaperone-like α-crystallin with several target proteins has been shown [11,14–17]. It binds to aggregation-prone partially unfolded states, having structural properties similar to those of molten globules [16,17]. In addition to its anti-aggregation properties, it is also found to protect the activity of some enzymes upon heat-induced inactivation. α-Crystallin has been shown to protect catalase against thermal inactivation [15]. Activities of thermolabile enzymes, *Nde*I and sorbitol dehydrogenase, have also been shown to be preserved at high temperatures in the presence of α-crystallin [18,19]. α-Crystallin-mediated protection of some enzymes from other inactivating modifications, such as fructation, carbamylation and steroids [20–22], has also been reported, although the molecular mechanism of this process is not understood. A deluge of studies addresses the molecular details of the chaperone activity of α-crystallin in the protection of other proteins from undergoing stress-induced denaturation and aggregation [23–26]. α-Crystallin was shown to prevent the aggregation of intermediates in the unfolding [27], as well as the refolding [28,29] pathways of proteins. Despite the demonstration of this anti-aggregation property of sHSPs in general, and in α-crystallin in particular, there are only a few reports which indicate that sHSPs can reactivate thermally or chemically denatured proteins [30,31]. Recently, it was demonstrated that α-crystallin could transiently interact with and help reactivate the early unfolding intermediates on the heat-induced unfolding pathway of citrate synthase [32]. It was, however, not capable of reactivating the aggregation-prone late intermediates with which it formed a stable complex. Ganea and Harding [33] have reported an increase in the recovery of the activity of glyceraldehyde-3-phosphate dehydrogenase when refolded from its denatured state in guanidinium chloride in the presence of α-crystallin. Rawat and Rao [34] have observed the interaction of the kinetic molten-globule state of xylose reductase with α-crystallin on its refolding from guanidinium chloride. However, detailed information on the structure of the target protein and the nature of its interaction with α-crystallin needs to be clearly elucidated.

To provide an insight into the molecular aspects of α-crystallin in the refolding process of globular proteins, we have studied refolding of ζ-crystallin from its different denatured states in urea. ζ-Crystallin is a 140 kDa tetrameric protein comprising four identical subunits, each with a molecular mass of 35 kDa [35,36]. We chose ζ-crystallin as a model target protein for studying molecular chaperones because (i) it aggregates at 42 °C

Abbreviations used: ANS, 8-anilinonaphthalene-l-sulphonic acid; Hsp, heat-shock protein; sHSP, small heat-shock protein.

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[37], which is close to physiological heat-shock temperature, (ii) it possesses quinone oxidoreductase activity [38], which can be easily assayed, and (iii) it binds the nucleotide cofactor NADPH [34], which can be used as a spectroscopic reporter to assess the changes in the structure of the target protein. In order to investigate the structure of the non-native target protein that is recognized by α-crystallin in the process of reactivation, it is essential to delineate the unfolding intermediates in the denaturation pathway of the chosen target protein. This study describes the details of the urea-induced unfolding pathway of the tetrameric enzyme ζ-crystallin. This also holds significance because no reports are available on the folding of quinone oxidoreductases, the enzyme family to which ζ-crystallin belongs. The results presented in this study demonstrate the ability of α-crystallin to assist reactivation of the urea-denatured ζ-crystallin, resulting in significant enhancement of its refolding yield.

MATERIALS AND METHODS

Materials

8-anilinonaphthalene-l-sulphonic acid (ANS) was purchased from Aldrich. Urea was procured from Sigma. Sephacryl S-200 and Blue Sepharose CL-6B were purchased from Amersham Pharmacia Biotech. Phenanthrenequinone and NADPH were purchased from Merck and Sisco Research (Mumbai, India) respectively. All other reagents used were of analytical grade.

Preparation of α-crystallin

α-Crystallin was purified from calf eye lenses as described previously [11]. Recombinant human αA- and αB-crystallins were prepared by cloning and overexpressing the protein in *Escherichia coli* and purification as described previously [39].

Preparation of ζ-crystallin

ζ-Crystallin was purified from guinea-pig eye lenses by the method described by Rao and Zigler [35]. In brief, the soluble lens homogenate was passed through a Blue Sepharose CL-6B column equilibrated with 10 mM Tris/HCl buffer, pH 7.4, containing 0.5 mM EDTA. The bound ζ-crystallin was eluted with the above buffer supplemented with 1 M NaCl. The protein was purified to homogeneity further by gel-filtration chromatography on a Sephacryl S-200 column.

Unfolding studies

Unfolding experiments were carried out at room temperature (25 °C) by incubating ζ-crystallin (at the concentrations indicated in the Figure legends) in 20 mM potassium phosphate buffer, pH 7.8, containing 100 mM NaCl and different concentrations of urea, for 1 h. To investigate the effect of α-crystallin on its denaturation profile, in one set of samples we have included bovine α-crystallin or human recombinant αA- or αB-crystallins at a 2:1 mass ratio of α-crystallins/ζ-crystallin.

Refolding studies

ζ-Crystallin was denatured by incubating 1.32 mg/ml protein either in the absence or in the presence of 2.64 mg/ml α-crystallins or other proteins (controls), such as BSA, thyroglobulin, lysozyme and β-crystallin, in 20 mM potassium phosphate buffer, pH 7.8, containing 100 mM NaCl and different concentrations of urea, for 1 h. Refolding was initiated by rapidly diluting 10 μl of the urea-denatured samples, prepared as above, into 990 μl of 20 mM potassium phosphate buffer, pH 7.8, containing 100 mM NaCl. The samples were mixed thoroughly by vortexing, and the enzyme activity was measured 1 h after initiation of refolding.

Assay for ζ-crystallin activity

ζ-Crystallin possesses quinone oxidoreductase activity, which was assayed by using phenanthrenequinone as a substrate and providing the cofactor NADPH. The activity of ζ-crystallin in the unfolding and refolding reactions was monitored by measuring the decrease in the A_{340} due to oxidation of the NADPH [38]. The 1-ml assay mix contained 13 μg of ζ-crystallin and 20 μM NADPH in 20 mM potassium phosphate buffer, pH 7.8, containing 100 mM NaCl. The reaction was initiated by addition of 25 μM phenanthrenequinone and measured spectrophotometrically for 2 min, during which the decrease in NADPH absorption was linear. The activity of the native enzyme at the same concentration was taken as 100 %, and the activity of the unfolded or refolded enzyme was expressed as a percentage of the activity of the native enzyme.

Intrinsic tryptophan fluorescence of ζ-crystallin

Fluorescence spectra were recorded using a Hitachi F4010 fluorescence spectrophotometer. Intrinsic tryptophan fluorescence spectra of the urea-denatured ζ-crystallin (0.1 mg/ml), prepared as described above, were recorded between 310 and 400 nm by exciting the sample at 295 nm. The excitation and emission band passes were set at 5 nm and 3 nm respectively. All the spectra were recorded in corrected spectrum mode.

