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

Christopher H. EVANS
Department of Orthopaedic Surgery, 986 Scaife Hall, University of Pittsburgh, Pittsburgh, PA 15261, U.S.A.

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Tervalent cations of the lanthanide (rare-earth) elements reversibly inhibit bacterial collagenase (clostridiopeptidase A; EC 3.4.24.3). Sm³⁺, whose ionic radius is closest to that of Ca²⁺, is the most effective inhibitor, completely suppressing clostridiopeptidase activity at a concentration of 100 μM in the presence of 5 mM Ca²⁺, Er³⁺ and Lu³⁺, which both have ionic radii smaller than either Ca²⁺ or Sm³⁺, inhibit less efficiently, and La³⁺, which is slightly larger than Ca²⁺ or Sm³⁺, inhibits only weakly. These findings indicate a closely fitting, stereospecific, Ca²⁺-binding pocket in clostridiopeptidase, which excludes ions that are only slightly larger than Ca²⁺ [ionic radius 0.099 nm (0.99 Å)]. By contrast, trypsin, an enzyme whose activity does not depend on Ca²⁺, 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 μM, Sm³⁺ 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 Sm³⁺ enhances the affinity of this enzyme for its substrate; inhibition of hydrolysis of Pz-peptide may result from the excessive stability of the enzyme–Sm³⁺–substrate complex. Inhibition by Sm³⁺ is competitive with regard to Ca²⁺. The apparent dissociation constant, K_d, of Ca²⁺ is 0.27 mM, where the K_i for Sm³⁺ is 12 μM.

Clostridiopeptidase is more thermolabile in the absence of Ca²⁺. With Sm³⁺, thermostabilization 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 Ca²⁺ 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 (Seifert et al., 1959). It is a metalloproteinase, containing Zn(II) and requiring Ca²⁺, 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. Phenylazobenzyloxy-carbonyl-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 Ca²⁺. Their ionic sizes and electronic configurations permit them to substitute for Ca²⁺ in a specific manner. Like Ca²⁺, 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 Ca²⁺ (see review by Reuben, 1975). The tervalent charge of lanthanide ions increases the stability of their co-ordination complexes relative to Ca²⁺. The substitution of tervalent lanthanide ions for Ca²⁺ 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

ErCl₃·6H₂O and LuCl₃·6H₂O were obtained from Alfa Products (Danvers, MA, U.S.A.); LaCl₃·6H₂O was from Fisher Scientific (Pittsburgh, PA, U.S.A.); Sm₂O₃, 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-CaCl₂ 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 A₅₆₆ 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-CaCl₂ 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 A₃₅₀ 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 LaCl₃·6H₂O and ErCl₃·6H₂O were made up in distilled water. LuCl₃·6H₂O and Sm₂O₃ 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 Ca²⁺ and Sm³⁺ 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.

Ca²⁺ or Sm³⁺ 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 Ca²⁺ or Sm³⁺, Ca²⁺ 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 Ca²⁺ (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 Ca²⁺, 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 Ca²⁺ 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.

Kₘ values were determined by Lineweaver–Burk plots. The Kᵢ for Sm³⁺ 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). \(K_v\) 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 (100μg).

**Assays with collagen or gelatin**

Tritiated type I collagen purified from the skin of newborn guinea pigs that had received intraperitoneal injection of \(^{3}H\)Iglucose was used as substrate. Before each assay, 100μl portions containing 100μg 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 100μg of reconstituted collagen fibrils or 100μg of gelatin (both approx. 5000 c.p.m.) and clostridiopeptidase (10μg) in 30 mM-Tris/HCl buffer, pH 7.0, containing 0.2 mM-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 0.45μm. 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 (10μg).

**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 (100μg in 250μl) 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 0.45μm) and washed exhaustively with 20 mM-Tris/HCl buffer, pH 7.0, containing 0.2 mM-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 10μg of clostridiopeptidase in 30 mM-Tris/HCl buffer, pH 7.0, containing 0.2 mM-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 10μg 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 10μM (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.
Reversibility of inhibition of lanthanide ions

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 La\(^{3+}\) < Sm\(^{3+}\) < Er\(^{3+}\) < 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 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
Lanthanide–collagenase interactions

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).

