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In Vitro Renaturation of Alkaline Family G/11 Xylanase via a Folding Intermediate: α-Crystallin Facilitates Refolding in an ATP-Independent Manner

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Abstract In this study, alkaliphilic family G/11 xylanase from alkali-tolerant filamentous fungi *Penicillium citrinum* MTCC 6489 was used as a model system to gain insight into the molecular aspects of unfolding/refolding of alkaliphilic glycosyl hydrolase protein family. The intrinsic protein fluorescence suggested a putative intermediate state of protein in presence of 2 M guanidium hydrochloride (GdmCl) with an emission maximum of 353 nm. Here we studied the refolding of GdmCl-denatured alkaline xylanase in the presence and the absence of a multimeric chaperone protein α-crystallin to elucidate the molecular mechanism of intramolecular interactions of the alkaliphilic xylanase protein that dictates its extremophilic character. Our results, based on intrinsic tryptophan fluorescence and hydrophobic fluorophore 8-anilino-1- naphthalene sulfonate-binding studies, suggest that α-crystallin formed a complex with a putative molten globule-like intermediate in the refolding pathway of xylanase in an ATP-independent manner. A 2 M GdmCl is sufficient to denature alkaline xylanase completely. The hydrodynamic radius (R_H) of a native alkaline xylanase is 4.0, which becomes 5.0 in the presence of 2 M GdmCl whereas in presence of the higher concentration of GdmCl R_H value was shifted to 100, indicating the aggregation of denatured xylanase. The α-crystallin·xylanase complex exhibited the recovery of functional activity with the extent of ~43%. Addition of ATP to the complex did not show any significant effect on activity recovery of the denatured protein.

Keywords Alkaline xylanase · Denaturation · Refolding · α-Crystallin · Guanidium hydrochloride

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Introduction

In the recent years, considerable attention is being paid on cellulase-free xylanases (1, 4-β-D-xylan-xylanohydrolase, E.C. 3.2.1.8) from extremophilic sources due to their ability to withstand the hazardous reaction conditions in paper and pulp and several other biotechnological industries [1–4]. The understanding of the three-dimensional structural organizations of this protein, thus, has got immense interest of scientific study, which, in turn, will provide the molecular aspects of intramolecular interactions involved in the compact three-dimensional structure of the protein. We use an alkaliphilic family G/11 xylanase from *Penicillium citrinum* MTCC 6489 [3] as a model enzyme to gain insight into the unfolding and refolding mechanism of an alkaliphilic glycosyl hydrolase. The folding pathway of a protein is a complicated process, which involves a succession of a definite number of intermediate conformational states [5, 6]. The native conformation is determined by the totality of inter atomic interactions and hence by the amino acid sequence, in a given environment. Despite of the in vivo protein folding which requires the assistance of a preexisting machinery of molecular chaperone proteins, the folding of protein in vitro condition account a good speculation of different conformational intermediate states which are generated during the folding process [5, 7–9]. Denaturation and renaturation are thermodynamic processes, involving a change in free energy and the large change in conformation between the denatured and native states [5]. Misfolding and aggregation can happen due to the association of the exposed hydrophobic surfaces during the refolding process, which poses a serious problem in the industrial process of producing recombinant proteins [8–12]. The problem of aggregation in vivo is circumvented by the chaperones, which interact with the high-energy state protein intermediates and catalyzes correct protein folding by preventing the non-specific protein aggregation. Elucidating the mechanistic details underlying the efficient refolding of proteins by chaperones to its lowest energy state now appears to be a good speculation for defining how proteins fold in vivo [5, 6, 13–15]. Since, α-crystallin is a prominent member of the small heat shock protein (sHSP) family [16–21] and has chaperone function like other sHSPs to suppress the aggregation of heat, UV-irradiated and chemically denatured proteins [11, 21], we use this protein in the refolding process of the denatured xylanase in the present study.

Xylanase (MW ~25 kDa and pI 3.6) from alkali-tolerant *P. citrinum* has been purified and characterized previously in our laboratory [3, 4]. In this study, we show that xylanase looses its activity mostly in presence of 2 M guanidium hydrochloride (GdmCl) resembling an extended conformation. α-Crystallin binds to the unfolded state of the protein and facilitates the enzyme to refold by its chaperone activity in an ATP-independent manner.

