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Interaction of recombinant human cystatin C with the cysteine proteinases papain and actinidin

Peter LINDAHL,* Magnus ABRAHAMSON† and Ingemar BJÖRK*‡

*Department of Veterinary Medical Chemistry, Swedish University of Agricultural Sciences, Uppsala Biomedical Center, Box 575, S-751 23 Uppsala, Sweden, and †Department of Clinical Chemistry, University Hospital, University of Lund, S-221 85 Lund, Sweden

The interaction between recombinant human cystatin C and the cysteine proteinases papain and actinidin was studied by spectroscopic, kinetic and equilibrium methods. The absorption, near-u.v. c.d. and fluorescence-emission difference spectra for the cystatin C–proteinase interactions were all found to be similar to the corresponding spectra for chicken cystatin. The kinetics of binding of cystatin C to the two enzymes were best described by a simple reversible one-step bimolecular mechanism, like the kinetics of the reaction of chicken cystatin with several cysteine proteinases. Moreover, the second-order association rate constants at 25 °C, pH 7.4 and I 0.15, of 1.1×10^7 and 2.4×10^6 $M^{-1} \cdot s^{-1}$ for the reactions of cystatin C with papain and actinidin respectively, were similar to the corresponding rate constants for the chicken inhibitor and close to the value expected for a diffusion-controlled rate. The dissociation equilibrium constants, approx. 11 fM and approx. 19 nM for the binding of cystatin C to papain and actinidin respectively, were also comparable with the dissociation constants for chicken cystatin. The affinity between cystatin C and several inactivated papains or actinidins decreased with increasing size of the inactivating group in a manner similar to that in earlier studies with the chicken inhibitor. Together, these results strongly indicate that the mechanisms of the reactions of cystatin C and chicken cystatin with cysteine proteinases are identical or highly similar, but differ from that of reactions between serine-proteinase inhibitors and their target enzymes. The model for the proteinase–inhibitor interaction, based on the X-ray structure of chicken cystatin, therefore should be largely applicable also to human cystatin C.

INTRODUCTION

The cystatin superfamily of protein inhibitors of cysteine proteinases in mammalian organisms comprises three individual families, I, II and III (Barrett *et al.*, 1986). The inhibitors of the first two of these families, the cystatins, are small proteins (M_r approx. 11000–14000) present mainly in tissues and body secretions, whereas the members of the third family, the kininogens, are larger proteins (M_r approx. 60000–120000) found in blood plasma. The cystatins of family I are polypeptides of about 100 amino acid residues without disulphide bridges. Individual members of this family are cystatins A and B (also called stefins A and B). The family II cystatins, which have about 120 residues with two disulphide bridges, comprise cystatins C and S and variants of the latter inhibitor. An avian analogue of cystatin C is also present in chicken egg-white. All cystatins inhibit proteinases belonging to the papain family of cysteine proteinases.

Cystatin C is the predominant family II cystatin in mammalian organisms. It is present in most body fluids, including blood plasma, and appears to be the physiologically most important inhibitor of endogenous cysteine proteinases in many such fluids (Abrahamson *et al.*, 1986). It occurs in particularly high concentrations in seminal fluid and cerebrospinal fluid (Abrahamson *et al.*, 1986). A variant of cystatin C is found in amyloid deposits around cerebral arteries in a hereditary form of angiopathy leading to fatal haemorrhage (Ghiso *et al.*, 1986; Löfberg *et al.*, 1987), and the concentration of cystatin C in cerebrospinal fluid is greatly decreased in patients with this disorder (Grubb *et al.*, 1984). However, the relation of these findings to the aetiology of the disease is unclear.

To date, most studies of the structure of the family II cystatins and of their mechanism of interaction with cysteine proteinases have been done with chicken cystatin. The inhibitor has been shown to form a tight (K_d = 5 nM–60 fM) equimolar complex with a number of proteinases (Anastasi *et al.*, 1983; Nicklin & Barrett, 1984; Lindahl *et al.*, 1988; Björk *et al.*, 1989; Björk & Ylinenjärvi, 1990). The crystal structure of chicken cystatin (Bode *et al.*, 1988) suggests that the proteinase-binding site comprises three regions, the *N*-terminal region around Gly-9, the Gln-Leu-Val-Ser-Gly sequence at residues 53–57 and the region around Trp-104, in agreement with other evidence (Ohkubo *et al.*, 1984; Barrett *et al.*, 1986; Abrahamson *et al.*, 1987*a*; Lindahl *et al.*, 1988; Moreau *et al.*, 1990; Nycander & Björk, 1990). This proposal has been essentially confirmed by the recently determined X-ray structure of a complex between cystatin B, a family I cystatin, and papain (Stubbs *et al.*, 1990). The kinetics of binding of chicken cystatin to several cysteine proteinases are compatible with the complex being formed by a simple reversible bimolecular reaction with an association rate constant close to that of a diffusion-controlled rate (Björk *et al.*, 1989; Björk & Ylinenjärvi, 1990). Inhibitor and proteinase thus appear to interact with minimal conformational changes of either protein, in agreement with computer docking experiments (Bode *et al.*, 1988).

