α -Crystallin binds to the aggregation-prone molten-globule state of alkaline protease: Implications for preventing irreversible thermal denaturation

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Abstract

α -Crystallin, the major eye-lens protein with sequence homology with heat-shock proteins (HSPs), acts like a molecular chaperone by suppressing the aggregation of damaged crystallins and proteins. To gain more insight into its chaperoning ability, we used a protease as the model system that is known to require a propeptide (intramolecular chaperone) for its proper folding. The protease ("N" state) from *Conidiobolus macrosporus* (NCIM 1298) unfolds at pH 2.0 ("U" state) through a partially unfolded "I" state at pH 3.5 that undergoes transition to a molten globule- (MG) like "I_A" state in the presence of 0.5 M sodium sulfate. The thermally-stressed I_A state showed complete loss of structure and was prone to aggregation. α -Crystallin was able to bind to this state and suppress its aggregation, thereby preventing irreversible denaturation of the enzyme. The α -crystallin-bound I_A state exhibited native-like secondary and tertiary structure showing the interaction of α -crystallin with the MG state of the protease. 8-Anilinoanthracene-9-sulphonate (ANS) binding studies revealed the involvement of hydrophobic interactions in the formation of the complex of α -crystallin and protease. Refolding of acid-denatured protease by dilution to pH 7.5 resulted in aggregation of the protein. Unfolding of the protease in the presence of α -crystallin and its subsequent refolding resulted in the generation of a near-native intermediate with partial secondary and tertiary structure. Our studies represent the first report of involvement of a molecular chaperone-like α -crystallin in the unfolding and refolding of a protease. α -Crystallin blocks the unfavorable pathways that lead to irreversible denaturation of the alkaline protease and keeps it in a near-native, folding-competent intermediate state.

Keywords: Protease; molten globule; α -crystallin; thermal denaturation; aggregation; protein folding

Molecular chaperones function by binding to specific structural features that are exposed only in the early stages of assembly, thereby inhibiting unproductive pathways that otherwise would act as kinetic dead-end traps and produce incorrect structures (Ellis and van der Vies 1991). Gro-EL and Gro-ES from *Escherichia coli* and heat-shock proteins

(HSPs) such as HSP25 and HSP27 are among the well-studied chaperones (Hendrick and Hartl 1993; Clark and Muchowski 2000). Apart from molecular chaperones and some other accessory proteins such as protein isomerases catalyzing *cis-trans* isomerization of peptide bonds or disulfide exchange (Lang et al. 1987; Freedman 1984), there is one more mechanism of assisted protein folding that was initially shown in serine proteases such as subtilisin (Zhu et al. 1989), α -lytic protease (Silen and Agard 1989), and carboxypeptidase Y (Winther and Sorensen 1991). When denatured, these proteases are unable to refold spontaneously even when placed in conditions that favor folding and thus conflict with the self-assembly hypothesis. They are synthesized as precursors containing an amino-terminal pro-

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Abbreviations: APC, alkaline protease from *Conidiobolus*; ANS, 8-anilinoanthracene-9-sulphonate; SAAPF-pNA, N-succinyl-L-alanyl-L-proline-p-nitroanilide.