ANS-binding studies

10 μl of a methanolic solution of 20 mM ANS was added to the urea-denatured ζ-crystallin samples (0.1 mg/ml), prepared as described above. The emission spectra of the samples were recorded between 400 and 600 nm using a Hitachi F4010 fluorescence spectrophotometer by exciting at 365 nm. The excitation and emission band passes were both set at 3 nm. All the spectra were recorded in corrected spectrum mode.

NADPH-binding studies

Binding of NADPH results in the quenching of the intrinsic fluorescence of ζ-crystallin. To study the ability of the native and urea-denatured ζ-crystallin (0.1 mg/ml), prepared as described above, to bind its cofactor, NADPH was added at final concentration of 10 μM to the sample, and the fluorescence spectra of the sample recorded between 310 and 550 nm upon exciting at 295 nm. All spectra were recorded in the corrected spectrum mode.

The association constant K_a gives a measure of the binding of NADPH to the enzyme. It was calculated for the native and the denatured ζ-crystallin in 1 M urea, as described below. ζ-Crystallin (0.1 mg/ml) in 20 mM potassium phosphate buffer, pH 7.8, either in the absence or in the presence of 1 M urea, was titrated with increasing concentrations of NADPH. The range of concentrations of NADPH used did not contribute to the inner filter effect. The formation of the enzyme–NADPH complex was followed by the progressive quenching of the protein fluorescence at 314 nm when excited at 280 nm. Using this quenching of the intrinsic fluorescence of the protein as a function of NADPH concentration, the K_a was calculated by the method described by Stintson and Holbrook [40]. The slope of the plot of $1/(1-\delta)$ against [NADPH]/δ provided the value of the K_a, and the x-axis

intercept gave an estimate of the number of cofactor binding sites, E_0 [41]. The value of δ was calculated from the formula

$$\delta = (F - F_B)/(F_U - F_B)$$

where F is the fluorescence intensity of the sample at a given concentration of NADPH. F_U and F_B refer to the fluorescence intensities of the protein where all its ligand-binding sites are free and where all its ligand-binding sites are saturated respectively.

CD studies

CD spectra were recorded using a JASCO J-715 spectropolarimeter. All spectra reported are the average of five accumulations. Far- and near-UV CD spectra of the native and urea-denatured ζ-crystallin (1 mg/ml) were recorded using 0.02-cm- and 1-cm-pathlength cuvettes respectively. Binding of NADPH to the native and urea-denatured ζ-crystallin was also monitored by the induced CD spectrum of the bound NADPH between 280 and 400 nm. The final concentration of NADPH in the sample was 1 mM.

The fraction of protein denatured in different concentrations of urea (f_D) was calculated [42] using the formula

$$f_D = (\theta_N - \theta)/(\theta_N - \theta_U)$$

where θ is the observed ellipticity at a given urea concentration, θ_U the ellipticity when the protein is completely unfolded, and θ_N the ellipticity of the native protein.

Size-exclusion chromatography

The oligomeric status of ζ-crystallin in different concentrations of urea was determined using a Superose 12 HR 10/30 prepacked FPLC column (10 mm × 300 mm, bed volume 24 ml; Amersham Pharmacia Biotech). The molecular mass standards used were aldolase (158 kDa), BSA (67 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa). The standards were run in their native conditions in order to discern the size of the test protein in various concentrations of urea with respect to a standard curve. The effect of NADPH on the oligomeric status of the urea-denatured ζ-crystallin was also investigated by incubating the urea-denatured sample with 1 mM NADPH for 5 min before loading on to the column. The Stokes radius was determined for the protein in different concentrations of urea as described by Uversky [43].

RESULTS AND DISCUSSION

In order to study the ability of α-crystallin to assist the renaturation of ζ-crystallin from its denatured states, we initially investigated the inactivation and reactivation of the enzyme as a function of urea concentration. Figure 1 shows the activity of the enzyme at different concentrations of the denaturant. The enzyme activity decreased sharply as a function of urea concentration; almost 60 % of the measured activity was lost in 1 M urea. In 2 M urea, the enzyme was completely inactivated. The activity regained upon renaturation of the enzyme from different concentrations of urea is also shown in Figure 1. The urea concentrations shown on the abscissa refer to the concentrations of the denaturant from which the protein was refolded. On refolding from urea concentrations below 2 M, the enzyme could regain almost complete activity. As is evident from Figure 1, the reactivation yield decreases sharply when the enzyme is refolded from urea concentrations above 2 M and has negligible activity when refolded from 3 M urea.

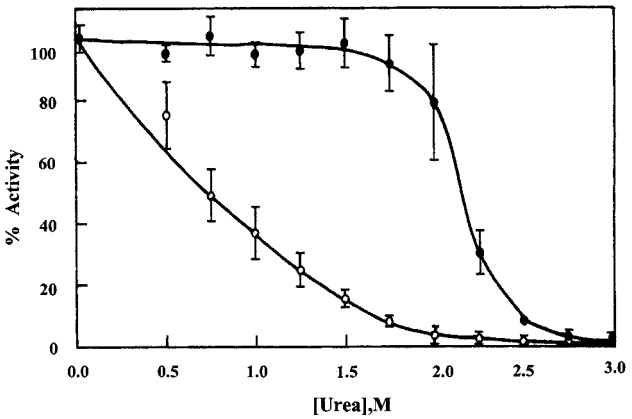


Figure 1 Unfolding and refolding of ζ-crystallin

Enzyme activity of ζ-crystallin in different concentrations of urea (○). The activity regained when refolded from the given urea concentrations by rapid dilution into buffer (●). The enzyme concentration for the activity measurements was 13 μg/ml (see the Materials and methods section). Results are expressed as the mean ± S.D. for four independent experiments.

The effect of molecular chaperones on the denaturation process of other proteins appears to be complex in nature. Molecular chaperones, such as the heat-shock proteins Hsp90 and Hsp70, are known to slow down the inactivation rate of citrate synthase on its heat denaturation, whereas Hsp25 does not have any effect [31]. On the other hand, GroEL is found to accelerate the inactivation process [44]. A recent study [32] from our laboratory shows that αA- and αB-crystallins decrease the rate of inactivation of the enzyme significantly. In contrast to the effect of α-crystallin on heat-induced denaturation of proteins, we found that the presence of α-crystallin in the urea-denaturation experiments in this study does not affect the denaturation process of ζ-crystallin.

