Glycine flanked by hydrophobic bulky amino acid residues as minimal sequence for effective subtilisin catalysis

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The specificity of alkaline mesentericopeptidase (a proteinase closely related to subtilisin BPN') for the C-terminal moiety of the peptide substrate (P_1' specificity) has been studied in both hydrolysis and aminolysis reactions. *N*-Anthraniloylated peptide *p*-nitroanilides as fluorogenic substrates and amino acid or peptide derivatives as nucleophiles were used in the enzymic peptide hydrolysis and synthesis. Both hydrolysis and aminolysis kinetic data suggest a stringent specificity of mesentericopeptidase and related subtilisins to glycine as P_1' residue and predilection for bulky hydrophobic P_2' residues. A synergism in the action of S_1' and S_2' subsites has been observed. It appears that glycine flanked on both sides by hydrophobic bulky amino acid residues is the minimal amino acid sequence for an effective subtilisin catalysis.

INTRODUCTION

Subtilisins are bacterial extracellular serine proteinases with broad P_1 specificity (nomenclature of Schechter & Berger, 1967). The secondary interactions of these proteinases with the *N*-terminal moiety of peptide effectors (acyl or P_1 specificity) have been studied with peptidylchloromethanes and peptide esters and *p*-nitroanilides (Phillip & Bender, 1983). These studies indicate an extended binding site comprising at least five S_i subsites.

In contrast with the well-studied P_1 specificity, less is known for the interactions of subtilisins with the C-terminal moiety of the peptide substrates (leaving-group or P_1' specificity). A favourable effect of these interactions has been observed in peptide hydrolysis catalysed by subtilisin BPN' (Moriyama & Oka, 1977) and the subtilisin-like proteinase thermolysin (Brömme *et al.*, 1986). Any information concerning S_i' – P_1' interactions became particularly valuable with the fast development of enzymic peptide synthesis (Fruton, 1982; Jakubke *et al.*, 1985). P_1' specificity or nucleophile specificity of the enzyme determines the preparative yield in kinetically controlled enzymic peptide synthesis (Petkov & Stoineva, 1984).

In the present paper we report data for the P_1' specificity of alkaline mesentericopeptidase. This proteinase is closely related to subtilisin BPN', as judged by primary structure (Svendsen *et al.*, 1986) and primary (P_1) specificity (Stambolieva & Chakarova, 1978). To get the necessary information we studied the enzymic hydrolysis of *N*-anthraniloylated peptide *p*-nitroanilides. Recently we found (Bratovanova & Petkov, 1987) that these peptide substrates are highly sensitive intramolecularly quenched fluorogenic substrates. We obtained additional information concerning the S_i' – P_1' interactions by determination of the aminolysis/hydrolysis ratio of nucleophiles designed to model the P_1' part of peptide substrates.

MATERIALS AND METHODS

Enzymes

Crystalline alkaline mesentericopeptidase was isolated from culture broth of *Bacillus mesentericus* (Karadjova *et al.*, 1970). Bovine pancreatic α -chymotrypsin (chymotrypsin A_4) and thermolysin were obtained from Boehringer Mannheim.

Active-site concentrations of the enzyme stock solutions (α -chymotrypsin and alkaline mesentericopeptidase) were determined by active-site titration with *N*-trans-cinnamoylimidazole (Schonbaum *et al.*, 1961).

Substrates

Ac-Phe(NO₂)-OMe was obtained as described previously (Petkov & Stoineva, 1984).

Ant-Ala-Phe-OMe was prepared from the corresponding free base by acylation with benzotriazol-1-yl anthranilate as described by Bratovanova & Petkov (1987). The free base H-Ala-Phe-OMe was obtained by acylation of H-Phe-OMe, HCl with Z-Ala-Cl and subsequent hydrogenolysis (Pd/C catalyst) of the resulting benzyloxycarbonyl derivative. Recrystallization from ethyl acetate/light petroleum produced the pure compound (yield 82%).

Ant-Ala-Phe-OH was obtained by enzymic hydrolysis of the corresponding methyl ester. A 20 mmol portion of Ant-Ala-Phe-OMe was suspended in 10 ml of 0.2 M-sodium carbonate/bicarbonate buffer, pH 9.3, and 10 mg of alkaline mesentericopeptidase was added. After the hydrolysis was over (30–40 min), the product was precipitated on acidification to pH 2. Recrystallization from ethyl acetate/light petroleum gave the pure product (yield 45%).

Amino acid and peptide *p*-nitroanilides were obtained by acylation of *p*-nitroaniline or amino acid *p*-nitroanilides with the corresponding benzyloxycarbonyl-

Abbreviations used: Ac, acetyl; Ant, anthraniloyl; Z, benzyloxycarbonyl.

