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ON THE CATALYTIC AND BINDING SITES OF PORCINE ENTEROPEPTIDASE

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Summary

The active site of porcine enteropeptidase (EC 3.4.21.9) was investigated in order to characterize better both catalytic and binding sites. The participation of a serine and a histidine residue in the catalytic process was fully confirmed and the two residues were located on the light chain of the enzyme. The binding site was found to be composed of at least 2 subsites, S_1 and S_2. The subsite S_1 (similar to the trypsin-binding site) is responsible for the interactions with the small substrates of trypsin and the lysine side chain of trypsinogen, while subsite S_2 (probably a cluster of lysines) is responsible for the interactions with the polyanionic sequence found in all trypsinogens. Binding of substrate by subsite S_2 led to an increased efficiency of the catalytic site which can be correlated to the known high specificity of enteropeptidase.

Introduction

Like trypsin, enteropeptidase (EC 3.4.21.9) is known to hydrolyze basic bonds in small esters and to be readily inhibited by iPr_2P-F and Tos-LysCH_2Cl [1,2]. However, it essentially differs from the pancreatic enzyme when the substrate is a peptide or a protein, by an absolute requirement for several dicarboxylic acid residues just before the basic bond to be cleaved. This unusual property is probably related to the biological role of enteropeptidase which is to promote a fast and highly specific activation of trypsinogen in the duodenum [1].

On the structural level, the above-reported findings are consistent with the view that the catalytic sites of enteropeptidase and trypsin are similar, but that the binding site of the first is composed of at least 2 subsites, S_1 and S_2 [3].

Abbreviations: Bz-ArgOEt = *N*-α-benzoyl-L-arginine ethyl ester; BAPNA = *N*-α-benzoyl paranitroanilide; iPr_2P-F = diisopropylfluorophosphate; NPGB = nitrophenyl guanidinobenzoate; Tos-Lys CH_2Cl = *N*-α-tosyl-L-lysine chloromethyl ketone.

Subsite S_1, responsible for the interactions with small substrates and inhibitors would be similar to that of trypsin. By contrast, substrate S_2 would be involved in the necessary interactions of enteropeptidase with protein substrates.

The purpose of the present work is to investigate more closely the essential serine and histidine residues already shown to participate in the catalytic site of enteropeptidase [1] and to explore the topography of its binding site with the aid of competitive inhibitors and chemical modifications.

Materials and Methods

Enzymes and proteins

Enteropeptidase was purified as previously reported [2] from porcine duodenal mucosa. Bovine cationic trypsinogen and trypsin were purchased from Worthington (1 and 3× crystallized, respectively).

Determination of enzyme activities

Quantitative assay techniques were already worked out in our Laboratory for enteropeptidase using Bz-Arg-OEt or trypsinogen as substrates [1,4]. They were found not suitable for kinetic investigations in the presence of inhibitors. The sensitivity of the first was not sufficient and the second had the disadvantage that most enteropeptidase inhibitors also inhibited trypsin formed during the reaction. Therefore a new technique employing BAPNA [5,6] had to be developed. The enzyme sample (20—30 μg) in 50 μl of water was incubated at 25° C with 0.15 ml of 30 mM DL- or L-BAPNA (Cyclo Chemicals) in dimethylsulfoxide and 0.8 ml of a 20 mM Tris · HCl buffer pH 8.0, 0.28 M in NaCl. The reaction was stopped after 30 min by addition of 10 μl of 0.1 M benzamidine hydrochloride (Mann). Any precipitate was removed by centrifugation at 600 × *g* for 10 min and the absorbance of the supernatant was read at 410 nm. Blank assays without enzyme were run in parallel. The molar extinction coefficient of *p*-nitroaniline resulting from BAPNA hydrolysis was taken as 8800 $cm^{-2} \cdot mol^{-1}$ and 1 enteropeptidase unit was defined as the amount of enzyme yielding 1 μmol of this compound per min under the conditions of the assay. Fig. 1 shows that the production of *p*-nitroaniline is linear up to 30 min and the amount generated after 30 min is proportional to the amount of enteropeptidase. The specific activity of the pure enzyme using DL-BAPNA as substrate was found to be 6 times higher than with Bz-Arg-OEt (45 units/mg instead of 8). By bringing the temperature up to 37° C the rate of hydrolysis was only increased 1.3-fold.

