The latent collagenase and gelatinase of human polymorphonuclear neutrophil leucocytes

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Two metallo-proteinases of human neutrophil leucocytes, collagenase and gelatinase, were studied. Collagenase specifically cleaved native collagen into the TC_A and TC_B fragments, whereas gelatinase degraded denatured collagen, i.e. gelatin, and the TC_A fragments produced by collagenase. On subcellular fractionation by zonal sedimentation, collagenase was found to be localized in the specific granules, separate from gelatinase, which was recovered in smaller subcellular organelles known as C-particles. Neither enzyme was present in the azurophil granules, which contain the two major serine proteinases of neutrophils, elastase and cathepsin G. Collagenase and gelatinase were separated by gel filtration from extracts of partially purified granules. Both enzymes were found to occur in latent forms and were activated either by trypsin or by 4-aminophenylmercuric acetate. Gelatinase was also activated by cathepsin G, which, however, destroyed collagenase. Both enzymes were destroyed by neutrophil elastase. Activation resulted in a decrease by 25000 in the apparent mol.wt. of both latent metallo-proteinases.

Human polymorphonuclear neutrophil leucocytes contain both a specific collagenase (Lazarus et al., 1972; Kruze & Wojtecka, 1972; Oronsky et al., 1973; Murphy et al., 1977) and a gelatinase (Sopata & Dancewicz, 1974). These enzymes act specifically on collagen and on its denatured form, gelatin, respectively. Maximal activities are obtained at pH values near neutrality and are dependent on the presence of bivalent cations. Both enzymes occur in latent forms, since their activities are enhanced by treatment with either trypsin or 4-chloromercuribenzoate. Collagenase of human neutrophils is localized in the specific granules, separate from the serine proteinases, which are stored in the azurophil granules (Murphy et al., 1977).

We have studied the possible role of these metallo-proteinases in leucocyte function by assessing their subcellular localization, their activities on collagen and gelatin, the extent and nature of their latency and the ability of neutrophil serine proteinases to function as activators. Preliminary observations suggested that the cytosol of neutrophils contains inhibitors of the collagenolytic activity of granule extracts (Kopitar & Lebez, 1975);

Abbreviations used: Dip-F, di-isopropyl phosphorofluoridate; SDS, sodium dodecyl sulphate.

consequently the capacity of the cytosol to inhibit the two metallo-proteinases was assessed.

Materials and methods

The materials used have been described previously (Bretz & Baggiozini, 1974; Sellers et al., 1977). Purified human neutrophil elastase and cathepsin G were gifts from Dr. J. Saklatvala and Dr. A. Barrett, Strangeways Laboratory. These enzymes were purified to homogeneity by a modification of the method of Baugh & Travis (1976).

Methods

If not otherwise stated all procedures were carried out at 4°C.

Preparation and fractionation of neutrophils from human blood. Neutrophils were isolated from pooled buffy coats of donor blood by dextran sedimentation at room temperature, followed by hypo-osmotic lysis of the remaining erythrocytes (Bretz & Baggiozini, 1974). The cells were washed three times by centrifugation at 3300g-min (r威尼斯, 27.5 cm) and resuspension in saline (0.9% NaCl). The purified neutrophils (90–95% pure, 1 × 10⁹–3 × 10⁹ cells/ml, in saline) were compressed to 2MPa (20 bar) of
nitrogen by using a Parr Cell Destruction Bomb (Parr Instrument Co., Moline, IL, U.S.A.) and homogenized by decompression. Approx. 60% of the cells were disrupted by this procedure. A postnuclear supernatant was obtained by centrifugation of the homogenate at 10000g·min (r
, 27.5 cm). It was diluted to 1.5 vol. with 1 M-sucrose and fractionated by rate zonal sedimentation in a B-XIV rotor essentially as described by Bretz & Baggiolini (1974). Established methods (Bretz & Baggiolini, 1974) were used for the determination of protein, alkaline phosphatase, lysozyme and peroxidase in the gradient fractions.

Preparation of samples for assay of metalloenzymes. Activities were assessed either in resuspended particulate material after addition of 0.05% (v/v) Triton X-100 or in granule extracts. If not otherwise stated, the specimens were treated with 5 mM-Dip-F for 1 h at 37°C before assay. The particulate material either from postnuclear supernatants or from gradient fractions was collected after centrifugation at 3 × 10
 g·min (r
, 7.5 cm) and resuspended in saline. The respective supernatants were assayed for the presence of possible soluble inhibitors. Suspensions were freeze-dried and stored at −20°C. Pooled gradient fractions were used for separation and characterization of the two enzymes.

