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ORIGINAL PAPER

Fluorescence Study on Interactions of α–Crystallin with the Molten Globule State of 1, 4–β–D–Glucan Glucanohydrolase from *Thermomonospora* sp. Induced by Guanidine Hydrochloride

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Abstract In this paper, the interaction between α- crystallin and molten globule structure of 1,4–β–D–Glucan Glucohydrolase (TSC) from an alkalothermophilic *Thermomonospora* sp. was investigated mainly by fluorescence quenching spectra, circular dichroism and three dimensional fluorescence spectra under simulative physiological conditions. Denaturation studies using GdnCl indicated that TSC folds through a partially folded state that resembles molten globule at 1.8 M GdnCl. The chaperone activity of α- crystallin was employed to study refolding of TSC. Here we studied the refolding of GdnCl denatured TSC from its molten globule state (TSC-m complex) in the presence and absence of α-crystallin to elucidate the molecular mechanism of chaperone-mediated in vitro folding. Our results, based on intrinsic tryptophan fluorescence and ANS binding studies, suggest that α-crystallin formed a complex with a putative intermediate molten globule – like intermediate in the refolding pathway of TSC. Reconstitution of the active TSC was observed on cooling the α-crystallin • TSC -m complex to 4°C. Addition of α-crystallin to the molten globule – like intermediate of TSC (TSC-m complex) complex initiated the refolding of TSC with 69 % recovery of the biological activity of the enzyme.

Keywords ANS · Cellulase · Protein folding · Guanidine hydrochloride

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Introduction

The question of how protein folds into its unique, compact, highly ordered, and functionally active form is one of the most intriguing and perplexing questions of structural and cellular biology. Protein folding is determined by the information encoded by the amino acid sequence and proceeds *in vivo* by the same spontaneous mechanism [1]. The folding and assembly of a protein into its biologically active conformation is a complex succession of reactions involving the formation of secondary and tertiary structures and domains, and the oligomerization of folded monomers [2]. *In vivo* folding and assembly of proteins occur in a highly complex heterogeneous environment, in which high concentrations of proteins at various stages of folding and with potentially interactive surfaces coexist that may change the folding potentials inherent in the sequence [3]. Chemical denaturants like guanidine chloride (GdnCl) and urea have long been known to unfold proteins by stabilizing the unfolded state compared to the native state. Unfolding by GdnCl and urea has since then become the standard method to determine protein stability and to investigate the kinetics and mechanisms of protein folding reactions. Empirically, a linear correlation between the free energy for unfolding (ΔG^0) and denaturant concentration ([D]) was found in the region of the unfolding transition. The correlation coefficient of this linear free energy relationship is commonly termed m_{eq}-value

$$m_{eq} = \delta\Delta G^0/\delta[D]$$

It reflects the denaturing strength of the denaturant. Linear relationships were also found between denaturant concentration and the logarithm of the rate constants for protein folding (k_f) and unfolding (k_u) indicating that the activation free energies for folding $\delta\Delta G_f^0$ and unfolding

$\delta\Delta G_u^0$) are also linearly dependent on denaturant concentration. Accordingly, kinetic m-values were defined as:

$$m_f = \frac{\partial \Delta G_1^0}{\partial [D]} \text{ and } m_u = \frac{\partial \Delta G_u^0}{\partial [D]}$$

Refolding of the denatured proteins *in vitro* has been an important issue at the fundamental as well as at the biotechnological level. Denaturation and renaturation are thermodynamic processes, involving a change in free energy and large changes in conformation between the denatured and the native states [4]. A number of accessory proteins have been identified that affect the folding and subsequent assembly of proteins. These include the protein isomerases catalyzing cis-trans- isomerization of peptide bonds or disulfide exchange [5] and the polypeptide binding proteins termed as "molecular chaperones". The molecular chaperones are required for the successful folding, assembly, transport and degradation of proteins within the cell [6]. The molecular mechanism of refolding of proteins by chaperones now appears to be an important consideration for defining how proteins fold *in vivo*. α-Crystallin, the major protein of the vertebrate eye lens, consists of two types of highly homologous 20-kDa subunits, α- A and α- B [7, 8]. α-Crystallin shares both sequence and structural homology with small heat shock proteins (sHSPs) that are known to act as molecular chaperones [9]. The ability of α-crystallin to suppress aggregation of damaged proteins play a crucial role in maintaining the transparency of the ocular lens, and the failure of this function could contribute to the development of cataracts [10–12]. α-Crystallin has been reported to bind the temperature induced molten globule state of proteins and prevent photo-aggregation of γ- crystallin by providing hydrophobic surfaces [13, 14]. α-Crystallin has been reported to be functionally equivalent to sHSPs namely murine HSP25 and human HSP27 in refolding of α-glucosidase and citrate synthase *in vitro* [15]. Despite the growing interest in the chaperone action of α-crystallin, little is known about its mechanism of chaperoning. Elucidating the mechanistic details underlying the efficient refolding of proteins by chaperones now appears to be an important consideration for defining how proteins fold *in vivo*. Cellulases have raised enormous interest in the past decades in view of their biotechnological applications. However, the reports on unfolding/folding studies of this class of enzyme are scarce and especially of enzymes from extremophiles. For the functional in vitro analysis of α-crystallin, 1,4-β-D-Glucan Glucanohydrolase (TSC) from *Thermomonospora* sp. was used as the model system. Experimental evidence presented in this paper serves to implicate that the chaperone α-crystallin stabilizes the molten globule state of TSC and thus restrains the non-native conformer from exploring unproductive pathways.