We have investigated the role of α-crystallin in the refolding of ζ-crystallin. ζ-Crystallin was refolded together with α-crystallin from its different urea-induced denatured states. We included ζ-crystallin and α-crystallin at the ratio of 1:2 (w/w) in buffer containing different concentrations of urea. The samples were then subjected to refolding as described in the Materials and methods section. Since previous studies have shown that structural perturbation, either by temperature or by low concentrations of urea, enhances the chaperone-like activity of α-crystallin [11,29,45–47], and co-refolding of α-crystallin with β- or γ-crystallin is more effective in preventing their aggregation [47], we also co-refolded ζ-crystallin and α-crystallin from the said urea concentrations. As seen in Figure 1, ζ-crystallin refolds almost completely from its denatured states below 2 M urea, but the reactivation yield decreases sharply when refolded from urea concentrations above 2 M. The reactivation yield is < 10 % when it is refolded from 2.5 M urea (Figures 1 and 2). Figure 2 shows that when ζ-crystallin is refolded together with calf eye lens α-crystallin from 2.5 M urea, the activity regained is approx. 54 %. This shows that α-crystallin can interact with the refolding ζ-crystallin and can significantly enhance its renaturation. Similar enhancement of reactivation by α-crystallin was observed when ζ-crystallin was refolded from 2.25 M or 3 M urea (results not shown). However, α-crystallin could not improve the reactivation yield beyond 3 M urea.

As mentioned earlier, eye lens α-crystallin is a heteromultimeric protein composed of αA- and αB-crystallin with the subunit ratio varying from species to species [9]. The homomultimers of αA-

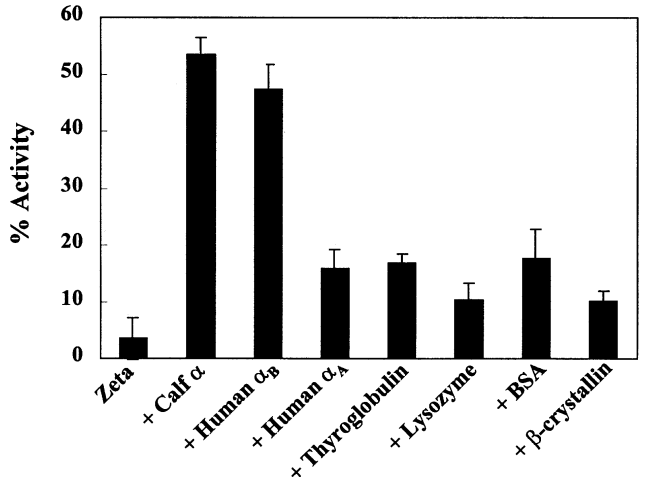


Figure 2 Effect of co-refolding of ζ-crystallin with α-crystallins

ζ-Crystallin was denatured in 2.5 M urea. Co-refolding of ζ-crystallin with control proteins has also been shown. The chaperone/ζ-crystallin ratio was 2:1 (w/w). The samples were refolded and then activity measurements were performed as described in the Materials and methods section. Results are expressed as the mean ± S.D. for four independent experiments.

and αB-crystallin are known to exhibit differences in their chaperone-like activity and structural stability [13]. We therefore investigated the effect of human recombinant αA- and αB-crystallin on the refolding process of ζ-crystallin. Although αA- and αB-crystallins share more than 80 % sequence similarity, αB-crystallin is found to be much more effective in increasing the regaining of ζ-crystallin activity than αA-crystallin; the reactivation in the presence of αB-crystallin is approx. 48 %, whereas it is approx. 15 % in the presence of αA-crystallin (Figure 2).

Kinetic competition between productive folding and intermolecular aggregation or misfolding determines the net yield of the refolded proteins [48]. Partitioning of the partially unfolded states of proteins with solvent-exposed hydrophobic surfaces is often the limiting factor for productive folding. Chaperone molecules offer spatially appropriate hydrophobic surfaces where the folding polypeptide chain can either bind stably or reversibly depending on several factors, such as complementarity of interacting surfaces, and stabilizing interactions, such as those contributed by charge and extent of exposed hydrophobic surfaces on the folding intermediate. Such interactions decrease or prevent the intermolecular aggregation and inactivation. It therefore appears that molecules, which can offer appropriate hydrophobic surfaces to interact with the folding intermediate, can influence its folding [49]. Additives such as detergents or other proteins, for example BSA, can also enhance the yield of refolded proteins by mimicking molecular chaperones [49,50]. Therefore we compared the ability of α-crystallin to improve the reactivation yield of ζ-crystallin with other proteins including BSA, lysozyme, thyroglobulin and β-crystallin (Figure 2). As seen in Figure 2, whereas these proteins enhance the reactivation yield marginally (10–17 %), α-crystallin and recombinant human αB-crystallin enhance the reactivation of ζ-crystallin quite substantially (54 % and 48 % respectively). The reactivation by αA-crystallin, however, is comparable with that of thyroglobulin (Figure 2). Therefore it appears that the reactivation of ζ-crystallin by calf eye lens α-crystallin and recombinant human αB-crystallin is specific. αA- and αB-crystallins have previously been shown to differ quite significantly in their chaperone-like activity in preventing aggregation of other proteins [13]. Our present study shows that they also differ significantly in their ability to improve the reactivation yield of ζ-crystallin.

Thus these results show that α-crystallin not only prevents aggregation, but also helps in the productive refolding of ζ-crystallin. Refolding yield, however, depends on the initial urea concentration in which the enzyme is denatured. We observe that when ζ-crystallin is refolded from high concentrations of urea (> 3 M), either in the absence or in the presence of α-crystallin, the activity regained is negligible. This suggests that α-crystallin can effectively interact with a partially unfolded structure and assist in its reactivation. Therefore we investigated the species generated in the unfolding pathway by monitoring spectral features of ζ-crystallin under various urea concentrations.

ζ-Crystallin, a quinone oxidoreductase, is a tetrameric assembly where each subunit binds one molecule of NADPH; the bound nucleotide molecule is involved in the transfer of reducing equivalents and hence is essential for its activity [38]. Binding of NADPH stabilizes the enzyme against thermal denaturation [37], whereas its role in the chemically induced unfolding of the protein is not known. Binding of the nucleotide molecule can be monitored easily by spectroscopic tools; it thus serves as an additional probe to investigate the unfolding pathway of the enzyme. It is important to note that in the unfolding studies presented here, we have used ζ-crystallin without NADPH. The apo-enzyme was incubated in different concentrations of urea (for a fixed time period), and the different partially unfolded states were studied for their ability to bind the cofactor and for consequent structural changes, if any, associated with their binding.

