Local pH-dependent conformational changes leading to proteolytic susceptibility of cystatin C

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Cystatin C, a cysteine protease inhibitor, was subject to hydrolysis at two sites when complexed with papain and in the presence of excess papain. A pH-dependent cleavage at His-86↑Asp-87 was observed, as well as a pH-independent one at Gly-4↑Lys-5. His-86↑Asp-87 hydrolysis increased with decreasing pH and was characterized kinetically. It could be described by a single ionization with $pK_a = 3.4 \pm 0.2$ and $(k_{cat.}/K_m)_{max.} = 1.4 (\pm 0.4) \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ at $I = 0.3 \text{ M}$. C.d. spectroscopy, also at $I = 0.3 \text{ M}$, demonstrated a conformational change with $pK_a = 3.2 \pm 0.2$, indicating that the pH-dependence of hydrolysis was due to a conformational change in cystatin C. At $I = 0.15 \text{ M}$, the pK_a of the conformational change observed by c.d. shifted to 4.1 ± 0.1 . This indicates that at physiological ionic strength of 0.15 M , a significant proportion of cystatin C complexed with protease

would be in a proteolytically labile conformation over the pH range 4.5 to 5, which is encountered in lysosomes. This may constitute a mechanism for clearing inappropriately localized cystatins. A pH-dependent conformational variability in this region of the inhibitor could explain the differences in the X-ray crystallographic and n.m.r. structures of the homologous chicken cystatin. The ionic-strength dependence of ionization indicates a hydrophobic stabilization of the ionizable group. The lack of pH-dependence of hydrolysis at Gly-4↑Lys-5, with $k_{cat.}/K_m = 220 \pm 41 \text{ M}^{-1} \cdot \text{s}^{-1}$ in the pH range 3.89 to 7.96 was unexpected in light of the normal, bell-shaped pH-dependence of papain-catalysed hydrolyses. This may reflect a different rate-limiting step of cystatin C hydrolysis.

INTRODUCTION

The cystatins are a superfamily of protein inhibitors of cysteine proteases that have been characterized in plants and animals [1] and protozoa [2,3]. Three types, or families, of cystatins have been identified. The type-I cystatins, or stefins, occur intracellularly, have molecular masses of approx. 11 kDa and do not possess disulphide bridges. The type-II cystatins, which are also referred to as the cystatin family, occur extracellularly, have molecular masses of approx. 13 kDa and two intramolecular disulphide bridges. Cystatin C (cC), a type-II cystatin, is present in most body fluids and appears to be the most physiologically important inhibitor of endogenous cysteine proteases [4]. Type-III cystatins, or kininogens, occur in blood plasma, have molecular masses of 68–120 kDa and contain three tandem repeats of a type-II-like domain.

Cystatins are potent non-covalent inhibitors of papain-like cysteine proteases, such as cathepsins B, H, L and S. Inhibition constants are generally in the subnanomolar range [1]; a dissociation equilibrium constant of $1.1 \times 10^{-14} \text{ M}$ has been estimated for cC with papain [5]. The inhibitory mechanism appears to be a simple bimolecular association [6]; cystatins bind in the active-site cleft of cysteine proteases, preventing substrate binding and hence proteolysis.

The X-ray crystallographic structures of two cystatins have been solved: an N-terminally truncated form of the type-II cystatin, chicken cystatin [7], and a type-I cystatin, human cystatin B (also called stefin B), in complex and papain [8].

