

Stromelysin is an activator of procollagenase

A study with natural and recombinant enzymes

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The latent forms of stromelysin and collagenase from human gingival fibroblasts were purified to homogeneity. These latent proenzymes underwent serial small reductions in M_r upon activation by treatment with either 4-aminophenylmercuric acetate or trypsin. Similar shifts in M_r and activation kinetics were observed upon identical treatments of either recombinant prostromelysin or procollagenase. Prostromelysin showed a lag between activation and M_r fall, suggesting an initial activation by conformational change. Collagenase activity was enhanced up to 12-fold by either natural or recombinant stromelysin in the presence of trypsin or 4-aminophenylmercuric acetate. Stromelysin caused a further apparent decrease in the M_r of procollagenase. Since these important connective-tissue-degrading enzymes are usually co-ordinately produced by cells, a cascade mechanism is proposed in which collagenase is activated by stromelysin.

INTRODUCTION

The connective tissue metalloproteinases collagenase, gelatinase and stromelysin (formerly called proteoglycanase) are known to be secreted in proenzyme form requiring extracellular activation [1]. Plasmin, kallikrein and cathepsin B have all been implicated as putative physiological collagenase activators [2]. More recently, endogenous metalloproteinase activators of collagenase have been described, although activation conditions have been variable [3,4]. We demonstrate that stromelysin, the metalloproteinase that is often produced with collagenase, can fulfil the role of an activator *in vitro* in the presence of another proteinase such as trypsin. Due to their similar structures and properties, natural collagenase and stromelysin are difficult to separate totally. We have therefore used recombinant collagenase and recombinant stromelysin to corroborate our observations without the presence of cross-contaminants.

MATERIALS AND METHODS

Natural collagenase and stromelysin purification

The latent proforms of these enzymes were purified from the medium of mononuclear cell cytokine-stimulated human gingival fibroblasts [5], as described for the corresponding rabbit enzymes [6,7]. The identity of the enzymes was confirmed by *N*-terminal amino acid sequencing [5] and substrate specificity. Collagenase had a specific activity of 320 units/mg and stromelysin 360 units/mg (casein substrate) after trypsin activation.

Metalloproteinase activation and assay

The enzymes were activated either alone or together by incubation with varying concentrations of trypsin (from 0.1 to 10 $\mu\text{g/ml}$; trypsin:enzyme ratio, 1:100–1:1)

followed by the addition of a 10-fold excess of soya-bean trypsin inhibitor. Temperature and time variations are as described. Treatment with 4-aminophenylmercuric acetate (APMA) was at 1–2 mM for varying times as indicated at 37 °C.

Collagenase was assayed by the [^{14}C]acetylated collagen diffuse-fibril assay at 35 °C for 4 h [8] and stromelysin was assayed using [^{14}C]acetylated casein [6] at 37 °C for 1 h or 4 h; 1 unit of collagenase degrades 1 μg of type I collagen $\cdot \text{min}^{-1}$ at 35 °C and 1 unit of stromelysin degrades 1 μg of casein $\cdot \text{min}^{-1}$ at 37 °C.

Preparation of recombinant collagenase and stromelysin

The two metalloproteinase cDNAs [5] were introduced individually into two expression vectors designed specifically for (i) transient expression in COS cells [9] or (ii) stable expression in C127 cells [10]. The COS-cell expression vector is a 'poison minus' derivative of pBR322 [11] containing the SV40 late promoter and the SV40 early polyadenylation regulatory elements. Vector DNA was purified from caesium chloride gradients and transfected into COS cells using DEAE dextran [12]. Serum-free supernatants were harvested 72 h after transfection. Vectors for stable expression were based on the bovine papilloma virus genome and were essentially as described for the expression of tissue inhibitor of metalloproteinase [13]. The metalloproteinase cDNAs, under the control of the mouse metallothionein I promoter and the early SV40 polyadenylation sequence, were introduced into C127 cells by the calcium phosphate co-precipitation method [14]. Metalloproteinase-producing cell lines were identified after 21 days and used to generate serum-free culture medium.

