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THE EFFECT OF LANTHANIDE IONS ON ENTEROPEPTIDASE-CATALYZED ACTIVATION OF TRYPSINOGEN

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Summary

Rare earths were found to be powerful inhibitors of enteropeptidase-catalyzed (enterokinase, EC 3.4.21.9) activation of trypsinogen. Inhibition was complete at a La^{3+} concentration of $12.5 \cdot 10^{-6}$ M in the assay system used and still detectable at a concentration of $1.25 \cdot 10^{-6}$ M.

Inhibition was observed with all lanthanides tested. No significant differences between individual metals could be established under the conditions of the inhibition assay.

Increasing ionic strength decreased enzyme activity and progressively diminished the inhibitory effect of rare earth ions suggesting an electrostatic basis for the mechanism of this inhibition.

La^{3+} did not significantly affect enteropeptidase-mediated hydrolysis of *N*-benzoyl-L-arginine ethyl ester. Its inhibitory effect on activation of trypsinogen by enteropeptidase, therefore, must be attributed to interaction with the zymogen rather than the enzyme.

Kinetic measurements show that inhibition by rare earths is noncompetitive in nature. Binding of lanthanides to the tetraaspartyl sequence near the amino-terminus of trypsinogen may prevent this group from interacting with a critical specificity subsite on the enzyme.

Introduction

It has been known for some time that Ca^{2+} accelerates the activation of trypsinogen by trypsin [1]. Recently Gomez et al. [2] have shown that lanthanide ions can isomorphously replace Ca^{2+} in the conversion of trypsinogen to trypsin. Several investigators have reported that Ca^{2+} also accelerates activation of trypsinogen by pig [3,4], rat [5] and human [6] enteropeptidase (enterokinase EC 3.4.21.9). It appeared to us of interest, therefore, to investigate the effect of

lanthanide ions on enteropeptidase-catalyzed conversion of trypsinogen to trypsin. This report presents the results of our work.

Materials and Methods

Enteropeptidase was obtained free of trypsin and other pancreatic enzymes by gel filtration of human duodenal juice as described previously [6]. Trypsinogen, once crystallized, salt free and trypsin, twice crystallized, salt free were purchased from Worthington Biochemical Corp., Freehold, New Jersey. Rare earth metal chlorides were obtained from ICN-K&K Laboratories, Irvine, California. Carbobenzoxy-glycylglycyl-L-arginine β-naphthylamide (Z-Gly-Gly-Arg-NNap) was purchased from Bachem, Inc., Marina del Rey, California and *N*-benzoyl-L-arginine ethyl ester (Bz-Arg-OEt) from Nutritional Biochemical Corp., Cleveland, Ohio.

Assay procedure for enteropeptidase

The single-stage method described earlier [6] was used throughout this work except where stated otherwise. One unit of enteropeptidase = 1 μmol trypsin (active site titrated) generated/min. Duplicate plastic tubes were charged with 10 to 40 μl of an aqueous solution of a lanthanide salt to give a final concentration of 1.25 to $50 \cdot 10^{-6}$ M, 0.5 ml substrate solution (40 mg Z-Gly-Gly-L-Arg-NNap/100 ml H_2O), 20 μl of an enteropeptidase solution (approx. 40 mIU/l) in 0.05 M Tris maleate buffer, pH 7.0 and 0.05 M Tris · HCl buffer pH 8.5 (Ca^{2+}-free) to give a final volume of 1.7 ml. The reaction was then initiated by adding 0.1 ml of a freshly prepared solution of trypsinogen (1 mg/20 ml 0.005 M Tris · HCl buffer, pH 5.7) and the mixture was incubated at 25°C for 15 min. The reaction was terminated by adding 0.2 ml 1 M citrate buffer, pH 4.5. Fluorescence readings were taken in an Aminco-Keirs spectrophotofluorometer: λ_{Ex}:335 nm, λ_{Em}:415 nm, uncorrected.

