Enzyme–substrate interactions in the hydrolysis of peptides by cathepsins B and H from rat liver

Dieter BRÖMME, Knut BESCHERER, Heidrun KIRSCHKE and Siegfried FITTKAU

Institute of Biochemistry, Faculty of Medicine, Martin-Luther-University Halle-Wittenberg, DDR-4020 Halle (Saale), Hollystrasse 1, PF 184, German Democratic Republic

The number of possible subsites of the rat liver cysteine proteinases cathepsin B and cathepsin H was determined in the *N*-terminal direction from the scissile bond. An elongation of the substrate peptide chain of up to four amino acid residues enhances the hydrolysis rate of both cathepsins. The greatest increase in activity was observed by elongation to the dipeptide substrate for cathepsin B and to the tetrapeptide substrate for cathepsin H. Both proteinases discriminate proline from their subsites S_1 and S_2 , but accept it well in S_3 . A quantitative distinction between the endopeptidase and the peptidyl dipeptidase activity of cathepsin B was feasible by using two model peptides:

(Z = benzyloxycarbonyl; X = NH_2 or OH; the arrow shows the cleavage site). Whereas the peptide acid, representing the peptidyl dipeptidase substrate, was hydrolysed by cathepsin B twice as fast as the peptide amide as an endopeptidase substrate, cathepsin H clearly had a preference for the amide substrate.

INTRODUCTION

Systematic approaches to characterizing the binding-site length and single subsites of the lysosomal cysteine proteinases cathepsin B (EC 3.4.22.1) and cathepsin H (EC 3.4.22.16) are rather rare. Studies with diazomethyl ketones emphasize, for cathepsin B, a preference for aromatic residues like phenylalanine in the P_2 position (Watanabe *et al.*, 1979; Shaw & Green, 1981). In contrast, the P_1 and P_3 positions in cathepsin B inhibitors do not markedly influence their efficiency. Aromatic residues, as well as smaller amino acids such as alanine or glycine, are well accepted. The specificity of possible subsites S_3 and S_4 has not yet been determined. The S_1 pocket appears to favour hydrophobic residues such as leucine or phenylalanine (Barrett *et al.*, 1982; Takahashi *et al.*, 1986b). The existence of a multisubsite-binding region in cathepsin H responsible for a specificity in its endopeptidase action was not investigated. Cathepsin H exhibits a rather broad specificity in its aminopeptidase activity (Chatterjee & Kalnitsky, 1986).

The present study was undertaken to investigate in more detail the length of the binding region of cathepsins B and H interacting with the *N*-terminal part of the substrate. Furthermore, the influence of proline residues in the substrate positions P_1 , P_2 and P_3 on the kinetic parameters was determined. By using the results of proline discrimination, two model peptides were synthesized that permitted one to distinguish between the endopeptidase and the peptidyl dipeptidase activity of

cathepsin B. The hydrolysis of these substrates by cathepsin H was also tested for.

MATERIALS AND METHODS

Enzymes

Rat liver cathepsins B and H in electrophoretically homogeneous form were prepared as described by Kirschke *et al.* (1977) and Barrett & Kirschke (1981). The molarity of enzyme stock solutions was determined by active-site titration with E-64 as described by Barrett *et al.* (1982).

Substrates

The synthesis of the alanine- and proline-containing peptides was carried out by a stepwise chain elongation using the mixed-anhydride method. In all cases the benzyloxycarbonyl (Z) group was used for reversible α -amino-group protection and the methyl ester group for the α -carboxy-group protection. The Z residue was removed by HBr/acetic acid treatment, whereas the methyl ester was removed by alkaline hydrolysis in the presence of 4-(dimethylamino)pyridine (Merck-Schuchardt). Z-Ala-pNA (m.p. 109–110 °C; $[\alpha]_D^{25} - 54.2^\circ$, *c* 1 in methanol) and Z-Pro-pNA (m.p. 162 °C; $[\alpha]_D^{25} - 104.4^\circ$, *c* 1 in acetic acid) were also synthesized by the mixed-anhydride method. The reacylation of the Z-protected alanine, (n = 2–5) nitroanilides and proline-