Experimental

Purification of TSC

TSC was produced purified by conventional protein purification methods of ammonium sulphate precipitation, followed by ion exchange chromatography and gel filtration chromatography as described in published results [16]. The enzyme was assayed according to the procedure described in [17].

Denaturation / renaturation studies of TSC

All denaturation and renaturation experiments were carried out in 50 mM sodium phosphate buffer, pH 7.0. TSC was incubated with varying concentrations of GdnCl for 1.5 h at 28°C, and the accompanying structural changes were investigated using fluorescence and circular dichroism measurements. Renaturation was initiated by rapidly diluting the sample (TSC at varying states of denaturation) in the same buffer with or without α-crystallin, and the carboxymethyl cellulase activity at various times of refolding was measured. Renaturation of TSC was also carried out by substituting α-crystallin with bovine serum albumin (0.6 mg/ml).

Circular dichroism and fluorescence studies

Circular dichroism (CD) spectra were recorded on a JASCO J600 model spectropolarimeter. Changes in the secondary structure of TSC induced by the denaturant GdnCl were monitored in the far UV region (200–250 nm) using a 1 mm path length cell. The enzyme concentration in these experiments was 0.5 mg/ml. Fluorescence spectra were recorded with a Perkin-Elmer LS 50B spectrofluorimeter equipped with a Julabo F20 water bath. The appropriate blanks, run under same conditions, were subtracted from the sample spectra. The protein solution was excited at 295 nm and the emission was recoreded in the range of wavelengths 300–400 nm. Both the excitation and emission spectra were obtained setting the slit-width at 5 nm, and speed 100 nm min^{-1}.Mean residue ellipticities [θ] (expressed as degree cm^2dmol^{-1}) were determined according to Labhardt [18].

Results and discussion

Folding intermediates of TSC

The CD spectrum of TSC in the far–UV region (200–260 nm) exhibited a strong negative ellipticities in the region 215–222 nm and a weaker one at 208 nm, characteristic of a protein having an α-helix (Fig. 1a). TSC was incubated with increasing concentrations of the denaturant GdnCl, and the changes in the negative CD band in