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>Ionic radius (nm)</th>
<th>Inhibition of clostridiopeptidase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sm³⁺</td>
<td>0.0964</td>
<td>98 100</td>
</tr>
<tr>
<td>Fe²⁺</td>
<td>0.0740</td>
<td>61  86</td>
</tr>
<tr>
<td>Fe³⁺</td>
<td>0.0640</td>
<td>77  84</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>0.0720</td>
<td>21  79</td>
</tr>
<tr>
<td>Al³⁺</td>
<td>0.0510</td>
<td>61  84</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>0.0660</td>
<td>0  0</td>
</tr>
<tr>
<td>AuCl₃*</td>
<td>*</td>
<td>0  0</td>
</tr>
<tr>
<td>Pb²⁺</td>
<td>0.120</td>
<td>20 73</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>0.0740</td>
<td>65 89</td>
</tr>
</tbody>
</table>

* 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. Sm³⁺ 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).

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Enzyme activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Standard assay</td>
<td>100</td>
</tr>
<tr>
<td>(2) -Ca²⁺ (Chelex)</td>
<td>4</td>
</tr>
<tr>
<td>(3) -Ca²⁺ (EDTA)</td>
<td>3</td>
</tr>
<tr>
<td>(4) +Sm³⁺ (100 µM)</td>
<td>5</td>
</tr>
<tr>
<td>(5) As (2) above, Ca²⁺ re-applied</td>
<td>95</td>
</tr>
<tr>
<td>(6) As (3) above, excess of Ca²⁺ added</td>
<td>96</td>
</tr>
<tr>
<td>(7) As (4) above, Sm³⁺ removed by Chelex, Ca²⁺ re-applied</td>
<td>94</td>
</tr>
<tr>
<td>(8) As (4) above, Sm³⁺ removed by EDTA; excess of Ca²⁺ re-applied</td>
<td>92</td>
</tr>
</tbody>
</table>

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.

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 thermostability is biphasic.
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 Ca²⁺ (Fig. 6). This effect is much more marked for the first phase of heating ('plateau') than the subsequent one. Sm³⁺ 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 Ca²⁺. However, the final thermoinactivation rate is greatly diminished in the presence of Sm³⁺ (Fig. 6).

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

As shown in Fig. 2(b) (broken lines), Lu³⁺ 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 Sm³⁺, washed exhaustively in Tris/HCl buffer, pH 7.0, and then used as substrate for clostridiopeptidase, each appears to have sequestered sufficient Sm³⁺ to diminish its subsequent susceptibility to clostridiopeptidase (results not shown).
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 0.27mM. Compared with an inhibitor constant, \(K_i\), for Sm\(^{3+}\) of 12 \(\mu\)M, this suggests that Sm\(^{3+}\) binds to the Ca\(^{2+}\)-binding site on clostridiopeptidase 22.5-fold more strongly than Ca\(^{2+}\). The \(K_a\) of the Pz-peptide reaction is lowered from 5.3mM to 0.37mM in the presence of Sm\(^{3+}\), indicating that clostridiopeptidase binds to Pz-peptide 14.6-fold more strongly with Sm\(^{3+}\) as a cofactor than with Ca\(^{2+}\), lowering the \(V_{\text{max}}\) some 11.8-fold as a consequence. These high \(K_a\) 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 \(V_{\text{max}}\) 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 \(\alpha\)-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 \(\mu\)M and the \(K_d\) of Ca\(^{2+}\) 0.31mM. For the inhibition of staphylococcal nuclease by lanthanide ions the \(K_i\) is 1–2 \(\mu\)M (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 [0.0990nm (0.990Å)]. Thus Sm\(^{3+}\) [radius 0.0964nm (0.964Å)], Er\(^{3+}\) [radius 0.0881nm (0.881Å)] and Lu\(^{3+}\) [radius 0.0850nm (0.850Å)] all have access to the Ca\(^{2+}\)-binding site, whereas La\(^{3+}\) [radius 0.1016nm (1.016Å)] 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\textsuperscript{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 Lu\textsuperscript{3+} ≫ Er\textsuperscript{3+} ≫ Sm\textsuperscript{3+} ≫ La\textsuperscript{3+}. Using a similar approach, Tew (1977) found Ca\textsuperscript{2+}-specific and non-specific sites of lanthanide binding to mitochondria.

Ca\textsuperscript{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\textsuperscript{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\textsuperscript{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).

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