The Contribution of N-Terminal Region Residues of Cystatin A (Stefin A) to the Affinity and Kinetics of Inhibition of Papain, Cathepsin B, and Cathepsin L[†]

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ABSTRACT: The affinity and kinetics of binding of three N-terminally truncated variants of the cysteine proteinase inhibitor cystatin A to cysteine proteinases were characterized. Deletion of Met-1 only minimally altered the inhibitory properties of the protein. However, deletion also of Ile-2 resulted in reduced affinities of 900-, ≥3-, and 200-fold for papain and cathepsins L and B, respectively. Further truncation of Pro-3 substantially increased the inhibition constants to ~0.5 μM for papain and cathepsin L and to 60 μM for cathepsin B, reflecting additionally 2×10^3-, 2×10^4-, and 400-fold decreased affinities, respectively. The reductions in affinity shown by the latter mutant indicate that the N-terminal region contributes about 40% of the total free energy of binding of cystatin A to cysteine proteinases. Moreover, Pro-3 and to a lesser extent Ile-2 are the residues responsible for this binding energy. The reduced affinities for papain and cathepsin L were due only to higher dissociation rate constants, whereas both lower association and higher dissociation rate constants contributed to the decreased affinity for cathepsin B. These differential effects indicate that the N-terminal portion of cystatin A primarily functions by stabilizing the complexes with enzymes having easily accessible active-site clefts, e.g., papain and cathepsin L. In contrast, the N-terminal region is required also for an initial binding of cystatin A to cathepsin B, presumably by promoting the displacement of the occluding loop and allowing facile interaction of the rest of the inhibiting wedge with the active-site cleft of the enzyme.

Cysteine proteinase inhibitors of the cystatin family are important regulatory proteins, present in all mammals. Their main physiological function is believed to be to protect the organism from the harmful effects of free cysteine proteinases by rapidly trapping these enzymes in tight complexes. Major target proteinases of the cystatins are cathepsins released from lysosomes during, e.g., inflammation (*1*) or secreted from cancer cells during metastasis (*2*), as well as exogenous cysteine proteinases from invading microorganisms (*3, 4*). Cystatins are divided into three subfamilies. Members of family I, or stefins, are small proteins of around 100 amino acids, which lack internal disulfide bonds and are present predominantly intracellularly. The inhibitors of family II contain 2 disulfides, have about 120 amino acids, and are found extracellularly, in body fluids. The members of family III are the larger multifunctional glycoproteins called kininogens, present in blood. The kininogens contain three family II-like domains, two of which have been shown to inhibit papain-like cysteine proteinases (*5*−*7*). Human cystatin A consists of 98 amino acid residues and belongs to family I. Among the target enzymes inhibited by cystatin A are cathepsins B, C, H, L, and S, which are of lysosomal origin (*8, 9*). Parasite enzymes, e.g., cruzipain and trypanopain-Tb (*3, 10*), and several plant proteinases, such as papain and actinidin (*8*), are also strongly inhibited by cystatin A.

The inhibitory activity of cystatins toward papain-like proteinases is due to the interaction of a wedge-shaped edge of the inhibitor with the active-site cleft of the enzyme. The inhibitory wedge is formed by three segments of the protein, viz., a region around a Gly residue in the N-terminal end of the chain and two hairpin loops, one central and one closer to the C-terminus. The crystal structure of chicken cystatin, a family II member (*11*), suggested that this wedge could be complementary to the cleft between the two domains of the papain molecule (*12*). This assumption was supported by computer docking experiments of the two proteins (*11*), and was corroborated by the X-ray structure of a complex between papain and cystatin B (also called stefin B), a family I cystatin (*13*). Although no other structures of complexes have been determined, the NMR solution structures of cystatin A (*14, 15*), chicken cystatin (*16*), and cystatin C (*17*) have highly similar folding, suggesting that the interaction mechanism is general for all cystatins.

