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ORIGINAL PAPER

Equilibrium unfolding of kinetically stable serine protease milin: the presence of various active and inactive dimeric intermediates

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Abstract Kinetically stable homodimeric serine protease milin reveals high conformational stability against temperature, pH and chaotrope [urea, guanidine hydrochloride (GuHCl) and guanidine isothiocynate (GuSCN)] denaturation as probed by circular dichroism, fluorescence, differential scanning calorimetry and activity measurements. GuSCN induces complete unfolding in milin, whereas temperature, urea and GuHCl induce only partial unfolding even at low pH, through several intermediates with distinct characteristics. Some of these intermediates are partially active (viz. in urea and 2 M GuHCl at pH 7.0), and some exhibited strong ANS binding as well. All three tryptophans in the protein seem to be buried in a rigid, compact core as evident from intrinsic fluorescence measurements coupled to equilibrium unfolding experiments. The protein unfolds as a dimer, where the unfolding event precedes dimer dissociation as confirmed by hydrodynamic studies. The solution studies performed here along with previous biochemical characterization indicate that the protein has α-helix and β-sheet rich regions or structural domains that unfold independently, and the monomer association is isologous. The complex unfolding pathway of milin and the intermediates has been characterized. The physical, physiological and probable therapeutic importance of the results has been discussed.

Keywords Serine protease milin · Kinetically stable protein · Sequential unfolding · Active dimeric intermediates · Circular dichroism

Abbreviations

ANS	8-Anilino-1-naphthalenesulfonic acid
BSA	Bovine serum albumin
CD	Circular dichorism
DSC	Differential scanning calorimetry
FPLC	Fast protein liquid chromatography
GuHCl	Guanidine hydrochloride
GuSCN	Guanidine isothiocynate
UV	Ultraviolet

Introduction

In spite of sincere efforts in the last few decades, the mystery related to protein folding has only been partially understood (Service 2008). The basic principles of protein folding have emerged mainly from unfolding studies of small, single-domain proteins (Go 1983; Kim and Baldwin 1982). However, many proteins, especially enzymes, are large with multiple putative folding domains and subunits. Most likely, these large proteins do not follow all of the folding principles derived from the studies with smaller proteins, and probably have more complex folding rules (Mei et al. 2005; Smith et al. 1991). Consequently, the

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study of small monomeric proteins is not sufficient to determine the folding principle of multimeric proteins with respect to subunit-subunit interactions or interactions between domains (Gittelman and Matthews 1990). The folding of multimeric proteins involves not only the intramolecular interactions as a polypeptide chain associates with itself (monomer folding), but also the intermolecular interactions as they associate with other monomers (subunit assembly) (Milla and Sauer 1994). How proteins control subunit-subunit interactions, and what additional stability is conferred by these intermolecular interactions, can be only addressed by understanding the mechanisms of folding of oligomeric proteins.

Several biophysical studies on multimeric enzyme systems have provided some insight into the folding and assembly of oligomeric enzymes (Herold and Kirschner 1990; Hornby et al. 2000; Ramstein et al. 2003; Ziegler et al. 1993). Inactive, partially folded intermediates have been identified in equilibrium unfolding investigations of several multimeric enzymes, such as dimeric aspartate aminotransferase (Herold and Kirschner 1990), tetrameric lactate dehydrogenase (Barry and Matthews 1999) and tetrameric pyruvate oxidase (Risse et al. 1992). However, partially unfolded active equilibrium intermediates have not yet been reported. Such active and partially unfolded intermediates might provide information about the unfolding of such domains that do not have a direct effect on the active site of proteins. These domains may be responsible for providing stability and protection to the active site in kinetically stable proteins. Thus, it is important to identify partially folded and catalytically active intermediate states in the folding of single- or multisubunit enzyme systems as well (Fink 1995). Detailed information on the complexity of the folding mechanisms of multisubunit proteins is too limited to rationalize a meaningful pathway of their folding and assembly. The equilibrium unfolding transitions of most multi-domain proteins are generally non-cooperative and populated with equilibrium intermediates (Zhuang et al. 1994). Mostly, the occurrence of equilibrium intermediates reflects in multiphasic transitions as measured by a single probe or non-coincidental transitions as monitored by different probes (Kuwajima 1989; Silinski and Fitzgerald 2002). An understanding of the structural and thermodynamic properties of such intermediate states may provide insight into the factors involved in guiding the pathway of folding (Clark et al. 1993; Guidry et al. 2000).

Proteases form a significant part of the biotechnology industry and are of immense physiological importance (Rao et al. 1998). However, very little information is available regarding their folding mechanism, except for papain, which is a monomeric cysteine protease (Edwin and Jagannadham 1998, 2000; Edwin et al. 2002; Sharma and Jagannadham 2003). Folding behavior of oligomeric serine proteases, such as activated lymphocytes and natural killer cells, cytoplasmic granule localized Granzyme A (Hink-Schauer et al. 2003), disulphide linked dimeric serine protease factor IX (Chen et al. 1996) and HIV-1 protease (Pettit et al. 2003), etc., are poorly reported, and this is especially true for plant serine proteases. Moreover, structural insight into various plant-originated serine proteases is also lacking. Investigation of multimeric plant serine proteases is of utmost importance to understand their folding mechanism. Such studies may provide insights into the structural and physical stabilities of the various industrially and medicinally important plant serine proteases.

Some plant proteases have been shown to be kinetically stable proteins (Xia et al. 2007). They seem to be trapped by a transition state energy barrier in their highly stable native conformation. The unfolding transitions of such proteins are not easily overcome so that unfolding of such proteins is difficult relative to other proteins, and they are resistant to harsh denaturing conditions, especially SDS and other commonly used denaturants (Jones et al. 1975; Manning and Colon 2004; Reynolds et al. 1967). Kinetically stable proteins show differences in the migration of their protein bands on SDS-PAGE when electrophoresed with and without heating (Xia et al. 2007). It has been shown that the majority of the kinetically stable proteins are enzymes belonging to the α/β class of proteins and are mostly multimeric in nature (Manning and Colon 2004; Spelbrink et al. 2005; Xia et al. 2007). It has also been reported that rigid protein structure is the physical basis of kinetic stability and resistance to various chaotropic agents (Jaswal et al. 2002; Yuan et al. 1999; Zaks and Klibanov 1988). Protection of the surface residues from solvent increases the rigidity and stabilization of specific regions of proteins. These are potential effects of oligomerization that may increase kinetic stability (Spelbrink et al. 2005; Xia et al. 2007). This also supports the evolutionary pressure of forming dimers or domains for protease from the latex (Cunningham et al. 1999). The protein-folding mechanism of kinetically stable proteins is poorly understood and reported.

