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Peptide Inhibitors and Activators of Carboxypeptidase B

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A number of peptides containing D-residues at the penultimate or C-terminal position were tested for their ability to affect the carboxypeptidase-B-catalysed hydrolysis of basic and non-basic peptides and esters. The effect of these modifiers, *i.e.* activation or inhibition is dependent on the nature of the modifier. The factors that determine the activity of the modifiers are: (a) the length of the peptide; (b) the basic or non-basic nature of the C-terminal and/or the penultimate residue and the optical configuration of these residues; (c) the substrate tested. The wide variations observed in the mode of activation and/or inhibition and the lack of correspondence between the K_i values is compatible with previous data on smaller inhibitors and larger peptide substrates, that the peptide and ester substrates of carboxypeptidase B occupy different loci on the enzyme.

In exploring the active site of enzymes, it is very advantageous to make use of competitive inhibitors which are lined up on the active site in a manner similar to the substrates. On the basis of previous information of the binding site of carboxypeptidase B [1, 3], it was expected that peptides containing D-residues at the penultimate or C-terminal position should behave as competitive inhibitors. In the present work, a number of related peptides were tested for their ability to inhibit the carboxypeptidase B hydrolysis of basic and non-basic peptides and esters. Kinetic studies are presented showing the nature of inhibition or acceleration observed.

MATERIALS AND METHODS

Carboxypeptidase B (porcine pancreas) was purchased from the Worthington Biochemical Corp. (N. J.); hippuryl-L-arginine, hippuryl-L-phenyllactate, hippuryl-L-argininic acid and D-arginine were obtained from Cyclo Chemical Corp.; benzyloxycarbonyl-L-alanyl-L-alanyl-L-alanine [Z-(L-Ala)$_3$] from Miles-Yeda (Israel).

SYNTHESIS OF PEPTIDES

All the peptides were synthesized by the *N*-hydroxysuccinimide ester method [4] essentially as described in the preceding paper [2]. Elongation of the peptide chain was carried out by the same procedure after removal of the benzyloxycarbonyl group as follows. The blocked pepitde was dissolved in anhydrous methanol. A 10% Pd/C suspension in water was then added, in an amount comprising 10% of the methanolic solution. Hydrogenation was carried out at room temperature for 12–24 h. The course or reaction was followed by thin-layer chromatography on silica gel, using 1-butanol–glacial acetic acid–water (25:6:25, v/v/v) as the solvent. The catalyst was then filtered and the solution was evaporated to dryness. The residue was dissolved in a minimal amount of water and precipitated by addition of ethanol.

All compounds were found to be pure on thin-layer chromatography on silica gel. After drying over H_2SO_4 *in vacuo*, elementary analysis showed that some of the peptides analyzed as hydrates, so that amino acid analysis was carried out to determine the exact composition of each peptide. The analytical data are shown in Table 1.

Protein Concentrations

These were determined by the absorbance at 278 nm, using a millimolar absorptivity of 73 $mM^{-1} \cdot cm^{-1}$ [5]. A stock solution of the enzyme (0.10 mM) was diluted before each set of runs and kept at 25 °C. These solutions were used within an hour.

Substrates and Inhibitors

Stock solutions (0.02 M) of peptides and esters were prepared in 0.1 M NaCl–0.05 M Tris buffer

Abbreviations. The abbreviations for peptides follow the CBN rules, see *Eur. J. Biochem. 27*, 201–207 (1972); Z-, benzyloxycarbonyl; Bz-, benzoyl and -OSuc, the *N*-hydroxysuccinimide ester.

Enzyme. Carboxypeptidase B (EC 3.4.2.2).