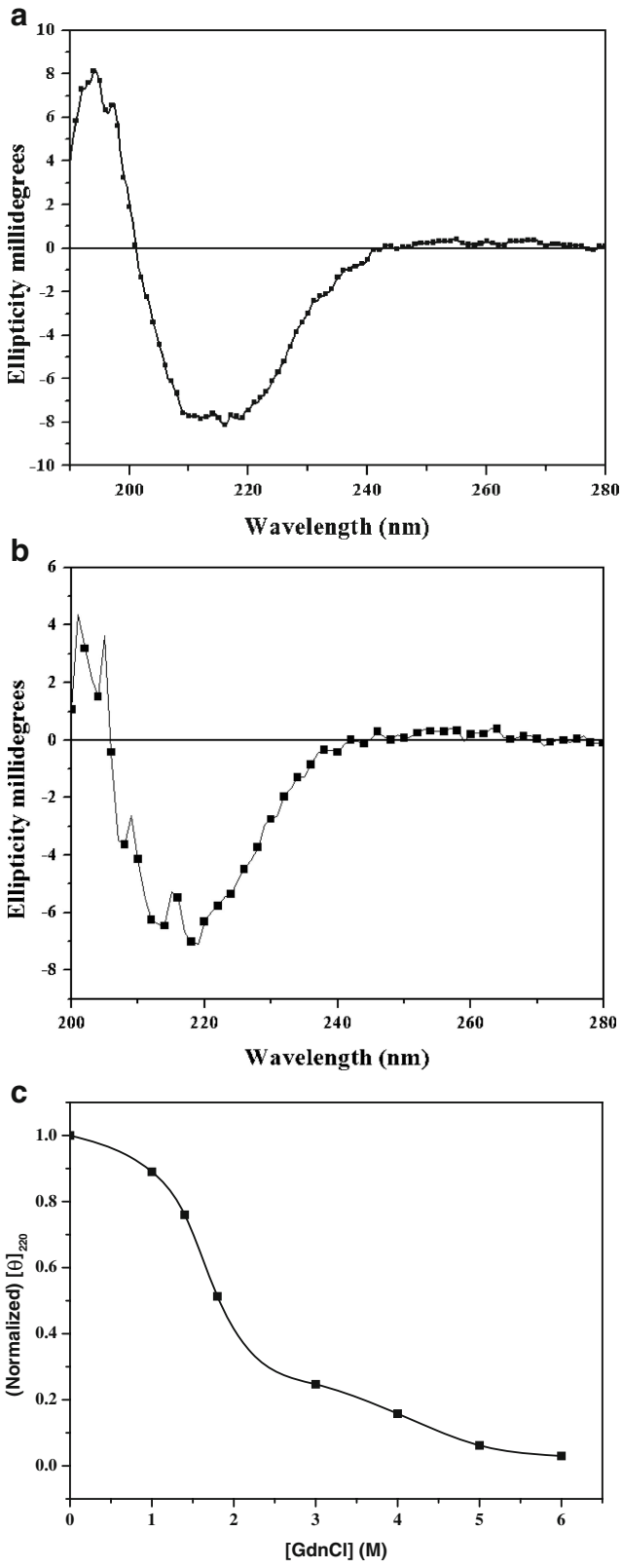

Fig. 1 **a** CD spectra of native TSC. Far-UV CD spectra recorded for native TSC from 280–190 nm at 25°C. **b** CD spectra of TSC-m. Far-UV CD spectra recorded for TSC incubated with 1.8 M GdnCl from 280–190 nm at 25°C. **c** Dependence of mean residue ellipticity of TSC at 220 nm on GdnCl. TSC was incubated with varying concentrations of GdnCl for 1.5 h at 28°C in 50 mM sodium phosphate buffer, pH 7, and the CD spectra in the far UV region (200–250 nm) were recorded. Protein concentration was 0.5 mg/ml, and a 1 mm path length cell was used. The ellipticity values obtained were normalized with respect to that in the absence of GdnCl

the far UV region were monitored. The mean residue ellipticities obtained at 220 nm ($[\theta]_{220}$) were normalized with respect to that in the absence of GdnCl and plotted against the respective GdnCl concentration. A decrease in the negative ellipticity was observed with the addition of GdnCl, and at 1.8 M GdnCl the $[\theta]_{220}$ decreased by almost 51.3 % of that in the absence of GdnCl (Fig. 1b). Further increase in the denaturant concentration resulted in a loss of negative ellipticity until there was a total loss of structure of the CD band in 6 M GdnCl indicating a considerable loss of secondary structure (Fig. 1c). At higher concentrations of the denaturant, a decrease in the intensity of the fluorescence was observed which was accompanied by a red shift in the λ_{em} indicating unfolding of TSC (Fig. 2a). 6 M GdnCl converted TSC into unfolded polypeptides, and this state has been referred to as TSC-u. The molten globule state is a productive on-pathway intermediate in the real folding reactions in many globular proteins and has a denatured state with native-like secondary structure. ANS has been widely used to detect the formation of molten globule- like intermediates in the folding pathways of several proteins. ANS is not fluorescent in aqueous solutions (λ_{em} 511 nm); however, binding to hydrophobic pockets of protein its emission maximum shifts to shorter wavelengths i.e. blue shift and the emission intensity is enhanced. We used the fluorophore ANS to determine the relative amount of exposed hydrophobic surfaces in the folding intermediates of TSC. As shown in Fig. 2b the binding of ANS to TSC was measured as a function of GdnCl. A maximum increase in the ANS fluorescence (λ_{em} 505 nm) was observed at 1.8 M GdnCl indicating maximum exposure of hydrophobic surfaces in this state of TSC. It was thus evident from the CD studies that at 1.8 M GdnCl TSC retains substantial amount of secondary structure but very little tertiary structure. Altogether the CD and ANS binding studies revealed that at 1.8 M concentration of GdnCl, TSC partially unfolded to its molten globule state, which has been referred to as TSC-m.

Chaperone assisted renaturation of TSC

Attempts to refold TSC from the TSC-u state in the absence and presence of α-crystallin were unsuccessful (Fig. 3a). Similar results were obtained for progressively less denatured states (TSC denatured with 2–4 M GdnCl). Further investigations were carried out to study the influence of the α-crystallin on the renaturation of TSC-m. The refolding of TSC-m was initiated at 28°C in the absence / presence of α-

