Interactions of trivalent lanthanide ions with bacterial collagenase (clostridiopeptidase A)

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(Received 19 August 1980/Accepted 25 February 1981)

Tervalent cations of the lanthanide (rare-earth) elements reversibly inhibit bacterial collagenase (clostridiopeptidase A; EC 3.4.24.3). Sm^{3+} , whose ionic radius is closest to that of Ca^{2+} , is the most effective inhibitor, completely suppressing clostridiopeptidase activity at a concentration of $100\text{ }\mu\text{M}$ in the presence of 5 mM-Ca^{2+} . Er^{3+} and Lu^{3+} , which both have ionic radii smaller than either Ca^{2+} or Sm^{3+} , inhibit less efficiently, and La^{3+} , which is slightly larger than Ca^{2+} or Sm^{3+} , inhibits only weakly. These findings indicate a closely fitting, stereospecific, Ca^{2+} -binding pocket in clostridiopeptidase, which excludes ions that are only slightly larger than Ca^{2+} [ionic radius 0.099 nm ($0.99\text{ }\text{\AA}$)]. By contrast, trypsin, an enzyme whose activity does not depend on Ca^{2+} , requires lanthanide concentrations 50–100-fold greater for inhibition. Furthermore, the relative efficiency of inhibition of trypsin by lanthanides increases as the lanthanide ions become smaller and the charge/volume ratio increases. At a concentration of $50\text{ }\mu\text{M}$, Sm^{3+} lowers the apparent K_m for the hydrolysis of Pz-peptide by clostridiopeptidase from 5.4 mM to 0.37 mM and the apparent V_{max} from 0.29 Wünsch–Heidrich unit to 0.018 unit. Thus Sm^{3+} enhances the affinity of this enzyme for its substrate; inhibition of hydrolysis of Pz-peptide may result from the excessive stability of the enzyme– Sm^{3+} –substrate complex. Inhibition by Sm^{3+} is competitive with regard to Ca^{2+} . The apparent dissociation constant, K_d , of Ca^{2+} is 0.27 mM , where the K_i for Sm^{3+} is $12\text{ }\mu\text{M}$. Clostridiopeptidase is more thermolabile in the absence of Ca^{2+} . With Sm^{3+} , thermoinactivation of the enzyme at 53°C or 60°C is initially accelerated, but then becomes retarded as heating continues. Lanthanide ions bind to gelatin and collagen. In so doing, they appear to protect these substrates from lysis by clostridiopeptidase through mechanisms additional to supplanting Ca^{2+} at its binding site on the enzyme. Collagen and gelatin sequester sufficient lanthanide ions to gain partial protection from clostridiopeptidase in the absence of an extraneous source of these inhibitors.

Clostridiopeptidase A (EC 3.4.24.3) is a neutral collagenolytic enzyme produced by the bacterium *Clostridium histolyticum* (Seifter *et al.*, 1959). It is a metalloproteinase, containing Zn(II) and requiring Ca^{2+} , that degrades collagen into short peptides by endopeptidolytic activity. A number of synthetic peptides of the general structure Pro-Xaa-Gly-Pro, where Xaa may be any amino acid, are also cleaved by clostridiopeptidase A. Phenylazobenzyloxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg (Pz-peptide) (Wünsch & Heidrich, 1963) is a peptide of this type.

