Biochemical and Biophysical Research Communications **258,** 6–10 (1999)
Article ID bbrc.1999.0570, available online at http://www.idealibrary.com on IDEAL®

Competitive Inhibition of Cathepsin C by Guanidinium Ions and Reexamination of Substrate Inhibition[1]

Blaž Cigić[2] and Roger H. Pain
Department of Biochemistry and Molecular Biology, J. Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia

Received March 5, 1999

Cathepsin C, a lysosomal dipeptidyl aminopeptidase, is competitively and reversibly inhibited by guanidinium ions with a $K_i \approx 1.5$ mM. Loss of activity is not the result of conformational change, subunit dissociation or altered mobility of the enzyme, but rather reflects a specific binding of guanidinium ions to the active site. The finding that cathepsin C is not inhibited by substrate has allowed the kinetic parameters in the presence of guanidinium ion to be determined. Guanidinium significantly decreases the K_m of substrate hydrolysis, without changing V_{max}. In a novel application of the transferase reaction, the K_m of the nucleophile substrate has been determined (11 mM) and found not to be affected by guanidinium, indicating its inhibition of substrate binding to the S, but not the S′, site. Inhibition is suggested to be the result of shielding a negative charge on the enzyme important for interaction with the substrate.

Cathepsin C (EC 3.4.14.1) is a lysosomal cysteine proteinase whose main function is protein degradation and activation of proenzymes (1). The oligomeric composition of cathepsin C (2), the large part of the proregion in the mature enzyme (2-5) and activation by certain monovalent anions (6), of which chloride is the most effective, are the most significant characteristics of the enzyme. On the basis of the reported sigmoidal activation by chloride (7), the enzyme was proposed to be under allosteric regulation.

In the course of studying the structure and function of cathepsin C, inhibition by millimolar concentrations of guanidinium ions was observed. GdmCl unfolds most proteins at molar concentrations, however smaller conformational changes (8,9), subunit dissociation (10,11) or binding of guanidinium ion to the active site residues (12,13), resulting in the loss of activity, can occur in sub-molar concentrations of guanidinium chloride. In order to explain the observed inhibition, the stability of the enzyme and binding of Gdm^+ ion to cathepsin C were studied. In the course of these experiments the inhibition by substrate was reexamined.

EXPERIMENTAL PROCEDURES

Materials. Cathepsin C was purified from human kidney (14); cathepsin H from porcine spleen (15) was a gift from Ivica Klemenčič. Urea and GdmCl were from Serva. Gly-Phe-4-methoxy-β-naphthylamide (Gly-Phe-4MβNA), Pro-Phe-OH, Pro-Phe-NH_2.Cl and Lys-Tyr-OH.acetate were from Bachem Germany. All other chemicals were of analytical grade.

Fluorimetric determination of enzyme activity. Hydrolysis of Gly-Phe-4MβNA was followed at 25°C using a Perkin Elmer LS-50B spectrofluorimeter with excitation at 360 nm and emission at 415 nm.

Spectroscopic characterization of conformational changes. Fluorescence of cathepsin C was measured with excitation at 280 nm and emission at 300-400 nm. The enzyme was incubated 16 hours in the presence of GdmCl prior to measurement. Near UV CD spectra were measured using an AVIV CD Spectrometer 62A DS. For quenching experiments, cathepsin C was dissolved in 400 mM acrylamide in 200 mM sodium phosphate pH 6 and titrated with 6 M GdmCl dissolved in 200 mM sodium phosphate buffer pH 6 and with buffer alone. After 5 minutes incubation fluorescence emission was monitored at 355 nm with excitation at 295 nm. The final concentration of acrylamide was 280 mM**.** The activity of the enzyme was not affected by the quencher in the range 0 to 500 mM. Fluorescence of the blanks was determined in the same way, except that cathepsin C was dissolved in sodium phosphate buffer in the absence of acrylamide. Stopped flow fluorescence measurements of enzyme activity were performed on a DX.17MV Sequential Stopped-flow Spectrofluorimeter with excitation at 335 nm and emission cut-off filter at 400 nm.

