Partial purification of collagenase and gelatinase from human polymorphonuclear leucocytes

Analyses of their actions on soluble and insoluble collagens

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The separation and further purification of human polymorphonuclear-leucocyte collagenase and gelatinase, using modifications of the method of Cawston & Tyler [(1979) *Biochem. J.* **183**, 647–656], are described. The final preparations yielded collagenase of specific activity 260 units/mg and gelatinase of specific activity 13 000 units/mg. Gelatinase was purified to apparent homogeneity in a latent form, and analysis of the activation of ^{125}I -labelled latent enzyme by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and gel-filtration techniques suggested that no peptide material was lost on conversion into the active form. The purified natural inhibitors α_2 -macroglobulin, tissue inhibitor of metalloproteinases ('TIMP') and amniotic-fluid inhibitor of metalloproteinases all inhibited the two polymorphonuclear-leucocyte metalloproteinases, but the last two inhibitors were slow to act and complete inhibition was difficult to attain. Collagenase degraded soluble types I and III collagen equally efficiently, but soluble type II collagen less well. Gelatinase alone had little activity on these substrates, although it enhanced the action of collagenase. Gelatinase was capable of degrading soluble types IV and V collagen at 25°C, whereas collagenase was only active at higher temperatures when the collagens were susceptible to trypsin activity. By using tissue preparations of insoluble collagens (type I, II or IV) the activity of leucocyte collagenase was low and gelatinase activity was negligible, as measured by the solubilization of hydroxyproline-containing material. The two enzymes together were two to three times more effective in the degradation of these insoluble collagens.

A characteristic feature of inflammatory conditions is the massive infiltration of connective tissues by polymorphonuclear leucocytes, often followed by the breakdown of the extracellular matrix. Important components of this matrix are both the interstitial collagens (types I, II and III; Miller *et al.*, 1976) and the collagens associated with basement membranes and cytoskeletal structures (types IV and V; Bornstein & Sage, 1980). It is known that the serine proteinase elastase, from the leucocyte azurophil granules, can degrade these macromolecules (Starkey *et al.*, 1977; Davies *et al.*, 1978; Mainardi *et al.*, 1980) as well as other matrix components such as proteoglycans (Roughley, 1978). However, polymorphonuclear leucocytes also contain a complement of metalloproteinases that are present in

other subcellular compartments, a collagenase in the specific granules and a gelatinase in the C-particles (Murphy *et al.*, 1980). In the present paper we describe the separation and purification of these two metalloproteinases. Some biochemical properties of the two enzymes are also described, including an analysis of their ability to degrade both soluble and insoluble collagens, as well as other matrix macromolecules.

Materials and methods

Materials

Most of the chemicals used have been described by Cawston & Tyler (1979). *N*-Succinimidyl 3-(4-hydroxy-5- ^{125}I iodophenyl)propionate (Bolton and Hunter reagent; 2000 Ci/mmol; IM. 861) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Porcine pancreatic trypsin was

Abbreviations used: SDS, sodium dodecyl sulphate; TIMP, rabbit bone inhibitor of metalloproteinases.

purchased from Sigma. Pure human α_2 -macroglobulin was generously donated by Dr. A. J. Barrett and Dr. G. S. Salvesen of this Laboratory. The rabbit bone inhibitor of metalloproteinases (TIMP) and the human amniotic fluid inhibitor were prepared as described by Cawston *et al.* (1981b) and Murphy *et al.* (1981a) respectively. Soluble type II collagen from bovine articular cartilage and type III collagen from human placenta were donated by Dr. G. M. Shellwell and Dr. A. J. Bailey of the Meat Research Institute, Langford, Bristol, U.K. 1 α ,2 α ,3 α (ABC-like; type V) collagen from human neonatal femoral head hyaline cartilage and types III and V collagen from human amniotic membrane were donated by Dr. R. Burgeson, Department of Paediatrics, University of California Los Angeles, Los Angeles, CA, U.S.A. Insoluble bovine tendon collagen (Type I) was purchased from Sigma. Bovine nasal and articular cartilage, as well as eyes for anterior-lens-capsule dissection, were obtained from freshly slaughtered bullocks at the local abattoir. Type IV collagen was solubilized from bovine anterior lens capsules, essentially by the method of Schwartz & Veis (1978), using pepsin (100 μ g per 200 capsules; about 400 mg of collagen). Rabbit plasma fibronectin was the gift of Dr. R. A. D. Bunning of this Laboratory, and mouse EHS sarcoma laminin was the gift of Dr. R. Timpl, Max Planck-Institut für Biochemie, Martinsreid, Germany. Dr. F. Woessner kindly supplied 2,4-dinitrophenyl-L-Pro-L-Leu-L-Ile-L-Ala-L-Gly-L-Arg-amide (obtainable from the Protein Research Foundation, Osaka, Japan). Purified bovine cartilage proteoglycan aggregate was prepared and incorporated into polyacrylamide beads as described by Nagase & Woessner (1980). Rabbit bone collagenase was purified by Mr. W. A. Galloway, Strangeways Research Laboratory, by using the method of Cawston & Tyler (1979).

Methods

Fractionation of granules and extraction. Specific granules and C-particles were obtained by subcellular fractionation of human neutrophil homogenates as described previously (Bretz & Baggiolini, 1974; Murphy *et al.*, 1980). Approx. 4×10^{10} neutrophils were used in each fractionation experiment. Purified granules were extracted with 0.05% Triton X-100-containing buffer as described previously (Murphy *et al.*, 1980) and the clear supernatant was treated with 5 mM-di-isopropyl phosphorofluoridate at 37°C for 1 h.