As first suggested by Williams (1970) and Darnall & Birnbaum (1970), members of the lanthanide series of elements (the rare earths) are useful probes in studying enzymes that require Ca^{2+} . Their ionic sizes and electronic configurations permit them to

substitute for Ca^{2+} in a specific manner. Like Ca^{2+} , they have no crystal-field-stabilization energy, they have negligible f -orbital-directing steric forces, and they engage in no significant covalent bonding. Their paramagnetic and spectral properties allow characterization of the sites on enzymes, or other macromolecules, that bind Ca^{2+} (see review by Reuben, 1975). The trivalent charge of lanthanide ions increases the stability of their co-ordination complexes relative to Ca^{2+} . The substitution of trivalent lanthanide ions for Ca^{2+} either activates or inhibits a given enzymic reaction. Thus lanthanide ions activate the conversion of trypsinogen into trypsin (Darnall & Birnbaum, 1970), but inhibit staphylococcal nuclease (Furie *et al.*, 1973). As with bacterial α -amylase, there may be activation at low

lanthanide ion concentrations, but inhibition at high concentrations due to non-specific binding (Darnall & Birnbaum, 1973).

The present paper describes experiments in which lanthanide ions were employed to investigate some aspects of the enzymology of clostridiopeptidase A.

Experimental

Materials

$\text{ErCl}_3 \cdot 6\text{H}_2\text{O}$ and $\text{LuCl}_3 \cdot 6\text{H}_2\text{O}$ were obtained from Alfa Products (Danvers, MA, U.S.A.); $\text{LaCl}_3 \cdot 6\text{H}_2\text{O}$ was from Fisher Scientific (Pittsburgh, PA, U.S.A.); Sm_2O_3 , bovine trypsin and phenylazobenzyl-carbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg (Pz-peptide) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); Chelex-100 was from Bio-Rad Laboratories (Richmond, CA, U.S.A.); clostridiopeptidase A was from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). ^3H -labelled guinea-pig skin collagen (type I) was a generous gift from Dr. Ian Freeman (Department of Ophthalmology, University of Pittsburgh School of Medicine, Pittsburgh, PA, U.S.A.).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was conducted with a Bio-Rad dual-vertical-slab gel system (model 220). All chemicals used during electrophoresis were purchased from Bio-Rad Laboratories. Other chemicals (analytical grade) were obtained from standard suppliers and used without further purification.

Purity of clostridiopeptidase A

The 'purified' clostridiopeptidase of a number of commercial suppliers was tested for purity by assaying for non-specific proteolytic activity, with azocasein as substrate, and by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Azocaseinase activity was determined with azocasein (0.6 mg) as substrate in 30 mM-Tris/HCl buffer, pH 7.0, containing 0.2 M-NaCl and 5 mM- CaCl_2 in a final volume of 200 μl . A solution of the commercially available collagenase (50 μl ; 20 μg of protein) was added and incubation at 37°C was continued for 3 h. The reaction was stopped with 3% (w/v) trichloroacetic acid (1 ml) and the A_{366} of the soluble products was measured.

For sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, 50 μg of protein was heated at 100°C in a solution containing 2% (w/v) sodium dodecyl sulphate, 0.1 M-dithiothreitol, 10% (v/v) glycerol and 0.01% Bromophenol Blue. Proteins were separated by slab gel electrophoresis in 10% polyacrylamide gels for 16 h at 10 mA current and at constant voltage, stained with 0.25% Coomassie Blue in 50% (w/v) trichloroacetic acid for 20 min,

and destained in water / acetic acid / methanol (73 : 7 : 20, by vol.).

Assay of clostridiopeptidase A

A standard assay mixture contained clostridiopeptidase (10 μg) and Pz-peptide (100 μg) in 30 mM-Tris/HCl buffer, pH 7.0, containing 0.2 M-NaCl and 5 mM- CaCl_2 in final volume of 250 μl . Incubation proceeded at 37°C, and the reaction was stopped by the addition of 1 ml of 3% (w/v) citric acid. Ethyl acetate (2 ml) was added, and the A_{320} of the organic phase was measured. One unit of Pz-peptidase activity releases 1 μmol of Pz-Pro-Leu/min (Wünsch & Heidrich, 1963).