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peptide usually preceded by a presequence or a signal peptide (Wells et al. 1983). The propeptide is required for the formation of the active enzyme (Wong and Doi 1986; Zhu et al. 1989). In addition to mediating folding, prosequence strongly inhibits the native enzyme, indicating that it functions at a late step on the folding pathway by helping to overcome a kinetic barrier. Because propeptides perform a function similar to that of a large family of HSPs (Shinde and Inouye 1994), they have been broadly classified as “molecular chaperones.” However, they differ from the latter in their highly specific nature and the absolute requirement for folding of the protein to which they are “covalently” attached and hence are further classified as “intramolecular chaperones” (Shinde and Inouye 1993). In vitro studies have shown that covalent linkage between the propeptide and subtilisin is not needed during the folding reaction (Zhu et al. 1989). The functioning of propeptide, despite its covalent linkage with the target protein and its sequence homology with HSPs, prompted us to investigate the interaction of a protease with α -crystallin, which has been assigned the function of a chaperone (Horwitz 1992). Earlier studies have shown that α -crystallin specifically binds aggregation-prone partially unfolded states (Das and Surewicz 1995a) with structural properties similar to those of the molten globules (MGs) (Rajaraman et al. 1996; Lindner et al. 1997). α -Crystallin has been shown to protect many enzymes such as catalase (Hook and Harding 1997), *Nde I* (Hess and Fitzgerald 1998), sorbitol dehydrogenase (Marini et al. 2000), and citrate synthase (Rajaraman et al. 2001) from thermal inactivation. Recently, it was shown that α -crystallin assists the reactivation of guanidine hydrochloride-denatured glyceraldehydes-3-phosphate dehydrogenase (Ganea and Harding 2000) and urea-denatured quinone oxidoreductase (Goenka et al. 2001). α -Crystallin is able to prevent the aggregation of intermediates on the unfolding (Das and Surewicz 1995), as well as refolding, pathways of proteins (Raman et al. 1997). However, the mechanism of action of the chaperone α -crystallin at present is not fully understood, and more information is needed on the nature of the target protein and its interaction with the chaperone. The present investigation was undertaken to assess the chaperoning power of α -crystallin in the unfolding and refolding of an alkaline protease from *Conidiobolus macrosporus* NCIM 1298 (APC). The intermediates on the unfolding pathway of proteases are less studied compared with those on the folding pathway, and there are no reports on the binding of proteases to the chaperones other than the propeptide, which is a highly specific intramolecular chaperone. The aim of our present studies has been to investigate whether under conditions favoring unfolding, a molecular chaperone such as α -crystallin will interact with the protease to prevent its irreversible denaturation and assist its refolding. The biotechnological promise of proteases (Rao et al. 1998) makes them an ideal candidate for studying

structure–function relationships. A highly active alkaline protease from *Conidiobolus* was attributed a novel role of physiological regulation of conidial discharge via its auto-proteolysis (Phadtare et al. 1992). It was able to replace trypsin in animal cell culture (Chiplonkar et al. 1985) and subtilisin Carlsberg in resolving racemic mixture of amino acids (Sutar et al. 1991), although it is structurally distinct from subtilisin (Phadtare et al. 1996). Studies on alkaline protease would assist in promoting its industrial applications (Bhosale et al. 1995). Recently, we have shown that the APC has a single Trp and Cys residue in its active site in addition to the characteristic triad of Asp, His, and Ser residues (Tanksale et al. 2000). In this communication, we have for the first time shown that α -crystallin binds to the aggregation-prone unfolding intermediate of APC and keeps it in a folding-competent state by preventing its aggregation.

Results

Acid-induced unfolding of the alkaline protease

The APC shows optimum activity at pH 10.0 and maximum stability at pH 7.5. The pH-activity profile indicated that the enzyme is stable up to pH 5.0. There was a rapid loss in activity with a further decrease in pH, and complete inactivation of the enzyme occurred at pH 3.5. The circular dichroism (CD) spectrum of the native enzyme at pH 7.5 (N state) in the aromatic region showed a sharp negative peak at 274 nm (Fig. 1A). The negative ellipticity decreased considerably at and below pH 3.5, indicating loss of tertiary structure. The spectrum of the N state in the far-UV region showed two minima at 212 and 219 nm (Fig. 1B). There was negligible change in ellipticity at 219 nm up to pH 4.0. The ellipticity of the enzyme decreased at pH 3.5 (I state) compared with that of the N state. No secondary structure was observed at pH 2.0 (U state), indicating complete un-

Fig. 1. Circular dichroism (CD) spectra of the alkaline protease from *Conidiobolus* (APC): (A) Near-UV and (B) far-UV CD spectra of the enzyme at concentrations 5.5 and 0.6 μ M, respectively. (1) “N” state, (2) “I” state, and (3) “U” state.

folding of the protein. Thus, the I state is characterized by the loss of activity, loss of rigid tertiary structure, and presence of considerable native-like secondary structure.