Gel-filtration chromatography. The di-isopropyl phosphorofluoridate-treated extract was chromatographed on Ultrogel AcA 34. A single peak of gelatinase activity of apparent mol.wt. 250 000 was eluted before a single peak of collagenase activity of apparent mol.wt. 95 000, as described previously

(Murphy *et al.*, 1980). Fractions containing gelatinase activity were pooled, as were fractions containing collagenase activity.

Further purification of collagenase. DEAE-cellulose, heparin-Sepharose and Zn²⁺-iminodiacetic acid-Sepharose (zinc chelate-Sepharose) chromatography were carried out sequentially (Cawston & Tyler, 1979). Collagenase did not bind to any of these matrices, but gelatinase activity was largely removed on DEAE-cellulose and contaminating proteins bound to the two subsequent columns.

Further purification of gelatinase activity. The gelatinase pool was further fractionated on DEAE-cellulose and zinc chelate-Sepharose under the same conditions as collagenase. Gelatinase activity bound to DEAE-cellulose and was eluted by NaCl at a conductivity of 5 mS. Most of the enzyme did not bind to the zinc chelate column, but, by using the conventional elution procedures, a number of contaminating proteins were shown to bind.

Rechromatography on Ultrogel AcA 34. Portions of the pools of both the collagenase and gelatinase eluted from zinc chelate-Sepharose were chromatographed on a calibrated Ultrogel AcA 34 column (1.5 cm $\times$ 76 cm). ¹²⁵I-labelled gelatinase, both before and after activation, was also chromatographed on this column. The column was eluted at 10 ml/h and 1 or 2 ml fractions were collected. The column was calibrated as described by Murphy *et al.* (1980).

Sephadex G-50 chromatography. A Sephadex G-50 column (0.5 cm $\times$ 45 cm), equilibrated with the gel-filtration column buffer, was used to analyse ¹²⁵I-labelled low-molecular-weight material generated by either the chemical or proteolytic activation of ¹²⁵I-labelled latent gelatinase. The exclusion volume, V_0 , and the total inclusion volume, V_t , of the column were assessed by using Dextran Blue and potassium ferricyanide respectively; 2 ml samples were loaded in all cases and the column was eluted at 8 ml/h; 0.5 ml fractions were collected.

Protein determination. The protein contents of the column eluates were monitored by measuring A_{280} . Enzyme pools were assayed for protein content by the fluorescamine method of Weigle *et al.* (1972).

Enzyme assays. Collagenase activity was determined by the fibril assay using [¹⁴C]acetylated rat skin collagen (Werb & Burleigh, 1974; Cawston & Barrett, 1979). For comparison, the diffuse-fibril assay of Cawston & Barrett (1979) was also used. Gelatinase activity was assayed by using heat-denatured type I collagen (Murphy *et al.*, 1980). Casein-degrading activity was assayed with [¹⁴C]-acetylated casein (Cawston *et al.*, 1981b). Latent enzymes were activated with 4-aminophenylmercuric acetate (0.3 mM; Murphy *et al.*, 1980).

Effect of inhibitors. Interaction of the activated enzymes with α_2 -macroglobulin, TIMP and human

amniotic fluid inhibitor were studied in two ways: (a) preincubation of enzyme and inhibitor at 37°C for different times before assay with the appropriate substrate; (b) inclusion of inhibitor with enzyme in a 20h assay (collagenase only). The amounts of human amniotic fluid inhibitor and TIMP are expressed as units of inhibitory activity against a standard preparation of rabbit skin collagenase (Murphy *et al.*, 1977).

Iodination of proteins. A ^{125}I -labelled acylating reagent was used to iodinate enzyme samples (Bolton & Hunter, 1973).

Polyacrylamide-gel electrophoresis and autoradiography. SDS/polyacrylamide-gel electrophoresis of ^{125}I -labelled enzyme samples was performed by the method of Laemmli & Favre (1973). After separation the proteins were stained with Coomassie Brilliant Blue G (Fairbanks *et al.*, 1971) and the gels were then dried and autoradiographed. Gelatinase samples (10 μg) were also analysed by electrophoresis on polyacrylamide gels, under conditions that preserved enzyme activity, with the same buffer systems but containing 0.5% Brij 35 instead of SDS.

Degradation of soluble collagens. Purified solubilized types I, II, III, IV and V collagens, gelatin, laminin and fibronectin were incubated with either collagenase or gelatinase, or both and the products examined by SDS/polyacrylamide-gel electrophoresis as described previously (Murphy *et al.*, 1980, 1981b).

Degradation of insoluble crude-tissue collagens. Bovine articular and nasal cartilage and anterior lens capsule were dissected, washed with 0.9% NaCl/0.02% NaN_3 , followed by distilled water, blotted, and frozen in liquid N_2 . The frozen tissues were pulverized in a Spex mill and immediately freeze-dried.

Either leucocyte collagenase (2.5–10 units) or gelatinase (75–300 units), or both, were added to 2mg of tissue powder in a final volume of 1ml, including 50mM-Tris/HCl, pH 8.0, containing 200mM-NaCl, 5mM- CaCl_2 , 0.3mM-4-aminophenyl-mercuric acetate and 0.02% NaN_3 . 1,10-Phenanthroline (2mM) was added to parallel incubations to check that the enzyme activities involved were metal-ion-dependent. Further control incubations consisted of: (a) buffer alone; (b) 20 μg of trypsin to determine the amount of material susceptible to non-specific proteolytic activity; and (c) 50 μg of bacterial collagenase to determine the total solubilizable hydroxyproline content of the powdered tissues. Incubations were carried out for 48h at 37°C in a revolving roller rack. At the end of this period, undigested tissue powder was sedimented at 10000g for 10min in a micro-haematocrit centrifuge (Gelman-Hawksley). The supernatant was removed and the pellet solubilized by incubation at

65°C with papain [1mg/ml in KH_2PO_4 (88mM)/ Na_2HPO_4 (12mM) buffer, pH 6.0, containing 1.33mM-EDTA, 0.02% NaN_3 and 2mM-cysteine; 1ml]. In some cases, 30–50 μl samples of the supernatant were analysed by SDS/polyacrylamide-gel electrophoresis.