Milin provides a perfectly suitable model to answer many of these questions and to verify several hypotheses and preliminary findings outlined above since it is a plant serine protease that is dimeric and kinetically stable. Our group has identified the novel protease and reported its preliminary biochemical characterization (Yadav et al. 2006). The therapeutic importance of milin in the control of the endemic disease schistosomiasis was reported as well (Yadav and Jagannadham 2008). We have also established the kinetically stable as well as differentially glycosylated homodimeric nature of milin (Yadav and Jagannadham 2009; Yadav et al. 2009). In the present

report, conformational studies of the kinetically stable plant serine protease milin have been performed to gain insight into the folding behavior of the protein. Equilibrium unfolding of milin was followed under diverse conditions by monitoring the enzyme activity as a functional probe, while ANS binding, far and near-ultraviolet (UV) circular dichroism, tryptophan fluorescence and differential scanning calorimetry were used as structural probes. The oligomerization state of intermediates and unfolded protein was investigated by size exclusion chromatography. The global structure of the protein, its protomer association, secondary structural organization and core could be envisaged from an analysis of in vitro solution studies. The probable mechanism of unfolding of the protease was analyzed as well.

Materials and methods

Materials

Milin was purified from freshly collected latex of *Euphorbia milii* (Yadav et al. 2006). The protease milin has nearly 7% glycosylation with a total of 276 amino acids of which 3 are tryptophan and 14 are tyrosine residues. The homogeneity of milin was confirmed on SDS-PAGE, and the protein concentration was determined using its extinction coefficient of $\varepsilon_{280}^{1\%} = 11.10$. Urea, guanidine isothiocynate (GuSCN) and guanidine hydrochloride (GuHCl) were procured from Sigma Chemical Company, USA. ANS was from Aldrich, USA. All other chemicals were of the highest purity available commercially. Concentration of urea and GuHCl were determined using refractometry (Pace 1990). The samples were prepared in Millipore water and filtered through 0.45-μM filters.

Methods

Activity

The proteolytic activity of milin under different conditions was measured using denatured casein/hemoglobin as substrates (Yadav et al. 2006). An appropriate blank without the enzyme was used as control. Milin was incubated for 24 h in desired conditions before activity measurement to confirm attainment of equilibrium (Yadav et al. 2006).

Circular dichroism and fluorescence measurements

Circular dichroism and fluorescence measurements were performed as described previously (Yadav et al. 2009). Appropriate reference samples or blanks were used in each case. The circular dichorism (CD) scan was performed after 24 h of incubation of the protein in desired conditions. This incubation time was found to be appropriate to achieve equilibrium.

Chemical and thermal unfolding of milin

Unfolding of milin was monitored by equilibrating protein samples at a given concentration of the denaturant (GuSCN, GuHCl and urea) for 24 h at room temperature before optical or enzymatic measurements.

The fraction of unfolded protein (F_u) at any denaturant concentration was calculated using the equation:

$$F_u = (F_{obs} - F_n)/(F_u - F_n)$$

where F_n and F_u represent the value of F characteristic of folded and unfolded states, respectively. F_{obs} represents the value of F in a particular condition.

The original transition curves obtained using raw data, without calculation of fraction unfolded, were used appropriately to avoid confusion and complexity in cases where unfolding was incomplete.

Differential scanning calorimetry

Calorimetric measurements were performed with a Microcal MC-2 differential scanning calorimeter at the pH mentioned in the respective figure. Protein solutions of 1.25 mg/ml were extensively dialyzed against 0.01 M glycine-HCl buffer at either pH 2 or 3 where no aggregation was observed. The protein concentration and pH of the samples were rechecked. All solutions were degassed under vacuum before being loaded into the calorimeter cells. The calorimetric experiments were conducted at a scan rate of 60°C/h. Buffer baselines were obtained under the same conditions and subtracted from the sample curves.

ANS binding

The extent of exposure of hydrophobic surfaces in the enzyme was measured by its ability to bind to the fluorescent dye ANS using experimental conditions described before (Sundd et al. 2002).

Light scattering

Relative light scattering measurements were performed to monitor the aggregation of protein, if any, during folding studies. The protein sample was excited at 350 nm, and the emission was recorded at the same wavelength with excitation and emission slit widths of 5.0 and 2.5 nm, respectively. The sample buffer was used as a control to assess the relative light scattering.

Gel filtration

Gel filtration experiments were carried out on Superdex 75 (Amersham Pharmacia Biotech) attached to a BioRad Duoflow FPLC system. The column was equilibrated with 50 mM potassium phosphate buffer, pH 8.0, at a flow rate of 0.5 ml/min at room temperature. Milin (0.1 mg) in 50 mM phosphate buffer pH 8.0 with 100 mM NaCl was loaded on a column. For GuHCl and GuSCN denaturation studies, the column was pre-equilibrated in 50 mM phosphate buffer pH 8.0 containing the desired concentration of the denaturants. The protein solution was equilibrated at the same concentration of denaturants for 24 h before loading on the gel filtration column. The column was eluted isocratically with the same buffer with or without denaturants. The column was pre-calibrated with standard molecular weight markers under similar conditions.

Results

The effect of different chaotropes on the activity, conformation, structure-function relationship and folding of milin were investigated under equilibrium conditions. For such investigations, several biochemical and biophysical techniques including enzyme activity assays, far and near UV circular dichroism, tryptophan fluorescence, ANS binding, size exclusion chromatography and differential scanning calorimetry measurements were employed.

