Mouse macrophage elastase

Purification and characterization as a metalloproteinase

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Macrophage elastase was purified from tissue-culture medium conditioned by inflammatory mouse peritoneal macrophages. Characterized as a secreted neutral metalloproteinase, this enzyme was shown to be catalytically and immunochemically distinct from the mouse pancreatic and mouse granulocyte elastases, both of which are serine proteinases. Inhibition profiles, production of nascent *N*-terminal leucine residues and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of degraded elastin indicated that macrophage elastase is an endopeptidase, with properties of a metalloproteinase, rather than a serine proteinase. Macrophage elastase was inhibited by α_2 -macroglobulin, but not by α_1 -proteinase inhibitor. Macrophage elastase was resolved into three chromatographically distinct forms. The predominant form had mol.wt. 22000 and was purified 4100-fold. Purification of biosynthetically radiolabelled elastase indicated that this form represented <0.5% of the secreted protein of macrophages. Approx. 800% of the starting activity was recovered after purification. Evidence was obtained for an excess of an endogenous inhibitor masking more than 80% of the secreted activity.

Elastin is a connective-tissue component of a variety of tissues and organs. In addition to constituting the major part of the intimal layer of blood vessels, it provides elasticity to lung connective tissue, yellow tendon, cartilage, breast and skin. Agents that mediate destructive changes in the structural integrity of elastin will necessarily play a major role in such chronic diseases as emphysema, atherosclerosis, and arthritis. Despite its obvious importance, little is known about the turnover of fully cross-linked elastin, and very few enzymes are known to degrade it. Proteinases that can successfully use elastin as a substrate are of bacterial and mammalian origin. The bacterial proteinases, thermolysin and *Pseudomonas* elastase, are metalloproteinases (Mandl & Cohen, 1960; Morihara, 1964; Mull & Callahan, 1965; Wretling & Wadstrom, 1977). Of the mammalian proteinases, pancreatic elastase is a classic serine proteinase (Shotton, 1970; Hartley & Shotton, 1971) homo-

logous to trypsin. A second mammalian elastase can be purified from granules of polymorphonuclear leucocytes (Janoff, 1972; Schmidt & Havemann, 1974; Ohlsson & Olsson, 1974; Starkey & Barrett, 1976*a,b*). This enzyme is also a serine proteinase and is inhibitable by α_1 -proteinase inhibitor.

A third mammalian elastase is a neutral proteinase secreted by inflammatory macrophages (Werb & Gordon, 1975*a*). It is probable that these cells mediate connective-tissue degradation during chronic inflammation. Other proteolytic activities have been identified in medium conditioned by activated macrophages, including enzymes mediating the degradation of collagen (Werb & Gordon, 1975*b*) and cartilage proteoglycans (Hauser & Vaes, 1978), activation of latent collagenase (Horwitz *et al.*, 1976), and plasminogen activator (Unkeless *et al.*, 1974).

In the present paper we describe an initial purification scheme and characterization of mouse macrophage elastase as a metalloproteinase. During purification, three forms of the enzyme were resolved, and we obtained evidence that the elastase was separated from an endogenous inhibitor. This neutral proteinase also degrades other proteins, including cartilage proteoglycans, fibrin and fibrinogen.

Abbreviations used: α_2 M, α_2 -macroglobulin; dansyl-, 5-dimethylaminonaphthalene-1-sulphonyl-; iPr₂P-F, diisopropyl phosphorofluoridate; DME medium, Dulbecco's modified Eagle's medium, high-glucose formulation; DME-LH medium, DME medium containing 0.2% lactalbumin hydrolysate; SDS, sodium dodecyl sulphate; Ac(Ala)₄CH₂Cl, acetyltetra-alanylchloromethane.

Part of this work was presented in abstract form at meetings of the Federation of American Societies for Experimental Biology (Banda & Werb, 1979) and at the International Congress of Biochemistry, held in Toronto, Canada, in 1979.

Experimental procedures

Materials

DME medium (complete and methionine-free) was obtained from the Tissue Culture Facility, University of California, San Francisco, CA, U.S.A. Tissue-culture flasks (T75; Co-star), were purchased from Microbiological Associates (Los Angeles, CA, U.S.A.). Penicillin, streptomycin, foetal-calf serum and Dulbecco's phosphate-buffered saline were purchased from Grand Island Biological Co. (Grand Island, NY, U.S.A.). NH_4HCO_3 and sucrose were purchased from Mallinckrodt (Paris, KY, U.S.A.). Human α_1 -proteinase inhibitor (α_1 -antitrypsin) and thermolysin were purchased from Worthington (Freehold, NJ, U.S.A.). Tris (Trizma base), *Micrococcus lysodeikticus*, egg-white lysozyme, soya-bean trypsin inhibitor, bovine pancreatic α -chymotrypsinogen, bovine erythrocyte carbonic anhydrase, bovine serum albumin, myoglobin, horse heart cytochrome c, SDS, colchicine, $\text{iPr}_2\text{P-F}$, 1,10-phenanthroline, phenylmethanesulphonyl fluoride, EDTA, EGTA, 4-chloromercuribenzoate, *N*-ethylmaleimide, β -mercaptoethanol, dithiothreitol and mersalyl were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Elastin (<400 mesh, type E60) was purchased from Elastin Products, St. Louis, MO, U.S.A. All electrophoresis reagents (electrophoresis grade) were purchased from Bio-Rad (Richmond, CA, U.S.A.). NaB^3H_4 (242 mCi/mmol), [^{35}S]SDS (43 mCi/mmol) and [$1\text{-}^3\text{H}(\text{n})\text{I}$] $\text{Pr}_2\text{P-F}$ (1 mCi/mmol) were purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). [^{35}S]Methionine (>1200 Ci/mmol) and ^{14}C -labelled protein molecular-weight markers were purchased from Amersham Corp. (Arlington Heights, IL, U.S.A.). DEAE-Sephadex A-25 was purchased from Pharmacia (Piscataway, NJ, U.S.A.). Ultrogel Aca54 was purchased from LKB (Pleasant Hill, CA, U.S.A.). IgSorb was purchased from the Enzyme Center (Boston, MA, U.S.A.). Freund's adjuvant was purchased from Difco (Detroit, MI, U.S.A.). All dansylation reagents were purchased from Pierce (Rockford, IL, U.S.A.). $\text{Ac}(\text{Ala})_4\text{CH}_2\text{Cl}$ was a gift from Dr. J. C. Powers, Department of Chemistry, Georgia Institute of Technology, Atlanta, GA 30332, U.S.A. Purified mouse $\alpha_2\text{M}$ was a gift of Dr. R. White, State University of New York, Stony Brook, NY 11794, U.S.A. The tissue inhibitor of metalloproteinases from rabbit bone was a gift of Dr. Gillian Murphy,

Strangeways Research Laboratory, Cambridge, U.K. Phosphoramidon was obtained from the U.S.-Japan Co-operative Cancer Research Program, New York University, NY 10016, U.S.A. Mouse pancreatic-elastase activity was obtained by homogenizing pancreatic tissue in 0.1% Triton X-100. Mouse granulocyte-elastase activity was obtained by lysing, in 0.1% Triton X-100, granulocytes that were obtained 16 h after intraperitoneal injection of 0.1 ml of complete Freund's adjuvant. Rabbit fibroblast collagenase was prepared and assayed as described previously (Werb & Aggeler, 1978).

