Probing the functional role of the N-terminal region of cystatins by equilibrium and kinetic studies of the binding of Gly-11 variants of recombinant human cystatin C to target proteinases

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The interaction between cystatin C variants, in which the evolutionarily conserved Gly-11 residue was substituted by Ala, Glu or Trp, and the cysteine proteinases, papain, ficin, actinidin and cathepsin B, was characterized. The substitutions reduced the affinity of binding in a manner consistent with the Gly residue of the wild-type inhibitor, allowing the N-terminal region to adopt a conformation that was optimal for interaction with target proteinases. Replacement of Gly-11 by Ala resulted in only a 5- to 100-fold reduction in binding affinity. Comparison with the affinities of wild-type cystatin C lacking the N-terminal region indicated that even this small structural change affects the conformation of this region sufficiently to largely abolish its interaction with the weakly binding proteinases, actinidin and cathepsin B. However, the substitution allows interactions of appreciable strength between the N-terminal region and the tightly binding enzymes, papain or ficin. Replacement of Gly-11

with the larger Glu and Trp residues substantially decreased the affinity of binding to all enzymes, from 10^3 - to 10^5 -fold. These substitutions further affect the conformation of the N-terminal region, so that interactions of this region with papain and ficin are also essentially eliminated. The decreased affinities of the three cystatin C variants for papain, ficin and actinidin were due exclusively to increased dissociation rate constants. In contrast, the decreased affinity between cathepsin B and the Ala-11 variant, the only one for which rate constants could be determined with this enzyme, was due almost entirely to a decreased association rate constant. This behaviour is analogous to that observed for forms of cystatin C lacking the N-terminal region and supports the conclusion that the mode of interaction of this region with target proteinases varies with the enzyme as a result of structural differences in the active-site region of the latter.

INTRODUCTION

Cystatin C is the predominant low-molecular-mass cysteine proteinase inhibitor of the cystatin superfamily in mammalian organisms [1]. It inhibits endogenous lysosomal cysteine proteinases (e.g. cathepsins B, H and L) and also several exogenous enzymes, such as papain and similar plant proteinases [2]. The inhibition occurs by the inhibitor forming a tight (20 nM–10 fM), equimolar complex with the proteinase, blocking the active site of the latter [2–4]. The crystal structures of the homologous inhibitor, chicken cystatin [5], and of a complex between another mammalian inhibitor, cystatin B (also called stefin B) and papain [6], have led to the conclusion that three regions of the amino acid sequence of cystatins comprise the proteinase binding site of the inhibitors. In cystatin C, these regions are the N-terminal region around Gly-11, which is an evolutionarily conserved residue in cystatins, a Gln-Ile-Val-Ala-Gly sequence at residues 55–59 and the region around Trp-106. In chicken cystatin, the corresponding residues form a wedge-shaped edge of the molecule that is complementary to the active-site cleft of papain [5], and presumably also to that of other target proteinases, consistent with the tight binding.

The importance of the N-terminal region of cystatin C and chicken cystatin for the binding of cysteine proteinases has been demonstrated by removal of this region by proteolytic cleavage before or after the conserved glycine residue. Such N-terminal truncation, which can be done without affecting the structure of the remainder of the molecule, results in a greatly decreased

affinity of the inhibitors for target enzymes [3,7–11], consistent with the N-terminal region contributing about 40% of the free energy of binding. Moreover, studies by site-directed mutagenesis have demonstrated the importance of the conserved Gly-11 residue of cystatin C for inhibition of endopeptidases [12]. In this work, we have further investigated the role of the N-terminal region of cystatin C in binding of target proteinases by equilibrium and kinetic studies of the interaction of such Gly-11 variants with several cysteine proteinases.

MATERIALS AND METHODS

Recombinant human cystatin C and cystatin C variants in which Gly-11 was replaced with Trp, Ala or Glu (G11W-, G11A- and G11E-cystatin C respectively) were expressed in *Escherichia coli* and purified by ion-exchange chromatography on Q-Sepharose (Pharmacia LKB Biotechnology, Uppsala, Sweden), followed by gel chromatography on Sephadex G 75 (Pharmacia LKB), as described earlier [4,12,13]. The purified proteins showed only one band in SDS/PAGE under reducing conditions. The phosphorylated form of chicken cystatin (form 2) was obtained from egg white [14,15].

