Inhibition of cathepsin B by its propeptide: Use of overlapping peptides to identify a critical segment

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Received 3 July 1996; revised version received 15 July 1996

Abstract **Ten overlapping 15-mer peptides (peptidyl amides) spanning the proregion of rat cathepsin B (residues 1p–60p) were constructed to identify minimal segments having inhibitory activity towards the mature enzyme, that could be used to develop a new generation of peptide-derived inhibitors specifically targeting the active site of the corresponding proteinase. Three synthetic peptides, containing the pentapeptide Leu-Cys-Gly-Thr-Val (residues 41p–45p) in their sequence, inhibited cathepsin B with K_i values in the micromolar range. Alkylation of the thiol group of Cys-42p of peptide PB8 (36p–50p) resulted in its rapid proteolytic degradation, suggesting that this residue is essential for inhibition. The inhibition constant was slightly improved ($K_i = 2$ μM) using a longer peptide (26p–50p) which was completely resistant to cleavage even after a prolonged incubation. Alkylation of its cysteinyl residue also resulted in rapid cleavage of the peptide chain. Peptides derived from the rat cathepsin B prosequence also inhibited human cathepsin B with similar K_i values. Unlike rat cathepsin B, which cleaves peptide PB8 at the G47p–G48p bond after prolonged incubation, the human enzyme cleaved both PB8 and PB11 at the Lys-40p–Leu-41p bond, in agreement with the different kinetic properties of these two proteinases. New probes with improved specificity for cysteine proteinases may therefore be designed based on the sequences of their propeptides.**

Key words: Cysteine proteinase; Cathepsin; Propeptide

1. Introduction

Most of the proteinases from all four classes (metalloproteases, aspartic, serine, and cysteine proteases) are synthesized as pre-pro-enzymes that are converted to their mature active forms by proteolytic cleavage of the proregion. Whereas the activation of some serine proteinases that have short proregions, like trypsin and chymotrypsin, is well known, it is less clear for enzymes with longer proregions (> 60 residues). The proregions of these proteinases are essential for such physiological functions as intracellular trafficking, correct folding of the enzyme during maturation, and inhibition of the mature enzyme [1–5]. The inhibition of proteinases by their respective proregions is a rather specific process [6,7], and this relationship can be used to develop a new generation of specific peptide inhibitors. The cysteine proteinases of the papain family are particularly appropriate targets for such a strategy, since no specific synthetic substrates and inhibitors are available to follow the activity of individual members.

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Abbreviations: Acm, 5-acetamidomethyl; AMC, 7-amino-4-methylcoumarin.

Mature cathepsin B is involved in a variety of biological processes, such as the intracellular degradation of endocytosed and endogenous proteins, bone resorption, and in pathologic events such as rheumatoid arthritis, tumor metastasis, or muscular dystrophy (see [8] for review). The proenzyme has a propeptide of ≈60 residues, which is significantly shorter than the propeptides of papain and cathepsin L [9]. Maturation of cathepsin B occurs via an autocatalytic unimolecular mechanism [10]. The resulting 6-kDa propeptide and the 30-kDa single chain enzyme form a non-covalent complex, which may stabilize the processed proteinase at neutral pH [11]. The rat cathepsin B proregion is a potent slow-binding reversible inhibitor of the mature enzyme [12] that may control its proteolytic activity physiologically. The recent elucidation of the 3D structures of human and rat procathepsin B is an essential step towards understanding the way the mature enzyme is inhibited by its propeptide. Three major areas in the protease take part in the interaction; two of them, a prosegment binding loop (PBL) and an occluding loop crevice, are remote from the active site, while the third is the substrate binding cleft [13,14]. However, whether all are required for inhibition or whether a minimal segment in the proregion can specifically interact with the corresponding enzyme is not known. We have attempted to answer this question by constructing overlapping peptides spanning the whole proregion of rat cathepsin B, and measuring their capacities to inhibit mature rat and human enzymes.