Macrophage collection

Mouse peritoneal macrophages were obtained by peritoneal lavage with phosphate-buffered saline, containing 100 units of heparin/ml, 4 days after 1.0 ml intraperitoneal injections of 3% Brewer's thioglycollate medium (Werb & Gordon, 1975a). Approx. 1.5×10^7 exudative cells were obtained from each animal. A complete enzyme purification protocol required medium conditioned by about 3×10^8 cells.

Cell culture

Cells were washed free of phosphate-buffered saline, resuspended in DME medium supplemented with 10% (v/v) foetal-calf serum, and cultured at a final density of 1.5×10^7 – 2×10^7 cells per T75 flask. Cells were allowed to adhere for 2–4 h, then washed twice with Hanks' balanced salt solution. To each flask we then added 10 ml of DME-LH medium containing $2 \mu\text{M}$ -colchicine. All media contained penicillin (50 units/ml) and streptomycin ($50 \mu\text{g}/\text{ml}$). Cells were cultured for 48 h, after which the conditioned medium was collected and frozen at -20°C . The conditioned medium was replaced with fresh DME-LH medium + $2 \mu\text{M}$ -colchicine. This process was repeated until 500–600 ml of conditioned medium were obtained.

Biosynthetic labelling with [^{35}S]methionine

Macrophages were grown as described above for 24 h in DME-LH medium + $2 \mu\text{M}$ -colchicine. The medium was removed and the cells were washed twice in Hanks' balanced salt solution and replaced with methionine-free DME medium containing $2 \mu\text{M}$ -colchicine and $25 \mu\text{Ci}$ of [^{35}S]methionine/ml. After 8 h the medium was collected. The cells were washed twice in Hanks' balanced salt solution and replaced with DME-LH medium + $2 \mu\text{M}$ -colchicine. After 48 h this medium was collected and pooled with the radioactive medium and processed for purification. Elastase activity was determined with the [^3H]elastin assay modified for a double-isotope analysis.

Preparation of [^3H]elastin

Radioactive elastin was prepared by modification of a NaBH_4 reduction procedure (Takahashi *et al.*, 1973; Gordon *et al.*, 1976; Stone *et al.*, 1977). Amino acids and reducible cross-links are the major labelling sites with this method (Rucker *et al.*, 1979). Dry elastin powder (2.5 g) was moistened with ethanol and suspended in 50 ml of water. The pH was adjusted to 9.2 with 100 mM-NaOH, and NaB^3H_4 (25 mCi), dissolved in 3 mM-NaOH, was added. After 10 min, 250 mg of non-radioactive NaBH_4 , dissolved in 3 mM-NaOH, was added and allowed to mix in a very well ventilated fume hood for 2 h. **Care should be exercised because millicurie amounts of ^3H gas are released as by-products of the reaction.** The pH was then adjusted to 3.0 by the careful addition of acetic acid. Foaming was suppressed by the addition of a few drops of Antifoam-B emulsion (Sigma). After 30 min of additional mixing the elastin was collected by centrifugation at 10000 g for 30 min. The pellet was repeatedly washed with water and collected by centrifugation until the supernatant radioactivity approached background values. [^3H]Elastin was stored at -80°C . Incorporated radioactivity was determined after complete solubilization with pancreatic elastase and was counted in a toluene/Cellosolve-based scintillation fluid (Hall & Cocking, 1965). The specific radioactivity of the labelled elastin used in these experiments was 5.3×10^5 d.p.m./mg. Non-specific proteolytic activity was determined with 1-chloro-4-phenyl-3-L-tosylaridobutan-2-one ('TPCK')-treated trypsin (absorbed with 5 mg of elastin/mg to remove contaminating pancreatic elastase). The trypsin-sensitive radioactivity (5 μg of trypsin/200 μg of elastin) did not exceed 1.5% of the total available radioactivity released by pancreatic elastase.

Elastase assay

Elastase activity was measured by determining the amount of radioactivity released after incubation of enzyme preparations at 37°C with 200 μg of [^3H]elastin in 100 mM-Tris/HCl buffer, pH 7.8, containing 5 mM- CaCl_2 and 0.02% NaN_3 . Final reaction volume was 300 μl . Working stock solutions of [^3H]elastin (2 mg/ml in 3-fold-concentrated assay buffer) were stored at 4°C for 1–2 weeks. At the end of incubation, assay tubes were centrifuged for 3 min in a Beckman Microfuge. The radioactivity remaining in 100 μl of supernatant was measured by liquid-scintillation spectrometry. One unit of elastase activity was defined as the solubilization of 1.0 μg of elastin/h at 37°C . By varying the assay time at 37°C (3–20 h) and the volume of elastase preparation in the reaction mixture (5–200 μl), this assay detected elastase activity over the range of 0.5–10000 units/ml.

Elastase assay with SDS

Previous work has shown that elastinolytic activity is enhanced by the use of elastin in the form of a complex with SDS (referred to here as 'SDS-elastin') (Kagan *et al.*, 1972; Kagan, 1978). We therefore used this SDS-modified substrate for selected determinations (where indicated). The substrate was prepared by adding SDS to a working stock suspension of [^3H]elastin to give a final SDS/elastin weight ratio of 0.2. The SDS was mixed with the elastin at room temperature for at least 20 min before use in an assay. A ratio of 0.2 gave a total SDS concentration (bound and free) of 133.3 $\mu\text{g}/\text{ml}$. Under the assay conditions described, the free concentration of SDS (i.e., in the supernatant) determined by using [^{35}S]SDS was 99 $\mu\text{g}/\text{ml}$ and the measured ratio of bound to free SDS was 0.34.

Adsorption of macrophage elastase to elastin

The selective removal of elastase by binding to insoluble elastin was performed by using a modification of the method of Kagan *et al.* (1972). Partially purified preparations of macrophage elastase were incubated with insoluble elastin to give a final suspension of 10 mg/ml. Incubations were performed at 4°C for 5 min in 0.1 M-Tris/HCl/5 mM- CaCl_2 , pH 8.0. The elastin and any adsorbed elastase were removed by centrifugation at 8000 g for 5 min in a Beckman Microfuge. The supernatants were concentrated by precipitation with *M. lysodeikticus* and trichloroacetic acid and applied to an SDS/polyacrylamide gradient gel run under reducing conditions.