Table 1. *Analytical data of the isomers of Z-(Ala)n-Arg*

Compound	Formula	Neutral equiv.		Calculated			Found			Alanine / arginine	Melting point
		Calcd	Found	C	H	N	C	H	N		
				%	%	%	%	%	%		°C
Z-D-Ala-OSuc	$C_{15}H_{16}O_5N_2$	320	322	—	—	8.75	—	—	8.6		120
Z-(L-Ala)$_2$-OSuc	$C_{18}H_{21}O_6N_3$	391	393	55.2	5.4	10.7	55.4	5.5	10.9		143-145
Z-L-Ala-D-Arg	$C_{17}H_{25}O_5N_5 \cdot 5\,H_2O$	469	—	43.4	7.5	14.9	43.3	7.5	14.7	1.0:1.0	
Z-D-Ala-L-Arg	$C_{17}H_{25}O_5N_5 \cdot 3\,H_2O$	433	—	44.8	7.1	16.2	44.7	7.3	16.1	1.0:1.0	
Z-(L-Ala)$_2$-D-Phe	$C_{23}H_{27}O_6N_3$	441	440	62.5	6.1	9.5	62.6	6.3	9.4		196
Z-(L-Ala)$_2$-D-Arg	$C_{20}H_{30}O_6N_6$	450	—	53.3	6.7	18.6	53.5	6.8	18.9	2.0:0.99	
Z-L-Ala-D-Ala-L-Arg	$C_{20}H_{30}O_6N_6 \cdot 2\,H_2O$	486	—	49.4	7.0	17.2	49.6	7.1	17.1	2.0:0.98	
Z-L-Ala-D-Ala-L-Ala	$C_{17}H_{23}O_6N_3 \cdot H_2O$	383	385	53.2	6.5	11.0	53.4	6.4	11.1		114-115
Z-(L-Ala)$_3$-D-Arg	$C_{23}H_{35}O_7N_7 \cdot 3\,H_2O$	575	—	48.0	7.1	17.0	47.8	7.0	16.9	2.9:0.98	
Z-L-Ala-L-Ala-D-Ala-L-Arg	$C_{23}H_{35}O_7N_7 \cdot 3\,H_2O$	575	—	48.0	7.1	17.0	48.1	7.1	16.8	3.0:0.98	

Table 2. *Effect of modifiers on the carboxypeptidase-B-catalyzed hydrolysis of peptides*
The inhibition was all of a competitive type. $\bar{K}_i = 1/K_i$

Modifier	Bz-L-Gly-L-Arg		Z-L-Ala-L-Ala-L-Ala	
	Type of interaction	$\bar{K}_i$	Type of interaction	$\bar{K}_i$
		M^{-1}		M^{-1}
Z-L-Ala-D-Arg	Inhibition	3700	Inhibition	1050
Z-L-Ala-D-Ala	Activation	—	Activation	—
Z-L-Ala-L-Ala-D-Arg	Inhibition	4000	Inhibition	2700
Z-L-Ala-L-Ala-D-Phe	Activation	—	Activation	—
Z-L-Ala-D-Ala-L-Arg	Inhibition	9000	Inhibition	1140
Z-L-Ala-D-Ala-L-Ala	Activation	—	Activation	—
Z-L-Ala-L-Ala-D-Ala-L-Arg	Inhibition	1090	Inhibition	210
Z-L-Ala-L-Ala-D-Ala-L-Ala	Activation	—	Activation	—

pH 7.9 and were extracted with 0.1% dithiazone in carbon tetrachloride to avoid contamination by adventitious metal ions. All further dilutions were performed with the same buffer previously extracted with dithizone.

The pH of solutions was measured on a radiometer model-26 pH meter equipped with a GK2312C glass-calomel combination electrode.

Activity Measurements

All rate measurements conducted by means of spectrophotometric assay were carried out on a Cary model-16K recording spectrophotometer equipped with a thermostated cell compartment at 25 $\pm$ 0.1 °C. The values of k_{cat} and K_m were calculated from Lineweaver-Burk plots of initial velocities. The inhibition constants were obtained from Lineweaver-Burk plots and from Dixon plots.