2. Material and methods

2.1. Peptide synthesis

Unless otherwise stated, all amino acids were of the L-configuration, and were purchased from NovaBiochem (FranceBiochem, Meudon), or Neosystem (Strasbourg, France). Peptides were prepared simultaneously using a Zinsser solid-phase multisynthesizer (Advanced ChemTech GmbH, Frankfurt, Germany). Synthesis was performed, using a Rink Amide MBHA ((4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethylphenoxyacetamidonorleucyl)-4-methylbenzhydrylamine) resin (NovaBiochem), by Fmoc chemistry [15]. After deprotection and cleavage, peptidyl amides were purified by gel filtration on a Sephadex G-25 column (Pharmacia) equilibrated in 5% acetic acid, and their homogeneity checked by reverse-phase chromatography on a C18 OD 300 cartridge (Brownlee), using a 30-min linear (0–60%) acetonitrile gradient in 0.1% trifluoroacetic acid. Elution was monitored by an Applied Biosystems 1000S diode array detector (Perkin Elmer-France, Roissy).

An alkylated form of peptide PB8, peptide $PB8_{Acm}$, was prepared by Fmoc chemistry (synthesizer Applied Biosystems 431 A). An *S*-acetamidomethyl group (Acm) was used to block the cysteinyl thiol group. After cleavage and deprotection, peptide $PB8_{Acm}$ was purified by gel filtration on Sephadex G-25, as described above. Alternatively, the *S*-acetamidomethyl group was removed by mercuric acetate according to Lamthanh et al. [16], then checked by MALDI-TOF mass spectroscopy (Brüker). Deprotection of $PB8_{Acm}$ gave its reduced form, peptide PB8. The purity of the peptides was checked by RP-HPLC on

PII S0014-5793(96)00822-8

a C18 OD 300 Brownlee cartridge, using a 30 min linear (0–60%) acetonitrile gradient in 0.1% trifluoroacetic acid. Peptide molecular weights were determined by mass spectroscopy. Peptides PB11 (residues 26p–50p) and $PB11_{Acm}$ were prepared by the same procedure.

2.2. Enzymes

Rat liver cathepsin B was purified as previously described [17]. Recombinant human cathepsin B was a gift from Dr. John S. Mort (McGill University, Montreal, Canada). Cathepsin B was assayed on Z-Phe-Arg-AMC (Bachem Biochimie, Voisins-le-Bretonneux, France), and the enzymatic activity was recorded in a spectrofluorometer, with wavelengths of 350 and 460 nm for excitation and emission. Assays were carried out in activating buffer: 0.1 M phosphate buffer pH 6.0, containing 1 mM EDTA, 2 mM dithiothreitol, 0.1% Brij 35. Cysteine proteinases were titrated with L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido(4-guanidino)butane (E-64) [18].

2.3. Kinetic measurements

All peptides were screened for their inhibitory capacity by microfluorimetry (Dynatech microreader). Cathepsin B (1.2 nM) was incubated with peptides (10 μM), in the activating buffer, at 37°C for 10 min, in a microfluor plate, in a final volume of 100 μl; the enzymatic reaction was triggered by adding Z-Phe-Arg-AMC (20 μM), and the residual enzymatic activity was recorded. The screening was repeated with a peptide concentration of 1 μM. Any peptides showing significant inhibition under the above conditions were selected, and their inhibitory constants (K_i) calculated by the method of Dixon [19].

2.4. Peptide stability

The stability of the peptides in the presence of cathepsin B was checked by incubating proteinase (4.8 nM) and peptides (30 μM) in the assay buffer (0.1 M phosphate buffer pH 6.0, 1 mM EDTA, 2 mM dithiotreitol, 0.1% Brij 35), at 37°C for 0–240 min (molar ratio enzyme/peptide: 1:6250). Samples were fractionated by reverse-phase chromatography on a C18 Brownlee cartridge, using a linear 0–60% gradient of acetonitrile in 0.075% TFA. The elution profiles were analyzed by Spectacle (PC 1000 version 3.0) (Thermo Separation Products, Les Ulis, France), and the hydrolysis products were identified by MALDI-TOF mass spectroscopy.