In the present work we have characterized the interaction between human cystatin C and two cysteine proteinases, papain and actinidin, by spectroscopic, kinetic and equilibrium methods. The amounts of protein required for these studies necessitated the use of a recombinant inhibitor, expressed in *Escherichia coli* (Abrahamson *et al.*, 1988). The results show that cystatin C behaves similarly to chicken cystatin in all aspects investigated, in particular with regard to the kinetics of proteinase binding. The reaction mechanisms for the two inhibitors are thus

Abbreviations used: E-64, [*N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]amido-4-guanidinobutane.

‡ To whom correspondence should be addressed.

apparently identical or highly similar, but differ from the mechanism of reactions between serine-proteinase inhibitors and their target enzymes. The similarity between the inhibition mechanisms for the two cystatins suggests that the model for the proteinase–inhibitor interaction, based on the X-ray structure of chicken cystatin, can be extended also to human cystatin C.

MATERIALS AND METHODS

Recombinant human cystatin C, expressed in *E. coli*, was purified essentially as described previously (Abrahamson *et al.*, 1988; Dalbøge *et al.*, 1989). The periplasmic fraction from fermented bacteria was obtained by osmotic shock, and periplasmic proteins were concentrated by precipitation with 50%-saturated $(NH_4)_2SO_4$. Following desalting on Sephadex G-25 (Pharmacia, Uppsala, Sweden), recombinant cystatin C was purified by Q-Sepharose (Pharmacia) chromatography in 0.02 M-ethanolamine/HCl buffer, pH 9.5, containing 5 mM-benzamidinium chloride, 10 mM-EDTA and 0.1% NaN_3. Cystatin C appeared in the break-through fraction and was concentrated by $(NH_4)_2SO_4$ precipitation (50% saturation). Monomeric cystatin C was isolated by a final gel chromatography on Sephadex G-75 (Pharmacia) in 0.05 M-ammonium bicarbonate buffer, pH 9.5, and was then freeze-dried. The protein was stored at −20 °C in the dried state or at −70 °C as a 1–5 mg/ml solution in 0.05 M-Tris/HCl buffer, pH 7.4, containing 0.1 M-NaCl and 100 μM-EDTA. Forms 1 and 2 of chicken cystatin were purified and stored as described previously (Anastasi *et al.*, 1983; Lindahl *et al.*, 1988).

Papain (EC 3.4.22.2) and actinidin (EC 3.4.22.14) were purified from papaya latex and kiwi fruit respectively (Burke *et al.*, 1974; Brocklehurst *et al.*, 1981; Lindahl *et al.*, 1988; Björk & Ylinenjärvi, 1990). The enzymes (containing 0.9–1.0 mol of thiol group/mol of protein) were stored as their inactive *S*-(methylthio) derivatives and were activated by treatment with 1 mM-dithiothreitol for 15 min before use (Roberts *et al.*, 1986; Lindahl *et al.*, 1988; Björk & Ylinenjärvi, 1990). Inactivation of papain and actinidin (25–100 μM) with iodoacetamide or iodoacetic acid at pH 8.0 or with *N*-ethylmaleimide or *N*-n-butylmaleimide at pH 7.4 was performed at reagent concentrations of 1–1.8 mM (in excess of the concentration required to neutralize the dithiothreitol used for the activation) for 5, 5, 65 and 90 min respectively. The reagents were then removed by gel chromatography on Sephadex G-25.

Near-u.v.-absorption difference spectra, near-u.v. and far-u.v. c.d. spectra and fluorescence-emission spectra of cystatin C, proteinases and the cystatin C–proteinase complexes were measured essentially as reported previously (Lindahl *et al.*, 1988; Björk & Ylinenjärvi, 1990). In the analyses of the cystatin C–proteinase complexes the low-M_r cysteine-proteinase inhibitor [*N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]amido-4-guanidinobutane (E-64) (Barrett *et al.*, 1982) was added at a molar ratio of E-64 to enzyme of 0.06–0.2:1 immediately after the two proteins were mixed. This addition was done to prevent proteolysis by possibly contaminating cysteine proteinases in the papain and actinidin preparations that were not inactivated by cystatin C (Abrahamson *et al.*, 1987*a*; Buttle *et al.*, 1990). Difference spectra were calculated from measured c.d. and fluorescence-emission spectra as described by Lindahl *et al.* (1988).

Titrations of active or inactivated proteinases with cystatin C 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). The titrations were done at enzyme concentrations of 0.1–2 μM, and the fluorescence was measured at the wavelength of the maximum change (350–356 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.) on a Macintosh Plus computer.

The kinetics of binding of cystatin C to active or inactivated cysteine proteinases were studied under pseudo-first-order conditions (i.e. with an excess of inhibitor) by stopped-flow fluorescence measurements, as detailed previously (Björk *et al.*, 1989).

