Biochem. J. (1991) 273, 621–626 (Printed in Great Britain)

Human cystatin C

Role of the *N*-terminal segment in the inhibition of human cysteine proteinases and in its inactivation by leucocyte elastase

Magnus ABRAHAMSON,*‖ Robert W. MASON,†‡ Heléne HANSSON,* David J. BUTTLE,‡ Anders GRUBB* and Kjell OHLSSON§

*Department of Clinical Chemistry, University of Lund, University Hospital, S-221 85 Lund, Sweden, †Department of Biochemistry and Nutrition, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0308, U.S.A., ‡Department of Biochemistry, Strangeways Research Laboratory, Worts Causeway, Cambridge CB1 4RN, U.K., and §Department of Surgical Pathophysiology, University of Lund, Malmö General Hospital, S-214 01 Malmö, Sweden

Leucocyte elastase in catalytic amounts was observed to rapidly cleave the Val-10–Gly-11 bond of the human cysteine-proteinase inhibitor cystatin C at neutral pH. The resulting modified inhibitor had size and amino acid composition consistent with a cystatin C molecule devoid of the *N*-terminal Ser-1–Val-10 decapeptide. Leucocyte-elastase-modified cystatin C had more than 240-fold lower affinity than native cystatin C for papain. Removal of the *N*-terminal decapeptide of human cystatin C also decreased inhibition of human cathepsins B and L by three orders of magnitude, but decreased inhibition of cathepsin H by only 5-fold. A tripeptidyldiazomethane analogue of part of the *N*-terminal portion of cystatin C was a good inhibitor of cathepsins B and L but a poor inhibitor of cathepsin H. It therefore appears that amino acid side chains of the *N*-terminal segment of cystatin C bind in the substrate-binding pockets of cathepsins B and L but not in those of cathepsin H. It is argued that the *N*-terminal cystatin C interaction with cathepsin B is physiologically important and hence that leucocyte elastase could have a function as a regulator of extracellular cysteine-proteinase inhibitory activity at sites of inflammation.

INTRODUCTION

Human cystatin C is a low-M_r protein of 120 amino acid residues in a single polypeptide chain (Grubb & Löfberg, 1982). It inhibits papain-like cysteine proteinases, such as human cathepsins B, H and L, by the formation of tight reversible complexes, with dissociation constants for formed complexes typically in the sub-nanomolar to nanomolar range (Barrett *et al.*, 1984). Cystatin C, which belongs to Family 2 of the cystatin superfamily (Barrett *et al.*, 1986*a*), is synthesized as a pre-protein with a signal peptide, indicating mainly extracellular functions of the inhibitor (Abrahamson *et al.*, 1987*a*). It has a widespread distribution in human extracellular fluids and is expressed in many human tissues (Abrahamson *et al.*, 1986, 1990). In addition, it is the human cystatin with highest affinity for cathepsin B and other human cysteine proteinases, and therefore appears to be one of the most physiologically important inhibitors of cysteine-proteinase activity in human extracellular fluids (Abrahamson *et al.*, 1986).

Because of their activities *in vitro*, it is assumed that inhibitors of the cystatin superfamily are involved in the control of mammalian cysteine proteinases (Barrett *et al.*, 1986*b*). Any mechanisms for the regulation of cysteine-proteinase inhibitory activities in extracellular fluids *in vivo* are not known, however. This contrasts with the serine-proteinase inhibitor α_1-proteinase inhibitor, which can be regulated by proteolytic processing mediated by cysteine proteinases, such as papain (Johnson & Travis, 1977) and, physiologically, cathepsin L (Johnson *et al.*, 1986).

It has previously been shown that the *N*-terminal 11 amino acid residues of cystatin C are important for its high-affinity binding to papain, a plant cysteine proteinase, and it has been suggested that the polypeptide chain region around the Gly-11 residue might serve as a substrate-like binding region for cysteine proteinases (Abrahamson *et al.*, 1987*b*). Peptides with amino acid sequences identical with that of the *N*-terminal segment of cystatin C are indeed good substrates for papain, being cleaved at the bond following residue Gly-11 after incubation with the enzyme (Abrahamson *et al.*, 1987*b*; Grubb *et al.*, 1990). A peptidyldiazomethane mimicking part of the proposed substrate-like-binding region of cystatin C displays irreversible inhibition of papain and a streptococcal cysteine proteinase and appears to have a potential as an antimicrobial drug (Björck *et al.*, 1989). No studies concerning the importance of the *N*-terminal portion of human cystatin C for the inhibition of human cysteine proteinases have been performed so far.

In the present investigation we obtained evidence *in vitro* that the serine proteinase leucocyte elastase could act as a regulator of cysteine-proteinase inhibitory activity in human biological fluids by cleaving a single *N*-terminal cystatin C bond. We have used native and leucocyte-elastase-truncated recombinant cystatin C, as well as two peptidyldiazomethanes, to elucidate the importance of the *N*-terminal portion of human cystatin C for the inhibition of human cysteine proteinases and thereby the effect that leucocyte-elastase-mediated removal of the *N*-terminal cystatin C segment would have.