Occurrence of the intermediate " I_A " state

The fluorescence spectrum of the N state excited at 295 nm had an emission maximum at 340 nm. The fluorescence intensity at the emission maximum (340 nm) remained unchanged up to pH 5.0 with a substantial decrease on further lowering of pH (Fig. 2A). Fluorescence spectra revealed a red shift of 10–14 nm in the emission maximum of the enzyme below pH 4.0 (Fig. 2B), which indicated complete exposure of Trp residues to the solvent as a result of acid-induced unfolding of the protein. These spectra were similar

to that of the protease denatured with 6 M guanidine hydrochloride. Addition of sodium sulfate (0.5 M) had no effect on the fluorescence intensity at pH 7.5 but decreased gradually at lower pH values. A clear transition was observed at pH 3.5, with the formation of a new intermediate state having a fluorescence intensity lower (73% of the total) than that of the N state and approximately twice that of the U state. Thus, a blue shift of 10 nm (λ_{max} 346 nm) accompanied by an increase in the fluorescence intensity was observed at pH 3.5 and 2.0 in the presence of salt. The Trp residues in these states are more exposed to solvent than the N state but much more buried compared with the U state, and they are similar to the "A" state observed in the case of many proteins (Fink et al. 1994). The salt-induced state of the protease at pH 3.5 was termed as the " I_A " state. Thus, the I state at pH 3.5 in the presence of salt undergoes transition into the I_A state.

Characterization of the I_A state

The extrinsic fluorophore 8-anilinonaphthalene sulphonate (ANS) was used to determine the relative amount of exposed hydrophobic surfaces in the unfolding intermediates. ANS fluorescence of the N state was negligible and was not affected by the presence of salt (Fig. 3A,B). A significant (fourfold) increase in ANS fluorescence was observed in the case of the I_A state compared with that of the I state, with a shift in the emission maximum from 510 to 478 nm indicating the burial of the ANS molecule in a hydrophobic environment. Increased ANS binding is one of the important characteristics of the MG state of a protein. Further insight into the unfolding process was obtained by determining the relative hydrodynamic volumes of different conformational states of APC by gel-filtration chromatography (data not shown). Native APC eluted from the column at a volume of 10 mL as a sharp peak indicated a defined structure, whereas the U and I states eluted at 8.0 mL as a broad peak, tailing considerably, indicated a substantial increase in the hydrodynamic volume of the polypeptide chain on unfolding as a result of acidification. The I_A state showed an additional peak at an elution volume of 9 mL. A comparison of elution volumes of these states indicated that the compactness of the I_A state was intermediate between the N and U states. The relative exposure of Tyr and Trp residues of APC under various unfolding conditions was monitored by second derivative absorption spectroscopy (data not shown). Aromatic residues of the enzyme were more exposed at pH 3.5 compared with those of the native enzyme. The increase in exposure was also accompanied by a spectral shift, indicating a change in polarity of the environment of these residues. The I_A state of the enzyme showed a decreased Tyr/Trp exposure, which was intermediate between that of the native and denatured states. The heat-induced denaturation of the APC at various pH values was

Fig. 2. pH dependence of the fluorescence intensity of APC: (A) Fluorescence intensity of APC (0.3 μ M) was monitored at 340 nm in the absence ($\circ$) and presence ($\bullet$) of 0.5 M Na₂SO₄ at 25°C. (B) Fluorescence emission spectra of APC (excitation 295 nm). (—) "N" state, (.....) "I" state, (---) "U" state, and (- - -) " I_A " state.