Materials and Methods

Materials

Birchwood xylan, dinitrosalicylic acid, and guanidium hydrochloride were purchased from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals used in this work were of analytical grade.

Microorganisms and Growth Condition

P. citrinum (MTCC-6489) is an alkali tolerant fungus. It was isolated from soil from the Dhapa situated near Kolkata, India. *P. citrinum* was cultivated in solid-state fermentation.

The enzyme was extracted from solid matrix and was purified as described previously [3, 4, 22]. The purity of the enzyme was confirmed by SDS-PAGE and gel filtration chromatography [3].

Purification of Xylanase

The *P. citrinum* was grown at 30°C for 96 h for the production of xylanase. The enzyme was purified to homogeneity from the extracellular culture filtrate by 0–80% ammonium sulfate precipitation (*w*/*v*), followed by phenyl-Sepharose matrix affinity chromatography. Native molecular weight was determined on gel filtration chromatography using Amersham Pharmacia Biotech Superdex-200 HR size-exclusion column [3].

Xylanase Assay

The xylanase assay was carried out by incubating 0.3 mL of appropriately diluted enzyme in 0.05 M potassium phosphate buffer, pH 7.0, with 0.3 mL of 1% birchwood xylan (*w*/*v*) in a final volume of 0.6 mL, at 50°C for 30 min. The released reducing sugar was determined by the dinitrosalicylic acid method using D-xylose as standard [3, 4]. One unit of xylanase activity was defined as the amount of enzyme that produced 1 μmol of xylose equivalent per minute from xylan under assay conditions. Protein concentration was determined according to the method of Bradford [23] using bovine serum albumin as standard.

Denaturation/Renaturation Studies of Xylanase

All denaturation and renaturation experiments were carried out in 50 mM potassium phosphate buffer, pH 7.0. Xylanase (2 μM) was denatured for 16 h in the presence of 6 M GdmCl. Renaturation was initiated by diluting 10 μL of the sample into a final volume of 1 mL of potassium phosphate buffer, pH 7.0 at 37°C. At different time intervals, 100-μL aliquots were withdrawn and assayed for xylanase activity. Renaturation of xylanase in presence of α-crystallin was also carried out at 37°C. Further refolding experiments were carried out to investigate the effect of ATP and its analogs such as AMP, ADP, etc. on the renaturation of xylanase.

Measurement of ANS Fluorescence

Fluorescence measurements were performed with a Hitachi F3010 automatic recording spectrofluorimeter with an excitation and emission bandwidth of 4 mm in a quartz cuvette. The samples were excited at 375 nm and fluorescence was recorded at 490 nm. ANS is not fluorescent in aqueous solutions (λ_{em} 525 nm); however, on addition of proteins containing hydrophobic pockets its emission maximum shifts to shorter wavelengths and the emission intensity is enhanced.

Dynamic Light Scattering

The Nano-ZS (Malvern) instrument, used for the experiment, is equipped with a 4 MW He–Ne Laser (λ=632 nm). The sample was taken in a 3 ml glass cuvette (path length 1 cm) with all transparent walls. Prior to the dynamic light scattering (DLS) study, protein samples were passed through a filter (pore size 0.22 μm). The operating procedure was programmed (using the DTS software supplied with the instrument) such that there are

average 20 runs, each being averaged for 10 s, and a particular hydrodynamic radius (R_H) is computed in each case and ultimately the result is presented as the distribution of R_H.

