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ORIGINAL PAPER

Protein unfolding studies of thiol-proteinase inhibitor from goat (*Capra hircus*) muscle in the presence of urea and GdnHCl as denaturants

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Abstract In recent years, many advances have been made in the understanding of functional and structural characteristics of protein evolution from denaturant-based studies that subject the protein to a change in the micro-environment. This paper reports the chemical denaturation of purified goat muscle cystatin (GMC) a thiol-proteinase inhibitor, using urea and guanidine hydrochloride (GdnHCl). The subtle conformational changes of GMC were monitored by intrinsic fluorescence, extrinsic fluorescence, and CD spectroscopic techniques. Further, the activity of GMC as a function of increasing concentration of denaturants was also studied. It was found that increasing the concentration of GdnHCl significantly enhances the inactivation and unfolding of the inhibitor (GMC). In urea-induced denaturation, the intrinsic and extrinsic fluorescence intensity reveals significant structural changes in the inhibitor. Further, it was found that at low concentrations of urea, up to 0.5–1.0 M, there was quenching of fluorescence intensity compared with the native form and a red shift of 5 nm was observed up to 5–8 M. The results presented in this paper suggest that GdnHCl-induced denaturation of GMC follows a simple two-state rule in which native → denatured state transition occurs in a single step. However denaturation with urea proceeds through an intermediate or non-native state.

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Introduction

Elucidation of the detailed mechanism of protein folding/unfolding has been a major challenge for structural biologists and protein chemists for many decades. Proteins control most of the events of life, and their functions mainly depend on specific interactions of the active forms of proteins with other molecules. The biologically active form of proteins must depend on its ability to acquire a stable three-dimensional structure (Thornton et al. 1999). This requires that they fold to a unique, globular conformation that is only marginally more stable than the large ensemble of unfolded states. The folded state is stabilized mainly by the burial and tight packing of peptide groups and non-polar side chains. Although the detailed mechanisms of folding are not completely known, significant advances have been made in the understanding of this complex process through both experimental and theoretical approaches (Dobson and Karplus 1999). It has been clearly established that the main rules of protein folding deduced from in-vitro experiments are also valid in the cellular environment. This modern view of protein folding enables better understanding of the aggregation processes that play a role in several pathologies, including those induced by prions and Alzheimer's disease (Prusiner 1998; Kelly 1996).

Proteins are known to accumulate different conformational states during their unfolding by various denaturants (Baldwin 1996; Privalove 1996; Fink et al. 1997). To understand the phenomenon of protein folding, all conformational states should be designed with regard to their structure and function, because such conformational states

might resemble the intermediate state of the in-vivo protein-folding pathway and thus be important in understanding the mechanism of protein folding (Arai and Kuwajima 2000). Urea and guanidine hydrochloride have been of pivotal importance in revealing the mechanisms of protein unfolding. Various studies have used urea/guanidine hydrochloride denaturation curves as a model to obtain an estimate of the conformational stability of proteins by measuring differences between the stability of the folded (native-N) and unfolded (denatured-D) states (Privalov 1979; Pace 1986; Park et al. 2003). Although the partially folded forms of various proteins have been characterized, it has not yet been possible to generalize the concept of "intermediate states" as universal equilibrium protein folding intermediates. However, characterization of protein folding intermediates is helpful in identifying and understanding various transition steps and forms located between the native and denatured states of a protein (Goto et al. 1990, 1993).

Cystatins are non-covalent competitive inhibitors of cysteine proteinases, for example cathepsins B, H, L and S (Ekiel et al. 1997) and are ubiquitously present in mammalian systems. There are three sub-types of the cystatin super family—Family I cystatins with molecular weight 11,000–12,000 lacking in disulfide loops and carbohydrate content; Family II cystatins having two disulfide loops and no carbohydrate content with molecular weight in the range of 13,000–14,000; and Family III cystatins or kininogens which have complex domain structures and are very-high-molecular-weight proteins found only in mammalian plasma (Barrett 1987). These inhibitors might protect the cells from unwanted proteolysis which may otherwise cause several pathologies (Shah and Bano 2009), for example rheumatoid arthritis (Trabant et al. 1991), osteoporosis (Delaisse et al. 1991), Alzheimer's disease (Bernstein et al. 1996), metastasizing cancer (Köppel et al. 1984), and microbial invasion (North et al. 1990).