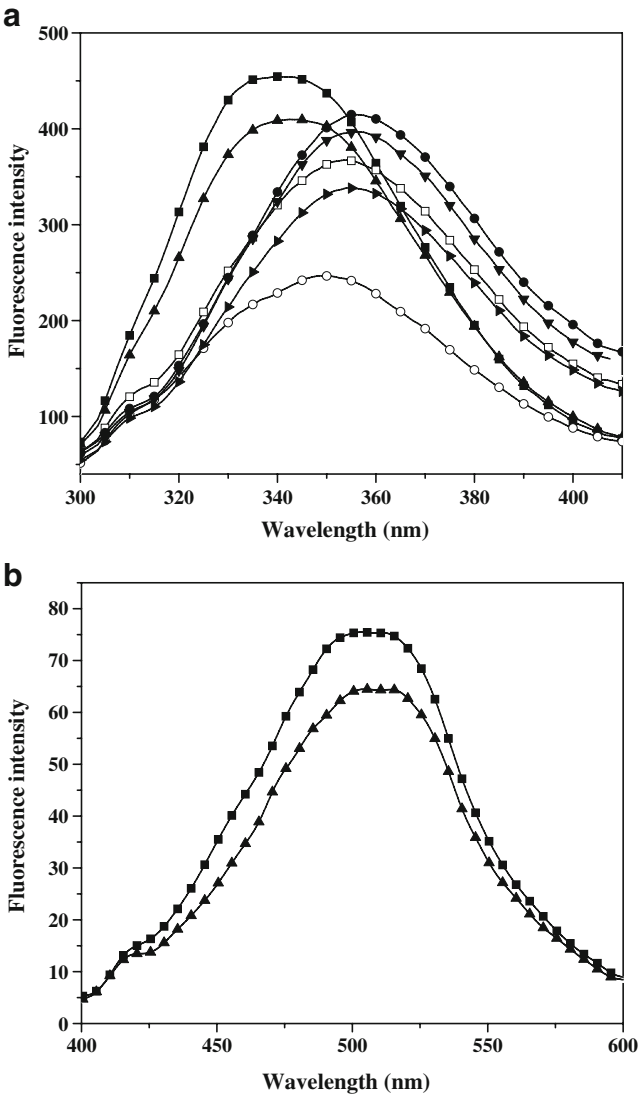

Fig. 2 **a** Tryptophanyl fluorescence of GdnCl treated TSC. TSC was incubated incubated with following concentrations of GdnCl for 1.5 h at 28°C in 50 mM sodium phosphate buffer, pH 7: 0 M (■); 1 M (▲), 1.8 M (●); 3 M (▼); 4 M (□); 5 M (►); 6 M (○). The samples were excited at 295 nm. **b** GdnCl-dependent exposure of hydrophobic surfaces of TSC measured by ANS fluorescence. ANS fluorescence intensity at 475 nm of 25 μM TSC [treated with GdnCl: 0 M (▲) and 1.8 M (■)] on incubation with 10 μM ANS for 15 min. The concentrations indicated are the final concentrations. The samples were excited at 375 nm