Granule extracts were prepared by mixing suspensions of particulate material with 1 vol. of double-concentrated extraction medium and incubating for 30 min at 4°C with five to ten passages through a 25-G-1-gauge syringe needle. Particulate material was eliminated by centrifugation at 4 × 10
 g·min (r
, 13.2 cm). Fresh extraction medium was then added and the extraction procedure repeated twice. Two media were selected, 0.2 M-sodium acetate buffer, pH 4.5, containing 10 mM-CaCl
, and 50 mM-Tris/HCl buffer, pH 7.5, containing 150 mM-NaCl, 10 mM-CaCl
 and 0.05% (v/v) Triton X-100. Collagenase was extracted equally well in both media. The medium containing Triton X-100, however, extracted gelatinase more efficiently and was therefore used routinely.

Granule fractions and extracts were freeze-dried immediately on collection and stored at −20°C. Other treatments, such as direct freezing or freezing in the presence of 10 mM-EDTA, 5 mM-mercaptoethanol or 5 mM-Dip-F, invariably led to the subsequent activation of latent collagenase. Collagenase and gelatinase activities and the degree of gelatinase latency were not affected by storage at −20°C, either with or without previous freeze-drying.

Enzyme assays. Collagenolytic activity was assayed by using reconstituted fibrils of [14C]-acetylated rat type I collagen at pH 8.0 and 35°C for 15 h (Sellers et al., 1977; Cawston & Barrett, 1979). Gelatinolytic activity was assayed by a modification of the method of Harris & Krane (1972). [14C]-Acetylated gelatin was prepared by denaturation of the above-mentioned collagen at 45°C for 20 min. Incubations were made at pH 8.0 for 1 h, unless otherwise stated, at 37°C in a final volume of 250 µl containing 100 µg of gelatin. The degradation of gelatin to give trichloroacetic acid-soluble fragments was linear with respect to enzyme concentration (Fig. 1), as well as with incubation time up to 18 h (from 10 to 60% breakdown). Confirmation of endopeptidase activity was obtained by SDS/polyacrylamide-gel electrophoresis of degradation products as described below. One unit of enzyme is defined as the amount of enzyme that degrades 1 µg of substrate/min.

pH–activity relationships. Collagenase and gelatinase activities were assessed at different pH values in 0.1 M-Tris/HCl buffer. Solutions of collagen and gelatin were prepared in unbuffered 0.2 M-NaCl. The final pH of each incubation was checked after addition of all the components.

Activation of latent enzymes. The routine enzyme-activation procedure used 300 µM-4-aminophenylmercuric acetate, either in the assay medium or before the assay for 2 h at 37°C. Activation by proteinases was assessed with freshly prepared extracts corresponding to the C-particles and specific granules from about 5 × 10
 neutrophils (0.18 unit of collagenase, 3.5 units of gelatinase) in a final volume of 50 µl. Incubation with trypsin was

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Fig. 1. Dependence of gelatin degradation on enzyme concentration

Gelatin (100 µg) was incubated at 37°C for 1 h with different amounts of Dip-F-treated neutrophil granule extract as described in the Materials and methods section. The results are expressed as percentages of the total radioactivity soluble in 15% (w/v) trichloroacetic acid.
performed at 25°C. Trypsin was then inactivated by the addition of a 4-fold excess of soya-bean trypsin inhibitor and further incubation for 10 min. Incubations with neutrophil elastase and cathepsin G were carried out at 37°C for 2 h. The enzymes were then inactivated by the addition of 5 mM-Dip-F and further incubation for 30 min. Appropriate controls of granule extracts and serine proteinases alone were treated similarly. The amounts of activatable collagenase and gelatinase remaining were assessed after subsequent treatment with 4-aminophenylmercuric acetate as described above. In addition, all samples were tested in the presence of 2 mM-1,10-phenanthroline, which is known to inhibit completely both metallo-enzymes.

**Molecular-weight estimations.** Gel filtration was performed on a calibrated column of Ultrogel AcA-34 (1.5 cm diam., 135 ml bed volume) equilibrated with 50 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-NaCl, 10 mM-CaCl₂, 0.05% (w/v) Brij 35 and 0.02% NaN₃. Flow rate was 6 ml/h and 2 ml fractions were collected. Apparent molecular weights were estimated by the method of Andrews (1964).

**Analysis of the reaction products of collagen degradation.** Enzyme preparations were incubated with 100 µg of soluble type-I collagen at 25°C for 15 h in a total volume of 200 µl as described by Murphy et al. (1977), except that glucose was omitted from the incubation mixtures. Electrophoresis was carried out in a 7%-acylamide polyacrylamide-gel slab containing 0.1% (w/v) SDS, by the method of Laemmli & Favre (1973). The separated proteins were stained with Coomassie Brilliant Blue (Fairbanks et al., 1971).