Cystatin used in this study was purified from goat muscle (GMC). It is a high-molecular-weight (58.8 kDa) protein with no disulfide bonds and very low carbohydrate content. The properties of the cystatin revealed that it is a variant of cystatin type I and type II families. In this study a comprehensive analysis involving the various conformational changes in goat muscle cystatin (GMC) on interaction with GdnHCl and urea has been undertaken.

Materials and methods

Materials

Sephadex G-75, guanidine hydrochloride, and urea were purchased from Sigma Chemical (St Louis, USA). All other chemicals used were of analytical grade.

Purification of goat muscle cystatin

Purification of goat muscle cystatin (GMC) to homogeneity was accomplished by use of a three-step procedure—treatment with alkali, ammonium sulfate precipitation, and gel filtration column chromatography. The inhibitor was purified from 100 g of minced goat thigh muscle excised within 1 h post-slaughter. Muscle tissue was homogenized in extraction buffer (25 mM sodium phosphate buffer, 1% sodium chloride (NaCl), 3 mM ethylenediaminetetraacetic acid (EDTA), and 2% *n*-butanol) pH 7.5 in 1:5 ratio. After centrifugation (5,000 rpm for 15 min at 4°C), the supernatant was adjusted to pH 11.0 by 3 M sodium hydroxide (NaOH) and then incubated at 4°C for 20 min to remove unwanted proteins. The pH of the solution was brought back to 7.5 with glacial acetic acid. After centrifugation (8,500 rpm) for 30 min, the supernatant was fractionated between 40 and 70% ammonium sulfate saturation, the precipitated protein was dissolved in 25 mM sodium phosphate buffer, pH 7.5 and dialyzed against the same buffer containing 1% NaCl. The dialyzed protein was subjected to gel filtration on a Sephadex G-75 column (65 × 1.8 cm) equilibrated with 25 mM sodium phosphate buffer, pH 7.5. Finally, the column was eluted with 25 mM sodium phosphate buffer pH 7.5 at a flow rate of 20 ml/h. Proteins on the gel filtration column were resolved into single peaks with significant papain inhibitory activity, i.e. GMC. Further characterization was then performed on the purified inhibitor (GMC).

Electrophoresis

Homogeneity of the purified inhibitor GMC was checked by native PAGE. Native gels (7.5%) were run for the sequential steps of purification. The gels were stained with 0.1% Coomassie brilliant blue R-250.

Guanidine hydrochloride and urea denaturation of GMC

Cystatin (GMC) dissolved in sodium phosphate buffer (25 mM, pH 7.5) in the presence and absence of increasing concentrations of GdnHCl and urea was incubated for 2 h before the measurements were done.

Inhibitory activity assay of GMC treated with denaturants

The inhibitory activity of GMC was assessed by its ability to inhibit the caseinolytic activity of papain by the method of Kunitz 1947. The inhibitor was incubated with increasing concentrations of GdnHCl (0.5–6 M) and urea

(0.5–8 M) at 25°C for 2 h before the activity was measured.

Fluorimetry

Fluorescence measurements were carried out at 25°C on a Shimadzu model RF-540 spectrofluorimeter equipped with a DR-3 data recorder. The fluorescence was recorded in the wavelength region 300–400 nm after exciting the protein solution at 280 nm for total protein fluorescence. The slits were set at 5 nm for excitation and emission. The path length of the sample was 1 cm. The protein concentration used in the measurement was 2 μM. GMC in 25 mM sodium phosphate buffer, pH 7.5, was incubated in the presence of increasing GdnHCl (0·5–6 M) or urea (1–8 M) concentration for 2 h at 25°C before the spectra were recorded. Appropriate controls containing the denaturants for the study were run. Each spectrum was the average of at least three scans.

