Fragmentation of human polymorphonuclear-leucocyte collagenase

Vera KNÄUPER,*§ Anja OSTHUES,* Yves A. DECLERCK,† Keith E. LANGLEY,‡ Jörg BLÄSER* and Harald TSCHESCHE*

* University of Bielefeld, Faculty of Chemistry, Department of Biochemistry, Universitätsstrasse, W-4800 Bielefeld 1, Federal Republic of Germany,
† Childrens Hospital of Los Angeles, Division of Hematology/Oncology, 4650 Sunset Boulevard, Los Angeles, CA 90027, U.S.A.,
and ‡ Amgen Inc., Thousand Oaks, CA 91320, U.S.A.

Human polymorphonuclear-leucocyte collagenase (M_r 64000) shows autoproteolytic degradation to two major fragments of M_r 40000 and M_r 27000. N-terminal sequence data and investigation of the substrate specificity of the fragments demonstrate that the M_r-40000 fragment corresponds to the catalytic domain, whereas the M_r-27000 fragment shows no enzymic activity. The activity profile of the M_r-40000 fragment is comparable with the specificity of the intact active collagenase (M_r 64000), but the ability to cleave collagen was lost. The enzymic activity of this fragment can be inhibited by either tissue inhibitor of metalloproteinase (TIMP)-1 or recombinant TIMP-2 in a 1:1 molar ratio. The C-terminal part of the enzyme (M_r 27000), important for the binding reaction with collagen substrates, is involved in collagenolysis.

INTRODUCTION

Polymorphonuclear-leucocyte (PMNL) collagenase is synthesized as a preproenzyme, including a 20-amino-acid residue signal peptide, during maturation of PMNLs (Hasty et al., 1990; Devarajan et al., 1991). After biosynthesis and post-translational processing, the glycosylated proenzyme is stored inside the specific granules of the cells (Murphy et al., 1977). PMNL procollagenase is released upon chemotactic stimulation *in vitro* (Schettler et al., 1991) and during inflammatory processes *in vivo*. The proenzyme can be activated *in vitro* by proteinases or mercurials. Activation results in the removal of at least 79, 80 or 81 amino acid residues from the N-terminal part of the proenzyme (Knäuper et al., 1990a; Mallya et al., 1990; Mookhtiar and Van Wart, 1990; Bläser et al., 1991). Once activated, the enzyme plays a major role in the connective-tissue turnover in inflammatory processes. To date, only limited information on the activation process *in vivo*, the mechanism of inhibition and structure/function relationship of the PMNL enzyme, is available. In the present paper we report that active human PMNL collagenase is autoproteolytically processed into two major fragments, which are equivalent to the catalytic and C-terminal domains of the intact enzyme. The investigation of their enzymic activity and inhibition experiments using tissue inhibitor of metalloproteinases (TIMP)-1 and recombinant (r) TIMP-2 yielded information on the structure/function relationship of these domains.

MATERIALS AND METHODS

Materials

The glycan detection kit was purchased from Boehringer Mannheim (Germany). Hydroxamic acid–Sepharose was prepared by coupling Pro-Leu-Gly-NHOH (Bachem, Switzerland) to activated CH-Sepharose 4B as recommended by Pharmacia (Sweden). The chromatographic procedures were performed at 4 °C. All other reagents were of the purest grade available.

Purification of PMNL procollagenase, activation and isolation of active PMNL collagenase and fragments

PMNL procollagenase was purified as recently published (Knäuper et al., 1990a). Activation was achieved by incubation of 500 μg of PMNL procollagenase with 500 μg of trypsin for 15 min at 37 °C. The reaction was terminated by the addition of a 10-fold molar excess of trypsin kallikrein inhibitor (TKI) from bovine lungs (Knäuper et al., 1990a). Alternatively, 500 μg of PMNL procollagenase was activated by incubation with 1 mM $HgCl_2$ at 37 °C for 2 h as recently described (Bläser et al., 1991). The specific activities of trypsin and of $HgCl_2$-activated PMNL collagenase were determined, and were 3004 and 3388 units/mg. The active enzyme was dialysed against 20 mM Tris/HCl (pH 7.5)/10 mM $CaCl_2$/0.5 M NaCl before affinity chromatography on a hydroxamic acid–Sepharose column (1.8 cm × 18 cm). The active enzyme bound to the affinity support and was eluted by increasing the pH of the buffer to 9.0 as described by Moore and Spilburg (1986). Affinity-purified active PMNL collagenase (60 μg/ml) was dialysed against 20 mM Tris/HCl (pH 7.5)/10 mM $CaCl_2$/0.5 M NaCl before incubation for 24 h at 37 °C to allow fragmentation. The fragments were separated on the hydroxamic acid–Sepharose column (1.8 cm × 18 cm). The M_r-27000 fragment remained unbound and was isolated by collection of the run-through fractions. In contrast, the M_r-40000 fragment bound, and was eluted as described above for active PMNL collagenase.

Determination of collagenolytic, gelatinolytic and peptidolytic activity

Collagenolytic activity was demonstrated by degradation of soluble type I collagen at 25 °C monitored by SDS/PAGE, followed by gel scanning (Welgus *et al.*, 1981). One unit of collagenase cleaved 1 μg of collagen/min at 25 °C. Gelatinolytic activity was determined by the fluorescamine method as described by Evans and Ridella (1984). One unit of enzyme degraded 1 μg of gelatin/min at 37 °C. Peptidolytic activity was determined by proteolytic degradation of the synthetic octapeptide (dinitrophenyl-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg) (DNP-peptide) as described by Masui et al. (1977). One unit of enzyme degraded 1 μM substrate/min at 37 °C.