Fig. 3. Exposure of hydrophobic surfaces of APC on acid-induced unfolding measured by 8-anilino-1-naphthalene sulphonate (ANS) binding: (A) ANS (20 μ M) was added to the enzyme (0.3 μ M) and incubated for 20 min at 25°C in the absence (○) and presence (●) of 0.5 M Na₂SO₄. (B) ANS fluorescence emission spectra of APC (excitation at 369 nm). (1) "N" state, (2) "N" state in the presence of 0.5 M Na₂SO₄, (3) "I" state, (4) "U" state, (5) "I_A" state, and (6) U state in the presence of 0.5 M Na₂SO₄.

studied by monitoring the changes in Trp fluorescence. The cooperativity of thermal unfolding at different pH values was monitored by plotting the ratio of relative fluorescence intensity at 330 and 350 nm as a function of temperature (Fig. 4). At higher temperatures (60°C and above), a red shift of 7.5–15 nm was observed at all pH values from 7.5–2.0, indicating complete exposure of Trp residues to the solvent. The presence of salt at pH 7.5 to 3.7 did not alter the temperature transition. The I and U states showed an increase in $I_{330/350}$ ratio with an increase in temperature, whereas the I_A state was stable even at higher temperatures, revealing a poor cooperativity of only this state to thermal unfolding. However, the lack of red shift at the higher temperature can also result from intermolecular interactions that prevent exposure of Trp to water, as this state was shown to self-associate. The I_A state was regarded as the "MG state" of the *Conidiobolus* alkaline protease on the basis of its increased hydrophobicity, retention of secondary but lack of tertiary structure, patterns of hydrodynamic volume and second derivative absorption spectrum, and poor thermal cooperativity compared with those of the N, I, and U states.

Interaction of α -crystallin with the protease

To understand the anomalous thermal cooperativity of the I_A state, the aggregation status of different enzyme intermediates during thermal denaturation at 58°C was determined. No aggregation was observed from pH values of 4 to 7.5 in the presence or absence of salt at 58°C. The I state of APC when heated at 58°C showed no aggregation. In marked contrast, the I_A state showed considerable aggregation at 58°C with time (Fig. 5A). The ability of α -crystallin to recognize non-native intermediates on the unfolding pathway of APC was probed by monitoring the effect of α -crystallin on the aggregation of the I_A state. Complete prevention of aggregation occurred at a molar ratio of α -crystallin:APC of 2 : 1, showing that α -crystallin is very effective in recognizing and binding to non-native intermediates formed during the thermal denaturation of the proteins. The conformation of the thermally stressed I_A state of APC bound to α -crystallin was studied by CD (Fig. 5B) and fluorescence spectroscopy (Fig. 5C). The I_A state (Fig. 5B, curve 2) lost its secondary structure on heating (Fig. 5B, curve 3), which was retained to a considerable extent by binding with α -crystallin (Fig. 5B, curve 4). α -Crystallin could not retain the secondary structure of the I state after heat shock, which confirmed that it was able to recognize and bind only to the aggregation-prone MG state of the APC and prevented its heat-induced aggregation. The emission maximum of the native enzyme was 340 nm, whereas that of the aggregated I_A state was 336 nm (Fig. 5C, curve 3). The completely unfolded enzyme emitted at 355 nm. The thermally stressed I_A state of APC bound to α -crystallin showed an emission maximum of 344 nm, thereby showing that the Trp residues in this state are more exposed to the solvent compared with that of the N state but are less ex-

Fig. 4. Thermal cooperativity of APC at different pH values: The effect of increasing temperatures on shifts in the emission maximum of APC was assessed by monitoring the $I_{330/350}$ ratio at pH 7.5 (◆), pH 5.0 (■), pH 3.5 (▽), pH 2.0 (▲), and pH 3.5 in the presence of 0.5 M Na₂SO₄ (○).

Fig. 5. Interaction of the chaperone α -crystallin with APC: (A) The effect of α -crystallin on thermally induced aggregation of the I_A state. The I_A state of APC (0.3 μ M) (1) at 58°C and (2) in the presence of α -crystallin. (B) Far-UV CD spectra of the (1) N state, (2) I_A state, (3) I_A state at 58°C, and (4) α -crystallin-bound I_A state. (C) Trp fluorescence of (1) N state, (2) U state, (3) I_A state at 58°C, and (4) α -crystallin-bound I_A state.

posed than that of the U state. When the I state was heated in the presence of the chaperone, no change was observed in the fluorescence intensity, as well as in the emission maximum of the enzyme, either at 58°C or on cooling to room temperature (data not shown), showing that α -crystallin does not interact with the I state even under thermal stress.