Circular dichroism

CD measurements were made on a Jasco model J-720 spectropolarimeter equipped with a microcomputer. The instrument was calibrated with D-10-camphorsulfonic acid. All the measurements were carried out at 25°C by use of a thermostatically controlled cell holder attached to a Neslab RTE-110 circulating water bath with an accuracy of ±0.l°C. Far-UV CD spectra were taken at a protein concentration of 2.0 μM with an 1 mm path-length cell. Spectra were collected at a scan speed of 20 nm min^{-1} and with a response time of 4 s. The spectra obtained were normalized by subtracting the baseline recorded for the buffer having the same concentration of denaturants under similar conditions.

Binding of ANS to GMC

The binding of 1-anilinonapthalene-8-sulfonic acid (ANS) to GMC in the absence and presence of increasing concentrations of GdnHCl (0.5–6 M) and urea (1–8 M) was studied by exciting the dye at 380 nm and the emission spectra were recorded in the wavelength range 400–600 nm. The molar ratio of protein to ANS was 1:50. The protein concentration used was 2.0 μM.

Results

Purification of cystatin from goat muscle

Proteins precipitated between 40 and 70% ammonium sulfate saturation, were dialyzed and loaded on to a Sephadex G-75 column; single protein peaks with papain inhibitory activity were obtained (Fig. 1). The protein peak of GMC was purified by a factor of 117.31 with a percentage yield of 30.80, and furnished a single band under non-denaturing conditions. The purified inhibitor (58.8 kDa. Mr) had a stokes radius of 29.5 Å. GMC was found to be highly specific for thiol-proteases with no interaction with serine proteases.

Effect of GdnHCl/urea on inhibitory activity of GMC

Changes in the inhibitory activity of goat muscle cystatin (GMC) after incubation for 2 h with different concentrations of urea and guanidine hydrochloride are shown in (Figs. 2a and 3a). The activity of GMC initially increased 1.25 fold with an increasing concentration of urea up to 0.5 M. However, further increasing the urea concentration led to a decrease in the inhibitory activity. With GdnHCl

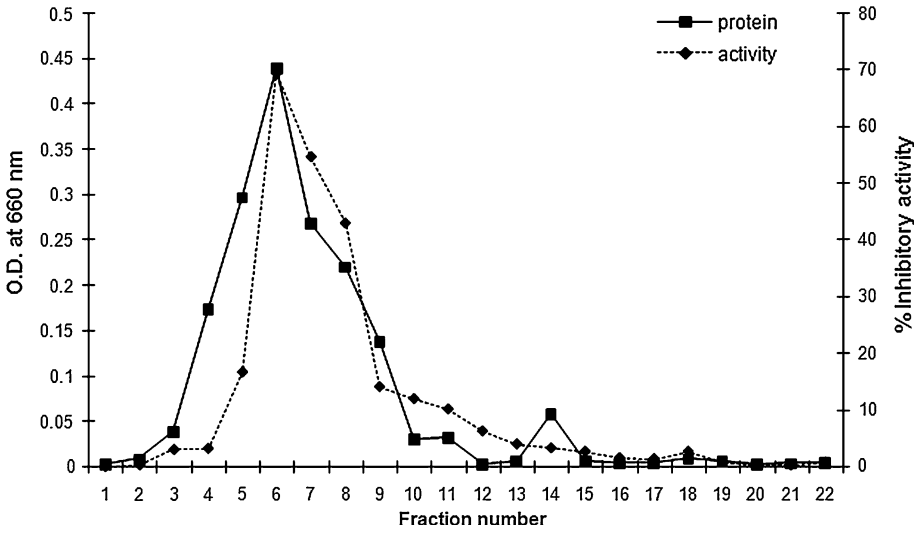


Fig. 1 The dialyzed fraction obtained after (40–70%) ammonium sulfate precipitation was further purified by gel permeation chromatography on a Sephadex G-75 column (65 × 1.8 cm) equilibrated at pH 7.5 by use of 0.025 M sodium phosphate buffer. Elution was performed in 5 ml fractions at a flow rate of 20 mL/h. Each fraction was assayed for thiol proteinase inhibitory activity and protein concentration