Abbreviations used: PMNL, polymorphonuclear leucocyte; TIMP, tissue inhibitor of metalloproteinases.
§ To whom correspondence should be addressed.

Sequence determination of procollagenase, active collagenase and fragments

Purified proteins were dialysed against 10 mM acetic acid and freeze-dried before N-terminal sequence determination using a microsequencer (model 810; Knauer, Berlin, Germany) with on-line amino acid phenylthiohydantoin separation (Reinke et al., 1991).

Purification of tissue inhibitors of metalloproteinases TIMP-1 and rTIMP-2 and inhibition experiments

Human TIMP-1 was purified from rheumatoid synovial fluid as described by Osthues et al. (1992). Transfected Chinese-hamster ovary cells containing the cDNA encoding human TIMP-2 were used for the isolation of rTIMP-2 as recently published (DeClerck et al., 1991). The activated enzyme (300 ng) or the M_r-40000 fragment (256 ng) was incubated for 30 min with various amounts of human TIMP-1 or rTIMP2 (for details see Figures 5a and 5b). The remaining peptidolytic activity was determined by degradation of the synthetic DNP-peptide.

Proteolytic inactivation of different serpins

Samples (200 μg) of human C1-inhibitor, α_1-proteinase inhibitor or α_2-antiplasmin were incubated with either 2 μg of active PMNL collagenase (M_r 64000), 1.5 μg of M_r-40000 fragment or 1 μg of M_r-27000 fragment at 37 °C for 20 h. The serpins were cleaved by either active collagenase or the M_r-40000 fragment as demonstrated by SDS/PAGE (results not shown). Inactivation of α_1-proteinase inhibitor or C1-inhibitor was demonstrated essentially as described previously for active PMNL collagenase (Knäuper et al., 1990b, 1991). The inactivation of α_2-antiplasmin was demonstrated by monitoring the residual inhibitory activity with human plasmin as target proteinase.

Collagen-binding experiments

Collagen–Sepharose was prepared by coupling acid-soluble type I collagen (bovine) to CNBr-activated Sepharose CL6B by the method described by Kohn and Wilchek (1984). The resulting affinity support (1.2 cm × 4 cm) was equilibrated with 20 mM Tris/HCl (pH 7.5)/5 mM $CaCl_2$/0.5 M NaCl/0.001 % NaN_3. A 10 ml portion of purified proenzyme, active collagenase or fragments (protein concn. 20 μg/ml) was applied to the column at a flow rate of 5 ml/h. The column was washed with equilibration buffer until the absorbance reached the baseline. Bound proteins were eluted with 20 mM Tris/HCl (pH 7.5)/5 mM $CaCl_2$/1 M NaCl/5 % dimethyl sulphoxide/0.001 % NaN_3; 2 ml fractions were collected and assayed for enzymic activity by using the synthetic octapeptide.

SDS/PAGE and blotting

SDS/PAGE was performed by the method of Laemmli (1970). The proteins were made visible by silver staining (Heukeshoeven and Dernick, 1985). Western blotting was performed at 150 mA on to a polyvinylidene difluoride membrane by using the Biometra fast blot system. Immunolabelling of PMNL collagenase and fragments was performed by incubation with a monospecific polyclonal anti-collagenase immunoserum. Immune complexes were detected by their reaction with a secondary antibody (goat anti-rabbit IgG; Sigma, Germany), which was conjugated with alkaline phosphatase (Bergmann et al., 1989).

Protein determination

Protein concentrations were determined by the Bradford assay (1976) with albumin standard solutions.

RESULTS

Homogeneity of the purified PMNL collagenase preparations

The PMNL procollagenase and active collagenase preparations were 99 % homogeneous as estimated by SDS/PAGE (Figure 1a, lanes 1 and 2) and N-terminal sequence determination (Table 1). The proenzyme preparation as well as the active enzyme were free of human PMNL gelatinase, since analysis of the enzyme preparations with an e.l.i.s.a., which specifically detects PMNL gelatinase, failed to measure any contaminating PMNL gelatinase (Bergmann et al., 1989). PMNL collagenase was incubated with α-casein at a 1:200 enzyme/substrate ratio at 37 °C for 24 h to demonstrate that the enzyme preparation was free of stromelysin-like enzymic activity. The purified human PMNL collagenase did not degrade α-casein (results not shown), and thus it can be concluded that the enzyme preparations were not contaminated with either PMNL gelatinase or a stromelysin-like matrix metalloproteinase.

Fragmentation of active PMNL collagenase (M_r 64000)