To rule out the contribution of α -crystallin to the spectrum of the complex, the protease labeled with isatoic an-

hydride was used to study the interaction of the thermally stressed I_A state of APC and α -crystallin. Isatoic anhydride reacts with the nucleophilic groups of the proteins to yield o-aminobenzoyl protein conjugates (Lau et al. 1998). The derivatized proteins show an absorption band centered at 330 nm together with an emission band covering the spectral range of 360–500 nm. The emission maximum of the fluorescent labeled APC (410 nm) does not interfere with that of the unlabeled α -crystallin (336 nm) and is suitable for shedding some light on the conformation of APC in the presence of α -crystallin after the heat shock. Heat treatment of the I_A state of the labeled protein led to quenching of fluorescence and a blue shift ($\lambda_{\max}$ 400 nm) as a result of aggregation (Fig. 6A). Mixing of APC with α -crystallin before heat treatment, however, restored the emission maximum to 409 nm that was similar to that of the native protein ($\lambda_{\max}$ 410 nm). These results confirmed that α -crystallin prevents the denaturation of the protease during thermal

Fig. 6. Interaction of the chaperone α -crystallin with APC: (A) Fluorescence spectra of the isatoic anhydride-labeled APC (1) native enzyme, (2) α -crystallin bound I_A state, and (3) I_A state at 58°C. (B) ANS fluorescence spectra of (1) α -crystallin at 58°C, (2) I_A state of APC added to α -crystallin at 58°C, (3) I_A state of APC at 58°C, (4) complex of α -crystallin and I_A state cooled to 42°C, (5) cooled to 26°C, and (6) mixture of α -crystallin and I_A state at 26°C.

stress and keeps it in a conformation similar to that of the N state.

The complex of α -crystallin and the I_A state of APC was assessed using ANS fluorescence. ANS fluorescence of the multimeric α -crystallin was enhanced on heating to 58°C, indicating exposure of hydrophobic surfaces. Quenching in the ANS fluorescence of α -crystallin at 58°C on addition of the heated I_A state of APC instead of the expected additive increase in the emission intensity indicated the binding of α -crystallin to APC and involvement of hydrophobic interactions in the formation of the complex (Fig. 6B). A steady increase in ANS fluorescence was observed on gradual cooling of this complex from 58°C to 42°C and further to 26°C, which shows that the accessible hydrophobic surfaces of the α -crystallin-APC complex were significantly enhanced on cooling. The emission intensity of ANS fluorescence of the mixture of α -crystallin and the I_A state of APC at 26°C was comparatively more than that of the complex, revealing that α -crystallin forms a complex with the MG state of the APC only under thermal stress, and that hydrophobic interactions are involved in the formation of the complex.

Refolding of the alkaline protease

Dialysis of the unfolded protein against the buffer of pH 7.5 resulted in the aggregation of the protein with a lack of secondary or tertiary structure, as detected by CD and fluorescence spectra, respectively (data not shown). The degree of aggregation was comparatively less when the pH was adjusted to 7.5 by dilution in the presence of salt, as shown by absorbance of the protein at 340 nm. When the protease was unfolded in the presence of α -crystallin (in a molar ratio of 1 : 2) and refolded by dilution in the presence of salt, the absorbance at 340 nm was negligible, indicating the absence of aggregating protein species. The $\lambda_{\max}$ of such refolded enzyme shifted to 342 nm (closer to that of the N state), indicating regain of substantial tertiary structure (Fig. 7A, curve 3). The $\lambda_{\max}$ of ANS fluorescence also shifted to 490 nm (Fig. 7B, curve 2), indicating internalization of hydrophobic surfaces. Structural characterization showed that the refolded enzyme has considerable secondary structure (Fig. 7C, curve 2). During refolding, the I_A state showed near-native conformation when unfolded in the presence of chaperone-like α -crystallin, whereas the I state showed aggregation on refolding. Thus the I_A state is more susceptible to refolding than the I state. When α -crystallin was added to the already denatured protein and then dialyzed or diluted, there was no decrease in aggregate formation. Thus the addition of α -crystallin during unfolding prevents aggregation and enables the protein to regain a native-like structure. However, this intermediate does not display any activity.

