Fragments of human fibroblast collagenase

Purification and characterization

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On purification, human fibroblast collagenase breaks down into two major forms (M_r 22000 and M_r 27000) and one minor form (M_r 25000). The most likely mechanism is autolysis, although the presence of contaminating enzymes cannot be excluded. From *N*-terminal sequencing studies, the 22000- M_r fragment contains the active site; differential binding to concanavalin A shows the 25000- M_r fragment is a glycosylated form of the 22000- M_r fragment. These low- M_r forms can be separated by Zn^{2+} -chelate chromatography. An activity profile of this column, combined with data from substrate gels, indicates no activity against collagen in the 22000- M_r and 25000- M_r forms, but, rather, activity against casein and gelatin. The 27000- M_r form has no activity. The 22000/25000- M_r form can act as an activator for collagenase in a similar way to that reported for stromelysin. The activity of the 22000/25000- M_r form is not inhibited by the tissue inhibitor of metalloproteinases (TIMP). The 27000- M_r C-terminal part of the collagenase molecule therefore appears to be important in maintaining the substrate-specificity of the enzyme, and also plays a role in the binding of TIMP.

INTRODUCTION

Vertebrate collagenase (matrix metalloproteinase 1) is a neutral metalloproteinase capable of cleaving collagen at a single locus, to yield characteristic one-quarter–three-quarter products (see, e.g., Gross & Nagai, 1965). A family of neutral metalloproteinases exists including collagenase, gelatinase (matrix metalloproteinase 2) and stromelysin (matrix metalloproteinase 3), which have different activities but closely related primary sequences (Goldberg *et al.*, 1986; Whitham *et al.*, 1986; Collier *et al.*, 1988; Saus *et al.*, 1988).

Collagenase is synthesized as a proenzyme, including a 19-amino acid-residue signal peptide. The major secreted protein is a proenzyme of M_r 51929, which can be *N*-glycosylated (via Asn-120 and Asn-143) to a minor form of M_r approx. 57000. Procollagenase can be activated, at least *in vitro*, by proteinases (e.g. trypsin or plasmin) and mercurials (e.g. 4-aminophenylmercuric acetate). Activation results in the removal of 80 amino acid residues from the *N*-terminal end; the active enzyme has M_r 42570 and contains both glycosylation sites and two cysteine residues. Activation of human procollagenase with 4-aminophenylmercuric acetate also gives a minor cleavage approximately half-way through the molecule. A sequence of 14 amino acid residues is present that is homologous to the Zn^{2+} -binding region of thermolysin; this region is conserved in all the matrix metalloproteinases, and is likely to be the active-site region of these enzymes (Goldberg *et al.*, 1986; Whitham *et al.*, 1986).

The structure–function relationship in the matrix metalloproteinases is of obvious importance in understanding their action *in vivo*. The way in which proenzymes are activated and active enzymes are inhibited is also crucial to delineating their role in connective-tissue turnover and destruction. In the present

paper we report that purified active human fibroblast collagenase is capable of autolysis. This yields two major fragments, one of which contains the active site of the enzyme. The separation, activities and inhibition of these fragments were studied and the information this yields on the functions of the *N*-terminal and *C*-terminal domains is discussed.

MATERIALS AND METHODS

Materials

Chemicals for electrophoresis were obtained from BDH Chemicals, Poole, Dorset, U.K.; 4-aminophenylmercuric acetate was from Aldrich Chemical Co., Gillingham, Dorset, U.K.; chelating Sepharose, heparin-Sepharose and concanavalin A-Sepharose were from Pharmacia, Milton Keynes, Bucks., U.K.; pre-stained M_r markers were from Bio-Rad Laboratories, Watford, Herts., U.K.; peroxidase-conjugated antibodies were from Dako, High Wycombe, Bucks., U.K.

All other chemicals were analytical-grade reagents from Fisons, Loughborough, Leics., U.K.

