Differential changes in the association and dissociation rate constants for binding of cystatins to target proteinases occurring on N-terminal truncation of the inhibitors indicate that the interaction mechanism varies with different enzymes

Ingemar BJÖRK,*§ Ewa POL,* Elke RAUB-SEGALL,* Magnus ABRAHAMSON,† Andrew D. ROWAN‡ and John S. MORT‡

*Department of Veterinary Medical Chemistry, Swedish University of Agricultural Sciences, Uppsala Biomedical Center, Box 575, S-751 23 Uppsala, Sweden, †Department of Clinical Chemistry, University of Lund, University Hospital, S-221 85 Lund, Sweden, and ‡Joint Diseases Laboratory, Shriners Hospital for Crippled Children and the Department of Surgery, McGill University, Montreal, Quebec, Canada H3G 1A6

The importance of the N-terminal region of human cystatin C or chicken cystatin for the kinetics of interactions of the inhibitors with four cysteine proteinases was characterized. The association rate constants for the binding of recombinant human cystatin C to papain, ficin, actinidin and recombinant rat cathepsin B were 1.1×10^7, 7.0×10^6, 2.4×10^6 and 1.4×10^6 $M^{-1} \cdot s^{-1}$, whereas the corresponding dissociation rate constants were 1.3×10^{-7}, 9.2×10^{-6}, 4.6×10^{-2} and 3.5×10^{-4} s^{-1}. N-Terminal truncation of the first ten residues of the inhibitor negligibly affected the association rate constant with papain or ficin, but increased the dissociation rate constant approx. 3×10^4- to 2×10^6-fold. In contrast, such truncation decreased the association rate constant with cathepsin B approx. 60-fold, while minimally affecting the dissociation rate constant. With actinidin, the truncated cystatin C had both an approx. 15-fold lower association rate constant and an approx. 15-fold higher dissociation rate constant than the intact inhibitor. Similar results were obtained for intact and N-terminally truncated chicken cystatin. The decreased affinity of human cystatin C or chicken cystatin for cysteine proteinases after removal of the N-terminal region is thus due to either a decreased association rate constant or an increased dissociation rate constant, or both, depending on the enzyme. This behaviour indicates that the contribution of the N-terminal segment of the two inhibitors to the interaction mechanism varies with the target proteinase as a result of structural differences in the active-site region of the enzyme.

INTRODUCTION

Human cystatin C and its avian analogue, chicken cystatin, are the best-characterized members of family II of the cystatin superfamily of cysteine proteinase inhibitors (Barrett et al., 1986). The two proteins have highly similar amino acid sequences of 120 and 116 residues respectively with two disulphide bonds. They inhibit mammalian cysteine proteinases, e.g. cathepsin B, H, L and S, and also some plant enzymes, such as papain and actinidin, by forming tight equimolar complexes with the enzymes, thereby blocking the active site of the latter. The X-ray structures of chicken cystatin and of a complex of a related human inhibitor, cystatin B (also called stefin B), with carboxymethylpapain have provided fundamental insights into the nature of this interaction (Bode et al., 1988; Stubbs et al., 1990). These structures show that in chicken cystatin the Gln-Leu-Val-Ser-Gly sequence at residues 53–57, the region around Trp-104 and probably also the N-terminal region are involved in binding to target proteinases. The homologous amino acid sequences of chicken cystatin and human cystatin C, as well as the similar kinetic and equilibrium characteristics of the reactions of the two inhibitors with proteinases (Lindahl et al., 1992a), indicate that the corresponding segments of cystatin C constitute an analogous binding region of this inhibitor.

The N-terminal regions of human cystatin C and chicken cystatin include an evolutionarily conserved glycine residue, Gly-11 in cystatin C and Gly-9 in the chicken inhibitor. Removal of the N-terminal region by proteolytic cleavage before or after this residue leads to a marked reduction in the affinity of both inhibitors for target proteinases, in agreement with the deductions from the X-ray crystallographic data (Abrahamson et al., 1987, 1991; Machleidt et al., 1989; Popovič et al., 1990; Lindahl et al., 1992b). Truncation of chicken cystatin after Gly-9 thus increases the dissociation equilibrium constant for binding to papain from approx. 60 fM to approx. 150 nM, indicating that residues in the N-terminal region contribute approx. 40% of the total free-energy change of the interaction (Lindahl et al., 1992b). In previous studies of the binding of an N-terminally truncated form of human cystatin C to cathepsin B, the decreased affinity was attributed primarily to a reduced association rate constant (Abrahamson et al., 1991). In contrast, characterization of reactions of analogous truncated forms of chicken cystatin with papain instead implicated an increased dissociation rate constant as the cause of the weaker binding (Lindahl et al., 1992b). In this work, we resolve this apparent discrepancy by showing that the reduced affinity after N-terminal truncation of both cystatin C and chicken cystatin is due to either a decreased association rate constant or an increased dissociation rate constant, or both, depending on the target enzyme. This behaviour indicates substantially different contributions of the N-terminal segment of the two inhibitors to the mechanism of interaction with different cysteine proteinases as a result of structural variations in the active-site region of the enzymes.

