Characterization by rapid-kinetic and equilibrium methods of the interaction between *N*-terminally truncated forms of chicken cystatin and the cysteine proteinases papain and actinidin

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The interaction between five *N*-terminally truncated forms of chicken cystatin (starting at Leu-7, Leu-8, Gly-9, Ala-10 and Asp-15) and the cysteine proteinases papain and actinidin was studied by spectroscopic, kinetic and equilibrium methods. The u.v. absorption, near-u.v. c.d. and fluorescence emission difference spectra for the interactions with papain were all similar to the corresponding spectra for intact cystatin. The second-order association rate constants at 25 °C, pH 7.4, $1/0.15$, for the binding of the truncated forms to papain varied about 2-fold, from 6×10^6 to $1.5 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$, and were comparable to the value of $9.9 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ for intact cystatin. In contrast, the rate constants for the dissociation of the complexes with papain increased markedly with increasing extent of truncation, from $7.5 \times 10^{-6} \text{ s}^{-1}$ for Leu⁷ cystatin (a truncated form of cystatin having Leu-7 as its *N*-terminal amino acid) to 1.6 s^{-1} for Ala¹⁰-cystatin, whereas the dissociation rate constants for the latter form and Asp¹⁵-cystatin were similar. Consequently, the binding affinities between the truncated cystatins and papain decreased in an analogous manner, as was also shown for the interaction with actinidin by equilibrium measurements. Studies of the binding of the truncated cystatins to inactivated papains indicated that small substituents on the active-site cysteine of the enzyme can be accommodated in the complex without any loss of affinity when the *N*-terminal segment of the inhibitor is removed. Taken together, the results suggest that in the *N*-terminal region of chicken cystatin only residues preceding Ala-10 participate in the interaction with proteinases. Of these residues, Leu-7 and Leu-8 together account for about two-thirds of the unitary free energy of binding contributed by the *N*-terminal region, the relative importance of the two residues being dependent on the target proteinase. Both Gly-9 and residues *N*-terminal of Leu-7 further stabilize the interaction but contribute substantially smaller binding energies than do the two leucine residues.

INTRODUCTION

Cystatins are structurally related cysteine proteinase inhibitors with M_r values of 12000–14000. Four such inhibitors, cystatins A, B, C and S, have been identified in human tissues and secretions, and analogues of some of these inhibitors have also been found in other animals, as well as in plants (Barrett *et al.*, 1986; Rawlings & Barrett, 1990). Chicken cystatin (Fossum & Whitaker, 1968; Keilová & Tomásek, 1974), an analogue of human cystatin C readily available by purification from chicken egg-white (Anastasi *et al.*, 1983), has been studied as a model for the general mode of action of such inhibitors. Chicken cystatin forms tight, equimolar complexes with a number of cysteine proteinases, blocking the active site of the enzymes (Anastasi *et al.*, 1983; Nicklin & Barrett, 1984; Lindahl *et al.*, 1988; Björk & Ylinenjärvi, 1990). The binding kinetics are consistent with the complexes being formed by simple, reversible, bimolecular reactions with association rate constants close to that of a diffusion-controlled rate (Björk *et al.*, 1989; Björk & Ylinenjärvi, 1990). Recent studies have also shown that the kinetics of binding of human cystatin C to cysteine proteinases are compatible with this mechanism (Lindahl *et al.*, 1992). Inhibitor and proteinase thus appear to interact with minimal conformational changes of either protein, consistent with a computer docking model, based on the X-ray crystallographic structures of chicken cystatin and papain (Bode *et al.*, 1988). This model suggests that the reactive site of the inhibitor comprises the region around Gly-9, the Gln-Leu-Val-Ser-Gly segment at the middle of the sequence and the region around Trp-104, in agreement with earlier proposals

(Ohkubo *et al.*, 1984; Barrett *et al.*, 1986; Abrahamson *et al.*, 1987; Lindahl *et al.*, 1988). These regions, which are conserved in many cystatins, form a hydrophobic, wedge-shaped edge at one end of the molecule that is highly complementary to the active-site cleft of papain and fits almost perfectly, without any conformational adjustments, into this cleft. The general features of this model have been recently confirmed by crystallographic studies of the structure of a complex between cystatin B (also called stefin B) and *S*-(carboxymethyl)papain (Stubbs *et al.*, 1990).