Methods

Enzyme purification. Human collagenase was purified from the culture medium of WI38 fetal lung fibroblasts (large-scale cell culture was kindly performed by Dr. C. Mannix, Beecham Pharmaceuticals, Great Burgh, Surrey, U.K.). Purification was achieved by using Zn^{2+} -chelate chromatography, heparin-Sepharose and a peptide–hydroxamic acid-Sepharose affinity matrix (Moore & Spilburg, 1986).

The separation of the low- M_r collagenase fragments was achieved by using Zn^{2+} -chelate chromatography with stepwise elution with increasing salt and decreasing pH, essentially as described by Cawston & Tyler (1979).

Enzyme assays. Collagenase activity was measured with [^3H]acetylated collagen in the diffuse fibril assay (Cawston & Barrett, 1979). One unit of collagenase digests 1 μg of collagen/min at 37 °C.

Gelatinase activity was measured essentially by the method of Harris & Krane (1972). ^3H -labelled gelatin was freshly prepared by heat denaturation of [^3H]collagen. One unit of gelatinase digests 1 μg of gelatin/min at 37 °C.

Stromelysin activity was measured with [^3H]acetylated casein as a substrate, essentially as described by Cawston *et al.* (1981). One unit of caseinase digests 1 μg of casein/min at 37 °C.

All these activities are due to metalloproteinases, since they can be abolished by the addition of 1,10-phenanthroline.

Electrophoresis. SDS/polyacrylamide-gel electrophoresis was performed by the method of Laemmli & Favre (1973). Gels were fixed in 40% (w/v) trichloroacetic acid and stained with Coomassie Brilliant Blue G-250.

Gelatinase and caseinase substrate gels were prepared essentially as described by Heussen & Dowdle (1980). Samples were reduced with a final concentration of 4 mg of dithiothreitol/ml and boiled; after electrophoresis, shaking the gel in 2.5% (v/v) Triton X-100 allowed the proteins to renature by removing the SDS.

Western blotting. Proteins were separated by SDS/polyacrylamide-gel electrophoresis and electroblotted on to nitrocellulose paper. This was then incubated with rabbit anti-(human collagenase) IgG, followed by horseradish-peroxidase-conjugated pig anti-(rabbit IgG) antibody. Colour was developed with 4-chloro-1-naphthol.

RESULTS

Breakdown of collagenase

Purification of active human collagenase (see the Materials and methods section) consistently yielded both whole molecules (M_r 43000/45000) and a defined set of lower- M_r bands (M_r 27000, 25000 and 22000) (Fig. 1a). A polyclonal antibody to human collagenase, previously shown to be monospecific, recognized all these bands on immunoblotting (Fig. 1b), and hence they were breakdown products of collagenase rather than contaminants.

Attempts to prevent this breakdown were made by using proteinase inhibitors; the serine-proteinase inhibitor di-isopropyl phosphorofluoridate had no effect, whereas the specific high-affinity collagenase inhibitor *N*-[3-(*N*'-hydroxycarboxamido)-2-(2-methylpropyl)-propanoyl]-*O*-methyl-L-tyrosine methylamide (IC_{50} 10 nM), used at 5 μM , was capable of stopping breakdown, maintaining the enzyme at M_r 43000/45000 (as Fig. 1a, lane 1). Therefore the most likely mechanism for the breakdown of collagenase is autolysis.

The M_r values of the collagenase breakdown products were determined by SDS/polyacrylamide-gel electrophoresis. Gels containing 12%, 14% and 16% acrylamide were scanned on an LKB Ultrosan XL densitometer, and M_r values were determined by a plot of $\log M_r$ versus distance migrated. The two major fragments had M_r 26750 $\pm$ 180 and 22120 $\pm$ 320, and the minor fragment had M_r 25210 $\pm$ 360.