ζ-Crystallin has a single tryptophan residue at position 303. The changes in the micro-environment of its sole tryptophan residue in the presence of various concentrations of urea can be monitored by exciting the protein at 295 nm, a wavelength at which other aromatic amino acids do not absorb significantly and do not contribute to the fluorescence. Figure 3 shows the changes in the intrinsic tryptophan fluorescence of the protein as a function of urea concentrations. ζ-Crystallin exhibits a maximum emission at 324 nm (Figure 3A). The maximum emission gradually shifts towards the red end of the spectrum as a function of urea concentration. It is shifted to 350 nm by 4 M urea, indicating complete exposure of the tryptophan residue to the bulk water. Although the sigmoidal nature of the urea concentration versus wavelength of maximum emission profile is indicative of a two-state transition with an inflection point at approx. 1.5 M urea (Figure 3B), changes in fluorescence intensity with urea concentration (Figure 3B, inset) suggest that the unfolding pathway of ζ-crystallin may be populated with one or more intermediates, particularly around 1 M and 2 M urea. This possibility was further probed by their ability to bind NADPH.

Binding of NADPH results in the quenching of the intrinsic tryptophan fluorescence of ζ-crystallin in a concentration-dependent manner [51]. In addition to quenching the tryptophan fluorescence of the protein in the presence of NADPH, the sample also exhibits a fluorescence band between 400 nm and 550 nm (Figure 3C). NADPH by itself exhibits fluorescence in this region. The fluorescence quenching of the protein by NADPH may involve two possible mechanisms: (i) fluorescence resonance energy transfer (Förster energy transfer, [52]) or (ii) a change in the tryptophan's environment induced by NADPH binding. The first mechanism involves quenching of the donor (tryptophan residue) fluorescence accompanied by enhancement of the acceptor (bound NADPH) fluorescence. This mechanism is ruled out in the case of NADPH binding to the native ζ-crystallin, because the resultant spectrum of ζ-crystallin in the

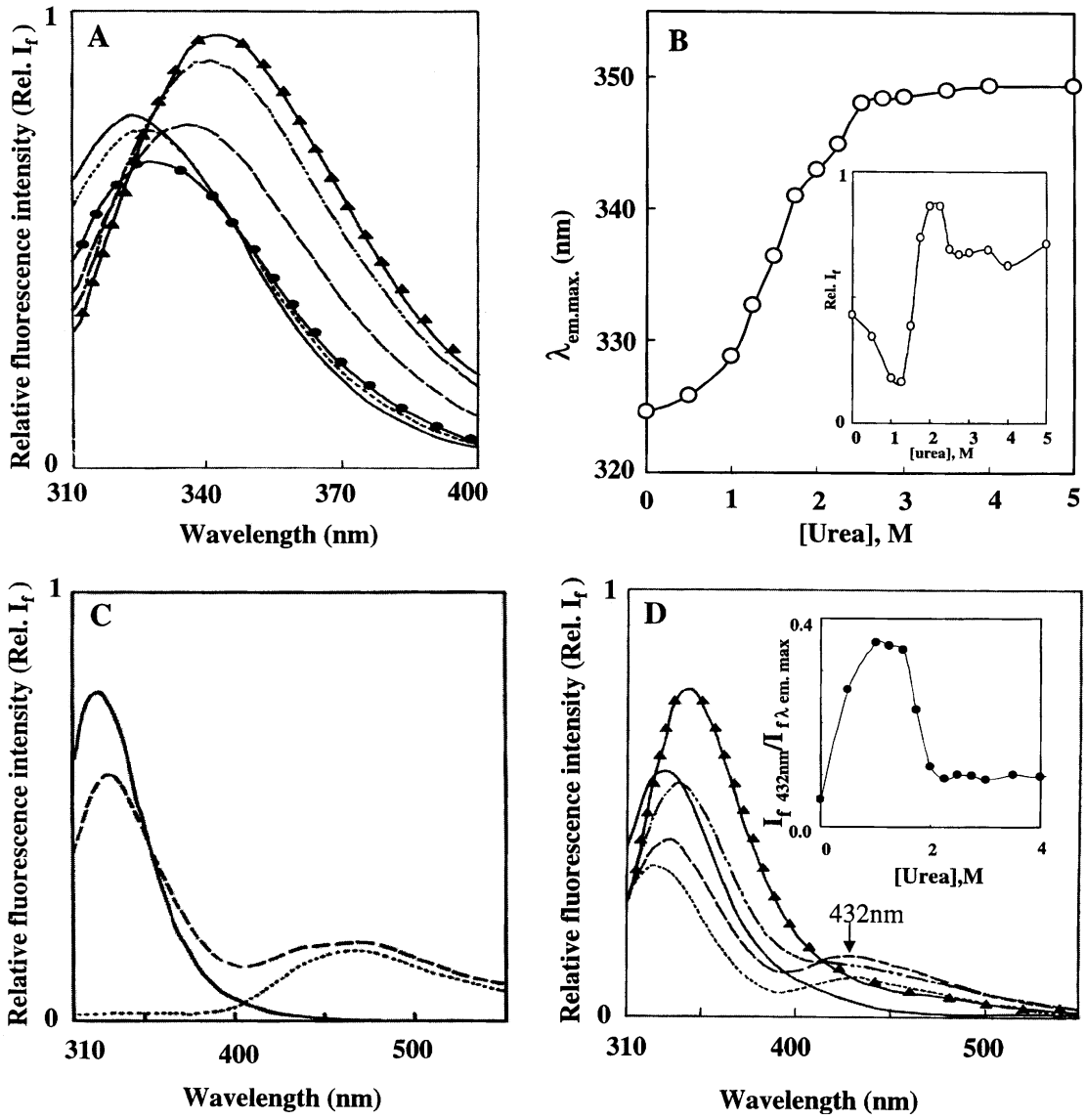


Figure 3 Urea-induced unfolding and NADPH binding of ζ-crystallin monitored by fluorescence spectrscopy

(**A**) Intrinsic tryptophan fluorescence spectra of ζ-crystallin (0.1 mg/ml) in 20 mM potassium phosphate buffer, pH 7.8, containing 100 mM NaCl (——), and in buffer containing 0.5 M urea (- - -), 1 M urea (●), 1.5 M urea (– – –), 1.75 M urea (– - –) and 2 M urea (▲). (**B**) Variation of the wavelength of emission maximum as a function of urea concentration. Inset, changes in the relative fluorescence intensity of ζ-crystallin as a function of urea concentration. (**C**) Quenching of the intrinsic tryptophan fluorescence of ζ-crystallin by NADPH. Fluorescence spectra of ζ-crystallin (0.1 mg/ml) in the absence (——) and in the presence (– – –) of 10 μM NADPH. The fluorescence spectrum of NADPH alone (- - -) is also shown. (**D**) Fluorescence spectra of urea-denatured ζ-crystallin in the presence of NADPH after subtraction of the spectral contribution of NADPH, in the absence of urea (——), and in 0.5 M urea (- - -), 1.5 M urea (– – –), 1.75 M urea (– - –) and 2 M urea (▲). Inset, variation of the ratio of fluorescence intensity at 432 nm to the fluorescence intensity at the wavelength of emission maximum ($\lambda_{em.max.}$) of ζ-crystallin (fluorescence band between 310 and 350 nm) in different concentrations of urea. All samples were excited at 295 nm.