Other enzyme assays

All column fractions were assayed for lysozyme activity by measuring lysis zones in agarose plates containing *M. lysodeikticus* (Schumacher & Schill, 1972; Gordon *et al.*, 1976). Selected column fractions and starting materials were assayed for direct fibrinolytic and plasminogen-dependent release of peptides from ^{125}I -labelled fibrin (Unkeless *et al.*, 1973; Gordon *et al.*, 1976).

Dialysis and concentration of conditioned medium

All procedures were carried out at 4°C . Pooled conditioned medium was dialysed against three changes of 20 vol. of 10 mM- NH_4HCO_3 and was then freeze-dried. The conditioned medium was redissolved in approx. 4 ml of cold water and dialysed overnight against 4 litres of 15 mM-Tris/HCl buffer, pH 7.6. After dialysis, a protein precipitate was removed by centrifugation (26000 g-min) in a Beckman Microfuge and discarded. The conductivity of the supernatant fluid was measured with a Radiometer CDM3 conductivity meter and adjusted when necessary to 1.8 mS.

Anion-exchange chromatography on DEAE-Sephadex A-25

The concentrated conditioned medium was applied to a column (0.9 cm × 27 cm) of DEAE-Sephadex A-25 equilibrated with 0.02 M-Tris/HCl buffer, pH 7.6 (conductivity 2.0 mS). The sample was loaded and eluted at a constant flow rate of 9.4 cm/h (6 ml/h). The column was washed with 5 column vol. of starting buffer and then eluted with a linear NaCl gradient (0–0.7 M, 80 ml total vol.) in starting buffer. Fractions (2.0 ml) were collected and assayed for protein and enzyme activity. Peaks of elastase activity were pooled separately and freeze-dried.

Gel filtration on Ultrogel AcA54

Samples were prepared by dissolving in water freeze-dried pooled fractions from DEAE-Sephadex chromatography. The final sample volume, approx. 2.0 ml, was adjusted to 10% (v/v) glycerol and applied to an Ultrogel AcA54 column (1.5 cm × 87 cm) equilibrated and eluted with a buffer containing 50 mM-Tris/HCl/150 mM-NaCl/50 mM-CaCl₂, pH 7.6, at a flow rate of 5 cm/h (8.8 ml/h). Fractions (2.0 ml) were collected. The Ultrogel AcA54 column was calibrated with Blue Dextran, bovine serum albumin (K_{av} 0.13, mol.wt. 66 000), ovalbumin (K_{av} 0.23, mol.wt. 46 000), myoglobin (K_{av} 0.37, mol.wt. 17 600), cytochrome *c* (K_{av} 0.42, mol.wt. 12 400), egg-white lysozyme (K_{av} 0.69, mol.wt. 14 300) and K₃Fe(CN)₆ (mol.wt. 329). With the exception of egg-white lysozyme, this column resolved the protein markers such that a plot of K_{av} against the logarithm of the molecular weight was linear ($r = 0.992$). The K_{av} of lysozyme indicated an anomalous mol.wt. of 2600. Active fractions were frozen at –80°C. Protein concentrations of DEAE-Sephadex and Ultrogel fractions were determined by measuring their A_{280} , assuming a protein solution of 1 mg/ml gave an A_{280} of 1.0. The protein concentrations of starting materials and peak fractions were determined by the method of Lowry *et al.* (1951).

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide gels were prepared and run by a modification of the method of Laemmli (1970). Samples were applied to a 3% (w/v)-polyacrylamide stacking gel on a linear 8–20% (w/v) polyacrylamide gel slab (0.75 cm thick × 10 cm long × 10 cm wide). Gels were run at room temperature at 20 mA/gel. Dilute protein samples were concentrated before electrophoresis by precipitation with cold 5% (w/v) trichloroacetic acid plus 150 µg of *M. lysodeikticus*/ml of sample. The entire precipitate was resuspended in sample buffer and applied to the gel. After electrophoresis the protein bands were fixed in the gel by 20% (w/v) trichloroacetic acid at

4°C and stained with 0.05% Coomassie Brilliant Blue R250 dissolved in 20% (v/v) methanol. Radiolabelled proteins were located by autoradiography on prefogged Kodak X-Omat R film at –80°C (Laskey & Mills, 1975). Dried stained gels and autoradiographs were scanned on a Canalco model G microdensitometer by using a point light source.

Ultracentrifugation analysis

Sucrose velocity gradients were run at 4°C in a Beckman L2-65B ultracentrifuge with an SW60 rotor at 60 000 rev./min ($r_{av} = 120.3$ mm) for 20 h. Each 4.0 ml tube contained a linear 10–20% (w/v) sucrose gradient with a 0.2 ml 32%-sucrose cushion. Six gradients were poured simultaneously with an air-driven gradient maker (Steier, 1980). Sucrose solutions (w/v) were made up in 50 mM-Tris/HCl pH 7.6, containing 150 mM-NaCl and 10 mM-CaCl₂. Samples (100 µl) were loaded at the top of the gradients. After centrifugation, fractions (approx. 120 µl) were collected through the bottom of the tube. Macrophage lysozyme present in the samples was used as an internal marker. The isokinetic gradients were calibrated with horse heart cytochrome *c* (1.71 S), egg-white lysozyme (2.11 S), soya-bean trypsin inhibitor (2.30 S), bovine pancreatic α -chymotrypsinogen (2.60 S), bovine erythrocyte carbonic anhydrase (2.80 S), human α_1 -proteinase inhibitor (3.40 S), bovine serum albumin (4.41 S), and sheep haemoglobin from lysed sheep erythrocytes (4.13 S). All sedimentation coefficients for these markers were taken from published data (Smith, 1970). Each marker was run separately and its position determined by reading the A_{220} of 120 µl fractions in a Gilford 250 spectrophotometer.

Determination of N-terminal amino acids of elastin by dansylation

Insoluble elastin (500 µg) was incubated with thermolysin, macrophage elastase form B and pancreatic elastase at an enzyme/substrate ratio of 1:100 in 0.1 M-Tris/HCl/0.05 M-CaCl₂, pH 8.0, at 37°C for 15 h. The resulting N-terminal amino acids were labelled by the dansyl chloride procedure and resolved by two-dimensional t.l.c. on 5 cm × 5 cm polyamide sheets (Gray, 1972). Under these conditions all 20 dansyl-amino acids and dansyldesmosine were resolved.