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Table 1. Physical constants and elemental analyses of the newly synthesized anthraniloyl-peptide *p*-nitroanilides Ant-Ala-Phe-P₁'-P₂'-NHPH(NO₂)

-P ₁ '-P ₂ '-NHPPh(NO ₂)	M.p. (°C)	[α] _D ²⁵ (c 0.4 in EtOH) (°)	Formula	Analyses			
					C	H	N
-Gly-NHPPh(NO ₂)	116–117	12.0	C ₂₇ H ₂₈ N ₆ O ₆	Calc.	60.90	5.26	15.79
				Found	60.92	5.38	15.59
-Ala-NHPPh(NO ₂)	128–129	32.0	C ₂₈ H ₃₀ N ₆ O ₆	Calc.	61.54	5.49	15.38
				Found	61.57	5.67	15.21
-Val-NHPPh(NO ₂)	135–136	−48.0	C ₃₀ H ₃₄ N ₆ O ₆	Calc.	62.70	5.92	14.63
				Found	62.75	6.06	14.59
-Gly-Val-NHPPh(NO ₂)	155–156	−32.5	C ₃₂ H ₃₇ N ₇ O ₇	Calc.	60.80	5.80	15.50
				Found	60.92	6.02	15.38

amino acid chlorides and subsequent hydrogen-bromidolysis of the resulting benzyloxycarbonyl derivatives.

Anthraniloyl peptide *p*-nitroanilides were obtained by enzymic peptide synthesis.

Ant-Ala-Phe-Gly-NHPH(NO₂), Ant-Ala-Phe-Ala-NHPH(NO₂) and Ant-Ala-Phe-Gly-Val-NHPH(NO₂) were obtained by kinetically controlled chymotrypsin aminolysis of Ant-Ala-Phe-OMe with the corresponding amino acid or peptide *p*-nitroanilides. A 0.2 mmol portion of the latter and 0.2 mmol of Ant-Ala-Phe-OMe were dissolved in ethyl acetate (10 ml), and 10 ml of 0.1 M-sodium carbonate/bicarbonate buffer, pH 9.3, was added, followed by 10 mg of α-chymotrypsin. After 30–40 min the precipitate formed was collected by filtration, washed and recrystallized from ethyl acetate/light petroleum.

Ant-Ala-Phe-Val-NHPH(NO₂) was prepared by thermodynamically controlled thermolysin synthesis. A 0.25 mmol portion of Ant-Ala-Phe-OH was dissolved in 1.5 ml of 0.2 M-sodium maleate buffer, pH 7.2, containing 40% (v/v) dimethylformamide. A 0.25 mmol portion of H-Val-NHPH(NO₂) dissolved in 1.5 ml of the same buffer was then added, and the reaction was initiated by 1.5 mg of thermolysin. After 30–40 min the precipitated product was collected by filtration, washed with buffer and recrystallized from ethyl acetate/light petroleum.

The purity of the compounds was checked by t.l.c. on silica-gel plates (Merck) in the solvent system chloroform/acetic acid/light petroleum (1:1:1, by vol.). The characteristic analytical data for the newly synthesized compounds are shown in Table 1.

Nucleophiles

H-Gly-Phe-OH was a product of Serva. The tripeptide nucleophiles H-Gly-Phe-Phe-NH₂, H-Gly-Phe-Leu-NH₂ and H-Gly-Phe-Leu-NH₂ and the dipeptide H-Tyr-Phe-NH₂ were prepared by chymotrypsin aminolysis of Z-Gly-Phe-OMe and Z-Tyr-OMe and subsequent hydrolysis. The resulting peptides were h.p.l.c. pure. The rest of the nucleophiles were prepared as described previously (Petkov & Stoineva, 1984).

Kinetic studies

The enzymic hydrolysis of the anthraniloyl-peptide *p*-nitroanilides was monitored spectrofluorimetrically. The fluorescence of the intramolecularly quenched fluoro-

phore Ant-Ala-Phe-OH, released during the hydrolysis, was monitored at 425 nm upon excitation at 340 nm with a Perkin-Elmer LS 5 spectrofluorimeter. All measurements were carried out in 25 mM-Tris/HCl buffer, pH 8.0, containing 50 mM-CaCl₂ and 2.5% (v/v) dimethylformamide. The enzymic hydrolysis of all substrates conformed to Michaelis-Menten kinetics. The kinetic parameters *k*_{cat} and *K*_m were calculated from double-reciprocal plots of the initial-rate data. On the other hand, the specificity constants *k*_{cat}/*K*_m were obtained directly from the pseudo-first-order fluorescence traces.