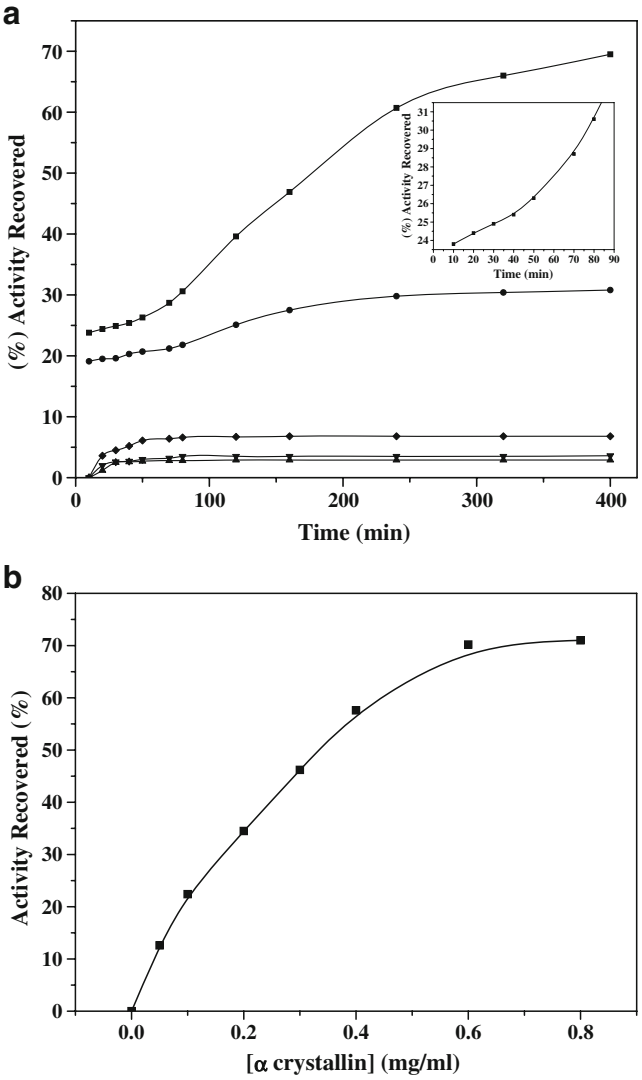

Fig. 3 **a** Time course of renaturation of TSC in the absence or presence of α-crystallin. TSC at a concentration of 25 μM was incubated with 6 or 1.8 M GdnCl for 1.5 h at 28°C to yield TSC-u and TSC-m, respectively. The renaturation process was initiated at the same temperature by diluting 10 μl of the sample into a final volume of 1 ml of 50 mM sodium phosphate buffer, pH 7, with or without α-crystallin (final concentration 0.6 mg/ml). After 30 min the samples were kept at varying temperatures, and 100-μl aliquots of the refolding solution were withdrawn at various times of refolding and assayed for TSC activity. The refolding of TSC-m in the presence of α-crystallin when the refolding solution was shifted from 28°C to the following temperatures: 4ºC (■), 28°C (●), and 37°C (♦). (▲) and (▼) represents the refolding of TSC-u and progressively less denatured states of TSC (TSC denatured with 2–4 M GdnCl) in the absence and presence of α-crystallin under the experimental conditions described above. The percentage activity recovered was determined with reference to native TSC. [*Inset: The early time course of α-crystallin-assisted renaturation demonstrating the lag phase (first 40 min) at 4ºC* (■).] **b** Reactivation of TSC-m at varying concentrations of α-crystallin. TSC-m was renatured (as described in the legend to Fig. 2a) in the presence of varying concentrations of α-crystallin. After 30 min the samples were kept at 4ºC, and aliquots withdrawn after 6 h were assayed for TSC activity. The percentage activity recovered is with respect to native TSC

crystallin; after 30 min the samples were shifted to varying temperatures and the CMCase activity recovered at different time intervals was measured.

As shown in Fig. 3a, TSC-m lacked the ability to spontaneously reconstitute active TSC. However, in the presence of 0.6 mg/ml of α-crystallin the process of refolding of TSC-m at 4°C followed a sigmoidal time course. There was no measurable CMCase activity for the first 40 min (lag phase). The rate of activation beyond the lag phase was slow, and a maximum of 69.5 % of activity of CMCase activity was recovered in 6 h.

The α-crystallin-mediated renaturation of TSC-m was examined as a function of the chaperone concentration. As shown in Fig. 3b, 22.4% of the original CMCase activity was recovered at the lowest concentration of α-crystallin (0.1 mg/ml). The extent of renaturation increased in concentration-dependent manner, and a maximum 69.5% of the original activity was recovered at α-crystallin concentration of 0.6–0.8 mg/ml. The concomitant increase in the extent of renaturation with an increase in α-crystallin can be attributed to simple mass action effects, wherein an increase in the α-crystallin concentration would increase the collisional frequency so as to favor the formation of α-crystallin•TSC-m complex as opposed to forming non-native TSC.

To test the specificity of α-crystallin, the renaturation of TSC-m was also investigated in the presence of bovine serum albumin alone (0.6 mg/ml), under the conditions described for renaturation with α-crystallin. It was observed that unlike α-crystallin bovine serum albumin failed to mediate the reconstitution of active TSC (data not shown). α-Crystallin mediated reconstitution of active TSC was obtained only from its TSC-m state (Fig. 3a), indicating that the chaperone probably traps the TSC in a conformation resembling the molten globule.

α-Crystallin forms a complex with folding intermediate

Fluorescence studies were performed to confirm that the TSC bound to α-crystallin exists in the molten globule state. Native TSC exhibited an emission maximum at 339 nm, whereas in 6 M GdnCl the emission maximum was shifted to 357 nm which corresponds to the fluorescence maximum of tryptophan in aqueous solution during unfolding process. The TSC bound to α-crystallin exhibited an emission maximum at 344 nm indicating that the tryptophans in the bound form of TSC are more exposed to the solvent than the native enzyme. These results revealed that the conformation of TSC bound to α-crystallin is neither native-like nor completely unfolded but a partially folded intermediate resembling the molten globule. Thus by confiscating the molten globule state of TSC in the form of a stable binary complex, α-crystallin is able to suppress their interaction that would otherwise lead to aggregation.

Temperature dependence of the exposure of hydrophobic surfaces of α-crystallin •TSC -m complex

The thermodynamic properties of the unfolding reactions of globular proteins are now known accurately as a function of temperature through calorimetric studies. The unfolding reactions of different proteins display certain common properties. The enthalpy of unfolding depends on the temperature at which unfolding occurs, which can be varied by adjusting pH or guanidine hydrochloride concentration. "Hydrophobic interaction" refers to the process in which a hydrophobic side chain of an unfolded protein is taken out of H_20 and is buried in the interior of a protein through folding. The temperature dependence of the hydrophobic interactions in protein folding has been studied earlier [19, 20]. Maximum stabilization of these interactions is observed at high temperature where the enthalpy is the dominating factor in determining the stability, and as the temperature is decreased the interactions are weakened.