The kinetics of dissociation of the complexes between cystatin C and papain or *S*-(methylthio)-papain were evaluated by displacement of cystatin C from the complexes (10 μM) by an excess (90–230 μM) of chicken cystatin (form 2), essentially in the same manner as in earlier studies with chicken cystatin (Björk *et al.*, 1989). However, the rate of appearance of the complexes between the displacing chicken cystatin 2 and the active or inactivated papain liberated from the complex with cystatin C, rather than the rate of appearance of liberated cystatin C, was monitored by chromatography on the Mono-Q (Pharmacia) ion-exchange column. The reason for this was that cystatin C was not well separated from its complex with papain either on a Mono-Q or a Mono-S column. Immediately after cystatin C and active papain had been mixed to form a complex, but before addition of the displacing chicken cystatin, E-64 was added at a molar ratio of reagent to enzyme of 2.0:1. Similarly, methyl methanethiolsulphonate was added to the complex of cystatin C with *S*-(methylthio)-papain. As in the spectroscopic studies, these additions were made to inactivate possibly contaminating cysteine proteinases not bound to cystatin C. In the analyses with active papain, excess E-64 was then removed by gel chromatography on Sephadex G-25 to prevent formation of an E-64 derivative of the dissociated papain, which would have bound only weakly to chicken cystatin 2 (Björk & Ylinenjärvi, 1990).

Cystatin C liberated from its complex with actinidin was prepared by incubation of the complex for 30 min with a 10-fold excess of chicken cystatin 2, followed by isolation of the displaced inhibitor by h.p.l.c. on an ion-exchange column (Björk *et al.*, 1989; Björk & Ylinenjärvi, 1990). The *N*-terminal sequence of the liberated cystatin C and the behaviour of the protein in SDS/PAGE under reducing conditions were analysed by procedures described previously (Björk *et al.*, 1989; Björk & Ylinenjärvi, 1990).

Amino acid analyses were carried out on an LKB 4151 Alpha Plus amino acid analyser (LKB, Bromma, Sweden) after hydrolysis of the samples in 6 M-HCl at 110 °C for 24 and 72 h. Half-cysteine and methionine were determined as cysteic acid and methionine sulphone respectively (Moore, 1963).

Protein concentrations were obtained by absorption measurements at 280 nm. An absorption coefficient of 0.83 ± 0.03 litre·g^{-1}·cm^{-1} (mean ± range, $n = 2$) was determined for cystatin C by quantitative amino acid analyses, and an M_r of 13400 (Grubb & Löfberg, 1982; Abrahamson *et al.*, 1987*b*) was used in calculations of molar concentrations of the inhibitor. Absorption coefficients and M_r values used for other proteins were 0.87 litre·g^{-1}·cm^{-1} and 13100 for chicken cystatin (Anastasi *et al.*, 1983; Schwabe *et al.*, 1984), 2.39 litre·g^{-1}·cm^{-1} and 23400 for papain (Brocklehurst *et al.*, 1973; Husain & Lowe, 1969) and 2.12 litre·g^{-1}·cm^{-1} and 23500 for actinidin (McDowall, 1970; Carne & Moore, 1978). The absorption coefficients for the active enzymes were used also for inactivated enzymes (Björk & Ylinenjärvi, 1989).

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

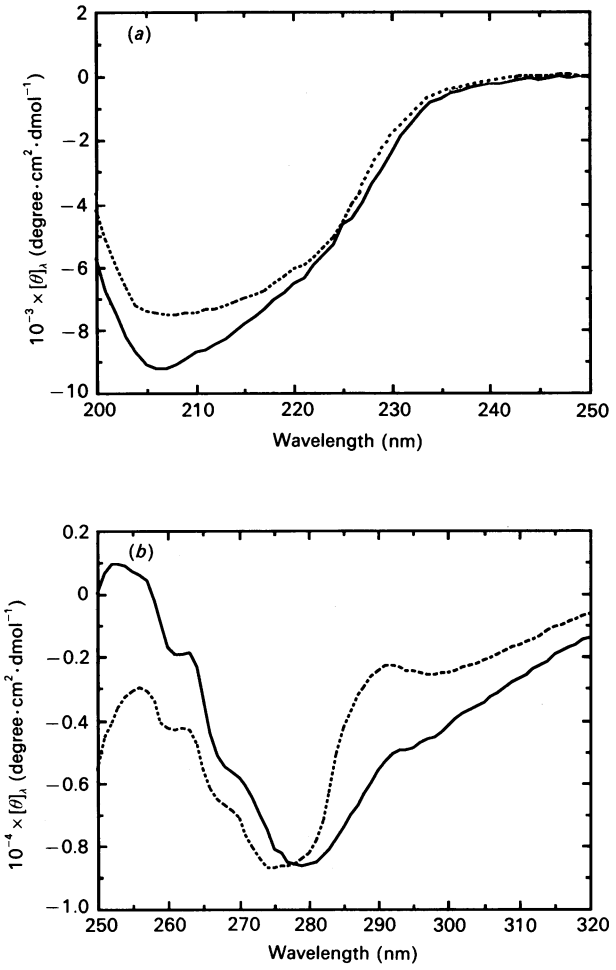

Fig. 1. C.d. spectra of cystatin C and chicken cystatin

(*a*) Far-u.v. wavelength region; (*b*) near-u.v. wavelength region. ——, Cystatin C; -----, chicken cystatin 1. The cell pathlengths were 0.1 and 1 cm and the protein concentrations 15 and 75 μM in (*a*) and (*b*) respectively.