Determinations of hydroxyproline solubilized from insoluble collagen. The hydroxyproline content of the digested pellets and supernatants was determined by the dimethylaminobenzaldehyde method (Burleigh *et al.*, 1974). In each case the amount of collagen solubilized was calculated as the percentage of hydroxyproline measured in the supernatant fraction relative to the total hydroxyproline content of the supernatant and pellet. The approximate collagen content (calculated from the hydroxyproline content) relative to total dry weight of the substrates used were: bovine nasal cartilage, 55%; articular cartilage, 60%; tendon, 70%; lens capsule, 85%.

Degradation of other substrates. Proteoglycan-bead degradation was assayed by the method of Nagase & Woessner (1980) and plasminogen-activator and fibrin-degrading activity by the method of Christman *et al.* (1977). The synthetic substrate 2,4-dinitrophenyl-L-Pro-L-Leu-L-Gly-L-Ile-L-Ala-L-Gly-L-Arg-amide was assayed as described by Masui *et al.* (1977) as modified by Woessner (1979).

Results

Enzyme purification

The purification of metalloproteinases from human polymorphonuclear neutrophil leucocytes is technically difficult for two main reasons: (a) the metalloproteinase contents are relatively low; (b) both enzymes are degraded by the serine proteinases, which are present in large amounts in the azurophil granules (Murphy *et al.*, 1980) and are usually a minor contaminant of granule fractions (Dewald *et al.*, 1975). The problems were minimized by isolating specific granules and C-particles (Murphy *et al.*, 1980) and treating the subsequent extracts with di-isopropyl phosphorofluoridate in order to block serine proteinases.

Collagenase and gelatinase activities were then separated by Ultrogel AcA 34 chromatography, as in our former study (Murphy *et al.*, 1980), and then purified separately by the procedures outlined in the Materials and methods section. Purification was monitored by assaying for protein content and for enzymic activities against native collagen, gelatin and casein.

Purification of collagenase

In preliminary experiments, collagenase was found to become inactivated on adsorption to many column matrices. A purification procedure was

therefore selected that avoided the binding of the enzyme while ensuring maximum retention of other proteins. A typical purification protocol is given in Table 1. The specific activity of the final preparation was 260 units/mg in the fibril assay and 500 units/mg in the diffuse-fibril assay of Cawston & Barrett (1979). The collagenase/caseinase activity ratio did not change appreciably during purification. Gel filtration of the final preparation yielded a single peak of collagenase, with constant ratios of collagenase/gelatinase and collagenase/caseinase activity across the peak (results not shown). Although the starting material for the procedure contained mainly latent collagenase, there was a progressive activation during the course of purification (Table 1) and on subsequent storage. The preparation was only 20% latent 7 days after the final purification step.

Purification of gelatinase

The peak of gelatinase activity eluted from Ultrogel AcA 34 was purified by using chromatographic steps similar to those used for collagenase (Table 2), with the omission of heparin-Sepharose chromatography. Re-chromatography of the purified gelatinase on Ultrogel AcA 34 gave a single peak of gelatinase activity, with constant ratios of gelatinase/collagenase and gelatinase/caseinase activities (results not shown). After radio-

labelling with ^{125}I , the A_{280} peaks and the ^{125}I -labelled protein present corresponded with enzyme activity (see below and Fig. 1a). Analysis of the enzyme, with and without ^{125}I -labelling, by electrophoresis at alkaline pH, was carried out. The enzyme migrated as a very diffuse band, although ^{125}I radioactivity, gelatin-degrading activity and Coomassie Blue staining corresponded to each other (result not shown). The enzyme that could be eluted from the gel gave the same pattern on subsequent SDS/polyacrylamide-gel electrophoresis as that given by the starting material. The behaviour of ^{125}I -labelled gelatinase on SDS/polyacrylamide gels is described in detail below.

Gelatinase latency

Because the latent leucocyte gelatinase is a more stable species than other latent mammalian metallo-proteinases, it was possible to purify the enzyme in a completely latent form and to study the activation process in detail. Activation of the purified latent gelatinase eluted from zinc chelate-Sepharose by treatment with either trypsin ($5\text{ }\mu\text{g/ml}$, 37°C , 15 min) or 4-aminophenylmercuric acetate (0.1 mM , 37°C , 2 h) showed that it was more readily activated than the crude latent enzyme (Murphy *et al.*, 1980).

It has been proposed (Murphy & Sellers, 1980) that collagenase and other mammalian metallo-proteinases are latent as a consequence of their

Table 1. *Purification of collagenase from human polymorphonuclear-leucocyte granule extract*

The individual stages of the purification were performed as described in the Materials and methods section, and collagenase activity was monitored by using the collagen-fibril assay. Abbreviation used: n.d., not determined.