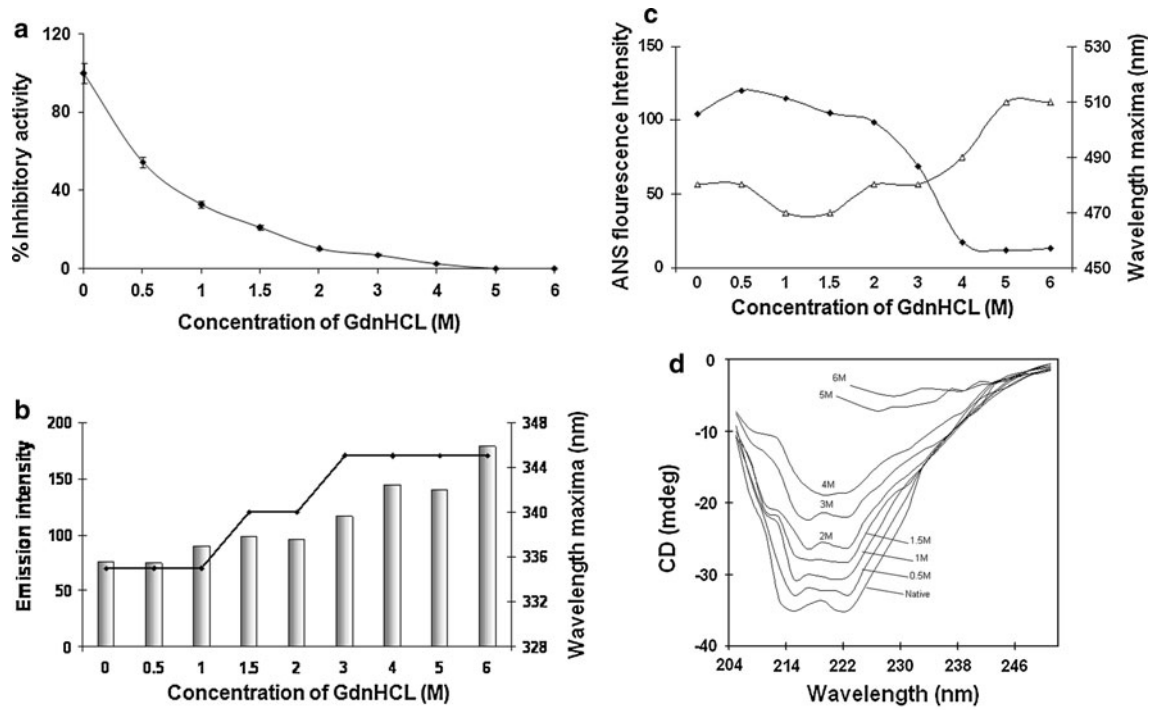


Fig. 2 **a** Inactivation of GMC in the presence of GdnHCl. Native GMC (1.066 μM) was incubated with increasing concentrations of GdnHCl (1–6 M). GMC was assayed for loss of inhibitory activity by the caseinolytic assay of Kunitz (1947). **b** Dependence of the wavelength of maximum fluorescence emission (*line*) and emission intensity (*bars*) on GdnHCl concentration. **c** Dependence of fluorescence emission (*filled diamonds*) and wavelength maxima (*open triangles*) of ANS bound to GMC in the absence and presence of different concentrations of GdnHCl. **d** Secondary structure analysis of GMC in the presence of GdnHCl, showing changes in the far UV-CD spectrum of GMC denatured in increasing concentrations of GdnHCl (1–6 M). The concentration of GMC used was 2 μM

(Fig. 2a) at a concentration of 4 M the enzyme completely lost its inhibitory activity. The protein was found to be completely inactivated at 5–6 M GdnHCl.