Although the structure of chicken cystatin was solved with a truncated form of the inhibitor, starting at Gly-9, the docking model suggested that the preceding residue, Leu-8, may bind to the hydrophobic S_2 -subsite at the active site cleft of papain (Bode *et al.*, 1988, 1990). This suggestion was supported by inhibition studies of *N*-terminally cleaved forms of the inhibitor with papain (Abrahamson *et al.*, 1987; Machleidt *et al.*, 1989). However, these studies could not discriminate between the contributions made by Leu-7 and Leu-8, and the importance of residues preceding Leu-7 was not elucidated. Inhibition studies with truncated forms of human cystatin C were also consistent with the residues preceding the conserved glycine in this inhibitor, Leu-9 and Val-10, contributing to the binding (Abrahamson *et al.*, 1987, 1991; Popovic *et al.*, 1990). In contrast, *N*-terminal truncation of cystatin B (Thiele *et al.*, 1990) has been reported to have little or no effect on the inhibition of papain when the deletion includes the conserved glycine.

In this work, we have further characterized the importance of individual residues in the *N*-terminal region of chicken cystatin

Abbreviations used: Leu⁷-cystatin (etc.) denotes a truncated form of cystatin having Leu-7 as its *N*-terminal amino acid (see Table 1).

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above. SDS/PAGE (Laemmli, 1970) was done on 15% (w/v) gels with and without reducing agent. *N*-Terminal sequences were analysed as described by Björk *et al.* (1989).

Near-u.v. absorption difference spectra, near-u.v. and far-u.v. c.d. spectra, and fluorescence emission spectra of truncated forms of cystatin, proteinases, and the inhibitor–proteinase complexes were measured as reported previously (Lindahl *et al.*, 1988; Björk & Ylinenjärvi, 1990). Difference spectra were calculated from measured c.d. and fluorescence emission spectra as described by Lindahl *et al.* (1988).

Titration of active or inactivated proteinases with truncated forms of cystatin for the determination of binding stoichiometries or affinities were monitored by the decrease of fluorescence emission intensity accompanying the interactions (Lindahl *et al.*, 1988; Björk & Ylinenjärvi, 1989). Stoichiometries were determined at proteinase concentrations of 1–2 μM , whereas affinities were measured at concentrations of enzyme comparable to the dissociation equilibrium constants of the complexes formed. The fluorescence was measured at the wavelength of the maximum change (350 nm). Stoichiometries and dissociation constants were obtained by non-linear least-squares regression of the titration curves (Nordenman & Björk, 1978; Lindahl *et al.*, 1988) with the MultiFit program (Day Computing, Cambridge, U.K.) in a Macintosh Plus computer.

Batch titrations of actinidin with Gly⁹- and Ala¹⁰-cystatin for the determination of inhibition constants were monitored by the rate of increase of fluorescence emission intensity accompanying actinidin hydrolysis of the substrate 'carbobenzoxy-L-phenyl-alanyl-L-arginine 4-methylcoumaryl-7-amide' (Peptide Institute, Osaka, Japan). Different concentrations (0–40 μM) of the truncated inhibitors were mixed with 150 nM-actinidin in a final volume of 0.8 ml. After addition of 8 μl of 1 mM-substrate in 50% dimethyl sulphoxide, i.e., to a final substrate concentration of 9.9 μM , the rate of substrate hydrolysis was monitored for 3 min at 25 °C. The excitation and emission wavelengths were 370 and 440 nm and the corresponding bandwidths were 4 and 16 nm respectively. Substrate hydrolysis never exceeded 5%. The inhibition constant, K_i , was obtained by non-linear least-squares regression of the steady-state rates in the presence of inhibitor to the equation (Salvesen & Nagase, 1989):

$$v_i = v_0/[1 + ([I]/K_{i,\text{app}})]$$

In this equation, v_i and v_0 are the observed rates in the presence and absence of inhibitor respectively, $[I]$ is the inhibitor concentration, and $K_{i,\text{app}} = K_i(1 + [S]/K_m)$, where $[S]$ is the substrate concentration. The K_m value of actinidin for the substrate was estimated by similar measurements without inhibitor with substrate concentrations varying from 0 to 200 μM . The data were analysed by non-linear regression to the Michaelis–Menten equation.