RESULTS

C.d. spectra of cystatin C

The far-u.v. c.d. spectrum of cystatin C (Fig. 1*a*) had a minimum at approx. 207 nm and a shoulder at 215–220 nm, features that were characteristic also of the spectrum of chicken cystatin, shown for comparison. However, the ellipticity of the cystatin C spectrum at the wavelength minimum was about 20% lower than that of the spectrum of the chicken inhibitor. The near-u.v. c.d. spectrum of cystatin C was also comparable with that of chicken cystatin (Fig. 1*b*), although the two spectra differed somewhat, particularly around 290 nm and below 265 nm.

Stoichiometry of interaction with proteinases

Active papain and actinidin and the inactive *S*-(methylthio) derivative of papain were titrated with cystatin C at protein concentrations much higher than the dissociation equilibrium constants of the complexes formed. The titrations were monitored by the changes in intrinsic fluorescence accompanying the binding (see below). These analyses showed that the inhibitor bound to the three proteinases with stoichiometries of 0.96–1.05 (Fig. 2), values experimentally indistinguishable from 1.0. The recombinant cystatin C was thus fully active in binding cysteine proteinases.

Spectroscopic changes on interaction with active proteinases

The changes of near-u.v. absorption, c.d. and fluorescence induced by the interaction of cystatin C with active papain or actinidin were analysed at molar ratios of inhibitor to enzyme of 1.2–1.6:1. These ratios were sufficient to give over 99% saturation of the enzymes with the inhibitor in most analyses under the conditions used, as calculated from the equilibrium constants obtained in this work (see below and Table 1). However, only about 95% saturation was reached in the measurements of fluorescence-emission spectra of the cystatin C–actinidin complex. The spectra for the cystatin C–proteinase complexes are

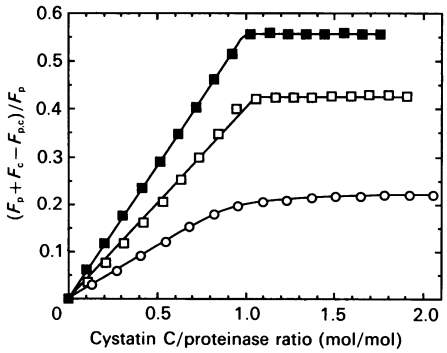

Fig. 2. Titrations of papain, *S*-(methylthio)-papain and actinidin with cystatin C, monitored by measurements of tryptophan fluorescence

□, Papain (1 μM); ■, *S*-(methylthio)-papain (1 μM); ○, actinidin (2 μM). Excitation was at 280 nm and fluorescence emission was measured at 350 nm. F_p, fluorescence of proteinase; F_c, fluorescence of added cystatin; $F_{p,c}$, fluorescence of the proteinase/cystatin mixture. The continuous lines represent the computer fits of the data to the theoretical binding equation.

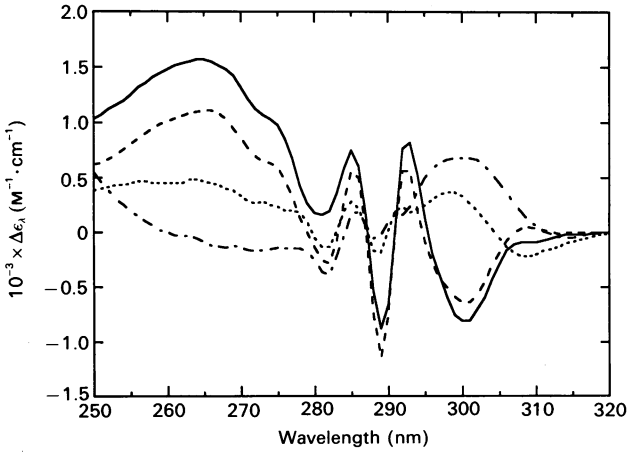

Fig. 3. Near-u.v.-absorption difference spectra measured between complexes of cystatin C or chicken cystatin with papain or actinidin and the free proteins

——, Cystatin C and papain; – – – –, chicken cystatin 1 and papain; -----, cystatin C and actinidin; – – – –, chicken cystatin 1 and actinidin. The proteinase concentrations were 14–15.8 μM, and the molar ratios of cystatin to proteinase were 1.2–1.3:1.

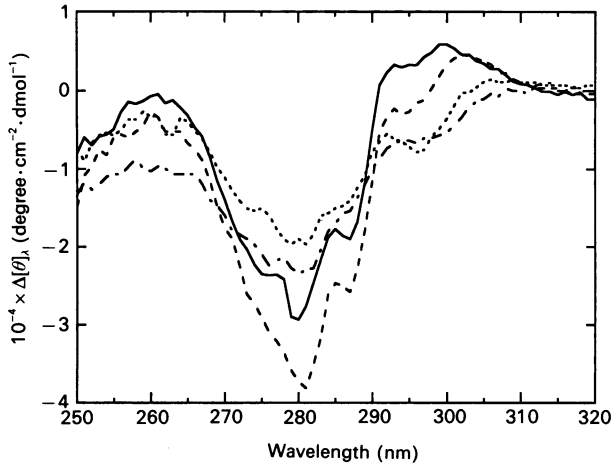

Fig. 4. Near-u.v. c.d. difference spectra between complexes of cystatin C or chicken cystatin with papain or actinidin and the free proteins

——, Cystatin C and papain; – – – –, chicken cystatin 1 and papain; – – – –, cystatin C and actinidin; – – – –, chicken cystatin 1 and actinidin. The difference spectra were calculated from separately measured c.d. spectra of the complexes and the free proteins (Lindahl *et al.*, 1988). The proteinase concentrations were 13.4–15 μM, and the molar ratios of cystatin to proteinase were 1.2–1.6:1.