Proteolytic activity of milin under various denaturing conditions

Milin was assayed for its proteolytic activity under several denaturing conditions (Fig. 1). It was functionally stable over a wide range of pH (3.0–10.0). However, the residual activity of milin decreased sharply below pH 3.0, while 20% activity was still retained at pH 1.0. Milin was 100% active up to 70°C at neutral pH. However, a gradual decrease of proteolytic activity of milin was observed with increasing concentration of different chaotropes. Nearly 70% proteolytic activity was retained in 8 M urea and up to 2 M GuHCl at neutral pH. However, the activity of milin was completely lost in ≥4 M of GuHCl at neutral pH (Fig. 1). Milin is thus functionally stable under various conditions. It was interesting to note that while lower concentrations of urea and GuHCl (up to 2 M) affected proteolytic activity identically (30% reduction), a further increase in GuHCl concentration resulted in progressive loss of activity while the same was constant at higher concentrations of urea. This probably indicates that lower concentrations of the chaotropes perturb the active site physically, and higher concentrations of GuHCl cause a

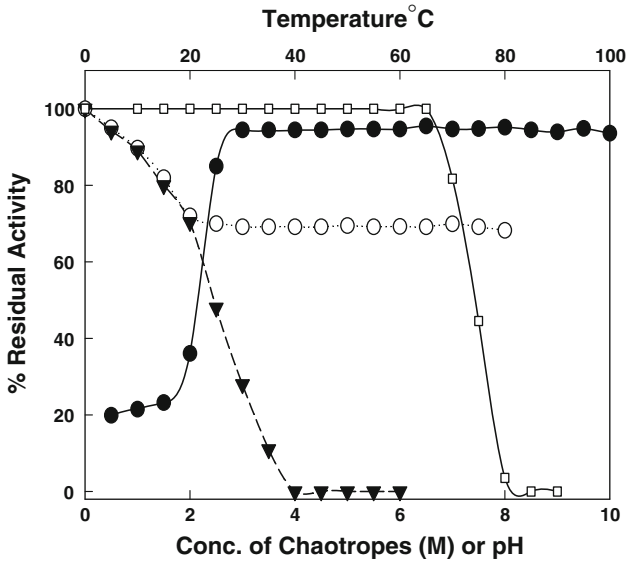


Fig. 1 Effect of increasing concentration of chaotropes, pH and temperature on the proteolytic activity of milin. Appropriate aliquots containing 5 μg of the enzyme were assayed for pH (*filled circle*) and temperature (*open square*) stability as described (Yadav et al. 2006). The activity was also assayed in different concentrations of urea (*open circle*) and GuHCl (*filled inverted triangle*) after 24 h of incubation at 37°C. The activities of enzyme in the absence of the chaotropes, pH 7.0 and at 37°C were taken as 100%

solvent effect or ionization effect. Solvents may affect activity of proteins because of the disruption of water structure around the macromolecule (Schiffer and Dotsch 1996). However, the ionic denaturants/pH stimulate the ionization of different amino acids, which together with the solvent disrupts the water balance around proteins (Watt et al. 2007). There is thus a probability of the active site being at or near the surface of the molecule.

Spectroscopic characterization of milin

Since milin retained its functional activity under several denaturing conditions, we investigated its conformational behavior under the same conditions. As also mentioned previously (Yadav and Jagannadham 2009), the tertiary structure of milin under native conditions was probed by near UV CD (Fig. 2a). The spectra showed two positive peaks centered at 278 and 288 nm along with a negative peak of similar magnitude at 300 nm. This indicated high structural organization in the protein and the presence of tryptophan in the apolar environment. An almost identical spectrum was observed at pH 2.0, indicating that milin retained its native structure at this pH. The loss of proteolytic activity at pH 2.0 (Fig. 1) was thus due to ionization of the active site rather than loss of native structure, as also observed with GuHCl described above. Typical molar ellipticity at wavelengths of 278, 288 and 300 nm was 170,

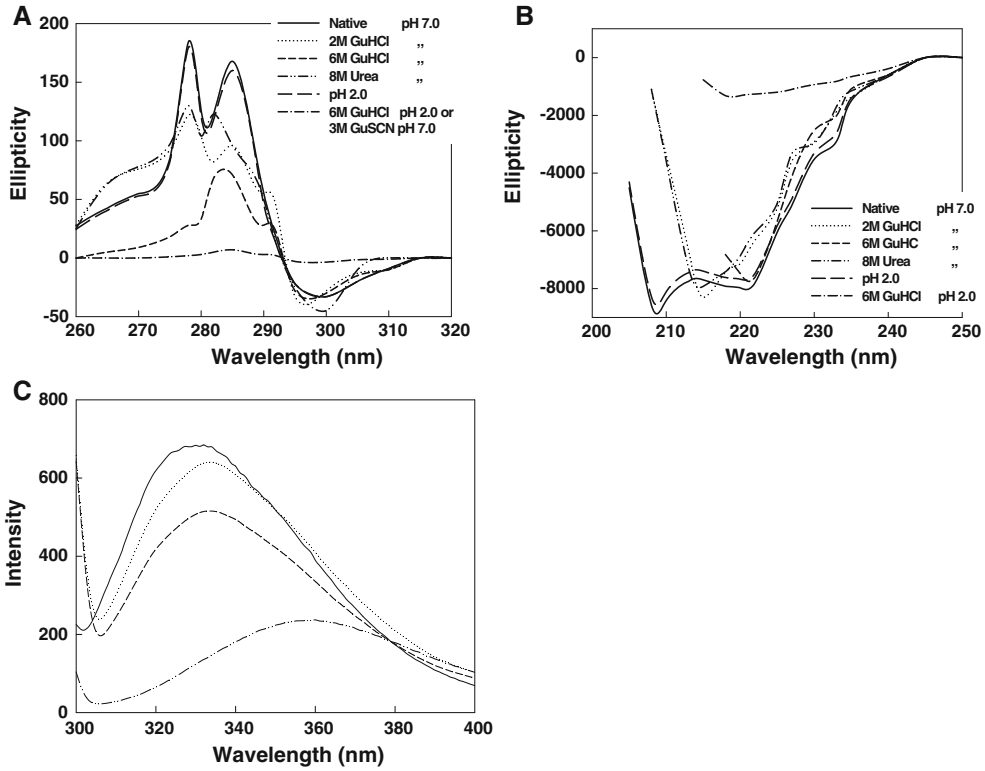


Fig. 2 **a** Near UV CD spectra and **b** far UV CD spectra of milin. The denaturation conditions were pH 7.0 (*black lines*), pH 2.0 (— — —), 8 M urea at pH 7.0 (— · · —), 2 M GuHCl at pH 7.0 (·····), 6 M GuHCl at pH 7.0 (- - - -) and 6 M GuHCl at pH 2.0/3 M GuSCN (- · · · -). The samples were incubated under given conditions or along with chaotropes for 24 h at room temperature before the measurements. The protein concentrations for near UV and far UV CD measurements were 1 and 0.1 mg/ml, respectively. **c** Intrinsic fluorescence spectrum of milin. The protein was incubated at pH 7.0 or pH 2.0 (*black lines*), 8 M urea at pH 7.0 or 2 M GuHCl at pH 7.0 (·····), 6 M GuHCl at pH 7.0 (— — —) and 6 M GuHCl at pH 2.0 (— · · —) for 24 h at room temperature before the measurements. The protein concentration was 0.01 mg/ml for the measurements and was excited at 292 nm