Abbreviations used: Ac-, acetyl-; Suc-, succinyl-; Z-, benzyloxycarbonyl-; -pNA, 4-nitroanilide; TNBA, trinitrobenzenesulphonic acid; DMF, dimethylformamide; DMSO, dimethyl sulphoxide; all amino acids used were of the L-configuration; E-64, L-3-carboxy-*trans*-2,3-epoxypropyl-leucylamido-(4-guanidino)butane; for the discussion of the interactions between the proteinase and the substrate, the nomenclature of Schechter & Berger (1967) was used.

containing tripeptide *p*-nitroanilides was carried out with succinic anhydride (or acetic anhydride for the preparation of Ac-Ala-pNA) in DMF or pyridine after deprotection. Pro-NH₂·HCl (m.p. 180–182 °C; $[\alpha]_D^{25}$ –65°, *c* 1 in water) was prepared as described by Chambers & Carpentier (1955). All synthesized peptides were purified by silica-gel chromatography, and the purity of the substrates was checked by amino acid analysis in a Microtechna (Prague, Czechoslovakia) model AAA881 amino acid analyser and t.l.c. on silica sheets (Kavalier, Czechoslovakia) in various developing systems. After chromatography, peptide spots were revealed by treatment with ninhydrin and chlorine/*o*-tolidine. Characteristic analytical data for the synthesized substrates are shown in Table 1.

Assay with *p*-nitroanilide substrates

The incubation mixture containing an appropriate amount of enzyme in 50 mM-phosphate buffer, pH 6.0 (cathepsin B) or pH 6.5 (cathepsin H), 2 mM-dithiothreitol (Reanal, Budapest, Hungary) and 2 mM-Na₂·EDTA was incubated 5 min at 40 °C in a volume of 200 µl and the reaction was started with 50 µl of substrate solution in the appropriate phosphate buffer. The progress of the reaction was monitored photometrically at 405 nm in a thermostatically controlled Eppendorf recording photometer at 40 °C. The concentration of *p*-nitroaniline liberated from the substrates was calculated by using an ϵ_{405} value of 9600 M⁻¹·cm⁻¹. The substrate concentrations varied between 0.1 and 20 mM.

TNBA assay

Hg²⁺-inactivated cathepsins B and H were activated under conditions analogous to those described for *p*-nitroanilide substrates. The reaction was started by the addition of substrate. After various time intervals (up to 5 min), 50 µl aliquots were withdrawn and the reaction was stopped by addition of 50 µl of ice-cold 10% (w/v) trichloroacetic acid. The extents of hydrolysis of Z-Pro-Ala-Ala-Ala-Pro-NH₂ and Z-Pro-Ala-Ala-Ala-Pro-OH respectively were measured with a modified TNBA method as described by Langner *et al.* (1971). The TNBA colour yield of the cleavage products was based on calibration curves of Ala-Pro (Serva) and Ala-Pro-NH₂ respectively. The substrate concentrations for the kinetic studies varied between 1 and 8 mM, and, in the

pH-dependence study, a substrate concentration of 2 mM was used. In order to exclude the possibility of formation of dioxopiperazine derivative of the liberated cleavage product Ala-Pro-NH₂ under neutral and slightly alkaline pH conditions, the product was incubated under conditions analogous to those used for enzyme assay over the tested pH range. No differences in the TNBA colour yield were observed. The cleavage sites in the peptides were characterized by using Ala-Pro and Ala-Pro-NH₂ as standards in high-voltage paper electrophoresis at pH 1.9 (1500 V; separation time, 90 min; solvent, acetic acid/formic acid/water, 15:5:80, by vol.) and t.l.c.

Regression analysis of kinetic parameters

For the analysis of the parameters K_m and V_{max} , at least seven substrate concentrations in duplicate were used. In all cases data were in agreement with Michaelis–Menten kinetics. The K_m and V_{max} values with their S.D. values were determined by non-linear regression analysis on a KC 85/1 microcomputer (Robotron, Dresden, German Democratic Republic). In addition, the linear Hanes plots gave correlation coefficients mostly in excess of 0.99. The inhibition constant, K_i , of Suc-Ala-Pro-Ala-pNA for cathepsin H was determined from a Dixon plot employing four substrate concentrations [0.07–0.4 mM-Arg-pNA (Bachem) as substrate].