Inhibition studies

Various concentrations of inhibitors were added in 100 µl volumes to 100 µl of enzyme solution. Elastase forms A, B and C were used in these studies. After 30 min incubation at room temperature, 100 µl of elastin substrate was added and assayed in the usual manner. iPr₂P-F was dissolved

in dimethyl sulphoxide, phenylmethanesulphonyl fluoride in propan-2-ol, and 1,10-phenanthroline in 100% ethanol. In these cases, inhibition was expressed as percentage inhibition with respect to solvent controls. *N*-Ethylmaleimide and 4-chloromercuribenzoate were initially dissolved in 0.2 M-NaOH and neutralized with HCl. All other inhibitors and enzyme solutions were diluted in 0.01 M-Tris/HCl, pH 7.8. In experiments designed to evaluate the ability of divalent metals to protect enzyme activity from chelation by EDTA and EGTA, a dilute solution of enzyme was mixed with various concentrations of CoCl_2 , MgCl_2 , CaCl_2 and ZnCl_2 for 2 h before the addition of the chelator and elastin substrate. In experiments designed to investigate the reversal of chelation, the enzyme and chelators were pre-incubated for 2 h before addition of metal and substrate.

α_2 -Macroglobulin

Human $\alpha_2\text{M}$ was purified from fresh haptoglobin type 1-1 serum by Cibacron Blue affinity chromatography (Virca *et al.*, 1978). The purified inhibitor gave a single line of identity against rabbit anti-(human whole serum) antiserum and rabbit anti-(human $\alpha_2\text{M}$) antiserum and was >95% active when calibrated by inhibition of trypsin.

Preparation of immune sera against macrophage elastase

Rabbit anti-(mouse macrophage elastase) was prepared by injecting peak fractions (approx. 50 μg) of macrophage elastase (form B), emulsified in Freund's complete adjuvant, into four sites on the back of a female New Zealand White rabbit. After 3 weeks the animal was 'boosted' with elastase emulsified in Freund's incomplete adjuvant and then bled 7 days later. Immune immunoglobulin fraction was prepared by precipitation with 2 M- NH_4SO_4 . Immunoglobulins were isolated from preimmune normal rabbit serum in the same manner and used as a control.

Results

Purification of multiple forms of macrophage elastase

Colchicine treatment of mouse macrophage cultures. Colchicine increased the total amount of elastase secreted by cultured macrophages 2–8-fold, in agreement with previously published observations (Gordon & Werb, 1976), and decreased secretion of plasminogen activator and lysozyme by more than 50%. The enrichment of elastase activity constituted the first stage of purification of macrophage elastase.

Dialysis and freeze-drying. Conditioned medium was dialysed against 10 mM- NH_4HCO_3 before being concentrated by freeze-drying. Dialysis increased total activity 5-fold (Table 1) (see 'Inhibition studies on macrophage elastase' below). Dialysis removed dialysable proteins and peptides (including 59% of the lysozyme) from the conditioned medium. The retained material was reconstituted with water to 1% of its original volume and further dialysed against 15 mM-Tris/HCl buffer, pH 7.6, to decrease its conductivity before application to an ion-exchange column. After the second dialysis a precipitate was removed by centrifugation. This precipitate routinely contained less than 5% of the elastase activity recovered in the supernatant. However, the supernatant contained only about 50% of the elastase activity of the dialysed medium, but contained 59% of the protein recovered after the first dialysis. Thus 89% of the initial protein was removed before ion-exchange chromatography.

DEAE-Sephadex chromatography. Three ionically distinct forms of elastase were eluted during anion-exchange chromatography (Fig. 1). The first peak (Pool I) did not bind to the column under the starting conditions used. When this peak was re-run on an identical DEAE-Sephadex column, it eluted similarly, thus demonstrating that the lack of affinity was not an artifact produced by overloading of the bed. A second peak (Pool II) eluted with the

Table 1. Summary of purification of macrophage elastase

Stage	$10^{-3} \times$ Elastase activity (units)	Protein (mg)	Specific activity (units/mg)	Enrichment (fold)
Crude conditioned medium	1.1	803	1.4	1
Dialysed medium	6.3	210	30	22
Concentrated dialysed medium	3.0	96	31	23
DEAE-Sephadex*	5.3	35	151	110
Ultrogel AcA54†	8.8	4.7	1872	1367

* Based on total activity and protein recoveries from the sum of DEAE-Sephadex Pools I, II, and III.

† Based on all activity and associated protein from Ultrogel AcA54 of DEAE Pools, I, II, and III. Protein recoveries and specific activity include values from Ultrogel pool III, which had the lowest specific activities. Peak fraction recoveries are shown in Table 2.

Fig. 1. Anion-exchange chromatography of macrophage elastase on DEAE-Sephadex A-25

Elastase activity was eluted by a linear NaCl gradient in 0.02 M-Tris/HCl equilibration buffer, pH 7.6, at 9.4 cm/h (6 ml/h). Elastase assay time was 8.0h. Fractions labelled I, II, and III were pooled separately. —, Elastase; ····, A_{280} ; ---- relative lysozyme activity; - - -, conductivity.

beginning of the gradient between conductivities of 2.5 and 15 mS. Because the tail of Pool II still contained elastase at enriched specific activities, the fractions eluted from 15 to 30 mS were collected as Pool III. The majority of the eluted protein appeared after 30 mS. As indicated in Table 1, over 150% of starting activity was recovered after this purification step.

Separation on Ultrogel AcA54. We took advantage of the anomalous behaviour of some proteins during gel filtration on Ultrogel AcA54. Although the separation of elastase on Ultrogel AcA54 was complex and not due entirely to molecular sieving (see below for macrophage lysozyme), this resin gave high recovery and enrichment of enzyme activity. It also resolved multiple forms of elastase not seen with other forms of filtration media.

The three peaks of activity developed by gel filtration on Ultrogel AcA54 will be referred to as forms A (K_{av} 0.13), B (K_{av} 0.33) and C (K_{av} 0.61). The general elution pattern indicated that the B-form was the major form of macrophage elastase and that it was weakly retained by the DEAE-Sephadex column. Chromatography of Pool I gave a peak with 81% of the elastase activity as form B on the shoulder of the major protein peak (Fig. 2) and a lesser peak with 19% of the activity eluting as A-form. Chromatography of Pool II gave 4% as A-form, 77% as B-form and 19% as C-form. The elastase activity in DEAE-Sephadex Pool III eluted from Ultrogel AcA54 with equal activity resolved as each of the three forms. These peaks rechromatographed at their original elution volumes; however,

loss of activity after rechromatography prevented the repeated use of Ultrogel AcA54 for further purification. All three forms of macrophage elastase were stable for at least 3 months at -80°C in gel-filtration buffer and withstood repeated freezing and thawing.