Intrinsic fluorescence studies of GMC in the presence of denaturant (GdnHCl and urea)

Fluorescence spectroscopy is widely employed and is an excellent spectroscopic probe to investigate conformational changes in the tertiary structure of proteins and peptides. The aromatic amino acids tryptophan, tyrosine, and phenylalanine act as intrinsic fluorescence probes of protein conformation, dynamics, and intermolecular interactions. Of these three, tryptophan is the most popular probe (Chen and Barkley 1998). Modification of the microenvironment of aromatic residues of GMC by denaturants has been studied by monitoring changes in the intensity and wavelength of emission maxima as a function of denaturant concentration. Fluorescence spectra of GMC in the presence of GdnHCl (Fig. 2b) show unfolding of the inhibitor as manifested by the red shift (10 nm) of the emission maximum. The transition curve for urea denaturation (Fig. 3b) is different from that of GdnHCl. At lower concentrations of urea (0–3 M) the emission maximum was not changed but at higher concentrations (4–8 M) a red shift (5 nm) was observed.

ANS fluorescence studies of GMC

Changes in the ANS fluorescence are generally used to detect non-native conformations of globular proteins (Semisotonov et al. 1991). The hydrophobic fluorescent dye ANS was used to probe intermediate conformations of goat muscle cystatin (GMC). Figure 3c shows ANS fluorescence spectra of GMC in the presence of different concentration of urea. Upon pre-incubation of GMC with urea, the ANS fluorescence intensity of the inhibitor decreased at low concentrations (0.5–1 M). Moreover high concentrations of denaturant resulted in an increase in ANS fluorescence intensity with a red shift up to 10–20 nm. A further increment in the concentration caused a sharp decrease in fluorescence intensity. With GdnHCl (Fig. 2c) a fall in extrinsic fluorescence was observed with a red shift of 30 nm.

Changes in the secondary structure of GMC

Far UV–CD studies of GdnHCl and urea-induced unfolding of cystatin were carried out to investigate the effect of these denaturants on the secondary structure of the protein. Figures 2d and 3d show the effect of GdnHCl and urea on the ellipticity of the native protein. There was a gradual