Purification stage	Collagenase:				Activity ratio	
	Total activity (units)	Specific activity (units/mg)	Overall recovery (%)	Latency (%)	Collagenase	Collagenase
					Gelatinase	Caseinase
Initial extract	3336	8.8	—	87	0.056	1.5
Ultrogel AcA 34 eluate	1349	43	40	82	0.15	2.2
DEAE-cellulose eluate	1109	n.d.	33	n.d.	14.4	1.8
Heparin-Sepharose eluate	1288	54	39	79	13.1	3.2
Zinc chelate-Sepharose eluate	1191	260	36	63	11.3	2.5

Table 2. *Purification of gelatinase from human polymorphonuclear-leucocyte granule extract*

The individual stages of the purification were performed as described in the Materials and methods section.

Purification stage	Gelatinase activity		Overall recovery (%)	Activity ratio	
	Total (units)	Specific (units/mg)		Gelatinase	Gelatinase
				Collagenase	Caseinase
Initial extract	59 318	156	—	18	27
Ultrogel AcA 34 eluate	50067	5215	84	30	40
DEAE-cellulose eluate	49801	19 920	84	1757	55
Zinc chelate-Sepharose eluate	21 896	13 033	62	1663	49

Fig. 1. Gel-filtration analysis of the activation of ^{125}I -labelled latent gelatinase

Purified latent gelatinase ($10\mu\text{g}$) was ^{125}I -labelled with the Bolton and Hunter reagent and chromatographed on Ultrogel AcA 34. The peak of latent enzyme activity (M_r 250 000) and radioactivity was pooled and portions (2ml) were rechromatographed on the same gel: (a) without further treatment; (b) after treatment with 4-aminophenylmercuric acetate; (c) after treatment with trypsin (see under 'Methods' for details). Portions of the eluate ($100\mu\text{l}$) were counted for radioactivity, and the data are plotted as c.p.m. (●). The radioactivity values for the low-molecular-weight fractions of (b) and (c) are also plotted on a larger scale (■).

forming a complex with an inhibitor. The purified latent gelatinase was therefore used to investigate whether an inhibitor or 'peptide' were released during the process of enzymic or chemical activation. Since only small amounts of purified gelatinase were available, the present study was facilitated by ^{125}I -labelling the enzyme. This treatment did not affect either the latency of the enzyme, the subsequent activation procedure, or the enzymic activity. Gel filtration of ^{125}I -labelled latent gelatinase on Ultrogel AcA 34 gave a single peak of radioactivity (Fig. 1a) of M_r 250 000, which coincided with a peak of latent enzyme, as assayed after activation with 4-aminophenylmercuric acetate (result not shown). When a similar amount of labelled active enzyme (activated by a 2 h incubation with 4-aminophenylmercuric acetate), was subjected to gel filtration, most of the radioactivity was again recovered in a single peak that was slightly retarded with respect to the latent enzyme and corresponded to an M_r of 225 000 (Fig. 1b). This peak coincided with the peak of gelatinase activity and thus corresponded to the activated enzyme. A small proportion of the radioactivity (4% of that of the original sample) was recovered in the region of the inclusion volume where peptide material would be expected. This material was concentrated with an Amicon UM2 filter (cut-off M_r : 1000) in a Diaflo ultrafiltration apparatus, and the retained fraction, containing about 50% of the radioactivity, was chromatographed on a Sephadex G-50 column. A peak of radioactivity at the column V_0 and a peak at the column V_t were obtained (Fig. 2). The latter peak corresponded to free iodide and the former to any peptide material that might have been generated. The V_0 peak of radioactivity was pooled and counted and found to correspond to 0.3–0.5% of the original radioactivity applied to the column. However, if the activated gelatinase peak from the column was rechromatographed, a comparable peak of 'low-molecular-weight' radioactivity was again obtained, presumably because of autolysis. On concentration and chromatography on Sephadex G-50, a similar peak of radioactivity eluted at the V_0 of the column, corresponding to 0.4% of the original enzyme radioactivity. Gel-filtration analysis of the trypsin-activated ^{125}I -labelled latent gelatinase yielded a major peak of radioactivity of M_r 225 000, corresponding to the active enzyme and a peak of lower molecular weight accounting for 12% of the sample radioactivity (Fig. 1c). Upon concentration of the material under the latter peak by ultrafiltration as described above and chromatography of the concentrate (8.7% of total radioactivity) on Sephadex G-50, two peaks of radioactivity were obtained, comparable with those obtained by 4-aminophenylmercuric acetate activation. Most of this radioactivity (93% of the

Fig. 2. Sephadex G-50 chromatography of ^{125}I -labelled low-molecular-weight material generated by the activation of latent gelatinase by 4-aminophenylmercuric acetate

The low-molecular-weight fractions from Ultrogel AcA 34 chromatography of 4-aminophenylmercuric acetate-activated labelled latent gelatinase (Fig. 1b) were concentrated and a portion (2 ml) chromatographed on Sephadex G-50. Portions (100 μl) of each fraction were counted for radioactivity ($\bullet$). The shaded area ($\blacksquare$) indicates the fractions pooled for assessment of the total radioactivity content of the putative peptide material produced by activation of the latent enzyme.

concentrate) was in the low-molecular-weight region, indicating that no material of M_r greater than 2000 was present. It is possible that the peptide(s) that are dissociated from the latent enzyme by trypsin may be broken down further. Interestingly, however, under these conditions trypsin does not seem to attack the active gelatinase.

