PORCINE CARBOXYPEPTICASE B: MULTIPLE SUBSTRATES BINDING MODES

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The kinetics of porcine carboxypeptidase B catalyzed hydrolysis of hippurylarginine, hippurylargininic acid, Z·Ala_3 and hippurylphenyllactic acid have been examined in order to elucidate the substrate specificity of the enzyme. Activity toward both basic and non-basic (i.e., hydrophobic type) substrates has been confirmed. However, it would appear that these substrates are not all bound in the same manner to the same site. The pH dependencies of the kinetic parameters revealed characteristic differences among the two types of substrates. Furthermore, inhibition studies with L-argininic acid, ε-aminocaproic acid, Z-D-Ala-L-Arg and β-phenylpropionic acid support this postulate. These studies provide insite regarding possible pathways for the evolution of the carboxypeptidases.

In addition to its known specificity toward basic substrates, porcine carboxypeptidase B recently has been shown (1) to possess an intrinsic activity with specificity similar to that of carboxypeptidase A. In order to determine whether or not the details of the hydrolysis of the A and B-type substrates are identical, we have examined the kinetics and inhibition of hydrolysis of four selected peptide and ester substrates, hippurylarginine, hippurylargininic acid, carbobenzoxy-L-alanyl-L-alanyl-L-alanine (Z·Ala_3) and hippurylphenyllactate (1).

EXPERIMENTAL

Carboxypeptidase B (Code: COBC) was purchased from the Worthington Biochemical Corp.; hippuryl-L-arginine and hippuryl-L-phenyllactate from Cyclo Corp.; Z·Ala_3 from Miles-Yeda, Rehovot; hippuryl-L-argininic acid was prepared as described previously (1). All other chemicals were of the best grade available. Buffers were extracted with 0.1% dithizone in carbon

Abbreviations: CP, Carboxypeptidase; Z, Carbobenzoxy

tetrachloride to avoid contamination by adventitious metal ions.

A stock solution of enzyme (1×10^{-4}M) was diluted before each set of runs and kept at 25°. These solutions were used within 1 hr. Temperature was controlled to 25 ± 0.1° by means of a Haake thermostated circulator. Rate measurements were conducted by means of a spectrophotometric assay using a Cary Model 16K recording spectrophotometer, and 3 ml of substrate solution in 1 cm cuvets in a thermostated cell compartment. The hydrolyses of hippuryl-L-arginine, hippuryl-L-argininic acid and hippuryl-L-phenyllactate were followed at 254 nm (2)(3). The concentration of CP-B in these assay mixtures were 1×10^{-8}M; 2.5×10^{-9}M and 2×10^{-8}M, respectively. The hydrolysis of Z·Ala_3 was followed at 225 nm (4), using 0.7 ml of substrate solution in 0.2 cm light path cuvets. The enzyme concentration in this assay mixture was 2.2×10^{-7}M. All substrates were dissolved in 0.05M Tris - 0.1M NaCl buffers. The values of kcat and Km were calculated from Lineweaver-Burk plots. The substrate concentration was varied approximately 5-7 fold both above and below Km.

RESULTS AND DISCUSSION

The initial rates of the CP-B catalyzed hydrolysis of peptides and esters are shown as a function of substrate in the double-reciprocal plots in Figure 1. The ester substrates, hippurylargininic acid and hippuryl-phenyllactic acid, exhibit obvious substrate inhibition, similar to that observed with CP-A hydrolysis of hippurylphenyllactate (5). However, the peptide substrates, hippurylarginine (2) and Z·Ala_3 exhibit typical uninhibited (Michaelis-Menten) kinetics. All activities of CP-B were independent of ionic strength in the region between 0.1M - 0.5M NaCl.