Results

Denaturation of Alkaliphilic Xylanase by Guanidium Hydrochloride

Xylanase (2 μM) was treated with varying concentrations (1–6 M) of guanidium hydrochloride (GdmCl) for 4 h. The denaturation profile of xylanase was monitored (Fig. 1) by measuring the intrinsic tryptophan fluorescence intensity at graded concentrations of GdmCl. In presence of 2 M of GdmCl, highest fluorescent intensity was observed. At the higher concentrations of GdmCl, tryptophan fluorescence was observed to decrease. The enzyme also lost 90% of its maximum activity in the presence of 1 M GdmCl. At higher concentrations of GdmCl, the activity was completely lost (Fig. 2). This state has been referred to as unfolded state of xylanase (Xyn-u). The fluorophore ANS was used to determine the relative amount of exposed hydrophobic surfaces in the folding intermediates of alkaline xylanase. ANS is not fluorescent in aqueous solutions (λ_{em} 525 nm); however, on addition of proteins containing hydrophobic pockets its emission maximum shifts to shorter wavelengths and the emission intensity is enhanced [24]. As shown in Fig. 3A, the binding of ANS to xylanase was measured as a function of GdmCl. A maximum increase in the ANS fluorescence was observed at 2 M GdmCl indicating maximum exposure of hydrophobic surfaces in this state of xylanase. At higher concentrations of the denaturant, a decrease in the intensity of the dye fluorescence was observed which was accompanied by a shift in the λ_{em} maxima toward red indicating unfolding of xylanase (Fig. 3B). ANS has been widely used to detect the formation of molten globule-like intermediates in the folding pathways of several proteins [24, 25]. This state is characterized to be as compact as the native protein with solvent-accessible hydrophobic regions and appreciable amount of secondary structure but no rigid tertiary

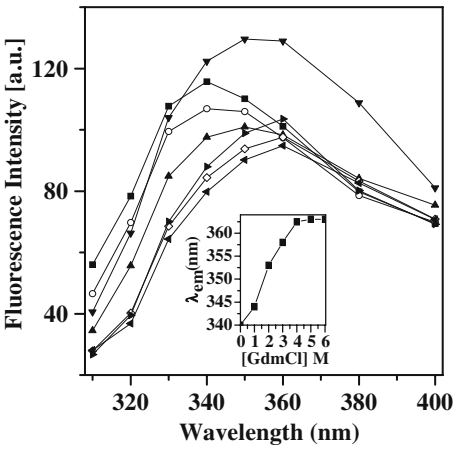


Fig. 1 Xylanase (2 μM) was incubated with different concentrations of GdmCl (0 M (*empty circle*), 1 M (*filled square*), 2 M (*filled inverted triangle*), 3 M (*filled triangle*), 4 M (*filled right triangle*), 5 M (*diamond*), 6 M (*filled left triangle*)) for 4 h. Denaturation profile of GdmCl-treated xylanase was observed by measuring the tryptophan fluorescence intensity. In all the cases, the samples were excited at 295 nm. *Inset*, figure represents the change in λ_{em} maxima of tryptophan fluorescence of xylanase with increasing concentration of GdmCl

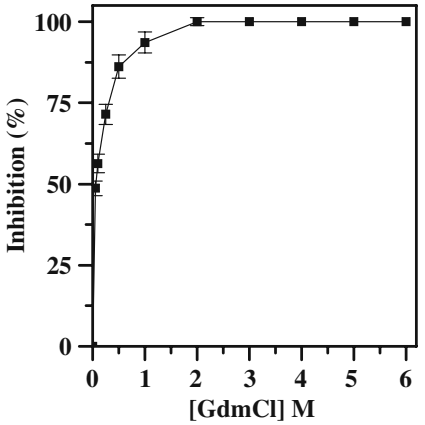


Fig. 2 Xylanase (2 μM) was incubated with different concentrations of GdmCl (1–6 M) for 4 h. Residual activity of xylanase was then measured by DNS method

structure. ANS-binding studies revealed that at 2 M concentration of GdmCl, xylanase partially unfolded to its molten globule state, which has been referred to as XR-m.

To investigate the possible changes in hydrodynamic radius of xylanase in presence of 2 M GdmCl, DLS study was performed (Fig. 4). The hydrodynamic radius of native xylanase is 4.0 nm, which was shifted to 5.0 nm indicating the generation of a more extended structure in the presence of 2 M GdmCl. This is the radius of molten globule state of alkaliphilic xylanase. At the higher concentration of GdmCl, R_H value of alkaline xylanase was observed at ~100 nm (data not shown), which reflects the aggregation of the protein probably through exposed hydrophobic surfaces.