It is noteworthy that the kinetic parameters of the enteropeptidase-catalyzed hydrolysis of L-BAPNA can be calculated using DL-BAPNA as substrate. The basic assumption in this respect is that, as already reported in the case of trypsin [5], the K_m of the substrate L-BAPNA is equal to the K_i of the competitive inhibitor D-BAPNA. The equation of the velocity of hydrolysis in the presence of a competitive inhibitor is:

$$\frac{1}{v} = \frac{1}{V} + \frac{K_m}{V}\left(1 + \frac{I}{K_i}\right)\frac{1}{S} \qquad (1)$$

Taking into account that $K_m = K_i$ and that the concentrations of S and I are

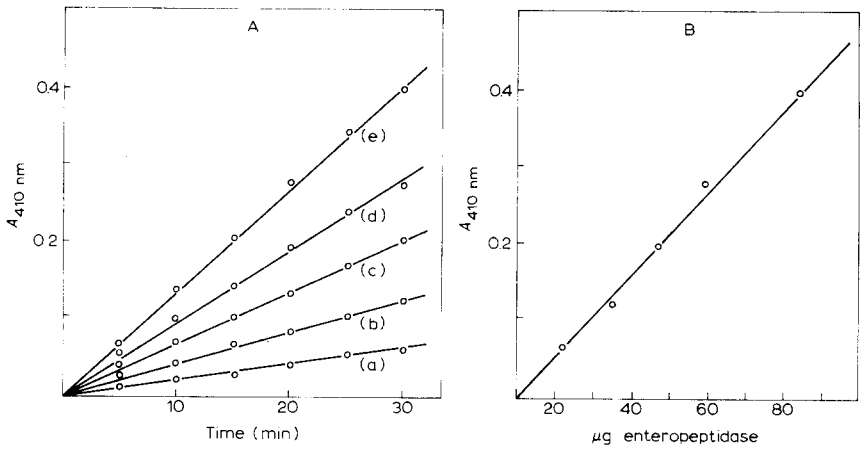

Fig. 1. Hydrolysis of DL-BAPNA by enteropeptidase. A, kinetic study of the liberation of *p*-nitroaniline by 12.5 (a), 25.0 (b), 37.5 (c), 50.0 (d) and 75.0 (e) μg of pure enteropeptidase. B, proportionality between enzyme concentration and liberation of *p*-nitroaniline after 30 min.

the same when the racemic derivative is used, Eqn. 1 becomes:

$$\frac{1}{v} = \frac{2}{V} + \frac{K_m}{V} \times \frac{1}{S} \qquad (2)$$

The original assumption K_m (L-BAPNA) = K_i (D-BAPNA) was later experimentally checked when L-BAPNA became commercially available.

The parameters related to enteropeptidase are seen (Table I) to be slightly less favorable than for trypsin. But the observed differences are so small that BAPNA can for all practical purposes be considered as a good substrate for both enzymes.

Aminopeptidase activity was evaluated as previously described [2] by spectrophotometry with the aid of L-alanine *p*-nitroanilide. Trypsin activity towards Bz-Arg-OEt was measured by titrimetry [4].

Synthesis of [^{14}C]Tos-Lys-CH_2Cl

This compound was synthesized by the Department of Organic Chemistry of the Commissariat à l'Energie Atomique (CEA), Saclay, France. The two-step procedure employed includes condensation of ^{14}C-labelled diazomethane with *N*-α-tosyl-*N*-ε-carbobenzoxy-L-lysine chloride and reaction of the resulting

TABLE I

KINETIC PARAMETERS OF DL AND L-BAPNA HYDROLYSIS BY ENTEROPEPTIDASE AND TRYPSIN

Substrate	Enteropeptidase		Trypsin	
	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})
L-BAPNA	13.6	2.0	4.8	0.6 *
DL-BAPNA	12.7	0.7	4.9	—

* Data from Erlanger [5] at pH 8.15 and 15°C.

diazoketone with anhydrous HCl. After filtration through Sephadex G-10 in 10 mM HCl and crystallization in an ethanol-diethyl ether mixture, the product yielded a single spot by paper chromatography in *n*-butanol/formic acid/water (75 : 15 : 10, v/v). A single spot was also obtained by thin layer chromatography on Silicagel-G plates in the systems *n*-butanol/acetic acid/water and ethyl acetate/formic acid/water (40 : 10 : 20 and 70 : 20 : 10, v/v). *N*-α-tosyl-*N*-ε-carbobenzoxy-L-lysine was synthesized in our laboratory by the method of Swallow et al. [7].