RESULTS AND DISCUSSION

Cathepsin B and cathepsin H are lysosomal cysteine proteinases with a mixed type of action. Whereas cathepsin B shows, in addition to its endopeptidolytic action, a peptidyl dipeptidase activity, cathepsin H acts as an endopeptidase and as an aminopeptidase (Barrett & Kirschke, 1981).

Number of subsites in the cathepsins B and H

In order to determine the number of possible subsites interacting with the *N*-terminal direction from the scissile bond of a substrate, a series of *N*-acylated alanylpeptide *p*-nitroanilides was tested. Alanine residues were used for chain elongation because of their minimal side-chain interactions with the subsites. Only the backbone and the β -carbon atoms of the alanine side chain, which are present in all amino acids but glycine,

Table 1. Analytical data of the synthesized peptides

Peptide	No.	M.p. (°C)	$[\alpha]_D^{25}$ (°) (concn./solvent)	Amino acid analysis
Ac-Ala-pNA	I	192–197	–76.5 (<i>c</i> 1 in acetic acid)	ND*
Suc-Ala ₂ -pNA	II	111	–61.4 (<i>c</i> 1 in acetic acid)	ND
Suc-Ala ₃ -pNA	III	245	–62.6 (<i>c</i> 2 in acetic acid)	ND
Suc-Ala ₄ -pNA	IV	268–269	–45.0 (<i>c</i> 0.5 in DMSO)	ND
Suc-Ala ₅ -pNA	V	350	–25.8 (<i>c</i> 0.5 in DMSO)	ND
Suc-Ala-Ala-Pro-pNA	VI	104–105	–99.8 (<i>c</i> 1 in acetic acid)	Pro 1(1)†; Ala 2.04(2)
Suc-Ala-Pro-Ala-pNA	VII	Not crystallized	–155.6 (<i>c</i> 1 in acetic acid)	Pro 1(1); Ala 1.89(2)
Suc-Pro-Ala-Ala-pNA	VIII	130–132	–104.5 (<i>c</i> 1 in acetic acid)	Pro 1(1); Ala 1.98(2)
Z-Pro-Ala ₃ -Pro-NH ₂	IX	Not crystallized	–189.3 (<i>c</i> 1 in water)	Pro 2(2); Ala 3.18(3)
Z-Pro-Ala ₃ -Pro-OH	X	Not crystallized	–163.4 (<i>c</i> in water)	Pro 2(2); Ala 3.04(3)

* ND, not determined.

† Theoretical amounts in parentheses.

interact with the binding region (Bizzozero *et al.*, 1982). Substrate elongation *N*-terminal to the scissile bond up to the tetrapeptide appears to enhance the hydrolysis rates of cathepsins B and H (Table 2). The pentapeptide (peptide V, Table 2) does not display a further increase in activity. The presence of four left-hand-side pockets, S_1 – S_4 , in cathepsin B is also discussed by Takahashi *et al.* (1986b). However, the increased hydrolysis rate is much lower for cathepsin B than for cathepsin H. The greatest effect on cathepsin B-catalysed hydrolysis was observed with the occupation of subsite S_2 by a second alanine residue (47-fold increase in k_{cat}/K_m). This is in agreement with the results obtained by other groups, who have characterized the S_2 – P_2 interaction as the most important one of cathepsin B (Barrett & Kirschke, 1981) and, in addition, papain (EC 3.4.22.2) (Berger & Schechter, 1970). A further elongation of the peptide chain to the tri- and tetra-peptide gives only a doubling of the k_{cat}/K_m value. The effect of substrate chain elongation on the activity of cathepsin H is quite different from that on cathepsin B. In contrast with cathepsin B, the greatest increase in k_{cat}/K_m (30-fold) is

observed by the elongation of the tri- to the tetra-peptide. The increase in reactivity is above all due to a decrease in K_m . Obviously the efficiency of the endopeptidase activity of cathepsin H is dependent on subsite–substrate interactions more remote from the scissile bond.