In contrast with the proenzyme (M_r 85000), activated (by trypsin or $HgCl_2$) PMNL collagenase (M_r 64000) was not stable at 37 °C, and subsequently fragmentation into two breakdown products of apparent M_r 40000 and M_r 27000 took place (Figure 1a, lane 3). The M_r-40000 fragment was relatively resistant to silver staining (yellow bands were generally observed), probably due to the glycosylation of the molecule, which was demonstrated by using the Boehringer glycoconjugate detection kit (Figure 1b, lane 4). Deglycosylation experiments using endoglycosidase F demonstrated a dramatic decrease in the apparent M_r to 20000 (results not shown), which corresponds to the calculated M_r of the protein core of the catalytic domain (residues 80–242 or 80–247). In contrast, the M_r-27000 fragment was not detected by the glycoconjugate detection kit (Figure 1b, lane 5), and it can be concluded that this fragment is not glycosylated. A polyclonal antiserum to human PMNL collagenase, previously shown to be monospecific (Bergmann et al., 1989), recognized these bands on immunoblotting (Figure 1c), demonstrating that they were breakdown products of the active enzyme. Fragmentation of active PMNL collagenase could be inhibited by TIMP-1 and rTIMP-2 (Figure 1a, lanes 4 and 5) indicating an autoproteolytic fragmentation mechanism. The fragments were separated by affinity chromatography (Figure 1d), further investigated by N-terminal sequence determination and aligned to the human PMNL and fibroblast collagenase sequences (Table 1). The M_r-40000 fragment showed the N-terminal sequence of the active enzyme corresponding to the residues 82–93 of human fibroblast collagenase. This part of the molecule corresponded to the catalytic domain of PMNL collagenase and showed proteolytic activity against DNP-octapeptide and various substrates (see below). The M_r-27000 autoproteolytic degradation product corresponded to the C-terminal part of the PMNL collagenase molecule, and showed an inhomogeneous N-terminal sequence, which was produced by autoproteolytic cleavage of the Gly^{242}–Leu^{243} and Pro^{247}–Ile^{248} peptide bonds, indicating collagenase-specific cleavage. Alignment of the determined sequences according to their highest similarity to the amino acid sequence of human fibroblast collagenase (Goldberg et al., 1986) revealed similarity to residues 243–257 of the fibroblast enzyme. The fibroblast collagenase showed fragmentation at the

Figure 1 (a) Autoproteolysis of $HgCl_2$-activated human PMNL collagenase; (b) demonstration of glycosylation of PMNL procollagenase, collagenase and the M_r-40000 fragment; (c) Immunoblot of human PMNL procollagenase, active enzyme, M_r-40000 and M_r-27000 fragments; (d) demonstration of homogeneity of PMNL procollagenase, active collagenase, M_r-40000 and M_r-27000 fragments

(**a**) Lane 1: 500 ng of purified PMNL procollagenase after 24 h at 37 °C. Lane 2: 200 ng of purified active PMNL collagenase. Lane 3: autoproteolytic degradation products of 1 μg of active PMNL collagenase after 24 h at 37 °C in the presence of 1 mM $HgCl_2$. Lane 4: inhibition of autoproteolytic degradation of $HgCl_2$-activated PMNL collagenase (500 ng) by 180 ng of TIMP-1 after 24 h at 37 °C. Lane 5: inhibition of autoproteolytic degradation of $HgCl_2$-activated PMNL collagenase (500 ng) by 200 ng of rTIMP-2 after 24 h at 37 °C. SDS/PAGE was done in 9% gels, which were silver-stained. M_r markers are indicated. The positions of TIMP-1 (upper arrow) and rTIMP-2 (lower arrow) are indicated. (**b**) Lane 1: M_r markers. Lane 2: 100 ng of PMNL procollagenase. Lane 3: 100 ng of partially activated PMNL procollagenase. Lane 4: 100 ng of purified M_r-40000 fragment. Lane 5: 100 ng of purified M_r-27000 fragment. The sugar chains of the purified enzymes and domains were oxidized by the metaperiodate method and chemically labelled with Digoxigenin-X-hydrazide before SDS/PAGE and Western blotting. The glycoproteins were detected by incubation with anti-Digoxigenin–alkaline phosphatase conjugate, followed by substrate incubation with 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium salt. (**c**) Lane 1: PMNL procollagenase. Lane 2: active PMNL collagenase. Lane 3: autoproteolytic degradation products of 1 μg of active PMNL collagenase after 24 h at 37 °C. The monospecific polyclonal antiserum recognizes the proenzyme, the active enzyme and both fragments. M_r markers are indicated. (**d**) Lane A: M_r markers. Lane 1: 200 ng of PMNL procollagenase. Lane 2: 100 ng of active PMNL collagenase. Lane 3: 500 ng of M_r-40000 fragment (catalytic domain). Lane 4: 100 ng of M_r-27000 fragment (C-terminal domain). SDS/PAGE was done in 10% gels, which were silver-stained.

Table 1 Comparison of autoproteolytic processing sites of human PMNL collagenase (↑) and fibroblast collagenase (↓)

	Sequence	Source
Proenzyme	1 10 F-P-V-S-S-K-E-K-N-T-K-T-V-Q...	PMNL collagenase (Knäuper et al., 1990a; Hasty et al., 1990)
	F-P-A-T-L-E-T-Q-E-Q-D-V-D-L... 1 10	Fibroblast collagenase (Goldberg et al., 1986)
Active enzyme and catalytic domain	80 90 M-L-T-P-G-N-P-K-W-E-R-T-N...	PMNL collagenase (Knäuper et al., 1990a; the present paper)
	F-V-L-T-E-G-N-P-R-W-E-Q-T-H... 81 90	Fibroblast collagenase (Clark and Cawston, 1989)
C-terminal domain	243 250 L-S-S-N-P-I-Q-P-T-G-P-S-T-P...	PMNL collagenase (the present paper)
	I-G-P-Q-T-P-K-A-C-D... 251 260	Fibroblast collagenase (Clark and Cawston, 1989)

Table 2 Comparison of the specific activities of PMNL procollagenase, active PMNL collagenase and M_r-40000 and M_r-27000 fragments

Abbreviation: U, unit.