3. Results and discussion

Ten 15-mer peptides (peptides PB1–PB10), spanning the rat procathepsin B sequence (residues 1p–60p) (Table 1), were synthesized as peptidyl amides by a multisynthesis procedure. Their capacities to inhibit rat cathepsin B were assayed by measuring the residual enzymatic activity on Z-Phe-Arg-AMC. Only three of these peptides (PB7–PB9) inhibited rat cathepsin B. Their K_i values were determined using the Dixon plot, and were all in the micromolar range, the lowest value being for peptide PB8 (Table 2). All 3 peptides behaved as competitive inhibitors of cathepsin B and no significant cleavage occurred during the course of the experiment, as shown by reverse-phase HPLC, using an identical peptide/cathepsin B molar ratio. However, a cleavage site was identified by mass spectrometry at the G47p–G48p bond, after incubation for 1 h. The sequence encompassing this scissile bond in the prosegment is reminiscent of that of the substrate-like sequence in human cystatin C [20], from which sensitive fluorogenic cathepsin B substrates have been constructed [21,22]. It is also similar to the autocatalytic activation site of some parasitic cysteine proteinases of the papain family [23]. The sequences of peptides PB7–PB9 all include the pentapeptide LCGTV corresponding to residues 41p–45p of the prosequence. One or more residue(s) in this sequence are therefore essential for the interaction with the catalytic site of the mature enzyme. This agrees with crystallographic data showing that interaction of the proregion with rat cathepsin B involves residues Leu-41p–Gly-47p which are deeply buried in the substrate binding site [13]. According to these data and those for human cathepsin B [13,14], Cys-42p, which is the residue closest to the oxyanion hole, significantly contributes to propeptide binding by interacting with several hydrophobic groups of cathepsin B. Alkylation of Cys-42p in peptide PB8 resulted in a loss of inhibition (peptide $PB8_{Acm}$). Peptide $PB8_{Acm}$ was also cleaved at the G47p–G48p site upon incubation with cathepsin B, but much more rapidly than peptide PB8. This may be because the 5-acetamidomethyl group of the $PB8_{Acm}$ peptide impairs fitting of the cysteinyl group due to steric hindrance. Alternatively, the more hydrophilic nature of $PB8_{Acm}$ peptide after alkylation, as deduced from its significantly shorter retention time on a C18 reverse phase cartridge (results not shown), could modify the interaction with the hydrophobic subsite. Competition would therefore occur between the LVGG substrate-like region and the inhibitory site. The K_i values obtained with peptides PB7–PB9 are greater than that in the nM range reported for a peptide spanning almost the entire proregion of cathepsin B (residues 1p–56p) [12]. This suggests that the other two regions in the prosegment, contributing to the tight binding of cathepsin B with its prosegment, are also involved in the inhibition. A 25-mer peptide (PB11) (residues 26p–50p), corresponding to peptide PB8 plus a 10-residue N-terminal extension, and including the region of interaction with the occluding loop crevice in ca-

Table 1
Overlapping synthetic peptides spanning the proregion of rat cathepsin B[a]

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          1p                                                          62p
          ↓                                                           ↓
          HDKPSFHPLS DDMINYINKQ NTTWQAGRNF YNVDISYLKK LCGTVLGGPK LPERVGFSED IN
(1)
PB1       HDKPSFHPLSDDMIN
PB2           FHPLSDDMINYINKQ
PB3                DDMINYINKQNTTWQ
PB4                     YINKQNTTWQAGRNF
PB5                          NTTWQAGRNFYNVDI
PB6                               AGRNFYNVDISYLKK
PB7                                    YNVDISYLKKLCGTV
PB8                                         SYLKKLCGTVLGGPK
PB9                                              LCGTVLGGPKLPERV
PB10                                                  LGGPKLPERVGFSED

(2)
PB11                              AGRNFYNVDISYLKKLCGTVLGGPK
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[a]Residue number of rat procathepsin B [13].

Table 2
Equilibrium dissociation constants for the inhibition of cathepsin B by rat pro-cathepsin B peptides

Peptides	K_i (μM)	
	Rat	Human
PB1	nsi	
PB2	nsi	–
PB3	nsi	–
PB4	nsi	–
PB5	nsi	–
PB6	nsi	–
PB7	12.5	–
PB8	4.6	14.6
PB9	11.6	–
PB10	nsi	–
PB11	2	2.8
PB8 Acm	nsi	nsi
PB11 Acm (b)	12.5	11.0

nsi, no significant inhibition. Kinetic measurements were performed as described in Section 2.