Analysis of the activation of latent gelatinase by SDS/polyacrylamide-gel electrophoresis was complicated by the partial dissociation of the enzyme in the presence of SDS and further dissociation under reducing conditions (Fig. 3). SDS-treated non-reduced latent gelatinase electrophoresed as two bands, a major band (M_r about 150 000) and a minor band (M_r 110 000), both of which underwent a decrease in M_r of about 10 000 upon activation of the enzyme with 4-aminophenylmercuric acetate before electrophoresis. Reduction of enzyme samples with 20 mM-2-mercaptoethanol before electrophoresis resulted in the smaller of these two bands being detected in small amounts ('latent' M_r about 110 000; 'active' M_r about 100 000) and a major band of M_r 25 000. Polyacrylamide gels up to 15% (w/v) were used in the present study, but no differences in the low- M_r bands of latent and active gelatinase could be observed. It is known that some metalloproteinases behave anomalously upon SDS/

Fig. 3. Autoradiograph of SDS/polyacrylamide-gel electrophoretic pattern of ^{125}I -labelled gelatinase, before and after activation

Purified labelled gelatinase (400 ng) was electrophoresed on an SDS/10%-polyacrylamide gel: (a) in the presence of 2-mercaptoethanol (20 mM) and SDS; (b) after activation with 4-aminophenylmercuric acetate in the presence of 2-mercaptoethanol and SDS; (c) in the presence of SDS alone; (d) after activation with 4-aminophenylmercuric acetate and in the presence of SDS alone. The values on the right-hand side are the molecular weights of standards: myosin, M_r 200 000; α_2 -macroglobulin, 180 000; phosphorylase α , 100 000; transferrin, 78 000; bovine serum albumin, 68 000; ovalbumin, 45 000; carbonic anhydrase, 29 000; soya-bean trypsin inhibitor, 22 000.

polyacrylamide-gel electrophoresis (Stricklin *et al.*, 1978; Cawston *et al.*, 1981a). These results were therefore checked by analysis of non-reduced and reduced, latent and active enzyme in the presence and absence of SDS by using filtration on Ultrogel AcA 34. This technique is far less sensitive for the study of macromolecules of similar M_r and was only used as a qualitative analysis of events. In the absence of SDS, as had already been shown, the latent and active species were eluted as single peaks. Reduction with 20 mM-2-mercaptoethanol before chromatography yielded 30% of the radioactivity eluting at about 150 000 M_r (with enzyme activity)

and 70% at an M_r of about 22 000 (inactive). No detectable differences in the elution on patterns of latent and active enzyme were observed under these conditions. Carboxymethylation also had no effect on the distribution of radioactivity between the two peaks. In the presence of SDS and under non-reducing conditions, both latent and activated forms eluted as one major peak of radioactivity of M_r about 110 000. Reduction had a similar effect on the samples as that described in the absence of SDS, with the major band eluting at M_r 26 000.

Inhibitors of collagenase and gelatinase

Both enzymes were inhibited by the chelating agents 1,10-phenanthroline and EDTA, and by thiol compounds such as cysteine, dithiothreitol and

penicillamine, which are known to interfere with the action of metalloproteinases. In contrast, serine-proteinase inhibitors such as di-isopropyl phosphorofluoridate, α_1 -proteinase inhibitor and soya-bean trypsin inhibitor had no effect on the enzymic activities degrading either collagen, gelatin or casein as substrates (results not shown).

A number of naturally occurring inhibitors known to inhibit metalloproteinases were also investigated.

(i) α_2 -Macroglobulin. Neutrophil collagenase was slowly inhibited by human α_2 -macroglobulin (Fig. 4a), 100% inhibition only being attained at very high levels of inhibitor (Fig. 4b). Gelatinase inhibition by α_2 -macroglobulin required lower levels of inhibitor, but was also slow (results not shown).

(ii) *TIMP and amniotic-fluid inhibitor*. Collagenase was inhibited by purified preparations of TIMP from bone culture media, but total inhibition was difficult to obtain (Fig. 5). The enzyme was similarly inhibited by the purified metalloproteinase inhibitor from human amniotic fluid (Fig. 5). The activity of both inhibitors against collagenase was not modified by the inclusion of 0.3 mM 4-aminophenylmercuric acetate in the assay.

Gelatinase inhibition by these two metalloproteinase inhibitors was slow. A maximum of 90% inhibition of 35 ng of gelatinase was obtained after 1 h preincubation with either 40 ng of TIMP or 140 ng of amniotic-fluid inhibitor. Subsequent treatment of enzyme-inhibitor complexes with either 4-aminophenylmercuric acetate (0.3 mM, 37°C, 2 h) or trypsin (100 μ g/ml, 37°C, 30 min) treatment also had no effect. These inhibitors did not form complexes with gelatinase that were stable to gel

Fig. 4. Inhibition of leucocyte collagenase by α_2 -macroglobulin

(a) Collagenase (960 ng; 0.25 unit) was preincubated with 165 μ g of α_2 -macroglobulin (○) and 55 μ g of α_2 -macroglobulin (●) for various times at 37°C before assaying for residual activity for 1 h in a diffuse collagen-fibril assay. (b) Collagenase (770 ng; 0.2 unit) was assayed in the presence of various amounts of α_2 -macroglobulin for 20 h in a collagen-fibril assay.

Fig. 5. Inhibition of leucocyte collagenase by TIMP and human amniotic-fluid inhibitor

Collagenase (1.2 μ g; 0.3 unit) was assayed in the presence of various amounts of TIMP (2 μ g/ml; 50 unit/ml) (○) and human amniotic-fluid inhibitor (27 μ g/ml; 190 unit/ml) (●) for 24 h in the fibril assay.

filtration or SDS/polyacrylamide-gel electrophoresis.