EXPERIMENTAL

Materials

Native human cystatin C, obtained by *Escherichia coli* ex-

Abbreviations used: Z-, benzyloxycarbonyl-; Bz-, benzoyl-; Suc, 3-carboxypropionyl-; Boc-, butyloxycarbonyl-; NH-Mec, 7-(4-methyl)coumaryl-amide; -NH-Np, *p*-nitroanilide; E-64, L-3-carboxy-2,3-*trans*-epoxypropionyl-leucylamido-(4-guanidino)butane.

‖ To whom correspondence should be addressed.

pression, was produced and purified as previously described (Abrahamson *et al.*, 1988). Leucocyte elastase (EC 3.4.21.37) was isolated from human leucocytes (Gauthier *et al.*, 1982). The leucocyte elastase preparation was electrophoretically pure and did not hydrolyse the synthetic substrate Suc-Ala-Ala-Pro-Phe-NH-Np even at 25 μM concentration. This assay would have revealed trace amounts of contaminating cathepsin G in the preparation. Papain (EC 3.4.22.2) isolated from crude papaya latex by affinity chromatography on Gly-Gly-Tyr(Bzl)-Arg–Sepharose (Blumberg *et al.*, 1970), activatable to 90%, was a gift from Dr. I. Björk and Dr. P. Lindahl, Uppsala, Sweden. Human cathepsins B, H and L were prepared as described previously (Rich *et al.*, 1986; Schwartz & Barrett, 1980; Mason *et al.*, 1985). Z-Leu-Val-Gly-CHN_2 and Boc-Val-Gly-CHN_2 (Grubb *et al.*, 1990) were gifts from Dr. Z. Grzonka, Gdansk, Poland. E-64 and Suc-Ala-Ala-Pro-Phe-NH-Np were obtained from Sigma Chemical Co. Other synthetic substrates were obtained from Bachem Feinchemikalien, Bubendorf, Switzerland, or Cambridge Research Biochemicals, Harston, Cambridge, U.K. All other chemicals used were of analytical grade and obtained from BDH Chemicals.

Protein analyses

Analytical agarose-gel electrophoresis in barbital buffer, pH 8.6, was performed by the procedure of Jeppsson *et al.* (1979). Electrophoretograms were scanned with a Joyce–Loebl Chromoscan 3 apparatus. Analytical PAGE on slab gels in the presence of SDS was done as described by Laemmli (1970), with separation gels containing 20% acrylamide. Amino acid analyses with a Beckman High Performance Analyzer System 6300 and automated amino acid sequence analyses with an Applied Biosystems 470A gas–liquid solid-phase Sequenator were performed by standard methods.

Concentrations of native recombinant cystatin C and papain were determined by A_{280} measurements ($\epsilon_{280} = 12200$ and 58500 $M^{-1} \cdot cm^{-1}$ respectively; Cejka & Fleischmann, 1973; Glazer & Smith, 1971). The concentration of active leucocyte elastase was determined as previously described (Gauthier *et al.*, 1982). The concentration of modified cystatin C was determined by amino acid analysis.

Leucocyte elastase interaction with cystatin C

The interaction of cystatin C with leucocyte elastase was studied at 37 °C in 50 mM-Tris/HCl buffer, pH 8.0, containing 100 mM-NaCl. Incubations were done in sterile capped Microfuge tubes, to protect against bacterial growth during long incubations. After incubation, the proteinase was inactivated by addition of di-isopropyl phosphorofluoridate to a final concentration of 1 mM. The resulting modified cystatin C was isolated and desalted by gel chromatography at 22 °C on a Superose 12 10/30 column (Pharmacia) in 50 mM-ammonium bicarbonate buffer, pH 8.0, with a flow rate of 18 ml/h. Alternatively, preparative agarose-gel electrophoresis (Johansson, 1972) was used as an additional purification step before gel chromatography. Estimation of the M_r of modified cystatin C by gel chromatography was performed on the Superose column as described above. BSA (M_r 67000), ovalbumin (M_r 43000), β-lactoglobulin (M_r 35000), human carbonic anhydrase B (M_r 28700), chymotrypsinogen (M_r 23250), bovine ribonuclease (M_r 12650), cytochrome *c* (M_r 12400) and aprotinin (M_r 6500) served as calibration standards.

Enzyme assays

The concentration of active cystatin was determined by titration of papain, which itself had been titrated with E-64. Titration of the truncated cystatin was performed by measuring residual papain activity against Bz-Arg-NH-Np (Buttle & Barrett, 1984). Such an insensitive assay for papain was chosen because the titrated enzyme–inhibitor complex only needed to be diluted 4-fold in the assay, so that papain would not be significantly released from the weakly bound cystatin. For kinetic studies, papain was assayed at 37 °C with Z-Phe-Arg-NH-Mec as substrate in 100 mM-acetate buffer, pH 6.5, containing 1 mM-dithiothreitol and 2 mM-EDTA. Human cathepsins B, H and L were assayed with Z-Arg-Arg-NH-Mec at pH 6.0 and 37 °C, with Arg-NH-Mec at pH 6.8 and 30 °C and with Z-Phe-Arg-NH-Mec at pH 5.5 and 30 °C respectively, as described previously (Rich *et al.*, 1986; Schwartz & Barrett, 1980; Mason *et al.*, 1985).