MATERIALS AND METHODS

Recombinant human cystatin C, expressed in *Escherichia coli*, and chicken cystatin were purified by published procedures

Abbreviation used: E64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane.

§ To whom correspondence should be addressed.

(Anastasi et al., 1983; Lindahl et al., 1988, 1992a; Abrahamson et al., 1988). Form 2 of chicken cystatin was used in all experiments in this study; the two forms of the inhibitor, 1 and 2, have been shown previously to interact with proteinases in an identical or highly similar manner (Nicklin and Barrett, 1984; Lindahl et al., 1988; Björk et al., 1989; Björk and Ylinenjärvi, 1989; Machleidt et al., 1989). An N-terminally truncated form of cystatin C with Gly-11 as the N-terminal residue was obtained by digestion with elastase (Abrahamson et al., 1991), whereas the corresponding form of chicken cystatin, having Gly-9 as the N-terminal residue, was produced by subtilisin cleavage (Lindahl et al., 1992b).

The purification, properties, storage and activation of papain (EC 3.4.22.2), ficin (EC 3.4.22.3) and actinidin (EC 3.4.22.14) have been reported elsewhere (Lindahl et al., 1988; Björk and Ylinenjärvi, 1990). Recombinant rat procathepsin B (EC 3.4.22.1) in which Ser-115 of the single glycosylation site of the mature enzyme had been mutated to Ala was expressed in yeast essentially as described previously (Rowan et al., 1992). The α-factor fusion construct used for the expression of procathepsin B in yeast produces the proenzyme as a secreted product. In the defined medium required for yeast growth with plasmid retention, active cathepsin B accumulates in the culture medium as a result of autoprocessing (Rowan et al., 1992); however, a substantial proportion of the enzyme is irreversibly inactivated because of oxidation of the active-site thiol group. Expression conditions were therefore modified to force accumulation of the recombinant protein as the proenzyme, and fully active cathepsin B was produced by subsequent processing of the proenzyme *in vitro*.

A culture of yeast strain BJ3501 (*MATαpep4::HIS3 prb1-Δ1.6R his3-Δ200 ura3-52 GAL can1*) containing the *URA3*-based shuttle vector for expression of rat procathepsin B construct was streaked on to fluorouracil-containing plates for selection of spontaneous fluorouracil-resistant colonies (Romanos et al., 1991). The resulting strains allowed growth on non-selective medium, with plasmid retention, as their uracil salvage pathway is no longer operative. When grown on the rich medium, YEPD (Sherman et al., 1986), autoprocessing of the secreted procathepsin B is prevented.

For purification of fully active cathepsin B, yeast culture medium was concentrated with an Amicon (Danvers, MA, U.S.A.) Spiral Concentrator, and protein was then precipitated by addition of 0.9 vol. of cold acetone. The pellet was dissolved in, and dialysed against, 1 mM disodium EDTA and was then dialysed extensively against water. Processing of procathepsin B to the mature enzyme was accomplished by adjusting the pH of the solution to 4.0 by addition of 0.5 M sodium formate, pH 3.0, adding pepsin to a concentration of 1 μg/ml and incubating the mixture at 40 °C for 30 min. The pH was then raised to 6.0 with 1.5 M Tris/HCl, pH 8.8, and cysteine was added to a concentration of 5 mM. The crude cathepsin B preparation was bound to DEAE-Sepharose (Pharmacia LKB Biotechnology, Uppsala, Sweden), equilibrated in 10 mM sodium phosphate/1 mM EDTA, pH 6.0, and the resin was collected by filtration. After washing with equilibration buffer, cathepsin B was eluted with the same buffer containing 0.25 M NaCl and loaded directly on to a thiopropyl-Sepharose (Pharmacia LKB) column previously equilibrated with 2,2′-dipyridyl disulphide. Unbound material was removed by washing with the equilibration buffer containing 0.25 M NaCl, and active cathepsin B was then eluted with the same buffer containing 20 mM cysteine and 5 mM dithiothreitol. Active fractions were concentrated by ultrafiltration and desalted on a Sephadex G-25 (Pharmacia LKB) column equilibrated in 1 mM EDTA and 1 mM methylmethanethiol sulphonate. The latter reagent reversibly inactivated the enzyme by converting the active-site thiol group into an *S*-methylthio derivative (Smith et al., 1975). Cathepsin B-containing fractions were pooled, dialysed against water and freeze-dried. With this procedure, 11 mg of fully active cathepsin B could be prepared from 5 litres of culture medium.