**Results**

In extracts of human neutrophil granules, collagen and gelatin were degraded at neutral pH by serine and metallo-proteinases (Table 1). When, in a fresh granule extract, the serine proteinases were blocked by Dip-F virtually no degradation was observed. Treatment of the extract with 4-aminophenylmercuric acetate uncovered the Dip-F-resistant activity, which was in turn inhibited by metal-ion-chelating agents. The fully activated metallo-proteinases accounted for one-third of the gelatinolytic and for more than half of the collagenolytic activities of the extracts (Table 1).

Subcellular fractionation was carried out with 20 times the amounts of material used by Murphy et al. (1977), so that collagenase and gelatinase could be assayed in single fractions. Fig. 2 shows the sedimentation profiles obtained in two experiments run at different centrifugal forces. Figs. 2(a), 2(b) and 2(c) show the distributions of marker enzymes, which were consistent with those obtained previously (Bretz & Baggioni, 1974). In both experiments the Dip-F-insensitive gelatinolytic and collagenolytic activities (Fig. 2d) were partially resolved from each other. Collagenase co-sedimented with the slower peak of lysozyme, which indicates the position of the specific granules, whereas gelatinase sedimented more slowly, but was recovered clearly resolved from alkaline phosphatase, in the upper half of the gradient. These results confirm our earlier findings that collagenase is a constituent of the specific granules (Murphy et al., 1977), and suggest that gelatinase belongs to the so-called C-particles (Bretz & Baggioni, 1974). Sedimentation at 10500 rev./min, which gives the best separation of the specific granules and lighter particles from the azurophil granules containing the serine proteinases (Dewald et al., 1975), was used to prepare the starting material for enzyme purification.

Both enzymes were activated fully by 4-aminophenylmercuric acetate (Fig. 3) and by phenylmercuric chloride (Table 2) and to various extents by other thiol-blocking agents (Table 2). Collagen-

<table>
<thead>
<tr>
<th>Pretreatment of granule lysate</th>
<th>Degradation of</th>
<th>Collagen</th>
<th>Gelatin</th>
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</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dip-F</td>
<td></td>
<td>21.5 (37)</td>
<td>1367 (66)</td>
</tr>
<tr>
<td>Dip-F + 4-aminophenylmercuric acetate</td>
<td></td>
<td>1.4 (2)</td>
<td>3 (&lt;1)</td>
</tr>
<tr>
<td>Dip-F + 4-aminophenylmercuric acetate + 1,10-phenanthroline</td>
<td></td>
<td>36.0 (61)</td>
<td>700 (34)</td>
</tr>
</tbody>
</table>

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Fig. 2. Subcellular distribution of collagenase and gelatinase in human neutrophils
Fractionation of postnuclear supernatants from $3 \times 10^8$–$4 \times 10^9$ cells by zonal sedimentation at 10500 rev./min (left-hand graphs) or 13500 rev./min (right-hand graphs) for 15 min. Graphs represent normalized distribution histograms as a function of the volume collected. The ordinate shows concentration in fractions relative to the concentration corresponding to uniform distribution throughout the gradient. Radial distance increases from left to right. The distributions of (a) alkaline phosphatase, (b) lysozyme and (c) peroxidase mark the sedimentation of membrane fragments, specific granules and azurophil granules respectively. The distributions of collagenase (continuous line) and gelatinase (broken line) are superimposed in the lower fields (d) to show resolution. Recoveries were 114 and 118% for gelatinase and 92 and 82% for collagenase.

Table 2. Activation of collagenase and gelatinase from human neutrophils by thiol-blocking reagents
A total granule extract pretreated with Dip-F was used as the enzyme source. Activators were added to the assays at optimal concentrations, higher concentrations usually being inhibitory. Activities are expressed relative to those in the presence of 4-aminophenylmercuric acetate. All activities were abolished by 1,10-phenanthroline (results not shown).