150 and −50 deg cm^2 dmol^{-1}, respectively. In 8 M urea, the near UV CD spectrum was also reduced by 30% (Fig. 2a), similar to its reduction in activity (Fig. 1), indicating that the loss in proteolytic activity in this case was indeed due to denaturation. The near UV CD spectrum in 2 M GuHCl at pH 7.0, which was equivalent to 8 M urea at pH 7.0 in terms of proteolytic activity, was similar with additional reduction in near UV CD intensity of the peak at 288 nm. Milin also retained 50% residual near UV CD spectra in 6.0 M GuHCl at pH 7.0, exhibiting a positive peak at 285 nm and no substantial change of 300 nm negative peak, thus indicating the presence of an intermediate. In search of a condition where the tertiary structure is completely lost, GuHCl denaturation at various pH was performed. Complete loss of CD signal was observed in 6 M GuHCl, at pH 2.0 (Fig. 2a). This state was defined as the unfolded (U) state. The acid ionized state (pH 2.0) was thus sufficiently perturbed for GuHCl to unfold it.

Far UV CD spectrum of native milin (Fig. 2b) showed two well-resolved negative peaks at 222 nm and 208 nm with higher magnitude of ellipticity at 208 nm (Yadav et al. 2009). The mean residue ellipticity ($[\theta]_{MRW}$) at 222 nm was 8.5 ± 0.20 χ 10^3 deg cm^2 dmol^{-1}, and the estimated α-helicity of milin was approximately 28%. Milin belongs to the α/β class of proteins as also shown previously (Yadav et al. 2009) as per the classification suggested by Manavalan and Johnson (Manavalan et al. 1984). Milin retained almost identical secondary structure at pH 2.0, indicating its high conformational stability (Fig. 2b). However, the far UV CD spectra of milin varied in different denaturing conditions. The far UV CD spectra centered at 215 nm with a small shoulder at 222 nm with similar ellipticity were observed in 8 M urea and 2 M GuHCl at neutral pH. However, CD spectra with a distinct peak at 222 nm peak with 8% reduction of ellipticity were observed in 6 M GuHCl at pH 7.0 (Manavalan and Johnson 1983). Due to high absorbance and incompatibility of the CD machine in 6 M GuHCl, the 208-nm peak could not be measured even when the path length was reduced to 0.5 mm. Hence, we have restricted our interpretation of changes in secondary structure in GuHCl to a minimum. Unfolded state was obtained in 6 M GuHCl, pH 2.0 (Fig. 2b). Care was taken to rule out the effect of aggregation, if any, on CD spectra through observation,

consistency in strength of CD signal and light-scattering experiments.

Intrinsic fluorescence properties of milin were identical when excited at either 278 or 292 nm. Milin exhibited a fluorescence emission maximum at 333 nm in native state at pH 7.0 (Yadav and Jagannadham 2009; Yadav et al. 2009), indicating that the three tryptophan residues are probably located in a highly stable hydrophobic ambience. Milin also retained its native-like fluorescence spectrum at pH 2.0 (Fig. 2c). Similarly, the wavelength shift was negligible (333–335 nm), and fluorescence intensities were reduced to 10–15 and 30–35% in 8 M urea/or 2 M GuHCl and 6 M GuHCl at neutral pH, respectively. These results indicated that tryptophan residues are buried in a core that is resistant to denaturation. The unfolded state with 25-nm red shift in wavelength (358 nm) and 70–75% reduction in fluorescence intensity was observed in the presence of 6 M GuHCl at pH 2.0. In brief, milin was conformationally stable in pH 2.0–10.0, up to 70°C, 8 M urea and 6 M GuHCl at neutral pH. However, milin attained different conformations under different environmental conditions. The protein showed a variety of intermediate states in which some are active.

Thermal stability of milin probed by differential scanning calorimetry

Calorimetric study of milin by differential scanning calorimetry (DSC) was carried out and corrected for the buffer-buffer tracing, obtained at pH 2.0 and 3.0, at a scan rate of 60°C/h (Fig. 3). Incomplete transition up to 100°C has been reported by our group at pH 7.0–10.0 (Yadav and Jagannadham 2009; Yadav et al. 2009). Milin aggregated at pH 4.0, 5.0 and 6.0, and hence, the transition scans could not be obtained at these pH values. At pH 3.0 the isotherm showed a single peak on deconvolution (Fig. 3), indicating a two-phase transition. A distinct shoulder on the thermal denaturation scan was observed at pH 2.0, which could be deconvoluted into two distinct peaks with different intensities (Reddy et al. 1999). The single scan deconvoluted endotherm at pH 2.0 showed two T_ms—one at 77.4°C and the other at 85.14°C (Fig. 3b). The thermal unfolding of milin was apparently irreversible at any pH as observed by the rescanning of the samples after reducing the temperature from the respective denaturation scan (data not shown).

Milin displays various molten globule states having strong ANS binding

Considering different intermediate states under different conditions, ANS binding in the presence of different chaotropes and at different pHs was measured (Fig. 4). ANS preferentially bound to milin at pH 2.0, 3 M GuHCl at pH 7.0, 4.5 M urea at pH 7.0 and 0.4 M GuSCN at pH 7.0 compared to the native and completely unfolded states. A blue shift of 40 nm for ANS emission maximum (from 520 to 480) was observed in comparison to ANS spectra for native and denatured milin (Fig. 4, inset).