The values of the aminolysis/hydrolysis ratio of different amino acid and peptide nucleophiles were determined spectrophotometrically as described previously (Petkov & Stoineva, 1984).

RESULTS AND DISCUSSION

The interpretation of kinetic data on secondary specificity is not always straightforward (Fruton, 1982). Caution has to be exercised on the reliability of the kinetic data and their relevance to the corresponding enzyme subsite-substrate portion interactions.

The substrate analysis of the acyl specificity of alkaline mesentericopeptidase (Stambolieva *et al.*, 1983) has shown that its S₁ subsite has a predilection for derivatives of amino acids with aromatic and bulky aliphatic side chains. The latter are particularly effective when alanine is at the P₂ position. Following these observations, we used the peptide substrates Ant-Ala-Phe-P₁'-P₂'-NHPH(NO₂) (P₁' = Gly, Ala, Val; P₂' = Val) to study the leaving-group specificity. Their hydrolysis can be easily monitored spectrofluorimetrically (Bratovanova & Petkov, 1987). On the other hand, the nature of the P₂' residue has been selected so as to avoid hydrolysis of the anilide bond. Actually, the t.l.c. analysis of the reaction products showed that the enzyme hydrolysis is restricted to the Phe-P₁' peptide bond.

Table 2 shows the kinetic parameters of the enzymic hydrolysis of these anthraniloylated peptide *p*-nitroanilides. The specificity constants *k*_{cat}/*K*_m for chymotrypsin hydrolysis of these substrates are also included in the Table. Our conclusions are principally based on these constants as the most reliable kinetic parameter. The variation of the *k*_{cat}/*K*_m values with the nature of the P₁' residue suggests a favourable interaction of the S₁' subsite with the smallest amino acid residue, glycine. Any

Table 2. Kinetic parameters of the hydrolysis of anthraniloyl-peptide *p*-nitroanilides catalysed by alkaline mesentericopeptidase and α -chymotrypsin

Measurements were carried out in 25 mM-Tris/HCl buffer, pH 8.0, containing 50 mM-CaCl₂ and 2.5% dimethylformamide at 25 °C; s.d. does not exceed 7%. $k_{\text{cat.}}/K_m$ values marked with an asterisk (*) were determined from pseudo-first-order traces.

P ₃ -P ₂ -P ₁ -P ₁ '-P ₂ '-P ₃ '	Alkaline mesentericopeptidase			α -Chymotrypsin
	$k_{\text{cat.}}$ (s ⁻¹)	K_m (mM)	$k_{\text{cat.}}/K_m$ (M ⁻¹ ·s ⁻¹)	$k_{\text{cat.}}/K_m$ (M ⁻¹ ·s ⁻¹)
Ant-Ala-Phe-Gly-NHPh(NO ₂)	0.057	0.020	2850 3150*	2040*
Ant-Ala-Phe-Ala-NHPh(NO ₂)	0.048	0.033	1450 1500*	2620*
Ant-Ala-Phe-Val-NHPh(NO ₂)			— 160*	5870*
Ant-Ala-Phe-Gly-Val-NHPh(NO ₂)	0.124	0.007	16700 18240*	8700*

Table 3. Reactivity of various amino acid and peptide nucleophiles in enzymic aminolysis of Ac-Phe(NO₂)-OMe

Reactions were carried out at pH 9.3 at 25 °C; s.d. is less than 5%.

H-P ₁ '-P ₂ '-P ₃ '-P ₄ '	Aminolysis/hydrolysis ratio	
	Alkaline mesenterico-peptidase	α -Chymotrypsin
H-Gly-NH ₂	800	3960*
H-Ala-NH ₂	50	4130*
H-Phe-NH ₂	< 1	3000
H-Val-NH ₂	< 1	4500
H-Leu-NH ₂	< 1	4280*
H-Gly-Phe-NHCH ₃	3250	4020*
H-Gly-Leu-NH ₂	2200	7340*
H-Gly-Val-NH ₂	1580	1250
H-Ala-Phe-NHCH ₃	560	4600
H-Ala-Lys-OH	480	140*
H-Ala-Ala-NH ₂	320	1100
H-Ala-Ala-OH	180	280*
H-Tyr-Phe-NH ₂	< 1	1040
H-Gly-Phe-Phe-NH ₂	4000	6600
H-Gly-Gly-Phe-OH	1740	200
H-Gly-Phe-Leu-NH ₂	1680	
H-Gly-Phe-Leu-NH ₂	1420	

* Petkov & Stoineva (1984).

increase in the size of the P₁' side chain brings about a large decrease in $k_{\text{cat.}}/K_m$. On the contrary, bulky P₁' residues improve the chymotrypsin catalysis.