The temperature-shift experiments (Fig. 4) revealed an increase in the α-crystallin-mediated reconstitution of TSC with the decrease in the temperature of the refolding solution implying that the hydrophobic interactions play a crucial role in the formation of α-crystallin•TSC-m complex. Attempts were made to correlate temperature-mediated alterations in the hydrophobic surfaces of the α-crystallin•TSC-m complex to reconstitution of active TSC, using ANS as a probe for apolar binding sites whose fluorescence is dependent on the hydrophobicity of the environment. As shown in Fig. 5, presence of α-crystallin•TSC-m complex incubated at 37°C resulted in a blue shift in the ANS fluorescence from 511 to 505 nm accompanied by an increase in fluorescence intensity; however, in the presence of the complex incubated at 4°C a 35% decrease in the dye fluorescence was observed compared with that at 37°C (Fig. 5). These results indicate

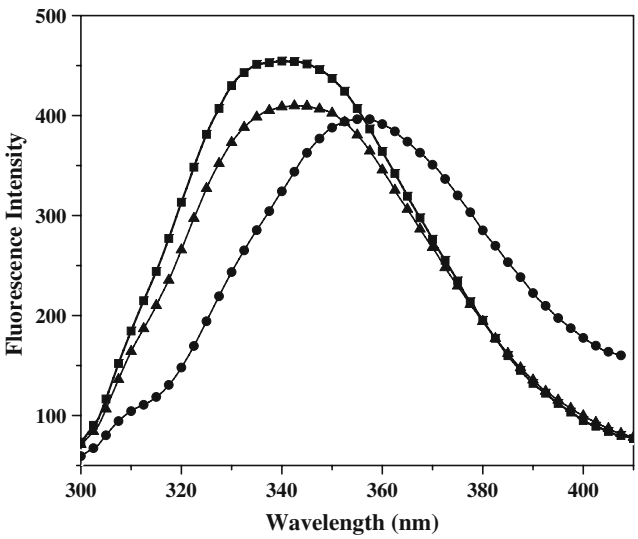

Fig. 4 Tryptophanyl fluorescence of free and α-crystallin bound TSC. TSC at a concentration of 25 μM was incubated with 1.8 M GdnCl for 1.5 h, and a further 10 μl of the sample was diluted into a final volume of 1 ml in 50 mM phosphate buffer, pH 7, containing 0.6 mg/ml α-crystallin at 37ºC. After 30 min incubation the refolding solution was diluted to 2 ml, and the tryptophanyl fluorescence was recorded. The fluorescence spectrum of α-crystallin bound TSC (▲) was obtained on subtracting the spectrum of α-crystallin•TSC-m complex from that of α-crystallin. (■) and (●) represent the fluorescence spectra of native and denatured TSC in the absence or presence of 1.8 M GdnCl, respectively. All samples were excited at 295 nm

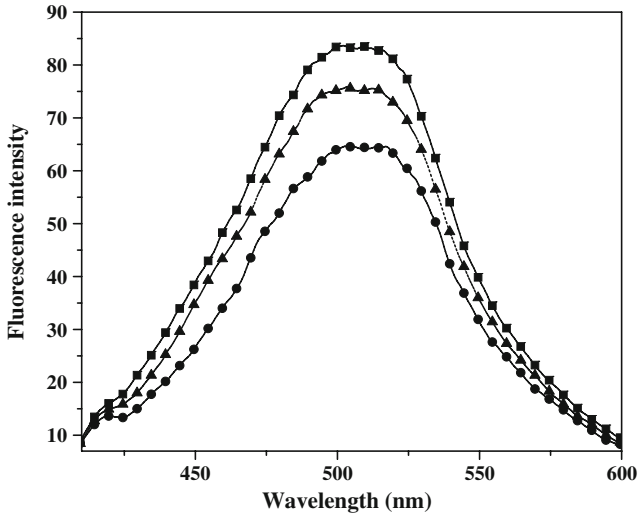

Fig.5 Temperature-dependent exposure of hydrophobic surfaces of α-crystallin•TSC-m complex measured by ANS binding. Renaturation of 25 μM TSC-m was initiated at 37°C in 50 mM phosphate buffer containing 0.6 mg/ml α-crystallin preincubated at the same temperature for 2 h. After 30 min the samples were incubated at 37°C (■) or shifted to 4°C (▲); furthermore, ANS (final concentration 100 μM) was added after 12 h, and the fluorescence was recorded at the respective temperatures at 1 h incubation, with the excitation wavelength fixed at 375 nm. (●) represents the fluorescence spectra of native TSC alone

that at 37°C the complex exists in a state with hydrophobic binding sites that are accessible to ANS; however, a decrease in the incubation temperature to 4°C probably mediates a conformational change in the complex that is accompanied by internalization of the hydrophobic surfaces previously exposed. This probably further acts to weaken the hydrophobic interactions holding the α-crystallin•TSC-m complex and thus reduces the affinity of α-crystallin for the substrate protein further, allowing reconstitution of active TSC. This observation also explains the cold labiality of α-crystallin•TSC-m complex and the inability of α-crystallin to reconstitute active TSC when refolding was initiated at 4°C. Fluorescent chemoaffinity labeling studies using *o*-phthalaldehyde corroborated α-crystallin mediated TSC renaturation. Incubation of the α-crystallin renatured TSC, with *o*-phthalaldehyde resulted in the formation of TSC-isoindole derivative as observed with the native enzyme (Fig. 6).

