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N-Peptidyl-*O*-carbamoyl amino acid hydroxamates: Irreversible inhibitors for the study of the S_2' specificity of cysteine proteinases

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A series of new inhibitors for cysteine proteinases with the general structure Z-Phe-Gly-NHO-CO-Aa (Aa = amino acids) was synthesized and tested as inhibitors of papain-like enzymes (cathepsins S, L, B and papain). Like *N*-peptidyl-*O*-acyl hydroxamates the inhibitors inactivate cysteine proteinases by a sulfenamidation of the active site cysteine residue. The most effective inhibitors display second order-rate constants of inactivation in the range of 10^3–10^4 $M^{-1} \cdot s^{-1}$. Since the structure of the *N*-peptidyl-*O*-carbamoyl amino acid hydroxamates allows the variation of the leaving group this class of inhibitors was used as a new tool for the evaluation of the S_2' specificity of cysteine proteinases.

Cysteine proteinase; Cathepsin; Substrate specificity; Hydroxamate; Inactivation

1. INTRODUCTION

N-Peptidyl-*O*-acyl hydroxamates are known as specific and potent inactivators of cysteine proteinases [1–3]. The variability of their *N*-acyl and *O*-acyl portions opens the opportunity to regulate effectively the affinity and reactivity towards the enzymes. The aliphatic and aromatic *O*-acyl residues tested so far were mainly used to control the reactivity of the leaving group by electronic effects. However, the introduction of an amino acid or peptide in the leaving group should allow the study of the S′ specificity of proteases.

Up to the present moment little is known about the S′ specificity of cysteine proteinases. Two recently published papers deal with the S_2' specificity of papain using two different approaches:
(i) peptide nucleophiles as deacylating agents [4] and
(ii) intramolecularly quenched fluorogenic peptide substrates which allow variations of P′ amino acid residues [5]. Both approaches select alanine as the the most effective residue in P_2' from a range of hydrophobic and non-hydrophobic residues. However, whereas the acylenzyme binds more efficiently hydrophobic bulky amino residues [4], the hydrolysis of peptide substrates is decreased with increasing hydrophobicity in the P_2' position [5].

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Abbreviations: Z-, benzyloxycarbonyl; -MCA, 4-methyl-7-coumarylamide; -NHO- is the hydroxylamine moiety; -Nbz, 4-nitrobenzoyl

These apparently conflicting results may be caused by different rate-determining steps.

In the present paper we report the inactivation of cysteine proteinases of the papain superfamily with several *N*-peptidyl-*O*-carbamoyl amino acid hydroxamates and compare the S_2' specificity of these proteases.

2. MATERIALS AND METHODS

2.1. *Enzymes*

Cathepsin L (EC 3.4.22.15) and cathepsin B (EC 3.4.22.1) were prepared from the lysosomal fraction of rat liver [6,7]. Cathepsin S (EC 3.4.22.27) was isolated from bovine spleen as previously described [8]. Papain (EC 3.4.22.2) was purchased from Serva, Heidelberg, Germany.

2.2. *Substrates and inhibitors*

Z-Phe-Arg-MCA and Z-Val-Val-Arg-MCA were synthesized as described in [9]. Z-Phe-Gly-NHOH was synthesized as previously reported [2]. The hydroxamic acid was transformed into a *N*-peptidyl-*O*-4-nitrophenylformate hydroxamate (Z-Phe-Gly-NHO-CO-ONPh) with 4-nitrophenyl chloroformate in dry tetrahydrofuran. The *N*-peptidyl-*O*-4-nitrophenylformate hydroxamate was finally coupled with a sodium salt of an amino acid suspension in dimethylformamide.

Homogeneity and integrity of the newly synthesized compounds were established by TLC, NMR, elemental analysis and mass spectroscopy (Z-Phe-Gly-NHO-NBz: Anal. Calcd. for $C_{26}H_{24}O_8N_4$: C, 58.98; H, 4.65; N, 10.77. Found: C, 58.81; H, 4,58; N, 10.67; Mass Calcd. 520.16. Found: 520.1; Z-FG-NHO-CO-Phe: Anal.Cald. for $C_{29}H_{30}O_8N_4$: C, 61.90; H, 5.38; N, 9.96. Found: C, 61.80; H, 5.32; N, 9.92; Mass Calcd. 562.21. Found: 562.1; Z-Phe-Gly-NHO-CO-Leu: Anal. Calcd. for $C_{26}H_{32}O_8N_4$: C, 59.07, H, 6.11, N, 10.60. Found: C, 59.23; H, 5,89; N, 10.55; Mass Calcd. 528.22. Found: 528.1; Z-Phe-Gly-NHO-CO-Val: Anal. Calcd. for $C_{25}H_{30}O_8N_4$: C, 58.34; H, 5.88; N, 10.89. Found: C, 587.24; H, 5.71; N, 10.59; Mass Calcd. 514.21. Found: 514.2; Z-Phe-Gly-NHO-CO-Ala: Anal. Calcd. for $C_{23}H_{26}O_8N_4$: C, 56.77; H, 5.39; N, 11.52. Found: C, 56.84; H, 5.46; N, 11.48; Mass Calcd. 486.17. Found: 486.2; Z-Phe-Gly-NHO-CO-Gly:

Anal. Calcd. for $C_{22}H_{24}O_8N_4$: C, 55.91; H, 5.12; N, 11.89. Found: C, 55.93; H, 5.13; N, 11.89. Mass Calcd. 472.16. Found: 472.1).

2.3. *Enzyme assays*

The inactivation of cysteine proteinases with substrate-analogous inhibitors proceeds according to Equation 1:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{–}I \overset{k_2}{\rightleftharpoons} E - I \quad (1)$$

where EI, E–I, k_1, k_{-1} represent the enzyme-inhibitor complex, the inactivated enzyme, and the rate constants of the non-covalent Michaelis–Menten complex. k_2 is the rate constant of the irreversible inactivation of the enzyme.

The inactivation rates (k_{obs}) in presence of substrate and for different inhibitor concentrations were determined for the cysteine proteinases according to Tian and Tsou [10]. By fitting the rate constants for different inhibitor concentrations to the Michaelis–Menten plot, the second-order rate constants k_2/K_i^{app} were obtained. The k_2/K_i^{app}-values were corrected to zero substrate concentration using Equation 2.

$$k_2/K_i = k_2/K_i^{app} \times (1 + [S]/K_m) \quad (2)$$

The progress curves for the inactivation of the cysteine proteinases in the presence of MCA-substrates were monitored at 22°C with a Shimadzu spectrophotometer UV-300 equipped with a fluorescence-detection unit at an excitation wavelength of 383 nm and with an emission filter of 450 nm.

The kinetic experiments were performed with a constant enzyme concentration (cathepsin L: 0.7 nM, cathepsin S: 2.0 nM, cathepsin B: 0.9 nM, papain: 3.1 nM) in 50 mM sodium acetate buffer (pH 5.5) for cathepsin L; in 50 mM potassium phoshate buffer (pH 6.5) for cathepsin S; in 50 mM potassium phosphate buffer (pH 6.0) for cathepsin B and in 50 mM Tris-HCl buffer (pH 7.5) for papain. The cathepsins L and B (stored in their Hg-inhibited form) were activated in the assay buffers containing 2.5 mM dithioerythreitol, and 2.5 mM EDTA × Na_2 for 5 min at 25°C, whereas cathepsin was preincubated for 15 min in its assay buffer containing 5 mM dithioerythreitol, 5 mM EDTA × Na_2 and 0.01% Triton X-100.

Substrates used were Z-Phe-Arg-MCA (1 μM for cathepsin L, K_m 2.8 μM [9], 50 μM for cathepsin B, K_m 250 μM [9], 10 μM for papain, K_m 50 μM (unpublished, D. Brömme)) and Z-Val-Val-Arg-MCA (10 μM for cathepsin S, K_m 17 μM [9]).

2.4. *Mass spectroscopy*

For the determination of the molecular mass of free papain and the papain–inhibitor adduct a triple quadrupole mass spectrometer (API III LC/MS system, Sciex, Thornhill, Ontario, Canada) was used as described in [11]. The molecular mass was calculated from the m/z peaks in the charge distribution profiles of the multiply charged ions [12]. Samples for mass spectrometric analysis were prepared in a buffer free mixture of papain (40μM) and inhibitor (Z-FG-NHO-CO-Phe 40 μM and 400 μM) in 10% acetonitrile. The formation of an inactive enzyme–inhibitor adduct was followed by measuring the residual activity.

3. RESULTS AND DISCUSSION

N-peptidyl-*O*-carbamoyl amino acid hydroxamates are irreversible inhibitors of cysteine proteinases. They belong to the class of *N*-*O*-diacyl hydroxamates, which are known as very potent inhibitors of lysosomal cysteine proteinases [1,2]. The inactivation of the enzyme follows a sulfenamidation of the active-site cysteine residue [13] as shown by mass spectroscopy (Fig. 1). The molecular mass obtained for the free enzyme was 23,422 ± 0.6 (calculated from the amino acid sequence: 23,422 Da) and for the papain-inhibitor adduct 23,774 ± 2.6 (calculated: 23,776 Da). The same type of enzyme–inhibitor adduct was already shown for the inactivation of papain with the Z-FG-NHO-CO-(2,4,6Me_3)Ph [13].