Separation of fragments

A separation of the breakdown fragments was carried out by using Zn^{2+} -chelate chromatography (see the Materials and methods section). A typical column profile is shown in Fig. 2(a); protein from each peak was subjected to SDS/12.5%-polyacrylamide-gel electrophoresis, showing that the first peak (fractions 8–30) contains 22000- M_r and 25000- M_r forms, whereas the

Fig. 1. (a) SDS/12.5%-polyacrylamide-gel electrophoresis of purified human collagenase and (b) immunoblot of human collagenase

(a) SDS/12.5%-polyacrylamide-gel electrophoresis of purified human collagenase. Lane 1, whole molecules run as a doublet of M_r 43000/45000; lane 2, autolysis products at M_r 27000, 25000 and 22000. Samples were reduced with dithiothreitol (4 mg/ml); gels were fixed in 40% (w/v) trichloroacetic acid and stained with Coomassie Brilliant Blue G-250. M_r markers (STD) are also shown. (b) Immunoblot of human collagenase (hCL). A monospecific antibody to human collagenase recognizes whole molecules and all three lower- M_r bands. Proteins from SDS/12.5%-polyacrylamide-gel electrophoresis were transferred to nitrocellulose and detected by using a monospecific antibody to human collagenase and then a horseradish-peroxidase-conjugated second antibody. Colour was developed with 4-chloro-1-naphthol. M_r markers (STD) are also shown.

Fig. 2. Separation of low- M_r collagenase fragments by Zn^{2+} -chelate chromatography

A sample of active collagenase spontaneously autolysed to lower- M_r fragments was loaded on to a column of chelating Sepharose saturated with Zn^{2+} . The column was eluted in a stepwise manner with increasing salt and decreasing pH (see the Materials and methods section). The column was monitored for (a) protein (A_{280}) and (b) collagenase ($\square$), gelatinase ($\triangle$) and caseinase ($\diamond$) activities.

final peak (fractions 70–76) contains the 27000- M_r form and any remaining whole molecules (Fig. 3). The central peak (fractions 40–60), on concentration, contains no protein and is due to a change in elution buffer.

N-Terminal sequencing

Having separated the two major lower- M_r forms, N-terminal sequencing was performed on each fragment by

Fig. 3. SDS/12.5%-polyacrylamide-gel electrophoresis showing separation of low- M_r fragments

Lane 1, sample applied to Zn^{2+} -chelating Sepharose column; lane 2, peak containing 22000/25000- M_r fragments (fractions 8–30); lane 3, peak containing 27000- M_r fragment (and any whole molecules) (fractions 70–76). Samples were reduced with dithiothreitol (4 mg/ml); gels were fixed in 40% (w/v) trichloroacetic acid and stained with Coomassie Brilliant Blue G-250. M_r markers (STD) are also shown.

sequential Edman degradation on an Applied Biosystems 470 A protein sequencer (kindly performed by Dr. N. S. Huskisson, A.F.R.C., Babraham, Cambridge, U.K.). The results, shown in Fig. 4, indicate that the peptides were formed by cleavage of the Pro-250-Ile-251 bond. The 22000- M_r fragment is the N-terminal part of the whole molecule, including the active site; the 27000- M_r fragment is the remaining C-terminal portion.

Activity of fragments

Since the 22000- M_r fragment contains the active site of collagenase, it is pertinent to look at the activity of these lower- M_r forms. Assays for collagenase, gelatinase and stromelysin activity were performed across the Zn^{2+} -chelating Sepharose column profile (Fig. 2b). Collagenase activity was present in the final peak, which contains the 27000- M_r fragment, and also whole molecules; since the 27000- M_r fragment does not contain the active site, this activity is probably due to the whole molecule. Both gelatinase and stromelysin (caseinase) activity was present in the initial peak, which contains 22000/25000- M_r forms; these activities were not present

Residue no. ...	90	100	110
Active human collagenase:	FVLPEGNPRWEQTHLTYSRIENYTPDLPRADV.....		
22000- M_r fragment:	FVLPEGNP...EQT....		
Residue no. ...	250	260	270
Active human collagenase:	RSQNPVQPIGPQTPKACDSKLTFTDAITTRIGEV.....		
27000- M_r fragment:	IGPQTPKA.DS.LTFTDAI.....		