Chaperone-Assisted Renaturation of Xylanase

α-Crystallin has been shown to have chaperone function in the folding/unfolding studies of many glycosyl hydrolases from different sources. In the present study, α-crystallin did not show any effect on the activity of native xylanase (Table 1), but it was employed to refold

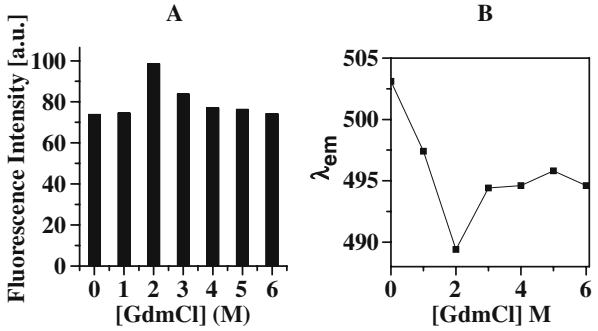


Fig. 3 Exposure of the hydrophobic surfaces of GdmCl-treated xylanase by ANS fluorescence. Xylanase (2 μM) was treated with 0–6 M GdmCl for 2 h at 25°C, and the relative exposure of the hydrophobic surface was monitored by ANS fluorescence. A 2-μM ANS was added followed by incubation in the dark for 5 min. Fluorescence of the ANS-bound partly/completely unfolded protein was monitored at excitation and emission wavelengths of 375 and 400–600 nm, respectively (**a**). **b** Represents the change in λ_{em} maxima of ANS fluorescence of xylanase with increasing concentration of GdmCl

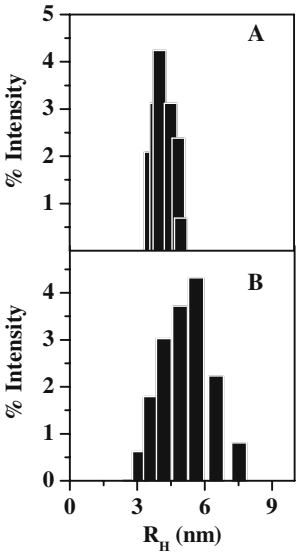


Fig. 4 Change of R_H with the addition of 2 M of GdmCl. R_H value of native xylanase (**a**) is 4.0 nm and that of partially unfolded (**b**) xylanase is 5.0 nm

partially unfolded xylanase (Xyn-m). A 0.3 mg/ml of α-crystallin in the refolding reaction was sufficient for the Xyn-m (Fig. 5A) to regain their highest refolded activity. It was observed that 44% of the maximum activity of xylanase was regained within 30 min (Fig. 5B) in the presence of 0.3 mg/ml of α-crystallin, when Xyn-m was used as the substrate in the renaturation reaction.

The α-crystallin-mediated renaturation of xylanase was examined as a function of the chaperone concentration. As shown in Fig. 5A, nearly 10% of the original xylanase activity was recovered at the lowest concentration of α-crystallin (0.05 mg/ml). The extent of renaturation increased in a concentration-dependent manner, and a maximum 43–45% of the original activity was recovered at α-crystallin concentration of 0.3–0.4 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-Xyn-m complex as opposed to forming non-native xylanase. To test the specificity of α-crystallin, the renaturation of Xyn-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 xylanase (data not shown).

Table 1 Effect of α-crystallin on xylanase activity.

α-Crystalline (mg/ml)	Activity (IU/ml)
0.1	14.3±1.2
0.2	13.1±1.05
0.4	14.1±0.92
0.6	14.2±0.9