Degradation of soluble collagens

Leucocyte collagenase degraded soluble native types I, II and III collagen (Fig. 6) at 25°C, when the typical 'three-quarter' products were obtained, indicating a single cleavage of the constituent α -chains in each case. Quantitative analysis of the relative rates of degradation of the different collagen types were not carried out, but the gel patterns indicated that the relative rates were $I = III > II$. At 37°C, however, further degradation of the respective chains occurred, due to the intrinsic gelatinolytic activity of the collagenase (results not shown). The leucocyte gelatinase had little detectable activity on these collagens at either 25 (Fig. 6) or 37°C, but the two enzymes together were more effective than collagenase alone, particularly at 37°C. In each case electrophoretic analysis of the reaction products at 37°C merely showed disappearance of the collagen α -chains and the accumulation of low-molecular-weight material at the electrophoretic front. The synergistic action of collagenase and gelatinase on soluble type I collagen at 25°C was reported previously (Murphy

et al., 1980), but in the collagen-fibril assay the activity of collagenase was not appreciably enhanced by the addition of gelatinase. Both collagenase and gelatinase degraded type I gelatin in a similar fashion (Fig. 7).

The activity of the leucocyte metalloproteinases towards soluble type IV collagen purified from bovine anterior lens capsules (see under 'Methods') was studied. This material was found to be substantially degraded by trypsin at 37°C (Sage *et al.*, 1979), whether extracted once with low levels of pepsin or after reduction, alkylation and a second pepsin extraction. At temperatures below 30°C, the collagen was less sensitive to trypsin. High levels of leucocyte collagenase degraded the type IV collagen under conditions similar to those used for trypsin at 25°C (Fig. 8). Gelatinase was more active than collagenase, degrading the type IV bands of M_r 150 000 and 100 000 (Fig. 8).

Type V collagen from human chorioamniotic membrane and the $1\alpha, 2\alpha, 3\alpha$ collagen from human neonatal cartilage were also sensitive to trypsin at

Fig. 6. Degradation of soluble type I, II and III collagens by leucocyte collagenase and gelatinase at 25°C

Native soluble collagen type I (a), type II (b) or type III (c) (25–30 μ g) were incubated for 20 h in a total volume of 50 μ l with: 1, collagenase (60 ng; 0.016 unit); 2, gelatinase (110 ng; 1.5 unit); 3, collagenase and gelatinase (as 1 and 2) together; 4, as for 3, but including 2 mM-1,10-phenanthroline; 5, no additions, and analysed by electrophoresis on an SDS/6.5%-polyacrylamide gel. α , β and γ indicate the collagen chains and α_A , β_A and γ_A the three-quarter-size digestion products.

Fig. 7. Degradation of gelatin (from type I collagen) by leucocyte collagenase and gelatinase at 37°C

Gelatin (100 μ g) was incubated for 1 h with: 1, no additions; 2, collagenase (190 ng; 0.05 unit); 3, gelatinase (8 ng; 0.1 unit); 4, gelatinase (12 ng; 0.15 unit), in a final volume of 200 μ l. Portions (30 μ l) were analysed by electrophoresis on an SDS/6.5%-polyacrylamide gel.

Fig. 8. Degradation of soluble type IV collagen by leucocyte collagenase and gelatinase at 25°C

Soluble type IV collagen (36 µg), prepared by pepsin extraction of bovine anterior lens capsule, was incubated for 20 h in a total volume of 30 µl with: 1, no additions; 2, gelatinase (120 ng; 1.6 unit) and 2 mM-1,10-phenanthroline; 3, gelatinase (20 ng; 0.3 unit); 4, gelatinase (60 ng; 0.8 unit); 5, gelatinase (120 ng); 6, gelatinase (240 ng); 7, gelatinase (600 ng); 8, collagenase (100 ng; 0.04 unit). Electrophoresis of the whole incubation mixture was carried out on an SDS/5.5%-polyacrylamide gel under reducing conditions. The migration positions of type I collagen α , β - and γ -components (approx. M_r 100 000, 200 000 and 300 000 respectively) and the three-quarter-size fragments generated by mammalian collagenase digestion (α_A , β_A and γ_A) are shown.

Fig. 9. Degradation of soluble type V and 1 α ,2 α ,3 α collagens by leucocyte collagenase and gelatinase

Incubation was for 20 h in a total volume of 30 µl before electrophoresis on an SDS/7.5%-polyacrylamide gel. (a) Type V collagen (18 µg); (b) 1 α ,2 α ,3 α collagen (12 µg), with: (1) no additions, (2) collagenase (190 ng; 0.05 unit), and (3) gelatinase (10 ng; 1.5 unit).

Degradation of insoluble collagens

The action of the leucocyte enzymes on insoluble collagens was estimated by testing their ability to solubilize hydroxyproline-containing material from powdered-tissue preparations of insoluble collagen. Bovine tissues were used, including tendon (predominantly type I collagen), nasal and articular cartilage (type II collagen) and anterior lens capsule (type IV collagen). Apart from lens capsule these preparations were shown to be relatively trypsin-resistant (Table 3). Collagenase solubilized the collagen of the tissue powders, but it was notable that 30–100 times more enzyme protein was required than in the soluble type I collagen assays (Table 3a). Solubilization of hydroxyproline was found to approximate to linearity with respect to enzyme concentration, but studies were limited by the small amounts of enzyme available. Gelatinase alone had little activity on the tissue collagens. However, when the two leucocyte enzymes were incubated together, the solubilization of hydroxyproline was potentiated by a factor of 2–3 with respect to the sum of their single activities. This effect was not linear with respect to the amount of gelatinase used, but tended to relate to the amount of

37°C. Leucocyte collagenase was active towards the collagens under these conditions. Below 30°C, the collagens were totally resistant to both trypsin and leucocyte collagenase activity. Gelatinase, however, was able to degrade them at lower temperatures, yielding specific degradation products (Fig. 9). The degradation of all soluble collagens by the leucocyte enzymes was inhibited by 2 mM-1,10-phenanthroline.