	Specific activity				
Substrate	PMNL procollagenase	$HgCl_2$-activated M_r-64000	Trypsin-activated M_r-64000	M_r-40000 fragment	M_r-27000 fragment
DNP-octapeptide	0 mU/mg	1028 mU/mg	747 mU/mg	862 mU/mg	0 mU/mg
Gelatin	0 U/mg	2309 U/mg	2105 U/mg	2010 U/mg	0 U/mg
Type I collagen*	0 U/mg	3388 U/mg	3004 U/mg	0 U/mg	0 U/mg

* Degradation of soluble type I collagen was performed at 25 °C. The cleavage products were quantified by scanning after SDS/PAGE as described by Welgus et al. (1981).

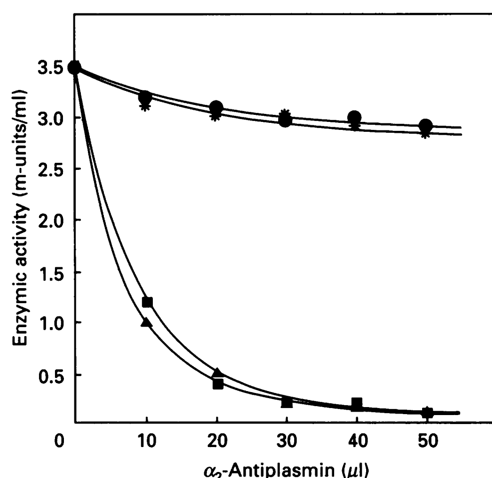

Figure 2 Demonstration of the loss of inhibitory capacity of α_2-antiplasmin after proteolysis by active PMNL collagenase and the M_r-40000 fragment

▲, Intact-α_2-antiplasmin after 24 h incubation at 37 °C in the presence of buffer; ●, inactivation of α_2-antiplasmin by active collagenase (M_r 64000) after 24 h at 37 °C; ✱, inactivation of α_2-antiplasmin by the M_r-40000 fragment after 24 h at 37 °C; ■, intact α_2-antiplasmin after 24 h incubation at 37 °C in the presence of the M_r-27000 fragment. The inhibition of human plasmin (10 μg) by α_2-antiplasmin (200 μg/ml) is shown.

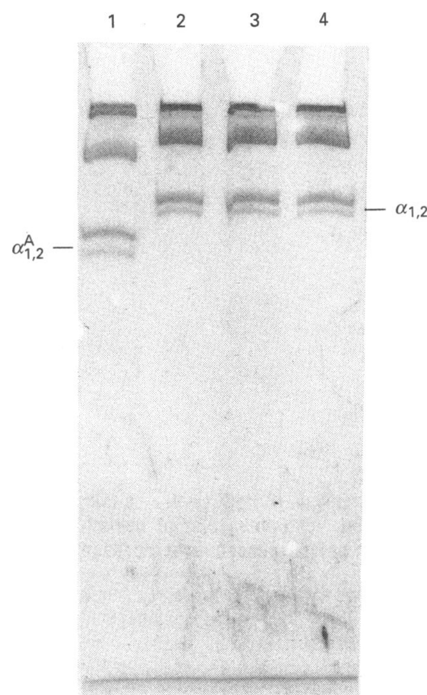

Figure 3 Demonstration of collagenolytic activity of active PMNL collagenase and purified fragments

Soluble bovine type I collagen (40 μg) was incubated at 25 °C (lanes 1–4) with purified active PMNL collagenase (lane 1), purified M_r-40000 fragment (lane 2), purified M_r-27000 fragment (lane 3) and alone (lane 4). The migration positions of $\alpha_{1,2}$ and $\alpha^A_{1,2}$ chains are indicated by arrows. SDS/PAGE was done in 6% gels, which were then stained with Coomassie Blue.

Pro^{250}–Ile^{251} locus (Clark and Cawston, 1989) eight or three residues downstream from the determined cleavage sites of PMNL collagenase, indicating similarities between both enzymes, but demonstrating differences in their autoproteolytic processing.

Comparison of the substrate specificities

The ability of active PMNL collagenase (M_r 64000) and the purified M_r-40000 and M_r-27000 fragments to digest various substrates was examined. Table 2 shows that the active enzyme and the M_r-40000 fragment were able to degrade gelatin and the synthetic DNP-octapeptide, displaying comparable specific activities. However, if one calculates the activity per molecule of enzyme, the specific activity is different. The M_r-40000 fragment retains only about 65% of the specific activity of the active collagenase. This is considerably lower and implicates an influence of the C-terminal domain on the rate of the catalysed reaction, which is in congruence with recently published data on fibroblast collagenase using casein as substrate (Murphy et al., 1992). Furthermore, the active PMNL collagenase (M_r 64000) as well as the M_r-40000 fragment were able to fragment various serine proteinase inhibitors of the serpin superfamily (results not shown). Inactivation of α-1-proteinase inhibitor, C1-inhibitor and α_2-antiplasmin was concomitantly observed and is shown for α_2-antiplasmin as an example (Figure 2). It can be concluded that the M_r-40000 fragment corresponds to the catalytic domain of PMNL collagenase, with regard to its N-terminal sequence and its ability to degrade gelatin, DNP-octapeptide and serpins. Similarly, the M_r-27000 fragment displayed no enzymic activity and corresponds to the hemopexin-like C-terminal domain.