The purification, properties, storage and activation of papain (EC 3.4.22.2), ficin (EC 3.4.22.3), actinidin (EC 3.4.22.14) and recombinant rat cathepsin B (EC 3.4.22.1) have been reported elsewhere [11,15]. The cathepsin B preparation was a gift from

Abbreviations used: E-64, [N-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]-amido-4-guanidobutane; G11W-, G11A- and G11E-cystatin C, cystatin C variants in which Gly-11 is replaced with Trp, Ala and Glu respectively.

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Dr. John S. Mort, Shriners Hospital, Montreal, Canada. The inactivated papain forms, *S*-(methylthio)-papain and *S*-(carbamoylmethyl)-papain, were obtained as in previous work [4,16].

Near-u.v. absorption difference spectra, near-u.v. and far-u.v. c.d. spectra and fluorescence emission spectra of G11W-cystatin C, papain and the complex between the inhibitor and the proteinase were measured as reported previously [4,15,17]. Difference spectra were calculated from measured c.d. and fluorescence emission spectra as described by Lindahl et al. [15].

Quenching of the tryptophan fluorescence of wild-type cystatin C or G11W-cystatin C (2 μ M) by increasing concentrations of iodide (0–0.4 M) was measured essentially as in earlier work [18]. The samples were excited at 280 nm and the emission was measured at wavelengths of 345 nm for wild-type cystatin C and 350 nm for G11W-cystatin C. The data were corrected for inner-filter effects and were plotted according to a modified Stern–Volmer equation [19].

Titration of active or inactivated cysteine proteinases with the cystatin C variants for the determination of binding stoichiometries or affinities were monitored by the changes of fluorescence emission intensity accompanying the interactions [4,11]. The stoichiometry titrations were done at enzyme concentrations of 1–2 μ M, whereas concentrations comparable to the dissociation constants (25–250 nM) were used in the affinity titrations. The fluorescence was measured at the wavelength of the maximum change (335–355 nm). Stoichiometries and dissociation constants were obtained by non-linear least-squares regression of the titration curves [15,20] with the KaleidaGraph program (Synergy Software, Reading, PA, U.S.A.).

Inhibition constants for the interaction of the cystatin C variants with cysteine proteinases were obtained from the equilibrium rates of cleavage of a fluorogenic substrate by the enzyme in the presence of increasing concentrations of inhibitor in a large excess over the enzyme [10]. The substrate was carbobenzoxy-L-phenylalanyl-L-arginine 4-methylcoumaryl-7-amide (Peptide Institute, Osaka, Japan) in experiments with papain, ficin and actinidin and carbobenzoxy-L-arginyl-L-arginine 4-methylcoumaryl-7-amide (Peptide Institute) in experiments with cathepsin B. Both substrates were used at concentrations of 10–20 μ M. The inhibitor concentration was usually varied from $\leq 0.5 \times K_{i,app.}$ (the apparent inhibition constant) to $\geq 3 \times K_{i,app.}$, except in measurements of $K_{i,app.}$ values $> 10 \mu$ M, in which case the range was from $\sim 0.2 \times K_{i,app.}$ to $\sim K_{i,app.}$. The enzyme concentration was at least ten-fold lower than that of the inhibitor. Substrate hydrolysis was $< 5\%$ in all analyses. The apparent inhibition constant was evaluated from equilibrium rates of substrate cleavage measured at different inhibitor concentrations and was corrected for substrate competition to give the true inhibition constant, as described previously [10,11]. Published K_m values of the substrate for papain and actinidin of 60 and 160 μ M respectively were used in these corrections [10,21]. For ficin, a K_m of $300 \pm 70 \mu$ M was estimated from measurements up to a substrate concentration of 200 μ M. No correction was made for cathepsin B, as the K_m of the substrate for cathepsin B is $\geq 500 \mu$ M [11].