Fig. 7. Refolding of APC in the presence of α -crystallin: (A) Trp fluorescence of APC (1) N state, (2) APC unfolded in the presence of α -crystallin and refolded by dilution in the presence of 0.5 M Na_2SO_4 at pH 7.5, and (3) U state. (B) ANS fluorescence spectra of APC unfolded and refolded in the (1) absence and (2) presence of α -crystallin. (C) Far-UV CD spectra of (1) native APC and (2) APC unfolded in the presence of α -crystallin and refolded by dilution to pH 7.5 in the presence of salt.

Discussion

A combination of various spectroscopic techniques has contributed to the following picture of the intermediates on the

acid-induced unfolding pathway of the *Conidiobolus* alkaline protease: At lower ionic strength, the process of unfolding can be explained by the three-state model represented as $N \rightarrow I \rightarrow U$, where N, I, and U are the native (pH 7.5), intermediate (pH 3.5), and unfolded (pH 2.0) states of the protease, respectively. The unfolding transition is complete at pH 2.0, as indicated by the complete loss of tertiary as well as secondary structure. Under conditions of extreme pH values, the main forces responsible for unfolding of the protein are the repulsions between the charged groups on the protein molecule (Goto and Fink 1989). APC is a basic protein with a pI of 9.8, and below this pH it unfolds as a result of repulsion between the positively charged groups. The I state is characterized by the loss of activity, considerable native-like secondary structure, and no rigid tertiary structure. However, it does not show either increased ANS binding or the lack of thermal cooperativity and, therefore, cannot be considered as an MG intermediate on the unfolding pathway of APC. Proteins show differential behavior on acid-denaturation (Zerovnik et al. 1997). Some proteins do not unfold at low pH values, some undergo transition to a compact MG state, and the third type unfolds first to an extended conformation and then undergoes transition to a compact state A on addition of anions. Addition of salt such as sodium sulfate to APC below pH 4.0 induced a new intermediate state, I_A , which is similar to the "A" state observed in the case of many type III proteins such as β -lactamase, α -amylase, ribonuclease A, cytochrome C, carbonic anhydrase, and lysozyme (Fink et al. 1994). The strong affinity of ANS to the I_A state (compared with the N, I, and U states) is caused by the absence of rigid packing of hydrophobic clusters in this state, resulting in a greater accessibility of the protein hydrophobic core for a solvent. Loss of tertiary structure, enhanced ANS binding, and increased hydrodynamic volume, in addition to its least thermal cooperativity, reveal the MG nature of this state. Thus, the process of acid-induced unfolding of the APC can be explained by a four-state model consisting of N state (pH 7.5), I state (pH 3.5), I_A state (pH 3.5 in the presence of 0.5 M Na_2SO_4), and U state and can be represented as

The MG state of proteins is known to contain unstable secondary structure and is thought to expose hydrophobic patches, resulting in a tendency to aggregate. Indeed, aggregation was detected for the I_A state of APC at higher temperatures. α -Crystallin has been reported to function as a molecular chaperone by suppressing aggregation of proteins undergoing denaturation (Horwitz 1992). However, there are no reports on the binding of α -crystallin to proteases. MG-like intermediates have been observed for subtilisin