Behaviour of lysozyme in the elastase-purification scheme. Lysozyme, a cationic macrophage secretion product, eluted with all three DEAE-Sephadex pools. The use of colchicine in tissue culture decreased the amount of lysozyme in the starting material, and two-thirds of the activity was removed by dialysis. However, the contamination remained significant. When each DEAE-Sephadex pool was chromatographed on an Ultrogel AcA54 column, lysozyme activity was fully separated from the elastase activity. Usually, the lysozyme activity eluted from this column in excess of 100% bed volume, although its mol.wt. is 14 000. Thus it was apparent that fractionation on Ultrogel AcA54 did not occur only by molecular exclusion. Because of this observation, elastase forms A, B, and C were analysed by other techniques before molecular weights were assigned.

Characterization of macrophage elastase

Molecular-weight characterization. SDS/polyacrylamide-gel electrophoresis of macrophage elastase forms B and C gave similar major bands, under reducing conditions, with an approx. mol.wt. 22 000 (Fig. 3). Unreduced forms B and C still gave single bands of mol.wt. 22 000 (results not shown). Heavily overloaded gels of half of each fraction of an

Fig. 2. Gel filtration of DEAE-Sephadex Pools I, II, and III on Ultrogel Aca54

The pools were from the same purification run as Fig. 1. Equilibration and elution were performed at 5 cm/h (8.8 ml/h) in 0.05 M-Tris/HCl/0.15 M-NaCl/0.05 M-CaCl₂ buffer, pH 7.6. Elastase assay time was 10h. —, Elastase; ····, A_{280} ; ----, relative lysozyme activity. (a) DEAE-Sephadex Pool I, (b) DEAE-Sephadex Pool II, (c) DEAE-Sephadex Pool III.

Ultrogel Aca54 elution of Pool II show that these same two bands, B and C, were considerably enriched in two areas of widely varying K_{av} . (0.33 and 0.61). From Ultrogel Aca54 chromatography

Fig. 3. SDS/polyacrylamide-gradient-gel electrophoresis of macrophage elastase

Crude conditioned medium before (a) and after (b) dialysis against 10 mM-NH₄HCO₃; (c) elastase form A reduced in β -mercaptoethanol, A_r; (d) elastase form B, reduced, B_r; (e) elastase form B, unreduced, B; (f) elastase form C, reduced, C_r. Elastase forms A and B were obtained from Ultrogel-Aca54 filtration of DEAE-Sephadex Pool II, and elastase form C was obtained from gel filtration of DEAE-Sephadex Pool III. The arrow indicates the molecular weight for form B. Each lane contained 10–20 units of elastase.

the expected molecular weight of the B-form was 25 000 and of C-form was 6000–7000. These results again emphasize that Ultrogel Aca54 did not separate solely by molecular size. From the gels depicting the elution of Pool I, it was evident that the major peak of this pool was not elastase, but a protein of considerably higher molecular weight, even though this protein eluted from the column with a K_{av} suggesting it was slightly smaller than elastase B-form (results not shown).

Form A has not been sufficiently purified to determine its molecular weight by migration in SDS/polyacrylamide gels. We have been unsuccessful

ful in attempts to elute activity from non-SDS, isoelectric-focusing or SDS/polyacrylamide electrophoretic gels. Despite our inability to localize the elastase band in the electrophoretic pattern of form A, the staining pattern was noticeably devoid of a protein band corresponding to the B- or C-form (Fig. 3). The tentative molecular weight of form A, as determined by its K_{av} from Ultrogel AcA54, was approx. 57 000.

Relative purity of the forms of macrophage elastase. After gel filtration on Ultrogel AcA54, the three forms of macrophage elastase were free from contamination by other macrophage enzymes, specifically, plasminogen activator, specific collagenase, 5'-nucleotidase and lysozyme activity. Form B had the highest specific activity (Table 2); specific activities of the B-form from five purifications were >6000 units/mg with the elastin substrate and represented enrichments of 3000–4000-fold over starting material. Most of the elastase activity (61%) was recovered as form B. The relative percentage of total activity recovered as form A was 18% and as form C was 21% (Table 2).

Form A was clearly the least homogeneous when examined by electrophoresis. In the most highly purified preparations, form B was >85% homogeneous (Fig. 4) and form C was >90% homogeneous (results not shown) as judged by densitometry of Coomassie Blue-stained gels containing amounts of proteins that gave a linear relationship between band staining and protein concentration.

Adsorption of elastase by elastin. Macrophage elastase binds tightly to elastin. To identify further the protein band associated with catalytic activity, we incubated elastase with elastin. The protein band of mol.wt. 22 000 was adsorbed from forms B and C in proportion to the elastase activity adsorbed (Fig. 5), and a protein band of mol.wt. 42 000 on SDS/polyacrylamide gels was adsorbed from form-A elastase. Poor recovery of elastase activity on elution prevented use of elastin as an affinity adsorbant during purification.

Determination of molecular size of elastase by sucrose-velocity-gradient analysis. Because of anomalous behaviour of cationic proteins on Ultrogel AcA54 chromatography, which gave three forms of macrophage elastase, and SDS/polyacrylamide gels, which gave two size classes of the enzyme, a third independent method for determining molecular size was used. Purified forms of the enzyme were not stable in either glycerol or sucrose velocity gradients. However, when freeze-dried and dialysed conditioned medium identical with the starting material used in the DEAE-Sephadex fractionation was analysed on sucrose velocity gradients, considerable activity was recovered (Fig. 6a). The major peak of activity was observed with a sedimentation coefficient of $3.0 \pm 0.2 S$, which was within the range of molecular weight determined by SDS/polyacrylamide-gel electrophoresis and gel filtration for form B (mol.wt. 22 000). A shoulder on the major peak had a

Fig. 4. Densitometric scan of a Coomassie Blue-stained SDS/polyacrylamide gel of purified macrophage elastase form B

Electrophoresis was performed under reducing conditions. The sample contained 4 μ g of protein.

Table 2. Relative amounts and peak activities of macrophage elastase

Form	K_{av} *.	$10^{-3} \times$ Mol.wt.†	$10^{-3} \times$ Peak specific activity (units/mg)	$10^{-3} \times$ Enrichment (fold)	Amount of each form (% of total)‡
A	0.13	42–57§	3.2	2.3	18
B	0.33	22	5.8	4.2	61
C	0.61	22	5.5	4.0	21

* Determined by elution from Ultrogel AcA54.

† Determined from SDS/polyacrylamide gradient gels.

‡ Expressed as % total recovery of each form of macrophage elastase. Total recovery of elastase activity was 8800 units (Table 1).

§ Range determined from K_{av} and protein absorption by elastin (see Fig. 5).

Fig. 5. Densitometric scan of a Coomassie Blue-stained SDS/polyacrylamide gel of (a) partially purified B-form of macrophage elastase, (b) supernatant of B-form absorbed with particulate elastin, (c) partially purified C-form and (d) supernatant of C-form absorbed with particulate elastin

sedimentation coefficient of $3.9 \pm 0.2S$. This was consistent with the molecular weight of form A estimated by gel filtration (57000).

