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Biphasic transition curve on denaturation of chicken cystatin by guanidinium chloride

Evidence for an independently unfolding structural region

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Far-ultraviolet circular dichroism and tryptophan fluorescence measurements showed that the reversible unfolding of the cysteine proteinase inhibitor, chicken cystatin, by guanidinium chloride is a two-step process with transition midpoints at ~3.4 and ~5.4 M denaturant. The partially unfolded intermediate had both far- and near-ultraviolet circular dichroism and fluorescence emission spectra comparable to those of the native protein. The largely retained tertiary structure suggests that the intermediate represents a species in which a separate region of lower stability has been unfolded, rather than an intermediate of the 'molten globule' type. Such a structurally independent region is apparent in the three-dimensional structure of the inhibitor.

Cysteine proteinase inhibitor; Cystatin; Protein denaturation; Stable intermediate; Circular dichroism; Fluorescence

1. INTRODUCTION

Chicken cystatin isolated from egg white is the best characterized member of the family II cystatins, which are extracellular cysteine proteinase inhibitors of ~120 amino acid residues with two disulfide bridges [1]. A homologous inhibitor, cystatin C, is the predominant member of this family in humans and presumably also other mammals [2]. Chicken cystatin and human cystatin C inactivate mammalian cysteine proteinases, e.g. cathepsins B, H and L, and also several structurally related plant enzymes, such as papain and actinidin, by forming tight (K_d = 5 nM–60 fM), equimolar complexes with the enzymes [1–5]. The crystal structure of chicken cystatin [6,7] suggests that the proteinase binding site comprises three regions of the polypeptide chain, which together form a wedge-shaped edge of the molecule that fits into the active-site cleft of papain with minimal conformational changes of either protein. The spectroscopic and kinetic characteristics of the interactions of chicken cystatin and cystatin C with those target proteinases that have been studied so far are highly similar [2,5,8], indicating that the two inhibitors bind to the enzymes in a closely related manner.

In this work we show that the reversible unfolding of chicken cystatin by guanidinium chloride is a two-step process. The stable intermediate on the unfolding pathway retains a considerable amount of the secondary and tertiary structure of the native protein and thus most likely represents a species in which a separate structural region of lower stability has been unfolded. These results, which can be interpreted in terms of the known X-ray structure of chicken cystatin [6,7], presumably can be extended also to human cystatin C because of the high similarity between the two proteins.

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2. MATERIALS AND METHODS

Chicken cystatin (form 1) and papain (EC 3.4.22.2) were purified and their concentrations determined as described earlier [3]. Guanidinium chloride ('ARISTAR' quality) was obtained from BDH Ltd., Poole, England. Concentrations of the denaturant were determined by densitometry [9].

Circular dichroism was measured at 25 ± 0.1°C with a Jasco J-41A spectro-polarimeter (Japan Spectroscopic Co., Tokyo, Japan). Measurements in the far-ultraviolet wavelength region were done in cells with 0.05- or 0.1-cm path-lengths and with cystatin concentrations of 0.25–0.5 g/l, whereas cells with 1 cm path-lengths and cystatin concentrations of 1.2 g/l were used in the near-ultraviolet region. The bandwidth was 2 nm in both regions.

Fluorescence measurements were done at 25 ± 0.1°C with an SLM 4800S spectrofluorimeter (SLM-Aminco, Urbana, IL) in the ratio mode. The excitation and emission wavelengths were 295 and 350 nm, with corresponding bandwidths of 4 and 8 nm, respectively. Cells with excitation and emission path-lengths of 0.4 and 1 cm, respectively, were used, and the cystatin concentration was 0.25 g/l. Emission spectra were corrected for the wavelength-dependence of the instrumental response.

Titrations of papain, at a concentration of 1 μM, with renatured cystatin for the determination of the binding stoichiometry were mon-

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itored by the decrease of fluorescence emission intensity accompanying the interaction, as described previously [3].

Polyacrylamide gel electrophoresis under non-denaturing conditions was done on 7% gels [10].

3. RESULTS

The unfolding of chicken cystatin in guanidinium chloride clearly is a two-step process with transition midpoints at ~3.4 and ~5.4 M, as shown by both far-ultraviolet circular dichroism and tryptophan fluorescence (Fig. 1). No further spectroscopic changes were observed after 24 h, when the results presented were obtained, indicating that equilibrium was attained within this time.

Far and near-ultraviolet circular dichroism spectra of cystatin at 6.5 M guanidinium chloride (Fig. 2) were those of a typical polypeptide chain in random conformation [11,12], indicating that the inhibitor is fully unfolded to a state approximating a disulphide-bonded random coil at this denaturant concentration. In contrast, the spectra of cystatin in the partially unfolded state, existing around 4 M guanidinium chloride, were comparable with those of the native protein, suggesting that the protein in this state retains a considerable amount of secondary and tertiary structure (Fig. 2). Corrected fluorescence emission spectra (not presented) were consistent with the latter conclusion, showing a shift of the wavelength of the emission maximum from that of the native protein (347 nm) of ≤ 1 nm at 4.2 M guanidinium chloride, compared with a red shift of ~5 nm for the fully unfolded protein at 6.5 M denaturant.