Chicken cystatin consists of a five-stranded anti-parallel β -sheet that is twisted and wrapped around a five-turn α -helix [7]. At the opposite end of the β -strands from the binding site is a short helical segment (Asp-77–Asp-85) that is somewhat separated structurally from the rest of the molecule. This is followed by residues Glu-86 to Met-89 which have no regular secondary structure and weak electron density. The wedge-shaped protease-binding region is formed by two residues at the N-terminus, Gly-9–Ala-10, and residues in two loops, Gln-53–Gly-57 and Pro-103–Trp-104, and is highly complementary to the active-site cleft of papain. There have also been n.m.r. studies of mutant, recombinant chicken cystatin performed [9], but no structure has yet been published. The n.m.r. structure is described as being very similar to the X-ray crystallographic structure, except in the region of the second α -helix, where the helix is not observed [10]. The structure of cystatin B is similar to chicken cystatin, but with an extra β -strand near the C-terminus [8]. There is a 23 residue deletion in cystatin B relative to chicken cystatin in the region of the second α -helix, so this helix is not present in cystatin B.

N-terminally truncated forms of cC and chicken cystatin have been isolated previously [11,12] and truncated forms have been prepared *in vitro* with a variety of proteases [13–15]. However, with the exception of a study where cC was degraded by cathepsin D at pH 3.5 [16], at which pH it would be unfolded (see the Discussion), cleavage of type-II cystatins at other sites has not been observed previously.

Papain was used in this study, as it has been in most studies of cystatin–protease interactions, as a model for the mammalian

Abbreviations used: cC, cystatin C; Gly-4↑Lys-5 and His-86↑Asp-87, hydrolysis of cC between residues Gly-4 and Lys-5 and His-86 and Asp-87 respectively; (H86↑D87)cC, cystatin C clipped between residues His-86 and Asp-87; E-64, 1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane; HFBA, heptafluorobutyric acid; Z-Gly-ONP, benzyloxycarbonyl-glycine-p-nitrophenyl ester.

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cathepsins because it is available in pure form and large quantities. Papain has the stereotypical cysteine protease specificity for large hydrophobic groups in the S_2 subsite and in general has similar specificity to cathepsin L [17]. Unlike the lysosomal cysteine proteases, cathepsins B, H and L [18], papain is stable above neutral pH, which allowed kinetics to be determined up to pH 8. Finally, the association of papain and cC is so tight [5] that there was no need to account for free cC in the degradation reactions.

The fate of cystatin–protease complexes that form extracellularly as lysosomal proteases are inhibited has been assumed to be endocytosis, followed by sorting to the lysosomes and degradation of both molecules [19]. This may require a mechanism for clearing cystatins from low-pH environments such as lysosomes. We present evidence here that cC complexed with papain undergoes a conformational change at pH values of less than 5, as would be encountered in a lysosome, that renders it labile to proteolysis. The site of pH-dependent hydrolysis, His-86†Asp-87, equivalent to His-84–Asp-85 of chicken cystatin, is located in the region of the molecule where the X-ray and n.m.r. structures of chicken cystatin differ and may indicate a region of conformational variability in both proteins.

EXPERIMENTAL

Proteins

Expression and purification of cC will be described elsewhere (P. J. Berti, I. Ekiel, P. Lindahl, M. Abrahamson and A. C. Storer, unpublished work). Briefly, cC was expressed in a manner similar to previously reported methods [20], into the periplasmic space of *Escherichia coli* strain MC1061 using a temperature-sensitive repressor, but with growth under anaerobic conditions. cC was purified from the osmotic-shock solution using affinity chromatography with E-64–papain–Sephadex as the affinity matrix and elution with sodium trichloroacetate. cC was > 99% pure, with < 1% oxidation of methionine to sulfoxide as determined by h.p.l.c. Mercuripapain [21] was activated with 3 mM dithiothreitol for 30 min, then desalted on G-15 Sephadex in 1 mM EDTA (sodium salt). Total papain concentration was determined using ϵ_{280} $5.6 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [22]; and the active papain concentration was determined as [thiol] with 5,5'-dithiobis(2-nitrobenzoic acid) [23].