Further information on the mechanism of inhibition has been provided by kinetic studies. Stopped-flow analyses of the interactions of papain and several similar cysteine proteinases with cystatins show that the binding is rapid and indicate that it occurs in one step, without major conformational changes of the molecules (*8, 18*−*22*). This behavior is consistent with the good fit between the structures of the interacting proteins (*11, 13*). In contrast, it has been

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Met-Ile-Pro-Gly*-Gly-	Cystatin A
Met-Met-Cys-Gly*-Ala-	Cystatin B
Ser-Ser-Pro-Gly-Lys-Pro-Pro-Arg-Leu-Val-Gly*-Gly-	Cystatin C
Ser-Glu-Asp-Arg-Ser-Arg-Leu-Leu-Gly*-Ala-	Chicken cystatin

FIGURE 1: Amino acid sequences of the N-terminal regions of some family I and II cystatins. The sequences are aligned with respect to the evolutionarily conserved Gly residue (marked with an asterisk).

demonstrated that the association of cathepsin B and cystatin C involves a two-step mechanism (*23*), probably reflecting the need of the inhibitor to displace the occluding loop that partially covers the active site of this enzyme (*24*) before a stable complex can be formed.

Several studies have demonstrated that the affinity of the family II inhibitors, chicken cystatin and cystatin C, for target enzymes is substantially decreased by truncation of N-terminal residues preceding the evolutionarily conserved Gly residue (Gly-11 in cystatin C; Figure 1) (*25*−*31*). Also, substitutions of the conserved Gly by other amino acids result in large decreases in binding affinity (*32, 33*). However, considerably less is known about the significance of the shorter N-terminal segment of the family I cystatins (Figure 1). Early studies proposed that this region in cystatin B was dispensable for high-affinity binding to papain (*34, 35*). However, truncation of the N-terminal residues preceding the conserved Gly-4 of a Met-65 to Leu variant of cystatin A was reported to result in a decrease in affinity for papain (*36*). Mutations of Gly-4 in cystatin A have also been shown to appreciably reduce the affinity of the inhibitor for target proteinases (*37, 38*).

In this investigation we have used a bacterial expression system for human cystatin A which produces intact and fully active inhibitor (*8*) to sequentially delete amino acid residues from the N-terminus. Affinities were determined for the interactions of the cystatin A truncation variants not only with papain but also with the physiologically relevant target enzymes cathepsins B and L. In addition, the kinetics of the interactions were characterized with the aim of elucidating the underlying mechanism responsible for the observed changes in affinity. Together, the results show that the N-terminal region of cystatin A contributes appreciable energy of binding to all three cysteine proteinases studied. However, the role of this region in the kinetics of binding of the inhibitor to cathepsin B is appreciably different from that in the kinetics of binding to the other two enzymes.

MATERIALS AND METHODS

Enzymes and Wild-Type Inhibitors. Papain (EC 3.4.22.2) was purified as detailed earlier (*28, 39*). Human liver cathepsin B (EC 3.4.22.1) was from Calbiochem (San Diego, CA). Cathepsin L (EC 3.4.22.15) from sheep liver (*40*) was a kind gift from Dr. R. W. Mason, Alfred I. duPont Institute, Wilmington, DE. Chicken cystatin was isolated from egg white (*39*). Wild-type cystatin A was expressed and purified as described previously (*38*).

Expression and Purification of Truncated Cystatin A Variants. Vectors for expression of the N-terminal truncation variants of cystatin A were constructed by the following strategy. A short segment was excised from the existing cystatin A expression vector, in which the cDNA for cystatin A is preceded by a His-tag[1] and an enterokinase cleavage site just before the N-terminus of the inhibitor (*38*). This fragment (74 bp) contained the His-tag, the enterokinase site, and the first three residues of cystatin A. The excised fragment was replaced with PCR products that lacked the codons for Met-1, Met-1 to Ile-2, and Met-1 to Pro-3 (Figure 1) for the three desired variants Δ M-, Δ MI-, and Δ MIP-cystatin A, respectively, but reconstituted the vector and the codons for the residues to be retained in the first two variants. The template for the PCR amplifications was the original expression vector. The upstream primer was the same for all mutants (5′-GCTCAGGCGACCATGGGCCATCATCATC). The downstream primers for Δ M-, Δ MI-, and Δ MIP-cystatin A were (5′-GCCCCCGGGTATCTTGTCGTCGTCGTCGATATGG), (5′-GCCCCCGGGCTTGTCGTCGTCGTCGATATGG), and (5′-CTTGTCGTCGTCGTCGATATGG), respectively. The DNA fragments generated by PCR were digested with *Nco*I and *Xma*I (only *Nco*I for the Δ MIP mutant), and were purified and ligated into the expression vector, which also had been digested with *Nco*I and *Xma*I (*Nco*I and *Sma*I for the Δ MIP mutant) and purified. The resulting constructs were sequenced over the mutated region and transformed into competent MC 1061 strains, and the His-tagged proteins were expressed essentially as in earlier work (*8, 38*).