Presence of proline residues in the substrate positions P_1 , P_2 and P_3

The introduction of proline residues into different positions of the substrate chain has long been known to influence the hydrolysis rate of proteinases. Suc-Ala₂-Pro-pA (peptide VI) is only weakly hydrolysed by cathepsins B and H (Table 3). In comparison, the trialanine peptide (peptide III) is a 226-fold better substrate of cathepsin H and a 1100-fold better one of cathepsin B. It is noteworthy that the rate constant, k_{cat} , is markedly decreased if proline is placed in the P_1 position. A proline residue in P_2 caused a complete resistance of the substrate towards cathepsin H. Though Suc-Ala-Pro-Ala-pNA (peptide VII) is not hydrolysed to a measurable extent by cathepsin H, the inhibition

Table 2. Kinetic parameters for cathepsin B (at pH 6.0) and cathepsin H (at pH 6.5)-catalysed hydrolysis of peptides of increasing chain length at 40 °C in 1% (v/v) DMF

The V_{max} and K_m values were determined as described in the Materials and methods section. The molar enzyme concentration for the calculation of k_{cat} was determined by titration with E-64. Abbreviations: Cat, cathepsin; ND, not determined.

Enzyme	$P_5 - P_4 - P_3 - P_2 - P_1 - P'_1$	Peptide no.	[S] (mM)	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (M ⁻¹ ·s ⁻¹)
Cat B	Ac-Ala-pNA	I	1–5	ND	ND	0.9*
	Suc-Ala-Ala-pNA	II	1–10	31.2±6.2	1.3±0.21	42
	Suc-Ala-Ala-Ala-pNA	III	1–10	16.9±3.8	1.5±0.24	89
	Suc-Ala-Ala-Ala-Ala-pNA	IV	0.1–1	4.1±1.1	0.7±0.12	170
	Suc-Ala-Ala-Ala-Ala-Ala-pNA†	V	0.1–1	2.0±0.4	0.3±0.05	150
Cat H	Ac-Ala-pNA	I	1–5	ND	ND	0.6*
	Suc-Ala-Ala-pNA	II	1.5–8	10.2±2.1	0.07±0.01	6.9
	Suc-Ala-Ala-Ala-pNA	III	1–10	7.0±1.9	0.3±0.05	43
	Suc-Ala-Ala-Ala-Ala-pNA	IV	0.1–0.5	0.7±0.2	0.9±0.12	1286
	Suc-Ala-Ala-Ala-Ala-Ala-pNA†	V	0.1–0.5	0.7±0.2	0.8±0.13	1143

* Calculated from the slope of the Lineweaver–Burk plot.

† In 4% (v/v) DMF.

Table 3. Hydrolysis of proline-containing tripeptides by cathepsin B (at pH 6.0) and cathepsin (at pH 6.5) at 40 °C in 1% (v/v) DMF

Abbreviations: Cat, cathepsin; NH, not hydrolysed.

Enzyme	$P_4 - P_3 - P_2 - P_1 - P'_1$	Peptide no.	[S] (mM)	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (M ⁻¹ ·s ⁻¹)
Cat B	Suc-Ala-Ala-Ala-pNA	III	1.0–10	16.9±3.8	1.5±0.24	89
	Suc-Ala-Ala-Pro-pNA*	VI	2.5–20	16.6±3.6	0.001±0.0002	0.06
	Suc-Ala-Pro-Ala-pNA	VII	0.8–5	21.7±4.3	0.4±0.07	18
	Suc-Pro-Ala-Ala-pNA	VIII	0.8–5	13.7±2.6	0.5±0.06	36
Cat H	Suc-Ala-Ala-Ala-pNA	III	1.0–10	7.0±1.9	0.3±0.05	43
	Suc-Ala-Ala-Pro-pNA*	VI	2.5–30	27.8±6.6	0.004±0.0006	0.14
	Suc-Ala-Pro-Ala-pNA*	VII	7.0–20 as [I]	3.0 K_1 †	NH	
	Suc-Pro-Ala-Ala-pNA	VIII	0.8–8	2.9±0.3	0.15±0.01	52

* In 4% (v/v) DMF.