Kinetic analyses

The rate of degradation of cC by papain was followed by h.p.l.c. Substrate (cC–papain complex) concentrations were $1 \mu\text{M}$ to $10 \mu\text{M}$, free enzyme concentrations were 50 nM to $5 \mu\text{M}$. Substrate, enzyme and products were separated on a C-4 reverse-phase protein column with 62:38 (v/v) to 48:52 (v/v) [0.1% heptafluorobutyric acid (HFBA)/ H_2O]/(0.1% HFBA/acetonitrile) over 28 min at a flow rate of 2 ml/min at 35 °C. HFBA was from Pierce Chemical Co. (Rockford, IL, U.S.A.). The hydrolysis products were isolated and identified by N-terminal sequencing and ionspray m.s. [24]. The N-terminal sequence of purified cC was confirmed by automated Edman degradation performed on a Model 470A gas-phase sequencer equipped with an on-line Model 120A phenylthiohydantoin analyser (Applied Biosystems). The general protocol of Hewick et al. [25] was employed, using the O3RPTH program.

Reactions were performed at 37 °C in 100 mM sodium citrate/sodium phosphate buffer [26] containing 200 mM NaCl (pH 3.77 to 7.96). At pH 4.00, rates were also determined with 100 mM sodium acetate/200 mM NaCl. cC and papain were mixed at neutral pH in 200 mM NaCl, then an equal volume of 200 mM

sodium citrate/sodium phosphate buffer containing 200 mM NaCl was added to reach the desired pH. An aliquot was taken to determine $[\text{papain}]_{\text{free}}$. The $[\text{papain}]_{\text{free}}$ was assayed by the rate of benzyloxycarbonyl-glycine-*p*-nitrophenyl ester (Z-Gly-ONP) hydrolysis as determined by change in A_{320} at pH 4.0 ($0.0428 \text{ s}^{-1} \cdot \text{nM}^{-1}$) or A_{325} at pH 6.0 ($0.0480 \text{ s}^{-1} \cdot \text{nM}^{-1}$). The blank rate was determined by adding excess E-64 to the assay mixture. For h.p.l.c. samples, the reaction was stopped by addition of E-64 to a final concentration of $50 \mu\text{M}$ and placing the aliquot on ice. For reactions at pH values greater than 5.0, acetic acid was added to a final level of 10% to prevent widening of the papain peak on h.p.l.c., presumably due to slow structural changes affecting its mobility on the column [27]. Initial reaction velocities (v_0) were determined from the rate of substrate depletion and/or product formation (for Gly-4†Lys-5 only) using Cornish-Bowden's direct linear plot method [28]. The kinetic constants were determined directly from $v_0/[\text{papain}]_{\text{free}}$ with Cornish-Bowden's algorithm [29]. Selwyn's test [30] was used to test for enzyme inactivation. Selwyn's test is a plot of $([S]_0 - [S]_i)$ versus $[E]_0 \cdot t$, where $[S]_0$ and $[S]_i$ are substrate concentrations at time = 0 and i . Reactions run with different enzyme concentrations, $[E]_0$, at a given $[S]_0$ give superimposable curves if there is no enzyme inactivation.

C.d. analysis

C.d. spectra were collected with a Jasco J-600 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) at 37.0 ± 0.1 °C from 260 to 196 nm with 2 nm bandwidth in 0.02-cm-pathlength cells. Protein concentrations were $15 \mu\text{M}$ (0.55 g/l) for cC–papain complex, and $30 \mu\text{M}$ for cC (0.4 g/l) and papain (0.7 g/l) alone in 10 mM citrate/phosphate with 150 mM or 300 mM NaCl. The cC–papain complex was made with 1.02 equivalents of cC to prevent proteolysis. Secondary structure predictions from c.d. data were performed using software supplied by Japan Spectroscopic Co.

RESULTS

cC complexed with papain and incubated in the presence of excess papain underwent hydrolysis at two peptide bonds: at His-86†Asp-87 in a pH-dependent manner, and at Gly-4†Lys-5 in a pH-independent manner. If the reaction was allowed to continue after His-86†Asp-87 hydrolysis was essentially complete, the (1–86)cC and (87–120)cC peaks on h.p.l.c. disappeared slowly. (1–86)cC was largely converted into (4–86)cC before complete degradation, with no other intermediate forms detected.