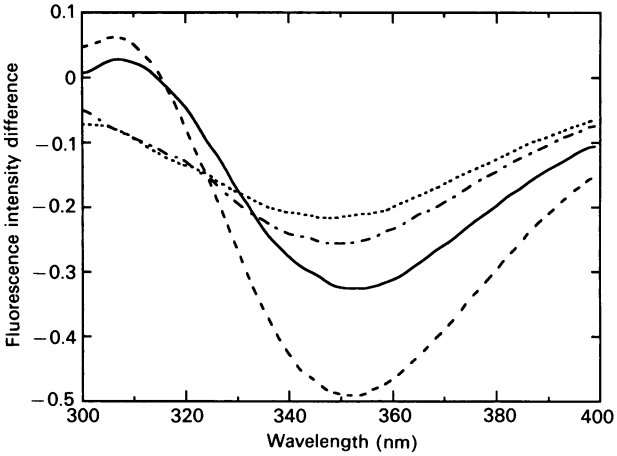

Fig. 5. Fluorescence-emission difference spectra between complexes of cystatin C or chicken cystatin with papain or actinidin and the free proteins

——, Cystatin C and papain; – – – –, chicken cystatin 1 and papain; – – – –, cystatin C and actinidin; – – – –, chicken cystatin 1 and actinidin. The difference spectra were calculated from separately measured corrected emission spectra of the complexes and the free proteins (Lindahl *et al.*, 1988). Before these calculations, all spectra were normalized to a fluorescence intensity of 1.0 for the free proteinases at the wavelength of the emission maximum. The proteinase concentration was 1 μM, and the molar ratios of cystatin to proteinase were 1.2–1.3:1. Excitation was at 280 nm.

presented together with the corresponding data obtained previously for chicken cystatin (Lindahl *et al.*, 1988; Björk & Ylinenjärvi, 1990) to facilitate comparisons. The absorption difference spectra (Fig. 3), near-u.v. c.d. difference spectra (Fig. 4) and fluorescence-emission difference spectra (Fig. 5) measured for the cystatin C–proteinase interactions were all found to be

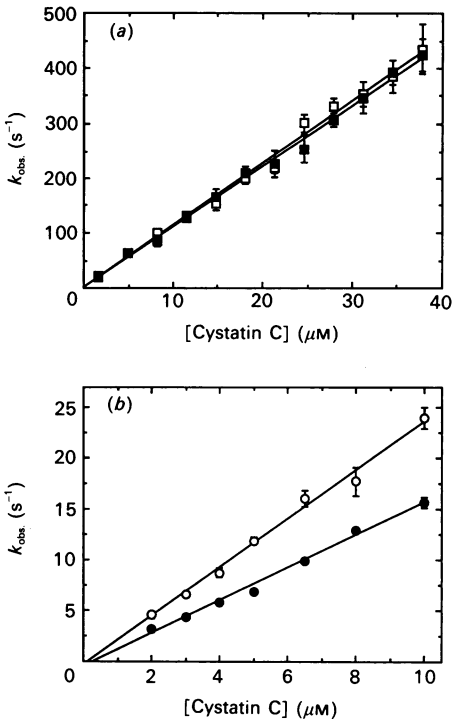

Fig. 6. Observed pseudo-first-order rate constants ($k_{obs.}$) for the binding of cystatin C to active or *S*-(methylthio) forms of papain or actinidin as a function of cystatin C concentration

(*a*) □, Papain; ■, *S*-(methylthio)-papain; (*b*) ○, actinidin; ●, *S*-(methylthio)-actinidin. The analyses were done at 25 °C, pH 7.4 and *I* 0.15. The molar ratio of cystatin C to proteinase was maintained at 10:1 in all reactions. The vertical bars represent the 95% confidence intervals computed from three to five individual experiments.

similar to the corresponding spectra for chicken cystatin. However, some differences were apparent, most notably the lower magnitudes of the c.d. and fluorescence-emission difference spectra for cystatin C than of those for chicken cystatin.