Peptidase activity was determined spectrophotometrically using 1 mM hippurylarginine (at 254 nm) [6]. The hydrolysis of the Z-(L-Ala)$_3$ was followed at 225 nm using 0.7 ml 10 mM Z-(L-Ala)$_3$ in 0.2-cm light-path cuvettes. Carboxypeptidase B concentrations in the assay mixture were 10—20 nM in the former and 200 nM in the latter.

Esterase activity was measured spectrophotometrically at 254 nm [6] using 0.5 mM hippuryl-L-argininic acid and hippuryl-L-phenyllactate. Enzyme concentrations in the assay mixtures were 6 nM and 12 nM respectively.

RESULTS AND DISCUSSION

As shown in Table 2, the effect of the modifiers on the peptidase activity is dependent on which D-isomer is present in the ultimate or penultimate positions of the modifier. Thus, the presence of D-arginine in the C-terminal, or the combination of a D-amino acid residue in the penultimate and L-arginine in the ultimate positions, will cause competitive inhibition in the hydrolysis of the two peptides substrates, *i.e.* Bz-L-Gly-L-Arg and Z-(L-Ala)$_3$. On the other hand, if the C-terminal is not basic, *i.e.* alanine or phenylalanine, then introduction of D-isomer at either penultimate or C-terminal residue will cause acceleration of the two peptides. The effect

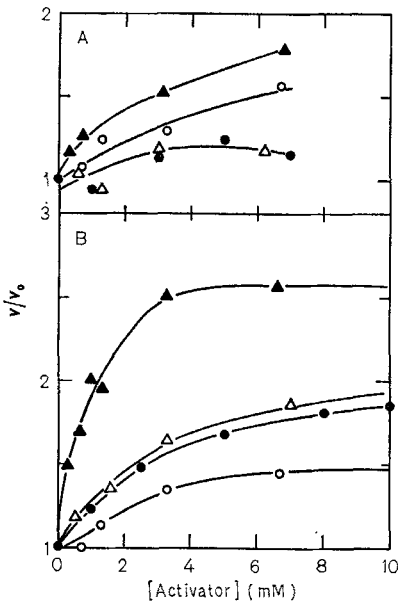

Fig. 1. *Activation of carboxypeptidase-B-catalysed hydrolysis of peptides: (A) Z-(L-Ala)$_3$ at 5 mM and (B) Bz-L-Gly-L-Arg at 0.5 mM.* The activators are: (●) Z-L-Ala-D-Ala-L-Ala; (○) Z-L-Ala-D-Ala; (△) Z-(L-Ala)$_2$-D-Ala-L-Ala and (▲) Z-(L-Ala)$_2$-D-Phe

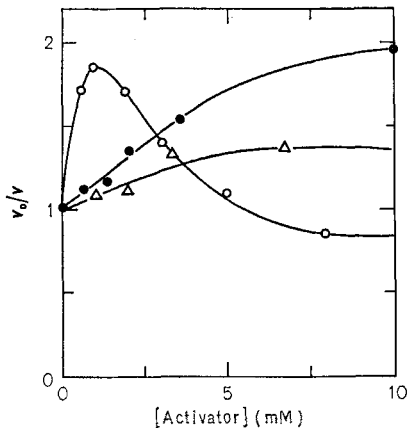

Fig. 2. *Activation of carboxypeptidase-B-catalysed hydrolysis of the ester hippuryl-L-phenyllactate at constant concentration of 0.5 mM.* The activators are: (△) Z-L-Ala-D-Ala; (○) Z-L-Ala-D-Ala-L-Ala and (●) Z-L-Ala-L-Ala-D-Phe