Although processing of the proenzyme was initiated by addition of pepsin, sequencing of the pure cathepsin B preparation showed the presence of a six-residue N-terminal extension, indicating that most of the conversion was due to autoprocessing (Rowan et al., 1992). As shown previously (Rowan et al., 1993), autoprocessing is also responsible for removal of the six-residue C-terminal extension present in the proenzyme but absent from the mature protein. Before use, cathepsin B was reactivated at concentrations of 1–10 μM with 1 mM dithiothreitol at room temperature for 10 min in buffer (0.05 M Mes, 0.1 M NaCl, 100 μM EDTA, pH 6.0). The activated protein was shown, by active-site titration with the low-molecular-mass cysteine proteinase inhibitor, E64 (Barrett and Kirschke, 1981), to be more than 90% active.

Fluorescence emission spectra of intact or truncated cystatins, cysteine proteinases and complexes of cystatins with proteinases were measured essentially as reported previously (Lindahl et al., 1988, 1992a; Björk and Ylinenjärvi, 1990). In the analyses of the complexes, E64 was added at a molar ratio to the enzyme of 1:5 immediately after the two proteins had been mixed to prevent proteolysis by possible contaminating cysteine proteinases in the enzyme preparation that were not inactivated by the cystatins (Lindahl et al., 1992a). Difference spectra were calculated from fluorescence emission spectra as described by Lindahl et al. (1988).

The stoichiometry of binding of chicken cystatin to cathepsin B was determined by a competitive titration of the enzyme (3 μM) with the inhibitor in the presence of papain labelled with 2-(4′-acetamidoanilino)naphthalene-6-sulphonic acid (1.5 μM), the fluorescence increase due to complex-formation of cystatin with the labelled papain being monitored (Lindahl et al., 1991). Titrations of cysteine proteinases (100 nM) with truncated cystatins for the determination of binding affinities were monitored by the changes in tryptophan fluorescence emission intensity accompanying the interactions (Lindahl et al., 1988, 1992a,b; Björk and Ylinenjärvi, 1990).

The kinetics of binding of intact or truncated cystatins to cysteine proteinases were studied by stopped-flow fluorescence measurements under pseudo-first-order conditions (i.e. with an excess of inhibitor), as detailed previously (Björk et al., 1989; Björk and Ylinenjärvi, 1990). The inhibitor concentration range was 0.5–5 μM for reactions with papain and ficin, 1–15 μM for reactions with actinidin and 1–10 μM for reactions with cathepsin B.

The kinetics of association of intact or truncated cystatins with cathepsin B were also analysed by continuous measurements of the loss of enzyme activity in the presence of a substrate. Cystatin (final concentrations of 10–100 nM and 10–250 nM for intact cystatin C and chicken cystatin respectively and of 0.5–5 μM and 2.5–28 μM for truncated cystatin C and chicken cystatin respectively) and the fluorogenic substrate, carbobenzoxy-L-arginyl-L-arginine 4-methylcoumaryl-7-amide (Peptide Institute, Osaka, Japan; final concentration 20 μM), were mixed in an acrylic cuvette. Cathepsin B, at a molar ratio to the inhibitor of at most 1:10, was added, and the fluorescence increase due to release of product from the substrate was recorded as a function of time in an SLM 4800S spectrofluorimeter (SLM-Aminco, Urbana, IL, U.S.A.) with excitation and emission wavelengths of 370 and 440 nm respectively. The inhibitor concentrations were

such that more than 60 % of the enzyme activity was inhibited at equilibrium. Substrate hydrolysis never exceeded 5 %. The data were fitted by non-linear least-squares regression to the equation:

$$[P] = v_\infty t - \frac{(v_\infty - v_0)(1 - e^{-k_{obs.}t})}{k_{obs.}} \quad (1)$$

(Cha, 1975) where [P] is the concentration of product formed by hydrolysis of the substrate, t is time, v_0 and v_∞ are the initial and final rates of substrate cleavage respectively, and $k_{obs.}$ is the observed pseudo-first-order association rate constant for the reaction of inhibitor with enzyme. The second-order association rate constant for this reaction, k_{ass}, was obtained from the slope of a plot of $k_{obs.}$ versus inhibitor concentration. No correction for substrate competition was made, as the K_m of the substrate for cathepsin B was estimated to be $\geqslant 500\ \mu M$, corresponding to $\leqslant 4\%$ correction, from the linear increase of the rate of substrate hydrolysis observed up to a substrate concentration of 200 μM. This lower limit for K_m is in agreement with earlier studies yielding a value of 1.1 mM, albeit under somewhat different solvent conditions (Hasnain et al., 1992).