Association rate constants (k_{+1}) and equilibrium constants (K_i) for reversible enzyme–inhibitor complexes (eqn. 1) were determined from continuous-rate assays as described (Nicklin & Barrett, 1984; Baici & Gyger-Marazzi, 1982):

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI \quad K_i = k_{-1}/k_{+1} \qquad (1)$$

Apparent second-order rates of inactivation for irreversible inhibitors (k_{+2}') were determined by continuously monitoring the activity of enzyme in the presence of inhibitor (Crawford *et al.*, 1988) (eqn. 2):

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI \xrightarrow{k_{+2}} EI \qquad (2)$$

$$\xrightarrow[k_{+2}' (= k_{+2}/K_i)]{}$$

RESULTS

Modification of cystatin C as a result of leucocyte-elastase action

Initial experiments to examine the effect of leucocyte elastase on cystatin C were performed by mixing cystatin C (final concentration 1 mg/ml, or approx. 75 μM) with isolated leucocyte elastase in 50 mM-Tris/HCl buffer, pH 8.0 in an enzyme/cystatin C molar ratio of 1:70. Samples were taken after various incubation times at 37 °C between 1 and 30 min, and the reaction was stopped by addition of di-isopropyl phosphorofluoridate. Analysis by charge-separating agarose-gel electrophoresis showed the rapid conversion of cystatin C into a modified form with a more anodal electrophoretic mobility (Fig. 1). Similar time-course experiments (now at 2 mg/ml cystatin C concentration) were performed with leucocyte elastase/cystatin C molar ratios ranging from 1:70 to 1:1000. The electrophoretograms were stained with Coomassie Blue and scanned in order to assess the rate of cystatin C modification at different enzyme concentrations (Fig. 2, top). In the conditions of the experiment, the half-life for cystatin C modification varied between approx. 1.5 min (at a molar ratio of 1:70) and 40 min (ratio 1:1000).

Prolonged incubation of leucocyte elastase/cystatin C mixtures at 37 °C (Fig. 2, bottom) revealed that the initially modified cystatin was stable for at least 2 h (for the highest enzyme concentration), but also that after longer incubation the modified cystatin C eventually was degraded. No further intermediate breakdown products could be detected on agarose-gel or SDS/polyacrylamide-gel electrophoretograms, however. The modified inhibitor formed in an 1:1000 leucocyte elastase/cystatin C incubation mixture was not significantly degraded after 73 h (Fig. 2, bottom). Control experiments with cystatin C alone in the pH 8 buffer at 37 °C revealed no breakdown of the inhibitor, even after 96 h incubation.

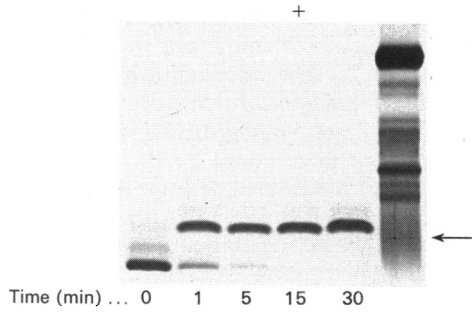

Fig. 1. Agarose-gel electrophoresis of a cystatin C/leucocyte elastase mixture

Cystatin C (final concentration 1 mg/ml) was incubated with leucocyte elastase at a 70:1 molar ratio at 37 °C and pH 8.0. Samples were taken after the times indicated and the elastase was inactivated by the addition of di-isopropyl phosphorofluoridate. Agarose-gel electrophoresis was performed in barbital buffer, pH 8.6, and the gel was stained with Coomassie Blue. An electrophoretogram of human blood plasma (far right) was included for comparison. The anode (plus sign) and point of sample application (arrow) are indicated.