Lanthanide solutions

Stock solutions (5 mM) of $\text{LaCl}_3 \cdot 6\text{H}_2\text{O}$ and $\text{ErCl}_3 \cdot 6\text{H}_2\text{O}$ were made up in distilled water. $\text{LuCl}_3 \cdot 6\text{H}_2\text{O}$ and Sm_2O_3 were initially dissolved in a minimum amount of warm conc. HCl, adjusted to pH 3–4 with NaOH and diluted with distilled water to a final concentration of 5 mM.

Removal of Ca^{2+} and Sm^{3+} from clostridiopeptidase

The chelating resin Chelex-100 was used for this purpose. All solutions were passed through a column of this material before use. Plastic vessels employed in this part of the work were first soaked three times in a solution of detergent and 25 mM-EDTA and then thoroughly rinsed in de-ionized water.

Ca^{2+} or Sm^{3+} ions were stripped from a solution of the enzyme in metal-ion-free buffer (20 mM-Tris/HCl buffer, pH 7) by passage through a column of Chelex-100.

To re-activate clostridiopeptidase after removal of Ca^{2+} or Sm^{3+} , Ca^{2+} ions were added to a final concentration of 5 mM and the mixture was incubated for 18 h at 15°C. The enzyme was subsequently assayed in the standard manner.

A quicker method, giving comparable results, was to add EDTA (2 mM) to a solution of the enzyme. Activity could then be regenerated by adding Ca^{2+} (10 mM) and incubating the mixture for 18 h at 15°C.

Kinetic analyses

In determining the effects of varying the concentrations of Pz-peptide or Ca^{2+} , preliminary experiments were first conducted to determine under which conditions the concentrations of these compounds were rate-limiting. From these studies (results not shown), concentrations of Ca^{2+} or Pz-peptide in the range 0–0.4 mM were selected for use. Assays were run in triplicate for 10 min, over which time the reaction rates were linear.

K_m values were determined by Lineweaver–Burk plots. The K_i for Sm^{3+} was obtained from plotting

the reciprocal of the reaction velocity against Sm^{3+} concentration at different Ca^{2+} concentrations and noting the point of intersection of the lines thus obtained (Dixon, 1953). K_d values for the binding of Ca^{2+} to clostridiopeptidase in the presence or in the absence of Sm^{3+} were determined by extrapolation of reciprocal plots of the reaction rates against the concentration of Ca^{2+} .

Thermoinactivation curves

A standard reaction mixture lacking Pz-peptide, with or without Ca^{2+} or Sm^{3+} , was maintained at 53°C or 60°C. Every minute (at 53°C) or 30s (at 60°C), samples were withdrawn and rapidly cooled at 4°C. All samples were then subjected to the Ca^{2+} -re-activation procedure described above under 'Removal of Ca^{2+} and Sm^{3+} from clostridiopeptidase' and assayed for residual activity for 30min at 37°C after the addition of Pz-peptide (100 µg).

Assays with collagen or gelatin

Tritiated type I collagen purified from the skin of newborn guinea pigs that had received intraperitoneal injection of [^3H]glycine was used as substrate. Before each assay, 100 µl portions containing 100 µg of collagen fibrils were reconstituted by incubation overnight at 25°C in Tris/HCl buffer, pH 7.2, containing 5 mM- CaCl_2 .

For gelatinase assays, collagen was denatured at 65°C for 15 min. Each assay tube contained 100 µg of reconstituted collagen fibrils or 100 µg of gelatin (both approx. 5000 c.p.m.) and clostridiopeptidase (10 µg) in 30 mM-Tris/HCl buffer, pH 7.0, containing 0.2 M-NaCl and 5 mM- CaCl_2 . The mixtures were incubated at 37°C for 1 h and then filtered through a 25 mm Millipore filter of pore size 0.45 µm. Each filter was washed with 30 ml of Tris/HCl buffer, pH 7.0, and dried, and the radioactivity of the undigested collagen or gelatin retained on the filter was measured by liquid-scintillation counting. The integrity of the collagen substrate was monitored to ensure that less than 10% of its radioactivity was solubilized by trypsin (10 µg).