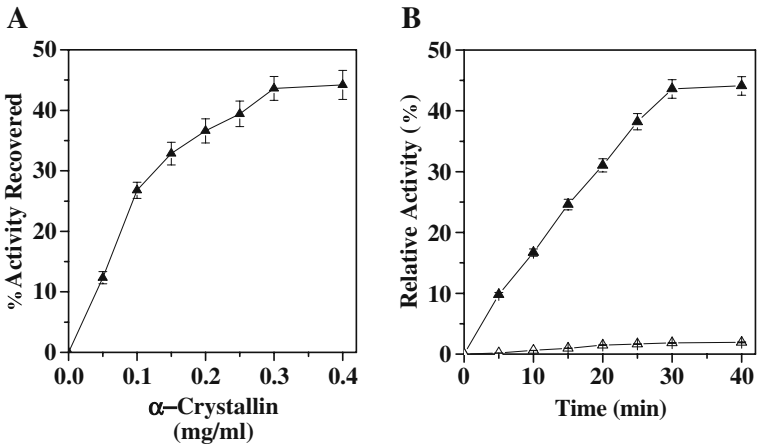


Fig. 5 **a** Reactivation of xylanase at varying concentrations of α-crystallin. Xylanase was renatured in the presence of different concentrations of α-crystallin. After 30 min, aliquots were withdrawn and assayed for xylanase activity. **b** The refolding of xylanase was monitored with or without the presence of α-crystallin as measured by enzymatic activity. The molar concentration of xylanase was 1 μM. Refolding study was done in the presence (*filled triangle*) and in the absence (*empty triangle*) of α-crystallin at the concentration of 0.3 mg/mL

α-Crystallin Forms a Complex with Folding Intermediate

Fluorescence studies were performed to confirm that the xylanase bound to α-crystallin exists in the molten globule state. The tryptophanyl fluorescence of native, denatured, and α-crystallin-bound xylanase is shown in Fig. 6. Native xylanase exhibited an emission maximum at 340 nm, whereas in 6-M GdmCl, the emission maximum was shifted to 363 nm, which corresponds to the fluorescence maximum of tryptophan in aqueous

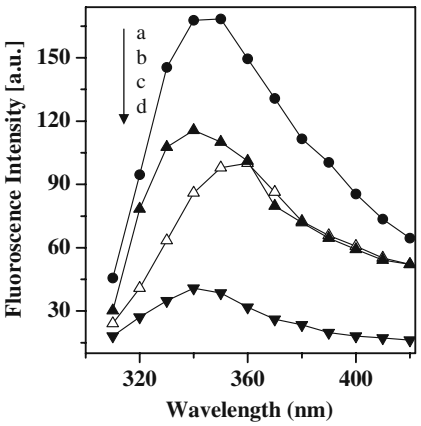


Fig. 6 Conformational properties of chaperone-associated xylanase. Xylanase at a concentration of 2 μM was incubated with 6 M GdmCl for 16 h, and further diluted in 50 mM phosphate buffer, pH 7.0, containing 0.2 mg/mL α-crystallin at 37°C. After incubation for 30 min, the tryptophan fluorescence was recorded. α-Crystallin-bound Xylanase (*trace a*), Trp fluorescence of native Xylanase (*trace b*), denatured Xylanase (*trace c*), and α-crystallin (*trace d*). All samples were excited at 295 nm

solution. The xylanase bound to α-crystallin exhibited an emission maximum at 345 nm indicating that the tryptophans in the bound form of xylanase are more exposed to the solvent than the native enzyme. The increase in fluorescence intensity of the α-crystallin-bound xylanase may be attributed to the denaturant-induced changes in the microenvironment of the tryptophans of xylanase or may be due to interactions of the unfolded protein with α-crystallin. Altogether, these results revealed that the conformation of xylanase bound to α-crystallin is neither native-like nor completely unfolded but a partially folded intermediate resembling the molten globule state. Thus by sequestering the molten globule state of xylanase in the form of a stable binary complex, α-crystallin is able to suppress their interaction that would otherwise lead to aggregation.

Xylanase bound by α-crystallin showed significant increase in ANS fluorescence, indicating exposure of hydrophobic surfaces in the folding intermediate of xylanase (Fig. 7). In contrast, the fluorescence of native as well as the denatured enzyme showed lower extent of binding to ANS. This intermediate was probably similar to the "molten globule" states proposed as an accessible conformation for several proteins.

Effect of Adenine Nucleotides on α-Crystallin-Mediated Renaturation

The role of ATP in the renaturation of the proteins rhodanese, ribulose-bisphosphate carboxylase/oxygenase, and β-protein [24] by the chaperone GroEL has been reported. Recently, evidence for the binding of ATP to α-crystallin was provided by ^{31}P NMR spectroscopy and fluorescence studies [24]. Hence, studies were undertaken to find out if ATP played any role in the chaperone function of α-crystallin. For this functional in vitro analysis, refolding of Xyn-u was initiated in a buffer containing α-crystallin at 37°C and further incubated in the absence/presence of ATP. As shown in Table 2, α-crystallin mediated reconstitution of active xylanase was observed in the absence of ATP. Though the enzyme activity was regained ~3% higher in presence of ATP-associated α-crystallin than α-crystallin only, but ATP did not show much significant effect on the refolding yield of