Two-stage assay

A plastic tube was charged with 0.25 ml Tris/maleate buffer, 0.05 M, pH 5.3, 0.6 ml of H_2O or a lanthanide solution (0—3 mM) and 0.1 ml of an enteropeptidase solution in 0.05 M Tris/maleate, pH 7.0. The reaction was initiated by adding 0.1 ml of a trypsinogen solution (13. 5 mg/5 ml Tris · HCl buffer, 0.005 M, pH 5.7) and the mixture incubated at 25°C for 15 min. At the end of this period 10 μl of the incubation mixture was added to each of three plastic tubes containing 0.6 ml Tris · HCl buffer, 0.05 M, pH 8.5 in 0.6% NaCl and 0.4 ml substrate solution (80 mg Z-Gly-Gly-L-Arg-NNap/100 ml H_2O). After incubating for 15 min at 25°C the reaction was terminated by adding 0.1 ml 1 M citrate buffer, pH 4.5 to each tube and the fluorescence was read as above.

Determination of enteropeptidase activity with N-benzoyl-L-arginine ethyl ester (Bz-Arg-OEt)

Hog enteropeptidase was found by Maroux et al. [7] to hydrolyze the trypsin substrate Bz-Arg-OEt. The activity of the human enzyme was measured also with this substrate according to the spectrophotometric method of Schwert and Takenaka [8]. Enteropeptidase concentrate, 20 μl (purified, 14.5 I.U./l)

was added to 0.89 ml of a 1 mM solution of Bz-Arg-OEt in 0.02 M Tris buffer, pH 7.5, containing 0.02 mol NaCl/l and ΔA_{253} recorded at 25°C. In other experiments $LaCl_3$ solutions (10 µl) were added to the reaction mixture to give final concentrations of 50 and $100 \cdot 10^{-6}$ M La^{3+}.

Results

Fig. 1 illustrates the effect of lanthanide salts (La,Pr,Nd,Tb,Ho,Lu) in concentrations ranging from 1.25 to $50 \cdot 10^{-6}$ M on the activity of enteropeptidase in our one-stage assay system. No significant differences between the effect of various rare earths tested could be detected during the period of observation (15 min) and the values for a given concentration of lanthanides were averaged and plotted ±1 standard error of the mean. Line A was obtained with a trypsinogen concentration of 0.1 µM and line B with a trypsinogen concentration of 1 µM in the assay mixture. It can be seen that the inhibitory effect of the rare earths on enteropeptidase activity was not significantly influenced by a 10-fold increase in substrate concentration. Line C in Fig. 1 which was obtained with isoionic concentrations of NaCl in the absence of lanthanide salts shows that inhibition of enteropeptidase activity by rare earths is not due to an increase in ionic strength (see ref. 3—6), but reflects an inherent property of these metals.

We have shown in an earlier report [6] that under the conditions of our single-stage assay of enteropeptidase, trypsinogen is resistant to activation by trypsin. Addition of $50 \cdot 10^{-6}$ M La^{3+} to this system neither initiated activation of trypsinogen nor affected enzymatic activity of trypsin.