Interaction of PMNL procollagenase, active enzyme and fragments (M_r 40000 and M_r 27000) with type I collagen

The ability of the M_r-85000 procollagenase, the M_r-64000 active collagenase and the M_r-40000 and -27000 fragments to degrade type I collagen was investigated by SDS/PAGE (Figure 3). Only the active enzyme (M_r 64000) was able to degrade type I collagen

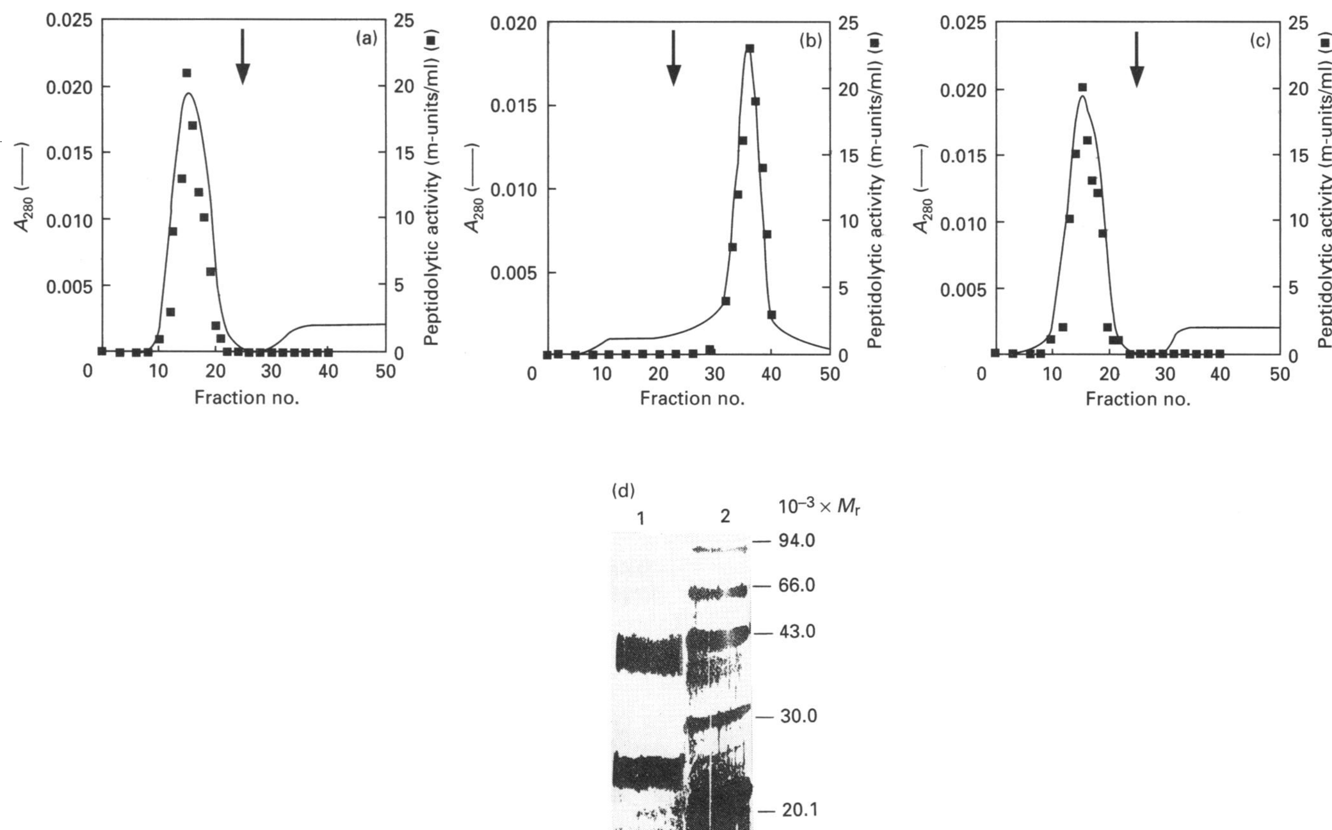

Figure 4 (a)–(c) A_{280} profiles of collagen–Sepharose chromatography used to demonstrate binding of activated PMNL collagenase; (d) SDS/PAGE showing the M_r-27000 fragment and the M_r-40000 fragment

(**a**) Chromatography of PMNL procollagenase (200 μg). Enzymic activity was demonstrated after $HgCl_2$ activation. (**b**) Chromatography of active PMNL collagenase (200 μg). (**c**) Chromatography of the M_r-40000 and M_r-27000 fragments (250 μg). The arrow denotes the time of buffer change as described in the Materials and methods section. (**d**) The M_r-27000 fragment was detected within the unbound material (see Figure 4**c**) by SDS/PAGE followed by silver-staining.

into the characteristic three-quarter fragments. Upon fragmentation to M_r 40000, the ability to cleave the native substrate, type I collagen, was lost, indicating that the C-terminal domain is important for substrate recognition, i.e. enzyme/substrate interaction. Neither the proenzyme nor either fragment was able to cleave the specific substrate. Thus the intact three-dimensional structure of the active enzyme obviously mediates the ability to cleave collagen. We therefore investigated whether the purified proenzyme, active collagenase or fragments were able to bind to a type I collagen–Sepharose column. The A_{280} profiles of type I collagen affinity chromatographies using PMNL procollagenase, active collagenase and fragments are summarized in Figures 4(a)–4(c) respectively. The enzymic activity was monitored by degradation of the synthetic octapeptide. In the case of PMNL procollagenase, the enzyme had to be activated before activity assay. Neither the proenzyme nor the M_r-40000 nor the M_r-27000 fragment bound to collagen–Sepharose, as demonstrated in Figures 4(a) and 4(c). The M_r-27000 fragment was detected in the run-through fractions by SDS/PAGE and silver staining (Figure 4d). About 90–95% of the M_r-27000 fragment was recovered in the flow-through fractions. Thus binding of the M_r-27000 fragment to collagen cannot be completely ruled out. Prolonged incubation at higher temperatures or lower salt concentrations and a more sensitive detection method such as e.l.i.s.a. or immunofluorescence techniques might lead to the detection of minor amounts of bound C-terminal domain, as demonstrated recently for fibroblast collagenase by Murphy et al. (1992). Only the active enzyme was able to bind to the specific substrate (Figure 4b), which is in congruence with the results of Hembry et al. (1986), who demonstrated the binding of active fibroblast collagenase to reconstituted collagen fibrils.