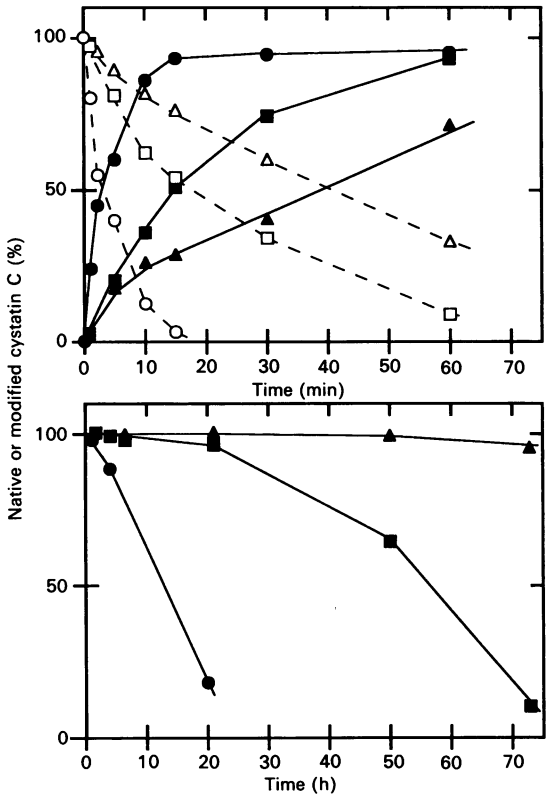

Fig. 2. Time-course experiments for the modification of cystatin C by leucocyte elastase

Mixtures of cystatin C (2 mg/ml) and leucocyte elastase were incubated at 37 °C and pH 8.0. Samples of the mixtures were taken after different periods of time and analysed by agarose-gel electrophoresis. The electrophoretograms were scanned and the areas under the peaks corresponding to native cystatin C (○, □ and △) and the modified inhibitor (●, ■ and ▲) were determined and related to the amount of native cystatin C at zero time (100%). The data shown were from incubation mixtures with elastase/cystatin C molar ratios of 1:70 (○ and ●), 1:300 (□ and ■) and 1:1000 (△ and ▲). The diagram is shown with two different time-scales, to highlight the rapid initial formation (upper) and the final slow degradation (lower) of modified cystatin C.

Isolation and physicochemical characterization of leucocyte-elastase-modified cystatin C

A 3 mg portion of cystatin C was incubated with leucocyte elastase at an 150:1 molar ratio for 3 h, after which no native cystatin C could be detected by agarose-gel electrophoresis of the sample. Modified cystatin C was isolated and desalted in one step by gel chromatography on Superose. *N*-Terminal sequence analysis of the isolated protein gave the single sequence Gly-Gly-Pro-Met-. This sequence only appears in the cystatin C sequence once, from native cystatin C residue Gly-11 onwards (Grubb & Löfberg, 1982). The approximate M_r of the modified protein as determined by gel chromatography on Superose 12 was 13300. Native cystatin C was eluted from the same column at a volume corresponding to an M_r of 13700. SDS/PAGE in reducing conditions gave M_r estimations for modified and native cystatin C of 14500 and 15300 respectively (Fig. 3). These results, together with amino acid analysis of the modified inhibitor (Table 1), were all consistent with leucocyte-elastase-modified cystatin being a cystatin C molecule devoid of the *N*-terminal Ser-1–Val-10 decapeptide. The observed modification of cystatin C by leucocyte elastase must therefore be due to cleavage of the single cystatin C Val-10–Gly-11 bond.

Papain-inhibitory activity of modified cystatin C

Three different preparations of modified cystatin C were used for inhibition studies. The modified inhibitor in two of these was isolated as described above; the protein in the third had been subjected to an additional purification step comprising preparative gel electrophoresis before gel chromatography. The equilibrium constant for dissociation (K_i) of the modified inhibitors in these, as well as of native cystatin C, from complexes with papain were assessed from continuous rate assays with Z-Phe-Arg-NH-Mec as substrate. The K_i value for native recombinant cystatin C was below the limit of sensitivity in the assay, < 5 pM, as was found for cystatin C isolated from human urine (Barrett *et al.*, 1984). The mean K_i value for cystatin C modified by leucocyte elastase was calculated to be 1.2 nM. Thus the initial rapid cleavage by leucocyte elastase of cystatin C results in a more than 240-fold decrease in the papain affinity of the inhibitor.

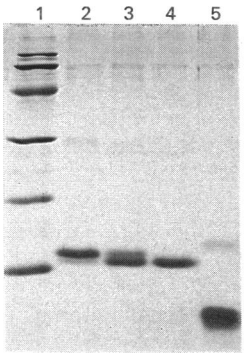

Fig. 3. SDS/PAGE of native and modified cystatin C

Proteins were subjected to electrophoresis in 20% polyacrylamide. Isolated modified cystatin C is shown in lane 4. Samples from a cystatin C/elastase mixture (molar ratio 300:1), incubated for 1 and 15 min (containing approx. 95% and 40% native cystatin C respectively) are in lanes 2 and 3. Size markers are shown in lane 1 (phosphorylase *b*, M_r 94000; BSA, M_r 67000; ovalbumin, M_r 43000; carbonic anhydrase, M_r 30000; soya-bean trypsin inhibitor, M_r 20100; α-lactalbumin, M_r 14400) and 5 (aprotinin, M_r 6500).

Table 1. Amino acid composition of leucocyte-elastase-modified cystatin C

Three samples of the modified inhibitor were hydrolysed in 6 M-HCl at 110 °C for 24 h, 48 h and 72 h, and the values obtained are compared with theoretical values for cleaved and uncleaved cystatin C. Unless otherwise stated (see the footnotes), the experimental values are means. Abbreviation: N.D., not determined.