The expressed His-tagged proteins were purified as described previously (*38*). The His-tag was removed by incubating the fusion protein, in 70 mM Tris-HCl, 2 mM $CaCl_2$, pH 7.4, with enterokinase (Biozyme, Blaenavon, U.K.) at an enzyme/fusion protein weight ratio of 0.002−0.004 for 20−72 h at 37 °C. After inactivation of the enterokinase by addition of *N*-tosyl-L-phenylalanyl chloromethyl ketone to a concentration of 10 μM, the solution was reapplied to a Ni^{2+} column (Novagen, Inc., Madison, WI) or a TALON column (Clontech, Inc., Palo Alto, CA), to remove uncleaved fusion protein and released His-tag.

As removal of the His-tag by enterokinase was ineffective in the case of Δ MI-cystatin A, this mutant was instead obtained by either of two methods. In the first of these, the His-tagged Δ MI variant was digested with clostripain (Sigma, St. Louis, MO) at an enzyme/fusion protein weight ratio of 0.05 for 72 h at 25 °C in 20 mM Tris-HCl, 1 mM $CaCl_2$, and 5 mM dithiothreitol, pH 8.0. After inactivation of the clostripain by addition of *N*-tosyl-L-lysyl chloromethyl ketone to 20 μM, the sample was reapplied to the TALON column, and the flow-through fraction was collected. This fraction was dialyzed against 20 mM Tris-HCl, pH 7.0, and applied to a Mono Q (Pharmacia Biotech, Uppsala, Sweden) ion-exchange column, which was eluted by a linear gradient to 0.2 M NaCl in the same buffer. Alternatively, His-tagged wild-type cystatin A (*38*) was incubated with human leukocyte elastase (a generous gift from Dr. Christer Peterson, Department of Clinical Chemistry, University of Uppsala) at an enzyme/cystatin weight ratio of 0.02 for 48 h at 30 °C

[1] Abbreviations: app, subscript denoting an apparent equilibrium or rate constant measured in the presence of an enzyme substrate; Δ M-, Δ MI-, Δ MIP-, and Δ MIPG-cystatin A, N-terminally truncated forms of cystatin A lacking Met-1, Met-1 to Ile-2, Met-1 to Pro-3, and Met-1 to Gly-4, respectively; E-64, [*N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]-amido-4-guanidobutane; His-tag, 10 consecutive histidine residues fused to an expressed protein; k_{ass}, bimolecular association rate constant; k_{diss}, dissociation rate constant; K_i, inhibition constant; k_{obs}, observed pseudo-first-order rate constant; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

in 50 mM Tris-HCl, pH 8.0, containing 100 mM NaCl, 1 mM EDTA, and 0.05% (v/v) Triton X-100. The elastase was then inactivated by the addition of phenylmethanesulfonyl fluoride to 1 mM. The sample was applied to a Bio-Gel Phenyl-5-PW column (Bio-Rad, Richmond, CA), equilibrated with 100 mM potassium phosphate, pH 7.0, containing 1.5 M $(NH_4)_2SO_4$, and the Δ MI-cystatin A was eluted by a linear gradient from 1.5 to 0 M $(NH_4)_2SO_4$ in the phosphate buffer. All purified truncated cystatin A mutants were dialyzed extensively against 50 mM Tris-HCl, 100 mM NaCl, 0.1 mM EDTA, pH 7.4.