Synthesis of the amide of p-aminobenzamidine and 6-aminohexanoic acid

1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide (1.2 mmol) was added over a 5-min period to 2 ml of a solution at pH 4.7 containing 0.3 mmol of *p*-aminobenzamidine and 0.5 mmol of 6-aminohexanoic acid. After 18 h at 25° C, the mixture was loaded on a column (1 × 10 cm) of CM-cellulose (Whatman microgranular CM-32) equilibrated with a 50 mM ammonium acetate buffer pH 5.0. The column was washed with 200 ml of buffer and then eluted by a linear gradient concentration of NaCl from 0 to 0.5 M. The product was separated at this stage from the excess *p*-aminobenzamidine and other reaction by-products and then desalted by passage through the same column and eluted with 0.1 M formic acid.

Results

The catalytic site of enteropeptidase

The essential serine. The existence of an essential serine residue located in the light chain of enteropeptidase [1,2] was confirmed starting from the reduced-carboxymethylated derivative of the [^{32}P]iPr_2*P*-F-treated enzyme. The light chain was then separated on Sephadex G-200, dialyzed and lyophilized (yield: 15 mg from 80 mg of pure enzyme). The chain was dissolved in 1 ml of a 0.1% ammonium bicarbonate buffer pH 7.8 and digested for 24 h at 37° C by 2 mg of thermolysin (Worthington). The resulting peptide mixture was fractionated by electrophoresis-chromatography on paper. Five radioactive peptides were separated, among which the smallest had the following amino acid composition: Asx (0.99), Glx (0.84), Gly (1.37), Pro (0.81), Ser (0.68). The four other peptides contained these and other amino acids. Phosphoserine could be identified in all peptides after acid hydrolysis. The amounts of peptides were too low to permit sequencing assays. Additional information about the essential serine could be obtained by reaction with NPGB. This reagent has been shown by Chase and Shaw [8] to be a good trypsin active site titrant because of the extremely low rate of the deacylation step. The method was standardized with a trypsin sample purified by affinity chromatography (specific activity against Bz-Arg-OEt: 60 units per mg) and then applied to pure enteropeptidase in which 0.99 active sites per mole of enzyme were found. This result confirms the participation of a single serine residue in the catalytic site of enteropeptidase. This also makes it likely that the catalysis by enteropeptidase proceeds via a transient acyl-enzyme derivative.

The essential histidine. The pH dependence of the kinetic parameters (K_m and k_{cat}) of the enteropeptidase-catalyzed BAPNA hydrolysis also confirms the

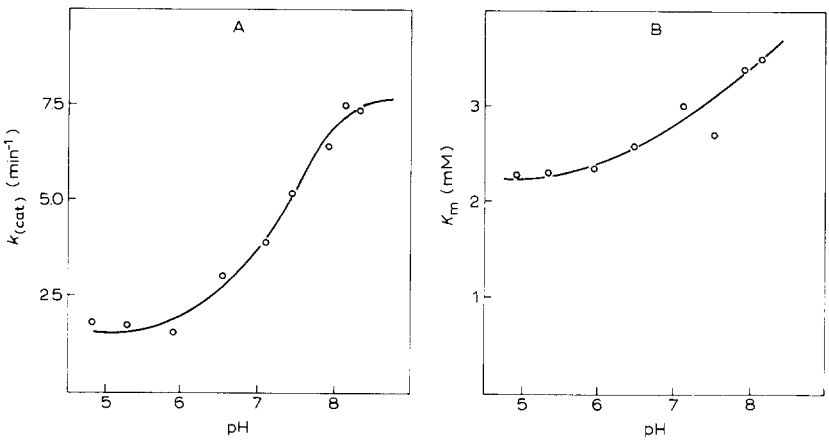

Fig. 2. pH dependence of L-BAPNA hydrolysis by enteropeptidase. The reaction was performed in sodium phosphate buffers of varying concentrations and constant ionic strength (I = 0.02). The ionic strength was held constant in all assays because of the extreme sensitivity of enteropeptidase towards this parameter (see later and ref. 4). The concentration of dimethyl sulfoxide and enteropeptidase in the assays were 15% and 10^{-4} M, respectively.