Gel exclusion chromatography. A calibrated Superdex S-200 column, total volume 22 ml and void volume 8.5 ml, was used to determine the molecular mass of cathepsin C in the presence and in the absence of 500 mM GdmCl.

Spectrophotometric determination of k_{cat} and K_m. Hydrolysis of Gly-Phe-4MβNA was followed by the increase in absorbance at 345 nm for the range of substrate concentration 0.01 to 1.1 mM. Reductively activated cathepsin C was added to a final concentration of 2 nM to 200 mM phosphate buffer, pH 6.0 containing 50 mM NaCl together with, in experiments with inhibitor, 20 mM guanidinium phosphate. The concentration of dimethylsulfoxide in the assay,

[1] This work was supported by the Ministry of Science and Technology of the Republic of Slovenia.
[2] To whom correspondence should be addressed. Fax: +386 61 273594. E-mail: blaz.cigic@ijs.si.
Abbreviations used: 4MβNA, 4-methoxy-β-naphthylamide, Gdm^+, guanidinium ion.

0006-291X/99 $30.00

after adding 10 mM substrate in dimethylsulfoxide, was 10%. k_{cat} and K_m were obtained by curve fitting according to the Michaelis-Menten equation.

Chromatographic determination of k_{cat} and K_m. The hydrolysis of Pro-Phe-NH_2 was followed chromatographically. The reaction was started by adding reductively activated enzyme (100 nM final concentration) to the substrate dissolved in 200 mM sodium phosphate buffer at pH 7.5, containing 50 mM NaCl, 10% dimethylsulfoxide and, in experiments with inhibitor, 20 mM guanidinium phosphate. Aliquots of 15 μl were transferred after 1, 3 and 5 minutes to 135 μl of 1% TFA to stop the reaction. Substrates and products of the reaction were separated on a Chrompack C10 column (3 mm × 100 mm) using an HPLC system (1100, Hewlett Packard with diode array detector) with a gradient of acetonitrile in the aqueous buffer containing 0.1% TFA. The positions of the peaks and molarity of the peptides in the reaction mixture were determined using Pro-Phe-NH_2 and Pro-Phe-OH as standards, monitoring the absorbance at 215 nm. The hydrolysis rate at each concentration of substrate is the average of rates obtained after 1, 3 and 5 minutes. k_{cat} and K_m values were obtained as for Gly-Phe-4MβNA.

Transferase assays. These were carried out with 10 mM Pro-Phe-NH_2 and various concentrations (2.5–150 mM) of Lys-Tyr-OH in the reaction mixture. The procedure was the same as for the hydrolysis of Pro-Phe-NH_2. Absorbance was measured simultaneously at 215 nm and 274 nm during the chromatographic separation. The concentration of product, Pro-Phe-Lys-Tyr-OH, was determined from the absorbance at 274 nm, using Lys-Tyr-OH as a standard, on the assumption that both have the same molar extinction coefficient at 274 nm. k_{cat} and K_m values were obtained as for Gly-Phe-4MβNA.

RESULTS AND DISCUSSION

Cathepsin C is inhibited by millimolar concentrations of Gdm^+ ions. During initial studies on denaturation and renaturation of cathepsin C, activity was found to be lost in the presence of millimolar concentrations of Gdm^+ ions. Only 10% activity remained in the presence of 20 mM GdmCl. Sulfate, an anion known to counteract the denaturing effect of Gdm^+ (16), gave no significant protection against loss of activity (Fig. 1). In the presence of 100 mM concentrations of two other denaturants, urea and calcium ion, cathepsin C retained 95% and 80% respectively of its activity against 20 μM Gly-Phe-4MβNA, strongly suggesting that the observed inhibition is not due to the denaturing action of guanidinium.

The inhibition is not a common feature of enzymes from the papain superfamily, in that papain (assayed with carbobenzoxy-Phe-Arg-4-methylcoumaryl-7-amide) retained 100% and cathepsin H, a cysteine monoaminopeptidase, (assayed with Arg-β-naphthylamide) 90% of activity in the presence of 100 mM Gdm^+. These results suggest the presence of a specific site for the guanidinium ion on cathepsin C.