Protein Concentrations. Concentrations of the inhibitors and papain were determined from absorption measurements at 280 nm with molar absorption coefficients of 11 400 $M^{-1}\cdot cm^{-1}$ for chicken cystatin (*39*), 8880 $M^{-1}\cdot cm^{-1}$ for all variants of cystatin A (*8*) and 55 900 $M^{-1}\cdot cm^{-1}$ for papain (*39*). The active-site concentration of cathepsin L was determined by titration with E-64 (*40*). The concentration of cathepsin B was provided by the manufacturer.

Experimental Conditions. All reactions between the cystatin A variants and proteinases were performed at 25.0 ± 0.2 °C. The buffers used were as follows: for papain, 50 mM Tris-HCl, 100 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 0.01% (w/v) Brij 35, pH 7.4; for cathepsin L, 100 mM sodium acetate, 1 mM EDTA, 1 mM dithiothreitol, and 0.01% (w/v) Brij 35, pH 5.5; for cathepsin B, 50 mM Mes−NaOH, 100 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol and 0.1% (w/v) poly(ethylene glycol), pH 6.0. The enzymes were activated by incubation in the reaction buffers for 10 min at 25 °C.

Circular Dichroism. Circular dichroism spectra were acquired at room temperature (22 ± 2 °C) in a Jasco J-41A spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan). Measurements were done in the far-ultraviolet region (200−250 nm) with protein concentrations of about 0.2 g/L. The path length of the cells was 0.1 cm, and the bandwidth was 2 nm.

Inhibition Constants. Inhibition constants (K_i) for the binding of the mutants to the cysteine proteinases papain, cathepsin L, and cathepsin B were determined from the equilibrium rates of cleavage of a fluorogenic substrate by the enzyme at different concentrations of the inhibitor, as described earlier (*28, 33*). The substrate was carbobenzoxy-L-phenylalanyl-L-arginine 4-methylcoumaryl-7-amide (Peptide Institute, Osaka, Japan) for papain and cathepsin L and carbobenzoxy-L-arginyl-L-arginine 4-methylcoumaryl-7-amide (Peptide Institute) for cathepsin B. Substrate concentrations were 1−10 μM and substrate hydrolysis never exceeded 5%. The inhibitor concentration was at least 10-fold higher than that of the enzyme and was varied from (0.2−1) × $K_{i,\,app}$ (the apparent inhibition constant) to (2−10) × $K_{i,\,app}$. For slow reactions, the equilibrium rates of substrate hydrolysis were evaluated by nonlinear least-squares regression analyses of the progress curves (*28*). Values of $K_{i,\,app}$ were obtained by nonlinear regression analyses of plots of the ratio between the inhibited and uninhibited rates of substrate hydrolysis against inhibitor concentration and were corrected for substrate competition to give K_i with K_m values reported elsewhere (*28, 32, 40*).

Association Kinetics. The kinetics of binding of the mutants to the cysteine proteinases were analyzed by continuous measurements of the loss of enzyme activity in the presence of a fluorogenic substrate, either in a conventional fluorimeter (F-4000; Hitachi, Tokyo, Japan) or in a stopped-flow apparatus (SX-17MV; Applied Biophysics, Leatherhead, U.K.) essentially as in earlier work (*22, 28, 33, 38*). The initial concentration of the inhibitor was varied over a 10−20-fold range and was at least 10-fold higher than that of the enzyme; i.e., pseudo-first-order conditions were used. The substrates, their concentrations, and the maximum extent of substrate hydrolysis were the same as in the determinations of inhibition constants. The observed, apparent pseudo-first-order rate constants ($k_{obs,\,app}$) were calculated by nonlinear least-squares regression analyses of the progress curves (*28*). Second-order association rate constants (k_{ass}) were obtained from the slope of plots of $k_{obs,\,app}$ versus inhibitor concentration, after correction for substrate competition (*28, 33*).

Dissociation Kinetics. The dissociation rate constant (k_{diss}) of the complex between Δ M-cystatin A and papain was determined by trapping the papain released from this complex with an excess of chicken cystatin and measuring the appearance of the tight chicken cystatin−papain complex by chromatography, essentially as detailed (*8*). The initial concentrations of the Δ M-cystatin A−papain complex were 2.5−4.0 μM, and the molar ratio of the displacing chicken cystatin to this complex was varied from 10- to 15-fold.