When Pool I from DEAE-Sephadex chromatography was analysed on a sucrose velocity gradient, the resulting activity profile was nearly devoid of the 3.9S form (Fig. 6b). This result was supported by

Fig. 6. Ultracentrifugation analysis of macrophage elastase in sucrose gradients

Top of the gradient is to the right. Marker positions are: i, sheep haemoglobin, 4.13S; ii, egg-white lysozyme, 2.11S; and iii, horse heart cytochrome c, 1.71S. Positions A and B are the peaks of elastase activity. (a) Is the average from two tubes containing concentrated conditioned medium and assayed with [3H]elastin. (b) Is the sedimentation of material identical with DEAE-Sephadex Pool I. Elastase activity is expressed as a percentage of the total activity eluted from the gradient. The total elastase activities recovered were (a) 116 units and (b) 63 units.

the elution profile of Pool I on Ultrogel filtration (Fig. 2a), thus demonstrating that the higher-molecular-weight form A could be substantially purified away from the active form B by DEAE-Sephadex chromatography. These velocity-sedimentation data also demonstrated that two distinct size classes of elastase activity, consistent with the A and B/C forms, were present in dialysed crude conditioned medium before chromatographic separation.

Biosynthetic labelling of elastase. To establish whether macrophages biosynthesize elastase, cells were allowed to incorporate [^{35}S]methionine, con-

ditioned medium was collected, and the elastase was purified by the scheme reported above. Total radioactivity applied to the DEAE-Sephadex A-25 column was 8.28 μ Ci, which represented 4.47% incorporation by approx. 1.4×10^8 cells. The total radioactivity recovered after gel filtration on Ultrogel AcA54 was >40 nCi, with 2.25 nCi recovered in the elastase peak. At a mol.wt. of 22 000 (arrowed shoulder in Fig. 7a), elastase forms B and C represented about 0.5% of the total [35 S]-methionine-labelled protein. In the absence of colchicine, this protein was <0.1% of the total secreted 35 S-labelled proteins (results not shown). Figs. 7(b) and 7(c) show that the radiolabelled form B was purified to >85% homogeneity. The two contaminants seen in Fig. 7(c) can be identified as the two major labelled species from Pool II.

pH optimum and requirement for calcium in assays for macrophage elastase. The pH optimum of form B, when assayed with elastin in either the absence or presence of SDS, was 8.0 with considerable activity in the range 7.4–7.8. Solubilization of 3 H-labelled elastin peptides with form B of elastase was linear for at least 24 h.

Calcium was required for catalytic activity of macrophage elastase. When form B was dialysed against 25 mM-Tris/HCl, pH 7.6, no elastase activity was detected. The addition of Ca^{2+} to a final concentration of 5 mM restored activity.

Dansyl N-terminal amino acids and size of cleavage fragments resulting from elastinolysis. The N-terminal amino acids resulting from the cleavage of insoluble elastin by macrophage elastase and thermolysin were determined by dansylation. The two metalloproteinases, macrophage elastase and thermolysin, both produced primarily N-terminal leucine residues, whereas pancreatic elastase gave a variety of N-terminal amino acids, as would be expected from its cleavage to the carboxy side of alanine residues. Macrophage elastase differed from thermolysin and pancreatic elastase because it did not generate significant N-terminal valine.

The sizes of the peptides released from insoluble [3 H]elastin after degradation by pancreatic and macrophage elastases were compared by SDS/polyacrylamide-gel electrophoresis (Fig. 8). Although proteolysis of elastin by these proteinases apparently made a continuous range of cleavage products, macrophage elastase produced predominantly high-molecular-weight peptides with noticeable peaks corresponding to a mol.wt. of 90 000–100 000. Pancreatic elastase did not have the predominance of larger peptides, but rather had peaks corresponding to a mol.wt. of 20 000–30 000.

The continuous size range of elastin cleavage products produced by the endopeptidases, pancreatic elastase and macrophage elastase, was due to the unique structure of the elastin particle.

Fig. 7. Densitometric scan of an autoradiograph of SDS/polyacrylamide gradient gels of macrophage elastase biosynthetically labelled with [35 S]methionine in culture

(a) Conditioned medium; (b) DEAE-Sephadex Pool II before gel filtration; (c) peak fraction of elastase form B from gel filtration of DEAE-Sephadex Pool II. The arrow indicates the molecular weight of elastase (form B). The elastase peak in panel (c) represents 85% of the radioactive material, determined by the relative weights of the peaks. Migration of molecular-weight standards is shown at the top of the gel (values are $10^{-3} \times$ mol.wt.).

Fig. 8. Densitometric scan of an autoradiograph of SDS/polyacrylamide-gel separation of cleavage products of insoluble [^3H]elastin

Light line (P) represents peptides produced by pancreatic elastase. Heavy line (M) represents peptides produced by macrophage elastase form B. Migration of molecular-weight standards is indicated at the top of the scan (values are $10^{-3} \times \text{mol.wt.}$)

The size of the cleavage fragment is due not only to the distance between susceptible residues but to the existence of intra- and inter-molecular cross-links between residues. Because crosslinks are not regularly situated within the elastin fibre, uniform cleavage would not be expected. However, macrophage elastase produced larger peptides than did pancreatic elastase, suggesting that macrophage elastase did not recognize as many susceptible residues as did pancreatic elastase. In the [^3H]elastin assay, ice-cold 22% (w/v) trichloroacetic acid precipitated >90% of the soluble radioactivity resulting from cleavage by both pancreatic and macrophage elastase.

Substrate specificity. Even after 48 h incubation, macrophage elastase did not degrade the acetyl-trialanine 4-nitroanilide and benzyloxycarbonyl-alanine 2-naphthol esters, two chromogenic substrates commonly used with granulocyte and pancreatic elastases (Visser & Blout, 1972; Bieth *et al.*, 1974). However, the activity of most proteinases is not restricted to one substrate. In addition to cleaving fully cross-linked, lathyrictic, aortic, and

oxalate-solubilized elastin, macrophage elastase forms A, B and C degraded a variety of proteins, including mouse immunoglobulins, bovine fibrinogen, bovine cartilage proteoglycans, casein and myelin basic protein (Banda & Werb, 1979, 1980a,b; M. J. Banda & Z. Werb, unpublished work).