The rate of dissociation of complexes between intact cystatins and cathepsin B was measured by a method in which cystatin dissociated from the complex was trapped by a large excess of *S*-(carbamoylmethyl)papain (Björk and Ylinenjärvi, 1990). This inactivated papain binds more rapidly and tightly to both cystatin C and chicken cystatin than does cathepsin B (Nicklin and Barrett, 1984; Björk and Ylinenjärvi, 1989; Abrahamson et al., 1991; Lindahl et al., 1992a) and thus prevents the liberated cystatin from reassociating with cathepsin B. The complexes were formed by incubating cathepsin B (final concentration 100–400 nM) with cystatin C or chicken cystatin in a 1.5:1 molar ratio to the enzyme for 5 min in an acrylic cuvette. The enzyme substrate *N*-α-benzoyl-L-arginine *p*-nitroanilide was then added to a concentration of 350–700 μM together with *S*-(carbamoylmethyl)papain at a molar ratio to the complex of 10–30:1, and the increase in absorbance at 410 nm with time caused by the liberated enzyme cleaving the substrate was recorded. The slow dissociation necessitated the use of an inefficient substrate for cathepsin B to ensure that the substrate consumption at the end of the experiment was less than 5 %. The first-order dissociation rate constant for the cystatin–proteinase complex ($k_{diss.}$) was obtained by non-linear least-squares regression of the absorbance increase as a function of time, as described previously (Björk and Ylinenjärvi, 1990).

The kinetics of dissociation of the complex between cystatin C and ficin were evaluated by trapping the enzyme dissociated from the complex (10 μM) by an excess (100–300 μM) of chicken cystatin (form 2), essentially in the same manner as in previous studies of the cystatin C–papain complex (Lindahl et al., 1992a). The dissociation rate constant was obtained from the initial rate of appearance of the complex between the displacing cystatin 2 and the liberated ficin, monitored by chromatography on a Mono Q (Pharmacia LKB) ion-exchange column.

Inhibition constants for the interaction of truncated cystatins with cathepsin B were obtained from the equilibrium rates of cleavage of a substrate by the enzyme in the presence of increasing concentrations of inhibitor in a large excess over the enzyme. Cystatin (final concentrations of 9–500 nM and 1–14 μM in experiments with truncated cystatin C and chicken cystatin respectively) and the substrate carbobenzoxy-L-arginyl-L-arginine 4-methylcoumaryl-7-amide (final concentration 10–20 μM) were mixed in a cuvette. Cathepsin B was added to concentrations of 0.5–1 nM and 2 nM in experiments with truncated cystatin C and chicken cystatin respectively, and the fluorescence increase was recorded as in the analyses of association rate constants in the presence of the substrate. The rate of substrate cleavage by the uninhibited enzyme was determined in separate experiments without cystatin. Substrate hydrolysis was less than 5 % in all analyses. The equilibrium rates of substrate cleavage in experiments with inhibitor were obtained by fitting the data to eqn. (1), and the inhibition constant (K_i) was evaluated from such rates measured at different inhibitor concentrations, as described previously (Lindahl et al., 1992b). No correction for substrate competition was necessary for the same reason as in the measurements of association rate constants.

All analyses were carried out at 25 °C. The buffer in studies of interactions of cystatins with papain, ficin and actinidin contained 0.05 M Tris/HCl, pH 7.4, 0.1 M NaCl and 100 μM EDTA, whereas interactions with cathepsin B were analysed in buffer containing 0.05 M Mes, pH 6.0, 0.1 M NaCl, 100 μM EDTA, 0.5 mM dithiothreitol and 0.1 % (w/v) poly(ethylene glycol).

Protein concentrations were obtained by absorption measurements at 280 nm. Absorption coefficients and relative molecular masses for the intact and truncated cystatins and for papain, ficin and actinidin have been given previously (Björk and Ylinenjärvi, 1990; Lindahl et al., 1992a,b). An absorption coefficient of 2.25 litre·g^{-1}·cm^{-1} was determined by quantitative amino acid analyses for the form of recombinant rat cathepsin B used, and a relative molecular mass of 28200 was calculated for this form from the amino acid sequence (Takio et al., 1983; San Segundo et al., 1985).

RESULTS

Interaction of intact human cystatin C with ficin and of intact human cystatin C or chicken cystatin with cathepsin B

The interactions of intact recombinant human cystatin C and chicken cystatin with papain and actinidin, as well as that of intact chicken cystatin with ficin, have been studied in detail previously (Lindahl et al., 1988, 1992a; Björk et al., 1989; Björk and Ylinenjärvi, 1990). In the present work, the interactions between intact cystatin C and ficin and between intact cystatin C or chicken cystatin and recombinant rat cathepsin B were characterized in a similar manner to enable comparisons with the corresponding interactions of the truncated inhibitors with the enzymes.