Binding of lanthanide ions to collagen and gelatin

To assess the effects of lanthanide binding to collagen or gelatin, solutions of the rare-earth ions were added to radioactive collagen or gelatin (100 µg in 250 µl) to a final lanthanide ion concentration of 1 mM. After 20 min incubation at room temperature, the solutions were filtered through 25 mm Millipore filters (pore size 0.45 µm) and washed exhaustively with 20 mM-Tris/HCl buffer, pH 7.0, containing 0.2 M-NaCl. The filters were then placed in the bottoms of scintillation vials. Added to each of these was 1 ml of a reaction 'cocktail' containing 10 µg of clostridiopeptidase in 30 mM-Tris/HCl buffer,

pH 7.0, containing 0.2 M-NaCl and 5 mM- CaCl_2 . After an incubation at 37°C for 1 h, digested fragments from the collagen or gelatin were washed through the filter and the radioactivity of the residual material was measured.

Trypsin assays

Trypsin assays were conducted with azocasein as substrate in the manner described above under 'Purity of clostridiopeptidase A' but with an incubation time of only 30 min and 10 µg of trypsin per assay.

Results

Purity of enzyme preparations

Only the Worthington product (catalogue no. LS00 05282) proved pure enough for these experiments. Its specific azocaseinase activity was always less than 2.5 munits/mg [1 unit of azocaseinase activity degrades 1 mg of azocasein/h at 37°C (Starkey, 1977)]. The specific Pz-peptidase activity was 9.3 units/mg [1 unit of Pz-peptidase activity degrades 1 µmol of Pz-peptide/min at 37°C (Wünsch & Heidrich, 1963)], and the specific collagenase activity was 5982 units/mg [1 unit of collagenase activity degrades 1 µg of fibrillar collagen/min at 37°C (Harris & Cartwright, 1977)]. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of 5–50 µg of the product revealed only one band staining with Coomassie Blue. The product was uncontaminated by brown pigment.

In view of the high specific activity of this product, and the absence of significant azocaseinase activity or detectable proteinaceous contaminants, it was considered pure enough for the requirements of the present work.

Inhibition of clostridiopeptidase by various lanthanide ions

At a concentration of 1 mM-lanthanide and with Pz-peptide as substrate and 5 mM- Ca^{2+} present, all the lanthanide ions tested completely inhibited clostridiopeptidase; only Sm^{3+} retained any inhibitory properties at 10 µM (Fig. 1). At intermediate lanthanide concentrations, differences in the inhibitory properties of the four lanthanide ions became apparent. As shown in Fig. 2(b) (continuous lines), inhibition of Pz-peptidase activity is related to ionic radius. At a lanthanide concentration of 100 µM, Sm^{3+} , which is closest in size to Ca^{2+} , gives complete inhibition; Er^{3+} and Lu^{3+} are smaller than Sm^{3+} and give progressively less inhibition, and La^{3+} , which is slightly larger than Sm^{3+} , is a very poor inhibitor. With soluble collagen as a substrate (Fig. 2b; broken lines), this pattern is disrupted by the anomalously high inhibition produced by Lu^{3+} , which may result from its interaction with collagen

Fig. 1. Inhibition of clostridiopeptidase as a function of lanthanide ion concentration

Pz-peptidase assays were conducted as described in the text, in the presence of $10\text{ }\mu\text{M}$ – 1 mM lanthanide ion and 5 mM Ca^{2+} . The lanthanide ions tested were La^{3+} (■), Lu^{3+} (□), Er^{3+} (●) and Sm^{3+} (○).

(see the Discussion section). Clostridiopeptidase activity was not stimulated by lanthanide concentrations in the range 1 – $10\text{ }\mu\text{M}$ (results not shown).