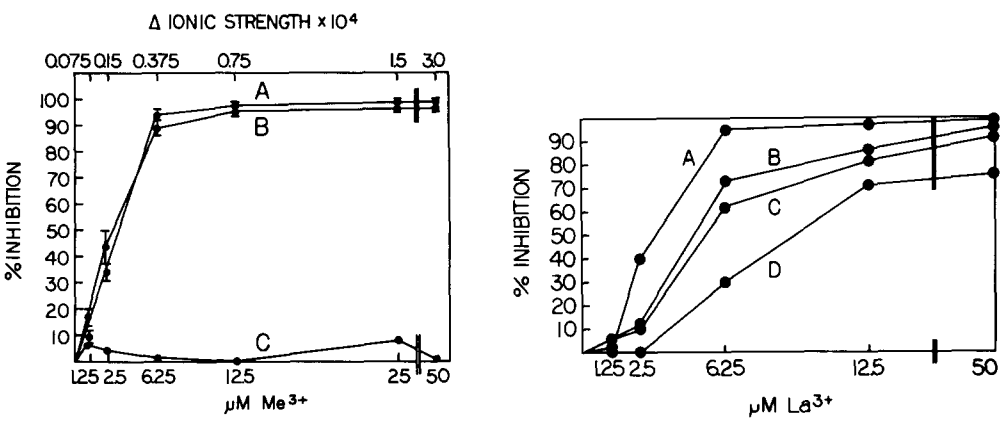

Fig. 1. Inhibition of enteropeptidase-catalyzed activation of trypsinogen by rare earths (La, Pr, Nd, Tb, Ho, Lu). Inhibition is plotted as percent difference between readings without and readings with rare earth metal vs. concentration of metal or increase in ionic strength respectively. Line A: tryspinogen concentration 10^{-7} M, Line B: 10^{-6} M, line C: isoionic concentrations of NaCl; trypsinogen: 10^{-6} M. Details in text.

Fig. 2. Effect of increasing ionic strength on inhibition of enteropeptidase activity by La^{3+}. Inhibition is plotted as in Fig. 1. Trypsinogen concentration: 10^{-6} M. Line A: assay conditions as in Fig. 1. Line B, C and D: assay in the presence of 0.2, 0.3 and 0.6% NaCl respectively.

Fig. 2 illustrates the effect of increasing ionic strength on inhibition of enteropeptidase by La^{3+}. It has been reported by several investigators including ourselves that enteropeptidase activity decreased with increasing ionic strength [3—6]. In the experiments represented by Fig. 2 the addition of 0.2 and 0.3% NaCl to the system reduced enzyme activity by about 40% and addition of 0.6% NaCl by about 80%. Inhibition by increasing concentrations of La^{3+} is plotted as percent difference between readings without and with La^{3+} in assay mixtures containing 0.2, 0.3 and 0.6% NaCl respectively. It will be noted that increasing concentrations of NaCl (increasing ionic strength) diminish the inhibitory effect of La^{3+} on enteropeptidase activity.

Lineweaver-Burk plots obtained from kinetic studies demonstrate that La^{3+} does not significantly affect K_m of enteropeptidase, but decreases V. Determinations were performed in 50 mM NaCl because experiments at low ionic strength showed poor reproducibility. Apparent K_m obtained by linear regression analysis of the experimental data was $4.8 \cdot 10^{-7}$ M for the assay without La^{3+} and $5.2 \cdot 10^{-7}$ M for the assay with $25 \cdot 10^{-6}$ M La^{3+}. V in the presence of La^{3+} was one half of that in the absence of La^{3+}.

The effect of La^{3+} on enteropeptidase activity in the Kunitz-assay is shown in Fig. 3. The trypsinogen concentration in this assay is 10 [10] times higher (10 μM) than in our single-stage method and the pH of the incubation mixture is 5.7 vs. 8.5 in our procedure. It can be seen that much higher concentrations of La^{3+} ($7.5 \cdot 10^{-4}$ M) are needed under these conditions to inhibit enteropeptidase activity than in our system ($3.5 \cdot 10^{-6}$ M for 50% inhibition). In contrast to the experiments described above, inhibition in the Kunitz-system can be attributed partly to an increase in ionic strength as shown by the curve (NaCl) obtained with concentrations of NaCl isoionic with corresponding concentrations of La^{3+}.

Experiments in which the effect of La^{3+} on enteropeptidase-catalyzed hydrolysis of Bz-Arg-OEt was investigated required 500—1000 times greater concentrations of enzyme than our single-stage assay with trypsinogen. Concentrations as high as 10^{-4} M La^{3+} had no detectable influence on the catalytic activity of enteropeptidase in the hydrolysis of Bz-Arg-OEt.

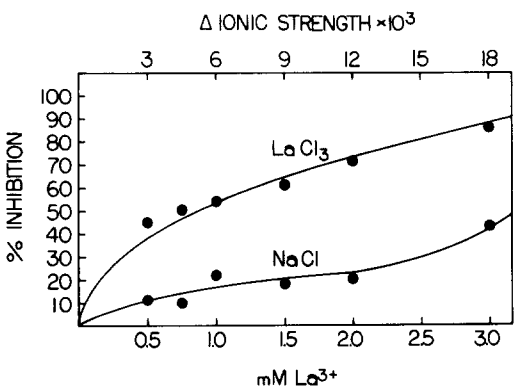

Fig. 3. Effect of La^{3+} on enteropeptidase-catalyzed activation of trypsinogen in the Kunitz two-stage system [10]. Inhibition is plotted as in Figs. 1 and 2. Details are given in text.