The kinetics of binding of cystatin C variants to papain, ficin and actinidin and of wild-type cystatin C to the inactivated papain derivative, *S*-(carbamoylmethyl)-papain, were studied by stopped-flow fluorescence measurements under pseudo-first-order conditions (i.e. with an excess of inhibitor), as detailed previously [4,11,17,22]. The inhibitor concentration range was 0.5–5 μ M for reactions with papain and *S*-(carbamoylmethyl)-papain, 0.3–7 μ M for reactions with ficin and 1–15 μ M for reactions with actinidin.

The kinetics of association of G11A-cystatin C with cathepsin B were analysed by continuous measurements of the loss of enzyme activity in the presence of the fluorogenic substrate, carbobenzoxy-L-arginyl-L-arginine 4-methylcoumaryl-7-amide (final concentration 20 μ M), essentially as in previous work [11]. The cathepsin B concentration was 1–10 nM and that of the inhibitor was varied from 0.1 to 1 μ M. The observed pseudo-first-order association rate constant at each inhibitor concentration was obtained by non-linear least-squares regression of the data [11], and the second-order association rate constant was obtained from the slope of a linear plot of these pseudo-first-order rate constants versus inhibitor concentration. No correction for substrate competition was made, as in the measurements of inhibition constants.

The rate of dissociation of complexes between G11W- or G11E-cystatin C and papain was measured by a method in which the inhibitor dissociated from the complex was trapped by a large excess of inactive *S*-(methylthio)-papain and the concurrent appearance of active enzyme was monitored [11,17]. The complex was formed by incubating papain (final concentration 0.5–2 μ M) for 2 min with the cystatin C variant in a 1.5:1 molar ratio to the enzyme. The papain substrate *N*- α -benzoyl-L-arginine *p*-nitroanilide was then added to a concentration of 500 μ M together with *S*-(methylthio)-papain at a molar ratio to the complex of 10–100:1, and the increase in absorbance at 410 nm with time due to the liberated enzyme cleaving the substrate was recorded. The substrate consumption at the end of the experiment was $< 8\%$. The first-order dissociation rate constant for the cystatin–proteinase complex was obtained by non-linear least-squares regression of the absorbance increase as a function of time, as described previously [17].

The kinetics of dissociation of complexes between G11A-cystatin C and papain or ficin and between wild-type cystatin C and *S*-(carbamoylmethyl)-papain were evaluated by trapping the enzyme dissociated from the complex (10 μ M) by an excess (100–200 μ M) of chicken cystatin (form 2), essentially in the same manner as in previous studies of the wild-type cystatin C–papain complex [4]. The dissociation rate constant was obtained from the rate of appearance of the complex between the displacing cystatin 2 and the liberated enzyme, monitored by chromatography on a Mono-Q (Pharmacia LKB) ion-exchange column.

All analyses were done at $25.0 \pm 0.2^\circ\text{C}$. The buffer in studies of interactions of the cystatin C variants with papain, ficin and actinidin was 0.05 M Tris/HCl/0.1 M NaCl/100 μ M EDTA, pH 7.4, whereas interactions with cathepsin B were analysed in 0.05 M Mes/0.1 M NaCl/100 μ M EDTA/0.5 mM dithiothreitol/0.1 % (w/v) poly(ethylene glycol), pH 6.0.

Protein concentrations were obtained by absorption measurements at 280 nm. A molar absorption coefficient of $1.81 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ was obtained for G11W-cystatin C from a specific absorption coefficient of 1.34 ± 0.02 (range, $n = 2$) litre $\text{g}^{-1} \cdot \text{cm}^{-1}$, determined by quantitative amino acid analyses, and a relative molecular mass of 13 500. The molar absorption coefficient for wild-type cystatin C, $1.11 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (4), was used also for the G11A and G11E variants. Absorption coefficients and relative molecular masses used for papain, ficin, actinidin and cathepsin B have been reported previously [11,17].

RESULTS

C.d. spectra of cystatin C variants and stoichiometry of binding to papain

The far-u.v. c.d. spectra of G11A- and G11E-cystatin C were identical within experimental error ($\pm 5\%$) with the spectrum of

Figure 1 Near-u.v. absorption difference spectra measured between complexes of wild-type or G11W-cystatin C with papain and the free proteins

Wild-type cystatin C, —; G11W-cystatin C, ----. The papain concentration was 15 μ M and the molar ratio of cystatin to papain was 1.4:1.