† The K_1 value was determined in a Dixon plot with the substrate Arg-pNA at pH 6.5 and 40 °C.

constant, K_1 , lies within the range of the Suc-Ala₃-pNA substrate. Cathepsin B hydrolyses the P₂ proline substrate with a rate of about 20% of that for the Suc-Ala₃-pNA peptide; the K_m values for both substrates are, however, similar. This indicates that the non-reactivity or the decreased hydrolysis rate of this substrate is not manifested at the complex-formation stage, but in a later stage of catalysis. In position P₃ the proline residue has the lowest (or not) influence on the hydrolysis rates of cathepsin B and cathepsin H. Takahashi *et al.* (1986b) supposed a discrimination of proline at the subsites S₁, S₁' and S₂' of cathepsin B from porcine spleen. However, Towatari & Katunuma (1983) described a cleavage site between Arg⁹ and Pro¹⁰ in neurotensin by cathepsin B from rat liver. The finding of a cleavage site in this position (the breakdown of an imino bond by cathepsin B) is surprising, because so far only two exopeptidases are known to be capable of splitting an Xaa-Pro bond (Walter *et al.*, 1980; Yoshimoto *et al.*, 1983).

The discrimination of proline residues from the subsites S₁ and S₂ and their acceptance in S₃ of both cysteine proteinases is different from the behaviour of serine proteinases towards proline residues. Several serine proteinases, such as α -chymotrypsin (EC 3.4.21.1) (Bauer, 1976), pancreatic elastase (EC 3.4.21.36) (Thompson & Blout, 1973), subtilisin BPN' (EC 3.4.21.14) (Moriyama & Oka, 1977), α -lytic proteinase (EC 3.4.21.12) (Bauer *et al.*, 1981) and thermolysin (EC 3.4.21.-) (Brömme *et al.*, 1986) discriminate proline more or less from their subsites S₁ and S₃, but accept it well in S₂. According to X-ray studies on subtilisin (Robertus *et al.*, 1972), proline residues in S₁ and S₃ prevent the formation of hydrogen bonds between the substrate and the subsite of the enzyme. This may reveal a different substrate-binding mode and architecture of the binding region in these two cysteine proteinases. Moreover, cathepsin L (EC 3.4.22.15) appears to exclude proline residues in S₁ and S₂ (Kirschke & Shaw, 1981).

Endo- and exo-peptidase activity of cathepsin B

Cathepsin B was first characterized as an endopeptidase (Barrett, 1977), but later its peptidyl dipeptidase activity was demonstrated. Cathepsin B sequentially cleaves C-terminal dipeptides from glucagon (Aronson & Barrett, 1978), from fructose-1,6-bisphosphate aldolase (Bond & Barrett, 1980), from neurotensin (Towatari & Katunuma, 1983) and from some enkephalins (Takahashi *et al.*, 1986b) and opioid oligopeptides (Marks *et al.*, 1986). Takahashi *et al.* (1986a)

supposed that one of two recently isolated cathepsin B isoenzymes from porcine spleen acts only as an exopeptidase.

In the hope of gaining more insight into the two different modes of cathepsin B action, two model peptides were synthesized (peptides IX and X in Table 4). The proline residues in P₃ and P₂' enforce a unique binding mode for cathepsin B and cathepsin H. This is somewhat in contrast with the expectation of Takahashi *et al.* (1986b), which excludes proline residues from subsite S₂'. The hydrolysis of these two pentapeptides is restricted to a single bond (Ala³-Ala⁴) by both cathepsins, which was proved by t.l.c. and high-voltage paper electrophoresis with Ala-Pro and Ala-Pro-NH₂ as references. The pentapeptide amide (compound IX) represents the endopeptidase substrate, the peptide acid (compound X) the peptidyl dipeptidase substrate. In order to investigate the supposed interaction between the arginine residue in subsite S₂' of cathepsin B and the P₂' substrate position (Takio *et al.*, 1983), the pH-dependence of the cathepsin B-catalysed hydrolysis of the pentapeptides was determined and compared with that of cathepsin H. For cathepsin H both substrates, the peptide amide as well as the peptide acid, have the same pH optima, at about pH 6.5 (Fig. 1). However, the pH optima for cathepsin B do not converge. Cathepsin B has its maximal activity hydrolysing the peptide acid close to pH 6.0, whereas the peptide amide is hydrolysed with the highest efficiency at pH 4.5. This acid pH optimum for the endopeptidase substrate is not attributable to contamination with an aspartic proteinase like cathepsin D (EC 3.4.23.5), because the ratio between the activities at pH 4.5 and pH 6.0 is not altered in the presence of 10⁻⁶ M-pepstatin. In contrast, both activities were completely inhibited by a peptide methyl ketone Z-Ala₂-Phe-Ala-CH₃, a highly efficient inhibitor of cathepsin B (results not shown).