Amino acid	Amino acid composition (residues/molecule)		
	Native cystatin C*	Modified cystatin C†	Des-(1-10)-cystatin C‡
Asx	12	12.1	12
Thr§	7	7.0	7
Ser§	8	6.5	6
Glx	12	11.8	12
Pro	8	5.4	5
Gly	8	7.3	7
Ala	10	9.0	10
Cys	4	N.D.	4
Val	10	7.6	9
Met	3	2.5	3
Ile	2	1.8	2
Leu	8	7.0	7
Tyr	4	3.7	4
Phe	5	4.8	5
Lys	7	6.2	6
His	3	3.5	3
Arg	8	6.2	7
Trp	1	N.D.	1
Total	120	102.4	110

* Theoretical amino acid composition for native cystatin C, from the amino acid sequence of human cystatin C (Grubb & Löfberg, 1982, 1985).

† Calculated assuming seven residues of leucine per molecule.

‡ Theoretical amino acid composition for cystatin C devoid of the *N*-terminal Gly-1–Val-10 decapeptide.

§ Values obtained after extrapolation to 0 h hydrolysis.

Importance of the *N*-terminal segment of cystatin C in the inhibition of human cysteine proteinases

The observed effect of *N*-terminal truncation on the papain-inhibitory activity of cystatin C prompted us to study the cystatin C interaction with human cysteine proteinases. The affinities for cathepsins B, H and L of native recombinant cystatin C, as measured by determining equilibrium constants (K_i) for dissociation of formed complexes (Table 2*a*), were found to be similar to those found for the native inhibitor isolated from human extracellular fluids (Barrett *et al.*, 1984). Removal of the *N*-terminal decapeptide of cystatin C resulted in a considerable decrease in the affinity of the inhibitor for human cathepsins B and L, but had a negligible effect on the inhibition of cathepsin H, indicating that the *N*-terminal portion of human cystatin C increases the specificity of this inhibitor for cathepsins B and L, but is not necessary for the inhibition of cathepsin H.

Association rates (k_{+1}) for interactions of native and *N*-terminally truncated cystatin C with cathepsins B, H and L were determined (Table 2*b*). The truncated form of cystatin C displayed approx. 350-fold slower association to cathepsin B than the native inhibitor. The association rates of both native and truncated cystatin C for cathepsins L and H were very fast. The rate constants for these interactions could therefore only be approximately estimated, and any conclusions with regard to an effect caused by removal of the *N*-terminal decapeptide could not be drawn.

Synthetic diazomethyl ketone analogues of the *N*-terminal portion of cystatin C (Fig. 4) were found to be rapid irreversible inhibitors of cathepsins B and L but were poor inhibitors of cathepsin H (Table 2*c*). The tripeptidyl inhibitor Z-Leu-Val-Gly-CHN_2 was a better inhibitor of cathepsins B and L than was the dipeptidyl inhibitor Boc-Val-Gly-CHN_2, showing that both the amino acid residues in positions P_2 and P_3 contribute in the binding of the Leu-Val-Gly peptide to the enzymes. These results correspond to the known substrate-specificities of these enzymes for protein and synthetic substrates, in that cathepsins B and L can hold hydrophobic residues in their S_2 and S_3 substrate-binding pockets (Barrett & Kirschke, 1981).

DISCUSSION

The results presented in this paper show that leucocyte elastase can specifically cleave the single *N*-terminal Val-10–Gly-11 bond of cystatin C, giving rise to a stable truncated inhibitor. The modified inhibitor has drastically decreased affinity for the cysteine proteinase papain, as well as for the human enzymes cathepsins B and L. Similar results have been observed for another truncated form of cystatin C, lacking the 11 most *N*-terminal amino acid residues (Abrahamson *et al.*, 1987*b*). This truncated cystatin C, being the result of cleavage of the cystatin C Gly-11–Gly-12 bond by the plant cysteine proteinase papaya proteinase IV (Buttle *et al.*, 1990*a*), displayed more than 1000-fold decreased affinity for papain. The inhibition data therefore demonstrate that the *N*-terminal segment of cystatin C plays an important role in the binding and inhibition of cysteine endopeptidases. The finding that truncation of cystatin C results in only minor changes in inhibition of cathepsin H suggests that the *N*-terminal portion of the inhibitor has little affinity for this enzyme, even though cathepsin H, like cathepsins B and L, has endopeptidase activity (Mason, 1989). With respect to inhibition by cystatin C, cathepsin H is thus more similar to dipeptidyl peptidase I, another lysosomal aminopeptidase, which is equally well inhibited by *N*-terminally truncated or native cystatin C (Abrahamson *et al.*, 1987*b*).