Ficin

The binding of cystatin C to ficin was accompanied by a fluorescence increase of approx. 30 % with a maximum of the difference spectrum at 334 nm. The fluorescence enhancement was somewhat lower than that measured for the binding of chicken cystatin to the enzyme, although the wavelength of the maximum change was similar (Björk and Ylinenjärvi, 1990).

The association rate constant for the reaction of cystatin C with ficin was measured by stopped-flow fluorimetry. The observed pseudo-first-order rate constant increased linearly with inhibitor concentration, the slope of this plot yielding the second-order association rate constant (Table 1a). The dissociation rate constant for the cystatin C–ficin complex was obtained by trapping the dissociated ficin with an excess of chicken cystatin. The value obtained (Table 1a) was independent of the concentration of the displacing cystatin, indicating that this concentration was sufficiently high to result in essentially irreversible binding of all the liberated enzyme and thus to give the true dissociation rate constant of the complex (Björk and Ylinenjärvi, 1990). The association rate constant was somewhat lower and the dissociation rate constant somewhat higher than

Table 1 Association rate constants ($k_{ass.}$), dissociation rate constants ($k_{diss.}$) and dissociation equilibrium constants (K_d) for the interaction between intact or N-terminally truncated recombinant human cystatin C (a) or chicken cystatin (b) and papain, ficin, actinidin or recombinant rat cathepsin B

Values measured in this work are given with their 95% confidence intervals (calculated for small-size samples from the standard errors; Havilcek and Crain, 1988) and with the number of measurements shown in parentheses. The buffer for papain, ficin and actinidin contained 0.05 M Tris/HCl, pH 7.4, 0.1 M NaCl and 100 μM EDTA and that for cathepsin B contained 0.05 M Mes, pH 6.0, 0.1 M NaCl, 100 μM EDTA, 0.5 mM dithiothreitol and 0.1% (w/v) poly(ethylene glycol).

	$k_{ass.}$ ($M^{-1} \cdot s^{-1}$)		$k_{diss.}$ (s^{-1})		K_d (M)	
Proteinase	Intact	Truncated	Intact	Truncated	Intact	Truncated
(a) Cystatin C						
Papain	1.14×10^{7}*	$(1.15 \pm 1.0) \times 10^{7}$ ($n = 10$)	1.3×10^{-7}*	0.3†	1.1×10^{-14}*	$(2.9 \pm 0.6) \times 10^{-8}$ ($n = 3$)
Ficin	$(7.0 \pm 0.2) \times 10^{6}$ ($n = 10$)	$(9.6 \pm 0.4) \times 10^{6}$ ($n = 10$)	$(9.2 \pm 2.6) \times 10^{-6}$ ($n = 5$)	0.3†	1.3×10^{-12}†	$(3.0 \pm 0.7) \times 10^{-8}$ ($n = 4$)
Actinidin	2.4×10^{6}*	$(1.5 \pm 0.2) \times 10^{5}$ ($n = 11$)	4.6×10^{-2}*	0.8 ± 0.2 ($n = 11$)	1.9×10^{-8}*	5×10^{-6}†
Cathepsin B	$(1.4 \pm 0.1) \times 10^{6}$ ($n = 10$)‡ $(1.1 \pm 0.3) \times 10^{6}$ ($n = 10$)§	$(2.1 \pm 0.2) \times 10^{4}$ ($n = 10$)	$(3.5 \pm 0.5) \times 10^{-4}$ ($n = 8$)	3×10^{-4}†	3×10^{-10}†	$(1.3 \pm 0.2) \times 10^{-8}$ ($n = 10$)
(b) Chicken cystatin						
Papain	9.9×10^{6}*	1.08×10^{7}*	5.7×10^{-7}*	0.13*	6×10^{-14}*	1.2×10^{-8}*
Ficin	9.6×10^{6}*	$(7.6 \pm 0.6) \times 10^{6}$ ($n = 10$)	4.6×10^{-7}*	0.6†	5×10^{-14}*	$(7.8 \pm 2.7) \times 10^{-8}$ ($n = 4$)
Cathepsin B	$(7.9 \pm 1.2) \times 10^{5}$ ($n = 8$)‡ $(5.2 \pm 0.7) \times 10^{5}$ ($n = 12$)§	$(3.8 \pm 1.0) \times 10^{2}$ ($n = 11$)	$(3.3 \pm 0.6) \times 10^{-3}$ ($n = 5$)	7×10^{-4}†	4×10^{-9}†	$(1.8 \pm 0.1) \times 10^{-6}$ ($n = 8$)

* From previous work (Björk et al., 1989; Björk and Ylinenjärvi, 1990; Lindahl et al., 1992a,b).
† Calculated value.
‡ Measured by stopped-flow fluorescence.
§ Measured by monitoring loss of enzyme activity in the presence of substrate.

the corresponding values for the interaction of chicken cystatin with ficin, resulting in a lower affinity (Table 1).