Electrophoretic analysis of M_r changes

Enzymes before and after activation, as detailed above and in the Figure legends, were assayed for activity or

Abbreviation used: APMA, 4-aminophenylmercuric acetate.

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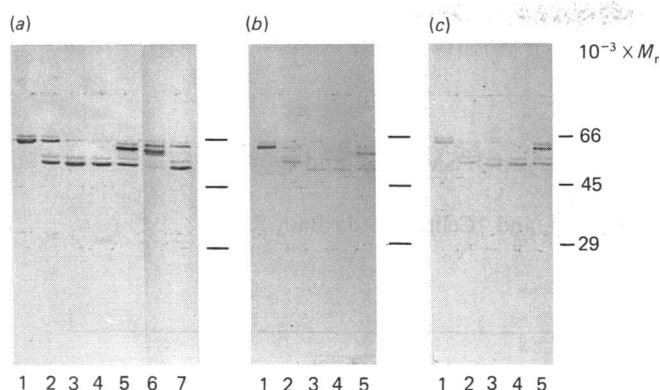


Fig. 1. Activation of prostromelysins

Prostromelysin preparations (a) purified from human gingival-fibroblast culture medium, (b) secreted by C127 cells transfected with the stromelysin-containing vector and (c) secreted by COS cells transfected with the stromelysin-containing vector, were treated as follows: lane 1, no treatment; 2, incubation with 1 mM-APMA for 2 h at 37 °C; 3, incubation with 10 µg of trypsin/ml for 30 min at 37 °C; 4, as 3, but with addition of soya-bean trypsin inhibitor after 30 min and further incubation for 2 h at 37 °C; 5, incubation with 100 µg of trypsin/ml for 10 min at 4 °C; 6, incubation with 10 µg of trypsin/ml for 5 min at 4 °C; 7, as 6, but with addition of soya-bean trypsin inhibitor after 5 min and further incubation for 2 h at 37 °C. The samples were electrophoresed on a 10 % polyacrylamide gel in SDS and reducing conditions, electro-blotted onto nitrocellulose and visualized using a sheep anti-(rabbit stromelysin) antibody and a peroxidase-labelled rabbit anti-(sheep IgG).

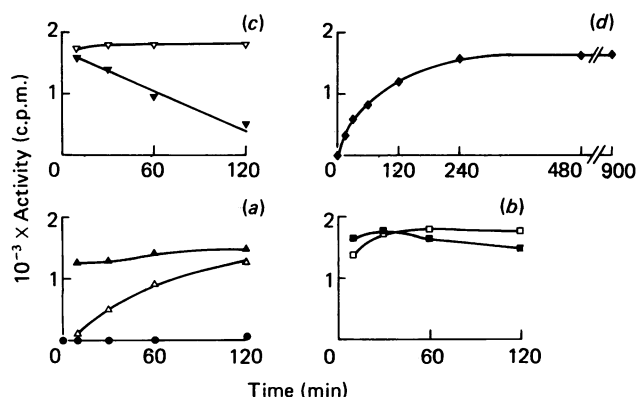


Fig. 2. Activation of C127 prostromelysin

Prostromelysin secreted by C127 cells transfected with the stromelysin-containing vector was incubated with (a) 0.1 µg of trypsin/ml (●) or 1 µg of trypsin/ml (△); (b) 10 µg of trypsin/ml (□); (c) 100 µg of trypsin/ml at 37 °C (▼) or 4 °C (▽) in a volume of 20 µl; or (d) with 2 mM-APMA at 37 °C (◆). Activity elicited was assayed by degradation of [¹⁴C]casein in a 1 h assay at 37 °C, after addition of soya-bean trypsin inhibitor. Stromelysin incubated without trypsin or APMA had no activity.

treated with 20 mM-EDTA, reduced with 500 mM-2-mercaptoethanol and run on 10 % polyacrylamide mini gels in the presence of SDS. Gels were electro-transferred to nitrocellulose and the enzyme bands visualized using either anti-(human collagenase) or anti-(rabbit