of various activators on the hydrolysis of Bz-L-Gly-L-Arg and Z-(L-Ala)$_3$ at constant subtsrate concentration is shown in Fig. 1. As can be seen there is an increase in the rate of hydrolysis of both substrates with increasing concentration of the modifier. This activation implies that the modifiers do not interact directly with the active site occupied by the substrates, since this would result with competitive inhibition, but with another site(s) on the enzyme. The enzyme · modifier complex thus formed is more active towards the peptide substrates than the free enzyme. The curves shown in Fig. 1 are indicative of a saturation reaction, *i.e.* at a high modifier concentration the enzyme is present mainly as a complex, the activity of which is given by the plateau of the curve. The reactivity of the complex, however, depends both on the nature of the modifier and on the substrate being hydrolyzed. Thus, the enzyme · modifier complex formed with Z-L-Ala-D-Ala-L-Ala and Z-(L-Ala)$_2$-D-Ala-L-Ala is more active towards the hydrolysis of the basic substrate Bz-L-Gly-L-Arg than towards the hydrolysis of the non-basic peptide, Z-(L-Ala)$_3$. The diversity in kinetic characteristics of the different enzyme · modifier complexes towards the different substrates will be further shown in regards to hydrolysis of ester substrates. As shown in Fig. 2, the complex formed with Z-L-Ala-D-Ala-L-Ala behaves differently showing biphasic effect towards the hydrolysis of hippurylphenyllactate. The modifier increases hydrolysis up to 1 mM whereas ar a higher concentration the hydrolysis rate drops. This can be caused by more than one mode of binding of the modifier probably at different binding sites.

Table 3 summarized the effect of the modifiers on the esterase activity of carboxypeptidase B. Here we can see that the response is much more complex than that of the peptidase activity (Table 2). The three non-basic modifiers studied, Z-L-Ala-D-Ala, Z-L-Ala-D-Ala-L-Ala and Z-(L-Ala)$_2$-D-Phe activate the esterase activity towards hippurylphenyllactate. The two tripeptides competitively inhibit the hydrolysis of hippurylargininic acid, while the dipeptide exhibits non-compeptitive inhibition. In the two series of arginyl-peptides, the two tetrapeptides, Z-(L-Ala)$_3$-D-Arg and Z-(L-Ala)$_2$-D-Ala-L-Arg, competitively inhibit the hydrolysis of both basic and non-basic ester substrates. In all other cases, the effect of the modifier was unpredictable : competitive, non-competitive or mixed inhibition. It should by mentioned that all of the modifiers investigated are either completely resistant to hydrolysis by carboxypeptidase B or degraded at a very slow rate which is negligible under the conditions employed. Recently, Wunsch *et al.* [7], reported the hydrolysis of peptides having D-arginine at the C-terminal but under considerably more drastic conditions, *i.e.* 20 h, 37 °C and 1:100 (w/w) ratio of enzyme to substrate.

The inhibition constants obtained for the various competitive inhibitors for the peptidase activity of carboxypeptidase B are given in Table 4. In general, dipeptides and tripeptides are better inhibitors; moreover, the presence of a C-terminal arginine causes the compounds to be competitive inhibitors, presumably due to anchoring of the derivative to part of the area of the active center. The effect of the length of inhibitor is similar to that seen in peptide substrates [2], where optimal activity is observed

Table 3. *Effect of modifiers on the esterase activities of carboxypeptidase B*
The ratio v/v_0 is calculated from initial rates of hydrolysis of 1 mM hippurylphenyllactate and 1.67 mM hippurylargininic acid by carboxypeptidase B in the absence (v_0) and presence (v) of 1 mM modifier. The mode of inhibition was determined from the linear portion of the corresponding Lineweaver-Burk plots [1] consisting of 6–8 experimental points