Miscellaneous Procedures. The stoichiometries of binding of the recombinant forms of cystatin A to papain were determined by either of two procedures, in both of which a constant amount of enzyme was titrated with increasing concentrations of inhibitor and the residual enzyme activity was measured. In the first of these, used for Δ M- and Δ MIP-cystatin A, the conditions were as given previously (*38*). In the alternative method, used for Δ MI-cystatin A because of a reduced amount of this variant available, 20 nM activated papain was incubated for 5 min with increasing concentrations (4−60 nM) of the inhibitor. The fluorogenic substrate, carbobenzoxy-L-arginyl-L-arginine 4-methylcoumaryl-7-amide, was then added to 10 μM, and the residual activity of the enzyme was determined by continuously measuring the rate of product formation for 2−10 min. Substrate hydrolysis never exceeded 2%. The data were analyzed as in the earlier procedure (*38*).

SDS−PAGE under reducing conditions was run on 16.5% (w/v) gels with the Tricine buffer system (*41*). N-Terminal sequences and relative molecular masses were determined as described in (*8*).

RESULTS

Expression of N-Terminally Truncated Cystatin A Variants. Variants of cystatin A lacking one, two, or three residues from the N-terminal end of the protein (Figure 1) were expressed in a bacterial system. Purification of the His-tagged variants gave 5−10 mg of the tagged proteins per liter of culture medium. Removal of the His-tag by digestion with enterokinase was not as effective as in previous work with Gly-4 mutants (*38*), despite the use of higher ratios of enzyme to fusion protein and longer incubation times. Typically, 70% of the tag of the Δ M mutant was cleaved after digestion for 20 h at 37 °C with enterokinase at a weight ratio to the fusion protein of 0.002, and the yield for the Δ MIP mutant was 30% after cleavage for 72 h at 37 °C with a ratio of 0.004. However, the latter conditions gave no detectable cleavage

Table 1: Inhibition Constants, Association Rate Constants, and Dissociation Rate Constants for the Interaction of Wild-Type and N-Terminally Truncated Forms of Cystatin A with Papain, Cathepsin L, and Cathepsin B[a]

enzyme	cystatin A form	K_i (M)	k_{ass} ($M^{-1}\cdot s^{-1}$)	k_{diss} (s^{-1})
papain	wild-type	1.8×10^{-13} [b] [1]	3.1×10^6 [b] [1]	5.5×10^{-7} [b] [1]
	Δ M	2.5×10^{-13} [c] [1.4]	$(3.3 \pm 0.04) \times 10^6$ (7) [0.9]	$(8.7 \pm 0.2) \times 10^{-7}$ (3) [1.6]
	Δ MI	$(1.6 \pm 0.2) \times 10^{-10}$ (9) [900]	$(3.4 \pm 0.03) \times 10^6$ (6) [0.9]	5.4×10^{-4} [d] [1000]
	Δ MIP	$(2.9 \pm 0.08) \times 10^{-7}$ (11) [2×10^6]	$(3.4 \pm 0.05) \times 10^6$ (7) [0.9]	0.99[d] [2×10^6] 0.9 ± 0.2 (7)
cathepsin L	wild-type	$\leq 1 \times 10^{-11}$ (3) [1]	5.2×10^6 [b] [1]	$\leq 5 \times 10^{-5}$ [d] [1]
	Δ M	$\leq 1 \times 10^{-11}$ (3) [1]	$(5.2 \pm 0.06) \times 10^6$ (6) [1]	$\leq 5 \times 10^{-5}$ [d]
	Δ MI	$(3.4 \pm 0.5) \times 10^{-11}$ (10) [≥3]	$(7.4 \pm 0.08) \times 10^6$ (7) [0.7]	2.5×10^{-4} [d] [≥5] $(3.6 \pm 1) \times 10^{-4}$ (7)
	Δ MIP	$(7.1 \pm 0.3) \times 10^{-7}$ (7) [$\geq 7 \times 10^4$]	ND	ND
cathepsin B	wild-type	9.1×10^{-10} [b] [1]	3.9×10^4 [b] [1]	3.5×10^{-5} [b] [1]
	Δ M	$(4 \pm 1) \times 10^{-9}$ (9) [4]	$(2.2 \pm 0.02) \times 10^4$ (9) [2]	9×10^{-5} [d] [3]
	Δ MI	$(1.6 \pm 0.2) \times 10^{-7}$ (7) [200]	$(1.5 \pm 0.04) \times 10^3$ (6) [30]	2.4×10^{-4} [d] [7]
	Δ MIP	$(6.0 \pm 0.8) \times 10^{-5}$ (8) [7×10^4]	ND	ND