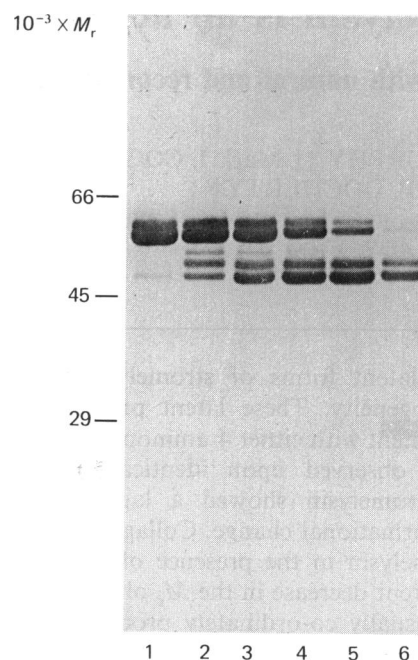


Fig. 3. Activation of C127 prostromelysin by APMA

Prostromelysin was activated with APMA for varying lengths of time (as described in Fig. 2): lane 1, 0 min; 2, 30 min; 3, 1 h; 4, 2 h; 5, 4 h; 6, 15 h. The samples were then treated with 20 mM-EDTA and electrophoresed under reducing conditions on 10 % polyacrylamide gel containing SDS and electroblotted and visualized as described in Fig. 1 and the Materials and methods section.

stromelysin) [15] sera coupled to a peroxidase-labelled second antibody [7].

RESULTS AND DISCUSSION

Stromelysin activation

The pro-forms of either purified natural stromelysin from human gingival fibroblasts, or the recombinant enzyme secreted by COS or C127 cells, had an identical M_r of 57000 with a minor M_r 60000 component (probably due to glycosylation [16]; Fig. 1, lanes a1, b1, c1). These forms were completely inactive against casein or other substrates [6] but could be activated by trypsin (e.g. C127 stromelysin; Figs. 2a–2c) or less efficiently by APMA, to degrade these substrates (Fig. 2d). Trypsin activation was optimal over a wide range of concentrations; activation could be effected at 4 °C with marginally slower kinetics (Figs. 2a–2c). Only very high levels of trypsin inactivated stromelysin. Activation by APMA was slower, achieving 58 % (COS) to 90 % (C127 and natural) of the trypsin value. Although maximum APMA activation was attained in 4–8 h at 37 °C (Fig. 2), it was found that activation was dependent upon stromelysin concentration (73 % of optimal at a 10-fold dilution and 33 % at a 100-fold dilution of a 50 unit/ml preparation). Hence, very low concentrations of enzyme, such as the COS cell-culture media, were probably not maximally activated even after 15 h of incubation.

Stromelysin M_r changes

Analysis of the effect of these treatments on the prostromelysin by gel electrophoresis and immuno-