The kinetics of binding of truncated forms of cystatin to papain were studied under pseudo-first-order conditions (i.e. with an excess of inhibitor) by stopped-flow fluorimetry, as detailed previously (Björk *et al.*, 1989).

The kinetics of dissociation of the complexes between Leu⁷- and Leu⁸-cystatin (form 2) and papain were evaluated by displacement of the inhibitor from the complex (10 μM) by an excess (100 μM) of intact cystatin (form 1), essentially as in earlier studies with intact cystatin (Björk *et al.*, 1989). The amount of inhibitor liberated at different times, and in the analyses with Leu⁸-cystatin also the decrease of the amount of initial complex with time, were determined by chromatography on a Mono-Q (Pharmacia) ion-exchange column (Björk *et al.*, 1989). At least 50 and 85% of the dissociation of the complexes with Leu⁷- and Leu⁸-cystatin respectively were monitored.

Protein concentrations were obtained by absorption measure-

ments at 280 nm. An absorption coefficient of $0.87 \text{ l} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$ (Anastasi *et al.*, 1983) and an M_r of 13 100 (Schwabe *et al.*, 1984) were used for intact cystatin. The molar absorption coefficient of $1.14 \times 10^4 \text{ m}^{-1} \cdot \text{cm}^{-1}$ obtained from these values was used also in calculations of molar concentrations of the truncated forms of the inhibitor, as no aromatic amino acid residues were cleaved off in any of the digestions. Absorption coefficients of 2.39 and $2.12 \text{ litre} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$ (McDowall, 1970; Brocklehurst *et al.*, 1973), and M_r values of 23 400 and 23 500 (Husain & Lowe, 1969; Carne & Moore, 1978), were used for papain and actinidin, respectively. The absorption coefficients for active papain were used also for inactivated forms of papain (Björk & Ylinenjärvi, 1989).

All analyses were made in 0.05 M-Tris/HCl/0.1 M-NaCl/100 μM -EDTA, pH 7.4, unless otherwise indicated.

RESULTS

Analyses of isolated truncated forms of cystatin

Five *N*-terminally truncated forms of chicken cystatin were obtained by limited proteolysis with different enzymes. Sequence analyses gave the *N*-terminal residues indicated in Table 1 and showed no evidence of impurities or further cleavage. The isolated forms all appeared as single peaks at different elution positions in hydrophobic interaction chromatography (Fig. 1). All forms also gave one band with a mobility comparable to that of intact cystatin in SDS/PAGE with or without reducing agent. These results indicate that all five preparations contained single truncated forms of cystatin produced by cleavage in the *N*-terminal region only.

The far-u.v. c.d. spectra of the truncated cystatin forms, like that of intact cystatin, all had a minimum at approx. 207 nm and a shoulder at 215–220 nm and also had magnitudes identical, within $\pm 5\%$, with the spectrum of the intact inhibitor (results not shown). The removal of the *N*-terminal region thus did not detectably alter the conformation of the protein, in agreement with deductions from the X-ray structure that this region does not take part in stabilization of the tertiary structure of the inhibitor, but extends freely into solution (Bode *et al.*, 1988, 1990). The changes of the interaction with proteinases observed in this work therefore should reflect contributions of the *N*-

Fig. 1. Hydrophobic interaction chromatography of intact and *N*-terminally truncated forms of chicken cystatin

—, Intact cystatin; — — —, Leu⁷-cystatin; - - - - -, Leu⁸-cystatin; ·····, Gly⁹-cystatin; - · - · - ·, Ala¹⁰-cystatin; — · — · —, Asp¹⁵-cystatin. A quantity (100 μg) of each protein was applied to a $0.75 \text{ cm} \times 7.5 \text{ cm}$ Bio-Gel Phenyl-5-PW column. The column was eluted at a flow rate of 1 ml/min with a linear gradient (total volume 85 ml) from 0.1 M-potassium phosphate/0.6 M-(NH₄)₂SO₄, pH 7.0, to the same buffer without (NH₄)₂SO₄. The straight line denotes the (NH₄)₂SO₄ concentration.