The two-step unfolding process was fully reversible, as shown by circular dichroism and fluorescence measurements (Fig. 1). Moreover, cystatin renatured from 6.5 M guanidinium chloride by dialysis for ~20 h against 0.05 M TRIS/HCl/0.1 M NaCl/100 μM EDTA, pH 7.4, migrated like native cystatin in polyacrylamide gel electrophoresis under non-denaturing conditions, with no evidence of aggregation. The renatured inhibitor bound to papain with a stoichiometry of inhibitor to enzyme of 1.1:1 and with a similar fluorescence decrease as that accompanying the interaction between papain and native cystatin [3], showing that also the proteinase-binding activity of the inhibitor was regained.

4. DISCUSSION

The biphasic equilibrium denaturation curve of chicken cystatin in guanidinium chloride indicates that a stable, partially unfolded intermediate exists on the unfolding pathway. This intermediate has certain characteristics of the 'collapsed form' or 'molten globule' demonstrated for the unfolding of certain small proteins [13–16]. It thus has a high degree of secondary structure, as evident from its far-ultraviolet circular dichroism spectrum, and presumably is compact, as suggested by the fact that it was not detected by a gel chromatographic technique in previous unfolding studies of chicken cystatin [17]. However, the near-ultraviolet circular dichroism and fluorescence emission spectra of

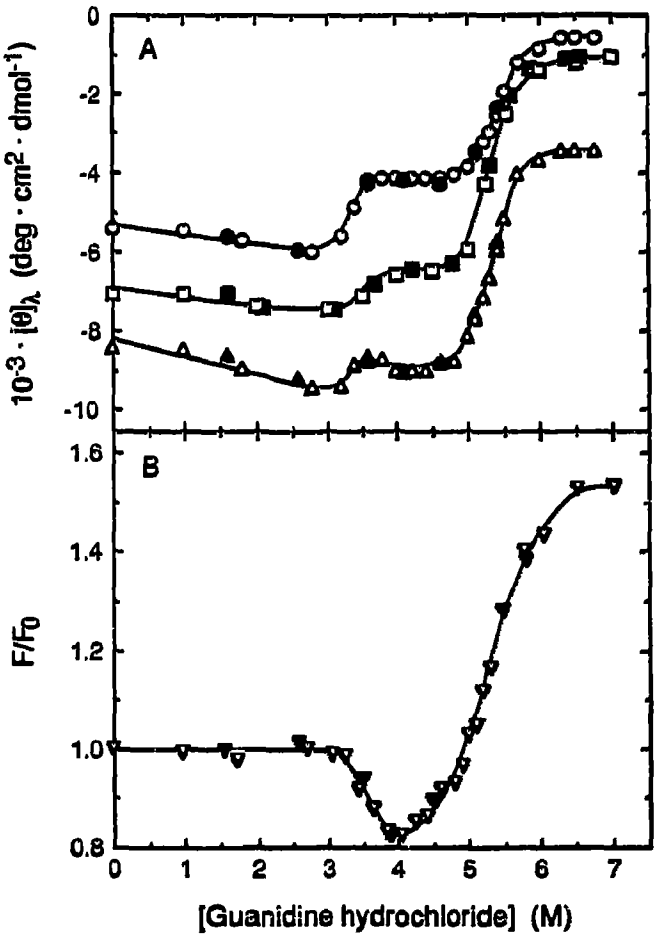

Fig. 1. Unfolding of chicken cystatin at different concentrations of guanidinium chloride and refolding of the denatured protein from 6.5 M guanidinium chloride, as monitored by (A) far-ultraviolet circular dichroism and (B) tryptophan fluorescence. *Unfolding:* buffer was mixed with cystatin (6–8.3 g/l) and guanidinium chloride (7.3–7.8 M) to the appropriate concentration of denaturant and a final protein concentration of 0.25 g/l. Measurements were made after ~24 h at 25°C by circular dichroism at wavelengths of 225 (○), 220 (□) and 212 (△) nm, and by fluorescence at excitation and emission wavelengths of 295 and 350 nm, respectively (▽). *Refolding:* cystatin, at a concentration of 1.0 g/l, was kept in 6.5 M guanidinium chloride for 5–10 min, at which time unfolding was complete, as shown by no further spectroscopic changes occurring. The solution was then diluted with buffer and 6.5 M guanidinium chloride to the appropriate concentration of denaturant and a final protein concentration of 0.25 g/l. Measurements were made after ~24 h at 25°C by circular dichroism at wavelengths of 225 (●), 220 (■) and 212 (▲) nm, and by fluorescence as described above (▼). All solutions in both the unfolding and refolding experiments contained 0.05 M TRIS/HCl/0.1 M NaCl, pH 7.4, as buffer. The circular dichroism results are given as mean residue ellipticity, based on a mean residue weight for cystatin of 113 [19]. The fluorescence results are expressed as the ratio between the fluorescence emission of the sample in the presence of guanidinium chloride (F) to that in the absence of the denaturant (F_0).