presence of NADPH after subtracting the fluorescence contribution of NADPH does not show any additional fluorescence in the 400–550 nm region. Thus, it appears that NADPH binding alters the structure of the protein such that its tryptophan environment is modified, resulting in quenching of fluorescence. However, the fluorescence quenching by NADPH in the presence of urea is interesting. Between 1.0 M and 1.75 M urea, the resultant spectra of ζ-crystallin in the presence of NADPH, after subtracting the fluorescence contribution of NADPH, exhibit significant fluorescence between 400 nm and 550 nm (Figure 3D). This shows that NADPH bound to the partially unfolded state(s) in this concentration range of urea exhibits enhanced fluorescence, probably involving the first mechanism. The additional fluorescence between 400 nm and 550 nm is not seen at higher urea concentrations. These results suggest that at and close to 1.5 M urea there are changes in the conformation of the nucleotide-binding site and/or changes in the mode of NADPH binding, indicating the presence of an intermediate state. We have determined also the K_a of the binding of NADPH in the absence and presence of the denaturant. The K_a for the native protein was found to be 0.958 μM^{-1} (see the Materials and methods section). At 1 M urea the binding is considerably weakened as is evident from the K_a value of 0.456 μM^{-1}. At higher concentrations of urea, the binding is too weak to

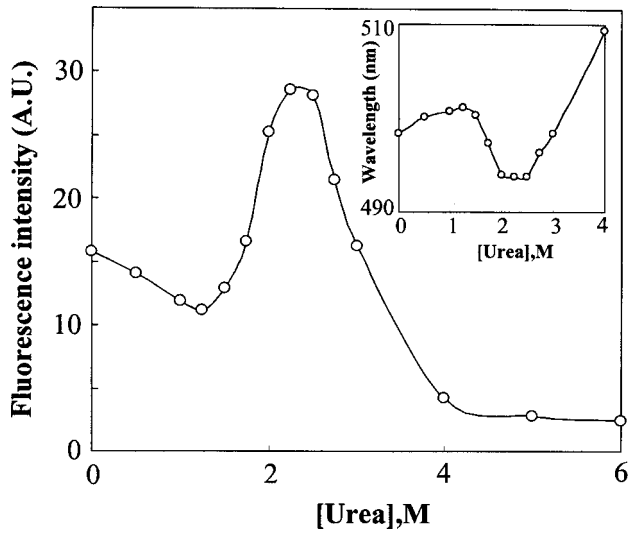


Figure 4 Binding of ANS to urea-denatured ζ-crystallin

Variation of fluorescence intensity of the protein-bound ANS (at the wavelengths of emission maximum) as a function of urea concentration. Inset, change in the wavelength of emission maximum ($\lambda_{em.max.}$) of ζ-crystallin-bound ANS with urea concentration. A. U., arbitrary units.

determine the K_a. We have further studied the unfolding of the protein using the hydrophobic fluorescent probe ANS. This probe can be used to detect partially unfolded/refolded intermediate states with exposed hydrophobic surfaces (as in the molten-globule state) [53]. On binding to hydrophobic surfaces, its fluorescence intensity increases accompanied by a blue shift in the emission maximum [54]. Figure 4 shows ANS-binding to ζ-crystallin in the presence of various concentrations of urea. As seen in Figure 4, the fluorescence intensity of ANS decreases gradually from 0–1 M urea, and then increases between 1 M and 2.25 M urea. The emission maximum is gradually red shifted until 1 M urea and then again blue shifted between 1 M and 2.25–2.5 M urea (Figure 4, inset). The decreased binding of ANS in 1 M urea suggests that the intermediate species, identified by both the NADPH binding (Figure 3D) and intrinsic tryptophan fluorescence results (Figure 3B, inset), exhibits fewer hydrophobic surfaces. The increased exposure of the hydrophobic surfaces of ζ-crystallin in 2.25–2.5 M urea may be due to the following possible reasons, dissociation of the tetrameric ζ-crystallin leading to the exposure of otherwise buried intersubunit surfaces having hydrophobic patches, and/or formation of a partially unfolded intermediate state of ζ-crystallin having highly exposed hydrophobic surfaces in 2.25–2.5 M urea. In order to obtain further insight into this aspect, we have studied the secondary, tertiary and quaternary structure of the protein by CD spectroscopy and gel-filtration chromatography.

Figure 5 (upper panel) shows the far-UV CD spectra of ζ-crystallin in different concentrations of urea. The CD spectra of native ζ-crystallin overlaps with that of the protein in 1.5 M urea, showing that there is no significant alteration in its secondary structure of the protein until 1.5 M urea. This result shows that the intermediate state observed in fluorescence studies around 1.0 M urea has not undergone significant secondary structural changes. Figure 5 (upper panel) also shows that ζ-crystallin exhibits a significant amount of secondary structural elements in 2–2.5 M urea. It loses its secondary structural elements completely beyond 3 M urea. Addition of NADPH to the sample