Table 2. Kinetic and equilibrium data for the interaction between *N*-terminally truncated forms of chicken cystatin and active papain

The analyses were done at 25 °C, pH 7.4, *I* 0.15. The values for intact cystatin, given for comparison, are taken from Lindahl *et al.* (1988) and Björk *et al.* (1989). Values measured in this work are given with their 95 % confidence limits, or, in the case of only two measurements, with the range. The number of measurements is given in parentheses. Association rate constants (k_{+1}) were obtained from slopes of plots of the observed pseudo-first-order rate constants, measured by stopped-flow fluorimetry, vs. inhibitor concentration. Dissociation rate constants (k_{-1}) were derived from displacement experiments or were calculated from k_{+1} and measured equilibrium constants (K_d). Values of K_d were obtained by non-linear least-squares regression of data from titrations, monitored by measurements of fluorescence emission of 350 nm, or were calculated from measured rate constants. The relative decrease of fluorescence emission, $(F_p + F_c - F_{p,c})/F_p$, at saturation of proteinase with inhibitor was derived from the non-linear regression analyses of the fluorescence titration data. Values of ΔG_u , the unitary free energy change for the binding reaction (Gurney, 1953; Karush, 1962), were calculated from the dissociation equilibrium constants.

Cystatin form	$10^{-6} \times k_{+1}$ ($M^{-1} \cdot s^{-1}$)	k_{-1} (s^{-1})	$(F_p + F_c - F_{p,c})/F_p$ at saturation	K_d (M)	ΔG_u ($kJ \cdot mol^{-1}$)
Intact	9.9	5.7×10^{-7}	0.67	6×10^{-14}	-85
Leu ⁷ -	14.8 ± 0.7 (10)	$(7.5 \pm 1.2) \times 10^{-8}$ (4)	0.49 ± 0.02 (5)	$5.1 \times 10^{-13} \dagger$	-80
Leu ⁸ -	12.4 ± 0.4 (13)	$(1.5 \pm 0.0) \times 10^{-3}$ (2)	0.46 ± 0.01 (2)	$1.2 \times 10^{-10} \dagger$	-66
Gly ⁹ -	10.8 ± 0.6 (12)	0.13*	0.44 ± 0.02 (6)	$(1.2 \pm 0.3) \times 10^{-8}$ (6)	-55
Ala ¹⁰ -	10.9 ± 0.4 (10)	1.6*	0.52 ± 0.03 (5)	$(1.5 \pm 0.7) \times 10^{-7}$ (4)	-49
Asp ¹⁵ -	6.0 ± 0.8 (11)	0.8*	0.53 ± 0.04 (4)	$(1.3 \pm 0.4) \times 10^{-7}$ (4)	-49

* Calculated from K_d and k_{+1} .

† Calculated from k_{+1} and k_{-1} .

terminal region of cystatin to the binding rather than a major alteration of the conformation of the protein as a result of the cleavage.

Stoichiometry of interaction with active papain

Titration, monitored by the changes in intrinsic fluorescence accompanying the binding (see below), of active papain with the truncated cystatin forms gave apparent binding stoichiometries of 1.10–1.15 mol of inhibitor/mol of enzyme. These values indicate that the preparations of the truncated cystatins may have contained small amounts of material that did not bind to the proteinase. However, the presence of this material would not have influenced the analyses in this work to any appreciable extent.