Inhibition by GdmCl is not due to denaturation. In order to examine further the mode of action of GdmCl, a number of conformational and enzymological probes have been used. The peak wavelength of tryptophan fluorescence for cathepsin C is 337 nm and no shift to higher wavelengths was found in the presence of GdmCl at concentrations less than 2 M, i.e. two orders

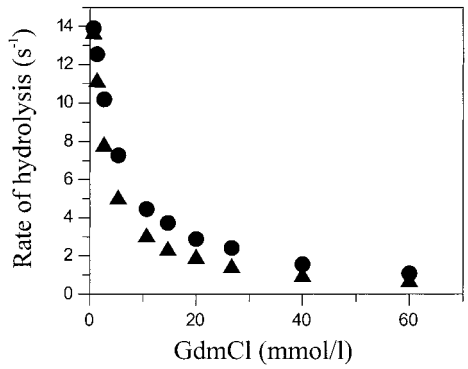

FIG. 1. Effect of Gdm^+ ions on the rate of hydrolysis by cathepsin C. Rates were measured using 20 μM Gly-Phe-4MβNA in 200 mM sodium phosphate, 50 mM NaCl, pH 6 in the absence (▲) and in the presence (●) of 200 mM Na_2SO_4.

of magnitude higher than that needed for more than 90% inhibition. The linearity of the increase in fluorescence intensity in the plateau region is characteristic of a non-specific solvent effect (17). Attempts to renature cathepsin C from GdmCl at concentrations higher than 2 M, either by dilution or by dialysis, have not resulted in regain of enzyme activity, indicating that general denaturation is irreversible. However dialysis or dilution from 200 mM GdmCl, where complete inhibition is observed, led to regain of full activity, indicating that the inhibition is reversible and distinct from general denaturation. This conclusion is further supported by the kinetics of inhibition which were measured by double jump experiments with stopped flow fluorescence. Equal volumes of Gly-Phe-4MβNA and activated cathepsin C were premixed. After 1 second incubation, equal volumes of the mixture and 40 mM GdmCl were mixed. The rate of substrate hydrolysis was constant in the interval 50 ms to 10 s at the level found above for enzyme in 20 mM GdmCl, indicating that the inhibition by GdmCl is very fast, with a half time less than 10 ms. The possibility remains, however, that submolar concentrations of Gdm^+ could lead to minor conformational changes, altered structural mobility of the enzyme or loss of quaternary structure.

Near UV CD provides a sensitive fingerprint of small changes in tertiary interactions and dynamics. The spectra of cathepsin C in the presence and in the absence of GdmCl (Fig. 2) did not differ significantly in shape or intensity.

Acrylamide can penetrate into a protein and quench the fluorescence of buried tryptophans, sensing changes in structure and mobility (18). Iodide quenching experiments show that approximately 45% of the tryptophans are inaccessible to solvent in cathepsin C. The fluorescence of the enzyme was measured in the presence and absence of acrylamide at a range of GdmCl concentrations. The fact that the ratio of the fluorescence in the presence and absence of GdmCl is unaf-

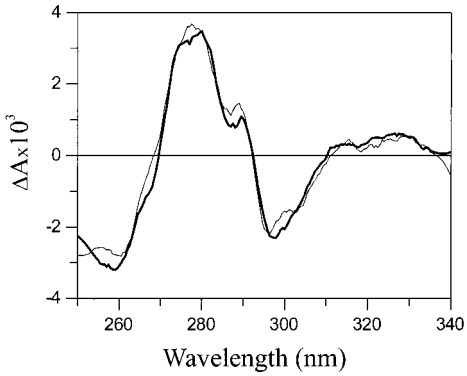

FIG. 2. Near UV CD spectra of cathepsin C in 200 mM sodium phosphate pH 6 in the absence (thick line) and in the presence of 800 mM GdmCl (thin line). Protein concentrations were 0.7 mg/ml and pathlength 10 mm.