Cathepsin B hydrolyses the peptide acid (peptide X) at pH 6.0 twice as fast as the peptide amide (peptide IX), which may indicate that cathepsin B acts preferentially as a peptidyl dipeptidase, although it is not restricted to one type of action. Cathepsin H clearly favours the peptide amide as substrate (Table 4). This is in agreement with the known endopeptidase activity of this enzyme.

If the kinetic parameters of cathepsin B are compared for both substrates (peptides IX and X) at pH 4.5 and pH 6.0, it is obvious that K_m and k_{cat} are decreased at the lower pH value (Table 4). Whereas the K_m values are nearly halved at pH 4.5 for both substrates, the k_{cat} values appear to be affected in a somewhat different manner. Going from pH 6.0 to pH 4.5 the k_{cat} value of

Table 4. Kinetic parameters for cathepsin (Cat) B- and cathepsin H-catalysed hydrolysis of Z-Pro-Ala-Ala-Ala-Pro-NH₂ and Z-Pro-Ala-Ala-Ala-Pro-OH at 40 °C

Enzyme	pH	P ₄ - P ₃ - P ₂ - P ₁ - P ₁ ' - P ₂ '	Peptide no.	[S] (mM)	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (M ⁻¹ ·s ⁻¹)
Cat B	6.0	Z-Pro-Ala-Ala-Ala-Pro-NH ₂	IX	1-8	8.0 ± 1.4	40 ± 7.2	5000
			X	1-8	17.9 ± 3.2	190 ± 24.7	10615
	4.5	Z-Pro-Ala-Ala-Ala-Pro-NH ₂	IX	1-8	4.6 ± 1.1	29 ± 2.3	6304
			X	1-8	8.0 ± 1.4	79 ± 13.4	9875
Cat H	6.5	Z-Pro-Ala-Ala-Ala-Pro-NH ₂	IX	1-8	3.5 ± 0.2	7.7 ± 0.2	2200
		Z-Pro-Ala-Ala-Ala-Pro-OH	X	1-8	7.4 ± 1.7	5.3 ± 0.5	716

Fig. 1. pH-activity profiles of cathepsins B- and H-catalysed hydrolysis of Z-Pro-Ala₃-Pro-NH₂ (▲) and Z-Pro-Ala₃-Pro-OH (□) at 40 °C

The relative activities of cathepsins B- and H-catalysed hydrolysis of Z-Pro-Ala-Ala-Ala-Pro-NH₂ (▲) and Z-Pro-Ala-Ala-Ala-Pro-OH (□) were determined with the TNBA method described in the Materials and methods section. The cleavage site in both substrates is between Ala³ and Ala⁴. The buffers used were 50 mM-sodium acetate (pH 3.5–5.5), 50 mM-phosphate (pH 6.0–7.0) and 50 mM-Tris/HCl (pH 7.5–8.0).

the peptide amide decreased by about 25%; however, in the case of the peptide acid, a decrease of 60% was observed. For the peptide acid a pK_a value of 3.85 was determined. Without considering the pH of the micro-environment in the active centre of the proteinase, the substrate is dissociated close to 100% at pH 6.0, whereas at pH 4.5 about 20% is available in the protonated form. The attraction between the positively charged arginine in S₂' and the α -carboxy group in P₂' should be then diminished. It is known from studies with serine proteinases that modifications on the P' part of a substrate influence the k_{cat} value in particular (Bauer, 1976; Obara *et al.*, 1979).

Conclusion

The efficiency of the endopeptidase activity of the cathepsins B and H from rat liver is influenced by four left-hand-side subsites, S₁–S₄. In both proteinases the pockets S₁ and S₂ discriminate proline in substrates, whereas a P₃ proline residue is well accepted. Cathepsin

B from rat liver is capable of acting as an endopeptidase and a peptidyl dipeptidase. The enzyme acts preferentially as a peptidyl dipeptidase if a free α -carboxy group is present and if the P₁, P₁' and possibly the P₂ position is not occupied by a proline residue.

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