Spectroscopic changes on interaction with active papain

The near-u.v. absorption, c.d., and fluorescence emission difference spectra for the interactions between the truncated cystatins and papain were all similar to the corresponding spectra for intact cystatin (results not shown). As the spectral changes have been shown to arise predominantly from interactions involving Trp-104 of the inhibitor (Lindahl *et al.*, 1988; Nycander & Björk, 1990), this similarity indicates that the region around this residue interacts with the enzyme in a comparable manner in all the truncated cystatin forms as in the intact inhibitor. These results therefore provide further evidence that the observed changes in the binding of the truncated forms to proteinases primarily are due to changes of the interaction in the *N*-terminal region of the inhibitor.

Kinetics of interaction with active papain

The kinetics of association of truncated forms of cystatin with active papain were monitored under pseudo-first-order conditions, with excess of inhibitor, by the fluorescence changes accompanying the interactions. For all truncated forms the approach of the fluorescence signal to its final value was a first-order process at all inhibitor concentrations. The observed pseudo-first-order rate constants increased linearly with the concentrations of truncated inhibitor throughout the concentration range covered (0.2–5 μM ; results not shown). The second-order association rate constants at 25 °C, pH 7.4, *I* 0.15, for the interactions, calculated from these plots, varied about 2-fold, from 6×10^6 to $1.5 \times 10^7 M^{-1} \cdot s^{-1}$ (Table 2), and were

comparable to the value of $9.9 \times 10^6 M^{-1} \cdot s^{-1}$ for the binding of intact cystatin to papain (Björk *et al.*, 1989). The intercepts of the regression lines on the ordinate were experimentally indistinguishable from zero, consistent with dissociation rate constants less than $2 s^{-1}$.

The rates of dissociation of the complexes between the Leu⁷- or Leu⁸- forms of cystatin and active papain were monitored by a displacement method similar to that used previously for intact cystatin (Björk *et al.*, 1989; Björk & Ylinenjärvi, 1989, 1990). The two truncated forms of cystatin 2 were displaced from their complexes with the enzyme by a large excess of intact cystatin 1, and the rate of the release was quantified by ion-exchange h.p.l.c. The resulting dissociation rate constants for the two truncated cystatins were both higher than that for intact cystatin, but differed markedly (Table 2). The values correspond to half-lives of approx. 26 h and 8 min for the complexes of papain with the Leu⁷- and Leu⁸- forms respectively as compared with a value of approx. 14 days for the intact cystatin–papain complex (Björk *et al.*, 1989). The value for Leu⁸-cystatin may be somewhat in error, due to the rapid dissociation of the complex of this form with the enzyme.

The dissociation rate constants for the complexes of the Gly⁹-, Ala¹⁰- and Asp¹⁵- forms of cystatin with papain (Table 2) could not be determined by the displacement method, because of too rapid dissociation, but were calculated from the association rate constants and the dissociation equilibrium constants obtained by fluorescence titrations (see below). The values were appreciably higher than the dissociation rate constants for the longer truncated cystatin forms, corresponding to half-lives for the complexes of 0.4–5.4 s.

Affinity of interaction with active papain

The dissociation equilibrium constants for the tight complexes between the Leu⁷- or Leu⁸- forms of cystatin and active papain were too low to be measured by equilibrium methods and were calculated from the association and dissociation rate constants (Table 2). In contrast, the affinity between Gly⁹-, Ala¹⁰- or Asp¹⁵-cystatin and papain could be assessed by fluorescence titrations (Table 2). The affinities of the different cystatin forms for papain were found to decrease progressively with loss of residues from the *N*-terminus, from intact cystatin to Ala¹⁰-cystatin, whereas the latter form and Asp¹⁵-cystatin bound with similar affinity to the proteinase.