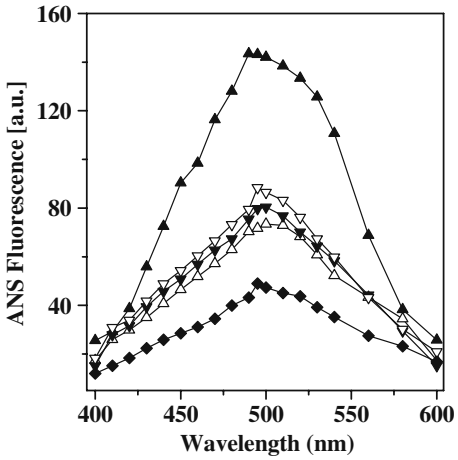


Fig. 7 Fluorescence spectra of ANS bound to (*filled triangle*) α-crystallin·xylanase complex; (*empty triangle*) native xylanase; (*filled inverted triangle*) GdmCl-denatured xylanase; (*empty inverted triangle*) refolded enzyme; and (*filled diamond*) α-crystallin. Forty micromolars ANS was added to each sample, and the fluorescence was recorded at 490 nm. The samples were excited at 375 nm

Table 2 Effect of adenine nucleotide on renaturation of xylanase activity.

Set	Activity Recovered (%)
Xyn-m+α-crystallin	43.6 ±1.98
Xyn-m+α-crystallin+ATP	46.8±2.05
Xyn-m+α-crystallin+ADP	43.5±1.78
Xyn-m+α-crystallin+AMP	43.2±1.86

alkaline xylanase. The effect of other adenine nucleotides like ADP, AMP on the refolding of xylanase was not observed (Table 2). This type of observation was found in case of sorbitol dehydrogenase [26].

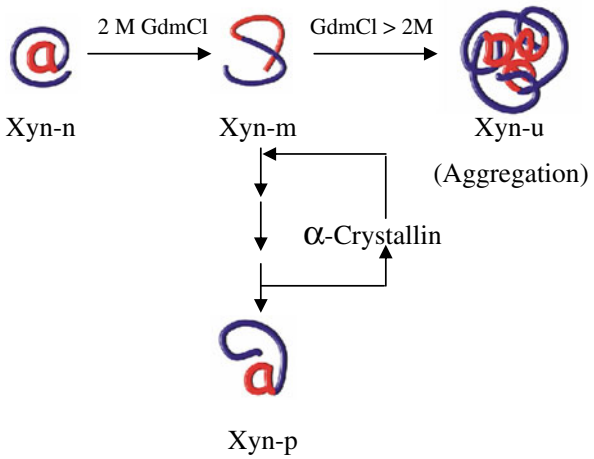
Discussion

The present investigation was carried out to gain some insight into the conformation of xylanase interacting with the chaperone α-crystallin and the mechanistic details underlying the reconstitution of active enzyme. The conditions for the unfolding of native xylanase were sought in the belief that the unfolded enzyme or its folding intermediates would serve as a substrate for the α-crystallin-mediated reconstitution of active xylanase. The denaturation studies using the structure-perturbing agent GdmCl revealed that the folding of xylanase involves an intermediate that resembles the molten globule in which the structure of the enzyme is altered enough to cause partial exposure of tryptophan residues with minimum loss of secondary structure. This is also supported by DLS study of native and GdmCl-treated xylanase. Since DLS measures the translational diffusion coefficient, which can easily be converted, to the R_H of the particles of interest, e.g., protein molecules in solution, therefore the tertiary and quaternary structure of protein in solution can be determined in terms hydrodynamic volume of the protein. This in turn reflects the present conformation and/or the number of species present in the solution. The extended conformation of 2 M GdmCl-treated alkaliphilic xylanase was observed. But at the higher concentration of GdmCl, high value of hydrodynamic radius clearly indicate the aggregation of denatured xylanase. Results presented in this study, based on the binding of the fluorescent probe ANS also suggested that xylanase possesses a hydrophobic region that is buried in the native state but is exposed during the refolding reaction.