The second order rate constants of the tested inhibitors with an amino acid in the P_2' position are 1–2 orders of magnitude lower than for the nitrobenzyl compound (compound 1). This is related to the weaker electronic withdrawing effect of the amino acids as leaving groups.

N-peptidyl-*O*-carbamoyl amino acid hydroxamates allow an extension of the leaving group with amino acids and thus the study of the S′ specificity of cysteine

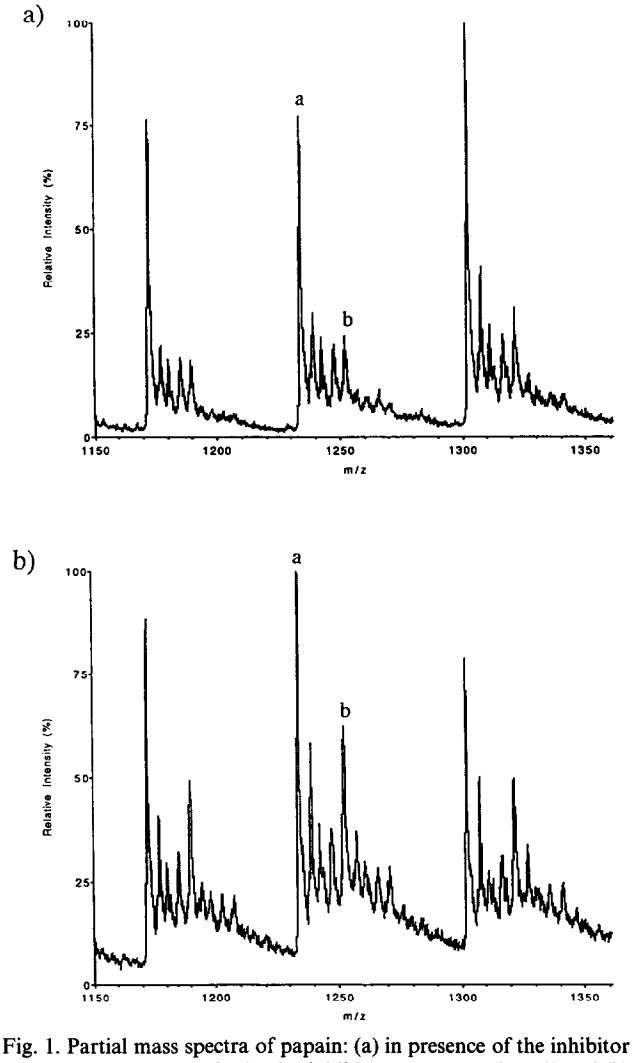

Fig. 1. Partial mass spectra of papain: (a) in presence of the inhibitor Z-Phe-Gly-NHO-CO-Phe (molar inhibitor–enzyme ratio = 1) and (b) in presence of the inhibitor Z-Phe-Gly-NHO-CO-Phe (molar inhibitor–enzyme ratio = 20). Peak a corresponds to the free enzyme (23,422 Da) and peak b to the sulfenamide (23,776 Da). The other minor peaks correspond to sodium and sulfate adducts of papain as reported in [13].

proteinases. Since the hydroxylamine carbonyl-moiety mimics a glycine residue (-NH-O-CO-) in P_1' position, the subsequent amino acids should interact with the S_2' subsite.

The data in Table I show that alterations in the P_2' position of the inhibitor effect the second-order-rate constants of inactivation. The most effective inhibitor for the cathepsins L and S and for papain is compound 2 with a phenylalanine in P_2'. Glycine (compound 6) in this position diminishes the inactivation constants by a factor of 10–30 for these enzymes. Fig. 2 clearly illustrates that with increasing hydrophobicity of the P_2'-residue the potential of the inhibitor increases too. Only cathepsin B is characterized by a different specificity. In general it displays a weaker discrimination between the tested P_2' residues and it prefers rather non-aromatic amino acid residues such as leucine and valine than phenylalanine and shows no detectable time-dependent inhibition with the glycine derivative. This may be related to the very different S' binding site in cathepsin B when compared with the other proteinases of the papain superfamily. The S' binding site in cathepsin B is closed by the so-called occluding loop, which explains the peptidyldipeptidase activity of the enzyme [14].