Modifier	Hippuryl-L-phenyllactate		Hippuryl-L-argininic acid	
	v/v_0	Mode of inhibition	v/v_0	Mode of inhibition
Z-L-Ala-D-Arg	0.42	Mixed	0.49	Mixed
Z-L-Ala-L-Ala-D-Arg	0.12	Non-competitive	0.33	Competitive
Z-L-Ala-L-Ala-L-Ala-D-Arg	0.55	Competitive	0.71	Competitive
Z-D-Ala-L-Arg	0.71	Mixed	0.20	Mixed
Z-L-Ala-D-Ala-L-Arg	0.69	Mixed	0.72	Mixed
Z-L-Ala-L-Ala-D-Ala-L-Arg	0.74	Competitive	0.28	Competitive
Z-L-Ala-D-Ala	1.08[a]	Activator	0.93	Non-competitive
Z-L-Ala-D-Ala-L-Ala	1.85[a]	Activator	0.84	Competitive
Z-L-Ala-L-Ala-D-Phe	1.16[a]	Activator	0.80	Competitive
Z-L-Ala-L-Ala-D-Arg	0.76	Non-competitive	0.73	—

[a] At substrate concentration of 0.5 mM.

Table 4. *Inhibition constants for competitive inhibitors of the hydrolysis of peptides by carboxypeptidase B*
Inhibition constants determined from Lineweaver-Burk plots at pH 7.9 and 25 °C; $\bar{K}_i = 1/K_i$

Inhibitor	$\bar{K}_i$	
	Bz-L-Gly-L-Arg	Z-(L-Ala)$_2$
	M^{-1}	M^{-1}
Z-L-Ala-D-Arg	3700	1050
Z-L-Ala-L-Ala-D-Arg	4000	2700
Z-L-Ala-L-Ala-L-Ala-D-Arg	1080	380
L-Arginine	2000	250
Z-D-Ala-L-Arg	3500	1070
Z-L-Ala-D-Ala-L-Arg	9000	1140
Z-L-Ala-L-Ala-D-Ala-L-Arg	1090	200

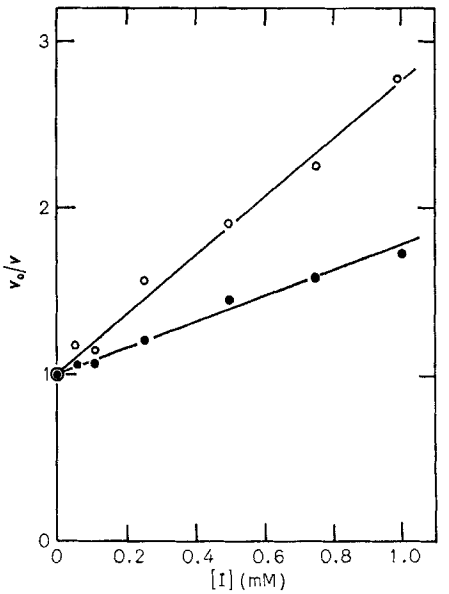

Fig. 3. *Inhibition of carboxypeptidase-B-catalysed hydrolysis of hippuryl-L-arginine (●) and Z-(L-Ala)$_3$ (○) by Z-(L-Ala)$_2$-D-Arg.* Concentration of substrates : 0.5 mM

with tripeptides, *i.e.* Z-(L-Ala)$_2$-L-Arg. In this work we employed the basic dipeptide hippurylarginine as substrate for the following reasons: (a) in the case of the tripeptide substrate, the low value of K_m (90 μM compared to 1 mM for hippurylarginine) and the high values of K_i (> 50 mM), did not allow accurate determination of the inhibition parameters; (b) since both basic substrates, dipeptide and tripeptide, show normal Michaelis kinetics, we assumed that the dipeptide could be used for the inhibition studies. Since $\bar{K}_i$ is a measure of the binding of the effector at the binding site of the enzyme, and if binding of the modifier and the substrate are at the same site, the resulting effect of the modifier should be competitive inhibition and the value of K_i should not depend on the nature of the substrate. The wide range of inhibition constants measured with the different substrates can be rationalized if we assume the existence of different active centers for the hydrolysis of the basic and non-basic ester substrate, as was previously suggested [1]. The modifiers which do not inhibit competitively are presumably bound to a secondary site, other than the active site. A tentative model which can account for the kinetic behaviour of the different modifiers is as follows: the modifier can be bound to the enzyme at a binding site other than either of the sites for basic and non-basic substrates. Binding of the modifier at this site causes a conformational change of the enzyme which might affect the two activities differently, *i.e.* each