thepsin B, was synthesized and its inhibiting capacity assayed. The K_i was only slightly improved ($K_i = 2$ μM) but the peptide was completely resistant to proteolysis, even after incubation overnight. It may appear surprising that a 25 residue long peptide is resistant to proteolysis by cathepsin B, the broad specificity of which has been well demonstrated [24]. It may be because the peptide is oriented in the reverse position compared to a natural peptide substrate. This reverse orientation has been described for the interaction between cathepsin B and its proregion in the rat procathepsin B crystal [13], and it also corresponds to the orientation of E 64 and its derivatives with papain and cathepsin B[25–28]. This explanation is supported by the observation that the same peptide with an alkylated cysteinyl residue ($PB11_{Acm}$) is more rapidly and unspecifically degraded by cathepsin B. Peptide PB11 therefore contains cleavage sites, but they are not accessible. This also demonstrates the pivotal role of the cysteinyl residue in the interaction. However, the K_i obtained using the PB11 peptide is still higher than that with the PCB1 peptide [12], suggesting that the peptidyl chain cannot adopt the anchoring structure needed to maintain intimate contacts with cathepsin B, even though it is correctly oriented at the cathepsin B surface.

The above experiments were repeated using human cathepsin B, the propeptide of which is similar to that of the rat and other mammals [29], especially in the region including the conserved Cys 42p residue. Peptide PB8, which is derived from the rat prosequence, inhibited the human enzyme with a slightly higher K_i, while the K_i was similar with PB11. However, the cleavage site revealed after prolonged incubation with the human enzyme was at the Lys-40p–Leu-41p bond in both peptides PB8 and PB11, which differs from that obtained with rat cathepsin B. This agrees with the somewhat different kinetic properties of the two proteinases that hydrolyse synthetic substrates at different rates [24]. We recently showed that a substrate with intramolecularly quenched fluorescence (Abz-LVGGP-EDDnp), derived from the substrate-like region of human cystatin C [20], is less efficiently hydrolysed by human cathepsin B than by its rat homologue (unpublished results). This explains the selective cleavage of the homologous sequence in peptides PB8 and PB11 by the rat proteinase after prolonged incubation. The close proximity of the Lys-40p–Leu-41p bond to the essential Cys-42p residue may explain why alkylation of the latter impairs cleavage by human cathepsin B.

These results show that the entire proregion of procathepsin B is not required for interaction with the mature enzyme, but that a short sequence containing a critical cysteinyl residue is sufficient to interact with, and inhibit both human and rat cathepsin B. However, this inhibition does not compare with that obtained with the whole propeptide, suggesting that other regions are essential to optimize peptide conformation and/or positioning at the surface of the mature enzyme. The segment upstream of the inhibitory sequence in the proregion, which acts as a short anchoring two-turn α-helix (residues 35p–41p) in rat procathepsin B [13], improves inhibition, probably by favouring the binding such as in the prosegment of procathepsin B. This explains the resistance of peptide PB11 to cleavage by the mature enzyme. The results obtained here using overlapping peptides spanning the cysteine proteinase proregion agree with those reported from crystal structures of human and rat procathepsin B [13,14]. This promising approach to designing more selective inhibitors of cysteine proteinases will be extended to the proregions of other papain-like proteinases, especially those of protozoan parasites like trypanosomes, that are involved in both spreading and host infection [30,31], but have no specific substrate or inhibitors that can selectively control their activity.

Acknowledgements: The authors thank Emmanuelle Bataillé for technical assistance. Recombinant human cathepsin B was a gift from Dr. John S. Mort (McGill University, Montreal, Canada). The English text was edited by Dr. Owen Parkes. This work was partially funded by a grant from la région Centre, and INSERM-CNPq. J.R.C. holds a post-doctoral fellowship from CNPq (Brazil).