Inhibition of trypsin by lanthanide ions

For comparative purposes, the susceptibility to lanthanide ions of trypsin was determined. Trypsin does not depend on Ca^{2+} for its activity (Darnall & Birnbaum, 1976), although it does have a specific Ca^{2+} -binding site that helps to stabilize the enzyme against autolysis. No important inhibitory effects were noted until a lanthanide concentration of 5 – 10 mM was reached, under which conditions the substrate, azocasein, was precipitated. Unlike clostridiopeptidase, inhibition increases with the charge/volume ratio of the lanthanide, in the order $\text{La}^{3+} < \text{Sm}^{3+} \leq \text{Er}^{3+} \ll \text{Lu}^{3+}$ (Fig. 2a).

Comparison of the inhibitory effects of Sm^{3+} with those of other metal ions and organic complexing agents

Sm^{3+} is a better inhibitor of Pz-peptidase activity than Fe^{2+} , Fe^{3+} , Cu^{2+} , Al^{3+} or Zn^{2+} (Table 1). Mg^{2+} and the solutions of AuCl_3 were not inhibitory at the concentrations tested.

Reversibility of inhibition by lanthanide ions

After exposure to inhibitory concentrations of Sm^{3+} , clostridiopeptidase was passed through a column of Chelex-100. The activity of the metal-free enzyme was regenerated by incubation for 18 h in

Fig. 2. Inhibition of clostridiopeptidase and trypsin by lanthanide ions of differing ionic radii

The inhibition of clostridiopeptidase by a $100\text{ }\mu\text{M}$ concentration of each of the lanthanide ions tested was compared with Pz-peptide (—) and collagen (---) as substrates. The size of the Ca^{2+} ion is indicated for comparison (in b). The inhibition of trypsin by a 10 mM concentration of each of the lanthanides tested is shown in (a).

the presence of 5 mM Ca^{2+} , and its reconstituted Pz-peptidase activity was assayed. Inhibition by Sm^{3+} is completely reversible (Table 2). These data also confirm that the inhibition that results from removal of Ca^{2+} is also completely reversible.

The same results are obtained when Sm^{3+} or Ca^{2+} is stripped from the enzyme by EDTA and activity regenerated by adding excess of Ca^{2+} , as described in the Experimental section (Table 2).

Kinetics of inhibition

Sm^{3+} lowers both the apparent K_m and $V_{\max}$ values of the Pz-peptidase reaction, as measured by the Lineweaver–Burk plots shown in Fig. 3. The

Table 1. *Inhibition of clostridiopeptidase by metal ions*
Pz-peptide was used as substrate, as described in the text. Ionic radii are taken from Weast (1974).

Metal ion	Ionic radius (nm)	Inhibition of clostridiopeptidase (%)		
		Concn. ...	0.1 mM	1 mM
Sm ³⁺	0.0964		98	100
Fe ²⁺	0.0740		61	86
Fe ³⁺	0.0640		77	84
Cu ²⁺	0.0720		21	79
Al ³⁺	0.0510		61	84
Mg ²⁺	0.0660		0	0
AuCl ₃ *	*		0	0
Pb ²⁺	0.120		20	73
Zn ²⁺	0.0740		65	89

* AuCl₃ does not form Au³⁺ on dissolution.

Table 2. *Effects on clostridiopeptidase of removing and adding Ca²⁺ and Sm³⁺*

Pz-peptide was used as substrate. Clostridiopeptidase was passed through a column of Chelex (condition 2) or had EDTA added to remove Ca²⁺ (3). Addition of excess of Ca²⁺ to either of these (5 and 6) restored enzymic activity. Sm²⁺ inhibited the enzyme (4), but activity was regained by passage through Chelex and addition of Ca²⁺ (7) or by addition of EDTA and an excess of Ca²⁺ (8).

