The lanthanide-enhanced affinity chromatography of clostridial collagenase

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Clostridiopeptidase A (EC 3.4.24.3) did not bind to a collagen affinity column in the absence of Ca^{2+} , but did so in the presence of lanthanide ions (Ln^{3+}). The sequestered enzyme could be eluted with EGTA. For the four Ln^{3+} ions tested, the order of efficiency in promoting enzyme binding, $\text{Sm}^{3+} > \text{Lu}^{3+} > \text{Er}^{3+} \gg \text{La}^{3+}$, reflected their relative abilities to inhibit clostridiopeptidase A. By using Sm^{3+} as an adjunct, it proved possible to separate a highly active preparation of collagenase from crude clostridial collagenase. Sodium dodecyl sulphate/polyacrylamide-gel-electrophoretic analysis of the preparation revealed a major protein of M_r 95000 and a minor component of M_r 82000. As both were stained by periodic acid/Schiff reagent, they were probably glycoproteins.

Previous studies have suggested that lanthanide ions (Ln^{3+}) competitively displace Ca^{2+} from clostridiopeptidase A (EC 3.4.24.3), producing a substituted enzyme that binds more tightly to its substrate, yet which is catalytically inactive (Evans, 1981). Ln^{3+} of smaller ionic radius than Ca^{2+} [0.100–0.118 nm (1.00–1.18 Å), co-ordination numbers 6–9] inhibit clostridiopeptidase A far more strongly than does the largest member, La^{3+} [0.103–0.122 nm (1.03–1.22 Å), co-ordination numbers 6–9]. Agents that inhibit an enzyme's activity while enhancing its substrate affinity have obvious potential as adjuncts to affinity chromatography. In this respect, Furie & Furie (1975) have confirmed the utility of Ln^{3+} as aids to the affinity-chromatographic purification of the enzyme that activates Factor X. Methods such as this would be of particular service to the affinity chromatography of clostridial collagenase, as previous attempts to do this have failed through the unusually high collagenolytic activity the enzyme retains at 4°C (Nagai & Hori, 1972).

The present paper describes the application of lanthanide-enhanced affinity chromatography to clostridial collagenase. It has twofold significance. First, it provides a direct and independent test of certain conclusions drawn from a previously described kinetic analysis (Evans, 1981). These are, that Ca^{2+} is required for the binding of enzyme to substrate, that Ln^{3+} can replace Ca^{2+} in this role and that La^{3+} does this much less effectively than the smaller Ln^{3+} . Secondly, the work examines whether the lanthanide-enhanced affinity chroma-

tography of clostridial collagenase has a possible role in the purification of this enzyme.

Experimental

Materials

Lanthanide hexahydrates were supplied by Alfa Products (Danvers, MA, U.S.A.). Pepsin-solubilized bovine skin collagen (type I) was purchased from the Collagen Corp. (Palo Alto, CA, U.S.A.). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed with a Bio-Rad dual-vertical-slab-gel system (Model 220), with chemicals obtained from Bio-Rad (Richmond, CA, U.S.A.). A Bio-Rad Econocolumn (15 cm $\times$ 1.5 cm int. diam.) in conjunction with a flow adaptor and a Pharmacia (Piscataway, NJ, U.S.A.) 'P-1' peristaltic pump were used for affinity chromatography. Clostripain and crude collagenase were bought from Worthington (Freehold, NJ, U.S.A.). A periodic acid/Schiff staining kit was purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.). From Sigma Chemical Co. (St. Louis, MO, U.S.A.) were obtained highly purified clostridiopeptidase A (type VII), phenylazobenzyloxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg (Pz-peptide), azocasein, bovine trypsin, EGTA, fluorescamine and all the other chemicals used in the present work.

Methods

Affinity chromatography. Collagen was incorporated into a 5%-(w/v)-polyacrylamide gel by

the method of Nagai & Hori (1972). The gel was finely diced with a scalpel and then forced through a 16-gauge hypodermic needle. The resulting slurry was transferred to the column and washed overnight with chromatography buffer (30mM-Tris/HCl/0.2M-NaCl/0.2% NaN_3 , pH 7.0), reversed flow being used to prevent clogging.