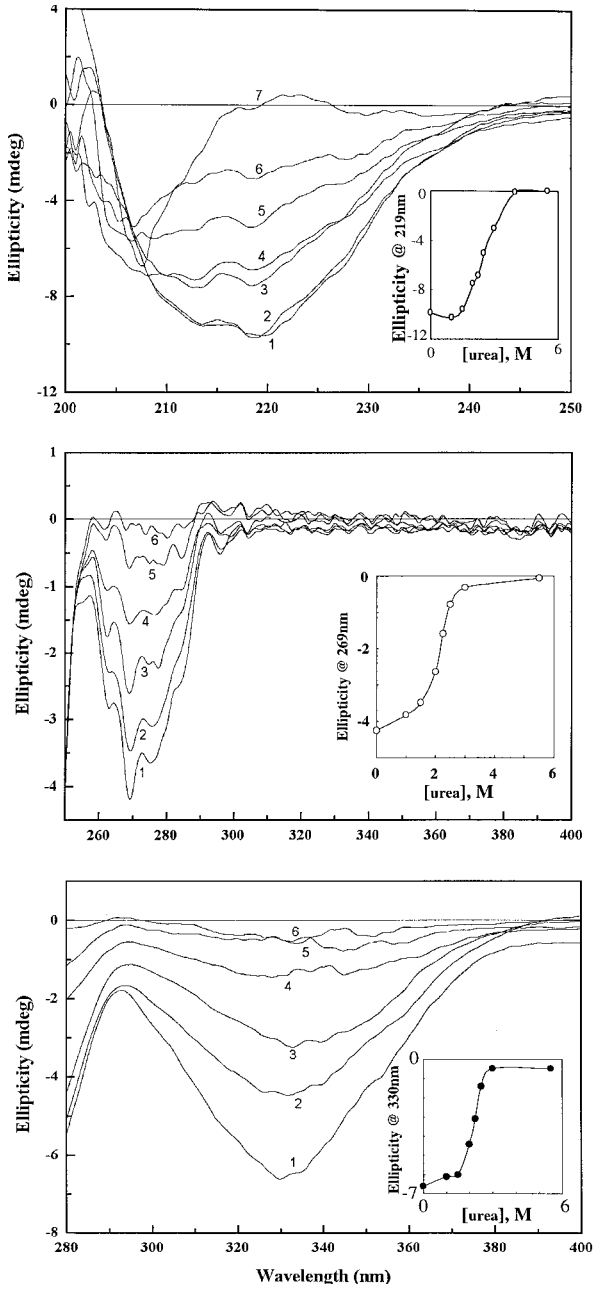


Figure 5 Urea-induced unfolding and NADPH binding of ζ-crystallin monitored by CD

Upper panel, far-UV CD spectra of ζ-crystallin in different concentrations of urea. Curves 1–7 represent the spectra of the protein in 0 M, 1.5 M, 2 M, 2.25 M, 2.5 M, 3 M and 5.5 M urea respectively. Inset, variation of ellipticity at 219 nm as a function of urea concentration. Middle panel, near-UV CD spectra of ζ-crystallin in different concentrations of urea. Curves 1–6 represent the spectra of the protein in 0 M, 1.5 M, 2 M, 2.25 M, 2.5 M and 3 M urea respectively. Inset, variation of ellipticity at 269 nm as a function of urea concentration. Lower panel, induced CD spectra of NADPH bound to urea-denatured ζ-crystallin. Curves 1–6 represent induced CD spectra in 0 M, 2 M, 2.25 M, 2.5 M, 3 M and 5.5 M urea respectively (see the Materials and methods section for details.) Inset, plot of the ellipticity at 330 nm in different concentrations of urea.

does not alter the far-UV CD spectra of the protein (results not shown).

The near-UV CD spectra of the ζ-crystallin in different concentrations of urea are shown in Figure 5 (middle panel). At

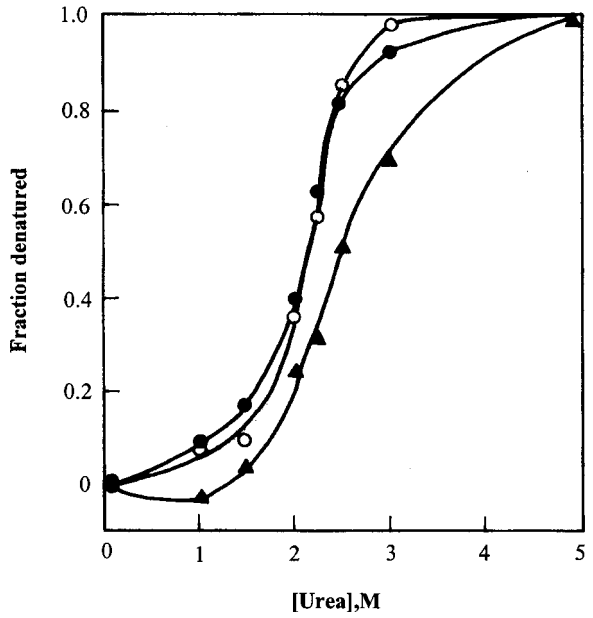


Figure 6 Fraction of ζ-crystallin denatured as a function of urea concentration

The fraction of ζ-crystallin denatured as a function of urea concentration was measured by far-UV CD (θ_{219}, data from Figure 5, upper panel) (▲), near-UVCD (θ_{269}, data from Figure 5, middle panel) (●), and by induced CD of the protein-bound NADPH (θ_{330}, data from Figure 5, lower panel) (○). See the Materials and methods section for calculations of the fraction denatured using ellipticity values.