Guanidine thiocyanate induces complete unfolding of milin at pH 7.0

In view of different conformational states and intermediates at varying pHs, we probed milin using chaotropes at neutral and acidic pH. Denaturation of milin was incomplete against the milder chaotrope urea and GuHCl at pH 7.0. GuSCN has been known to be a strong denaturant of

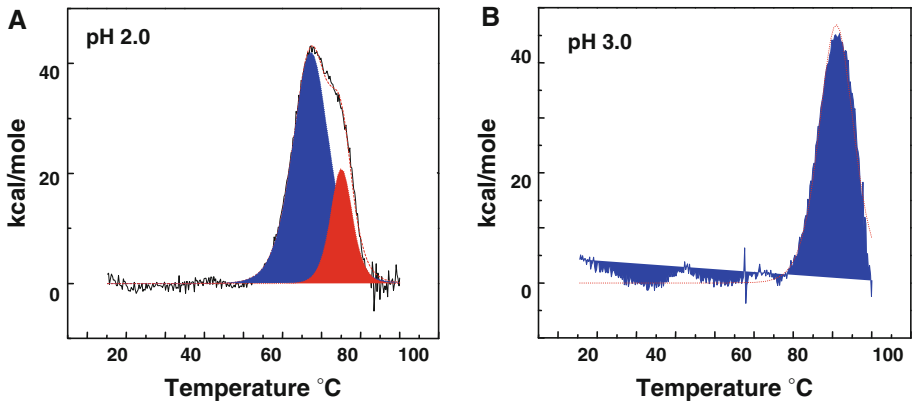


Fig. 3 DSC thermogram of milin as function of pH. The deconvoluted DSC thermogram at **a** pH 2.0 and **b** pH 3.0 shows melting temperature (T_m) of 77.4 and 85.14 and 91.40°C, respectively. The *blue* and *red* color zone represents the fitted curves. All the spectra after baseline correction are presented. The calorimetric scans were performed with a protein concentration of 1.25 mg/ml at a scanning rate of 60°C/h in each buffer. The enzyme was extensively dialyzed against 0.05 M respective buffers before scanning. Incomplete transitions were observed at pH 7.0–10.0

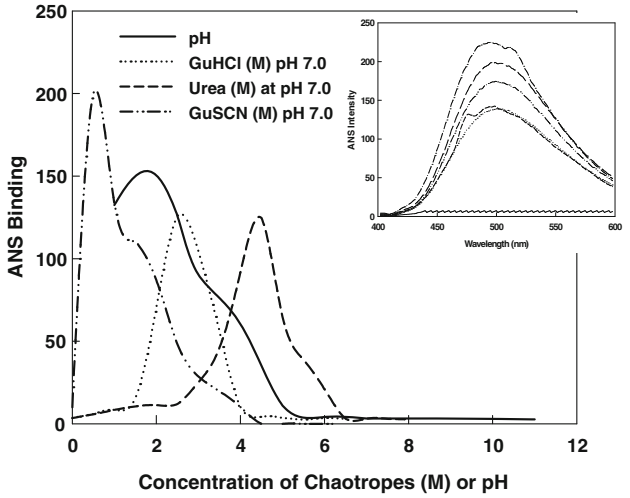


Fig. 4 ANS binding to milin during chaotrope/pH-induced transition. ANS spectra of milin in the presence of different concentrations of chaotropes (*inset*). Protein concentration used was 0.015 mg/ml. In all cases, the protein was incubated in dark with 100-fold molar excess of ANS for more than 30 min at room temperature, and ANS fluorescence was measured at 400–600 nm on excitation at 380 nm. The denaturing conditions are indicated in the figure

proteins. Complete unfolding of milin at neutral pH was achieved only with GuSCN (Fig. 5). The proteolytic activity of milin was lost in ≥0.5 M GuSCN, and the transition curve was highly cooperative in nature. Milin, however, resisted total loss of native structure till about 2.8 M GuSCN (Fig. 5) with 50% residual near UV CD ellipticity at 2 M GuSCN. Interestingly, the transition curve obtained by probing the near UV CD signal was biphasic in nature with the first transition coinciding with proteolytic activity. The intermediate at this stage was

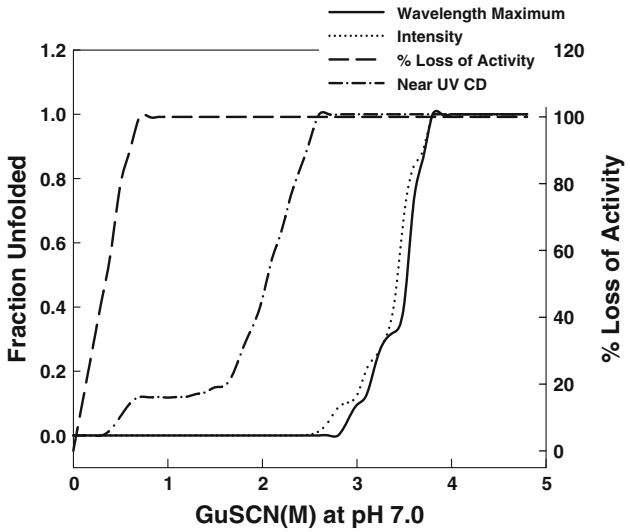


Fig. 5 GuSCN-induced unfolding at pH 7.0. The transition was complete at neutral pH. Far UV CD spectra were not obtained due to the instrument incompatibility. The denaturing conditions are indicated in the figure

quite stable and accumulated over ∼1 M GuSCN concentration followed by a sharp second cooperative transition with a C_m of 2.4 M. When denaturation was monitored by changes in fluorescence intensity or wavelength maxima, milin exhibited even higher stability with coincidental, cooperative transition reaching the unfolded state at ≥3.5 M GuSCN. The corresponding transition midpoint (C_m) was observed at 3.25 M GuSCN. The far UV CD spectra could not be measured due to instrumental incompatibility.

GuHCl induced unfolding of milin

Guanidine hydrochloride-induced unfolding of the kinetically stable protein milin at different pH was measured as a function of CD, absorbance and fluorescence. GuHCl-induced unfolding at pH 7.0 was incomplete as probed by far UV CD, near UV CD and fluorescence wavelength maxima (data not shown). However, interesting observations were made regarding the far UV CD spectra at different concentrations of GuHCl (Fig. 6a). In 2 M GuHCl, the spectrum was centered with maximum negative ellipticity at 215 nm. In 4 M GuHCl, a shoulder reappeared at 222 nm, while in 6 M GuHCl milin seemed to regain spectra characteristic of proteins belonging to the class of α/β proteins. However, as mentioned before, due to inability to obtain clean spectra below 210 nm in GuHCl, we restricted our analysis regarding possible switching of the secondary structural content of milin in this denaturant. To observe complete denaturation in GuHCl, similar experiments were performed at pH 2.0 (Fig. 6b). A complete loss of secondary structure and a red shift of the emission maxima from 333 to 358 nm were observed in GuHCl at pH 2.0. The transitions were coincidental and cooperative.