Collagenase was dissolved in chromatography buffer to a final concentration of 1mg/ml and dialysed overnight against a 1mM-solution of EGTA in the same buffer. Dialysis was then resumed against chromatography buffer lacking EGTA.

Before chromatography, the column was equilibrated with chromatography buffer in the presence or absence of Ln^{3+} . Each sample of collagenase (100 μg) was then infused into the column and 3ml fractions were collected. After the tenth fraction had been collected, the column was eluted with a solution of 0.5mM-EGTA in chromatography buffer and the next twenty fractions retained.

Enzyme assays. Pz-peptidase and azocaseinase were assayed as described by Evans (1981). Collagenase assays were conducted fluorometrically by a modification of the fluorescamine method of Macartney & Tschesche (1980) (Evans & Ridella, 1984).

A Ca^{2+} concentration of 25mM was used for all assays. This permitted measurement of full enzyme activity in samples that had previously been exposed to inhibitory concentrations of Ln^{3+} of EGTA, without altering the activity of untreated samples.

Protein concentrations were determined by the method of Lowry *et al.* (1951).

Electrophoresis. Proteins (5–20 μg) were heated at 100°C for 5min, in a solution containing 2% (w/v) sodium dodecyl sulphate, 0.1M-dithiothreitol, 10% (w/v) glycerol and 0.01% Bromophenol Blue. Samples were applied to 9%-(w/v)-polyacrylamide gels and subjected to electrophoresis in Tris/glycine buffer, pH 8.6, at 6mA, by the Laemmli (1970) method. Coomassie Blue (0.25% in methanol/water/acetic acid, 5:5:1, by vol.) staining of the gel for 2h was followed by destaining firstly in methanol/water/acetic acid (20:17:3, by vol.) and then aq. 5% (v/v) methanol.

Periodic acid/Schiff staining of gels was accomplished by using a kit from Fisher Scientific, used according to their instructions.

Results and discussion

Lanthanide-enhanced affinity chromatography of purified clostridiopeptidase A

The column was characterized with purified clostridiopeptidase A. None of the enzyme bound to the column in the absence of Ca^{2+} . However,

inclusion of 200 μM - Sm^{3+} in the chromatography buffer produced complete retention of the enzyme by the column. Activity that had been sequestered in this way could be eluted with a solution of 0.5mM-EGTA in chromatography buffer (results not shown).

Neither trypsin nor clostripain showed any affinity for the column, regardless of the absence or presence of Sm^{3+} (results not shown).

The amount of enzyme which bound to the column was clearly dependent on the concentration of Sm^{3+} included in the chromatography buffer (Fig. 1, closed circles). Complete retention of the enzyme required 150 μM - Sm^{3+} .

La^{3+} proved much less active than Sm^{3+} in bringing about the sequestration of clostridiopeptidase A by the collagen affinity column (Fig. 1, open circles).

Each of the four Ln^{3+} ions studied in the present work was tested for its ability to promote the binding of clostridiopeptidase A to the affinity column. The results, expressed as the percentage (mean $\pm$ S.E.M.; five separate determinations) of the total, applied enzyme which bound to the column in the presence of a 50 μM concentration of each La^{3+} ion, were as follows: La^{3+} , 16.3 $\pm$ 5.7%; Sm^{3+} , 73.8 $\pm$ 8.3%; Er^{3+} , 53.1 $\pm$ 7.8%; Lu^{3+} , 72.0 $\pm$ 9.1%. The order of effectiveness, $\text{Sm}^{3+} \geq \text{Lu}^{3+} > \text{Er}^{3+} \gg \text{La}^{3+}$, reflects their relative ability to inhibit the enzyme (Evans, 1981) and

Fig. 1. Retention of Ca^{2+} -free, purified clostridiopeptidase A by the affinity column in the presence of various concentrations of Sm^{2+} or La^{3+} .