Conditions	Enzyme activity (%)
(1) Standard assay	100
(2) –Ca ²⁺ (Chelex)	4
(3) –Ca ²⁺ (EDTA)	3
(4) +Sm ³⁺ (100 μM)	5
(5) As (2) above, Ca ²⁺ re-applied	95
(6) As (3) above, excess of Ca ²⁺ added	96
(7) As (4) above, Sm ³⁺ removed by Chelex, Ca ²⁺ re-applied	94
(8) As (4) above, Sm ³⁺ removed by EDTA; excess of Ca ²⁺ re-applied	92

uninhibited reaction has an apparent K_m of 5.4 mM, which is lowered to 0.37 mM in the presence of 50 μM-Sm³⁺. This concentration of Sm³⁺ also lowers the V_{max} of the reaction from 0.29 unit to 0.018 unit. This indicates an uncompetitive mode of inhibition. However, inhibition by Sm³⁺ is competitive with regard to Ca²⁺. Extrapolation of the reciprocal plots shown in Fig. 4 gives a dissociation constant K_d of 0.27 mM for the binding of Ca²⁺ to the enzyme in the absence of inhibitor. With 20 μM-Sm³⁺ present, the apparent K_d is raised to 0.48 mM. However, as indicated in Fig. 5, the inhibitor constant, K_i , is only 12 μM.

Fig. 3. *Lineweaver–Burk analysis of the inhibition of clostridiopeptidase by Sm³⁺*
Concentrations of Pz-peptide from 50 μM to 0.4 mM were used as substrate in the presence (O) or in the absence (●) of 50 μM-Sm³⁺. Assays were run for 15 min at 37°C, and initial velocities were calculated.

Effects of Ca²⁺ and Sm³⁺ on the thermostability of clostridiopeptidase

By chance, it was noted that Ca²⁺-free clostridiopeptidase that had been stored in the refrigerator over the summer holidays subsequently failed to be re-activated with Ca²⁺. This suggested a stabilizing role for Ca²⁺. To test this, the enzyme was heated at 53°C or 60°C for up to 10 min in the presence or in the absence of Sm³⁺ or Ca²⁺, and the residual activity was assayed after re-activation with Ca²⁺. At both temperatures thermoinactivation is biphasic.

Fig. 4. Kinetic analysis of the inhibition of clostridiopeptidase by Sm^{3+} at different concentrations of Ca^{2+} . Pz-peptidase activity was assayed at the concentrations of Ca^{2+} indicated, in the presence (O) or in the absence (●) of $50 \mu\text{M-Sm}^{3+}$. The apparent dissociation constant, K_d , of Ca^{2+} for the enzyme is given by the intersection with the abscissa.

Fig. 5. Graphical determination of K_i for inhibition of clostridiopeptidase by Sm^{3+} .

The reciprocal of the velocity of the Pz-peptidase reaction at the concentrations of Sm^{3+} and Ca^{2+} (O, 0.25 mM ; ●, 0.5 mM) indicated was determined. The $-K_i$ value is derived from the point of intersection of the two lines (Dixon, 1953).

Little loss of activity accompanies the first 1 min at 53°C or 30 s at 60°C . After this, the enzyme is rapidly inactivated, with first-order kinetics. Thermosensitivity is enhanced in the absence of Ca^{2+} (Fig. 6). This effect is much more marked for the first phase of heating ('plateau') than the subsequent one. Sm^{3+} ions have an odd effect. They elevate the initial rate of thermoinactivation to a value that is even greater than that occurring in the absence of Ca^{2+} . However, the final thermoinactivation rate is greatly diminished in the presence of Sm^{3+} (Fig. 6).

Effects of lanthanide ions on the gelatinase and collagenase activities of clostridiopeptidase

As shown in Fig. 2(b) (broken lines), Lu^{3+} inhibits the collagenase activity of clostridiopeptidase to a greater degree than it does its Pz-peptidase activity. A similar effect is seen with gelatin as the substrate.