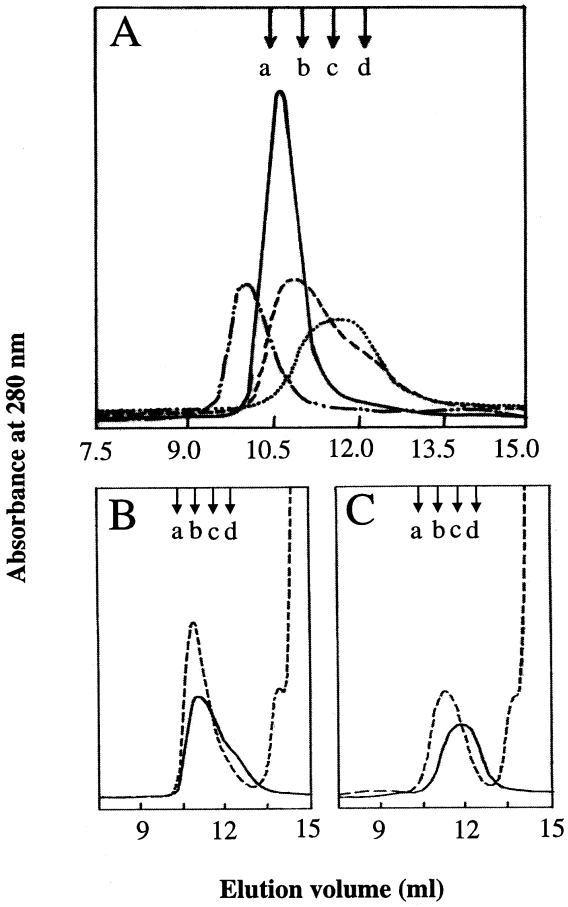


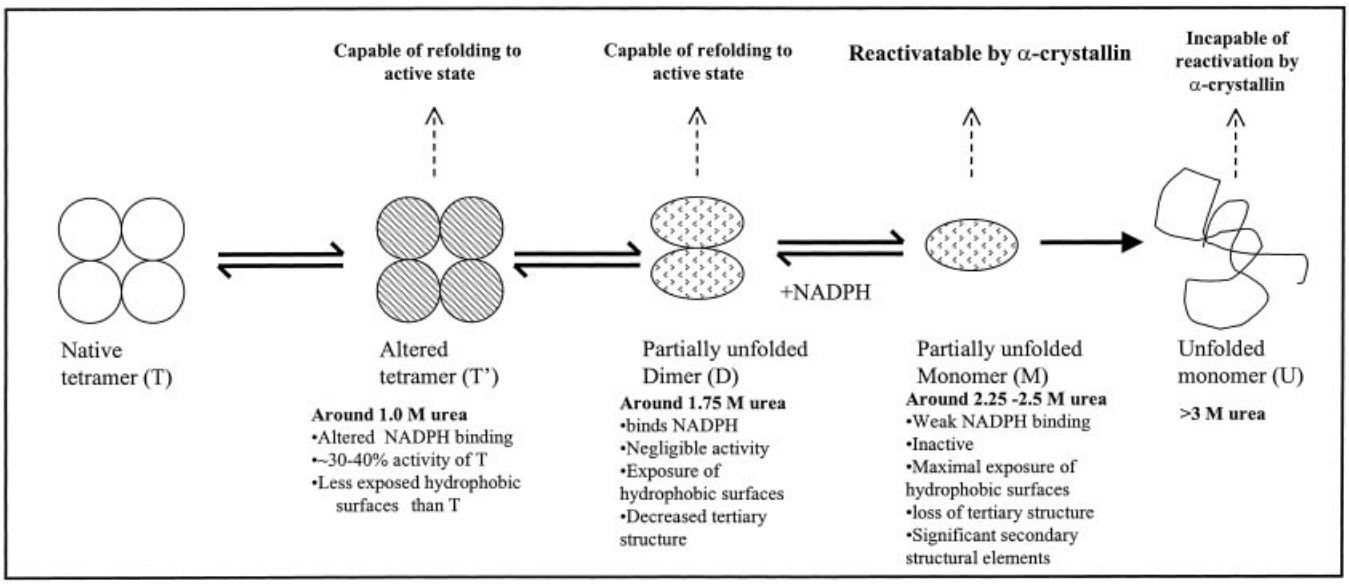
Figure 7 Elution profiles of ζ-crystallin in different concentrations of urea on a Superose 12 gel-filtration FPLC column

(**A**) ζ-Crystallin in buffer alone (——), in buffer containing 1.75 M urea (– – –), 2.5 M urea (- - - -) and 5.5 M urea (– - - –). The elution positions of standard molecular-mass markers are indicated by arrows: a, aldolase (158 kDa); b, BSA (67 kDa); c, ovalbumin (45 kDa); d, carbonic anhydrase (29 kDa). (**B**) Effect of NADPH on the oligomeric status of ζ-crystallin in 1.75 M urea. Curves (- - - -) and (——) represent the protein with and without NADPH respectively. (**C**) Elution profile of ζ-crystallin in 2.5 M urea with (- - - -) and without (——) NADPH. The high level of absorbance towards the end of the elution volume (reaching out of the scale) is due to free NADPH, which absorbs strongly at 280 nm.

a concentration of 1.5 M urea, the spectrum shows some decrease in the ellipticity. In 2.25–2.5 M urea, the protein exhibits highly decreased ellipticity, suggesting that the intermediate exhibits substantially loosened side-chain packing [Figure 5, middle panel (curves 3 and 4) and inset]. The tertiary structure is completely lost in 3 M urea. We have also studied the NADPH-binding ability of the protein in different concentrations of urea using induced CD of the bound NADPH (Figure 5, lower panel). NADPH in solution does not exhibit CD spectra, but on binding to ζ-crystallin it exhibits a negative CD spectrum between 300 nm and 400 nm [37] due to induced chirality of the molecule. The NADPH-binding ability of the protein is retained until about 1.5 M urea, then it progressively decreases and it is completely lost by 3 M urea (Figure 5, lower panel, inset). It is to be noted that, in this experiment, we have used a saturating concentration of NADPH to understand whether the binding site exists even at high concentrations of urea. This experiment, therefore, does not provide information on the affinity of the binding. This result shows that the species present around 2.25–2.5 M urea does not lose its NADPH-binding site, implying that the second intermediate(s) detected in ANS binding and intrinsic fluorescence experiments still has the NADPH-binding site intact.

Figure 6 compares the unfolding profile of ζ-crystallin in urea as monitored by near- and far-UV CD and the induced CD of NADPH upon binding to the protein. The change in the near-UV CD and the induced CD of NADPH provides information on the changes in the tertiary structural packing of the protein, whereas the changes in the far-UV CD report on the changes in secondary structure. It can be seen in Figure 6 that loss of tertiary structure precedes loss of secondary structure. Thus our results show that the intermediate(s) seen around 2.25–2.5 M urea exhibits largely decreased tertiary structural packing (at least around aromatic amino acid residues) with significant secondary structural elements. Comparison of these results with the ANS-binding studies (Figure 4) points to the existence of partially unfolded state(s) around 2.25–2.5 M urea, which has the characteristics of a molten globule. The molten-globule state, detected either as an equilibrium or kinetic intermediate state in the unfolding or refolding pathway of a globular protein, is characterized by native-like or significant secondary structural elements and little or no tertiary structure with solvent exposed hydrophobic surfaces [55].

Since ζ-crystallin is a homotetramer with subunit mass of 35 kDa, it is important to investigate quaternary structure of the partially unfolded state at 2.25–2.5 M urea. The permeation properties of the Superose 12 column have been shown to be practically independent of temperature, pH and denaturant concentration [43]. We have performed gel-filtration chromatography of ζ-crystallin using a Superose 12 FPLC column in the presence of different concentrations of urea. The elution profiles are shown in Figure 7(A). The elution positions of aldolase



Scheme 1 Representation of the urea-induced intermediates formed on the unfolding pathway of ζ-crystallin showing the states capable of spontaneous refolding and those requiring assistance