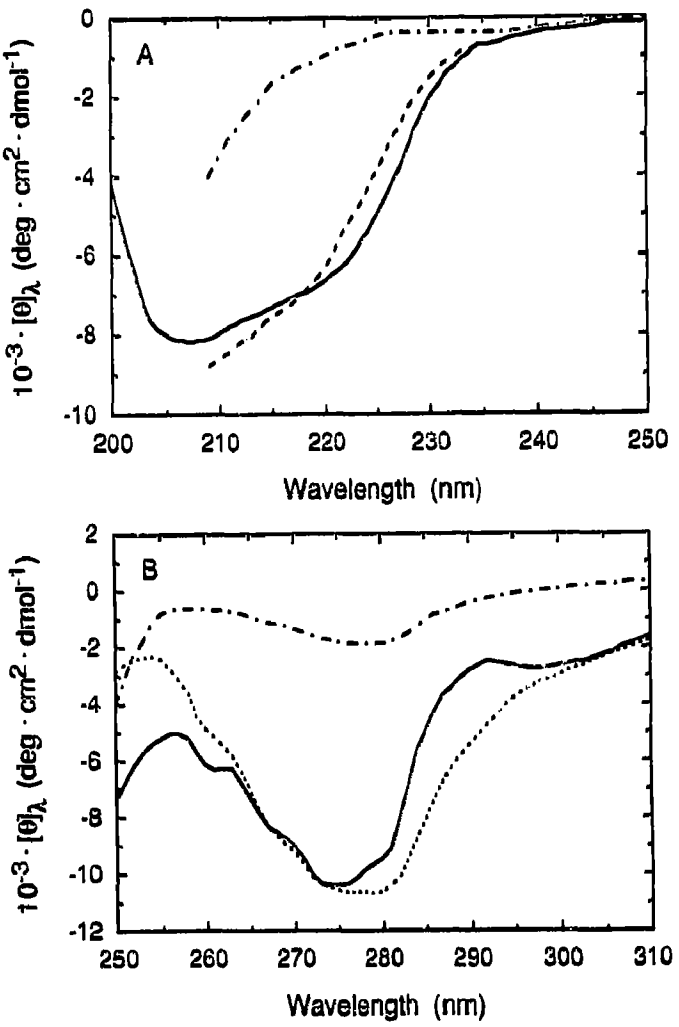

Fig. 2. Far-ultraviolet (A) and near-ultraviolet (B) circular dichroism spectra of chicken cystatin at different concentrations of guanidinium chloride. The guanidinium chloride concentrations were 0 (—), 4.0 (.....), 4.6 (---) and 6.5 (-.-.-.) M. Samples were prepared as described for the unfolding experiments in the legend to Fig. 1, except that the protein concentration in the measurements of the near-ultraviolet spectra was 1.2 g/l. Far-ultraviolet spectra for cystatin in guanidinium chloride could only be measured down to ~208 nm, due to the high absorbance of the solvent. The unit on the ordinate is mean residue ellipticity (see the legend to Fig. 1) in (A) and molar ellipticity in (B).

the intermediate are comparable with those of the native protein, a property not typical of the 'molten globule' state. Instead, this state is characterized by such spectra being similar to those of the fully unfolded protein, a finding that has been suggested to arise from a fluctuating tertiary structure.

A more plausible explanation for the nature of the stable intermediate of chicken cystatin is that it represents a form in which a distinct structural region of the protein with lower stability has been unfolded [18]. Although chicken cystatin is a small protein, such a region is nevertheless apparent in the three-dimensional structure of the inhibitor. The polypeptide chain between Pro-72 and Met-89 thus forms a slightly flexible tight turn, a 2.5-turn helical portion and a completely disordered four-residue segment, which together constitute a reasonably independent structural unit [6,7]. This region has substantial flexibility and, although disulphide-bonded to the main β-sheet of the protein, presumably has a lower stability than the remainder of the protein. We propose that the partially unfolded intermediate represents a form in which this region has been unfolded. Such a form should be compact and retain a considerable amount of the secondary and tertiary structure of the native protein and thus have the properties of the observed intermediate. A corresponding structurally independent region most likely also exists in human cystatin C because of the large structural and functional similarities between the two inhibitors.

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