(Eder et al. 1993) and α -lytic protease (Silen and Agard 1989) on the refolding pathway. Ours is the first report on the interaction of the intermolecular chaperone α -crystallin with the acid-induced MG state of the protease on the unfolding pathway. When the I state was heated at 58°C, it unfolded without aggregation in contrast with the I_A state. Both I and I_A states of APC heated at 58°C did not have any secondary structure. However, the far-UV CD spectrum of the α -crystallin-bound I_A state exhibited a considerable amount of secondary structure showing that α -crystallin forms a complex with the MG state of APC at higher temperatures and protects it from thermally induced irreversible denaturation as a result of aggregation. This is also supported by fluorescence studies. The emission maximum of α -crystallin-bound enzyme is 344 nm, which is intermediate between that of the N and U states. The interaction of α -crystallin and protease was also confirmed by fluorescent chemoaffinity labeling of the protease. The emission maximum of the labeled protease (410 nm) enabled the detection of conformational changes in the enzyme alone in the presence of α -crystallin, eliminating contribution of the latter. When the I state of APC at pH 3.5 was heated at 58°C, it unfolded without aggregation in contrast with the I_A state. Incubation of the I state of APC with α -crystallin before heat shock did not prevent its unfolding as detected by fluorescence and far-UV CD (data not shown), thereby revealing that a conformational state that is prone to aggregation seems to be a prerequisite for the binding of α -crystallin and indicating its highly specific nature of recognition of the target protein. It has been hypothesized that α -crystallin prevents aggregation of non-native structures by providing appropriately placed hydrophobic surfaces (Das and Surewicz 1995b; Lindner et al. 1997). Our results on the decrease in ANS fluorescence intensity of α -crystallin on addition of APC support this hypothesis. The observation that the hydrophobic surfaces of mixture are comparatively greater than that of the complex cooled to room temperature, which in turn are more than that of the α -crystallin alone, further supports the hypothesis that hydrophobic interactions are involved in the formation of the complex.

During refolding, the I state showed aggregation, whereas the I_A state showed near-native conformation when unfolded in the presence of α -crystallin. Thus our results on the unfolding and refolding of the protease indicate the major role of α -crystallin as a chaperone in the structural reconstitution of the protease when added before denaturation of the protein. Although α -crystallin prevents the thermally induced irreversible aggregation of the alkaline protease to keep it in a more ordered compact state, it is not able to restore the catalytic activity of the enzyme after refolding. This is also true for certain other proteins such as carbonic anhydrase (Rao et al. 1993). It would be interesting to study whether the refolded I_A state of the protease stabilized by the chaperone α -crystallin will be able to interact with the

intramolecular chaperone, that is, propeptide and regain its activity. Attempts to refold the APC in the presence of a propeptide of a serine proteinase from *Aspergillus fumigatus* were not successful (data not reported). However, a more detailed study is required for deciphering the specificities of the binding of propeptides to their target proteinases.

Materials and methods

Enzyme purification and assay

APC was purified according to the method of Tanksale (Tanksale et al. 2000). Protease activity was determined in 0.1 M carbonate–bicarbonate buffer at pH 10.0, 37°C, using casein (10 mg) (Kunitz 1947) and in 0.1 M Tris-HCl buffer at pH 7.8, 25°C, using sAAPF-pNA (Sigma, USA) (0.1 mM) as a substrate (Delmar et al. 1979). One unit of enzyme activity is defined as the amount of enzyme required to cause an increase of one absorbance unit at 280 nm for casein and at 410 nm for sAAPF-pNA per mL of reaction mixture per minute. Protein was determined by the method of Bradford (Bradford 1976) using BSA (Sigma) as a standard.

Equilibrium unfolding and refolding of the APC

All the denaturation and renaturation experiments were performed in 50 mM citrate-phosphate buffer of varying pH (2.0–7.5) in the presence or absence of salt (0.5 M sodium sulfate). For complete unfolding, APC was incubated in 50 mM citrate-phosphate buffer at pH 2.0 for 6 hr at 25°C. Refolding of the unfolded protease was initiated either by dialysis or by dilution under various conditions. Unfolded protease was dialyzed at 4°C against 50 mM sodium phosphate buffer at pH 7.5 in the absence or presence of salt, respectively. Refolding initiated by dilution was performed by neutralizing the acid-unfolded protein to pH 7.5 by addition of dibasic sodium phosphate from a 1 M stock solution and was supplemented with sodium sulfate to a final concentration of 0.5 M. In the α -crystallin- (Sigma) assisted refolding experiment, α -crystallin was added either to the unfolded protein before dialysis/dilution or before unfolding the protease in a molar ratio of 2 : 1. Equal amounts of α -crystallin or protease were treated under identical conditions and used as controls.