Table 3. *Solubilization of collagens from bovine tissues*

(a) Comparison of the activity of leucocyte and rabbit bone collagenases. The activities of purified collagenases on insoluble collagens, in comparison with reconstituted collagen fibrils (rat skin type I) are given as $\mu\text{g}/\text{min}$ per mg of enzyme, using the assay conditions given in the Materials and methods section. (b) The action of leucocyte collagenase and gelatinase alone and together is expressed as the percentage of the total hydroxyproline content released for each tissue preparation. Incubated controls and tissues incubated with trypsin are also given.

Enzyme	Substrate ...	Activity ($\mu\text{g}/\text{min}$ per mg of enzyme)				
		Reconstituted collagen fibrils (type I)	Tendon (type I)	Nasal cartilage (type II)	Articular cartilage (type II)	Anterior lens capsule (type IV)
(a)						
Leucocyte collagenase		260	4.2	8.7	3.5	6.4
Rabbit bone collagenase		28 000	250	—	93	50
(b)			Hydroxyproline content released (%)			
Leucocyte collagenase			16	24	15	44
Leucocyte gelatinase			4	4	2.5	16
Collagenase and gelatinase together			54	48	37	77
Blank			0.7	1	0.7	1
Trypsin			9	9	5	86

collagenase present, reaching a maximum at very low concentrations of gelatinase. This quantitative aspect could not be investigated in detail, nor could the mechanistic reason for the potentiation, owing to the small amounts of enzymes available. Trypsin did not have the same effect as gelatinase if added to incubations after collagenase digestion of tendon. Analysis of the products from solubilization by polyacrylamide-gel electrophoresis showed that fragments of M_r less than 25 000 were liberated in all cases. It was also noted that leucocyte collagenase preparations were less active than tissue collagenase in the solubilization of insoluble collagen (Table 3).

Degradation of other substrates

Neither collagenase nor gelatinase had significant degradative activity on either purified bovine cartilage proteoglycan aggregate (by either viscometric assay or the polyacrylamide bead-assay), rabbit plasma fibronectin, laminin or ^{125}I -labelled fibrinogen. Both enzymes were active towards 2,4-dinitrophenyl-L-Pro-L-Leu-L-Gly-L-Ile-L-Ala-L-Gly-L-Arg-amide, the low- M_r collagenase substrate. The purified preparation of gelatinase degraded 145 nmol/min per mg and collagenase degraded 973 nmol/min per mg (1100 mol of peptide/mol of collagen-degrading activity). Purified rabbit bone collagenase was more active, degrading 1700 nmol of peptide/min per mg (19 mol of peptide degraded/mol of collagen-degrading activity).

Discussion

Neutrophil collagenase was found to be relatively labile and it was not possible to achieve a very high degree of purification without unacceptably large

losses in activity. Its final specific activity of 500 units/mg (diffuse-fibril assay) is far below the values obtained in this laboratory for other mammalian collagenase [i.e. 53 000 units/mg for that of pig synovium (Cawston & Tyler, 1979) and 28 000 units/mg for that of rabbit bone (W. A. Galloway and J. J. Reynolds, unpublished work)]. In contrast, the gelatinase was obtained as a homogeneous preparation with a maximum specific activity of approx. 20 000 units/mg. Both metalloproteinases have minor activities against each other's major substrate and against casein. Pig synovial and rabbit bone collagenases are also known to have low, but significant, gelatin- and casein-degrading activities (Cawston & Tyler, 1979; T. E. Cawston & W. A. Galloway, unpublished work).

Previous studies of the latent forms of tissue-derived mammalian collagenases have shown that activation can be achieved by either limited proteolysis or organo-mercurial treatment, with a fall in apparent M_r of 10 000–20 000 in either case (Eeckhout & Vaes, 1977; Murphy *et al.*, 1977; Vater *et al.*, 1978). There has been considerable controversy as to the nature of the latent forms of the metalloproteinases: whereas we proposed that latent tissue metalloproteinases may be complexes of enzyme with the specific inhibitor, TIMP, others hold the view that they are proenzymes and that activation is the result of peptide cleavage (for a review, see Murphy & Sellers, 1980). It seemed possible that latent leucocyte metalloproteinases might be analogous to tissue-derived enzymes and be enzyme-inhibitor complexes, although no evidence for a specific inhibitor could be found in leucocytes

(Murphy *et al.*, 1980) and TIMP (the tissue-derived inhibitor) has poor activity against the leucocyte metalloproteinases, as demonstrated in the present paper. Since gelatinase could be purified in a fully latent form, it was possible to study the activation process in detail. A further advantage was the fact that ^{125}I labelling by acylation did not result in changes in enzyme activity or latency.

Treatment of labelled gelatinase with either a mercurial or trypsin resulted in the release of an active enzyme species with a 10% lower molecular weight. The active enzyme peak accounted for most of the radioactivity. However, the recovery of radioactivity corresponding to peptides in the molecular-weight range 10000–20000 was negligible. Since the distribution of the label in the protein is unknown, it could be argued that the 'peptide' released remained nearly unlabelled when the latent enzyme was acylated. The size of such a 'peptide' makes this possibility unlikely. These studies did indicate that gelatinase is probably an octomer associated by disulphide bonds as well as by non-covalent interactions. Variations in banding patterns and M_r values on electrophoresis or gel filtration under different conditions may be attributed to incomplete dissociation of the polymer. Although no labelled low-molecular-weight peptides were found after activation by 4-aminophenylmercuric acetate, an effect of this reagent on the size of the polymerized components could be demonstrated. Our present interpretation is that a marked conformational change must occur upon activation, within two groups each of four subunits. Consequently latent leucocyte gelatinase may be neither an enzyme-inhibitor complex nor a proenzyme. Whether these results can shed light on the nature of tissue metalloproteinases remains to be investigated. The latent gelatinase of human leucocytes has also been investigated by Sopata & Danciewicz (1974) and Sopata & Wize (1979). The results of these workers are broadly in agreement with the results in the present paper, including data from chemical-activation methods resulting in a decrease in molecular weight. These workers also proposed that latent gelatinase may be an enzyme-inhibitor complex, but were unable to isolate any inhibitor moiety after activation.