Inhibition studies on macrophage elastase

Effect of serine- and thiol-proteinase inhibitors. The forms of macrophage elastase were characterized by the effect of specific proteinase inhibitors. The effect of inhibitors of serine and thiol proteinases on form B is shown in Table 3. Neither of the inhibitors, $i\text{Pr}_2\text{P-F}$ or phenylmethanesulphonyl fluoride, which are directed at the active site of serine proteinases, inhibited the enzyme at a concentration of 1 mM, which was sufficient to inhibit the pancreatic-elastase controls. However, elastinolytic activity was very sensitive to the organic solvents of the inhibitors when present at concentrations of >0.5% (v/v). Macrophage elastase, form B, also did not bind [^3H] $i\text{Pr}_2\text{P-F}$. The thiol-proteinase inhibitors 4-chloromercuribenzoate, *N*-ethylmaleimide, and mersalyl did not inhibit macrophage elastase. At 1.0 mg/ml, an inhibitor of trypsin-like proteinases, soya-bean trypsin inhibitor, had no inhibitory effect on any form of the macrophage enzyme, whereas this concentration fully inhibits granulocyte elastase (Starkey & Barrett, 1976b). Form C was stimulated 4–5-fold by 0.2 mg of soya-bean trypsin inhibitor/ml and by 1.0 mM-lysine (results not shown), although the mechanism for this stimulation was not investigated. An inhibitor of pancreatic and granulocyte elastase directed at the active site, $\text{Ac}(\text{Ala})_4\text{CH}_2\text{Cl}$ (Powers & Tuhy, 1973; Ardelt *et al.*, 1976), had no effect on the macrophage elastase.

Effect of metalloproteinase inhibitors. Metal-ion chelators are potent inhibitors of metalloproteinases (Barrett, 1977; Knight, 1977). Macrophage elastase was inhibited by 1,10-phenanthroline and dithiothreitol, chelators of divalent cations, especially Zn^{2+} . EDTA was more inhibitory than EGTA (Table 4), suggesting that Ca^{2+} was not the only metal involved with macrophage elastase activity. When Co^{2+} and Mg^{2+} were mixed with macrophage elastase at a 3-fold higher concentration (mol/mol) than either EDTA or EGTA, they neither protected the enzyme from the effect of the chelators nor reversed the effect (Table 5). A 3-fold excess of Ca^{2+} easily prevented and reversed the inhibitory effect of EGTA, but had no effect on inhibition by EDTA. The inhibitory effect of EDTA was prevented and reversed by a $\text{Zn}^{2+}/\text{EDTA}$ (mol/mol) ratio of 0.33 and 1.0. However, a 3-fold molar excess of Zn^{2+} inhibited the elastase, as has been reported for Zn^{2+} metalloproteinases (Mori-hara *et al.*, 1965; Mori-hara & Tsuzuki, 1975; Kenny, 1977). These data

Table 3. *Effect of thiol and serine proteinase inhibitors on macrophage elastase (form B)*

Inhibitor	Concentration	Inhibition (%)*	
		Macrophage elastase	Pancreatic elastase
iPr ₂ P-F	0.1 mM	0	100
	1.0 mM	0	100
Phenylmethanesulphonyl fluoride	0.1 mM	0	0
	1.0 mM	0	97
Soya-bean trypsin inhibitor	0.2 mg/ml	0	16
	1.0 mg/ml	0	29
4-Chloromercuribenzoate	0.5 mM	0	ND†
	1.0 mM	0	ND
N-Ethylmaleimide	0.5 mM	0	ND
	1.0 mM	0	ND
Mersalyl	0.5 mM	0	ND
	1.0 mM	0	ND
Ac(Ala) ₄ CH ₂ Cl	0.1 mM	0	100
	1.0 mM	0	100

* Inhibitors were preincubated with enzyme for 20 min before addition of elastin substrate.

† ND, not determined.

are consistent with the classification of macrophage elastase as a metalloproteinase.

Phosphoramidon is a potent competitive inhibitor of thermolysin, a bacterial metalloproteinase (Suda *et al.*, 1973; Morihara & Tsuzuki, 1978), inhibiting with K_i 3.2×10^{-8} M (Nishino & Powers, 1980). However, in the present study we found that when elastin was used as a substrate for thermolysin, the inhibitory concentration of phosphoramidon was increased to 2.5×10^{-5} M, presumably because the size and nature of the elastin substrate decreased the efficacy of this competitive inhibitor. Phosphoramidon at 2.5×10^{-4} M fully inhibited thermolysin-mediated degradation of elastin, but did not inhibit macrophage elastase (Table 5). Phosphoramidon (10^{-4} M) also did not inhibit the metalloproteinase rabbit fibroblast collagenase (results not shown). A glycoprotein tissue inhibitor of metalloproteinases that inhibits rabbit bone collagenase, gelatinase and neutral metalloproteinase III (Sellers *et al.*, 1979) did not inhibit macrophage elastase at 3.0 units/ml.

Effect of serum proteinase inhibitors. Low concentrations of mouse, rabbit and human serum (0.05%) inhibited macrophage elastase activity (Table 6). Although α_1 -proteinase inhibitor did not inhibit macrophage elastase, purified human α_2 M, a naturally occurring endopeptidase inhibitor, was fully inhibitory at 0.1 μ M. Similar inhibition was observed with purified mouse α_2 M. It is noteworthy, however, that α_2 M inhibition was abolished when exogenous SDS was added to the assay mixture (Table 6).

Table 4. *Effect of metalloproteinase inhibitors on macrophage elastase*

Inhibitor	Concentration	Inhibition (%)
EDTA	0.1 mM	60
	1.0 mM	100
EGTA	1.0 mM	22
1,10-Phenanthroline	1.0 mM	97
Dithiothreitol	1.0 mM	72
Phosphoramidon*	0.25 mM	0
Tissue inhibitor†	3 units/ml	0

* This concentration completely inhibited thermolysin degradation of elastin.

† The tissue inhibitor of metalloproteinases from rabbit bone completely inhibited 1 unit of rabbit collagenase at this concentration.

Comparison of elastases from mouse pancreas, granulocytes, and macrophages. Elastase activities from mouse pancreas and from granules of mouse peritoneal polymorphonuclear leucocytes were inhibited by 1 mM-Ac(Ala)₄CH₂Cl and by α_1 -proteinase inhibitor, whereas mouse macrophage elastase was inhibited by the metal chelator 1,10-phenanthroline (Table 7). All three enzymes were sensitive to α_2 M. These observations constitute the first reported evidence for the production of three catalytically distinct forms of elastase from the same species and of two distinct elastases from inflammatory cells of the same species. Immunoglobulins from a rabbit antiserum raised against purified

Table 5. *Effect of bivalent cations on inhibition of macrophage elastase by chelators*

(a) Protection from chelation*

Metal compound	Me ²⁺ /EGTA (mol/mol)	Inhibition (%)	Me ²⁺ /EDTA (mol/mol)	Inhibition (%)
None	0.0	42	0.0	56
CaCl ₂	3.0	5	3.0	52
CoCl ₂	3.0	28	3.0	41
MgCl ₂	3.0	44	3.0	47
ZnCl ₂	0.1	14	0.1	0
	1.0	44	1.0	0
	3.0	72	3.0	77

(b) Reversal of chelation†

Metal compound	Me ²⁺ /EGTA (mol/mol)	Inhibition (%)	Me ²⁺ /EDTA (mol/mol)	Inhibition (%)
None	0.0	42	0.0	56
CaCl ₂	3.0	19	3.0	61
CoCl ₂	3.0	46	3.0	46
MgCl ₂	3.0	41	3.0	61
ZnCl ₂	0.1	2	0.1	4
	1.0	41	1.0	4
	3.0	82	3.0	84

* Elastase and bivalent metal ion (Me²⁺) were incubated for 2 h before addition of chelator and substrate; final concentration of chelator in assay was 1.0 mM.