The existence of molten globule-like intermediates has been demonstrated with several proteins, and these intermediates are known to be involved in various cellular functions such as membrane translocation of proteins [24], chaperone-assisted protein folding [6], and also in various genetic diseases [14, 15, 27]. Interest in such intermediates is strong since they have been proposed to be an obligatory intermediate formed early in the folding pathway [24]. A common feature of the molten globule state is the exposure of hydrophobic surfaces that lead to aggregation of proteins during folding. In vitro studies using xylanase revealed that the chaperone α-crystallin operates by interacting with the hydrophobic regions that appear on the surface of molten globule state of xylanase. This reduces the concentration of the free partially folded xylanase (Xyn-m) during renaturation and thus prevents loss of enzyme activity due to their hydrophobic aggregation.

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. One of the signals for inducing such structural changes is the hydrolysis of ATP as reported in case of the chaperone DnaK and GroEL. However, reports are also available wherein the chaperones GroEL, BiP [24] and in some cases α-crystallin [26] do not require ATP hydrolysis. Instead, the mere binding of the adenine nucleotide to the chaperone induces a topological change in the chaperone that weakens its interaction with the bound protein. This acts to release the protein, further allowing it to assume its native state. The present investigations reveal that the mechanism of chaperoning of α-crystallin essentially not requires the binding of ATP to the chaperone and not its hydrolysis.

A cascade of molecular chaperones has been reported to mediate folding of proteins in *E. coli*. The chaperone DnaK interacts with polypeptides in their extended conformation and prevents premature misfolding and aggregation after which GroEL stabilizes folding intermediates resembling the molten globule and mediates proper folding. The transfer of DnaK/DnaJ-bound protein to GroEL requires GrpE as the coupling factor [24]. Such a mechanism is likely to exist in eukaryotes also [24]. The present study reveals that α-crystallin is able to reconstitute xylanase via interaction with its non-native conformer characterized by an increased surface hydrophobicity but a low degree of unfolding, thus preventing the denatured xylanase from premature misfolding and aggregation. Further evidence is provided by the observation that α-crystallin prevents aggregation of lens proteins induced by oxidative stress and UV radiation. These conditions are not likely to unfold protein molecules completely but induce formation of partially folded state with hydrophobic surfaces that result in its aggregation [24]. Based on the data presented here, the refolding process of alkaliphilic xylanase by the chaperone function of α-crystallin has been proposed in a diagram (Scheme 1).



Scheme 1 Simplified model of α-crystallin-assisted refolding of alkaline xylanase. The abbreviations used are: *Xyn-n* native xylanase, *Xyn-m* xylanase in molten globule state, *Xyn-u* unfolded xylanase, *Xyn-p* partially folded xylanase. Aggregation occurs among unfolded intermediates with exposed hydrophobic patches (*blue lines*, hydrophilic solvent-exposed parts of the protein; *red lines*, hydrophobic patches)