Urea induced unfolding at pH 1.0

Milin was stable in 8 M urea from pH 7.0 to pH 2.0 so that complete transitions could not be obtained (Yadav and Jagannadham 2009; Yadav et al. 2009). Hence, unfolding experiments for milin in urea were performed at pH 1.0 (Fig. 7). The far UV CD spectra were centered at 215 nm in 4–6 M urea, but shifted towards 222 nm like native protein in 11 M urea, with a concomitant reduction in intensity (Fig. 7a). It is noteworthy that the strange shoulder at around 222 nm observed for native protein, the reasons for which are still unknown, is no longer seen in 11 M urea. Complete denaturation of milin was accomplished by heating the sample containing 11 M urea at 75°C for 15 min at pH 1.0 (data not shown). The corresponding transition curve obtained by following ellipticity at 222 nm revealed a biphasic transition with the first phase exhibiting spectra centered at 215 nm (Fig. 7b). The first

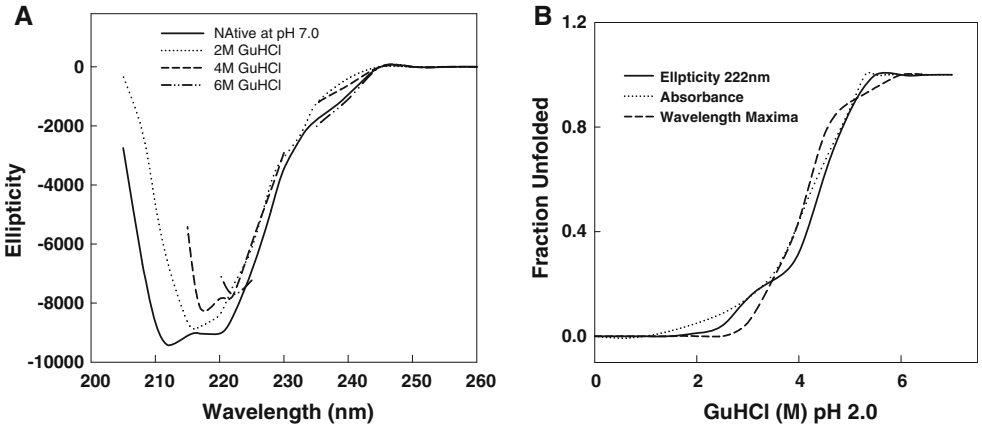


Fig. 6 GuHCl induced unfolding of milin **a** far UV CD scan at pH 7.0. The shapes of the spectra in different molarities of GuHCl are different, though the ellipticities represent mild reduction. **b** GuHCl induced unfolding of milin at pH 2.0. The denaturing conditions are indicated in the figure. The protein samples are incubated at given denaturant concentration for 24 h at room temperature

apparent transition midpoint (C_m) was at 3.5 M; however, the second apparent transition midpoint was at 7.5 M urea, though the transition was incomplete, indicating a possible third transition. The first and second intermediate states were stable in between 4.0–6.0 and 9.0–11 M urea, having mean residual ellipticity ($[\theta]_{MRW}$) of −7,400 and −5,800 deg cm^2 dmol^{-1}, respectively. The transition curve followed by fluorescence intensity was monophasic but incomplete as well. The fluorescence intensity reduced up to 55% with a red shift of 11 nm (333–345 nm) under the above conditions (Fig. 7b), while the unfolded state showed wavelength maxima at 358 nm. The decrease in far UV CD was only 35%. The maintenance of such a low-buffering pH was a problem, but was overcome by using a pH stabilizer (Dong and Lam 2005).

Size exclusion chromatography reveals dimeric intermediate

The gel filtration profiles of milin under native, 6 M GuHCl and 5 M GuSCN (all at pH 7.0) conditions are shown in Fig. 8. A single peak (∼64 kDa) was observed under native conditions for milin. A similar peak with slightly less retention time was observed in 6 M GuHCl at neutral pH. The shift in the peak was probably due to the increase of hydrodynamic volume of the protein molecule under the above conditions. However, in the presence of 5 M GuSCN, a peak corresponding to monomeric molecular weight of milin was observed, indicating a complete dissociation of the dimer into monomers. The light-scattering experiments excluded the possibilities of protein aggregation in 6 M GuHCl in neutral conditions.

Discussion

The environment in which a protein survives and functions is equally important as its sequence, as was evident in Anfinsen's classical ribonuclease experiments (Redfield and Anfinsen 1956) and others thereafter. Hence, investigation of a protein in solution can reveal much information. We have shown previously that milin is a dimeric serine protease that belongs to the α/β class of proteins and is kinetically stable (Yadav et al. 2009). The high conformational stability of milin has been reiterated throughout the current investigation. Thus, the protein retained

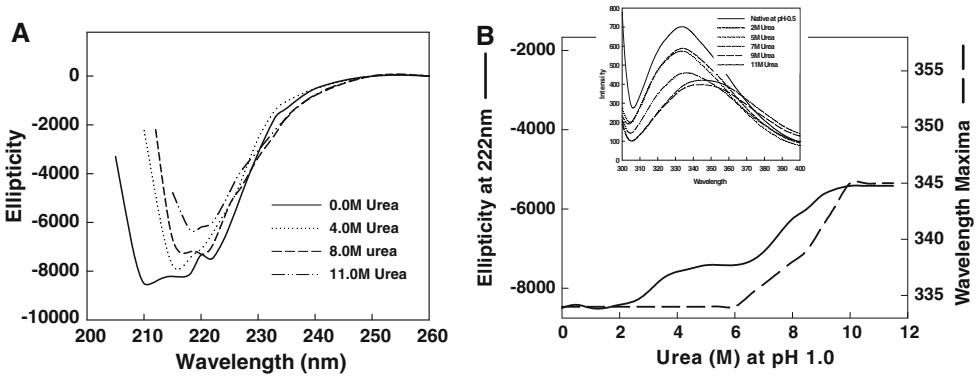


Fig. 7 Urea denaturation of milin at pH 1.0. Milin (0.1 mg/ml) at different concentrations of urea was incubated overnight at 37°C. **a** Far UV CD spectra were recorded by a using Jasco-J500A spectropolarimeter. **b** Far UV CD and fluorescence transition. Different denaturing conditions are given along with each figure separately. Fluorescence scans are represented in the *inset*