Table 3. Equilibrium data for the interaction between *N*-terminally truncated forms of chicken cystatin and active actinidin

The analyses were done at 25 °C, pH 7.4, *I* 0.15. The values for intact cystatin, given for comparison, are taken from Björk & Ylinenjärvi (1990). Values measured in this work are given with their 95 % confidence limits and with the number of measurements in parentheses. Most equilibrium constants (K_d) were obtained by fluorescence titrations, which also yielded values for $(F_p + F_c - F_{p,c})/F_p$ at saturation, as described in the legend to Table 2. However, the values for Gly⁹- and Ala¹⁰-cystatin were obtained as inhibition constants (K_i) from analyses of the inhibition of actinidin hydrolysis of a synthetic substrate, 'carbobenzoxy-L-phenylalanyl-L-arginine 4-methylcoumaryl-7-amide'. The $K_{i,app}$ values obtained from these analyses were corrected for substrate competition (see the Materials and methods section) with the use of a measured K_m value of actinidin for the substrate of 160 μ M. Values of ΔG_u were calculated as described in the legend to Table 2. N.D., not determined.

Cystatin form	$(F_p + F_c - F_{p,c})/F_p$ at saturation	K_d (nM)	ΔG_u (kJ · mol ⁻¹)
Intact	0.42	5	-57
Leu ⁷ -	0.34 ± 0.06 (4)	39 ± 17 (4)	-52
Leu ⁸ -	0.41 ± 0.05 (4)	250 ± 30 (4)	-48
Gly ⁹ -	N.D.	10 200 ± 2400 (14)	-38
Ala ¹⁰ -	N.D.	27 500 ± 9000 (8)	-36

Affinity of interaction with active actinidin

The dissociation equilibrium constants for the interaction of Leu⁷- or Leu⁸-cystatin with active actinidin were determined by fluorescence titrations (Table 3). However, the binding of Gly⁹- and Ala¹⁰-cystatin to the enzyme was too weak to be monitored by this method and, instead, inhibition constants were obtained from the effect of the two cystatin forms on the cleavage by actinidin of a fluorogenic substrate (Table 3). Such an analysis could not be done with Asp¹⁵-cystatin because of the limited amounts of this form available. The affinities of the different cystatin forms for actinidin were found to decrease with increasing extent of truncation of the *N*-terminal segment in the same general manner as the affinities for papain.

Affinity of interaction with inactivated papains

The dissociation equilibrium constants for the interactions between the truncated forms of cystatin and several inactivated papains were determined by fluorescence titrations (Table 4). As a rule, the affinities of the truncated inhibitor forms for the

inactivated papains were lower than those for the active enzyme, although both Ala¹⁰- and Asp¹⁵-cystatin bound to papain inactivated with the smallest blocking group, *S*-(methylthio)-papain, with similar affinity as that to intact papain (Tables 2 and 4). For each truncated form the affinities usually decreased with increasing size of the inactivating group (Table 4) in a similar manner to that found in earlier studies with intact cystatin (Björk & Ylinenjärvi, 1989). However, Ala¹⁰-cystatin bound considerably more weakly to *S*-(carboxymethyl)papain than to *S*-(*N*-ethylsuccinimidyl)papain. Like the affinity for active papain, the affinity of the truncated forms of the inhibitor for the same inactivated proteinase in general decreased with decreasing length of the *N*-terminal region of the inhibitor (Table 4). An exception was that Leu⁸-cystatin bound about 10-fold tighter to *S*-(carboxymethyl)papain than did Leu⁷-cystatin. Moreover, Gly⁹-, Ala¹⁰- and Asp¹⁵-cystatin bound with comparable affinities to *S*-(methylthio)papain, and the affinities of intact cystatin and Leu⁷-cystatin, as well as those of Gly⁹- and Ala¹⁰-cystatin, for *S*-(*N*-ethylsuccinimidyl)papain were also similar.