α-Crystallin operates by providing hydrophobic surfaces that interact with the molten globule state of TSC. The hydrophobic interactions play an important role in the formation of α-crystallin•TSC-m complex. Conformational changes have been proposed to play a major role in the binding of folding intermediates and in the discharge of polypeptides from molecular chaperones. The present investigation was carried out to gain some insight into the conformation of TSC interacting with the chaperone α-crystallin and to understand the mechanism underlying the reconstitution of active enzyme. The conditions for the unfolding of native TSC were chosen so that the unfolded enzyme or its folding intermediates would serve as a substrate for the α-crystallin-mediated reconstitution of active TSC. The work on denaturation studies using the structure-perturbing agent GdnCl revealed that the folding of TSC involves an intermediate that resembles the molten globule. Interest in such intermediates is strong since they have been proposed to be an obligatory intermediate formed early in the folding pathway. It has been reported that α-crystallin inhibits the aggregation of lysozyme and binds to aggregation- prone, molten-globule-like intermediate in the refolding pathway [21–23]. The present studies on TSC revealed that the chaperone α-crystallin operates by interacting with the hydrophobic regions that appear on the surface of molten globule state of TSC. This probably reduces the concentration of the free partially folded TSC (TSC-m) during renaturation and thus prevents loss of enzyme activity due to their hydrophobic aggregation. Lowering the temperature to 4°C induces a conformational change in the α-crystallin•TSC-m complex that is accompanied by a concomitant internalization of previously exposed hydrophobic surfaces. This acts to reduce the hydrophobic interactions involved in the formation of the complex and thus the affinity of the chaperone for the substrate protein further allowing reconstitution of the active TSC. α-crystallin-mediated reconstitution of TSC was observed when the refolding process was initiated at 28 and 37°C

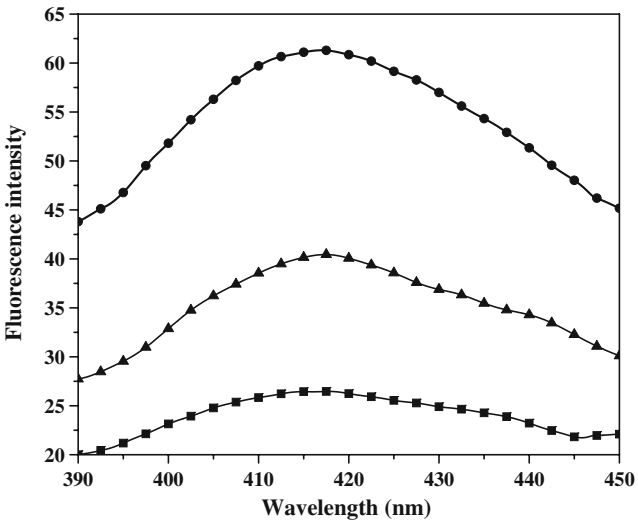

Fig. 6 Isoindole fluorescence of α-crystallin-renatured TSC on reaction with *o*-phthalaldehyde. TSC-m was renatured as described in legend to Fig. 2a. After 30 min the refolding solution was further incubated at 4°C for 6 h, when maximum TSC activity was recovered. Furthermore, 10 μl of 3 mM *o*-phthalaldehyde was added to 2 ml of the renatured TSC, and the spectra were recorded after 30 min, with the excitation wavelength fixed at 338 nm. Similar experiments were repeated for renaturation of TSC -m in the absence of α-crystallin. (▲) and (■) represent the isoindole spectra of TSC renatured from TSC-m state in the presence and absence of α-crystallin, respectively. (●) represents the isoindole spectra of native TSC

and not when initiated at 4°C which is attributed to the inability of the chaperone to prevent aggregation of TSC-m at low temperature. The dependence of protein aggregation reactions on temperature and concentration is known. The kinetic competition between refolding and aggregation has been reported to be a major determinant for lower yields or irreversibility in refolding of proteins *in vitro* [24]. This paper reveals that α-crystallin is able to reconstitute TSC via interaction with its non-native conformer characterized by an increased surface hydrophobicity but a remarkably low degree of unfolding. The enzyme is in molten globule state in 1.8 M GdnCl as at this concentration it loses its native three dimensional conformation (red shift in tryptophanyl fluorescence) but still maintaining its secondary structure (native like CD spectra). α-crystallin in particular binds to the molten globule state of the enzyme and assists its refolding by suppressing the formation of non-native conformers. The α-crystallin•TSC-m complex plays important role in the formation of near-native conformation of the enzyme which was corroborated by ANS binding studies and isoindole formation. Unfolding of cellulases from *Trichoderma reesei* were studied using Dithiothreitol, Brefeldin A and ionophore A23187 [25]. But the reports on chaperone mediated refolding of cellulase are scarce. The present investigation on α-crystallin adds to the information available on its chaperone function.