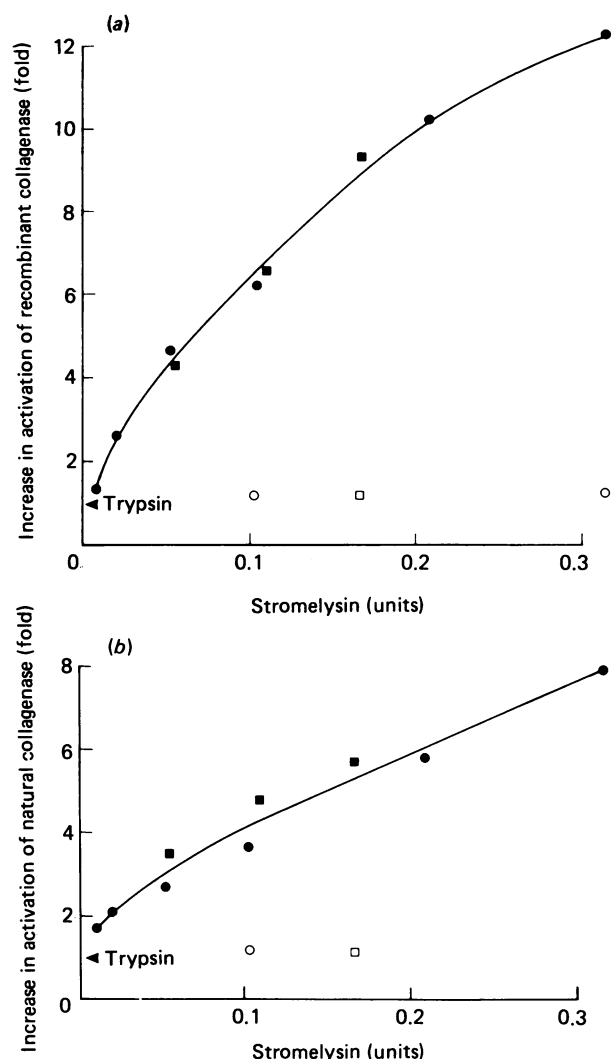


Fig. 4. Activation of procollagenases by stromelysin

Procollagenase preparations, (a) secreted by C127 cells transfected with the collagenase-containing vector and (b) purified from human-gingival-fibroblast culture medium, were incubated with $10 \mu\text{g}$ of trypsin/ml at 37°C for 30 min in the presence of varying amounts of (●) purified human fibroblast prostromelysin or (■) prostromelysin secreted by C127 cells transfected with the stromelysin-containing vector followed by the addition of excess soya-bean trypsin inhibitor. Open symbols denote the effect of the corresponding activated stromelysins in the absence of trypsin. Activity was assayed using ^{14}C -labelled type I collagen at 35°C for 4 h. Results are expressed as the fold increase elicited by stromelysin relative to the activity with trypsin alone.

blotting showed that a reduction in M_r occurred to yield two major species of M_r 50 000 and 48 000, slowly in the case of APMA (e.g. C127 stromelysin; Fig. 3) and very rapidly in the case of trypsin (Fig. 1, lanes a3, b3, 5; c3, 5). At longer incubation times with APMA, traces of an M_r 28 000 form of stromelysin were generated (Fig. 3). Plasmin also generated bands of the same M_r as those produced by trypsin (results not shown). It was noted that optimal activation (as documented in Fig. 2) did not require complete conversion of the upper doublet to the lower doublet (Fig. 1, lanes a6, a7; Fig. 3, lane 4). Using

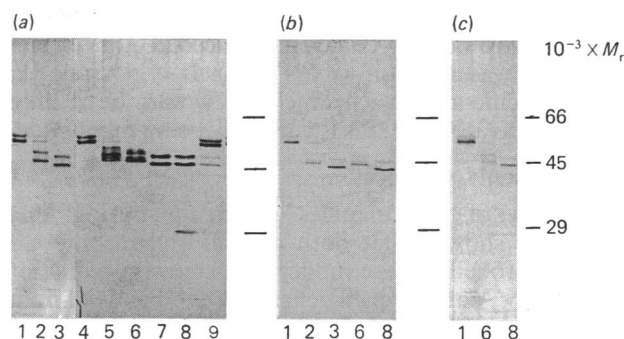


Fig. 5. Activation of procollagenases

Procollagenase preparations (a) secreted from C127 cells transfected with the collagenase vector, (b) purified from human-gingival-fibroblast culture medium, (c) secreted from COS cells transfected with the collagenase-containing vector, were treated as follows: lane 1, no treatment; 2, incubation with 1 mM-APMA at 37°C for 2 h; 3, as 2, but in the presence of purified stromelysin; 4, no treatment; 5, incubation with $10 \mu\text{g}$ of trypsin/ml at 4°C for 15 min, followed by the addition of soya-bean trypsin inhibitor; 6, as 5, but incubated with trypsin at 37°C for 30 min; 7, as 5, but in the presence of purified natural stromelysin; 8, as 6, but in the presence of stromelysin; 9, incubation with stromelysin at 37°C for 30 min. Samples were analysed as described in Fig. 1 and the Materials and methods section using an antibody to human collagenase.

collagenase, Stricklin *et al.* [17] have reported similar observations with activity detectable prior to an M_r loss. It has been proposed that conformational changes in the intact molecule occur, leading to subsequent self-cleavage. We found that the activity of stromelysin elicited was rather low, yielding specific activities of only 360 units/mg compared to values of 2400 units/mg for active rabbit-bone stromelysin [6] and 2900 units/mg for active rabbit-synovial stromelysin [18]. By sequence comparison, rabbit and human stromelysins are known to be very similar [5], with cleavage of the propeptide occurring at the sequence VGH↓F to yield the predominant active form of the enzymes. The reasons for the low activity of human stromelysin (both natural and recombinant) are under investigation.