(158 kDa), BSA (67 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa), which serve as molecular-mass markers, are indicated in Figure 7. We calculated the molecular mass of the native protein and the unfolding intermediates from the elution volume using the standard calibration curve. Since the elution properties of a protein in gel-filtration column are not only dependent on the molecular mass, but also on the hydrodynamic radius, direct correlation of elution volumes with molecular masses becomes difficult. Determination of Stokes radius and comparison with molecular-mass standards would allow a meaningful interpretation of the results. Therefore we determined the Stokes radius (R_s) using the observed elution volumes and the known values of standards proteins [43]. The R_s of ζ-crystallin in buffer alone was determined to be 44.3 Å, which is the predicted value for the tetrameric form of the protein. This is in agreement with the calculated molecular mass of the native protein as obtained from the standard curve, which is approx. 140 kDa. The elution profile of ζ-crystallin in 1 M urea overlaps with that of the protein in buffer alone, indicating no change in quaternary structure of the protein (results not shown). The elution profile in 1.75 M urea is composed of a peak with a shoulder indicating the presence of two species; from the standard molecular mass calibration curve, the major peak is found to correspond to a dimer and the shoulder corresponds to a monomer. The R_s value of the major peak species is 40.0 Å. The elution profiles at 2.25 M and 2.5 M urea overlap with each other. The elution profile at 2.5 M urea shown in Figure 7 exhibits a single symmetrical, but broad peak, corresponding to a monomer with an R_s value of 31 Å. The elution positions of the native and various denatured states of ζ-crystallin are in agreement with the calibration curve for the Superose 12 column as reported by Uversky [43], which shows a separation of about 2 ml for a molecular-mass range of 35–140 kDa. Further increase in urea concentrations (beyond 2.5 M) results in the gradual shifting of the elution position towards lower elution volumes. In 5.5 M urea, ζ-crystallin elutes at a lower elution volume than that of the native protein (Figure 7A) suggesting either expansion (or unfolding) of the monomer which is also corroborated by our spectroscopy studies. No further decrease in elution volume is observed beyond this concentration of urea. The Stokes radii of unfolded monomers of several multimeric proteins such as aldolase, glyceraldehyde-3-phosphate dehydrogenase, phosphoribosyl transferase and lactate dehydrogenase are known to be more than those of their native multimeric forms [43]. Similar gel-filtration profiles were observed for the guanidinium-chloride-induced unfolding of the tetrameric duck δ2-crystallin, where the fully unfolded monomeric protein eluted at a lower elution volume than the native protein [56]. It is important to note that the R_s values determined for the dimeric and monomeric species is higher than the expected values based on the molecular mass [43]. Our spectroscopy experiments show that these species are in partially unfolded state(s). The property of ζ-crystallin in 2.5 M urea (monomer) is similar to that of a molten-globule state of proteins. The R_s of the partially unfolded (molten-globule) states of monomeric proteins, such as human α-lactalbumin and bovine carbonic anhydrase, are known to be higher than those of the native state [43]. Thus our gel-filtration results are consistent with the spectroscopy observations at 2.5 M urea where ζ-crystallin exists in a partially unfolded molten-globule-like state.

We also investigated the effect of NADPH on the elution profiles. Figures 7(B) and 7(C) show that NADPH shifts the equilibrium to populate the dimeric species in 1.75 M and 2.5 M urea respectively, suggesting that NADPH stabilizes the dimer at these concentrations of urea. As described above, ζ-crystallin could be completely reactivated on refolding it from urea concentrations up to 2 M (Figure 1). The reactivation yield falls sharply if the protein is denatured above 2 M urea. Taken together, these results show that the dimeric species, which occur between 1.5 to 2 M urea, can readily refold back to active protein, whereas the monomer cannot. Since our gel-filtration experiments show that NADPH drives the equilibrium from monomer to dimer, one would expect that NADPH would promote the reactivation of ζ-crystallin when the denatured species at 2.0–2.5 M urea is subjected to refolding in its presence. In fact, we observed that NADPH significantly improves the regain of activity of the enzyme denatured in 2.0–2.5 M urea

(results not shown). However, it cannot assist the refolding of the completely unfolded protein. It appears that the cofactor NADPH improves the reactivation yield of ζ-crystallin by stabilizing the dimeric folding-competent state. Cofactors or substrates are known to stabilize many enzymes. ζ-Crystallin is known to be stabilized by its cofactor NADPH against heat-induced denaturation [37]. Citrate synthase is stabilized by its substrate oxaloacetate, the binding of which to early unfolding intermediates on the heat-induced unfolding pathway facilitates its refolding [57]. Substrate analogues of red kidney bean purple acid phosphatase are known to stabilize it against denaturant-induced inactivation as well as unfolding [58].

Our results have allowed us to propose a urea-induced unfolding pathway of ζ-crystallin, which is depicted in Scheme 1. The native tetramer (T) goes to the T′ state in 1 M urea. This first intermediate state (T′) remains tetrameric, and is characterized by altered cofactor binding and relatively less exposed hydrophobic surfaces compared to the native state (T). This state exhibits about 40% activity of the native state and can be refolded back to the fully active state. Further increase in the concentration of urea results in the dissociation, as well as partial unfolding of the molecule. Around 1.75 M urea it predominantly exists as a partially unfolded dimer (D) characterized by relatively higher exposure of hydrophobic surfaces, reduced tertiary structure as judged by fluorescence and near-UV CD, and possessing negligible activity. However, this state is still capable of binding NADPH. This partially unfolded dimeric state can also be refolded back to the active state. Around 2.25–2.5 M urea, a partially unfolded predominantly monomeric state (M) exists. This state is characterized by highly exposed hydrophobic surfaces and loss of tertiary structure with significant secondary structural elements. These properties are similar to those of a molten-globule state. This state cannot be refolded back to active form without assistance. Our present study shows that α-crystallin assists the reactivation of this unfolded state of ζ-crystallin. At concentrations of urea above 3 M, the enzyme completely looses secondary, as well as tertiary structure, and exists either as an expanded monomer or an aggregated species. α-Crystallin cannot assist the reactivation of the protein upon refolding it from this completely unfolded state.

Refolding of the monomeric, partially unfolded or molten-globule-like state of ζ-crystallin results in aggregation as measured by light scattering. The scatter (expressed as arbitrary units) at 365 nm after 5 min of the initiation of the refolding was found to be 95 units. α-Crystallin reduces this aggregation significantly, as seen by a scatter value of only 16 units (after subtracting the contribution of α-crystallin). Thus it appears that α-crystallin interacts with the monomeric molten-globule-like state of ζ-crystallin and helps it refold to its active state by preventing or decreasing the rate of off-pathway reactions.

α-Crystallin was shown previously to prevent aggregation of unfolding [10–17,23–27], as well as refolding [28,29] intermediates, and protect enzymes from inactivating stresses [15,18–22]. Our present study shows that α-crystallin, a member of the sHSP family, not only prevents aggregation of partially unfolded proteins, but can also help in the reactivation of enzymes from their inactive states. This is one of the few reports where the reactivation of enzymes by a member of the sHSP family has been demonstrated [30–34]. Considering all these observations, it may be appropriate to describe α-crystallin as a heat shock protein with molecular chaperone activity. The usage of the prefix 'chaperone-like' may not be necessary any more.

We conclude that the urea-induced unfolding pathway of ζ-crystallin involves three distinct intermediate states: (i) an altered tetramer, (ii) a partially unfolded dimer, and (iii) a partially unfolded monomer with highly exposed hydrophobic surfaces and molten-globule-like properties. The first two intermediates can be refolded to the active state; however, the third intermediate is not capable of refolding to the active state without assistance. α-Crystallin provides the required assistance and improves the reactivation of the third intermediate state. αB-Crystallin is more efficient than αA-crystallin in the above process. These studies should be of use for understanding assisted-protein folding.

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