It has also been shown that the *N*-terminal segment of chicken cystatin, another Family 2 cystatin, is important for binding of the inhibitor to papain (Abrahamson *et al.*, 1987*b*; Machleidt *et al.*, 1989). Theoretical docking experiments using the recently elucidated three-dimensional crystal structure of chicken cystatin and that of papain, indicate that the residue analogous to Gly-11 of human cystatin C could be positioned closes to the S_1 substrate-binding pocket of the enzyme in the enzyme–inhibitor complex (Bode *et al.*, 1988). The two preceding amino acid residues, at positions analogous to Leu-9 and Val-10 of human cystatin C, could, according to this docking model, interact with the enzyme substrate-binding pockets S_3 and S_2. Evidence has been given that these residues are indeed responsible for the increased papain affinity contributed by the *N*-terminal segment of chicken cystatin (Machleidt *et al.*, 1989). This is in full agreement with our results, which show that peptidyldiazomethanes based on the *N*-terminal portion of human cystatin C are efficient cysteine-proteinase inhibitors because of the interactions between amino acid residues equivalent to cystatin C residues Leu-9 and Val-10 and the enzyme substrate-binding pockets S_3 and S_2 respectively. Thus it appears that the *N*-terminal portion of cystatins is a good candidate for a part of the molecule conferring a certain degree of specificity to the interaction with potential target enzymes. Assuming that this is the case, one could make predictions of what effect the *N*-terminal portions of other cystatins may have on the binding of cysteine proteinases based on inhibition by synthetic active-site-directed inhibitors or hydrolysis of synthetic substrates. Alignment of cystatins according to the evolutionarily conserved glycine residue (Gly-11 in

Table 2. Inhibition of human cysteine proteinases by native and truncated cystatin C and by analogues of its *N*-terminal segment

The values given are means ± S.D. calculated from at least three determinations.

(*a*) Determination of equilibrium constants

	K_i (nM)		
	Cathepsin B	Cathepsin L	Cathepsin H
Native cystatin C	0.148 ± 0.011	< 0.005	0.35 ± 0.027
Truncated cystatin C	101 ± 4	2.11 ± 0.29	2.15 ± 0.27

(*b*) Determination of association rate constants for reversible interactions

	$10^{-6} \times k_{+1}$ ($M^{-1} \cdot s^{-1}$)		
	Cathepsin B	Cathepsin L	Cathepsin H
Native cystatin C	12 ± 1	140*	> 570†
Truncated cystatin C	0.033 ± 0.003	> 90†	> 90†

(*c*) Determination of association rate constants for irreversible interactions

	k_{+2}' ($M^{-1} \cdot s^{-1}$)		
	Cathepsin B	Cathepsin L	Cathepsin H
Z-Leu-Val-Gly-CHN_2	10639 ± 337	118203 ± 9674	104 ± 40
Boc-Val-Gly-CHN_2	2959 ± 215	6727 ± 399	345 ± 12

* k_{+1} was determined with $[I] \gg K_i$, and treating the inhibition as pseudo-irreversible (Crawford *et al.*, 1988).
† k_{+1} was determined as k_{-1}/K_i, but the maximum measurable value for k_{-1} was 0.2 s^{-1}, and hence the values given are minimal estimates of k_{+1}.

human cystatin C) shows that human cystatins A, S and SN have a proline residue in the position that should be equivalent to P_2 (Barrett *et al.*, 1986*b*). Z-Pro-Gly-CHN_2 is not an inhibitor of cathepsin B (Green & Shaw, 1981), suggesting that the *N*-terminal portions of these cystatins are not involved in binding to cathepsin B. Indeed, the affinity of cystatin A for cathepsin B is lower than that of cystatin C (Barrett *et al.*, 1986*b*).

Our data show that leucocyte elastase can cleave cystatin C at catalytic concentrations. Both proteins are secreted by cells into extracellular body fluids (Ohlsson & Olsson, 1978; Abrahamson *et al.*, 1986; Delshammar *et al.*, 1989), and hence it is possible that such cleavage may be physiologically relevant. The cystatin C variant found in amyloid deposits in brain arteries from patients with hereditary cystatin C amyloid angiopathy contains an amino acid substitution Leu-68→Gln, and also lacks the ten most *N*-terminal amino acid residues of normal cystatin C (Grubb & Löfberg, 1982, 1985; Ghiso *et al.*, 1986). The *N*-terminal modification could be the result of leucocyte-elastase cleavage of the bond Val-10–Gly-11 in a full-length variant cystatin C molecule.