Collagenase activation

Purified natural procollagenase or the recombinant collagenase from the transfected COS and C127 cells also had identical M_r values of 55 000 with a minor component of M_r 59 000 upon comparison by SDS/polyacrylamide-gel electrophoresis and blotting with a specific antiserum to collagenase (Fig. 5, lanes A1, B1, C1). They could also be activated by either APMA or trypsin treatment, behaving precisely as described by Stricklin *et al.* [17] with a fall in M_r of 10 000. The activity elicited was very low, in the region of 320 units/mg. A similar observation was made by Vater *et al.* [3] for rabbit procollagenase. However, the inclusion of either purified or recombinant human prostromelysin in the activation mixture enhanced the collagenase activity by up to 12-fold (Fig. 4). Addition of previously activated stromelysin to the collagenase after trypsin treatment had less effect on the final collagenase activity detectable (results not shown). Active stromelysin alone elicited similar collagenase

activities to those with trypsin alone. We found that the ratio of stromelysin:collagenase needed for efficient activation was high (in excess of 2). It is possible that higher-specific-activity stromelysin would be a more efficient activator and this is currently being investigated.

Collagenase M_r changes

Analysis of the changes occurring during these treatments showed that both natural and recombinant forms of procollagenase, underwent stepwise reductions in M_r . APMA and trypsin generated two bands of about 50000 and 45000 (Fig. 5, lanes *a2*, *a5*, *a6*, *b2*, *b6*, *c6*) which were converted to bands of 48000 and 43000 in the presence of stromelysin (Fig. 5, lanes *a3*, *a7*, *a8*, *b3*, *b8*, *c8*). An inactive M_r -28000 form was generated in some instances (Fig. 5, lane *a8*). The same band patterns were generated by both natural and recombinant stromelysin. Activated stromelysin alone appeared to produce very small changes in procollagenase of M_r approx. 500, with limited further conversion to 48000 and 43000 (Fig. 5, lane *a9*).

The rabbit procollagenase activator purified by Vater *et al.* [3] has an M_r and substrate range similar to the metalloproteinase stromelysin [6,18]. The finding that both human and rabbit stromelysin enhance the activity of human collagenase with an apparent reduction in M_r of 2000 (results not shown) provides evidence that the previously described activator is stromelysin.

The activation of prometalloproteinases appears to be a complex process; the role and nature of conformational changes and autocatalytic cleavages in the activation of both collagenase [17] and stromelysin require detailed analysis. Stromelysin cleavage alone is not sufficient to activate collagenase and it is evident that conformational changes in the latter are also required. The availability of cDNAs for the *in vitro* manipulation of the enzyme structure will further help in unravelling the details of the activation process.

The physiological relevance of these observations remains to be established. From unpublished observations and the work of Vaes & Eeckhout [2], Werb *et al.* [19], Gavrilovic *et al.* [20], and others, it appears that plasmin could be the physiological substitute for trypsin, acting as a stromelysin activator and a partial activator of collagenase. Stromelysin is generally synthesized and secreted co-ordinately with collagenase [1,15,21] and may be a more efficient collagenase activator *in vivo*, at the cell surface or under other specific pericellular conditions.

We thank Peter Angel (Kernforschungszentrum, Karlsruhe, Germany) for the collagenase cDNA and Sarah Whitham

(Celltech) for the stromelysin cDNA. We thank John Reynolds and Jelena Gavrilovic for helpful discussions and Jane Mead for typing the manuscript. G.M. is supported by funds from the M.R.C.

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