Kinetics and affinity of interaction with active proteinases

The kinetics of binding of cystatin C to active papain and actinidin were studied under pseudo-first-order conditions, i.e. at a molar ratio of cystatin to proteinase of 10:1, and were monitored by the fluorescence changes accompanying the interactions. In the reactions with papain, the cystatin concentration was increased as high as possible, the limit being set by the dead time of the stopped-flow instrument (approx. 2 ms), whereas the reactions with actinidin were studied only up to inhibitor concentrations of 10 μM (Fig. 6). For both enzymes, the approach of the fluorescence signal to its final value was a first-order process at all cystatin concentrations. Moreover, the observed pseudo-first-order rate constant increased linearly with cystatin concentration throughout the concentration ranges covered (Fig. 6). The second-order association rate constant at 25 °C, pH 7.4 and *I* 0.15 for the interaction of cystatin C with papain, calculated from these data, was 1.14×10^7 M^{-1}·s^{-1} (Table 1), similar to that of the binding of chicken cystatin to the enzyme (which is 9.9×10^6 M^{-1}·s^{-1}; Björk *et al.*, 1989). The corresponding rate constant for the reaction of cystatin C with actinidin was about 5-fold lower than for the reaction with papain (Table 1) and was comparable with the rate constant for the reaction of chicken cystatin with actinidin (Björk & Ylinenjärvi, 1990). The intercepts of the regression lines on the $k_{obs.}$ axis were experimentally

Table 1. Kinetic and equilibrium data for the interaction between cystatin C and active or inactivated forms of papain or actinidin

The analyses were done at 25 °C, pH 7.4 and *I* 0.15. Measured values are given with their 95% confidence limits, and with the number of measurements in parentheses. Association rate constants (k_{+1}) were obtained from the slopes of the plots in Fig. 5. Dissociation rate constants (k_{-1}) were obtained either from the rate of appearance of chicken cystatin 2–proteinase complexes in experiments in which cystatin C was displaced from its complexes with the proteinases by an excess of chicken cystatin 2, or by calculation from k_{+1} and measured equilibrium constants (K_d). Most values of K_d were obtained by non-linear least-squares regression of data from titrations (monitored by measurements of fluorescence emission at 350–356 nm) to the equation derived for formation of an equimolar complex between the two reactants. However, the dissociation equilibrium constants for the binding of cystatin C to papain and to *S*-(methylthio)-papain 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 cystatin C was derived by non-linear-regression analyses of the fluorescence titration data. For a full account of the inactivating reagents used and the structures of the inactivating groups see Table 1 in Björk & Ylinenjärvi (1989). Abbreviation: N.D., not determined.

Enzyme	Inactivating group	$10^{-6} \times k_{+1}$ ($M^{-1} \cdot s^{-1}$)	k_{-1} (s^{-1})	$(F_p+F_c-F_{p,c})/F_p$ at saturation	K_d (M)
Papain	–	11.4±0.9 (12)	$(1.3\pm0.3)\times10^{-7}$ (6)	0.43±0.04 (6)	1.1×10^{-14}†
	S-(Methylthio)-	11.0±0.7 (12)	$(2.4\pm0.4)\times10^{-6}$ (4)	0.55±0.02 (6)	2.2×10^{-13}†
	S-(*N*-Ethylsuccinimidyl)-	N.D.	N.D.	0.67±0.05 (3)	$(1.8\pm0.5)\times10^{-7}$ (3)
	S-(*N*-n-Butylsuccinimidyl)-	N.D.	N.D.	0.68±0.10 (3)	$(1.3\pm0.2)\times10^{-6}$ (3)
Actinidin	–	2.4±0.3 (7)	0.046*	0.21±0.01 (5)	$(1.9\pm1.0)\times10^{-8}$ (5)
	S-(Methylthio)-	1.6±0.2 (7)	0.21*	0.28±0.02 (5)	$(1.3\pm0.5)\times10^{-7}$ (5)
	S-(Carbamoylmethyl)-	N.D.	N.D.	0.60±0.03 (4)	$(1.4\pm0.5)\times10^{-6}$ (4)
	S-(Carboxymethyl)-	N.D.	N.D.	0.63±0.02 (3)	$(1.2\pm0.2)\times10^{-6}$ (3)

* Calculated from K_d and k_{+1}.
† Calculated from k_{+1} and k_{-1}.

indistinguishable from zero, consistent with very low dissociation rate constants.

The rate of dissociation of the complex between cystatin C and papain was monitored by a displacement method similar to that used previously for chicken cystatin (Björk *et al.*, 1989; Björk & Ylinenjärvi, 1989, 1990). Cystatin C was displaced from its complex with the enzyme by a large excess of chicken cystatin 2, and the cystatin 2–papain complex formed instead was quantified by ion-exchange h.p.l.c. Owing to the slow dissociation only the initial 10–15% of the reaction could be monitored, as the risk of bacterial growth precluded sufficiently long incubation times. An approximately linear increase in the amount of cystatin 2–papain complex was observed under these conditions. The dissociation rate constant for the complex of cystatin C with papain obtained from this initial rate (Table 1) was independent of the concentration of the displacing chicken cystatin, indicating that the observed rate constant approximates well the true dissociation rate constant of the complex (Björk *et al.*, 1989). The value corresponds to a half-life of approx. 60 days, somewhat longer than the half-life of approx. 14 days estimated for the chicken cystatin–papain complex (Björk *et al.*, 1989). The dissociation rate constant for the cystatin C–actinidin complex (Table 1) could not be determined by this method, because the dissociation was too rapid, but it was calculated from the association rate constant and the dissociation equilibrium constant obtained by fluorescence titrations (see below).