Cathepsin B

The stoichiometry of binding of chicken cystatin to recombinant rat cathepsin B, measured by a competitive titration with a fluorescent papain derivative, was 0.9:1. This value shows that essentially all enzyme molecules were able to bind to the inhibitor, similarly to what has been demonstrated earlier for papain, ficin and actinidin (Lindahl et al., 1988; Björk and Ylinenjärvi, 1990). The interactions of cystatin C and chicken cystatin with rat cathepsin B were accompanied by fluorescence decreases of approx. 6 and 10% respectively, with maxima at 355 and 340 nm respectively. The fluorescence changes were considerably smaller than those observed for the binding of the two inhibitors to other cysteine proteinases (Lindahl et al., 1988, 1992a; Björk and Ylinenjärvi, 1990; see also data for ficin above).

The fluorescence changes were too small to give data of sufficient precision in equilibrium titrations for the determination of stoichiometries or affinities but could be used to measure association rate constants for the interactions by stopped-flow fluorimetry under pseudo-first-order conditions (Table 1). Linear plots of the observed pseudo-first-order rate constants versus inhibitor concentration were obtained for both cystatin C and chicken cystatin. Measurements based on the loss of cathepsin B activity in the presence of a substrate corroborated the second-order association rate constants obtained by the stopped-flow method (Table 1). The association rate constants for the binding of either of the two inhibitors to rat cathepsin B were appreciably lower than those for the binding to papain or ficin and also somewhat lower than those for the interactions with actinidin. The rate constant for cystatin C was 3- to 10-fold lower than previously reported values for the reaction of this inhibitor with human cathepsin B (Barrett et al., 1984; Abrahamson et al., 1991), whereas the value for chicken cystatin was comparable with that published earlier for the reaction with the human enzyme (Nicklin and Barrett, 1984).

The dissociation rate constants of the complexes between cystatin C or chicken cystatin and rat cathepsin B were determined by trapping the inhibitor dissociating from the complexes by an excess of an inactivated papain and monitoring the cleavage of a substrate by the liberated enzyme. The values obtained (Table 1) were independent of the concentration of the displacing inactivated papain and thus well approximate the true dissociation rate constant of the complex, as discussed above. The dissociation rate constants of the cystatin–cathepsin B complexes were markedly higher than those of the complexes of the inhibitors with papain and ficin but appreciably lower than those of the complexes with actinidin. Substantially (3- to 10-fold) higher dissociation rate constants have been reported earlier for the interaction of cystatin C with human cathepsin B (Barrett et al., 1984; Abrahamson et al., 1991), whereas the value for chicken cystatin obtained in this work was comparable with that obtained previously for the interaction of this inhibitor with the human enzyme (Nicklin and Barrett, 1984).

Dissociation equilibrium constants were calculated from the association and dissociation rate constants (Table 1). The affinities of cystatin C or chicken cystatin for rat cathepsin B were much lower than those of the two inhibitors for papain or ficin.

However, cystatin C bound more tightly to rat cathepsin B than to actinidin, whereas the affinities of chicken cystatin for these two proteinases were comparable. The dissociation equilibrium constants for both inhibitors were similar to previously reported inhibition constants for the interactions with human cathepsin B, which range from 1.5×10^{-10} to 4.2×10^{-10} M for cystatin C (Barrett et al., 1984; Popovič et al., 1990; Abrahamson et al., 1991) and from 1.7×10^{-9} to 2.9×10^{-9} M for chicken cystatin (Nicklin and Barrett, 1984; Machleidt et al., 1991; Auerswald et al., 1992).

Kinetics of interaction of N-terminally truncated forms of human cystatin C and chicken cystatin with papain, ficin, actinidin and cathepsin B

Papain

The kinetics of binding of N-terminally truncated chicken cystatin to papain has been characterized previously (Lindahl et al., 1992b). The association rate constant for the reaction between similarly truncated cystatin C and papain was determined by stopped-flow fluorimetry (Table 1a). A dissociation equilibrium constant for the interaction was measured by fluorescence titrations (Table 1a), and the dissociation rate constant was calculated from this equilibrium constant and the association rate constant (Table 1a). Control experiments showed that association and dissociation rate constants essentially identical with those given in Table 1a, which were measured in the standard Tris buffer at pH 7.4, were obtained also in the Mes buffer containing dithiothreitol and poly(ethylene glycol) at pH 6.0 used for cathepsin B. Similarly to previous observations for truncated chicken cystatin (Lindahl et al., 1992b; Table 1b), the decreased affinity of cystatin C for papain after removal of the N-terminal region of the inhibitor was due exclusively to a greatly increased dissociation rate constant.