<table>
<thead>
<tr>
<th>Activator</th>
<th>Conc. (mM)</th>
<th>Collagenase</th>
<th>Gelatinase</th>
</tr>
</thead>
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<tr>
<td>4-Aminophenylmercuric acetate</td>
<td>0.3</td>
<td>(100)</td>
<td>(100)</td>
</tr>
<tr>
<td>Phenylmercuric chloride</td>
<td>0.5</td>
<td>108</td>
<td>102</td>
</tr>
<tr>
<td>Sodium tetrathionate</td>
<td>0.5</td>
<td>89</td>
<td>17</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>10</td>
<td>49</td>
<td>Not determined</td>
</tr>
<tr>
<td>Mercuric acetate</td>
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<td>36</td>
<td>65</td>
</tr>
<tr>
<td>Mercuric chloride</td>
<td>0.5</td>
<td>30</td>
<td>72</td>
</tr>
<tr>
<td>Lead acetate</td>
<td>0.5</td>
<td>21</td>
<td>1</td>
</tr>
</tbody>
</table>
Human neutrophil collagenase and gelatinase

![Graph](image)

Fig. 3. Activation of latent collagenase and gelatinase from human neutrophils by organomercurials and trypsin

Activation was studied in an extract of a preparation containing specific granules and lighter particles. The extract was treated with Dip-F (see the Materials and methods section) for the study of the effects of (a) 4-aminophenylmercuric acetate and (b) 4-chloromercuribenzoate. Dip-F treatment was omitted in the experiments (c) where activation by trypsin at 5μg/ml (-----) or 15μg/ml (-----) was tested. Trypsin was subsequently inactivated with soya-bean trypsin inhibitor as described in the Materials and methods section. Activities of collagenase (△) and gelatinase (■) are expressed as percentages of maximum activity obtained with 4-aminophenylmercuric acetate.

We reported previously that the collagenase of human neutrophil granules cleaves collagen into the typical three-quarter (TC_A) and one-quarter (TC_B) fragments at 25°C (Murphy et al., 1977), and that further fragmentation of these products was obtained on incubation with large amounts of specific granule extracts, and after treatment with either trypsin or 4-chloromercuribenzoate. This additional activity was also inhibited by metal chelators. The collagen breakdown products obtained with the partially purified enzymes are shown in Fig. 7. The fractions that were enriched in collagenase degraded native collagen to the three-quarter (TC_A) and one-quarter (TC_B) fragments, whereas those containing gelatinase had only negligible activity on collagen fibrils or on soluble collagen at 25°C. However, when both enzymes were present, additional collagen fragments were obtained, indicating that gelatinase degraded the TC_A fragment produced by collagenase. At 25°C the TC_A fragments showed a marked tendency to spontaneous activation (see the Materials and methods section) and was also activated by 4-chloromercuribenzoate, which, however, did not influence latent gelatinase (Fig. 3). Activation by trypsin and by the two major serine proteinases of human neutrophils, elastase and cathepsin G, was also studied. Trypsin activated gelatinase to the same extent as did 4-aminophenylmercuric acetate, but activated collagenase only partially (Fig. 3). Elastase apparently destroyed the latent enzymes, so that they could not be activated subsequently with 4-aminophenylmercuric acetate. Cathepsin G had a similar effect on collagenase, but was a good activator of latent gelatinase (Fig. 4).

Granule extracts in which collagenase and gelatinase had been activated with 4-aminophenylmercuric acetate were used to study the dependence of enzyme activity on pH. As shown in Fig. 5, both enzymes had maximum activity at about pH 8.

Gel filtration of the latent enzymes was performed on Ultrogel AcA-34 (Fig. 6). Collagenolytic and gelatinolytic activities were recovered in two single peaks, which were well resolved from each other. The average molecular weights of the latent and active forms of both enzymes obtained are given in Table 3. On activation, the mol.wts. of both enzymes decreased by about 25,000, although the decrease for gelatinase was not statistically significant. In the gel-filtration experiments, gelatinolytic activity coincided with activity against Azocoll (results not shown). Virtually no activity, however, was found against azocasein. Fractions containing the collagenolytic activity were inactive on either substrate. Furthermore, neither enzyme exhibited proteoglycan-degrading activity, as assessed by the cartilage-proteoglycan-bead assay method of Dingle et al. (1977).
are known to retain the native form of the collagen molecule, being denatured only at higher temperatures (35–37°C). Gelatinase appears to have a selective limited action on trimeric (γ), dimeric (β) and monomeric (α) TC₆ fragments, giving rise to single products, designated TC₆, in Fig. 7. In contrast, SDS/polyacrylamide-gel-electrophoretic analysis of the products of gelatinase action on fully

Fig. 4. Effects of neutrophil elastase and cathepsin G on latent collagenase and gelatinase
Granule extracts were preincubated for 2 h at 37°C with increasing amounts of (a) elastase and (b) cathepsin G and subsequently treated with Dip-F (see the Materials and methods section). Activities against collagen (△ and ▲) and gelatin (□ and ■) were determined both directly (△ and □) or after full activation with 4-aminophenylmercuric acetate (▲ and ■). Results from two separate experiments are given (broken and continuous lines).