The decrease in the second-order rate constants parallel to the decrease of hydrophobicity of the leaving group of the inhibitors for papain and the cathepsins S and L is mainly due to an increase in K_i. This reveals that especially the binding of the inhibitor is influenced by hydrophobicity of the P_2' residue. The S_2' specificity of the cathepsins S and L and papain is consistent with recently published results for papain using peptide substrates [5] and peptide nucleophiles [4]. In the case of

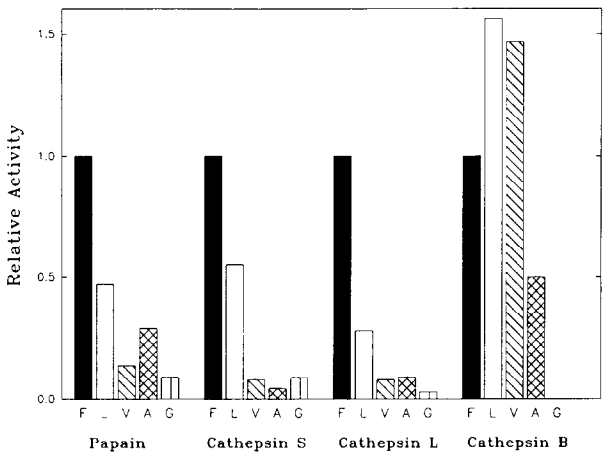

Fig. 2. Comparison of k_2/K_i values of the inactivation of papain and the cathepsins S, L and B by Z-Phe-Gly-NHO-CO-Aa (Aa: F = Phe, L = Leu, V = Val, A = Ala, G = Gly). The values are normalized to 1 for Z-Phe-Gly-NHO-CO-Phe.

substrates the k_{cat}/K_m values decrease with increased hydrophibicity of the P_2' residue. Here mainly the k_{cat} value is affected indicating the influence on the acylation rate. The authors [5] argued that increased hydrophobic interactions between the P_2' and S_2' papain binding site retard the release of the C-terminal product. On the other hand, the deacylation rate of papain was enhanced by using peptide nucleophiles with increased hydrophobicity in the putative P_2' position [4].

The effect of hydrophobic interactions between the ligand and the enzyme depends on the mechanism and the catalytic step which is affected and can therefore be

Table I

Inactivation of the cysteine proteinases cathepsin S, cathepsin L, cathepsin B and papain with *N*-peptidyl-*O*-carbamoyl amino acid hydroxamates (Z-Phe-Gly-NHO-CO-Aa)

		Cathepsin S			Cathepsin L		
	Aa	k_2 (s^{-1}) 10^3	K_i (μM)	k_2/K_i ($M^{-1} \cdot s^{-1}$)	k_2 (s^{-1}) 10^3	K_i (μM)	k_2/K_i ($M^{-1} \cdot s^{-1}$)
1	4-NO_2Ph	86 ± 1	0.9 ± 0.03	95,600	41 ± 4	0.044 ± 0.004	931,800
2	Phe	2.1 ± 0.1	0.25 ± 0.05	8,400	13.0 ± 1	0.19 ± 0.04	68,420
3	Leu	4.7 ± 0.4	1.01 ± 0.2	4,650	8.2 ± 0.8	0.42 ± 0.08	19,520
4	Val	–	–	710[a]	4.0 ± 0.1	0.71 ± 0.1	5,630
5	Ala	2.7 ± 0.2	7.5 ± 1.3	360	6.1 ± 0.3	0.97 ± 0.08	6,290
6	Gly	3.2 ± 0.1	4.4 ± 1.0	780	3.2 ± 0.1	1.65 ± 0.2	1,940
		Cathepsin B			Papain		
1	4-NO_2Ph	190 ± 30	12 ± 2	15,800	76 ± 13	15 ± 5	5,070
2	Phe	5.0 ± 0.4	6.7 ± 1.1	750	27.0 ± 4	25 ± 4	1,080
3	Leu	4.8 ± 0.4	4.1 ± 0.6	1,170	9.7 ± 0.6	19 ± 2	510
4	Val	1.1 ± 0.05	1.0 ± 0.2	1,100	3.0 ± 1.0	20 ± 3	150
5	Ala	2.4 ± 0.2	6.4 ± 1.7	375	5.6 ± 0.2	18 ± 2	310
6	Gly	–	3[b]		5.6 ± 0.2	57 ± 8	98

[a] No saturation kinetics achieved
[b] Competitive inhibition

of opposite character. This can be exploited in the design of inhibitors and substrates. Inhibitors of papain and the cathepsins S and L demand bulky hydrophobic residues in P_2', whereas substrates should contain smaller residue in this position.

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