The enzyme-inhibitory properties of truncated cystatin C resulting from leucocyte-elastase cleavage given here can be used to evaluate the contribution of the *N*-terminal segment of cystatin C to the physiological binding of the inhibitor to target proteinases. For an enzyme–inhibitor interaction to be physiologically significant, several kinetic criteria should be met. These include $[I] \gg [E]$, association rates (k_{+1}) must be fast and $[I] \gg K_i$ (Bieth, 1984). The concentration of cystatin C in various biological fluids is in the range 0.1–1 μM (Abrahamson *et al.*, 1986), although the local concentrations around cells and tissues may vary considerably. The concentration of the cysteine proteinases can be as high as millimolar in lysosomes, but is considerably less in extracellular fluids (Barrett & Kirschke, 1981). Therefore in most, if not all, biological fluids the condition $[I] \gg [E]$ will be met. The association rates for inhibition of cathepsins B, H and L by native cystatin C are very fast, such that the half-life of cathepsin B in most biological fluids will be less than 1 s, and the half-lives of cathepsins H and L less than 100 ms. The equilibrium constants for the interactions between native cystatin C and cathepsins B, H and L are all much lower than the concentration of cystatin C in biological fluids. We can therefore conclude that at equilibrium native cystatin C can be a physiologically significant inhibitor of the activities of all of these proteinases. Leucocyte-elastase-mediated removal of the *N*-terminal deca-

```
                          10        20
Native cystatin C   SSPGKPPRLVGGPMDASVEEEGVRRALD...
Truncated cystatin C          GGPMDASVEEEGVRRALD...
                    (Z)LVG(CHN2)
                    (Boc)VG(CHN2)
```

Fig. 4. Inhibitors used to elucidate the importance of the cystatin C *N*-terminal segment for cysteine-proteinase inhibition

The *N*-terminal amino acid sequences of the single polypeptide chains of full-length (native) and leucocyte-elastase-modified (truncated) human cystatin C are shown in the one-letter code. The synthetic peptidyldiazomethane inhibitors used are shown below, with the peptidyl parts aligned with the corresponding segments of the cystatin C polypeptide chain. Lettering in parentheses indicate components that are not amino acid residues.

peptide of cystatin C would not seriously affect the inhibition of cathepsins L and H, since [I] would still be $\gg K_i$ and the fast association rates would result in half-lives of the enzymes of less than 1 s. The physiological inhibition of cathepsin B would be affected, however. Truncation of the inhibitor results in a K_i close to [I] in several biological fluids, and the half-life of cathepsin B will be prolonged to much more than 1 s. Therefore we can conclude that, at least in the case of cathepsin B, the *N*-terminal segment of cystatin C is important for the inhibitor to play a physiologically significant role. Leucocyte elastase may thus down-regulate the inhibition of extracellular cathepsin B activity and therefore influence conditions in which the cystatin C/cathepsin B balance might be of importance, e.g. sepsis (Assfalg-Machleidt *et al.*, 1988), metastasing cancer (Pietras *et al.*, 1978; Köppel *et al.*, 1984), active bone resorption (Delaissé *et al.*, 1984) and at local inflammatory processes in rheumatoid arthritis (Mort *et al.*, 1984) and purulent bronchiectasis (Buttle *et al.*, 1990*b*). It should be noted that the inflammatory conditions mentioned are also accompanied by high extracellular concentrations of leucocyte elastase (Ohlsson & Tegner, 1975; Delshammar *et al.*, 1989; Buttle *et al.*, 1990*b*).

The work in Lund was supported by grants from A. G. Crafoord's Foundation, T. Nilson's Foundation, A. Österlunds Stiftelse, Direktör A. Påhlssons Stiftelse, G. & J. Kocks Stiftelser, Ferring A.B., Immuknowledge A.B., the Medical Faculty of the University of Lund and the Swedish Medical Research Council (Project nos. 05196 and 09291). The work in Malmö was supported by grants from the Medical Faculty of the University of Lund and the Swedish Medical Research Council (Project no. 03910).

REFERENCES

Abrahamson, M., Barrett, A. J., Salvesen, G. & Grubb, A. (1986) J. Biol. Chem. **261**, 11282–11289

Abrahamson, M., Grubb, A., Olafsson, I. & Lundwall, Å. (1987*a*) FEBS Lett. **216**, 229–233

Abrahamson, M., Ritonja, A., Brown, M. A., Grubb, A., Machleidt, W. & Barrett, A. J. (1987*b*) J. Biol. Chem. **262**, 9688–9694

Abrahamson, M., Dalbøge, H., Olafsson, I., Carlsen, S. & Grubb, A. (1988) FEBS Lett. **236**, 14–18

Abrahamson, M., Olafsson, I., Palsdottir, A., Ulvsbäck, M., Lundwall, Å., Jensson, O. & Grubb, A. (1990) Biochem. J. **268**, 287–294

Assfalg-Machleidt, I., Jochum, M., Klaubert, W., Inthorn, D. & Malchleidt, W. (1988) Biol. Chem. Hoppe-Seyler **369** (Suppl.), 263–269

Baici, A. & Gyger-Marazzi, M. (1982) Eur. J. Biochem. **129**, 33–41

Barrett, A. J. & Kirschke, H. (1981) Methods Enzymol. **80**, 535–561

Barrett, A. J., Davies, M. E. & Grubb, A. (1984) Biochem. Biophys. Res. Commun. **120**, 631–636