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References

1. Anfinsen CB (1973) Principles that govern the folding of protein chains. Science 181:223–230. doi:10.1126/science.181.4096.223
2. Jaenicke R (1987) Folding and association of proteins. Prog Biophys Mol Biol 49:117–237. doi:10.1016/0079-6107(87)90011-3
3. Jaenicke R, Rudolph R (1986) Refolding and association of oligomeric proteins Methods Enzymol. Enzyme Structure part L 131:218–250
4. Arai M, Kuwajima K (2000) Role of the molten globule state in protein folding. Adv Protein Chem 53:209–271. doi:10.1016/S0065-3233(00)53005-8 Protein folding mechanisms
5. Lang K, Schmid FX, Fisher G (1987) Catalysis of protein folding by prolyl isomerase. Nature 329:268–270. doi:10.1038/329268a0
6. Hendrick JP, Hartl FU (1993) Molecular chaperone functions of heat-shock proteins. Annu Rev Biochem 62:349–384. doi:10.1146/annurev.bi.62.070193.002025
7. Siezen RJ, Argos P (1983) Structural homology of lens crystallins. III. Secondary structure estimation from circular dichroism and prediction from amino acid sequences. Biochim Biophys Acta 748:56–67
8. Montfort RV, Slingsby C, Vierlingt E (2001) Structure and function of the small heat shock protein/α-crystallin family of molecular chaperones. Adv Protein Chem 59:105–156. doi:10.1016/S0065-3233(01)59004-X Protein folding in the cell
9. Klemenz R, Frohli E, Steiger RH, Schafer R, Aoyama A (1991) α B-Crystallin is a small heat shock protein. Proc Natl Acad Sci USA 88:3652–3656. doi:10.1073/pnas.88.9.3652
10. Horwitz J (1993) Proctor Lecture. The function of alpha-crystallin. Invest Ophthalmol Vis Sci 34:10–21
11. Kelley MJ, David I, Iwasaki N, Wright J, Shearer TR (1993) α-crystallin chaperone activity is reduced by calpain II in vitro and in selenite cataract. J Biol Chem 268:18844–18849
12. Lorimer GH, Baldwin TO (1998) Lens crystallin: chaperone like properties methods Enzymol. Molecular Chaperones 290:365–384
13. Raman B, Rao CM (1994) Chaperone-like activity and quaternary structure of alpha-crystallin. J Biol Chem 269:27264–27268
14. Groenen PJ, Merck KB, de Jong WW, Bloemendal H (1994) Structure and modifications of the junior chaperone alpha-crystallin: from lens transparency to molecular pathology. Eur J Biochem 225:1–19. doi:10.1111/j.1432-1033.1994.00001.x
15. Jakob U, Gaestel M, Engel K, Buchner J (1993) Small heat shock proteins are molecular chaperones. J Biol Chem 268:1517–1520
16. Jagtap S, Rao M (2005) Purification and properties of a low molecular weight 1, 4-β–D–Glucan glucohydrolase having one active site for carboxymethyl cellulose and xylan from an alkalothermophilic Thermomonospora sp. Biochem Biophys Res Commun 329:111–116. doi:10.1016/j.bbrc.2005.01.102
17. Jagtap S, Rao M (2006) Conformation and microenvironment of the active site of a low molecular weight 1,4–β–D–Glucan Glucanohydrolase from an alkalothermophilic *Thermomonospora* sp.: Involvement of a lysine and cysteine residue. Biochem Biophys Res Commun 347:428–432. doi:10.1016/j.bbrc.2006.06.100
18. Labhardt AM (1986) Folding intermediates studied by circular dichroism. Methods Enzymol 131:126–135. doi:10.1016/0076-6879(86)31038-3
19. Baldwin RL (1986) Temperature dependence of the hydrophobic interaction in protein folding. Proc Natl Acad Sci USA 83:8069–8072. doi:10.1073/pnas.83.21.8069
20. Surewicz WK, Olesen PR (1995) On the thermal stability of alpha-crystallin: a new insight from infrared spectroscopy. Biochemistry 34:9655–9660. doi:10.1021/bi00030a001
21. Bychkova VE, Pain RH, Ptitsyn OB (1988) The 'molten globule' state is involved in the translocation of proteins across membranes? FEBS Lett 238:231–234. doi:10.1016/0014-5793(88)80485-X
22. Raman B, Ramakrishna T, Rao CM (1997) Effect of the chaperone-like alpha-crystallin on the refolding of lysozyme and ribonuclease A. FEBS Lett 416:369–372. doi:10.1016/S0014-5793(97)01240-4
23. Das KP, Petrash JM, Surewicz WK (1996) Conformational properties of substrate proteins bound to a molecular chaperone α-crystallin. J Biol Chem 271:10449–10452. doi:10.1074/jbc.271.28.16934
24. Pace CN (1986) Determination and analysis of urea and guanidine hydrochloride denaturation curves. Methods Enzymol 131:266–280. doi:10.1016/0076-6879(86)31045-0
25. Pakula TM, Laxell M, Huuskonen A, Uusitalo J, Saloheimo M, Penttilä M (2003) The effects of drugs inhibiting protein secretion in the filamentous fungus *Trichoderma reesei:* evidence for down-regulation of genes that encode secreted proteins in the stressed cells. J Biol Chem 278:45011–45020. doi:10.1074/jbc.M302372200