Figure 2 Near-u.v. c.d. difference spectra between complexes of wild-type or G11W-cystatin C with papain and the free proteins

Wild-type cystatin C, —; G11W-cystatin C, ----. The difference spectra were calculated from separately measured c.d. spectra of the complexes and the free proteins [15]. The papain concentration was 15 μ M, and the molar ratio of cystatin to papain was 1.4:1. The unit on the ordinate is the difference in molar ellipticity.

wild-type cystatin C [4], indicating that substitution of Gly-11 with the larger Ala and Glu residues did not detectably affect the conformation of the protein. The spectrum of G11W-cystatin C had a similar shape and wavelength minimum to that of the wild-type inhibitor, but about a 40% lower magnitude. However, no conclusion regarding the conformation of G11W-cystatin C can be drawn from this finding, as local interactions between the extra tryptophan side chain and adjacent residues may have substantially influenced the far-u.v. c.d. spectrum of the protein [23,24].

Active papain and its inactive derivative, *S*-(methylthio)-papain, were titrated with G11A-, G11E- and G11W-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 and [4]). The three variants bound to both active and inactive papain with stoichiometries of 0.95–1.1, which are experimentally indistinguishable from 1.0. The recombinant cystatin C variants thus were all fully active in binding the proteinase.

Spectroscopic characterization of G11W-cystatin C and its interaction with papain

The G11W variant of cystatin C and its complex with papain were characterized by spectroscopic methods, as the introduction of a tryptophan residue in the N-terminal region might allow such studies to give pertinent information on the structure of the inhibitor and its binding to proteinases. The maximum of the fluorescence emission spectrum of G11W-cystatin C was at 349 nm, compared with 342 nm for the wild-type inhibitor. Moreover, iodide quenching of tryptophan fluorescence showed that $84 \pm 1\%$ (S.E., $n = 16$) of this fluorescence was accessible to the quencher in G11W-cystatin C, whereas only $63 \pm 4\%$ (S.E., $n = 16$) was accessible in the wild-type inhibitor.

The changes of near-u.v. absorption, c.d. and fluorescence induced by the binding of wild-type and the G11W variant of cystatin C to papain were compared under conditions giving > 99% saturation of enzyme with inhibitor (Figures 1–3). The u.v. absorption difference spectrum (Figure 1), and in particular the near-u.v. c.d. difference spectrum (Figure 2), for the G11W-cystatin C–papain interaction were similar to the corresponding spectra for wild-type cystatin C. In contrast, the fluorescence

Figure 3 Fluorescence emission difference spectra between complexes of wild-type or G11W-cystatin C with papain and the free proteins

Wild-type cystatin C, —; G11W-cystatin C, ----. The difference spectra were calculated from separately measured corrected emission spectra of the complexes and the free proteins [15]. Before these calculations, all spectra were normalized to a fluorescence intensity of 1.0 for free papain at the wavelength of the emission maximum. The papain concentration was 1 μ M and the molar ratio of cystatin to papain was 1.4:1. Excitation was at 280 nm.

emission difference spectra (Figure 3) were substantially different for the variant and wild-type inhibitors.

Equilibrium and kinetic characterization of interactions of cystatin C variants with papain, ficin, actinidin and cathepsin B

Dissociation equilibrium constants, as well as association and dissociation rate constants, were measured for the binding of the three cystatin C variants to papain, ficin, actinidin and cathepsin B (Table 1). The choice of method for these analyses was dictated by the characteristics of the particular interaction studied and the amounts of enzyme available. Most dissociation equilibrium constants $\geq \sim 10^{-9}$ M were determined as inhibition constants and, if possible, were corroborated by fluorescence titrations, with good agreement between the two methods. Lower dis-

Table 1 Dissociation equilibrium constants (K_d), inhibition constants (K_i), association rate constants (k_{ass}) and dissociation rate constants (k_{diss}) for the interaction between wild-type, N-terminally truncated or Gly-11 variants of cystatin C and papain, ficin, actinidin or cathepsin B

The values for wild-type cystatin C and N-terminally truncated cystatin C (lacking the first ten residues and thus having Gly-11 as N-terminal) are from previous work [4,10,11] and are shown for comparison. Values measured in this work are given with their standard errors and the number of measurements in parentheses, or with the range if the value is an average of two measurements. N.D., not determined.