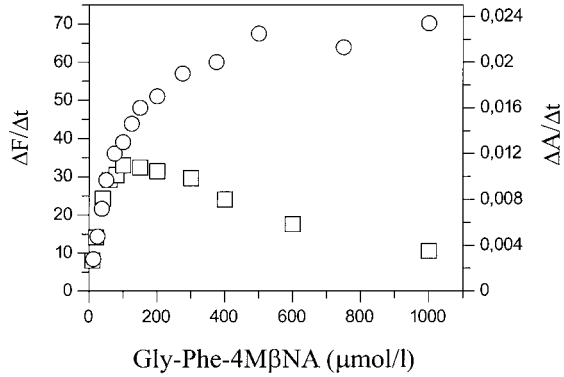

FIG. 4. Influence of probe on apparent rates of Gly-Phe-4MβNA hydrolysis. Rates determined fluorimetrically, $\Delta F/\Delta t$ with excitation at 335 nm following the emission at 415 nm (□) and spectrophotometrically, $\Delta A_{345}/\Delta t$ (○) are plotted as a function of substrate concentration.

fected by acrylamide (Fig. 3) argues strongly against even small conformational perturbations being induced by the inhibitor.

The possibility that low concentrations of GdmCl could be causing subunit dissociation was tested by size exclusion chromatography in the presence and absence of 500 mM GdmCl. The elution volume and shape of the peak were unchanged by guanidinium.

Substrate inhibition of cathepsin C. Taken together, the above results lead to the conclusion that Gdm^+ is acting as an enzyme inhibitor. Determination of the kinetic parameters were expected to be complicated in the light of the reported substrate inhibition of cathepsin C (2). When the rate of Gly-Phe-4MβNA hydrolysis was determined fluorimetrically in a 1×1 cm cuvette with excitation at 335 and emission at 415 nm similar results to those reported earlier were obtained (Fig. 4). However when monitored by the increase in absorbance at 345 nm, due to the liberated 4MβNA, a simple saturation curve was observed (Fig. 4). The molar extinction coefficient ε_{335} of Gly-Phe-4MβNA was found to be $\approx 10^3$, confirming that a pronounced inner filter effect (19) is the cause of the anomalous fluorescence results. In the absence of substrate inhibition in this concentration range it now became feasible to obtain meaningful kinetic parameters for Gdm^+ inhibition.

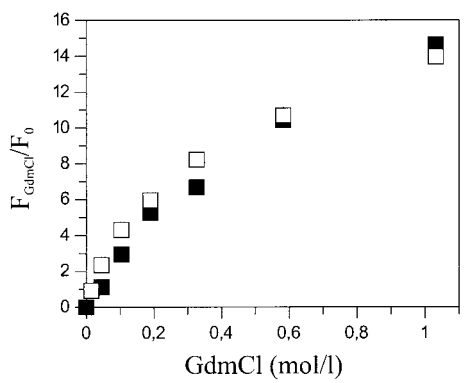

FIG. 3. Effect of GdmCl on acrylamide quenching of tryptophan fluorescence. Fluorescence at 355 nm in the presence of GdmCl divided by fluorescence in the absence of GdmCl (F_{GdmCl}/F_0) is plotted in the absence (□) and in the presence (■) of acrylamide.

Effect of Gdm^+ on the kinetics of substrate hydrolysis. Hydrolysis of Gly-Phe-4MβNA substrate was followed in the presence and in the absence of Gdm^+ (Fig. 5A). In the absence of Gdm^+, values for k_{cat} of 91 s^{-1} and K_m of 75 μM were obtained (Table I). The presence of 20 mM Gdm^+ in the assay led to an increase in K_m to 850 μM, whereas the k_{cat} of 98 s^{-1} was not significantly different from that determined in its absence (Table I). Bound Gdm^+ thus decreases the affinity of the enzyme for the substrate, without an appreciable effect on the turnover number.

The inhibition constant for Gdm^+ was determined from a Dixon plot (20), assuming a mechanism of competitive inhibition. Activity was assayed at a series of inhibitor concentrations, with constant substrate concentration, and 1/v plotted as a function of Gdm^+ concentration. From the point of intersection of three straight line plots (10 μM, 20 μM and 30 μM Gly-Phe 4MβNA) a K_i of 1.5 mM was obtained.