The variation of Km and kcat as a function of pH over the pH range 6-9 for the CP-B catalyzed hydrolysis of peptides and esters was investigated. The values of Vmax and Km were calculated by extrapolation of the linear segement of the Lineweaver-Burke plots. Bell-shaped profiles for Km were

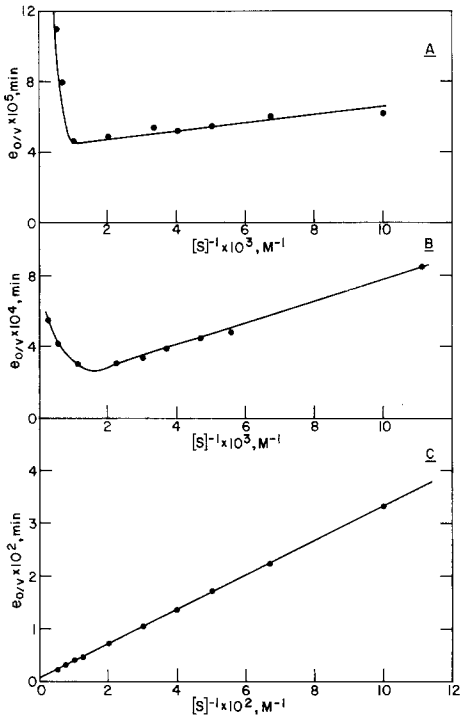

Figure 1. Lineweaver-Burk plots for carboxypeptidase B - catalyzed hydrolysis of hippurylargininic acid (A), hippurylphenyllactate (B) and Z·Ala_3 (C). Conditions of assays were 0.05M Tris - 0.1M NaCl (pH 7.9) and 25°.

observed with all four substrates (Figure 2). However, while the basic substrates (peptide and ester) yielded inverted bell-shaped curves with minimum Km values of 1.8×10^{-4}M and 3.6×10^{-5}M respectively at about pH 7.8 - 8.0, the non-basic ester and peptide substrates exhibited bell shaped curves with maximum Km value of 4×10^{-3}M at pH 7 - 7.2 and 3.3×10^{-2}M at pH 8 - 8.2 respectively. Likewise, the pH-dependence of kcat for the hydrolysis of the basic substrates, i.e., hippurylarginine and hippurylargininic acid is sigmoidal with apparent pKa's of 6.6 and 7.0 respectively, whereas, that for the non-basic substrates, i.e., Z·Ala_3 and hippurylphenyllactate is bell-shaped.

While the magnitude of Km for the basic ester hydrolysis is approximately 5-fold that of the Km of the basic peptide, as was also found by Folk et al. (6), the magnitude of Km for basic substrates is about

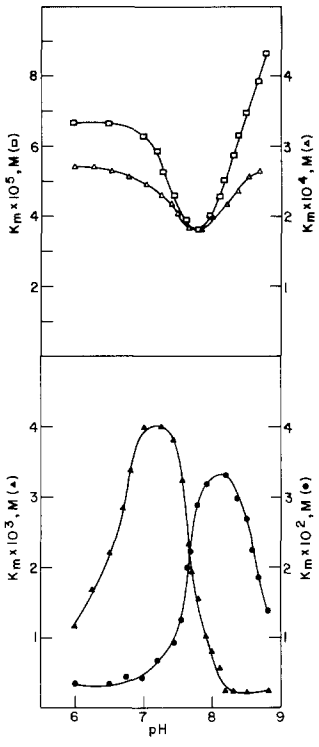

Figure 2. The pH dependence of Km for hippurylarginine (Δ), hippurylargininic acid (□), Z·Ala$_3$ (●) and hippurylphenyllactate (▲) hydrolysis. Conditions of assay: 0.05M Tris - 0.1M NaCl buffer, 25°.

20-200 fold smaller than that for the non-basic substrates. In this regard, it should be noted that assays of non-basic substrate cannot be carried out at optimum conditions, since for a better binding (Km) the assay should be performed at pH values (lower than 6.5 or above 8.5) that differs from that for obtaining optimal catalytic efficiency (the range of 7-8, dependent on whether it is a peptide or ester substrate).

It is, therefore, suggested that the two classes of substrates, the basic and the non-basic, i.e., carboxypeptidase A-like, are associated with the enzyme in a somewhat different manner. Preliminary kinetic investigations of solvent isotope effects in D_2O in the pD range of 6-9 have indicated differences towards the two ester substrates, thus supporting the proposition of different behaviour of CP-B catalyzing hydrolysis of basic and non-basic substrates.