Fig. 4. N-Terminal sequences of collagenase fragments

The fragments were separated on a Zn^{2+} -chelating Sepharose column, and sequenced by Edman degradation. Alignment with the amino acid sequence of human fibroblast collagenase shows the position of cleavage within the whole molecule. Unidentified residues in the collagenase fragments are shown as ‘.’.

Fig. 5. Electrophoresis on substrate gel, incorporating 1 mg of type I gelatin/ml

Lane 1, sample applied to Zn^{2+} -chelating Sepharose column; lane 2, 22000/25000- M_r fragments; lane 3, 27000- M_r fragment + whole molecule. Clear lysis zones indicate enzyme activity; gelatinase activity is present in whole-molecule collagenase and also in 22000/25000- M_r fragments, but not in the 27000- M_r fragment. The same pattern is seen when casein is incorporated into the gel. Lysis zones within the M_r markers (STD) are due to contaminating proteinases.

in the final peak, and were at a lower level than the collagenase activity.

As a further means of assigning the activities to each fragment, electrophoreses on substrate gels were performed for caseinase and gelatinase activity. The substrate gels show gelatinase and caseinase activity in the 22000/25000- M_r fragments, and in the whole molecule, but not in the 27000- M_r fragment (Fig. 5). The substrate gel technique cannot be used for collagenase activity, because the collagen is denatured by SDS. An alternative overlay technique is less sensitive, and cannot detect such small amounts of activity.

Fig. 6. SDS/12.5%-polyacrylamide-gel electrophoresis showing binding of 25000- M_r fragment to concanavalin A

Lane 1, sample applied to concanavalin A-Sepharose column, containing a mixture of 22000/25000- M_r fragments; lane 2, unbound material, showing that 25000- M_r fragment has bound to concanavalin A and is therefore glycosylated. M_r markers (STD) are also shown.

Table 1. Superactivation of procollagenase by 22000/25000- M_r active collagenase fragments

Pig collagenase and active collagenase were assayed in the presence and in the absence of active low- M_r fragments (approx. 7 μg). Procollagenase was activated with trypsin (20 $\mu\text{g}/\text{ml}$) for 15 min.

	Collagenase activity (units/ml)
Procollagenase	1.19
Procollagenase + 22000/25000- M_r fragment	6.67
Trypsin-activated collagenase	9.02
Trypsin-activated collagenase + 22000/25000- M_r fragment (during activation)	23.73
Trypsin-activated collagenase-22000/25000- M_r fragment (after activation)	21.70
22000/25000- M_r fragment alone	2.81

Glycosylation

The 22000- M_r and 25000- M_r fragments appear to have the same activities, and are eluted in the same fractions from the Zn^{2+} -chelating Sepharose column. Also, from N-terminal sequencing studies, only a single cleavage point is indicated; a peptide of M_r 25000 would not fit this datum. The 22000- M_r contains both known glycosylation sites for collagenase, and therefore we proposed that the 25000- M_r form was a glycosylated form of the 22000- M_r fragment. A sample containing both fragments was applied to a concanavalin A-Sepharose column, which was then washed and eluted with free sugar. SDS/polyacrylamide-gel electrophoresis of the fractions showed that only the 25000- M_r fragment binds to concanavalin A, indicating that this form is glycosylated (Fig. 6).

Collagenase activator

The 22000/25000- M_r fragments have some stromelysin-like activity; it has previously been noted that these forms can activate procollagenase in a similar way to stromelysin (personal communication from Dr. G. Murphy, Strangeways Research Laboratories, Cambridge, U.K.) (Murphy *et al.*, 1987). To study this, we used pig procollagenase, and activated with trypsin in the presence and in the absence of 22000/25000- M_r fragments. The results in Table 1 show that procollagenase activated in the presence of these fragments shows a large increase in activity; this increase is still seen if the fragments are added after activation. The increase in activity is greater than the combined activities of the active collagenase and the fragments. This is similar to the activation of procollagenase by stromelysin.