Fig. 5. pH–activity curves of neutrophil collagenase and gelatinase
The activities of a Dip-F-treated granule extract against collagen fibrils (a) and gelatin (b) were assayed at different pH values as described in the Materials and methods section.
denatured collagen, i.e. gelatin, revealed progressive degradation of the substrate to small peptides (results not shown). All these actions were abolished in the presence of 1,10-phenanthroline.

In postnuclear supernatants, the relative activities of collagenase and gelatinase were about 25% lower (average of 12 preparations) than in purified granules, suggesting the presence of inhibitors in the cytosol. Experiments in which increasing amounts of cytosol were added to granule extracts showed no clear concentration-dependent inhibition. When the enzymes were inhibited by cytosol, they could not be re-activated by either 4-aminophenylmercuric acetate or trypsin, suggesting that the latency described above is not due to an interaction with cytosolic constituents (Sellers et al., 1977).

**Discussion**

In the present paper we describe the presence in human neutrophils of two neutral metallo-proteinases, collagenase and gelatinase. Both enzymes are latent and can be activated by organomercurial compounds or by certain proteinases. Collagenase selectively cleaves collagen into the TCA and TCb fragments, and gelatinase degrades the TCA fragments, as well as denatured collagen, i.e. gelatin. Subcellular fractionation confirmed that collagenase is localized in the specific granules (Murphy et al., 1977) and indicated that gelatinase is a constituent of lighter organelles, which we have described previously as C-particles (Bretz & Baggioiini, 1974; Baggioiini et al., 1978).

An important methodological aspect of the present study was the use of large-scale preparation of neutrophil granules by zonal sedimentation as a first step in the purification of collagenase and gelatinase. By this method, our starting material for enzyme purification was virtually free of azurophil granules, and our granule extracts were therefore free of elastase and cathepsin G, which we subsequently found to affect both the degree of
delay and the total activity of the metallo-proteinases.

Collagenase and gelatinase are likely to be released from their stores when the neutrophils either engage in phagocytosis or are subjected to other stimuli, but the mechanisms by which the released enzymes are activated is unknown. Our experiments suggest that this may occur autocatalytically (with collagenase) and through the action of other proteinases. The spontaneous activation of collagenase was first described by Dancewicz et al. (1978). Our results also show that latent collagenase is destroyed by elastase and that only cathepsin G is able to activate latent gelatinase. Plasmin was described as a possible activator of collagenase by Eeckhout & Vaes (1977) and by Welb et al. (1977). As plasminogen activator is released by stimulated neutrophils (Granelli-Piperno et al., 1977), plasmin is likely to be formed in the pericellular environment. Other activators of neutrophil collagenase were described in synovial fluid (Kruze & Wojtecka, 1972; Sopata & Dancewicz, 1974; Dancewicz et al., 1978) and in medium of cultured rheumatoid synovia (Wize et al., 1975).

In our experiments the mol.wts. of activated collagenase and gelatinase were 25000 lower than those of their latent precursors, suggesting that a macromolecular inhibitor is released in the activation process. However, our attempts to detect an inhibitor analogous to those described for tissue collagenases (Reynolds et al., 1977; Sellers et al., 1979) were unsuccessful. We did not attempt to characterize the inhibiting principle that we observed in the cytosol, but we could exclude its involvement in the latency of collagenase or gelatinase.

The role of the two metallo-enzymes in neutrophil-mediated protein degradation cannot be outlined with certainty, since the mechanism of their activation is still speculative and the extent of their possible destruction by elastase and cathepsin G is unknown. Our experiments in vitro with neutrophil granule extracts show that the relative activities of the metallo-proteinases and the serine proteinases on native and denatured collagen are of the same order of magnitude. The sites of cleavage, however, are different. The metallo-enzymes selectively cleave peptide bonds in the helical region of the molecule, whereas the serine proteinases degrade the terminal non-helical peptides only. Neutrophil collagenase is more active on soluble than on reconstituted fibrils or on insoluble tissue collagen (Lazarus et al., 1972).

Elastase and cathepsin G, by contrast, attack the telopeptides of highly cross-linked structural collagen (Starkey et al., 1977), and are also able to degrade proteoglycans. The neutrophil serine proteinases could therefore play an important role in the destruction of cartilage and other connective-tissue structures. The metallo-proteinases may be more relevant to pathophysiological events during chemotaxis and diapedesis, when they are selectively released from stimulated neutrophils (Gallin & Wright, 1978; Dewald et al., 1980).

References

Baugh, R. S. & Travis, J. (1976) Biochemistry 15, 836–841
Human neutrophil collagenase and gelatinase