The percentage of the total applied Pz-peptidolytic activity that bound to the column at various concentrations of Sm^{3+} (●) or La^{3+} (○) was determined. Means $\pm$ S.E.M. for five separate determinations for Sm^{3+} , and three for La^{3+} , are given.

points to the existence of strict spatial restrictions on the Ca^{2+} -binding site(s) at which the Ln^{3+} are presumably attached. Such restrictions are likely to reside in the enzyme, rather than the collagen, as there is no equivalent major difference between the effects of Sm^{3+} and La^{3+} on solutions of collagen (Evans & Drouven, 1983). Levine & Williams (1982) have suggested that extracellular proteins with a strict and specific requirement for Ca^{2+} may bind this cation at sites tailored to the size of the Ca^{2+} . Clostridiopeptidase A appears to provide an extreme example of this.

Lanthanide-enhanced affinity chromatography of crude collagenase

Preliminary experiments revealed the need to increase the concentration of Sm^{3+} to $400\text{ }\mu\text{M}$ for maximum retention of the enzyme contained within the crude preparation (results not shown). Fractions were assayed for Pz-peptidase, collagenase and azocaseinase activity.

Azocaseinase activity was restricted to fractions containing material that did not bind to the column. Although Pz-peptidase activity was found in both binding and non-binding fractions, over 85% of the total Pz-peptidase activity was retained by the column and eluted with EGTA. The non-binding Pz-peptidase activity could be the pseudo-collagenase identified by Mitchell (1968). Collagenase activity was restricted to the eluted fractions (Fig. 2).

Eluates from several experiments were pooled, dialysed overnight against 10 mM -Tris/HCl/ 0.5 mM - CaCl_2 , pH 7.0, and freeze-dried.

Fig. 2. Lanthanide-enhanced affinity chromatography of a crude collagenase preparation

Sm^{3+} ($400\text{ }\mu\text{M}$) was included in the chromatography buffer. After application of the sample and collection of the first ten fractions, the column was eluted with a solution of 1 mM -EGTA in chromatography buffer. Fractions were assayed for Pz-peptidase (●), azocaseinase (○) and collagenase (▲).

The residue, now free of brown pigment, was redissolved in 1 ml of reaction buffer and portions were used to determine a specific activity of 310 mkat/kg ($18.6\text{ }\mu\text{mol}$ of Pz-peptide degraded/min per mg). As the starting material had a specific activity of only 23 mkat/kg , this represents a purification of more than 13-fold. Protein determinations revealed a 77% yield. The specific Pz-peptidolytic activity of 310 mkat/kg ($18.6\text{ }\mu\text{mol}$ of Pz-peptide degraded/min per mg) compares favourably with specific activities of 330 mkat/kg (Heindl *et al.*, 1980), $7.56\text{ }\mu\text{mol/min per mg}$ (Kula *et al.*, 1976), and $16.2\text{ }\mu\text{mol/min per mg}$ for the 'A- α ' collagenolytic fraction of Kono (1968). Although exceeding greatly the value of around 56 mkat/kg for the commercially available, 'purified' product, the specific activity remains below the minimum value of $66.5\text{ }\mu\text{kat/kg}$ suggested by Emöd & Keil (1977).

Other portions of redissolved residue were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The starting material contained at least seven different proteins ranging in apparent M_r from 95000 to below 21500. The eluted material showed one major protein of apparent M_r 95000 and a minor protein with an apparent M_r of 82000 (Fig. 3). Both of these stained with periodic acid/Schiff reagent, suggesting that they are glycoproteins. From Fig. 3, it appears that the major band has undergone a slight decrease in M_r during purification. This could have been due to proteolysis, deglycosylation or both.