Ficin

Stopped-flow fluorimetry was also used to determine the association rate constants for the interactions between N-terminally truncated cystatin C or chicken cystatin and ficin (Table 1). Interestingly, these reactions were accompanied by a decrease in tryptophan fluorescence of about 30%, in contrast with the approx. 30–45% increase observed with the intact inhibitors (Björk and Ylinenjärvi, 1990; see also above). Fluorescence titrations gave the dissociation equilibrium constants for the binding of the two truncated cystatins to ficin (Table 1), and the dissociation rate constants for the interactions were calculated from these values and the association rate constants (Table 1). As was found for papain, the reduced affinities of the truncated inhibitors for ficin were due entirely to markedly increased dissociation rate constants.

Actinidin

Both the association and dissociation rate constants for the binding of N-terminally truncated cystatin C to actinidin could be determined by stopped-flow fluorimetry. As in the previous experiments with this method, the slope of the linear plot of the observed pseudo-first-order rate constant versus inhibitor concentration yielded the association rate constant (Table 1a). However, in contrast with the previous experiments, the intercept on the ordinate was clearly distinguishable from zero, and the dissociation rate constant could thus be obtained from this intercept (Table 1a). The dissociation equilibrium constant was calculated from the two rate constants (Table 1a). As was shown earlier for the interaction between truncated cystatin C and papain, control experiments in Mes buffer at pH 6.0 gave similar results to those obtained in the standard Tris buffer at pH 7.4. The lower affinity of truncated cystatin C than of the intact inhibitor for actinidin was due to both an approx. 15-fold lower association rate constant and an approx. 15-fold higher dissociation rate constant.

A similar kinetic characterization of the interaction between chicken cystatin and actinidin was not possible, because of an insufficient fluorescence increase accompanying the binding and very rapid reactions being observed, presumably because of a high dissociation rate constant.

Cathepsin B

The association rate constants for the reactions of N-terminally truncated cystatin C or chicken cystatin with rat cathepsin B could not be determined with acceptable precision by stopped-flow fluorimetry, because of only small fluorescence changes accompanying the binding and low reaction rates. Instead, these rate constants were determined from the decrease with time of the rate of cleavage of a substrate by the enzyme under pseudo-first-order conditions, i.e. with excess inhibitor (Table 1). The magnitude of the association rate constant for the binding of truncated cystatin C to rat cathepsin B obtained by this method was corroborated by stopped-flow fluorimetry, which gave an approximate value of 2.5×10^4 $M^{-1} \cdot s^{-1}$. The rate constant for this reaction was also comparable with that reported previously for the reaction of truncated cystatin C with human cathepsin B (Abrahamson et al., 1991). The dissociation equilibrium constants for the interactions were measured as inhibition constants, obtained from the equilibrium rates of cleavage of a substrate by the enzyme in the presence of inhibitor, and the dissociation rate constants were calculated from the association rate constants and the dissociation equilibrium constants (Table 1). The dissociation rate constant, and consequently also the dissociation equilibrium constant, for the reaction of truncated cystatin C with rat cathepsin B was approx. 10-fold lower than the value obtained previously for the reaction with the human enzyme (Abrahamson et al., 1991). This difference notwithstanding, the conclusion was analogous, i.e. that the decreased affinities of both cystatin C and chicken cystatin for cathepsin B after truncation of the N-terminal regions of the inhibitors were due almost exclusively to greatly decreased association rate constants. In the case of truncated chicken cystatin, the dissociation rate constant was even approx. 5-fold lower than that of the intact inhibitor, which, by itself, would have led to a tighter binding.

DISCUSSION

Several studies have shown that removal of the N-terminal region of cystatin C or chicken cystatin by proteolytic cleavage markedly decreases the affinity of the inhibitors for cysteine proteinases (Abrahamson et al., 1987, 1991; Machleidt et al., 1989; Popovič et al., 1990; Lindahl et al., 1992b). Two previous investigations, which differed from each other with regard to both inhibitor and target enzyme, attributed this decrease to either a reduced association rate constant or an increased dissociation rate constant (Abrahamson et al., 1991; Lindahl et al., 1992b). The present study resolves this apparent discrepancy and shows that the two inhibitors behave identically on truncation of their N-termini, the relative contributions of the changes in individual rate constants to the reduced affinity being dependent only on the target enzyme. The decreased affinity thus

was shown to be due exclusively to an increased dissociation rate constant in the case of papain and ficin, to both a decreased association rate constant and an increased dissociation rate constant for actinidin and entirely to a decreased association rate constant for cathepsin B as target enzyme. Appropriate control experiments, together with the independence of the interaction between intact cystatins and papain on pH in the range 6–8 (Björk et al., 1989), indicated that the lower pH necessary in the studies with cathepsin B did not contribute to the observed differences in rate constants.