When collagen or gelatin is exposed to a 1 mM solution of Sm^{3+} , washed exhaustively in Tris/HCl buffer, pH 7.0, and then used as substrate for clostridiopeptidase, each appears to have sequestered sufficient Sm^{3+} to diminish its subsequent susceptibility to clostridiopeptidase (results not shown).

Fig. 6. Thermoinactivation of clostridiopeptidase in the presence or in the absence of Ca^{2+} or Sm^{3+} .

The thermosensitivity of clostridiopeptidase was determined at 53°C in the presence of 5 mM-Ca^{2+} (●), in the absence of Ca^{2+} (O) or in the presence of $100 \mu\text{M-Sm}^{3+}$ (Δ).

Discussion

Tervalent lanthanide ions inhibit clostridiopeptidase by displacing Ca^{2+} from its site of binding to the enzyme. Sm^{3+} is a better inhibitor than any of the other metal ions tested in the present work.

Inhibition of clostridiopeptidase by lanthanide ions is completely reversible. This is consistent with their ionic attachment to the enzyme; lanthanides do not readily form covalent linkages. Inhibition is competitive with regard to Ca^{2+} . The apparent dissociation constant, K_d , of Ca^{2+} for clostridiopeptidase is 0.27 mM. Compared with an inhibitor constant, K_i , for Sm^{3+} of 12 μM , this suggests that Sm^{3+} binds to the Ca^{2+} -binding site on clostridiopeptidase 22.5-fold more strongly than Ca^{2+} . The K_m of the Pz-peptidase reaction is lowered from 5.3 mM to 0.37 mM in the presence of Sm^{3+} , indicating that clostridiopeptidase binds to Pz-peptidase 14.6-fold more strongly with Sm^{3+} as a cofactor than with Ca^{2+} , lowering the $V_{\max}$ some 11.8-fold as a consequence. These high K_m values indicate a rather weak binding of clostridiopeptidase to Pz-peptide. Yagisawa *et al.* (1965) also employed artificial pentapeptide substrates and found similarly high K_m values. Thus clostridiopeptidase seems to prefer naturally occurring substrates to Pz-peptide (Nordwig, 1963).

Sm^{3+} ions also lower the $V_{\max}$ of the reaction. These uncompetitive kinetics suggest that the greater stability of the enzyme- Sm^{3+} -substrate complex impedes further progress of the enzymic reaction. The formation of such 'abortive complexes' with lanthanide ions has been postulated previously by Levitzki & Reuben (1973) for α -amylase and by Furie & Furie (1975) for the activation of Factor X. Whether lanthanide ions activate or inhibit an enzymic reaction requiring Ca^{2+} may depend on the stability of the enzyme-lanthanide ion-substrate complex compared with that for the Ca^{2+} -containing one.

Similar kinetic data for the inhibition of the activation of bovine Factor X have been obtained by Furie & Furie (1975), the K_i for competitive inhibition by Nd^{3+} being 1–4 μM and the K_d of Ca^{2+} 0.31 mM. For the inhibition of staphylococcal nuclease by lanthanide ions the K_i is 1–2 μM (Furie *et al.*, 1973). Whether the values reported in the present paper have been affected by possible interaction of the lanthanide ions or Ca^{2+} with the Tris buffer (Fischer *et al.*, 1979) is unknown. Another possible source of error would be impurities in the clostridiopeptidase preparation used in the present work. The preparation had negligible azocaseinase activity, but azocasein is not a very sensitive substrate. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis failed to reveal contaminating proteins; under the conditions employed,

this would be expected to reveal 5% or more, by weight, of a proteinaceous contaminant.