The cleavage sites were identified by N-terminal sequencing of the hydrolysis products isolated by h.p.l.c. (Figure 1). A small amount of (4–86)cC was co-purified with (1–86)cC and a small amount of (93–120)cC was co-purified with (87–120)cC. The presence of these further degradation products was probably caused by the extended incubation of cC with papain in order to

Figure 1 Sites of cleavage of cystatin C

Sites of the pH-dependent His-86†Asp-87 hydrolysis yielding (H86†D87)cC and the pH-independent Gly-4†Lys-5 hydrolysis are shown.

Figure 2 Selwyn's test for enzyme inactivation during His-86↑Asp-87 hydrolysis

Using 5 μM cC–papain complex as substrate, product formation, determined as $[\text{cC}]_0 - [\text{cC}]_i$ by h.p.l.c., was followed at pH 4.0, 37 °C in 200 mM NaCl, with 100 mM citrate/phosphate buffer and $[\text{papain}]_{\text{free}}$ values of 1.26 μM (○) or 0.70 μM (△); or with 100 mM acetate buffer and $[\text{papain}]_{\text{free}}$ values of 0.61 μM (◇) or 0.29 μM (▽). The curve is calculated from: $[\text{product}] = [\text{cC-papain}]_0 \cdot (1 - \exp\{-1 \cdot (k_{\text{cat}}/K_m)_{\text{pH}4} \cdot [\text{papain}]_{\text{free}} \cdot t\})$, and the $(k_{\text{cat}}/K_m)_{\text{pH}4} = 2.8 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ is calculated from $k_{\text{cat}}/K_m = (k_{\text{cat}}/K_m)_{\text{max}} / (1 + K_a/[\text{H}^+])$, using $(k_{\text{cat}}/K_m)_{\text{max}}$ and $\text{p}K_a$ from the text.

Figure 3 Test of steady-state assumption

Initial rates of His-86↑Asp-87 hydrolysis at pH 4.0, 37 °C with different $[\text{papain}]_{\text{free}}$ were determined. Dashed line is average $v_0/([\text{papain}]_{\text{free}} \cdot [\text{cC-papain}]_0)$ for $[\text{papain}]_{\text{free}} < 2 \mu\text{M}$.

generate large amounts of the degradation products. The identity of the major products was confirmed by ionspray m.s.

The hydrolyses at the two sites were characterized kinetically. Selwyn's test [30] was used to test for enzyme inactivation. At pH 4.0, different runs gave superimposable curves (Figure 2), indicating that: (a) there was no enzyme inactivation during the

Figure 4 pH-dependence of His-86↑Asp-87 hydrolysis

Initial rates of hydrolysis were determined with 5 μM cC–papain as substrate at 37 °C in 100 mM citrate/phosphate buffer, 200 mM NaCl. Curve is fitted to $\text{p}K_a = 3.4$, $(k_{\text{cat}}/K_m)_{\text{max}} = 1.4 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$.

course of the reaction, (b) the method of determining $[\text{papain}]_{\text{free}}$ was accurate, and (c) buffer (acetate versus citrate/phosphate) had no effect on kinetics.