Interaction with specific metalloproteinase inhibitors TIMP-1 and rTIMP-2

The ability of TIMP-1 and rTIMP-2 to inhibit active PMNL collagenase (M_r 64000) and the M_r-40000 fragment was investigated by using the synthetic octapeptide as substrate. Detailed analysis of TIMP-1 and rTIMP-2 inhibition of active PMNL collagenase (M_r 64000) showed that a 1:1 molar complex is formed (Figure 5a). Inhibition of the M_r-40000 fragment was observed by incubation with increasing amounts of either natural TIMP-1 or rTIMP-2 (Figure 5b). Analysis of inhibition by TIMP-1 and rTIMP-2 of the M_r-40000 fragment revealed that a fragment/inhibitor molar ratio of approx. 1:1 is found on extrapolation to 100% inhibition, indicating that the catalytic domain is responsible for enzyme/inhibitor interaction. The deviation from 100% inhibition was used to calculate the approximate K_d of enzyme–inhibitor complexes (Green and Work, 1953). The results are summarized in Table 3 and compared with recently published K_d values of fibroblast stromelysin, M_r-72000 gelatinase and fibroblast collagenase. The results obtained with PMNL collagenase and the M_r-40000 fragments are 10 times lower than the K_d values recently

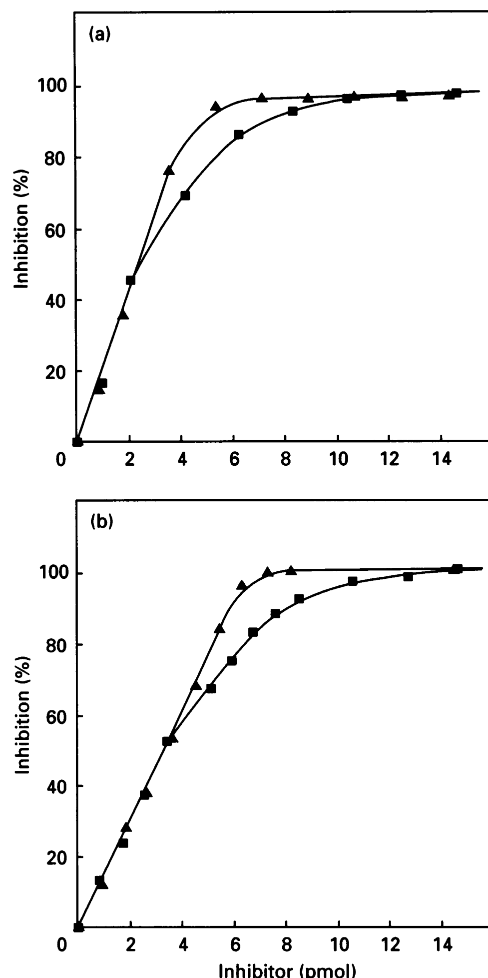

Figure 5 Inhibition of active PMNL collagenase (M_r 64000) (a) and M_r-40000 fragment (b) by TIMP-1 (■) and rTIMP-2 (▲)

Purified active PMNL collagenase or M_r-40000 fragment were incubated with increasing amounts of TIMP-1 or rTIMP-2 in a final volume of 100 μl of 20 mM Tris/HCl/5 mM $CaCl_2$/150 mM NaCl, pH 7.5, for 30 min at 37 °C. Synthetic octapeptide (100 μl) was placed into the reaction tubes for 90 min at 37 °C. The enzymic activity was calculated from the amount of cleaved peptide. Percentage inhibition was calculated in relation to the activity of active PMNL collagenase (M_r 64000) or M_r-40000 fragment free of TIMP-1 or rTIMP-2.

determined for TIMP-1 interaction with fibroblast collagenase or stromelysin (Welgus et al., 1985; Murphy et al., 1989). The rTIMP-2 complex with either PMNL collagenase or M_r-40000 fragment is a tighter-binding complex than TIMP-1/enzyme complexes. The K_d values determined for PMNL collagenase/rTIMP-2 and fragment/rTIMP-2 are very similar to those obtained for active fibroblast gelatinase complexed to TIMP-2 (Ward et al., 1991; Howard and Banda, 1991). Since inhibition of PMNL collagenase and M_r-40000 fragment with TIMP-1 or rTIMP-2 demonstrated similar K_d values, it may be concluded that the C-terminal domain of PMNL collagenase does not contribute to the binding reaction of both matrix metalloproteinase inhibitors.

Direct activation of PMNL procollagenase by the M_r-40000 fragment

As the M_r-40000 fragment corresponds to the catalytic domain and showed enzymic activity, we investigated the ability of the fragment to activate the PMNL proenzyme. A 4 μg portion of PMNL procollagenase was incubated with 8 μg of M_r-40000 fragment for 4 h either alone or in the presence of 0.5 mM $HgCl_2$ to achieve a possible super-activation of the PMNL procollagenase, as previously demonstrated for fibroblast collagenase (Clark and Cawston, 1989). The fragment activated

Table 3 Comparison of approximate K_d values of different complexes of matrix metalloproteinases with TIMP-1 and TIMP-2

References: [a] Welgus et al. (1985); [b] Ward et al. (1991); [c] Howard and Banda (1991); [d] Murphy et al. (1989).