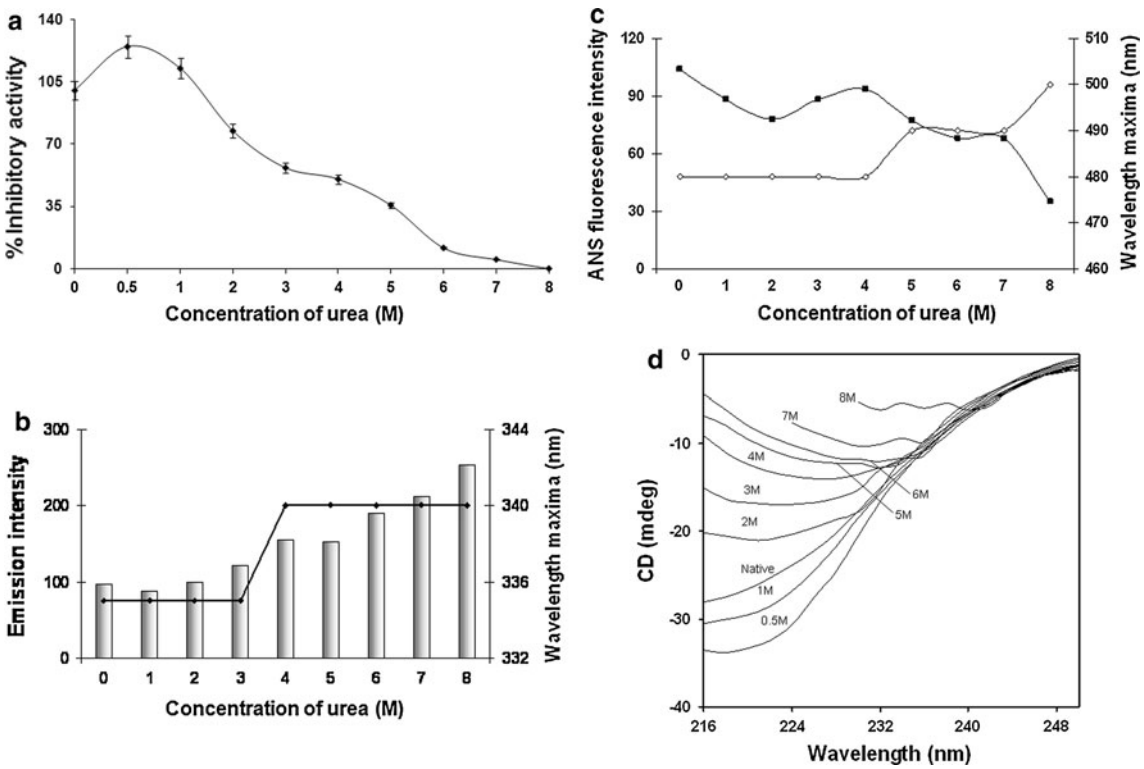


Fig. 3 **a** Dependence of the inhibitory activity of GMC on urea concentration. **b** Dependence of the wavelength of maximum fluorescence emission (*line*) and emission intensity (*bars*) on urea concentration. **c** Dependence of fluorescence emission (*filled squares*) and wavelength maxima (*open diamonds*) of ANS bound to GMC in the absence and presence of different concentrations of urea. **d** Far UV-CD spectra of GMC denatured in increasing concentrations of urea (0–8 M). The concentration of GMC used was 2 μM

decrease in ellipticity with increasing concentration of GdnHCl and the protein was almost unfolded in 5 M GdnHCl. The transition curve for urea shows an increase in ellipticity at 222 nm as compared with the native form in the range 0–1 M, whereas its ellipticity decreased with further increase in the concentration (2–8 M) and it was completely unfolded at high concentration.

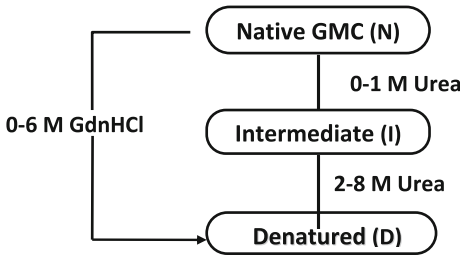
Discussion

The search for a better understanding of the process of protein folding/unfolding and the stability of the resulting structure has been long and difficult. This is not only because of the tremendous complexity of the structure of native proteins, but also because of our limited understanding of the solvent denaturation process (Pace 1986; Makhatadze and Privalov 1992; Creighton 1993). GdnHCl and urea are the most common chemical denaturants used for probing protein conformation, stability, and unfolding studies. These denaturants behave differently toward different proteins; for example, the enzyme activity of prostaglandin D-synthase increases in the presence of low concentrations of urea and GdnHCl, which is different from the classical behavior of denaturants (Inui et al. 2003). It is generally recognized that protein denaturation is a highly cooperative process, which for small globular proteins may be approximated by a two-state model and no significant intermediates are present during the transition N → D (Aune and Tanford 1969). However, recently reported results show that some intermediates exist during the unfolding of proteins. The intermediates between native and unfolded states have been referred to as molten globules in some cases (Ptitsyn 1995). For several proteins, when NMR data has been combined with hydrodynamic and small-angle-scattering data, the picture emerges that the protein chains are relatively compact under mildly denaturing conditions, forming intermediates (Shortle 1996); at higher concentrations of denaturant the conformation is expected to converge towards that of a random coil (Shortle and Ackerman 2001).In this study an attempt has been made to gain an insight into the structure–function relationship of goat muscle cystatin (GMC) to determine its behavior in the presence of the denaturants GdnHCl and urea.