Proteinase	Cystatin C form	K_d (M)	K_i (M)	k_{ass} ($\text{M}^{-1} \cdot \text{s}^{-1}$)	k_{diss} (s^{-1})
Papain	Wild-type	1.1×10^{-14}	N.D.	1.1×10^7	1.3×10^{-7}
	Truncated	2.9×10^{-8}	N.D.	1.2×10^7	0.3
	G11A	$1.0 \times 10^{-12*}$	N.D.	$(9.3 \pm 0.2) \times 10^6 \dagger$ ($n = 8$)	$(9.5 \pm 1.0) \times 10^{-6} \ddagger$ ($n = 3$)
	G11E	$2.5 \times 10^{-9*}$	$(1.7 \pm 0.2) \times 10^{-9}$ ($n = 6$)	$(9.1 \pm 0.3) \times 10^6 \dagger$ ($n = 8$)	$(2.3 \pm 0.2) \times 10^{-2} \S$ ($n = 7$)
	G11W	$8.5 \times 10^{-10*}$	$(8.0 \pm 2.0) \times 10^{-10}$ ($n = 19$)	$(1.3 \pm 0.1) \times 10^7 \dagger$ ($n = 10$)	$(1.1 \pm 0.1) \times 10^{-2} \S$ ($n = 6$)
Ficin	Wild-type	1.3×10^{-12}	N.D.	7.0×10^6	9.2×10^{-6}
	Truncated	3.0×10^{-8}	N.D.	9.6×10^6	0.3
	G11A	$6.0 \times 10^{-12*}$	N.D.	$(6.3 \pm 0.1) \times 10^6 \dagger$ ($n = 10$)	$(3.8 \pm 0.5) \times 10^{-5} \ddagger$ ($n = 2$)
	G11E	$(2.7 \pm 0.4) \times 10^{-8}$ ($n = 4$)	$(2.3 \pm 0.1) \times 10^{-8}$ ($n = 9$)	$(6.8 \pm 1.3) \times 10^6 \dagger$ ($n = 17$)	0.18*
	G11W	N.D.	$(2.9 \pm 0.2) \times 10^{-8}$ ($n = 9$)	$(9.5 \pm 0.4) \times 10^6 \dagger$ ($n = 10$)	0.28*
Actinidin	Wild-type	1.9×10^{-8}	N.D.	2.4×10^6	4.6×10^{-2}
	Truncated	5×10^{-6}	N.D.	1.5×10^5	0.8
	G11A	$(8.0 \pm 1.0) \times 10^{-7}$ ($n = 3$)	$(1.1 \pm 0.1) \times 10^{-6}$ ($n = 8$)	$(2.4 \pm 0.1) \times 10^6 \dagger$ ($n = 8$)	1.9*
	G11E	N.D.	$\geq 4 \times 10^{-5}$	N.D.	N.D.
	G11W	$1.6 \times 10^{-5*}$	N.D.	$(1.9 \pm 0.3) \times 10^6 \dagger$ ($n = 8$)	$30 \pm 3 \dagger$ ($n = 8$)
Cathepsin B	Wild-type	3.2×10^{-10}	N.D.	1.1×10^6	3.5×10^{-4}
	Truncated	N.D.	1.3×10^{-8}	2.1×10^4	3×10^{-4}
	G11A	N.D.	$(8.1 \pm 0.7) \times 10^{-9}$ ($n = 10$)	$(6.9 \pm 0.7) \times 10^4 \parallel$ ($n = 7$)	$5.6 \times 10^{-4*}$
	G11E	N.D.	$(1.8 \pm 0.1) \times 10^{-5}$ ($n = 5$)	N.D.	N.D.
	G11W	N.D.	$(9.6 \pm 0.6) \times 10^{-6}$ ($n = 4$)	N.D.	N.D.