References

[1] Baker, D., Sohl, J.L. and Agard, D.A. (1992) Nature 356, 263–265.
[2] Gallagher, T., Gilliland, G., Wang, L. and Bryan, P. (1995) Structure 3, 907–914.
[3] Neurath, H. (1989) Trends Biochem. Sci. 14, 268–271.
[4] Ohta, Y., Hojo, H., Aimoto, S., Kobayashi, T., Zhu, X., Jordan, F. and Inouye, M. (1991) Mol. Microbiol. 5, 1507–1510.
[5] Tao, K., Stearns, N.A., Dong, J., Wu, Q. and Sahagian, G.G. (1994) Arch. Biochem. Biophys. 311, 19–27.
[6] Baker, D., Silen, J.L. and Agard, D.A. (1992) Proteins Struct. Funct. Genet. 12, 339–344.
[7] Fusek, M., Mares, M., Vagner, J., Voburka, Z. and Baudys, M. (1991) FEBS Lett. 287, 160–162.
[8] Katunuma, N. (1989) in: Intracellular Proteolysis (Katunuma, N. and Kominami, E. eds.) pp. 3–23, Japan Scientific Societies Press, Tokyo.
[9] Chan, S.J., San Segundo, B., McCormick, M.B. and steiner, D.F. (1986) Proc. Natl. Acad. Sci. USA 83, 7721–7725.
[10] Mach, L., Mort, J.S. and Glössl, J. (1994) J. Biol. Chem. 269, 13030–13035.
[11] Mach, L., Mort, J.S. and Glössl, J. (1994) J. Biol. Chem. 269, 13036–13040.
[12] Fox, T., de Miguel, E., Mort, J.S. and Storer, A.C. (1992) Biochemistry 31, 12571–12576.
[13] Cygler, M., Sivaraman, J., Grochulski, P., Coulombe, R., Storer, A.C. and Mort, J.S. (1996) Structure 4, 405–416.
[14] Turk, D., Podobnik, M., Kuhelj, R., Dolinar, M. and Turk, V. (1996) FEBS Lett. 384, 211–214.
[15] Atherton, E. and Sheppard, R.C. (1989) Solid Phase Peptide Synthesis: A Practical Approach, IRL Press, Oxford.
[16] Lamthanh, H., Roumestand, C., Deprun, C. and Ménez, A. (1993) Int. J. Peptide Protein Res. 41, 85–95.
[17] Moreau, T., Esnard, F., Gutman, N., Degand, P. and Gauthier, F. (1988) Eur. J. Biochem. 173, 185–190.
[18] Barrett, A.J., Kembhavi, A.A., Brown, M.A., Kirschke, H., Knight, C.G., Tamai, M. and Hanada, K. (1982) Biochem. J. 201, 189–198.
[19] Dixon, M. (1953) Biochem. J. 55, 170–171.
[20] Grubb, A., Abrahamson, M., Olafsson, I., Trojnar, J., Kasprzykowska, R., Kasprzykowski, F. and Grzonka, Z. (1990) Biol. Chem. Hoppe-Seyler 371, 137–144.
[21] Lalmanach, G., Serveau, C., Brillard-Bourdet, M., Chagas, J.R., Juliano, L., Mayer, R. and Gauthier F. (1995) J. Protein. Chem. 14, 645–653.
[22] Serveau, C., Lalmanach, G., Juliano, M.A., Scharfstein, J., Juliano, L. and Gauthier, F. (1996) Biochem. J. 313, 951–956.
[23] Hellman, U., Wernstedt, C. and Cazzulo, J.J. (1991) Mol. Biochem. Parasitol. 44, 15–22.
[24] Barrett, A.J. and Kirschke, H. (1981) Methods Enzymol. 80, 535–560.
[25] Varughese, K.I, Ahmed, F.R., Carey, P.R., Hasnain, S., Huber, C.P. and Storer, A.C. (1989) Biochemistry 28, 1330–1332.
[26] Yamamoto, D., Matsumoto, K., Ohishi, H., Ishida, T., Inoue, M., Kitamura, K. and Mizuno, H. (1991) J. Biol. Chem. 266, 14771–14777.
[27] Kim, M., Yamamoto, D., Matsumoto, K., Inoue, M., Ishida, T., Mizuno, H., Sumiya, S. and Kitamura, K. (1992) Biochem. J. 287, 797–803.

[28] Turk, D., Podobnik, M., Popovic, T., Katunuma, N., Bode, W., Huber, R. and Turk, V. (1995) Biochemistry 34, 4791–4797.
[29] Karrer, K.M., Peiffer, S.L. and Di Tomas, M.E. (1993) Proc; Natl. Acad. Sci. USA 90, 3063–3067.
[30] MacKerrow, J.H. (1989) Exp. Parasitol. 68, 111–115.
[31] Mac Kerrow, J.H., Sun, E., Rosenthal, P.J. and Bouvier, J. (1993) Annu. Rev. Microbiol. 47, 821–853.