The affinity between cystatin C and papain was too tight to be determined by equilibrium methods, as was also previously found for the corresponding interaction between chicken cystatin and the enzyme (Lindahl *et al.*, 1988). Instead, the dissociation equilibrium constant for the cystatin C–papain complex was calculated from the association and dissociation rate constants (Table 1). The value obtained, 1.1×10^{-14} M, is about 5-fold lower than that of the chicken cystatin–papain complex (which is 6.0×10^{-14} M; Björk *et al.*, 1989). In contrast, the affinity between cystatin C and active actinidin could be determined by fluorescence titrations at protein concentrations of 0.1 μM, comparable with the dissociation equilibrium constants of the complex formed (Table 1). The binding was about 4 times weaker than that of chicken cystatin to actinidin (Björk & Ylinenjärvi, 1990).

Analyses of dissociated inhibitor

Cystatin C was displaced from its complex with actinidin by an excess of chicken cystatin 2. The *N*-terminal sequence of the liberated inhibitor was Ser-Ser-Pro-Gly-Lys-, identical with that of intact cystatin C (Grubb & Löfberg, 1982). Moreover, the liberated inhibitor migrated identically with untreated cystatin C in SDS/PAGE under reducing conditions, both when the two proteins were analysed separately and mixed together. The dissociation of the complex thus appears to produce intact inhibitor. Analogous analyses of cystatin C displaced from its complex with papain could not be done, as the low dissociation rate of this complex precluded the isolation of sufficiently large amounts of the liberated protein.

Kinetics and affinity of interaction with inactivated proteinases

The kinetics of binding of cystatin C to the *S*-(methylthio) derivatives of papain or actinidin were studied by stopped-flow measurements in the same manner as the kinetics of the reactions with the active enzymes. The approach of the fluorescence signal to its final value was a first-order process at all cystatin concentrations also in these analyses, and the observed pseudo-first-order rate constant increased linearly with cystatin concentration throughout the concentration ranges covered (Fig. 6). The second-order association rate constant for the interaction of cystatin C with *S*-(methylthio)-papain was 1.10×10^{7} $M^{-1} \cdot s^{-1}$, the same as with active papain, whereas the corresponding value for the reaction with *S*-(methylthio)-actinidin was almost half of that for the binding to the active enzyme (Table 1). As in the analyses with the active enzymes, the intercepts of the regression lines were experimentally indistinguishable from zero.

The rate of dissociation of the complex between cystatin C and *S*-(methylthio)-papain was monitored by the displacement method used for the corresponding analysis with active papain. In this case 50–70% of the reaction could be monitored, owing to the more rapid dissociation. A time-dependent appearance of

cystatin 2–proteinase complex compatible with a first-order reaction was observed. The dissociation rate constant obtained from these data (Table 1) corresponds to a half-life for the complex between cystatin C and *S*-(methylthio)-papain of approx 80 h, comparable with approx. 35 h for the complex of chicken cystatin with this inactivated papain form (Björk & Ylinenjärvi, 1989). The dissociation rate constant for the complex between cystatin C and *S*-(methylthio)-actinidin was calculated from the association rate constant and the dissociation equilibrium constant, as for active actinidin (Table 1).

The affinity between cystatin C and several inactivated papains or actinidins was determined by fluorescence titrations at protein concentrations of 0.2–2 μM, comparable with the dissociation equilibrium constants of the complexes. The affinity of cystatin C for the inactivated proteinases generally decreased with increasing size of the inactivating group (Table 1) in a manner similar to that observed in previous studies with chicken cystatin (Björk & Ylinenjärvi, 1989, 1990). The affinity between cystatin C and *S*-(methylthio)-papain was too tight to be determined by fluorescence titrations, as was also previously found for the corresponding interaction between chicken cystatin and this inactivated enzyme form (Björk & Ylinenjärvi, 1989). The dissociation equilibrium constant of 2.2×10^{-13} M calculated from the association and dissociation rate constants (Table 1) is somewhat lower than the value of 7.1×10^{-13} M obtained for the complex of chicken cystatin and *S*-(methylthio)-papain (Björk & Ylinenjärvi, 1989).

DISCUSSION

This work was done with recombinant human cystatin C rather than with the natural inhibitor. Previous studies have shown that the *E. coli*-derived cystatin C is identical with the inhibitor isolated from biological fluids in most respects (Abrahamson *et al.*, 1988). However, Pro-3 of the recombinant cystatin C is not hydroxylated (Abrahamson *et al.*, 1988), but is partially so in the natural inhibitor (Grubb & Löfberg, 1982). It is unlikely that this minor difference would cause any appreciable change in the mode of interaction of the inhibitor with its target enzymes, particularly as the natural and recombinant inhibitors have experimentally indistinguishable inhibitory activities (Abrahamson *et al.*, 1988). The results of this work therefore should be valid also for natural human cystatin C.