already postulated existence of a histidine residue in the catalytic site of the enzyme [1]. Fig. 2 shows that the K_m increases slightly over the entire pH range (4.9—8.3), but that the variation of k_{cat} is sigmoidal with an inflection point at pH 7.3. Similar results, observed at two ionic strength values (I = 0.02 and 0.3) and with DL or L-BAPNA, clearly indicated that the catalytic site of enteropeptidase is under the control of an ionizable group which should be deprotonated for maximal efficiency. Considering the pH interval in which activation occurs, this group is probably the imidazole ring of an histidine residue. The inhibition of enteropeptidase by Tos-Lys-CH_2Cl already mentioned in a preceding publication [1] is also consistent with the effective participation of a histidine residue in catalysis. In order to better characterize the position of this residue, the enzyme was incubated with [^{14}C]Tos-Lys-CH_2Cl under the conditions used for the unlabelled reagent [1]. The condensate was dialyzed against several changes of water and filtered through Sephadex G-25 to remove the unreacted material. Counts incorporated showed the fixation of 1.05 mol of Tos-Lys-CH_2Cl per mol of enzyme. Then, the light and heavy chains were separated by gel electrophoresis [9] after reduction-carboxymethylation in the presence of 8 M urea and 1% sodium dodecyl sulfate and the radioactivity was found only in the light chain. It may therefore be concluded that the light chain of enteropeptidase possesses two of the constituents of the catalytic site, namely the essential serine and histidine residues.

The binding site

Use of competitive inhibitors. The subsites comprising the binding site of enteropeptidase could be investigated with the aid of suitable competitive inhibitors. Several of these compounds were comparatively tested on enteropeptidase and trypsin with the results reported in Table II.

Table II indicates that known competitive trypsin inhibitors such as butyl-

TABLE II

COMPARATIVE STUDY OF SOME COMPETITIVE INHIBITORS OF ENTEROPEPTIDASE AND TRYPSIN

The substrate in all assays was DL-BAPNA

Inhibitors	Inhibition constants (mM)	
	Enteropeptidase	Trypsin
Butylamine	1.6	1.7 (ref. 10)
Benzamidine	0.022 (0.029+)	0.0184 (ref. 11)
p-Aminobenzamidine	0.0095	0.0082 (0.0085, ref. 6)
Amide of *p*-aminobenzamidine and 6-amino-hexanoic acid	>5	0.093
Tosyl-L-arginine methylester	2.2	—
Benzoyl-L-arginine methylester	2.0	—
Benzoyl-L-lysine methylester	3.4	—

amine, benzamidine and *p*-aminobenzamidine are also strongly inhibitory for enteropeptidase. These compounds are closely related to the lysine side-chain which is found in all substrates cleaved by trypsin and enteropeptidase and, consequently, they can be expected to interact only with the subsite S_1 of the latter enzyme. On a more quantitative basis, nearly the same K_i values were obtained for both enzymes. This finding strongly suggests that enteropeptidase subsite S_1 is very similar to, if not identical with, the binding site of trypsin. It is also of interest to note that, in sharp contrast with the other derivatives listed in Table II, the amide of *p*-aminobenzamidine and 6-amino-hexanoic acid is a much less efficient inhibitor of enteropeptidase than of trypsin. The atypical behavior of this compound will be discussed later.

Another comment concerning Table II is that enteropeptidase is inhibited to about the same extent by arginine and lysine derivatives and, that therefore, like trypsin, it probably makes no distinction between arginine and lysine bonds in protein substrates.