Inhibition

The inhibition of low- M_r collagenase fragment by tissue inhibitor of metalloproteinases (TIMP) was examined, since the contribution of the C-terminal peptide to TIMP binding is unknown. The data in Table 2 suggest that, although TIMP is capable of inhibiting the whole molecule, it has little or no effect on the activity of the 22000/25000- M_r fragment. In an all-human system, TIMP has previously been noted to bind less strongly

Table 2. Inhibition of collagenase and collagenase fragments by TIMP

Active human collagenase and its autolysis products were assayed in the presence and in the absence of TIMP (either in equivalent amounts or in excess) for either collagenase or stromelysin activity. Pool 1 contains 22000/25000- M_r fragments; pool 2 contains 45000- M_r whole molecules plus 27000- M_r fragments.

	Pool 1: stromelysin activity (units/ml)	Pool 2: collagenase activity (units/ml)
- TIMP	3.20	2.53
+ TIMP (equivalent)	2.90	1.66
+ TIMP (excess)	2.99	0.82

than in, for example, the pig (T. E. Cawston, unpublished work), and hence even the whole molecule is not inhibited fully.

DISCUSSION

Lower- M_r 'contaminants' of pure collagenase have previously been reported (e.g. for pig collagenase by Cawston & Tyler, 1979), but until recently no characterization of these products has been performed.

Purified human fibroblast collagenase breaks down, with cleavage of a Pro-Ile bond. A low- M_r inhibitor of collagenase (see the Results section) inhibits this breakdown, and therefore the most likely mechanism is autolysis. However, this inhibitor is not entirely specific, and can also inhibit stromelysin and gelatinase to some extent; contamination with trace amounts of these enzymes cannot be excluded. Human fibroblast stromelysin exists as two active forms of M_r 45000 and 28000 (Saus *et al.*, 1988); the high- M_r form is approximately the same as active collagenase, but there is no evidence from our data for the presence of an active 28000- M_r form. Active human gelatinase has M_r 66000 (Collier *et al.*, 1988), and again no evidence for this proteinase is seen.

Birkedal-Hansen *et al.* (1988) reported a low- M_r procollagenase formed on storage of the purified proenzyme, autolytic cleavage within the molecule occurring with cleavage of the same Pro-Ile bond as we have reported; the low- M_r procollagenase could be activated by proteolysis or 4-aminophenylmercuric acetate, but the activity was not discussed further. Low- M_r forms of stromelysin have been reported that retain activity and substrate-specificity (Chin *et al.*, 1985; Okada *et al.*, 1986), and a recombinant protein corresponding to the N-terminal of rat stromelysin also has caseinase activity (Sanchez-Lopez *et al.*, 1988). The C-terminal domain of rat stromelysin has previously been shown to have homology to hemopexin, a haem-binding plasma protein (Matrisian *et al.*, 1986), but no definite function has been assigned to it; this observation also holds true for collagenase. A cDNA has been isolated, corresponding to a human gene named *pump-1*; this codes for a putative metalloproteinase of 267 amino acid residues with homology to the N-terminal domain of stromelysin and collagenase (Muller *et al.*, 1988).

Collagenase and stromelysin co-purified from a rat mammary carcinoma cell line show conversion into lower- M_r forms on storage; an active fragment showed stromelysin-like but not collagenolytic activity, but since two enzymes were present initially it is difficult to draw conclusions from this result (Nethery & O'Grady, 1989).

The N-terminal fragment of collagenase contains the Zn^{2+} -binding region, which is presumed to be the active site of the enzyme; our studies show that this fragment retains some proteolytic activity, but with an altered substrate-specificity. Activity against casein, and to a lesser extent gelatin, is seen, but very little collagenolytic activity; the active fragment can also activate procollagenase. This suggests a switch to a stromelysin-like activity profile. The activity of the N-terminal fragment is not inhibited by TIMP. As discussed, collagenase appears to consist of two domains; our results indicate that the N-terminal domain is responsible for enzyme activity, whereas the C-terminal domain is involved in substrate-specificity and binding of TIMP. Specificity for collagen may be conferred if the C-terminal domain of collagenase can bind to collagen; it would then take on a dual role of binding substrate and inhibitor, though different regions within the domain must be responsible for each of these actions.