[a] The conditions of the measurements are described under Materials and Methods. Measured values are given with standard errors and with the number of measurements in parentheses. Calculated values and values obtained previously are given without errors. Relative values, defined as $K_{i,\,mutant}/K_{i,\,wild-type}$, $k_{ass,\,wild-type}/k_{ass,\,mutant}$, and $k_{diss,\,mutant}/k_{diss,\,wild-type}$, are given within brackets. Relative values >1 thus indicate changes of K_i, k_{ass}, and k_{diss} expected to result in decreased binding affinity. ND, not determined. [b] From previous work (*8*). [c] Calculated from k_{ass} and k_{diss}. [d] Calculated from K_i and k_{ass}.

of the Δ MI-cystatin A fusion protein, apparently because enterokinase does not cleave before Pro residues (*42*). Instead, alternative cleavage procedures, giving low yield and necessitating subsequent purification, had to be used for Δ MI-cystatin A. The recovery of this mutant by cleavage with elastase or clostripain was thus only 10 and 5%, respectively, of the initial His-tagged protein. The two cleavage procedures gave identical products. SDS−PAGE under reducing conditions showed that all mutants were >99% pure, and N-terminal sequence analyses confirmed that they had the correct N-terminus. The relative molecular masses of the cystatin A variants were in good agreement with those deduced from the amino acid sequence, e.g., with differences of less than 0.04% for all variants. The stoichiometries of binding to papain ranged from 0.9 to 1.1 for the wild-type inhibitor and the three variants, i.e., were experimentally indistinguishable from 1:1. These results indicate that the purified recombinant variants were intact and fully active.

Circular Dichroism. The far-UV CD spectra for all cystatin A variants were indistinguishable, within experimental error, from that of the wild-type, with identical magnitudes and minima at approximately 215 nm (not shown). No conformational changes detectable by CD were thus induced by the deletions of N-terminal residues of cystatin A.

Affinity and Kinetics of Binding to Papain, Cathepsin L, and Cathepsin B. The affinities of the Δ MI and Δ MIP variants of cystatin A for papain were determined as K_i's from the inhibited and uninhibited equilibrium rates of substrate cleavage by the proteinase (Table 1). However, the high affinity of the Δ M mutant prevented the use of this method, and K_i for this mutant was instead calculated from separate measurements of k_{ass} and k_{diss}. Values of k_{ass} for the reactions between the three variants and papain were obtained by stopped-flow measurements under pseudo-first-order conditions in the presence of an enzyme substrate (Table 1). In these analyses, the values of $k_{obs,\,app}$ increased linearly with increasing concentration of the inhibitors, within the concentration range covered, consistent with a simple bimolecular reaction. k_{diss} for the interaction with papain was determined by displacement experiments for the Δ M mutant and from the intercept on the ordinate of the plot of $k_{obs,\,app}$ vs inhibitor concentration for the Δ MIP mutant (Table 1). Values of k_{diss} were also calculated from K_i and k_{ass} for the Δ MI and Δ MIP mutants, the value for the latter mutant being in good agreement with the measured value.