Table 5. *Effect of the position of the N-blocking group in the inhibitors on the inhibition constants of peptides hydrolysis by carboxypeptidase B*
Inhibition constants determined from Lineweaver-Burk plots at pH 7.9, 25 °C; $\bar{K}_1 = 1/K_1$. $Q_{\bar{K}_1}$ is the ratio of the respective inhibition constants for a given pair of modifiers

Inhibitor pairs	$Q_{\bar{K}_1}$	
	Bz-L-Gly-L-Arg	Z-(L-Ala)$_3$
Z-L-Ala-L-Ala-D-Arg / Z-L-Ala-D-Arg	1.06	2.57
Z-L-Ala-D-Ala-L-Arg / Z-D-Ala-L-Arg	2.57	1.06
Z-L-Ala-L-Ala-D-Arg / L-Ala-L-Ala-D-Arg	3.45	9.3
Z-L-Ala-D-Ala-L-Arg / Z-L-Ala-L-Ala-D-Ala-L-Arg	8.25	5.7
Z-L-Ala-L-Ala-D-Arg / Z-L-Ala-L-Ala-L-Ala-D-Arg	3.7	7.1

of the active sites may exhibit higher or lower activity towards the specific substrate reflecting changes in the catalytic properties of the enzyme [8]. In addition since all C-terminal arginyl-peptides inhibit peptidase activity competitively but the inhibition constants are different when measured with basic or non-basic substrates, it is suggested that these peptides can be bound at the active site; the inhibition constant when measured with each of the substrates is thus a measure of the affinity of the modifier to the corresponding active site, *i.e.* the sites for hydrolysis of either basic or non-basic substrate. This view is supported by the linearity of the Dixon plots shown in Fig. 3 (see for example Worcel *et al.* [8]).

Comparison of the binding constants of pairs of arginyl-peptide modifiers which differ in length by one alanyl residue (Table 5) shows that N-blocked tripeptides are bound better than N-blocked di- or tetrapeptides. This is true for the hydrolysis of both basic and non-basic substrates. The data thus indicate that the active site for peptidase activity can best accomodate N-blocked tripeptides, or alternatively the presence of specific binding of the urethane bond at this position, in analogy to the binding of *N*-benzyloxycarbonyl and *N*-methyloxycarbonyl-tripeptides to carboxypeptidase A [9, 10]. The latter explanation is supported by the fact that removal of the blocking group of Z-(L-Ala)$_2$-D-Arg causes a marked reduction in binding constants, a 3.5-fold reduction when measured with the basic substrate and a 9.3-fold reduction when measured with the non-basic substrate. The possibility that the decrease in binding constants is also due to the introduction of a charge on the peptide should not be discounted. Work currently in progress in this laboratory is aimed at clarifying this point.

The phenomena described in the present work are qualitatively similar to that seen in the homologous enzyme, carboxypeptidase A [11—13]. However, quantitatively, *i.e.* in mode of acceleration and inhibition towards peptide and ester substrates as well as in the wide range of K_1 values, they differ. Furthermore, if all four substrates, *i.e.* Bz-L-Gly-L-Arg, Z-(L-Ala)$_3$, hippurylphenyllactate and hippurylargininic acid, were bound to carboxypeptidase B in a similar fashion and to the same binding site on the enzyme, one would expect the same type of activation or inhibition towards each of the substrates for any given modifier. The wide variations described in this work regarding the mode of inhibition and the lack of correspondence between the K_1 values is compatible with previous data on smaller inhibitors [1] and larger substrates [2], suggesting that binding of these substrates and inhibitors occurs in such a way that they occupy different loci on the enzyme.

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