DISCUSSION

This work substantiates the importance of the *N*-terminal region of chicken cystatin for the interaction with cysteine proteinases (Abrahamson *et al.*, 1987; Machleidt *et al.*, 1989), as has also been reported for human cystatin C (Abrahamson *et al.*, 1987, 1991; Popovic *et al.*, 1990). A major extension of the previous studies is that the part of the *N*-terminal region that interacts with the proteinase can be delimited and the strength of this interaction estimated. The similar affinities of Ala¹⁰- and Asp¹⁵-cystatin for active and *S*-(methylthio)papain thus show that only residues in the region *N*-terminal to Ala-10 participate in the interaction. The binding affinity measured for Ala¹⁰-cystatin therefore should represent the affinity of the interactions between the enzyme and the remainder of the binding site of the inhibitor, i.e. the Gln-Leu-Val-Ser-Gly and Pro-Trp hairpin loops (Bode *et al.*, 1988; Stubbs *et al.*, 1990). This affinity corresponds to about 60 % of the unitary free energy change (i.e. the free energy change after elimination of the decrease in entropy resulting from two molecules combining to form one larger one; Gurney, 1953; Karush, 1962) for the binding of the intact inhibitor to both papain and actinidin (Tables 2 and 3; Fig. 2). The *N*-terminal region therefore presumably contributes about 40 % of the total unitary free energy change for the binding of the inhibitor to the two enzymes.

Of the residues in the *N*-terminal region of cystatin, Leu-7 and

Table 4. Dissociation equilibrium constants (in nM) for the interaction between *N*-terminally truncated forms of chicken cystatin and inactivated forms of papain

The analyses were done at 25 °C, pH 7.4, *I* 0.15. The values for intact cystatin, given for comparison, are taken from Björk & Ylinenjärvi (1989). Values measured in this work are given with their 95 % confidence limits and with the number of measurements in parentheses. The dissociation constants were obtained as described in the legend to Table 2. The lower limit for the dissociation constant for the binding of Ala¹⁰-cystatin to *S*-(carboxymethyl)papain was estimated from a titration in which < 10 % saturation was reached. For a full account of the inactivating reagents used and the structures of the inactivating groups, see Table 1 in Björk & Ylinenjärvi (1989). N.D., not determined.

Cystatin form	Inactivating group...	K_d (nM)		
		<i>S</i> -(Methylthio)-	<i>S</i> -(Carboxymethyl)-	<i>S</i> -(<i>N</i> -Ethylsuccinimidyl)-
Intact		0.0007	0.5	170
Leu ⁷ -		N.D.	21 ± 6 (5)	100 ± 36 (3)
Leu ⁸ -		N.D.	1.9 ± 2.3 (3)	610 (1)
Gly ⁹ -		130 ± 12 (5)	1150 ± 160 (5)	1500 ± 120 (4)
Ala ¹⁰ -		180 ± 60 (3)	≥ 20000	1600 ± 700 (3)
Asp ¹⁵ -		160 ± 40 (4)	N.D.	N.D.

Fig. 2. Unitary free energy changes for the binding of intact or *N*-terminally truncated forms of chicken cystatin to papain and actinidin

Leu-8 together contribute the predominant part, about two-thirds, of the unitary free energy of binding of this region to both papain and actinidin (Tables 2 and 3; Fig. 2). The importance of the binding of Leu-8 to papain, possibly to the *S*₃ subsite of the enzyme, has been emphasized previously (Bode *et al.*, 1988; Machleidt *et al.*, 1989). However, this residue accounts for only about one-third of the unitary free energy of binding of the *N*-terminal region of cystatin to papain and only about half of this energy of binding to actinidin (Tables 2 and 3; Fig. 2). Leu-7 further increases the affinity for papain appreciably and contributes a larger portion of the free energy of binding of the *N*-terminal segment to this enzyme than does Leu-8 (Table 2; Fig. 2). The effect is most likely due to Leu-7 directly participating in the interaction with papain, presumably by binding to the *S*₃ subsite (Bode *et al.*, 1988; Stubbs *et al.*, 1990), although it cannot be excluded that an influence on the conformation of Leu-8 may be partly responsible. In contrast, Leu-7 has a smaller effect on the free energy of binding of cystatin to actinidin (Table 3; Fig. 2). The different contributions of the two leucine residues to the interaction of cystatin with papain and actinidin are in agreement with previous evidence that the two proteinases interact differently with the *N*-terminal region of the inhibitor (Björk & Ylinenjärvi, 1990).