K_i values for the interactions of the Δ MI- and Δ MIP-cystatin A mutants with cathepsin L were determined by equilibrium methods, as for papain. However, the high affinity of the Δ M mutant for cathepsin L again prevented accurate measurements of K_i by equilibrium methods and only allowed determination of a lower limit for the affinity (Table 1). However, even this limit implicated that the interaction between the truncated mutant and papain was stronger than for the wild-type, the K_i of which had been estimated in an earlier study (*8*). This observation prompted a reassessment of the affinity of wild-type cystatin A for cathepsin L by equilibrium methods, which gave the same limit of K_i as for the mutant, i.e., a higher affinity than that obtained previously (Table 1). The kinetics of inhibition of cathepsin L by the Δ M and Δ MI variants were studied as in the case of papain, although at lower protein concentra-

tions, resulting in slower reactions and obviating the need of stopped-flow measurements (Table 1). The low affinity of Δ MIP-cystatin A for cathepsin L made studies of the association kinetics of this interaction impossible because of the high concentrations of proteinase required for stopped-flow experiments. A value of k_{diss} for the binding of Δ MI-cystatin A to cathepsin L could be obtained from the intercept of the plot of $k_{obs,\ app}$ vs inhibitor concentration used for the determination of k_{ass} and was close to the calculated k_{diss} (Table 1). Values of k_{diss} for the interactions of the other two mutants with cathepsin L could not be determined because of the limited amounts of enzyme available.

K_i for the interactions between the cystatin A variants and cathepsin B was determined by equilibrium methods (Table 1), as in the case of the other enzymes. Values of k_{ass} for the binding of the Δ M and Δ MI variants to cathepsin B were measured by experiments in the presence of substrate, as for cathepsin L (Table 1), but the kinetics of the interaction of Δ MIP-cystatin A with this enzyme could not be studied because of the very low affinity. Values of k_{diss} were calculated from K_i and k_{ass} (Table 1).

DISCUSSION

The aim of this work was to characterize the contribution of N-terminal residues of cystatin A to proteinase inhibition by analyses of the affinity and kinetics of interaction of N-terminal deletion mutants of the inhibitor with several cysteine proteinases. The solution structure of wild-type cystatin A shows that the N-terminal five residues are very flexible, and no interactions between amino acids before Leu-6 and the rest of the protein can be detected (*14*). Moreover, analysis of the structure of two Gly-4 cystatin A mutants (to Ala and Trp) by NMR has demonstrated that the conformation of the inhibitor is negligibly altered by the mutations (*38*). These observations, together with the unaltered CD spectra of the cystatin A variants, strongly suggest that deletion of the first residues of cystatin A is not accompanied by changes in the three-dimensional structure of the protein. Instead, the observed changes of the interaction with proteinases should reflect primarily the contribution of the deleted residues to binding.

The successive deletion of amino acid residues from the N-terminus of cystatin A (Figure 1) in general resulted in weaker inhibitors of the three enzymes studied in this work, i.e., papain, cathepsin L, and cathepsin B. However, truncation of the N-terminal Met-1 in the Δ M-cystatin A variant gave indistinguishable inhibitory properties against papain and cathepsin L and only a 4-fold lowered affinity for cathepsin B, indicating that this residue is of minimal importance for the interaction. This finding is in accordance with evidence that the N-terminal Met-1 of cystatin B, a homologous family I cystatin, is not involved in interactions with papain in the crystal stucture of the cystatin B−papain complex (*13*). The removal of both Met-1 and Ile-2 in the Δ MI-cystatin A variant had considerably larger effects, decreasing the affinity for papain and cathepsin B 900- and 200-fold, respectively. The interaction with cathepsin L was also weaker compared with that of the wild-type, although the magnitude of this effect could not be ascertained, as it was only possible to determine an upper limit for the K_i of the wild-type inhibitor. It is evident, however, that the Δ MI variant is a better inhibitor of cathepsin L than of papain by a factor of about 5. The most drastic effects were observed for the Δ MIP mutant, which also lacks the Pro-3 residue preceding the evolutionarily conserved Gly-4. Compared with the Δ MI variant, the affinity of this mutant for papain, cathepsin L, and cathepsin B decreased another 2000-, 20 000-, and 400-fold, respectively. The affinities of the Δ MIP variant for papain and cathepsin B, in comparison with those of the wild-type inhibitor for these enzymes, indicated that the N-terminal region is responsible for about 40% of the total free energy of binding of cystatin A to both proteinases. The N-terminal region of cystatin A is thus indispensable for the ability of the inhibitor to bind tightly to the enzymes studied in this work and contributes comparable binding energy as the corresponding, longer region of family II cystatins (*25*−*31*). Moreover, it is apparent that the contribution of individual residues in this region to the affinity for proteinases decreases with the distance toward the N-terminus from the conserved Gly-4 residue in a manner resembling that for family II cystatins (*27, 28, 31, 43*). The importance of the N-terminal region of cystatin A for proteinase binding is further demonstrated by the decreased affinities of Gly-4 mutants of the inhibitor for target proteinases (*37, 38*).