Fluorescence studies

Fluorescence spectra were recorded on a Perkin-Elmer LS-50B spectrofluorometer equipped with a Julabo-F20 water bath. Trp fluorescence was recorded at an excitation wavelength of 295 nm and a slit width of 7.5 nm. The temperature-dependent structural changes at various pH values were studied by incubating the protein (0.3 μ M) in the temperature range from 10°C to 70°C for 30 min. The salt-dependent conformational transitions at various pH values were monitored by recording the Trp fluorescence in the presence of 0.5 M sodium sulfate. Ionic strength of the buffer component was 50 mM. ANS (Sigma) (20 μ M) was used as a fluorescent probe to detect the exposure of hydrophobic surfaces at an excitation wavelength of 369 nm.

Fluorescence labeling of the alkaline protease

APC (18 μ M) was treated overnight with a 50-fold molar excess of isatoic anhydride (Sigma) in 50 mM potassium phosphate buffer at pH 7.5 at room temperature. The excess of the reagent was removed by passing the mixture through the column of Sephadex G-10 (Sigma). The degree of labeling was determined spectrophotometrically using an extinction coefficient of 4600 M⁻¹ cm⁻¹ at 330 nm for the anthraniloyl chromophore (Churchich 1993).

Assay for aggregation

Protein aggregation was assessed either by monitoring the absorption at 340 nm or by the Rayleigh light scattering method. Both the excitation and emission wavelengths were set to 475 nm, and the change in scattering intensity of the protein (0.3 μ M) was monitored.

CD spectroscopy

The CD measurements were performed on a Jasco J715 spectropolarimeter fitted with a xenon lamp. Changes in secondary and tertiary structure induced by pH and/or salt and/or temperature were monitored in the far-UV (190–250 nm) and near-UV (250–300 nm) region, respectively, using a 10-mm path length cell. The protein concentrations used for far-UV and near-UV spectra were 0.3 μ M and 5.5 μ M, respectively. The spectra were averaged over five accumulations.

Size-exclusion chromatography

Analytical gel-filtration experiments were performed using a TSKG2000SW column (Pharmacia) connected to a Pharmacia-LKB HPLC system. APC (1.5 μ M) was incubated at varying pH values for 6 hr in the presence or absence of salt. The column was equilibrated for each sample by passing at least three bed volumes of buffer used for incubation of the sample, and a 50- μ L sample was injected into the column. Flow rate was maintained at 1 mL per minute.

Second-derivative absorption spectroscopy

Absorption spectra were recorded for APC (5.5 μ M) incubated for 6 hr under various denaturing conditions in the wavelength range of 250–300 nm on a Shimadzu UV-VIS spectrophotometer UV1601PC. The spectra were derivatized keeping a wavelength difference of 0.5 nm.

Interaction of α -crystallin with APC

The complex of α -crystallin and the I_A state of APC (in a molar ratio of 2 : 1) was prepared by incubating the mixture of the enzyme and α -crystallin in 50 mM citrate-phosphate buffer at pH 3.5, 58°C, for 20 min and then gradually cooling to room temperature. Near- and far-UV CD spectra of the complex and α -crystallin incubated under similar conditions were recorded. The spectrum of APC bound to α -crystallin was obtained by subtracting the spectrum of α -crystallin treated under similar conditions from that of the complex. Fluorescence spectra of the above samples were recorded at an excitation wavelength of 295 nm. ANS (20 μ M) was added to the complex of APC and α -crystallin, which was

cooled to room temperature, and fluorescence spectra were recorded using the excitation wavelength of 369 nm.

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