Nagai *et al.* (1975) reported that latent leucocyte gelatinase was activated by overnight dialysis against 3 M-sodium thiocyanate followed by dialysis against thiocyanate-free buffer. In preliminary experiments involving gel filtration of gelatinase which has been activated by either 4-aminophenylmercuric acetate, trypsin or sodium thiocyanate, we failed to detect material which proved inhibitory for the active enzyme.

In view of a possible similarity of the human neutrophil metalloproteinases with other mammalian

enzymes of this class, we decided to investigate the influence of natural inhibitors. The slow kinetics of inhibition by α_2 -macroglobulin, which was observed for both collagenase and gelatinase, is in agreement with previous observations by Werb *et al.* (1974). That TIMP and the human amniotic-fluid inhibitor only showed low activities supports the concept that the two neutrophil metalloproteinases differ very markedly from those found in mammalian connective-tissue cultures. These conclusions are in keeping with the data of Woolley *et al.* (1976), who showed that serum β_1 -anticollagenase inhibited tissue collagenase more readily than the collagenase of human neutrophils.

The main purpose of the present work was to separate the two neutrophil metalloproteinases completely, in order to study their actions on matrix components of connective tissues and gain insight into their possible pathophysiological role. The solubilized interstitial collagens are degraded by leucocyte collagenase at similar rates, in the manner of mammalian collagenases (Gross *et al.*, 1980). Horwitz *et al.* (1977) showed that leucocyte collagenase readily degraded soluble type I collagen, but their enzyme preparation had little activity towards soluble type III collagen. We have used type III collagen from various sources and always found it to be efficiently degraded by leucocyte collagenase. This would be expected, since the 'weak' cleavage point in the helix is even susceptible to trypsin (Miller *et al.*, 1976; Gross *et al.*, 1980). Woolley *et al.* (1975) showed that human rheumatoid synovial collagenase was less able to degrade soluble type II than type I collagen; leucocyte collagenase is apparently similar.

Leucocyte gelatinase has negligible activity on the intact native chains of interstitial collagens, but once they have been cleaved by collagenase, sufficient modification of their helical structure occurs to permit gelatinase to act. This action can even be observed, albeit at a lower rate, at 25°C. Thus the presence of gelatinase accelerates the action of collagenase. This was particularly noticeable in the study of collagen solubilized from tissue powders, where increased amounts of hydroxyproline were released from these substrates when both enzymes were used together. Collagenase had a low activity alone (although at least an order of magnitude less than its activity on reconstituted fibrils), but gelatinase alone had negligible activity. The vast difference in activity of collagenases on soluble collagens as compared with more-highly-cross-linked collagens has been noted previously for tissue collagenases (Harris & Farrell, 1972; Vater *et al.*, 1979). Leucocyte collagenase appears to be rather less active than tissue collagenase towards all forms of collagen (Table 3), even allowing for the fact that the former is not fully purified.

The solubilized IV and V collagens were only degraded by leucocyte collagenase under conditions where they were susceptible to trypsin, as described by others for the action of human fibroblast collagenase on type IV collagen (Sage *et al.*, 1979) and type V collagen (Sage *et al.*, 1981). It was noteworthy that gelatinase was active in degrading soluble types IV, V and 1 α ,2 α ,3 α (cartilage A,B,C-like) collagens; its action on the 1 α ,2 α ,3 α collagens was particularly marked at 25°C, yielding a limited cleavage product of slightly lower molecular size (Fig. 9), in a manner analogous to the action of collagenase on the interstitial collagens (Fig. 6). A similar activity has been observed by using gelatinase-containing fractions from gel-filtration chromatography of rabbit bone medium (Murphy *et al.*, 1981b). Uitto *et al.* (1980) have described a metal-dependent activity from crude human leucocytes that degrades cow lens basement-membrane collagen. Since their activity is partially sensitive to serine-blocking reagents, it is likely that the products described were generated by the action of leucocyte elastase (Mainardi *et al.*, 1980) as well as gelatinase. The only noticeable similarity between the pattern of products described by these workers and our own results is the disappearance of the 140 000- M_r band as analysed on SDS/polyacrylamide gels.

Neither leucocyte collagenase nor gelatinase had any effect on other connective components such as proteoglycan, fibronectin or laminin. Their precise role in the specific degradation of collagens, relative to the leucocyte serine proteinases, remains to be established. By using the data for collagen and gelatin breakdown by granule lysates (Murphy *et al.*, 1980) and assuming (a) that collagenase and gelatinase are not inactivated during purification and (b) that the contribution of each enzyme to the breakdown of the preferred substrate of the other is negligible, we have calculated that 10⁹ neutrophils contain approx. 0.2 mg of collagenase and 0.1 mg of gelatinase, in contrast with 4 mg of elastase and of cathepsin G (Barrett, 1978). Particularly noteworthy is the fact that these metalloproteinases exist in the leucocyte granules in a latent form. The nature of this latency and physiological mechanisms for their activation are still not understood. It is hoped that future studies will be oriented towards the elucidation of these problems.

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