* Calculated value.

† Measured by stopped-flow fluorescence.

‡ Measured by trapping dissociated enzyme with chicken cystatin 2 and analysing the appearance of the cystatin 2–enzyme complex by ion-exchange h.p.l.c.

§ Measured by trapping dissociated inhibitor with *S*-(methylthio)-papain and analysing the appearance of enzyme activity.

|| Measured by monitoring loss of enzyme activity in the presence of substrate.

sociation constants were not measured directly but were calculated from separately determined association and dissociation rate constants. Similarly calculated dissociation constants for weaker interactions agreed well with the directly measured values. Most association rate constants were measured by stopped-flow fluorescence, although reactions with cathepsin B were monitored by the loss of enzyme activity. Dissociation rate constants were measured by trapping the dissociated enzyme (for rate constants $\leq \sim 10^{-4} \text{ s}^{-1}$) or inhibitor (for rate constants higher than this value, but below $\sim 0.02 \text{ s}^{-1}$) and monitoring the appearance of trapped enzyme and liberated enzyme activity respectively. Still higher dissociation rate constants had to be calculated from measured dissociation equilibrium and association rate constants in most cases. However, the rate constant for the dissociation of G11W-cystatin C from its complex with actinidin could be determined by stopped-flow fluorimetry from a finite intercept on the ordinate of the plot of the observed pseudo-first-order rate constant versus inhibitor concentration. For some weak interactions, neither association nor dissociation rate constants could be obtained by the methods available.

A different kinetic behaviour than for all other interactions

between wild-type or mutant cystatin C and cysteine proteinases studied in this and earlier work [4,11,17,22] was observed for the binding of G11E-cystatin C to ficin. The plot of the observed pseudo-first-order rate constant, measured by stopped-flow fluorimetry, versus cystatin C concentration was not linear, as found for the other interactions, but reached a plateau at inhibitor concentrations $\geq \sim 2 \mu\text{M}$ (Figure 4). The binding of G11E-cystatin C to ficin is thus more complicated than the one-step, reversible, second-order reaction that is compatible with a linear plot and must occur in at least two steps. The data were fitted to the simplest mechanism for such a two-step reaction in which the reactants initially form a weak, Michaelis-type complex in rapid equilibrium, which is converted into a stable complex by an essentially irreversible conformational change (Figure 4; [25]). The equilibrium constant for the first step and the rate constant for the second step were obtained from this fit (see the legend to Figure 4), and the bimolecular association rate constant (Table 1) was calculated from the initial slope of the fitted curve. More complicated mechanisms, e.g. involving a reversible second step, are possible, but the data do not allow discrimination between these mechanisms and the simplest model used.

Figure 4 Observed pseudo-first-order rate constants (k_{obs}) for the binding of wild-type or G11E-cystatin C to ficin as a function of cystatin concentration

Wild-type cystatin C (○); G11E-cystatin C (●). The molar ratio of inhibitor to proteinase was maintained at 10:1 in all reactions. The vertical bars represent $\pm$ two standard errors computed from four to eight individual experiments. Note the different scales of the ordinates for the wild-type and variant inhibitors. The data for G11E-cystatin C were fitted by non-linear least-squares regression to the hyperbolic equation $k_{\text{obs}} = k_{+2}[I]/(K_1 + [I])$, derived for a two-step mechanism in which the enzyme and inhibitor initially form a weak complex in rapid equilibrium with a dissociation constant K_1 , followed by an irreversible conformational change with a rate constant k_{+2} [25]. $[I]$ is the concentration of inhibitor. This fit gave $K_1 = (0.40 \pm 0.06) \times 10^{-6}$ M and $k_{+2} = 2.7 \pm 0.09$ s $^{-1}$. The initial slope of this fit, i.e. k_{+2}/K_1 , defines the bimolecular association rate constant given in Table 1.

Table 2 Dissociation equilibrium constants (M) for the interaction between wild-type or Gly-11 variants of cystatin C and inactivated forms of papain

The values for wild-type cystatin C, two of which are from previous work [4], and the values for active papain (from Table 1) are shown for comparison. Other values measured in this work are given with their standard errors and the number of measurements in parentheses, or with the range if the value is an average of two measurements.