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References

1. Kulkarni, N., Shendye, A., & Rao, M. (1999). Molecular and Biotechnological Aspects of Xylanases. *FEMS Microbiology Reviews, 23*, 411–56.
2. Collins, T., Gerday, C., & Feller, G. (2005). Xylanases, xylanase families and extremophilic xylanases. *FEMS Microbiology Reviews, 29*, 3–23.
3. Dutta, T., Sengupta, R., Sahoo, R., Sinha Ray, S., Bhattacharjee, A., & Ghosh, S. (2007). A novel cellulase free alkaliphilic xylanase from alkali tolerant *Penicillium citrinum*: production, purification and characterization. *Letters in Applied Microbiology, 44*, 206–211.
4. Dutta, T., Sahoo, R., Sinha Ray, S., Bhattacharjee, A., Sengupta, R., & Ghosh, S. (2007). Probing the active site environment of alkaliphilic family 11 xylanase from *Penicillium citrinum*: Evidence of essential histidine residue at the active site. *Enzyme and Microbial Technology, 41*, 240–246.
5. Anfinsen, C. B. (1973). Principles that govern the folding of protein chain. *Science, 181*, 223–234.
6. Nath, D. & Rao, M. (2001). Artificial chaperone mediated refolding of xylanase from an alkaliphilic thermophilic *Bacillus* sp. *European Journal of Biochemistry, 268*, 5471–5478.
7. Gething, M. J. & Sambrook, J. (1992). Protein folding in the cell. *Nature, 355*, 33–45.
8. Fersht, A. R. & Daggett, V. (2002). Protein folding and unfolding at atomic resolution. *Cell, 108*, 1–20.
9. Onuchic, J. N. & Wolynes, P. G. (2004). Theory of protein folding. *Current Opinion in Structural Biology, 14*, 70–75.
10. Dash, C., Sastry, M., & Rao, M. (2005). Illustration of HIV-1 protease folding through a molten-globule-like intermediate using an experimental model that implicates α-crystallin and calcium ions. *Biochemistry, 44*, 3725–3734.
11. Das, K. P. & Surewicz, W. K. (1995). On the substrate specificity of α-crystallin as a molecular chaperone. *Biochemical Journal, 311*, 367–370.
12. Macario, A. J. L. & Macario, E. C. D. (2005). Sick chaperone cellular stress and disease. *New England Journal of Medicine, 353*, 1489–1501.
13. Nath, D., Rawat, U., Anish, R., & Rao, M. (2002). α-Crystallin and ATP facilitate the *in vitro* renaturation of xylanase: enhancement of refolding by metal ions. *Protein Science, 11*, 2727–2734.
14. Vallejo, L. P. & Rinas, U. (2004). Strategies for the recovery of active proteins through recovery of bacterial inclusion body proteins. *Microbial Cell Factories, 3*, 11–23.
15. Stefani, M. (2004). Protein misfolding and aggregation: new examples in medicine and biology of the dark side of the protein world. *Biochimica et Biophysica Acta, 1739*, 5–25.
16. Bukau, B. & Horwich, A. L. (1998). The Hsp 70 and Hsp 60 chaperone machine. *Cell, 92*, 351–366.
17. Ma, B., Tsai, C. J., & Nussinov, R. (2000). Binding and folding: in search of intramolecular chaperone-like building block fragment. *Protein Engineering, 13*, 617–627.
18. Fink, A. L. (1999). Chaperone mediated protein folding. *Physiological Reviews, 79*, 425–450.
19. Horwich, A. L., Weber-Ban, E. U., & Finley, D. (1999). Chaperone rings in protein folding and degradation. *Proceedings of the National Academy of Sciences of the United States of America, 96*, 11033–11040.
20. Klemenz, R., Frohli, E., Steiger, R. H., Schafer, R., & Aoyama, A. (1991). αB-crystallin is a small heat shock protein. *Proceedings of the National Academy of Sciences of the United States of America, 88*, 3652–3656.
21. Kumar, S., Kapoor, M., Sinha, S., & Reddy, G. B. (2005). Insights into hydrophobicity and the chaperone-like function of αA and αB crystallins. *Journal of Biological Chemistry, 280*, 21726–21730.
22. Dutta, T., Sahoo, R., Sengupta, R., Sinha Ray, S., Bhattacharjee, A., & Ghosh, S. (2008). Novel cellulases from an extremophilic filamentous fungi *Penicillium citrinum*: production and characterization. *Journal of Industrial Microbiology and Biotechnology, 35*, 275–282.
23. Bradford, M. M. (1976). A rapid and sensitive method for detecting microgram amounts of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248–254.
24. Biswas, A. & Das, K. P. (2004). Role of ATP on the interaction of α-crystallin with its substrates and its implication in molecular chaperone function. *Journal of Biological Chemistry, 279*, 42648–42654.
25. Narberhaus, F. (2002). α-Crystallin type heat shock protein: socializing minichaperone in the context of multichaperone network. *Microbiology and Molecular Biology Reviews, 66*, 64–93.
26. Marini, I., Moschini, R., Del Corso, A., & Mura, U. (2005). Alpha crystallin: an ATP independent complete molecular chaperone toward sorbitol dehydrogenase. *Cellular and Molecular Life Sciences, 62*, 599–605.
27. Soti, C. & Csermely, P. (2002). Chaperones come of age. *Cell Stress Chaperones, 7*, 186–190.