Properties of acetylated enteropeptidase. Since the role of subsite S_2 apparently is to recognize a cluster of carboxylates in large substrates, it may be assumed to be cationic in nature and consequently to be formed by lysine and/or by arginine side-chains. In order to see whether lysines were actually involved, pure enteropeptidase (8 mg, specific activity: 45 units/mg towards DL-BAPNA; 44 000 units/mg towards trypsinogen) was dissolved in 1 ml of a 0.1 M sodium phosphate buffer pH 7.0 and 25 μl of [^{14}C]acetic anhydride (from Amersham; 7 mCi/mmol) were added in 5 portions within 50 min, with stirring. The temperature was maintained at 0° C and the pH at 7.0 during the treatment. Then, the mixture was allowed to stand at 4° C for 2 h and, after dialysis against water, it was incubated for 2 h at 4° C and pH 8.0 with 1 M hydroxylamine in order to remove the majority of the acetate bound at places other than NH_2 groups. Counts incorporated in the dialyzed and lyophilized material indicated that 39 ± 2 acetyl groups were bound per mole of enzyme and, consequently, that all the 40 ϵ-NH_2 groups kown to be present in enteropeptidase [2] were probably acetylated. After acetylation, the residual activity to-

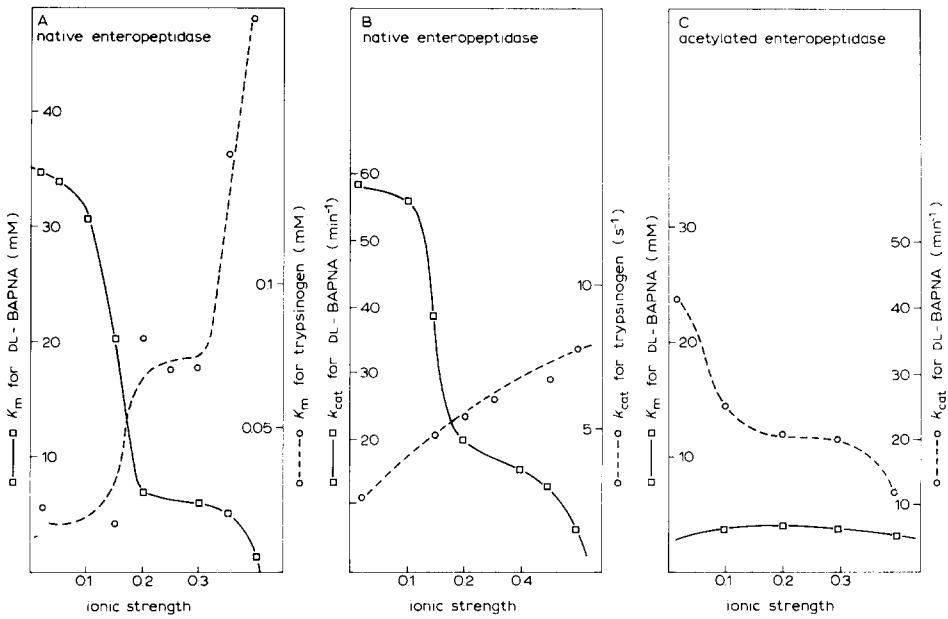

Fig. 3. Ionic strength dependence of trypsinogen activation and BAPNA hydrolysis by enteropeptidase. The experimental conditions under which the two reactions were performed are indicated respectively in ref. 4 and under Materials and Methods. A and B, respective variations of K_m and k_{cat} with native enteropeptidase. C, same variations with acetylated enteropeptidase.

wards trypsinogen was less than 2%. By contrast, the activity towards BAPNA was not only maintained but even increased, 1.8-fold. This suggests that the subsite S_2 may have a negative effect on enteropeptidase activity towards small substrates but its integrity is essential for trypsinogen activation. Enterokinase activity towards large and small substrates could be clearly differentiated in this manner.

Ionic strength effect. Trypsinogen activation by enteropeptidase is already known to be slowed down at high ionic strength due to a very sharp increase in K_m [4]. This early observation is confirmed by Fig. 3A. Fig. 3B further shows that the k_{cat} of the same activation increases only moderately with the ionic strength. By contrast, the two parameters related to BAPNA hydrolysis sharply decrease with the two characteristic plateaux observed for the K_m of trypsinogen activation.