Enzyme	TIMP-1	TIMP-2
PMNL collagenase (M_r 64000)	4.7×10^{-9} M	6.0×10^{-10} M
Catalytic domain (M_r 40000)	4.0×10^{-9} M	1.5×10^{-10} M
Fibroblast collagenase[a]	$< 10^{-9}$ M	–
M_r-72000 gelatinase[b]	–	1.0×10^{-11} M
M_r-72000 gelatinase[c]	–	7.1×10^{-10} M
Fibroblast stromelysin[d]	3.8×10^{-10} M	–

Table 4 Direct activation of PMNL procollagenase by the M_r-40000 fragment

	Specific collagenolytic activity (units/mg)
PMNL procollagenase	0
PMNL procollagenase + M_r-40000 fragment	1071
PMNL procollagenase + $HgCl_2$	3388
PMNL procollagenase + $HgCl_2$ + M_r-40000 fragment	3302
M_r-40000 fragment alone	0

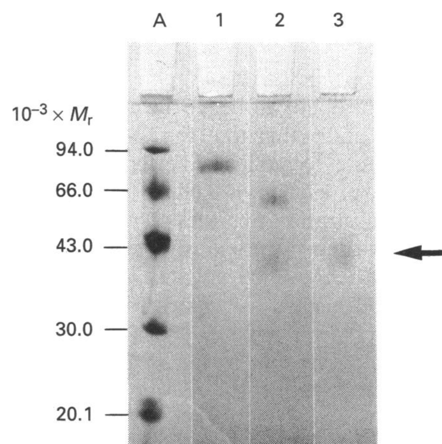

Figure 6 Activation of PMNL procollagenase by the M_r-40000 fragment

Lane A: M_r markers. Lane 1: PMNL procollagenase in the presence of buffer after 18 h at 37 °C. Lane 2: PMNL procollagenase in the presence of M_r-40000 fragment after 18 h at 37 °C. Lane 3: M_r-40000 fragment in the presence of buffer after 18 h at 37 °C, as indicated by the arrow. SDS/PAGE was done in gels, which were then silver-stained.

PMNL enzyme displayed a specific activity of 1071 units/mg. This activity is remarkably lower than that obtained after $HgCl_2$ or trypsin activation (see Tables 2 and 4) and is probably due to the generation of still latent intermediates. Activation of PMNL procollagenase by $HgCl_2$ in the presence of the M_r-40000 fragment did not result in the generation of an enzyme of high specific activity, but 100% activation was achieved. This might be one of the important differences between the collagenases. However, activation of PMNL procollagenase by the M_r-40000 fragment for 18 h at 37 °C resulted in the complete loss of the propeptide domain, as demonstrated by N-terminal sequence determination (Met^{80} or Leu^{81} N-terminus) and SDS/PAGE (Figure 6). We did not investigate whether the active PMNL collagenase (M_r 64000) itself was able to activate the proenzyme, since fragmentation of the active enzyme occurred during the incubation period. Thus it was impossible to distinguish whether activation was induced by the active enzyme or by the M_r-40000 fragment.

DISCUSSION

Purified active human PMNL collagenase is unstable, and breakdown of the entire molecule is observed. Cleavage of the Gly^{242}–Leu^{243} and Pro^{247}–Ile^{248} peptide bonds at the end of the catalytic domain leads to the generation of two fragments showing apparent M_r values 40000 and 27000. The most likely mechanism of this process is autoproteolysis, since the matrix metalloproteinase inhibitors TIMP-1 and rTIMP-2 were able to suppress this process. The possibility that traces of contaminating matrix metalloproteinases, such as PMNL gelatinase or a stromelysin-like matrix metalloproteinase, were responsible for fragmentation can be excluded, since addition of either active PMNL gelatinase or active stromelysin did not affect the rate of collagenase fragmentation (V. Knäuper, unpublished work). Similar self-cleavage processes of matrix metalloproteinases, fibroblast collagenase, stromelysin-1 and M_r-72000 gelatinase have been reported previously (Chin et al., 1985; Okada et al. 1986; Clark and Cawston, 1989; Marcy et al., 1991; Howard et al., 1991). Autoproteolysis of all these enzymes, including the PMNL collagenase, takes place in a proline-rich region, which is possibly a hinge region between the catalytic and C-terminal domains. The separation and purification of the domains allowed investigation of domain functions of different matrix metalloproteinases, which led to the demonstration of specific domain functions.

Autoproteolytic degradation of the collagenases results in the formation of a C-terminally truncated enzymically active fragment. The active PMNL collagenase and the M_r-40000 fragment share the ability to cleave the synthetic octapeptide, gelatin and certain members of the serpin superfamily, such as α_1-proteinase inhibitor, C1-inhibitor or α_2-antiplasmin (Knäuper et al., 1990b, 1991; Michaelis et al., 1990; Desrochers et al., 1992). This is obviously an intrinsic property of the catalytic domain. The ability to cleave native collagen into three-quarters and one-quarter fragments is lost upon fragmentation of the active enzyme. Similar results were obtained by using fibroblast collagenase fragments (Clark and Cawston, 1989), indicating a restricted analogy of domain functions between these enzymes. In contrast with the collagenases, the substrate specificity of C-terminally truncated stromelysin-1 remained unchanged, demonstrating that the C-terminal domain may not be involved in substrate-recognition processes of this matrix metalloproteinase (Okada et al., 1986; Marcy et al., 1991; Koklitis et al., 1991). The C-terminal domain of both collagenases participates in the recognition of type I collagen as substrate and mediates the ability to cleave type I collagen. Neither the catalytic nor the C-terminal domain, nor the proenzyme, are able to bind to or cleave the specific substrate. It has recently been claimed that the collagenases recognize the triple-helical region preceding the cleavable sequence, which consists of a hydrophobic region (Fields and Van Wart, 1992). We postulate that the binding site of PMNL collagenase for triple-helical collagen consists of parts of the catalytic and C-terminal domains. Crystallographic data of the active enzyme and the catalytic domain are needed to verify this hypothesis.