Discussion

Gomez et al. [2] recently have shown that rare earth ions are highly effective substitutes for Ca^{2+} in the activation of trypsinogen by trypsin at a concentration 100 times lower than that observed with Ca^{2+} and that acceleration of this activation is due to binding of lanthanide ions to the tetraaspartyl group of the zymogen. By contrast, the results presented here show that lanthanide ions are powerful inhibitors of enteropeptidase activity. Concentrations 100 times lower than those accelerating trypsinogen activation by trypsin [2] virtually abolished enteropeptidase activity (Fig. 1). The assay system reported earlier [6] was used in this work. It was preferred to the Kunitz procedure [10] because of its high sensitivity and greater simplicity; complicated side-reactions such as self-activation of trypsinogen and autodigestion of trypsin are competitively inhibited by the presence of a very large excess of the trypsin substrate Z-Gly-Gly-L-Arg-NNap [6]. Trypsin activity in this system was not affected by La^{3+} concentrations used in these experiments (cf. also ref. 2). Although inhibition of enteropeptidase by lanthanides was also evident in a modified Kunitz procedure (Fig. 3), the low pH (5.7) of the incubation mixture greatly diminished this effect and concentrations of La^{3+} 200 times higher than in our system were needed for 50% inhibition of the enzyme. At this concentration part of the inhibition was due to the increase in ionic strength by La^{3+} as demonstrated by the curve which was obtained with isoionic concentrations of NaCl (Fig. 3).

Inhibition of enteropeptidase activity by La^{3+} was not affected by a 10-fold increase in substrate concentration. Even at near equimolar concentration of trypsinogen ($1 \cdot 10^{-6}$ M) and La^{3+} ($1.25 \cdot 10^{-6}$ M) inhibition by the metal could still be detected (Fig. 1). Lower concentrations of La^{3+} (0.5 and $0.1 \cdot 10^{-6}$ M) neither inhibited nor accelerated enteropeptidase-mediated tryspinogen activation. This finding suggests that lanthanides either do not bind to trypsinogen at its high-affinity site for Ca^{2+} (cf. ref. 9) or that such binding has no perceptible influence on enteropeptidase activity. On the other hand, our results show that binding of lanthanides to the tetraaspartyl sequence [2] (the weak-affinity site for Ca^{2+}) inhibits enteropeptidase, evidently by preventing its interaction with this group which plays a critical role in the activation of the zymogen by this enzyme [7]. Our finding that lanthanides have no influence on enteropeptidase-catalyzed hydrolysis of Bz-Arg-OEt is consistent with this view and shows that these metals interact with the trypsinogen molecule rather than with the enzyme.

Recognition of the tetraaspartyl sequence of trypsinogen by enteropeptidase according to Maroux et al. [4,7] is mediated by electrostatic attraction to a positively charged subsite in the enzyme. This may explain not only the marked sensitivity of enteropeptidase to changes in ionic strength [3—6], but also the progressive weakening of the inhibitory effect of rare earths with increasing ionic strength of the medium as demonstrated by Fig. 2. This view also agrees with our observation that hydrolysis of Bz-Arg-OEt by human enteropeptidase does not exhibit similar ionic strength dependence [11].

It has been shown that blocking of the tetraaspartyl group of trypsinogen by binding with Ca^{2+} [1] or lanthanide ions [2] results in acceleration of the rate

of its activation by trypsin. K_m of the reaction decreases while V remains unchanged. By contrast, our kinetic studies demonstrate that rare earth metals do not significantly affect the affinity of enteropeptidase for the zymogen (K_m remains constant), but reduce the rate of trypsinogen activation by diminishing the breakdown of the enzyme-substrate complex as reflected in the decreased V of the reaction.

Acknowledgment

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