As $[\text{papain}]_{\text{free}}$ was often of the same order as that of the cC–papain complex, it was not possible to assume that the steady-state assumption was valid under the conditions being used. That is, it was not safe to assume that $d[\text{E} \cdot \text{S}]/dt \approx 0$, where $\text{E} \cdot \text{S}$ is the Michaelis complex. Under steady-state conditions, the initial velocity of the reaction should be proportional to the enzyme concentration, but this relationship breaks down when the steady-state assumption is not valid [31]. Since for all experiments $[\text{cC-papain}] \ll K_m$ (see below), it was possible to plot $v_0/([\text{papain}]_{\text{free}} \cdot [\text{cC-papain}])$ versus $[\text{papain}]_{\text{free}}$. The relationship was found to be proportional up to $[\text{papain}]_{\text{free}} \approx 2 \mu\text{M}$ at pH 4.0 (Figure 3). At $[\text{papain}]_{\text{free}} > 2 \mu\text{M}$ $v_0/([\text{papain}]_{\text{free}} \cdot [\text{cC-papain}])$ decreased due to breakdown of steady-state conditions.

The His-86↑Asp-87 hydrolysis displayed a pH-dependence, increasing at low pH values with $\text{p}K_a = 3.4 \pm 0.2$ and $(k_{\text{cat}}/K_m)_{\text{max}} = 1.4 (\pm 0.4) \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Figure 4). The calculated $K_m = 39 \mu\text{M}$ was much higher than $[\text{cC-papain}]_{\text{max}} = 5 \mu\text{M}$, so at a given pH, $k_{\text{cat}}/K_m = v_0/([\text{papain}]_{\text{free}} \cdot [\text{cC-papain}])$. When the data were fitted to two $\text{p}K_a$ s the calculated $\text{p}K_2 = 6.2 \pm 2.5$ was well separated from $\text{p}K_1$, had a large standard error associated with it and did not improve the fit to the data. A plot of $\log(k_{\text{cat}}/K_m)$ versus pH gave a straight line of slope = -1.28 , $r = 0.97$ (not shown), indicating that there is predominantly one ionization controlling the conformational change that allows the His-86↑Asp-87 cleavage to occur.

Clipped cC [(H86↑D87)cC] retained inhibitory activity, as $[\text{papain}]_{\text{free}}$ did not increase over the course of the reaction and (1–86)cC and (87–120)cC, purified separately by h.p.l.c., could be refolded together and purified by E-64–papain–Sepharose affinity chromatography (results not shown). Also, the similarity of k_{cat}/K_m for Gly-4↑Lys-5 hydrolysis of cC and (H86↑D87)cC (see below) indicates that there is no large change in the stability of the protein.

Figure 5 pH-dependence of Gly-4↑Lys-5 hydrolysis

Initial rates of Gly-4↑Lys-5 hydrolysis were determined with 5 μ M cC–papain complex for cC (○) and (H86↑D87)cC (▽) under the same conditions as in Figure 4. The rate of Gly-4↑Lys-5 hydrolysis with (H86↑D87)cC was determined by following the rate of conversion of (1–86)cC into (4–86)cC after His-86↑Asp-87 hydrolysis was essentially complete. The broken curve, illustrating the usual pH-dependence of papain-catalysed hydrolyses, is fitted to pK_a values of 3.7 and 4.5 in the ascending limb and 8.7 in the descending limb, with $(k_{cat}/K_m)_{max.} = 220 \text{ M}^{-1} \cdot \text{s}^{-1}$.

Figure 6 pH-dependence of cC–papain c.d. spectra

At $I = 0.3 \text{ M}$ (▽) with curve fitted to $pK_a = 3.2$, and $I = 0.15 \text{ M}$ (●) with curve fitted to $pK_a = 4.1$.

The hydrolysis of the Gly-4↑Lys-5 bond was pH-independent over the range examined, from pH 3.89 to 7.96, with $k_{cat}/K_m = 220 \pm 41 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Figure 5). The k_{cat}/K_m values of hydrolysis of Gly-4↑Lys-5 in intact cC and (H86↑D87)cC were the same. The calculated K_m value of 389 μ M was much greater than [cC–

papain] $_{max.} = 10 \mu$ M for that reaction; thus, as with His-86↑Asp-87 cleavage, at a given pH value $k_{cat}/K_m = v_0/([papain]_{free} \cdot [cC-papain])$.