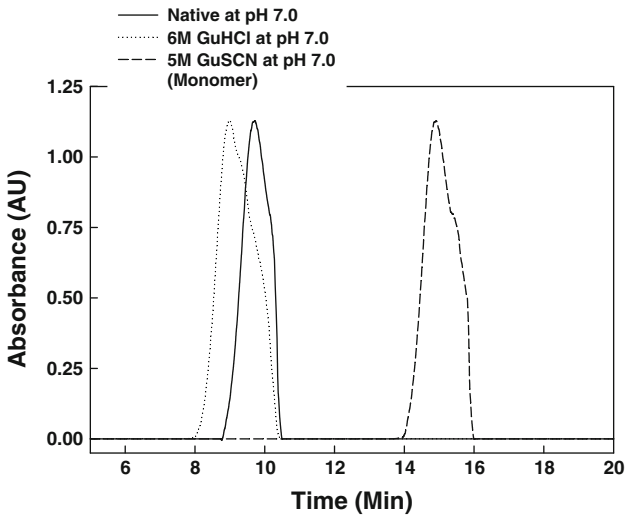
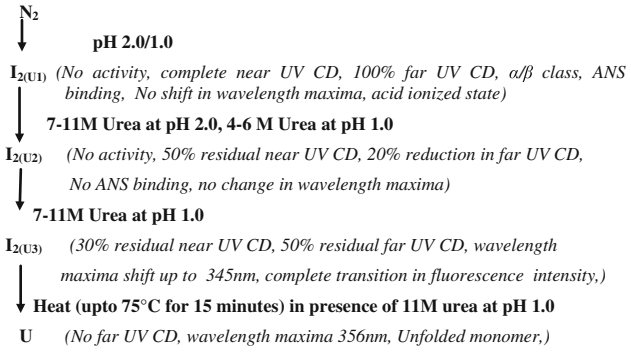


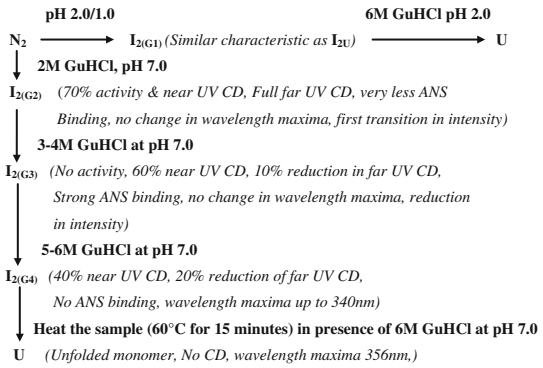
Fig. 8 Chaotropes induced denaturation/dissociation of milin by gel filtration. Concentration of protein used was 1 mg/ml, and the denaturant condition/concentration is indicated in each chromatogram

significant proteolytic activity on incubation at 70°C, in pH 3.0, in 2 M GuHCl, 8 M urea and 0.3 M GuSCN (Fig. 1). The tertiary structure of milin resisted denaturation in conditions where many proteins would become completely unfolded, e.g., in 2–6 M GuHCl, 8 M urea and pH 2.0 (Fig. 2a). The secondary structure of milin showed very negligible perturbation under all the above denaturing conditions, indicating rigid and compact packing for the protein (Fig. 2b). Tryptophan fluorescence under these conditions (Fig. 2c) showed an incomplete red shift in wavelength, indicating stability as well as the fact that the three intrinsic tryptophan probes are mostly buried in the protein core. The T_m for thermal unfolding was 91°C even at pH 3.0 (Fig. 3b). In urea at a low pH of 1.0, randomization of the structure was incomplete as well (Fig. 7). In the strong denaturant GuSCN, milin resisted denaturation of its tertiary and secondary structure till 2.8 and 4.0 M, respectively (Fig. 5). These observations clearly indicate that the protein is highly stable with a rigid core that contains most of the tryptophans present in the protein. Anomalous behavior of the protein in reducing and non-reducing SDS-PAGE already indicated that the dimers are not disulfide linked (Yadav et al. 2009). Thus, there is a distinct possibility that the dimers are associated through strong hydrophobic interactions. The active site seems to be perturbed more easily, indicating that it is situated away from the stable core.

It was also observed that different denaturing conditions yielded different functionally active and inactive intermediate states with varying properties. Thus, with urea as a denaturant, the following intermediates were observed, where N indicates native state and U indicates unfolded state; I designate intermediate, subscript 2 designates its dimeric state, subscript U is for urea, and the number adjacent to U represents the number of intermediates. The characteristic of each state is indicated within brackets.



Similar observations were also made with GuHCl as a denaturant, as shown below, where subscript $_G$ stands for GuHCl, and the number adjacent to $_G$ represents the number of intermediate.



GuSCN-induced unfolding of milin was complete at pH 7.0. Activity was lost at much lower concentrations of the denaturant compared to the tertiary structure, thus emphasizing that the active site is prone to perturbation. The near UV CD probed transition curve is biphasic with the first transition coinciding with the transition obtained by enzyme activity, indicating the presence of yet another stable intermediate, which significantly retained its native structure. The cooperative monophasic transition curve generated by monitoring fluorescence indicates a core where the fluorophores are shielded preferably in a transient intermediate. The unfolded state with complete loss of activity and lack of native-like characteristics were obtained at pH 7.0 only with GuSCN (at $\geq$4.5 M). ANS-binding experiments (Fig. 4) also hint at the existence of numerous intermediates.

The presence of such varied intermediates with distinct characteristics indicates a complex mechanism of unfolding for milin. Two possibilities can be visualized: (1) the dimers have different stabilities and dissociate into monomers first. This dimer unfolds independently, and the

domains in either the dimeric or monomeric states can add to the complexity in the same way that the two domains in papain unfold sequentially (Sharma and Jagannadham 2003); (2) the dimer assembly unfolds as a single unit before it dissociates, and the presence of domains and independent secondary structural arrangements results in further complexities. The experimental evidence presented here in combination with our earlier observations seems to favor the second hypothesis as explained below.