In contrast to these results, it has been reported that deletion of residues from the N-terminus of a Met-65 to Leu variant of cystatin A only affected the binding of the inhibitor to papain when the deletion included Pro-3 (*36*). Moreover, the decrease in affinity was only ∼200-fold for a Δ MIPG variant compared with the standard variant, whereas a $\sim 2 \times 10^6$-fold decrease in the affinity for papain was observed for the Δ MIP mutant in this work. These discrepancies are most certainly due primarily to difficulties in quantifying the very strong interactions of wild-type cystatin A and tight-binding variants of the inhibitor with papain by equilibrium methods. Values of K_i for papain of ∼0.2 pM were thus obtained in this work for wild-type and Δ M-cystatin A from separate determinations of k_{ass} and k_{diss}, whereas values of 1−2.5 nM were reported by Shibuya et al. (*36*) from equilibrium measurements. The affinities for papain of the variants with deletions including Pro-3 are more similar in the two studies, a K_i of 0.3 μM being obtained for Δ MIP-cystatin A in this work, compared with a value of 0.4 μM reported for Δ MIPG-cystatin A by Shibuya et al. (*36*). However, the uncertain binding constants for the other variants in the previous study render many conclusions regarding the role of residues in the N-terminal region of cystatin A in the binding to target proteinases somewhat doubtful.

The studies of the kinetics of formation and dissociation of the inhibitor−enzyme complexes, which were not characterized previously (*36*), provided further insight into how the N-terminal region of cystatin A participates in the binding to different proteinases. The values of k_{ass} for the binding of the inhibitor to papain and cathepsin L remained unaffected, within experimental error, on truncation of residues in this region, and the reduced affinity was only due to an increased k_{diss}; i.e., less stable complexes were formed. In contrast, the interactions of the truncated cystatin A variants with cathepsin B were affected by both a higher k_{diss} and a lower k_{ass}. These findings are akin to those of previous studies of the kinetics of binding of N-terminally truncated variants of family II cystatins to these enzymes (*26*−*28, 30*).

The mechanism proposed from these results for the binding of the N-terminal segment of cystatin A to papain and cathepsin L, which have exposed active-site clefts, is that this segment binds to the enzyme simultaneously with, or after, the other binding regions, i.e., the first and second binding loops. The N-terminal region thereby provides considerable stability to the complex by interactions of Ile-2 and Pro-3 with the S3- and S2-subsites, respectively, of the enzymes (*13*, *31*, *43*−*45*). The contribution of Pro-3 to the affinity is larger than that of Ile-2, especially in the binding to cathepsin L. It appears reasonable that the flexible N-terminal region of cystatin A (*14*), as well as those of chicken cystatin (*16*) and cystatin C (*17*), could bind in a second step, after an initial complex between the active-site cleft of the proteinase and the more rigid binding regions of the inhibitor, in particular the first binding loop, has been formed. However, stopped-flow kinetic studies at very high ligand concentrations have given no evidence for such binding, being consistent with a one-step mechanism for all these inhibitors (*8*, *18*−*22*).

The mechanism of binding of cystatin A to cathepsin B, in which the active-site cleft is partially covered by the "occluding loop" (*24*), presumably is different. In this reaction, the N-terminal region of the inhibitor is thus needed both for rapid association of the interacting proteins and for stabilization of the complex. These findings suggest that the complex is formed in two steps, analogous to the reaction between cystatin C and cathepsin B (*23*), and that in this case an initial binding of the N-terminal region of the inhibitor to the partially obstructed active-site cleft of the enzyme facilitates displacement of the occluding loop (*28*, *30*, *33*). This movement allows further interactions between the other regions of the inhibitory wedge of cystatin A and the enzyme, creating a more stable complex.

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