Comparison of these findings with those of other workers is complicated by the heterogeneity and multiple forms of clostridial collagenase that have been reported. Harper *et al.* (1965) isolated two clostridial collagenases whose M_r values were assessed by sedimentation equilibrium as 105000 and 57000. Kono (1968) purified three clostridial enzymes involved in the degradation of collagen, each of which had an M_r of about 100000. Isoelectric focusing has revealed four species of clostridial collagenase whose apparent M_r on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis depended on the concentration of acrylamide in the gels (Lwebuga-Mukasa *et al.*, 1976). On 5% gels, M_r values of 95000–150000 were recorded, whereas on 10% gels their apparent M_r values were decreased to between 72000 and 81000 M_r . Three of these species of collagenase had similar mobilities on 10% gels, with a convergence in M_r of about 81000, whereas the fourth ran as a protein of apparent M_r 72000. It is possible that the major band in the present work, with an apparent M_r on 9% gels of 95000, corresponds to their largest species, whereas the 82000- M_r band corresponds to their 72000- M_r protein, although it

Fig. 3. Sodium dodecyl sulphate/polyacrylamide-gel electrophoretic separation of proteins in a crude preparation of clostridial collagenase (a) and material eluted from the chromatography column (b)

In each case, 40 μ g of protein was applied to the gel. Electrophoresis and staining with Coomassie Blue was carried out as described in the text.

has yet to be shown that either of my bands is actually a collagenase.

To account for the anomalous behaviour of clostridial collagenases on gels, Lwebuga-Mukasa *et al.* (1976) suggested that these enzymes might be glycoproteins, a proposition supported in the present work.

Levdikova *et al.* (1963) proposed that clostridial collagenase is comprised of four monomers of M_r 25000. However, in neither the present study nor

that of Lwebuga-Mukasa *et al.* (1976) was a protein of M_r 25000 detected electrophoretically under denaturing conditions.

Whether or not clostridial collagenase was purified to homogeneity by lanthanide-enhanced affinity chromatography, it is clear from the increase in specific activity and decrease in the number of proteins present, that a substantial degree of purification had been achieved. In particular, the product contained no 'non-specific' (azocaseinase) activity, suggesting a role for the method in removing contaminating proteinases from preparations of collagenase.

References

- Emöd, I. & Keil, B. (1977) *FEBS Lett.* **77**, 51–56
- Evans, C. H. (1981) *Biochem. J.* **195**, 677–684
- Evans, C. H. & Drouven, B. J. (1983) *Biochem. J.* **213**, 751–758
- Evans, C. H. & Ridella, J. D. (1984) *Anal. Biochem.* **142** in the press
- Furie, B. C. & Furie, B. (1975) *J. Biol. Chem.* **250**, 601–608
- Harper, E., Seifter, S. & Hospelhorn, V. D. (1965) *Biochem. Biophys. Res. Commun.* **13**, 627–632
- Heindl, M.-C., Femandjian, S. & Keil, B. (1980) *Biochim. Biophys. Acta* **624**, 51–59
- Kono, T. (1968) *Biochemistry* **7**, 1106–1114
- Kula, M. R., Hatef-Haghi, D., Tauber-Finkelstein, M. & Shaltiel, S. (1976) *Biochem. Biophys. Res. Commun.* **69**, 389–396
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Levdikova, G. A., Orekhovich, N., Solovjeva, N. I. & Shpikiter, V. O. (1963) *Doklady Akad. Nauk. SSSR*, **153**, 725–727 (*Engl. Transl.* **153**, 1429–1431)
- Levine, B. A. & Williams, R. J. P. (1982) in *The Role of Calcium in Biological Systems* (Anghileri, L. J. & Tuffet-Anghileri, A. M., eds.), vol. 1, pp. 3–26, CRC Press, Boca Raton, FL
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Lwebuga-Mukasa, J. S., Harper, E. & Taylor, P. (1976) *Biochemistry* **15**, 4736–4741
- Macartney, H. W. & Tschesche, H. (1980) *FEBS Lett.* **119**, 327–332
- Mitchell, W. M. (1968) *Biochim. Biophys. Acta* **159**, 556–559
- Nagai, Y. & Hori, H. (1972) *Biochim. Biophys. Acta* **263**, 564–573