Size exclusion experiments demonstrate that monomers are obtained only when the protein is completely unfolded, viz. in 5 M GuSCN (Fig. 8). Other intermediates (e.g., in 6 M GuHCl, pH 7.0) were compact and eluted as a dimer. We have previously observed the molecular weight of milin corresponding to the dimeric state under normal SDS-PAGE conditions, which dissociate only under harsher conditions (Yadav et al. 2009). The strong dimeric association also explains the failure of milder chaotropes like urea to cause complete unfolding. The strong hydrophobic association of the monomers as discussed above is also emphasized by the inability of acidic pH to perturb tertiary or secondary structure or the tryptophan environment, arguing in favor of an intact dimeric state as well. This is supported by the DSC profiles at pH 3.0 and pH 2.0. The smaller peak at pH 2.0 obtained on deconvolution could be due to differences in glycosylation in the two subunits. DSC of deglycosylated milin could not be obtained because the protein aggregates. These observations support the hypothesis that unfolding of milin precedes dimer dissociation.

It is now well established in the literature that the different secondary structural entities (layers) of mostly α/β type kinetically stable enzymes unfold independently (Manning and Colon 2004; Xia et al. 2007). If milin also has a layered structural arrangement ($\alpha/\beta/\alpha$ being the most common type) and behaves similarly, the experimental findings fit into the above hypothesis and can explain the complex unfolding mechanism. Though we could not obtain unambiguous CD spectra showing such changes in secondary structure in Figs. 6 and 7 conclusively for technical reasons (very high voltage is obtained in presence of GuHCl), nonetheless the trend cannot be missed. It has to be noted that the CD spectrum can show a signal specific to the surface secondary structure, as reported for TNF-α (Kim et al. 1999). Thus, the monomers of dimeric milin might associate with each other to form a Rossman fold having an outer α type organization, middle β type and inner α type structural organization, where the two predominant regions of α-helices may or may not differ in their topology. The dimeric association can thus be hypothesized to be achieved in two ways: either isologous ($\alpha'/\beta/\alpha::\alpha/\beta/\alpha'$) or heterologous ($\alpha'/\beta/\alpha::\alpha'/\beta/\alpha$). The former arrangement having similar domains at the core seems more plausible. This is because the core group has much higher stability, and all the tryptophans are buried in it. Considering the presence of tryptophans mainly in the core, heterologous association would not allow such a placement. In heterologous arrangement of the dimer, if the tryptophans were present in the inner α-layer in one domain, they would be in the outer α-layer in another domain.

Milin resists dimeric dissociation until it is unfolded with the tryptophans being situated in the core. Three routes for unfolding may be possible—one that goes through acidic pH, one that is at neutral pH, and one in the presence of the strong denaturant GuSCN. The proteolytic activity is easily perturbed in acidic pH probably due to ionization of active site amino acids, where an acid ionized state $\left[\mathbf{I_{2(U1)}}\right.$ or $\left.\mathbf{I_{2(G1)}}\right]$ could be observed with an intact dimer. Further perturbation by 4–6 M urea could result in the loss of some domain resulting in an inactive molten globule (MG) like state $\left[\mathbf{I_{2(U2)}}\right]$ that still shows significant intrinsic fluorescence. The far UV CD spectra of this intermediate are centered at 215 with similar intensities. Even further perturbation (up to 11 M urea) results in loss of the 215-nm peaks $\left[\mathbf{I}_{2(U3)}\right]$, with the intermediate showing far UV CD centered at 222 nm in all probability. The fluorescence spectrum is partially red shifted, indicating that the tryptophans have started to be exposed. Finally, on heating the sample denatures with characteristics of the unfolded state and is monomeric. Similarly, 2 M GuHCl or 8 M urea at pH 7.0 (Yadav et al. 2009) reduced activity by 30% [$\mathbf{I_{2(G2)}}$], probably due to the combined solvent effect and perturbation of the outer domain. This intermediate also shows far UV CD spectra centered at 215 nm and retains the native-like fluorescence spectrum. The subsequent steps of unfolding could have different intermediates like $\left[\mathbf{I_{2(G3)}}\right]$ and $\left[\mathbf{I_{2(G4)}}\right]$ having properties described in the "Results" section viz. far UV CD, fluorescent and ANS binding properties. In case of the denaturant GuSCN, however, the perturbations are strong such that the equilibrium is pulled towards a single stable intermediate with the possibility of transient intermediates as well. This means that the overall unfolding mechanism has several dimeric intermediates in which some are even active (Dams and Jaenicke 1999). Structural characterization of in vitro protein intermediates is being pursued for numerous proteins with the aim of elucidating principles governing nascent polypeptide to functional protein conversion in vivo (Panse et al. 2000). Milin presents a novel pathway of unfolding.

Conclusions

The investigation shows that in vitro solution studies can be used to gain understanding of the folding mechanism of

protein architecture. The results also indicate that large proteins certainly do not follow the folding principles derived from studies with smaller proteins. The folding mechanism of large/multimeric proteins is more complex and sensitive to the environment, which dictates correct folding in the larger proteins. This may explain their importance and sensitivity in cellular metabolism and the physiological role, and their misfolding causes diseased states. The sensitivity of folding of plant latex proteases, such as milin, may also be the reason why most of these kinds of proteases are found in multiples in lattices, such that one or other isomer can fold properly in widely varying environmental conditions. On the other hand, once folded, these proteases are resistant to denaturation as well, which seems logical given their sensitivity to the folding environment. The presence of partially folded and partially active intermediates would allow these proteases to tide over environmentally difficult conditions and then renature under favorable conditions, especially that milin is a protease belonging to a xerophyte.

It is possible that evolving kinetic stability is nature's way to keep proteins stably folded, or in other words, it is a means of avoiding undesirable unfolding events. This arrangement could be a way of stress management as well for several proteins susceptible to environmental changes inside the cell or outside of the cells. Engineering protein stability is a common objective in biotechnology industries, and finding means to enhance kinetic stability could be a viable and reasonable strategy for the same. Investigation of milin shows that one way to achieve this goal is to stabilize the dimer association and promote formation of a strong core and independent structural domains that can fold/unfold sequentially. We have shown previously that milin has important medicinal applications, in that it can be used as an anti-schistosomiasis agent. The stability of native milin and its ability to form varied stable intermediates validate its therapeutic application, since such properties allow milin to be used in a wide variety of conditions and formulations.

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