The observed predilection of the subtilisin S₁' subsite for glycine could be questioned since the *p*-nitroanilide group is supposed to be involved in the S₂'-P₂' interactions. The data for the aminolysis/hydrolysis ratio for nucleophiles interacting with the S₁' subsite only (Table 3) are unambiguous: H-Gly-NH₂ is the best nucleophile in the aminolysis of Ac-Phe(NO₂)-OMe, H-Ala-NH₂ and other larger-size nucleophiles being almost

unreactive. Again, the reverse effect is observed in chymotrypsin aminolysis (Table 3). Therefore, in contrast with chymotrypsin, alkaline mesentericopeptidase possesses a stringent specificity for the P₁' residue.

A predilection for small-size P₁' residues of the S₁' subsite has been observed with subtilisin BPN' (Moriyama & Oka, 1977) and thermolysin (Brömme *et al.*, 1986) as well. Moriyama & Oka (1981) later studied the subtilisin BPN'-catalysed peptide synthesis using H-Ala-NH₂ and H-Leu-NH₂ as nucleophiles only and concluded that this enzyme is not a good catalyst in peptide synthesis. Data shown in Table 3 suggest that this is due to the stringent specificity of the S₁' subsite of subtilisin-like proteinases.

The close similarity between alkaline mesentericopeptidase and subtilisin BPN' (Svendsen *et al.*, 1986) enables us to discuss the experimental results in terms of the known three-dimensional structure of subtilisin BPN'-effector complexes. A speculated binding mode of a 'hypothetical' peptide substrate of subtilisin BPN' (Robertus *et al.*, 1972) has been more recently supported by the crystal structure of the complex between subtilisin BPN' and the *Streptomyces* subtilisin inhibitor (Hirono *et al.*, 1984). These studies indicate that the carbonyl group of P₁' points away from and the P₁' side chain points towards the mass of the enzyme. Just the opposite are the orientations of these groups in the chymotrypsin-inhibitor complex (Fersht *et al.*, 1973), accounting for the difference in the S₁'-subsite specificity of these enzymes.

As mentioned above, the *p*-nitrophenyl group of Ant-Ala-Phe-P₁'-NHPh(NO₂) is considered to interact with the S₂' subsite. The switch-on of the S₂'-P₂' interaction by the hydrophobic valine side chain greatly affects the hydrolysis of Ant-Ala-Phe-Gly-Val-NHPh(NO₂) catalysed by both alkaline mesentericopeptidase and chymotrypsin (Table 2). The secondary hydrolysis of the P₂'-NHPh(NO₂) bond prevents the study of this interaction by simple variation of the P₂' residue. This is possible, however, with the enzymic aminolysis of Ac-Phe(NO₂)-OMe. The determination of the aminolysis/hydrolysis ratio provides reliable data for the S₂'-subsite characterization (Petkov & Stoineva, 1984). In contrast with the S₁' subsite, this subsite accommodates bulky and hydrophobic P₂' side chains (Table 3). Moreover, S₂'-

P_2' interaction is effective provided that $S_1'-P_1'$ interaction is effective: in the case of a larger P_1' residue the nucleophile reactivity strongly decreases (H-Ala- P_2' -NH₂) or is negligible (H-Tyr-Phe-NH₂). Such a synergism in the action of proteinase subsites is probably the essential feature of proteinase secondary specificity.

Hydrogen-bond and hydrophobic interactions of the P_2' residue and subtilisin BPN' S_2' subsite have been proposed and later demonstrated kinetically (Moriyama & Oka, 1977) and crystallographically (Hirono *et al.*, 1984). It appears that these interactions are essential for the specificity-determining requirements of subtilisin-like enzymes. The P_2' residue is the C-terminal of the primary contact region of the microbial subtilisin inhibitor (Hirono *et al.*, 1984). $S_3'-P_3'$ interaction is probably not essential for alkaline mesentericopeptidase as well, since the effect of the P_3' residue is negligible (Table 3).

Comparison of the P_1' specificity of chymotrypsin-like and subtilisin-like enzymes reveals the structural basis of their specificity. The main difference is in their P_1' specificity: bulky P_1' residues promote chymotrypsin and hamper subtilisin catalysis. To work efficiently, the subtilisin S_1' subsite needs the assistance of the S_2' subsite. Therefore it appears that glycine flanked on both sides by hydrophobic bulky amino acid residues is the minimal amino acid sequence for effective subtilisin catalysis.

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