In addition to the two leucine residues, Gly-9 also contributes free energy of binding of cystatin to both papain and actinidin, possibly by virtue of a weak interaction with the reactive cysteine of the proteinase (Bode *et al.*, 1988). Moreover, residues *N*-terminal to Leu-7 further stabilize the interaction with the two enzymes. However, both Gly-9 and the outermost *N*-terminal residues account for a substantially smaller portion of the binding energy of the *N*-terminal region than do the two leucine residues (Tables 2 and 3; Fig. 2).

The markedly decreased affinities of the *N*-terminally truncated cystatin forms for active papain are due primarily to increased dissociation rate constants, as the association rate constants varied only about 2-fold. This observation is consistent with the proposal that the rate of association of chicken cystatin with proteinases is primarily diffusion controlled (Björk *et al.*, 1989). The result is also in agreement with studies of the binding of intact cystatin to inactivated forms of papain, in which the decreased affinities similarly were shown to be due to higher dissociation rate constants (Björk & Ylinenjärvi, 1989). Interestingly, the rate of association of Leu⁷- and Leu⁸-cystatin with papain is slightly higher than the rate for intact cystatin, perhaps due to the flexibility of the *N*-terminal segment of the intact inhibitor (Bode *et al.*, 1988, 1990) interfering somewhat

with the access of the binding region to the active-site cleft of the proteinase.

Characterization of the binding of the truncated forms of cystatin to inactivated papains provided additional information on the interaction between the *N*-terminal region of the inhibitor and the enzyme. The similar affinities of both Ala¹⁰- and Asp¹⁵-cystatin for active papain and *S*-(methylthio)papain indicate that small substituents on the reactive cysteine of the enzyme can be accommodated in the complex without any loss of affinity, when the *N*-terminal segment outside Ala-10 is removed. In contrast, such substituents on papain appreciably decrease the affinity of the interaction with intact cystatin (Björk & Ylinenjärvi, 1989), as well as with Gly⁹-cystatin (Table 4). The *N*-terminal region of the inhibitor thus appears to restrict the space around the reactive site cysteine of papain in the complex, Gly-9 presumably being mainly responsible for this effect (Bode *et al.*, 1988).

It is also apparent from the affinities of the truncated cystatins for inactivated papains that the *N*-terminal segment of cystatin becomes less important for the interaction upon increasing the size of the substituent on the active-site cysteine of the enzyme. Reduction of the length of this segment by 8 residues, from intact cystatin to Gly⁹-cystatin, only decreases the affinity for *S*-(*N*-ethylsuccinimidyl)papain by about 10-fold, as compared with about a 2×10^5 -fold decrease in the affinity for *S*-(methylthio)papain. Possibly, the bulkier substituents displace intact cystatin out of the tight fit with the proteinase so that the *N*-terminal segment of the inhibitor can interact only weakly with the enzyme. Therefore, removal of this segment has only a minor effect on the affinity of the interaction with papain inactivated by such large substituents.

The studies with inactivated papains also illustrate the importance of the charge on the substituent on the active-site cysteine of papain for the interaction (Björk & Ylinenjärvi, 1989). Leu⁸-cystatin thus binds more tightly to *S*-(carboxymethyl)papain than does Leu⁷-cystatin, in contrast to the reverse relative affinities of the two forms for intact papain. Most likely the carboxy group of *S*-(carboxymethyl)papain is located closer to the terminal amino group of the inhibitor in the complex with Leu⁸-cystatin than in the complex with Leu⁷-cystatin, allowing a strong ionic interaction. Moreover, Ala¹⁰-cystatin binds much more weakly to *S*-(carboxymethyl)papain than to *S*-(*N*-ethylsuccinimidyl)papain, which has a larger, but uncharged, inactivating group. In this case, the carboxy group reduces the binding affinity, presumably by repulsion from a negatively charged region of the inhibitor in a similar manner to that suggested for the interaction of *S*-(carboxymethyl)papain with intact cystatin (Björk & Ylinenjärvi, 1989).

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