Fig. 3 also indicates that the ionic strength effect on BAPNA hydrolysis is much less when acetylated enteropeptidase is employed. The K_m, in particular, remains low and almost constant over the whole range of ionic strength investigated. This last finding agrees well with the already reported negative effect of subsite S_2 on the hydrolysis of small subsites.

In conclusion, the assays performed at high ionic strength are in qualitative agreement with those carried out with acetylated enteropeptidase. In both cases, the subsite S_2 is presumably destroyed and this destruction inversely affects trypsinogen activation and BAPNA hydrolysis.

Discussion

The above results and those previously reported [1,2] provide us with a better understanding about the active site of enteropeptidase and its relation with that of trypsin. As in the trypsin molecule two catalytically important residues, a serine and a histidine, have been identified and located in the light chain of the enteropeptidase molecule.

The inhibition by the bifunctional reagent Tos-Lys-CH_2Cl and the values of inhibition constants of small inhibitors (Table II) are consistent with the view that, at least, a part of the binding site of enteropeptidase, designated subsite S_1, is related to that of trypsin [6]. The subsite S_1 is apparently responsible for the binding of small substrates of trypsin and for the interactions of the enzyme with the lysine side chain in the N-terminal region of trypsinogen.

When the substrate is larger (a protein for instance) it must possess, adjacent to the basic bond, a polyanionic structure [1] fitting the structural requirements of a second subsite S_2. This is particularly evident in the case of the amide of *p*-aminobenzamidine and 6-aminohexanoic acid when compared with *p*-aminobenzamidine.

Small substrates interact only with the subsite S_1 and are hydrolyzed at a low rate (k_{cat} from 5 to 20 min^{-1}). Trypsinogens interact with both subsites S_1 and S_2 and the catalytic constant rises to 300—400 min^{-1} [1,4]. Large substrates without the polyanionic sequence are not hydrolyzed because they have a low affinity and/or low catalytic constant.

In other words, the subsite S_2 is important because it controls the binding of large substrates to enteropeptidase and also a substantial part of the catalytic efficiency of the enzyme towards these substrates. It confers to enteropeptidase the properties of a "supertrypsin" with a high degree of specialization for trypsinogen.

The fact that the subsite S_2 must interact with a polyanionic sequence suggests that it is cationic in nature. Indeed exhaustive acetylation of enteropeptidase leads to a modified derivative active on small substrate, but 98% inactive on trypsinogen, suggesting the probable involvement of lysines in this subsite. Heating of the enzyme at 60° C for 7 min has been reported also to abolish its ability to activate trypsinogen while preserving the activity towards small substrates [12]. These results can probably be explained by postulating that a common effect of both acetylation and heating results in destruction of the subsite S_2 while the subsite S_1 and the catalytic site remain functional.

As in the case of acetylation, high ionic strength seems strongly and specifically to affect the subsite S_2. For both trypsinogen activation and BAPNA hydrolysis the Michaelis constants are strongly affected by changes in ionic strength. Their symmetrical variations suggest an effect on the enzyme itself rather than on the substrate or on the enzyme-substrate interactions. The catalytic constant of BAPNA hydrolysis drops when ionic strength increases. This would mean that there is a direct link between the integrity of the subsite S_2 and the efficiency of the catalytic site. The phenomenon is not seen with trypsinogen because, in spite of an increased Michaelis constant, maximum efficiency of the catalytic site is obtained at saturation of the enzyme by its substrate. In other words, the trypsinogen stabilizes the subsite S_2 in the configuration

that gives maximum efficiency of the catalytic site. Acetylation and ionic strength effects on the enteropeptidase activity again strongly suggest the existence of the two subsites S_1 and S_2.

Several attempts to adsorb specifically enteropeptidase on insoluble derivatives of *p*-aminobenzamidine [13] have failed. Although reports of affinity chromatography of enteropeptidase have been reported [14,15] our negative results can be explained by the lack of the polyanionic sequence in such derivaties. Non-specific adsorption of crude enteropeptidase fractions can be achieved at low ionic strength on *p*-aminobenzamidine linked to CNBr-activated Sepharose 4B [13]. Increasing the ionic strength further leads to elution patterns similar to those obtained on DEAE-cellulose [1].

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