Since the 4MβNA group spans the active site cysteine and is bound to the S′ sites however, it was not possible to resolve from this experiment whether Gdm^+ affects the binding of the Gly-Phe dipeptide moiety of the substrate to the S1 and S2 sites, of the chromophore label to the S′ sites, or both.

Use of a simple dipeptide amide as substrate enabled just the S sites to be studied. The aminopeptidase reaction, in which Pro-Phe-NH_2 is hydrolyzed by water, was monitored by HPLC analysis of the products and found to exhibit standard Michaelis-Menten kinetics in the absence of Gdm^+ (Fig. 5B, Table I). The K_m of

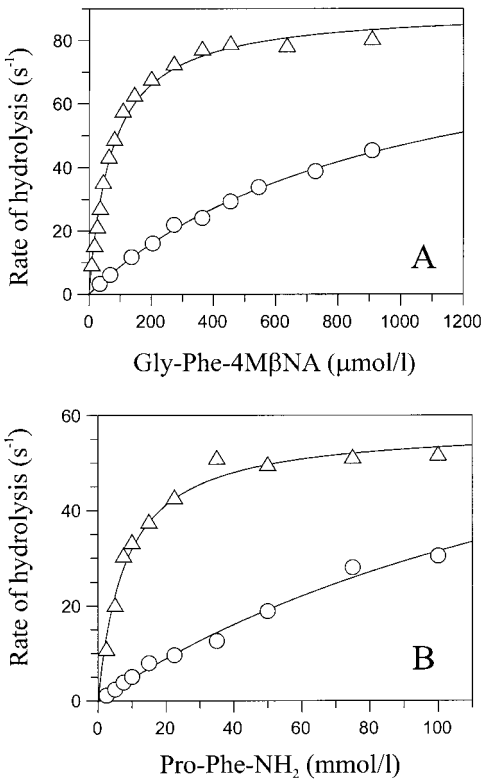

FIG. 5. Effect of Gdm^+ on substrate hydrolysis. (A) Hydrolysis of Gly-Phe-4MβNA measured by change of absorbance at 345 nm. (B) Hydrolysis of Pro-Phe-NH_2 was determined chromatographically. Rates of reaction at each substrate concentration were determined in the presence (○) and absence (△) of 20 mM Gdm^+.

8 mM is in the range of values determined for some other dipeptide amides (21,22,23). In the presence of 20 mM Gdm^+ however, the K_m value was more than 20 fold greater, whereas k_{cat} was only slightly higher than in the absence of inhibitor. It is evident from these results that guanidinium ion inhibits binding of substrate to the S site.

Effect of guanidinium ion on the transferase reaction. In order to discover whether there is any similar effect at the S′ site, the S′ and S sites of cathepsin C were studied separately using a transferase assay. In transferase reactions catalyzed by cathepsin C the nucleophilic attack of an amine on the carbonyl group of the activated peptide dominates over the aminopeptidase reaction at neutral and mildly alkaline pH (24). The activated dipeptide is bound to the S1 and S2 sites, whereas the dipeptide which acts as a nucleophile occupies the S1′ and S2′ sites. The dipeptide amide Pro-Phe-NH_2 was chosen as acylating agent since peptide amides with proline at the N-terminus are good acylating agents (23,25) but cannot act as nucleophiles in the transferase reaction catalyzed by cathepsin C (26). The dipeptide Lys-Tyr-OH was chosen as nucleophile since peptides with a basic amino acid at the N-terminus are poor acylating agents (21,25) and excellent nucleophiles (24,25), thus binding preferentially to the S′ sites. The low affinity of Lys-Tyr-OH for the S sites has been shown by the finding that peptides with an N-terminal Lys are weak inhibitors of the aminopeptidase reaction (27). The fact that Lys-Tyr-OH cannot form an activated intermediate with the enzyme excludes the formation of oligopeptides with N-terminal lysine. The sole products of the reaction therefore, Pro-Phe-OH and Pro-Phe-Lys-Tyr-OH, were separated from substrates on a C8 column and quantified as described in the experimental section.