Barrett, A. J., Fritz, H., Grubb, A., Isemura, S., Järvinen, M., Katunuma, N., Machleidt, W., Müller-Esterl, W., Sasaki, M. & Turk, V. (1986*a*) Biochem. J. **236**, 312

Barrett, A. J., Rawlings, N. D., Davies, M. E., Machleidt, W., Salvesen, G. & Turk, V. (1986*b*) in Proteinase Inhibitors (Barrett, A. J. & Salvesen, G., eds.), pp. 515–569, Elsevier, Amsterdam

Bieth, J. G. (1984) Biochem. Med. **32**, 387–397

Björck, L., Åkesson, P., Bohus, M., Trojnar, J., Abrahamson, M., Olafsson, I. & Grubb, A. (1989) Nature (London) **337**, 385–386

Blumberg, S., Schechter, I. & Berger, A. (1970) Eur. J. Biochem. **15**, 97–102

Bode, W., Engh, R., Musil, D., Thiele, U., Huber, R., Karshikov, A., Brzin, J., Kos, J. & Turk, V. (1988) EMBO J. **7**, 2593–2599

Buttle, D. & Barrett, A. J. (1984) Biochem. J. **223**, 81–88

Buttle, D. J., Ritonja, A., Dando, P. M., Abrahamson, M., Shaw, E. N., Wikstrom, P., Turk, V. & Barrett, A. J. (1990*a*) FEBS Lett. **262**, 58–60

Buttle, D. J., Burnett, D. & Abrahamson, M. (1990*b*) Scand. J. Clin. Lab. Invest. **50**, 509–516

Cejka, J. & Fleischmann, L. E. (1973) Arch. Biochem. Biophys. **157**, 168–176

Crawford, C., Mason, R. W., Wikstrom, P. & Shaw, E. (1988) Biochem. J. **253**, 751–758

Delaissé, J.-M., Eeckout, Y. & Vaes, G. (1984) Biochem. Biophys. Res. Commun. **125**, 441–447

Delshammar, M., Lasson, Å. & Ohlsson, K. (1989) Surgery **106**, 555–562

Gauthier, F., Fryksmark, U., Ohlsson, K. & Bieth, J. G. (1982) Biochim. Biophys. Acta **700**, 178–183

Ghiso, J., Jensson, O. & Frangione, B. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 2974–2978

Glazer, A. N. & Smith, E. L. (1971) Enzymes 3rd. Ed. **3**, 501–546

Green, G. D. J. & Shaw, E. (1981) J. Biol. Chem. **256**, 1923–1928

Grubb, A. & Löfberg, H. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 3024–3027

Grubb, A. & Löfberg, H. (1985) Scand. J. Clin. Lab. Invest. **45**, Suppl. **177**, 7–13

Grubb, A., Abrahamson, M., Olafsson, I., Trojnar, J., Kasprzykowska, R., Kasprzykowski, F. & Grzonka, Z. (1990) Biol. Chem. Hoppe-Seyler **371** (Suppl.), 137–144

Jeppsson, J.-O., Laurell, C.-B. & Franzén, B. (1979) Clin. Chem. **25**, 629–638

Johansson, B. G. (1972) Scand. J. Clin. Lab. Invest. **29**, Suppl. **124**, 7–19

Johnson, D. A. & Travis, J. (1977) Biochem. J. **163**, 639–641

Johnson, D. A., Barrett, A. J. & Mason, R. W. (1986) J. Biol. Chem. **261**, 14748–14751

Köppel, P., Baici, A., Keist, R., Matzku, S. & Keller, R. (1984) Exp. Cell Biol. **52**, 293–299

Laemmli, U. K. (1970) Nature (London) **227**, 680–685

Machleidt, W., Thiele, U., Laber, B., Assfalg-Machleidt, I., Esterl, A., Wiegand, G., Kos, J., Turk, V. & Bode, W. (1989) FEBS Lett. **243**, 234–238

Mason, R. W. (1989) Arch. Biochem. Biophys. **273**, 367–374

Mason, R. W., Green, G. D. J. & Barrett, A. J. (1985) Biochem. J. **226**, 233–241

Mort, J. S., Recklies, A. D. & Poole, A. R. (1984) Arthritis Rheum. **27**, 509–515

Nicklin, M. J. H. & Barrett, A. J. (1984) Biochem. J. **223**, 245–253

Ohlsson, K. & Olsson, A.-S. (1978) Hoppe-Seyler's Z. Physiol. Chem. **359**, 1531–1539

Ohlsson, K. & Tegner, H. (1975) Eur. J. Clin. Invest. **5**, 221–227

Pietras, R. J., Szego, C. M., Mangan, C. E., Seeler, B. J., Burtnett, M. M. & Orevi, M. (1978) Obstet. Gynecol. **52**, 321–327

Rich, D. H., Brown, M. A. & Barrett, A. J. (1986) Biochem. J. **235**, 731–734

Schwartz, W. N. & Barrett, A. J. (1980) Biochem. J. **191**, 487–497

Received 1 May 1990/12 July 1990; accepted 20 July 1990