The possible physiological relevance of the autolysis of collagenase is not yet known, but several possible roles can be proposed: the smaller size of the active fragment may enhance its ability to penetrate the extracellular matrix and gain access to substrate; it may be involved in the activation of collagenase at sites of increased collagen degradation; the active fragment is not inhibited by TIMP, and hence may still degrade matrix even if excess inhibitor is present.

Further characterization of the fragments and a study of their role *in vivo* would be valuable.

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REFERENCES

- Birkedal-Hansen, B., Moore, W. G. I., Taylor, R. E., Bhowan, A. S. & Birkedal-Hansen, H. (1988) *Biochemistry* **27**, 6751-6758
- Cawston, T. E. & Barrett, A. J. (1979) *Anal. Biochem.* **99**, 340-345
- Cawston, T. E. & Tyler, J. A. (1979) *Biochem. J.* **183**, 6447-656
- Cawston, T. E., Galloway, W. A., Mercer, E., Murphy, G. & Reynolds, J. J. (1981) *Biochem. J.* **195**, 159-165
- Chin, J. R., Murphy, G. & Werb, Z. (1985) *J. Biol. Chem.* **260**, 12367-12376
- Collier, I. E., Wilhelm, S. M., Eisen, A. Z., Marner, B. L., Grant, G. A., Seltzer, J. L., Kronberger, A., He, C., Bauer, E. A. & Goldberg, G. I. (1988) *J. Biol. Chem.* **263**, 6579-6587
- Goldberg, G. I., Wilhelm, S. M., Kronberger, A., Bauer, E. A., Grant, G. A. & Eisen, A. Z. (1986) *J. Biol. Chem.* **261**, 6600-6605
- Gross, J. & Nagai, Y. (1965) *Proc. Natl. Acad. Sci. U.S.A.* **54**, 1197-1204
- Harris, E. D. & Krane, S. (1972) *Biochim. Biophys. Acta* **258**, 566-576

- Heussen, C. & Dowdle, E. B. (1980) *Anal. Biochem.* **102**, 196–202
- Laemmli, U. K. & Favre, M. (1973) *J. Mol. Biol.* **80**, 575–599
- Matrisian, L. M., Leroy, P., Ruhlmann, C., Gesnel, M.-C. & Breathnach, R. (1986) *Mol. Cell. Biol.* **6**, 1679–1686
- Moore, W. M. & Spilburg, C. A. (1986) *Biochemistry* **25**, 5189–5195
- Muller, D., Quantin, B., Gesnel, M.-C., Millon-Collard, R., Abecassis, J. & Breathnach, R. (1988) *Biochem. J.* **253**, 187–192
- Murphy, G., Cockett, M. I., Stephens, P. E., Smith, B. J. & Docherty, A. J. P. (1987) *Biochem. J.* **248**, 265–268
- Nethery, A. & O'Grady, R. L. (1989) *Biochim. Biophys. Acta* **994**, 149–160
- Okada, Y., Nagase, H. & Harris, E. D. (1986) *J. Biol. Chem.* **261**, 14245–14255
- Sanchez-Lopez, R., Nicholson, R., Gesnel, M.-C., Matrisian, L. M. & Breathnach, R. (1988) *J. Biol. Chem.* **263**, 11892–11899
- Saus, J., Quinones, S., Otani, Y., Nagase, H., Harris, E. D. & Kurkinen, M. (1988) *J. Biol. Chem.* **263**, 6742–6745
- Whitham, S. E., Murphy, G., Angel, P., Rahmsdorf, H. J., Smith, B. J., Lyons, A., Harris, T. J. R., Reynolds, J. J., Herrlich, P. & Docherty, A. J. P. (1986) *Biochem. J.* **240**, 913–916

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