Rates of water hydrolysis and aminolysis were determined as functions of nucleophile concentration at constant concentration of acylating agent. Formation of Pro-Phe-Lys-Tyr-OH was used to evaluate the transferase reaction. In the absence of Gdm^+, values of K_m = 11 mM and k_{cat} = 27 s^{-1} were obtained (Table I) for the nucleophile. The addition of Gdm^+ led to a nearly 8-fold decrease in k_{cat} but, in contrast to the aminopeptidase reaction described above, K_m was only slightly changed (Table I). The fraction of substrate undergoing aminolysis is independent of Gdm^+ concentration (Fig. 6) over a wide range of nucleophile concentration. The facts that Gdm^+ markedly increases the K_m of the aminopeptidase reaction while having negligible effect on either the K_m of the transferase reaction (Table I) or the fraction of substrate undergoing aminolysis, shows that it does not affect the binding of the peptides bound to the S′ sites.

TABLE I

Effect of Gdm^+ on the Kinetics of Aminopeptidase and Transferase Activities of Cathepsin C

	50 mM Cl^-	50 mM Cl^- 20 mM Gdm^+
Aminopeptidase (Gly-Phe-4MβNA)[1]		
K_m (mM)	0.075	0.85
k_{cat} (s^{-1})	91	98
Aminopeptidase (Pro-Phe-NH_2)[2]		
K_m (mM)	8	170
k_{cat} (s^{-1})	58	83
Transferase (Lys-Tyr-OH)[3]		
K_m (mM)	11	6
k_{cat} (s^{-1})	27	3.7

[1] K_m and k_{cat} were calculated from experimental data shown in Fig. 4.

[2] Reductively activated cathepsin C was assayed for activity against Pro-Phe-NH_2 in 200 mM sodium phosphate buffer pH 7.5. Reaction rates were determined chromatographically.

[3] Reductively activated cathepsin C was assayed for transferase activity with 10 mM Pro-Phe-NH_2 and Lys-Tyr-OH (2.5 to 150 mM) in 200 mM sodium phosphate buffer pH 7.5. The product of the transferase reaction, Pro-Phe-Lys-Tyr-OH, was assayed chromatographically.

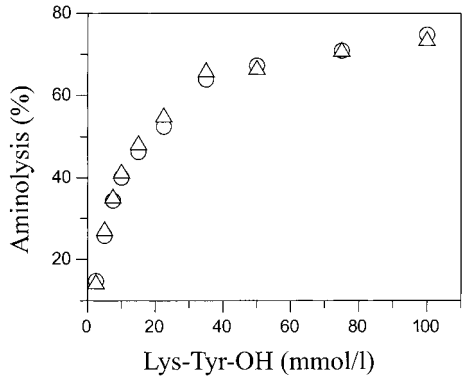

FIG. 6. Effect of Gdm^+ on the transferase reaction rates. Aminolysis, expressed as a percentage of the total consumption of substrate and determined from the ratio of the transferase reaction rate, and the sum of the transferase and aminopeptidase reaction rates, was determined over a range of Lys-Tyr-OH concentrations. Experiments in the presence 20 mM Gdm^+ (△) and in its absence (○) were performed as in Fig. 5B.

CONCLUSIONS

The activity of cathepsin C is lost at concentrations of GdmCl lower than those which induce conformational change, subunit dissociation or altered mobility of the enzyme. Guanidinium ion is shown to be a competitive inhibitor of cathepsin C, bound to the S site. Lack of inhibition by urea points to the importance of the positive charge for the inhibition. The negative charge at the S site is known to be important in defining the aminopeptidase activity of cathepsin H (28). Together with the family resemblance of cathepsins, the present results point to a negative charge at the S site being important for the dipeptidyl-aminopeptidase specificity of cathepsin C.

ACKNOWLEDGMENTS

We wish to thank Dr. Iztok Dolenc, who introduced us to the cathepsin C project, for many helpful discussions and Prof. Vito Turk for his support.

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