C.d. spectra of cC, papain and the cC–papain complex were collected. The spectra were similar to those obtained previously for cC [5], papain and chicken cystatin–papain complex [32]. In buffer containing 300 mM NaCl, the molecular ellipticity of the cC–papain complex at 207.4 nM underwent an increase that fits a single ionization, $pK_a = 3.2 \pm 0.2$ (Figure 6). At 150 mM NaCl, the ellipticities underwent a change that fits a single ionization, $pK_a = 4.1 \pm 0.1$. Neither cC nor papain on their own underwent these conformational changes. cC underwent an abrupt and large-scale conformational transition between pH values of 3.9 and 3.5 that suggested unfolding (results not shown). The spectra of papain on its own did not change down to pH 3.2. As denaturation of mature papain is irreversible, no attempt was made to characterize its unfolding.

Secondary structure predictions based on the c.d. spectra showed no clear trends. Due to absorbance of the carboxylates of the citrate buffer, it was not possible to collect spectra below 196 nm. When spectral data do not extend below 190 nm, it is generally possible to predict only α -helical structure [33]. There were no consistent changes in the predicted α -helix content with changing pH for the cC–papain complex (results not shown).

DISCUSSION

His-86↑Asp-87 hydrolysis

The rate of hydrolysis at His-86↑Asp-87 was pH-dependent, increasing at low pH values. Since the steady-state assumption was valid under the reaction conditions used, and there was no enzyme inactivation, it is likely that true Michaelis–Menten kinetics were observed. As $K_m \gg [S]_{max.}$, k_{cat}/K_m could be determined at each pH value. Under these circumstances, the increase in k_{cat}/K_m of hydrolysis at His-86↑Asp-87 with decreased pH accurately reflects the protonation of the ionizable group that affects this hydrolysis. The similarity of pK_a values determined from His-86↑Asp-87 hydrolysis and from c.d. spectra at $I = 0.3 \text{ M}$, 3.4 ± 0.2 and 3.2 ± 0.2 respectively, suggests that the same ionization was responsible for both observations.

There are three possible causes of the increased k_{cat}/K_m of His-86↑Asp-87 hydrolysis; (a) a conformational change in cC that renders His-86↑Asp-87 susceptible to hydrolysis, (b) protonation of a group on papain that increases activity, or (c) protonation of a side chain on cC that increases specificity of papain for that group. Given the observation by c.d. spectroscopy of a conformational change taking place with the same pK_a as that determined by the kinetics of hydrolysis, it is probable that the increase in His-86↑Asp-87 hydrolysis is caused by a conformational change in cC in the cC–papain complex. It is unlikely that protonation of an ionizable group on papain is responsible as the k_{cat}/K_m of Gly-4↑Lys-5 hydrolysis is constant over the pH range examined. It is also unlikely that a simple specificity effect would account for the observed kinetics. The ionizable groups of cC nearest the scissile bond were His-86 at P_1 and Asp-87 at P_1' . There is ample evidence from specificity studies that papain can accept deprotonated aspartic acid side chains at P_1' [17,34,35]. Although there are no kinetic data for histidine at P_1 , papain does not have any strong specificity for P_1 side chains and does hydrolyse peptide bonds with positively charged lysine and arginine side chains at P_1 [17,36]. It was not clear initially whether the observed k_{cat}/K_m values of His-86↑Asp-87 hydrolysis should be normalized for the pH-dependency of papain observed with small, synthetic substrates. There was no pH-dependent decrease in papain activity observed

with Gly-4↑Lys-5 hydrolysis, indicating that the pH-dependence with a protein substrate was not necessarily the same as with synthetic substrates. Secondly, fitting the recalculated $k_{\text{cat.}}/K_m$ values to three $\text{p}K_a$ values gave an unrealistically high $(k_{\text{cat.}}/K_m)_{\text{max.}}$ of $3.7 (\pm 1.0) \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$, which is more than 3-fold higher than the extremely good substrate benzyloxycarbonyl-Phe-Arg-(methylcoumarinyl-7-amide) [37]; therefore the data were not normalized.