Activity measurements show that at a GdnHCl concentration of 0.5 M the inhibitor was inactivated by approximately 46% in comparison with the fully active form of GMC, suggesting changes in the conformation of protein. GdnHCl starts behaving as a classical denaturant at higher concentration, when it leads to extreme unfolding of the enzyme, which ultimately causes complete loss of the inhibitory activity of GMC at 4 M GdnHCl. This result is supported by the fact that polar solvents, for example water, with a high polarizability value, cause a change in the microenvironment of the fluorophore by increasing solvation, reducing excited state complex energy, and producing a red shift, as in the case of GdnHCl. The charged molecule of GdnHCl enables competition with ionic pairs, destroying them and forming new interactions with denaturing agent, leading to the unfolded protein. Intrinsic fluorescence, extrinsic fluorescence, and CD spectroscopy results are indicative of a two-state transition of GMC in the presence of GdnHCl.

With urea, the enhanced activity of GMC at low concentrations (0.5–1 M) seems to be because of stabilization of GMC as a result of conformational and dynamic changes. It is generally believed that proteins are stabilized by hydrophobic effects, hydrogen bonding, and packing (Van der Waals) interactions. Urea and its derivatives are well known denaturants of proteins, because of their ability to weaken hydrophobic interactions in aqueous solutions. Because the hydrophobic force seems to be the main factor in stabilizing the globular proteins, any factor enhancing this force might be expected to stabilize a protein (Frank and Evans 1945; Tanford 1962). Urea, at low concentrations, might be able to achieve this role was suggested some time ago by Kumar et al. (2004) who observed a decrease in the cmc of SDS in presence of low concentration of urea. This was also agrees with previously reported findings that activation of different enzymes by denaturants was associated with conformational changes in the tertiary and secondary structure of the proteins (Inui et al. 2003; Wolosiuk and Stein 1990; Gull et al. 2007). Higher concentrations of urea reduce the hydrophobic forces and thus lead to protein denaturation. The changes noticed at very low concentrations of urea (0–1 M) could reflect the disruption of the associated/aggregated native protein as reported for other proteins. To rule out this possibility of low level aggregation/association of GMC in the absence of denaturant, GMC was subjected to non-denaturing polyacrylamide gel electrophoresis and turbidity analysis; both indicated the absence of aggregation. Further size exclusion chromatography was conducted and a single specific chromatographic peak was obtained with a stokes radius of 29.5 Å (corresponding to a molecular weight of 58.8 kDa) suggesting the absence of any aggregated form (data not shown).

Results obtained from secondary and tertiary analysis of the structure of GMC in urea solutions clearly indicate the existence of an intermediate state at low concentrations of the denaturant, as confirmed by the increasing ellipticity at lower concentration of urea (0.5–1 M) followed by a sharp decrease in ellipticity compared with the native state. Stabilization of the purified inhibitor (GMC) at low urea concentrations is in accordance with other previously reported results (Gull et al. 2007; Bhuyan 2002). High concentrations of urea lead to unfolding of GMC, as observed from the 5 nm red shift and increasing fluorescence intensity. ANS binding studies with GdnHCl result in a decrease in ANS fluorescence intensity with a red shift of 20 nm; this is indicative of disruption of hydrophobic patches because of unfolding of the inhibitor. Taking all these results into consideration, the mechanism of conformational alternation of GMC by urea and GdnHCl may be represented as:



Cystatins have important roles in normal body processes owing to their cysteine proteinase inhibitory activity and it is of utmost consequence that their conformation should be stable for maximum functional activity. The information obtained from GdnHCl and urea denaturation of proteins gives an idea about protein stability and can be extrapolated to physiological processes (Prusiner 1998; Kelly 1996). Thus the above observations have shed some light on the structural alterations and loss of function of GMC which can result from exposure of denaturants, thus effecting normal functioning of the protein. Such understanding of the folding/unfolding patterns of proteins is vital for analysis of many events involved in cellular regulation, the design of proteins with novel functions, and the development of novel therapeutic strategies for treating or preventing human diseases associated with the failure of proteins to fold correctly.

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