The X-ray structure of chicken cystatin shows that the N-terminal region of the inhibitor is highly flexible (Bode et al., 1988, 1990). Moreover, removal of this region does not detectably alter the three-dimensional structure of the rest of the protein (Bode et al., 1990; Lindahl et al., 1992b). The decreased affinity of the inhibitor for proteinases after such truncation therefore probably reflects loss of interactions involving the N-terminal region, rather than a perturbation of the remainder of the binding surface of the inhibitor. The pronounced similarity between cystatin C and chicken cystatin indicates that N-terminal truncation of cystatin C similarly affects the properties of this inhibitor. The findings of this paper therefore imply that the interactions of the N-terminal segment of cystatin C or chicken cystatin with target proteinases must contribute to the overall inhibition mechanism in different ways, depending on the structure of the active-site region of the proteinase.

In the case of papain, there is considerable evidence from the X-ray structures of the enzyme (Drenth et al., 1971) and of chicken cystatin, as well as from other work, that residues in the N-terminal region, specifically Leu-7 and Leu-8 in chicken cystatin and corresponding hydrophobic residues in cystatin C, interact with the S_3 and S_2 substrate-binding subsites respectively of the proteinase (Bode et al., 1988; Machleidt et al., 1989; Stubbs et al., 1990; Lindahl et al., 1992b). The similar rates of association of truncated and intact cystatins with papain show that this interaction does not contribute to the overall rate of the reaction of the inhibitor with the enzyme. Moreover, the binding of the N-terminal region may occur at about the same rate as that of the interaction of the other parts of the binding surface of the inhibitor with the proteinase, as kinetic studies with the intact inhibitors show no evidence of a two-step reaction (Björk et al., 1989; Lindahl et al., 1992a). However, the N-terminal region of the cystatins contributes substantially to the stability of the cystatin–papain complex by keeping the inhibitor anchored to the proteinase, thereby reducing the rate of dissociation of the two components. The interaction between the N-terminal region of the inhibitors and ficin presumably is similar to the corresponding interaction with papain, as this region also stabilizes the cystatin–ficin complexes by only decreasing the dissociation rate. However, no X-ray structure of ficin is available to support this contention further.

In contrast, the N-terminal region of the cystatins increases the rate of association of the inhibitors with actinidin and cathepsin B. The difference between papain and actinidin in this respect may be related to the finding that the structures of the two enzymes differ mainly in the S_2 subsite (Drenth et al., 1971; Baker, 1980). This site is lined with different side chains in actinidin from those found in papain, suggesting that it may bind the N-terminal region of cystatins differently from the corresponding site in papain. In addition to increasing the association rate, the N-terminal region also stabilizes the complex with actinidin by decreasing the dissociation rate constant as in the reaction with papain, albeit to a smaller extent.

The structure of cathepsin B (Musil et al., 1991) differs markedly from those of papain and actinidin by having an extra loop of about 20 residues that partially occludes the active-site cleft. Modelling experiments have suggested that this loop interferes substantially with binding of cystatins (Musil et al., 1991) in agreement with the comparatively weak complexes formed. In particular, the occluding loop would collide with the region around Trp-104 in chicken cystatin (corresponding to Trp-106 in cystatin C), preventing a close contact of this residue with Trp-221 near the active site of cathepsin B. This behaviour presumably is the reason for the low fluorescence decrease accompanying the binding of cystatins to cathepsin B, as the corresponding interaction with papain is responsible for most of the appreciably larger fluorescence quenching observed with this enzyme (Lindahl et al., 1988; Nycander and Björk, 1990). However, the modelling experiments revealed that much of the steric hindrance between cystatins and cathepsin B could be released by tilting and simultaneous rotation of the inhibitor. Such a process during actual complex-formation might be facilitated by initial binding of the N-terminal region of the cystatins to the S_2 subsite of cathepsin B, which has a similar structure to that in papain (Musil et al., 1991). Presumably such an effect would lead to a higher rate of association of the inhibitor with the enzyme by properly orientating the binding surface of the inhibitor with respect to that of the enzyme. However, it is apparent that once the N-terminal region of the cystatins has increased the rate of binding to cathepsin B, it does not participate to an appreciable extent in keeping the inhibitor attached to the enzyme, as its presence or absence minimally influenced the dissociation rate constant of the complex.

The two-step binding mechanism suggested for the binding of cystatins to intact cathepsin B by the X-ray structure of the enzyme and the data in this work might be expected to result in hyperbolic plots of the observed pseudo-first-order rate constant versus inhibitor concentration (Fersht, 1985). Such behaviour was not observed at the low concentrations that had to be used in these studies because of a limited supply of the enzyme. However, extension of the kinetic analyses to higher protein concentrations may give further evidence of the proposed mechanism of interaction of cystatins with cathepsin B.

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