The hydrolysis reactions and c.d. spectra were originally performed at $I = 0.3 \text{ M}$ to minimize potential effects of low ionic strength on the kinetics of interaction, as observed between papain and the homologous chicken cystatin [6]. With papain and chicken cystatin, the association rate constant decreased progressively at ionic strengths below 0.15 M , but was essentially constant above $I = 0.15 \text{ M}$. Unexpectedly, there was a strong ionic strength dependence on the conformational change observed by c.d. spectroscopy. At $I = 0.3 \text{ M}$, a conformational change with a $\text{p}K_a$ of 3.2 was observed; but closer to physiological ionic strength, at $I = 0.15 \text{ M}$, the conformational change had a $\text{p}K_a$ of 4.1. Generally, ionic interactions are weakened by increasing ionic strength, due to screening of electrostatic interactions, while hydrophobic interactions are strengthened, due to the higher energy of apposing a charge to a hydrophobic surface than bringing a neutral water molecule into contact with the hydrophobic group. The decrease in $\text{p}K_a$ of the conformational change by 0.9 pH unit with increasing ionic strength indicates that the deprotonated form of the group being protonated was being stabilized by stronger hydrophobic interactions within the protein.

The conformational change observed by kinetics and c.d. spectroscopy is controlled predominantly by one ionizable group. This conclusion is supported by the fact that both kinetic and c.d. data fit to one $\text{p}K_a$ and that a plot of $\log(k_{\text{cat.}}/K_m)$ versus pH gave a slope of -1.28 . There are many carboxyl groups on both proteins that would be ionized in the pH range examined; however, only the ionization of one of these groups effects the conformational change. There is no evidence for the identity of this group, although the side chain of Asp-87 of cC would be one candidate.

Structural significance

The structure of chicken cystatin, which is homologous to cC, has been determined by X-ray crystallography [7,38] and n.m.r. spectroscopy [9,10]. Although generally concordant, the structures determined by the two methods differ around residues 69–92 [10], which correspond to residues 71–94 in cC. In the X-ray crystallographic structure, these residues constitute an α -helix (77–85) followed by a region of poorly defined electron density for residues 86–90 [7]. Crystals of chicken cystatin were grown at pH 4.0 [38]. The n.m.r. structure, determined at pH 5.5 [9], does not possess an α -helix in this region. In the n.m.r. structure, residues 81–83 take part in the formation of the β -sheet, while residues 87–90 form a tight turn [10]. Given the evidence presented here for pH-dependent conformational variability in the equivalent region of cC, the observed differences between the X-ray and n.m.r. structures of chicken cystatin probably represent true conformational variability in this region of the protein and not an artifact of either technique.

The overall structure of (H86↑D87)cC is not drastically affected by the cleaved bond. It retains inhibitory activity, spontaneously refolds from the isolated peptides and the $k_{\text{cat.}}/K_m$ of Gly-4↑Lys-5 hydrolysis is not affected. This is consistent with the observation that reduction of the Cys-71–Cys-81 disulphide bond in chicken

cystatin, which is analogous to Cys-73–Cys-83 in cC, caused no detectable change in the protease-binding activity [39]. The fact that (H86↑D87)cC accumulates and can be quantified indicates that the next hydrolysis is the rate-limiting step of degradation. Once the second cut is made, the subsequent degradation into peptides is fast, with no intermediates accumulating. The conformational change in the region of residues His-86 and Asp-87 that is demonstrated by the His-86↑Asp-87 hydrolysis is necessary for the second hydrolysis step. It is possible, but not necessarily the case, that His-86↑Asp-87 cleavage is necessary to allow further degradation to occur.