Cystatin form	Inactivating group		
	None	S-(methylthio)	S-(carbamoylmethyl)
Wild-type	1.1×10^{-14} *	2.2×10^{-13} *	8.3×10^{-11} †
G11A	1.0×10^{-12} ‡	$(3.6 \pm 0.9) \times 10^{-9}$ ($n = 3$)	$(4.6 \pm 0.3) \times 10^{-8}$ ($n = 4$)
G11E	2.5×10^{-9} ‡	$(2.2 \pm 0.2) \times 10^{-8}$ ($n = 4$)	$(2.0 \pm 0.3) \times 10^{-7}$ ($n = 2$)
G11W	0.8×10^{-9} ‡	$(4.4 \pm 0.8) \times 10^{-9}$ ($n = 5$)	$(1.1 \pm 0.1) \times 10^{-7}$ ($n = 2$)

* From previous work.

† Calculated from an association rate constant of $(8.4 \pm 0.2) \times 10^6$ M $^{-1}$ s $^{-1}$ ($n = 9$), measured by stopped-flow fluorescence, and a dissociation rate constant of $(7.0 \pm 0.4) \times 10^{-4}$ s $^{-1}$ ($n = 2$), measured by trapping dissociated inactivated enzyme with chicken cystatin 2 and analysing the rate of appearance of the cystatin 2–enzyme complex by ion-exchange h.p.l.c.

‡ From Table 1.

wild-type cystatin C to S-(methylthio)-papain, necessary for comparison, has been measured previously [4], but the corresponding value for the binding to S-(carbamoylmethyl)-papain was determined in the present work from separately measured association and dissociation rate constants (Table 2).

DISCUSSION

The fluorescence emission spectrum and the iodide quenching of tryptophan fluorescence of G11W-cystatin C both suggest that the tryptophan replacing Gly-11 is in a highly exposed environment, even more exposed than the normally occurring Trp-106. The latter residue presumably is located in a similar position to the corresponding residue in the homologous inhibitor, chicken cystatin, i.e. well accessible to solvent on the surface of the protein [5]. This highly exposed location of Trp-11 in the mutant cystatin C is in keeping with the putative flexibility of the N-terminal region of the inhibitor [26,27].

The different fluorescence changes induced by the binding of wild-type and G11W-cystatin C to papain show that the tryptophan at position 11 of the variant inhibitor interacts with the proteinase. The N-terminal region thus participates to some extent in the binding to the enzyme [3,8,10,11] even after substitution of the conserved glycine residue by an amino acid with a bulky side chain. However, the binding occurs in such a manner that the interactions involving the tryptophan residue do not contribute appreciably to the observed changes of near-u.v. absorption and c.d. accompanying the binding. Instead, these latter changes arise mainly from interactions involving Trp-106, as in the wild-type inhibitor [4,15].

Dissociation equilibrium constants, but no rate constants, have been reported previously for the binding of the three cystatin variants to two of the proteinases used in this work, papain and cathepsin B [12]. In general, the values obtained in the two studies agree reasonably well. However, the dissociation equilibrium constant for the binding of G11A-cystatin C to papain was found to be about 20-fold lower in this work by calculation from separately determined rate constants. This difference presumably reflects the difficulties inherent in directly measuring equilibrium constants for tight interactions [12]. In addition, the equilibrium constants for the weak interactions between G11E- and G11W-cystatin C and cathepsin B, for which only upper limits were reported previously, were established in this work.