In addition to its function in the attachment of clostridiopeptidase to its substrate, the present findings suggest a second, stabilizing, role for Ca^{2+} . In the absence of Ca^{2+} , the enzyme is much more thermolabile. Thermoinactivation at 53°C or 60°C appears to occur in two stages, there being a plateau to the thermoinactivation curve. This deviation from simple first-order inactivation kinetics may result from the tetrameric subunit structure of clostridiopeptidase A. The dimer is inactive against soluble collagen but retains its ability to degrade insoluble collagen, whereas the monomers have no enzymic activity (see Nordwig, 1971). Thermal agitation of the enzyme into subunits with altered kinetics of hydrolysis of Pz-peptide could affect the kinetics of thermoinactivation in the observed manner. A simpler explanation would be contamination of the enzyme preparation by a more thermostable Pz-peptidase, but no such component appeared on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Thermolability increased in the absence of Ca^{2+} . This observation may be related to the part played by Ca^{2+} in protecting clostridiopeptidase from photoinactivation in the presence of Methylene Blue (Takahashi & Seifter, 1969). Most of the increased thermolability is due to a marked lowering of the 'plateau' on the thermoinactivation curve; the remaining enzyme activity is only slightly affected by the absence of Ca^{2+} . Sm^{3+} ions have a peculiar effect. They completely eliminate the 'plateau', thus making the enzyme initially even more thermolabile than it is in the absence of Ca^{2+} . Subsequently, however, Sm^{3+} markedly stabilized the enzyme. These findings suggest that thermoinactivation of clostridiopeptidase occurs in more than one step, which differ in their response to Ca^{2+} and Sm^{3+} . There is evidence that Ca^{2+} also enhances the resistance of vertebrate collagenase to thermodenaturation (McCroskery *et al.*, 1975). Lanthanide ions inhibit impure preparations of mammalian collagenase (C. H. Evans, unpublished work).

The relative abilities of the different lanthanide ions to inhibit clostridiopeptidase suggests that the pocket into which Ca^{2+} fits is not much wider than the radius of the Ca^{2+} ion [0.0990 nm (0.990 Å)]. Thus Sm^{3+} [radius 0.0964 nm (0.964 Å)], Er^{3+} [radius 0.0881 nm (0.881 Å)] and Lu^{3+} [radius 0.0850 nm (0.850 Å)] all have access to the Ca^{2+} -binding site, whereas La^{3+} [radius 0.1016 nm (1.016 Å)] is excluded and therefore produces only slight inhibition, presumably by non-specific binding. This specific interaction of the lanthanide ions with the Ca^{2+} -binding site of clostridiopeptidase may be compared with their non-specific effects on trypsin. (Although trypsin has a binding site for Ca^{2+} , which also accepts lanthanide ions, trypsin has no requirement

for Ca^{2+} for its proteolytic activity.) Here, lanthanide ion concentrations 50–100-fold greater are needed to produce large inhibition, and the order of efficiency of inhibition is $\text{Lu}^{3+} \gg \text{Er}^{3+} \gg \text{Sm}^{3+} > \text{La}^{3+}$. Using a similar approach, Tew (1977) found Ca^{2+} -specific and non-specific sites of lanthanide binding to mitochondria.

Ca^{2+} ions have the ability to act as a 'molecular glue', binding together otherwise disparate sites on proteins (Williams, 1970, 1979). Its ability to do this may explain the additional inhibitory mechanisms detected with collagen or gelatin as a substrate, where Lu^{3+} inhibits lysis to a degree greater than that expected by its inhibition of the Pz-peptidase reaction alone. Lanthanide ions bind to collagen (Evans & Mears, 1980). One consequence of these may be inter- or intra-helical stabilization. Steven (1967) provided evidence that Ca^{2+} stabilizes collagen fibrils *in vivo*, and such a role has been postulated for anti-arthritis drugs containing gold (Adam *et al.*, 1964). Whether agents based on the lanthanide elements will eventually assume a therapeutic role remains to be evaluated (Evans, 1979).

I thank Dr. A. B. Ferguson, Jr., for his strong support of this project. This work was partially funded by the Research Development Fund of the University of Pittsburgh. Dr. W. P. Tew provided much advice and useful discussion. Mrs. Diana Montgomery kindly typed the manuscript.

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