The conformational change in cC occurs at the opposite end of the molecule from the protease-binding region; as such, it appears to be a property of the cC molecule itself and would be expected not to depend on the identity of the bound protease. Specifically, the same conformational change would be expected if other cysteine proteases such as cathepsins B, H, L or S were used in place of papain.

Physiological significance

The location where cystatins could encounter low pH *in vivo* would be in lysosomes, which have an estimated pH of 4.5 to 5.0 [40]. Entry would be by endocytosis, whereby extracellular material is brought into vesicles or vacuoles which eventually merge with lysosomes [19]. The pH of the endocytosed material decreases from the extracellular pH down to the lysosomal pH as it is transported from endocytotic vesicles to lysosomes. There will be a certain amount of cystatins brought into the cell normally, but it is possible that there will be special conditions where the rate of cystatin intake is increased. For example, many types of intra-abdominal challenge can result in an inflammatory reaction that includes leukocytosis, causing the release of lysosomal enzymes [41]. Presumably these extracellular lysosomal cysteine proteases bind to cystatins and eventually the complexes are endocytosed. Also, there is considerable evidence that at least some cell types normally secrete and re-integrate lysosomal enzymes [42,43]. Cells in culture can quickly import and correctly process exogenous lysosomal enzymes [44,45], and at least some cell types normally secrete lysosomal cysteine proteases [46–48]. Given the high affinity of cystatins for cysteine proteases, lysosomal cysteine proteases appearing outside the cell would be expected to be quickly complexed. If these protease-inhibitor complexes were re-integrated, it would be advantageous to the cell to be able to degrade the cystatins and recycle the proteases.

Gly-4↑Lys-5 hydrolysis

Hydrolysis by free papain at Gly-4↑Lys-5 of cC in complex with papain indicates that in the complex at least residues Ser-1 to Gly-4 are relatively accessible to solvent and not in contact with the protease. This is consistent with the observations of Lindahl et al. [15], who found that the dissociation equilibrium constants for truncated forms of chicken cystatin with papain are not strongly affected by removing up to seven residues from the N-terminus. (des1–7)Chicken cystatin had an 8-fold increase in K_d relative to full-length chicken cystatin, while removal of residue Leu-8, which is believed to interact in a substrate-like manner with papain's S_2 subsite, caused a 2000-fold increase in K_d .

Over the pH range 3.89–7.96, $k_{\text{cat.}}/K_m$ of Gly-4↑Lys-5 hydrolysis was independent of pH. This is in contrast with the normal pH-dependence of papain-catalysed hydrolysis of low-molecular-mass substrates, which is characterized by two apparent $\text{p}K_a$ values of approx. 3.7 and 4.5 in the acid (ascending) limb and one apparent $\text{p}K_a$ of approx. 8.7 in the basic (descending)

limb [37,49]. The lack of pH-dependence may arise from a change in the rate-limiting step of the hydrolysis. If the usual steady-state assumption that $k_{-1} \gg k_{+2}$ for the mechanism:

is not true, then k_{+1} , the association rate constant, would become the rate-limiting step and would control the pH-dependence of the reaction. If this is the case, the Briggs–Haldane mechanism holds [50]. The Michaelis–Menten equation is still valid in this case, so the kinetic constants calculated above are valid. The combination of slow association and slow dissociation implies that there may be a conformational change occurring in forming the Michaelis complex of free papain with cC–papain. This would not be surprising given that the free papain must associate with cC residues close to where the papain molecule of the cC–papain complex is tightly bound to cC.

In conclusion, we have demonstrated two sites of cleavage of cC by papain. The pH-dependent hydrolysis at His-86†Asp-87 appears to be in a region of conformational variability in type-II cystatins. This variability may constitute a basis for degradation of inappropriately localized cystatins. The lack of pH-dependence for the Gly-4†Lys-5 hydrolysis may be due to the rate-limiting step of the reaction becoming the association of enzyme and substrate to form a Michaelis complex, that is, k_{+1} .

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