The c.d. spectra of cystatin C and chicken cystatin show that the two proteins have similar conformations, although cystatin C appears to have a somewhat higher content of ordered structure. Moreover, the overall similarity of the absorption, c.d. and fluorescence changes accompanying the binding of cystatin C and chicken cystatin to the two proteinases used in this work, papain and actinidin, indicates that the two inhibitors also interact with their target proteinases in a similar manner. However, some spectral differences were apparent, which presumably are related to the structure of cystatin C around Trp-106. The spectral changes accompanying the binding of proteinases to chicken cystatin thus have been shown to arise largely from interactions involving the analogous amino acid, Trp-104, in this inhibitor (Lindahl *et al.*, 1988). The sequences of the two inhibitors in this region differ primarily *C*-terminally to the tryptophan residue, cystatin C having the sequence Trp-Gln-Gly and chicken cystatin Trp-Leu-Asn, which may result in a somewhat altered orientation of the indole side chain. This possibility is supported by the difference around 290 nm in the near-u.v. c.d. spectra of the two inhibitors. However, minor differences in the mode of interaction with the proteinase in other parts of the proteinase-binding region may also influence the interactions involving the side chain of Trp-106.

Studies of the kinetics of binding of chicken cystatin to several cysteine proteinases have shown that these reactions are best described by the simple reversible bimolecular mechanism:

$$P + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} PI$$

where P is proteinase, I inhibitor and PI their complex (Björk *et al.*, 1989; Björk & Ylinenjärvi, 1990). The same conclusion is strongly indicated by the studies with cystatin C in this work. The pseudo-first-order rate constant for complex-formation, measured with excess inhibitor, thus was shown to increase linearly with inhibitor concentration up to the highest concentration that could be studied for reactions with both active papain and an inactivated form of the enzyme, *S*-(methylthio)-papain. Moreover, the rate constants measured for the association of these forms, and of the corresponding forms of actinidin, with cystatin C are of a magnitude approaching the value expected for a rate controlled by macromolecular diffusion, similarly to the values obtained for chicken cystatin. Reversibility of complex-formation was also demonstrated, the dissociation producing intact inhibitor, as shown for the actinidin complex, and occurring with rate constants of the same order of magnitude as those measured for the complexes with chicken cystatin. In the case of the latter inhibitor, the proposed simple one-step reaction model is supported by computer docking experiments based on the three-dimensional structures of the inhibitor and papain, which indicate that the two proteins can interact with negligible conformational adaptations (Bode *et al.*, 1988). However, the putative reaction mechanism differs from that demonstrated for reactions of serine-proteinase inhibitors with their target enzymes (Laskowski & Kato, 1980). The rates of these reactions approach a limiting value at high inhibitor concentrations under pseudo-first-order conditions, indicative of the reactions occurring in at least two steps and involving a conformational change (Luthy *et al.*, 1973; Quast *et al.*, 1974; Olson & Shore, 1982).

The affinities measured for the interactions of cystatin C with active and inactivated forms of papain and actinidin are comparable with the corresponding affinities of chicken cystatin for these enzyme forms (Björk *et al.*, 1989; Björk & Ylinenjärvi, 1990), in further support of a similar mode of interaction of the two inhibitors with the enzymes. However, minor differences are apparent. Cystatin C thus binds somewhat more strongly to active papain than the chicken inhibitor (K_d 1.1×10^{-14} M compared with 6×10^{-14} M), owing to a lower dissociation rate constant. This affinity corresponds to a unitary free-energy change (Gurney, 1953; Karush, 1962) for the binding reaction of approx. -89 kJ/mol, reflecting a very tight interaction. Although the dissociation of the cystatin C–papain complex is slow, it is nevertheless faster than that reported for the tightest complex between a small protein inhibitor of serine proteinases and its target enzyme studied so far, that between the basic pancreatic trypsin inhibitor and trypsin (Vincent & Lazdunski, 1972). Cystatin C also binds to the inactivated papain form, *S*-(methylthio)-papain, with a slightly higher affinity than chicken cystatin, whereas the affinities of the two inhibitors for *S*-(*N*-ethylsuccinimidyl)- and *S*-(*N*-n-butylsuccinimidyl)-papain are similar. In contrast, cystatin C binds somewhat more weakly than the chicken inhibitor to both active and inactivated forms of actinidin. The affinities of these interactions are approx. 10^3–10^6-fold lower than those of the binding of the two inhibitors to the corresponding forms of papain. In general, the affinity of cystatin C for inactivated proteinases was found to decrease with increasing size of the inactivating group in an analogous manner to that for chicken cystatin (Björk & Ylinenjärvi, 1989), in

further agreement with similar proteinase-binding mechanisms for the two inhibitors. The decreased affinity presumably is due primarily to an increased dissociation rate constant, as shown for chicken cystatin (Björk & Ylinenjärvi, 1989) and for the interaction of cystatin C with the *S*-(methylthio) derivatives of papain and actinidin in this work.

Together, the spectroscopic, kinetic and equilibrium evidence obtained in this work indicate that chicken cystatin and human cystatin C react with cysteine proteinases with a highly similar mechanism, differing from that of serine-proteinase inhibitors. Possibly, all cystatins of family II, and perhaps also those of the other families, interact with their target enzymes in the same general manner. Because of the similarity between the inhibition mechanisms for chicken cystatin and cystatin C, most results obtained so far with chicken cystatin, in particular the X-ray structure and the model for the proteinase–inhibitor interaction based on this structure, should be largely applicable also to human cystatin C.

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