Replacement of Gly-11, the evolutionarily conserved glycine residue in cystatin C, by alanine resulted in only a small reduction in the affinity of the inhibitor for active proteinases, from only ~ 5 -fold for ficin to ~ 100 -fold for papain. In contrast, substitution with the larger glutamic acid and tryptophan residues substantially decreased the affinity, from $\sim 10^3$ -fold for actinidin to $\sim 10^5$ -fold for papain. The effect of these substitutions on the affinity of the inhibitor for inactivated papains was appreciably different. Like wild-type cystatin C, all three variants bound with lower affinity to the inactivated papains than to the active enzyme, the affinity decreasing with increasing size of the inactivating group [4,16]. However, the largest part of the decrease in affinity for the inactivated enzymes, $\sim 10^4$ -fold for S-(methylthio)-papain and ~ 500 -fold for S-(carbamoylmethyl)-papain, was observed on replacement of the glycine residue with alanine, whereas substitution with glutamic acid or tryptophan only resulted in a further 2–5-fold reduction in affinity for both inactivated papains. These observations are consistent with the proposal that the conserved glycine residue, by conferring maximal flexibility on the N-terminal region of the inhibitor, is important for allowing this region to adopt a conformation

Interaction of cystatin C variants with inactivated papains

The dissociation equilibrium constants for the binding of the three cystatin C variants to two inactivated forms of papain, S-(methylthio)- and S-(carbamoylmethyl)-papain, were measured by fluorescence titrations (Table 2). The value for the binding of

appropriate for interaction with the target proteinase [12]. This flexibility presumably is reduced by substitution with larger residues, thus leading to lower affinity. However, steric interference between these bulkier side chains in the binding region of the inhibitor and the active-site region of the enzyme may also contribute to the decreased binding affinity, although apparently only to a moderate extent in the case of active enzymes [12]. In contrast, such steric interference presumably plays a prominent role in decreasing the affinity of the cystatin C variants for inactivated papains, consistent with the proximity of the conserved glycine residue to the reactive cysteine of the enzyme in the chicken cystatin–papain complex [5]. In this case, the presence of only an additional methyl group in G11A-cystatin C appears sufficient to appreciably interfere sterically with the binding of the proteinase because of the presence of the substituent in the active site of the latter.

Previous studies have shown that proteolytic cleavage of chicken cystatin or human cystatin C before or after the conserved glycine residue essentially abolishes the contribution made by the N-terminal region of the inhibitors to the energy of binding of target proteinases [10,11]. A comparison of the affinities of the three cystatin C variants for proteinases with the affinities of such an N-terminally truncated form of the inhibitor should therefore give a measure of the strength of the residual interactions in the N-terminal region after the substitution. Such a comparison (Table 1) indicates that replacement of Gly-11 with alanine allows interactions of substantial affinity between the N-terminal region and papain and ficin, both of which bind tightly to the wild-type inhibitor. In contrast, even this small structural change interferes sufficiently with the conformation of the N-terminal region to largely abolish the interaction of this region with the weakly binding proteinases, actinidin and cathepsin B. Substitution of Gly-11 with glutamic acid or tryptophan further affects the structure of the N-terminal region, so that interactions of this region with papain and ficin are also essentially eliminated. However, weak interactions presumably occur, as indicated by the fluorescence emission spectrum of the complex between G11W-cystatin C and papain discussed above. The effects caused by these latter substitutions are possibly large enough to interfere also with the interaction of adjacent regions of the binding surface of the inhibitor with actinidin and cathepsin B, as indicated by the lower affinities of the mutants than of truncated cystatin C for these proteinases.

The lower affinities of the three cystatin C variants than of the wild-type inhibitor for papain, ficin and actinidin were due exclusively to increased dissociation rate constants, the association rate constants being unaffected by the substitutions (Table 1). In contrast, the lower affinity between cathepsin B and G11A-cystatin C, the only variant for which rate constants could be determined with this enzyme, was due almost entirely to a decreased association rate constant. This behaviour is analogous to that observed for N-terminally truncated forms of cystatin C and chicken cystatin. The decreased affinities of these forms for target proteinases were thus due to increased dissociation rate constants for papain and ficin and to decreased association rate constants for cathepsin B, whereas both constants were affected

in the case of actinidin [11]. The effect on the rate constants for interaction with different proteinases thus appears to be similar regardless of whether the N-terminal region is removed by proteolysis or its structure is altered by mutagenesis of the conserved glycine residue. This observation supports the previous conclusion that the mode of interaction of the N-terminal region of cystatin C, and of chicken cystatin, with target proteinases varies with the enzyme as a result of structural differences in the active-site region of the latter [11].

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