In contrast with the results obtained using PMNL collagenase, it was elegantly shown that prostromelysin, active stromelysin, active fibroblast collagenase and the corresponding C-terminal domains are able to bind to reconstituted collagen fibrils (Allan et al., 1991; Murphy et al., 1992). The binding reaction of stromelysin and fibroblast collagenase is mediated by the C-terminal pexin-like domain. It has been recently proposed by Souza and Brentani (1992) that this binding reaction is mediated by the sequence motif SQNPVQP of fibroblast collagenase and SSNPIQP of PMNL collagenase, using the concept of sense–antisense peptide interactions. According to these suggestions, the catalytic domains of fibroblast collagenase as well as PMNL collagenase should bind to collagen via these conserved sequence motifs. This is in contrast with our results and those observed by Murphy et al. (1992).

It was proposed by Clark and Cawston (1989) that the C-terminal domain of fibroblast collagenase might be involved in inhibitor binding, since the catalytic domain was not inhibited significantly by a 2-molar excess of TIMP-1. In the case of the catalytic domain of human PMNL collagenase, we obtained linear inhibition using TIMP-1 and rTIMP-2. We conclude that the C-terminal domain of human PMNL collagenase is not involved in binding TIMP-1 or rTIMP-2. Similar results are obtained in the case of the active domain of stromelysin-1, r-matrilysin and matrilysin from rat uteri (Okada et al., 1986; Quantin et al., 1989; Woessner and Taplin, 1988), indicating that the catalytic domain is responsible for inhibitor binding. In contrast, the C-terminal domains of both the M_r-72000 and M_r-92000 progelatinases have been shown to possess a different function, as compared with collagenases and stromelysin. Their C-terminal domains obviously share the ability to bind specifically TIMP-1 (M_r-92000 progelatinase) or TIMP-2 (M_r-72000 progelatinase), as recently demonstrated by Howard and Banda (1991) and Goldberg et al. (1992). These progelatinase/TIMP complexes are potent matrix metalloproteinase inhibitors and can also be activated by mercurials to active enzymes. However, it should be noted that the specific activity of the activated complex is lower than that of inhibitor-free enzyme. Both proteolytically active gelatinase/TIMP complexes are remarkably stable. The enzyme is resistant to autoproteolytic fragmentation, when isolated as a complex, indicating that the inhibitor masks the hinge sequence where autoproteolysis occurs in a bimolecular reaction. The inhibitor-free active M_r-62000 gelatinase shows similar autoproteolytic fragmentation as described here for PMNL collagenase (Howard and Banda, 1991). Thus the C-terminal domains of both progelatinases have a different function as compared with the C-terminal domains of other members of the matrix metalloproteinase family. These results demonstrate that different specific functions of matrix metalloproteinases are mediated by their catalytic and C-terminal domains.

Activation of human PMNL procollagenase by the M_r-40000 catalytic domain is accompanied by the proteolytic removal of the complete propeptide domain. Thus proenzyme activation

follows the rules of the cysteine-switch activation mechanism (Van Wart and Birkedal-Hansen, 1990; Springman et al., 1990). It has been proposed that the propeptide of a latent matrix metalloproteinase interacts via its free Cys residue of the 'PRCGVPD'-sequence motif by complex-formation with the integral catalytic Zn centre of the enzyme. Disruption of the Cys–Zn interaction can be initiated by proteolytic or autoproteolytic cleavage in the propeptide domain, either 36 residues preceding, or within or behind the 'PRCGVPD'-sequence motif. The loss of the 'PRCGVPD' motif is essential in generating full enzymic activity in a mercurial-free system. This behaviour was demonstrated for all proenzymes of the matrix metalloproteinase family so far isolated and was confirmed by site-directed mutagenesis experiments (Grant et al., 1987; Sanchez-Lopez et al., 1988; Stetler-Stevenson et al., 1989; Suzuki et al., 1990; Knäuper et al., 1990a; Park et al., 1991; Windsor et al., 1991; Ogata et al., 1992).

Autolysis of matrix metalloproteinases seems to be a general phenomenon. Autoproteolytic fragmentation is observed around the conserved 'PRCGVPD' sequence motif of the propeptide domain and within the hinge region of the catalytic and C-terminal domain. It is proposed that a correlation exists between the sites of autoproteolytic attack and segment mobility in globular proteins, which was demonstrated for the metallo-endopeptidase from *Bacillus thermoproteolyticus* (thermolysin) (Fontana et al., 1986). It has been suggested that mobile segments in proteins appear to be of general significance in protein/protein recognition processes, which might also be true for all matrix metalloproteinases. In the collagenases the hinge region between the catalytic and the C-terminal domain seems to be responsible for enzyme/collagen binding. Furthermore, this part of active collagenase seems to be essential for collagenolysis, since fragmentation of the enzyme results in the loss of this characteristic function. The physiological relevance of the autoproteolytic fragmentation process is not yet known. A possible role of the M_r-40000 fragment might be the activation of procollagenase at sites of increased collagen turnover, which would be of physiological significance during inflammatory processes.

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