Considering the possibility of a hydrophobic site as well as ionic binding site leads to a possible explanation for these differences. The apparent Km for both kinds of substrates suggests a dependence upon two ionizable groups with pK's near neutrality. Thus, if these two groups are designated A and B, a minimum Km for the non-basic substrate will result when these two species will be in the form of (AH^++B) or (A+B), while a maximum Km will occur when they are in the form of (A+BH^+). For the basic substrates, the opposite situation occurs, i.e., a minimum Km pertains with (A+BH^+) while a maximum value of Km will be reached with (AH^++B) or (A+B). It's entirely speculative but tempting to suggest that at least one of the ionizable group might be a histidyl residue.

Examination of the data obtained with the various inhibitors (Table I), revealed that the types of inhibition obtained with a given inhibitor depended upon the particular substrate employed. For example, L-argininic acid, a basic inhibitor, was competitive toward the basic substrates as reported previously (6), but non-competitive toward the non-basic substrates. Likewise, the hydrolysis of hippurylarginine was inhibited competitively by all the inhibitors, whereas Z·Ala_3 was inhibited non-competitively. The type of inhibition exhibited by β-phenylpropionate as shown in Table I resembles its effects on CP-A (7). Thus, β-phenylpropionate is a non-competitive inhibitor of the hydrolysis of various non basic peptides by both CP-A and CP-B, while it inhibits competitively the hydrolysis of the ester, i.e., hippurylphenyllactate. If all four substrates were bound in the same way to the same binding site on the enzyme, one would expect the same type of inhibition toward each of these substrates for any single inhibitor, competitive, non-competitive or otherwise.

The kinetic and inhibition studies described here suggest that the basic and non-basic substrates are not bound in an identical manner, possibly there are two sites (hydrophobic and ionic), adjacent or overlapping, each of which exhibits the intrinsic binding requirements of carboxypeptidase B,

TABLE I

MODE OF INHIBITION[a] AND K_I VALUES FOR INHIBITION OF PEPTIDES AND ESTERS HYDROLYSIS BY CARBOXYPEPTIDASE B

INHIBITORS	PEPTIDES		ESTERS	
	Hippuryl-arginine	Z·Ala$_3$	Hippuryl-argininic acid	Hippuryl-phenylacetate
β-phenyl propionate	Comp. 9.2×10^{-4}M	Noncomp. 8.5×10^{-3}M	Comp. 3.8×10^{-3}M	Comp. 3.6×10^{-4}M
ε-aminocaproic acid	Comp. 15×10^{-4}M	mixed	Comp. 8×10^{-4}M	Noncomp. 15×10^{-4}M
Z-D-Ala-L-Arg	Comp. 2.9×10^{-4}M	Noncomp. 6.2×10^{-4}M	Noncomp. 1×10^{-5}M	mixed
L-Argininic acid	Comp. 2.5×10^{-4}M	Noncomp. 2.5×10^{-4}M	Comp. 2.1×10^{-4}M	Noncomp. 1×10^{-4}M

[a]Determined at pH 7.9. K_I's for competitive inhibition were obtained from Lineweaver-Burke plots and K_I's for noncompetitive inhibition from Dixon plots

and carboxypeptidase A. This will be in accord with the recent suggestion of Reeck et al. (8) that in CP-B an aspartic acid replaces Ile 255 present in the hydrophobic substrate binding pocket of CP-A. The aspartic acid, is thought to provide the anionic binding site for the basic substrates while the other part of the hydrophobic pocket serves as a perturbed but functional template for the non-basic substrates. The overlapping area of the sites may also constitute the catalytic site and include the metal (6), tyrosyl-"248" which was recently identified (9)(10)(11) and a carboxyl group (12)(13), all necessary for the four activities.

In view of the striking similarity in structure and function of carboxypeptidases (8), it is not surprising that the multiple modes of binding of substrates to CP-B as suggested here is similar to that of CP-A for which non-identical but overlapping multiple modes of substrate binding have been postulated (14). Furthermore, both the metal and at least one tyrosyl residue may play a common functional role among carboxypeptidases.

These chemical and functional similarities preserved in the two species of enzyme during evolution (8)(15) support the hypothesis that CP-B and CP-A have evolved from a common exopeptidase precursor. However, since CP-B has dual specificity, i.e., toward both basic substrates and CP-A-like substrates, one might postulate that the divergency proceeded from the exopeptidase via an intermediate-CP-B-like enzyme and finally to the present two CP. Some of the information specific for CP-A may have been preserved in CP-B though it's possible that the CP-A-like activity in CP-B is of the "degenerate-type".

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