† Chelator concentration in the preincubation mixture was 1.5 mM in the presence of 5 mM-Ca²⁺ contributed by the enzyme buffer; final concentration of chelator in assay was 1.0 mM.

Table 6. *Effect of serum proteinase inhibitors on macrophage elastase*

Inhibitor	Inhibitor concentration	Inhibition (%)
Mouse serum	0.5%	69
Rabbit serum	0.5%	72
Human serum	0.5%	63
α_1 -Proteinase inhibitor	1.0 mg/ml	0*
α_2 /M, human	0.01 mg/ml	11†
	0.10 mg/ml	100

* At 1.0 mg/ml, α_1 -proteinase inhibitor inhibited both granulocyte and pancreatic elastase.

† When assayed with SDS-elastin, inhibition of macrophage elastase activity by α_2 M was decreased to 0% with 0.01 mg/ml and 27% with 0.1 mg/ml.

macrophage elastase, form B, inhibited macrophage elastase forms A, B, and C, but did not inhibit mouse granulocyte and pancreatic elastases.

Endogenous elastase inhibitor and effect of SDS. A consistent observation through several purifications of macrophage elastase was the recovery of up to 800% of the initial elastase activity. This was likely due to elastase being purified away from an endogenous inhibitor or regulatory factor present in the conditioned medium. To test whether such an

inhibitory factor was present in excess, various volumes of crude conditioned medium were diluted with DME-LH medium and assayed for elastase activity. We found that 50 μ l of conditioned medium had more apparent elastinolytic activity than 200 μ l of the same medium. Dialysis of conditioned medium against 10 mM-NH₄HCO₃, before concentration by freeze-drying, resulted in 5-fold increases in activity. When dialysed conditioned medium and crude conditioned medium were mixed, only 36% of the predicted elastinolytic activity was recovered, suggesting the existence of excess inhibitor in the crude conditioned medium.

The catalytic activity of pancreatic elastase is enhanced by modification of the elastin substrate by the binding of SDS (Kagan *et al.*, 1972; Kagan, 1978). Additionally, SDS has been shown greatly to enhance elastin degradation by crude macrophage-conditioned medium (Werb & Gordon, 1975a). As determined with [³⁵S]SDS, the standard SDS-elastin assay had 99 μ g of SDS/ml free (i.e., not elastin bound) in the reaction mixture (see the Experimental procedures section). In the present study, we found that the elastase activity from conditioned medium was stimulated 5-fold by SDS, whereas dialysed medium was enhanced only 1.5-fold (Table 8). In the presence of SDS, the activity of crude conditioned medium, and of conditioned medium dialysed against 10 mM-NH₄HCO₃, was the same. In

Table 7. Evidence for three distinct mouse elastinolytic enzymes

Inhibitor	Concentration	Source of elastase*	Inhibition (%)		
			Pancreas	Granulocytes	Macrophages
α_1 -Proteinase inhibitor	1.0 mg/ml		100	100	0
α_2 -M	0.1 mg/ml		100	100	100
Ac(Ala) ₄ CH ₂ Cl	1.0 mM		100	97	5
1,10-Phenanthroline	1.0 mM		0	0	95
Immunoglobulins					
Immune†	1/10		0	0	92
Non-immune	1/10		0	0	0

* Mouse pancreatic and granulocyte elastases were prepared as described in the Experimental procedures section, and macrophage elastase was purified form B.

† Rabbit anti-(mouse macrophage elastase form B) was prepared as described in the Experimental procedures section. Proteinase solutions were incubated with a 1:10 dilution of antibody or immunoglobulins from control serum for 1 h at 37°C. Immune complexes were then removed by precipitation with heat-killed *Staphylococcus aureus* (IgSorb).

Table 8. Effect of SDS on macrophage elastase activity

Source of macrophage elastase	Elastase activity (units/ml)		Activity ratio (SDS/no SDS)
	No SDS*	SDS†	
Conditioned medium	1.9	15.4	8.1
Dialysed conditioned medium	10.5	15.1	1.4
Purified B-form	166.0	173.0	1.0

* Routine [³H]elastin substrate; see the Experimental procedures section for assay details.

† SDS-modified [³H]elastin substrate.

view of the reversal of inhibition of elastase by α_2 M by assay with SDS-elastin (Table 6), it is likely that the free SDS in the assay mixture acted by disrupting an elastase-inhibitor complex, rather than by directly increasing the catalytic activity of macrophage elastase. A preparation of the purified major form of macrophage elastase, B (mol.wt. 22 000), was not stimulated by SDS-elastin.

Discussion

The present study demonstrates that macrophages synthesize and secrete a neutral metallo-proteinase that will degrade elastin. Very few proteinases can degrade this substrate alone, although the combined efforts of more than one proteinase (e.g., trypsin and chymotrypsin) can be elastinolytic. Elastin was used as a substrate in the present study because the activity of other proteinases could be eliminated unambiguously. The present data show that macrophage elastase was capable of degrading elastin unassisted, and that macrophage elastase purified to >85% homogeneity retained full elastinolytic activity.

We have shown that macrophage elastase can be purified into multiple forms differing in molecular

size. The three forms A, B and C from gel filtration did not remain distinct by SDS/polyacrylamide-gel electrophoresis, which showed forms B and C to be of similar size (approx. mol.wt. 22 000). The putative mol.wt. 6000–7000 of form C by gel filtration is likely due to the interaction of form C with the Ultrogel AcA54 matrix, causing it to be retarded, as is lysozyme on this same column. The A-form, though relatively impure, had a mol.wt. of more than 30 000. The actual physiological form or forms have not been determined. However, the sucrose velocity gradients of dialysed and concentrated conditioned medium showed both 3.9 and 3.0S forms. These two forms are consistent with the sizes of the A-form and the B/C-forms, thus demonstrating that at least two size classes could be distinguished before chromatographic-separation steps.

It is necessary to establish that proteins produced by cells in small quantities are not cleaved from pre-existing proteins of exogenous origin, such as serum. The biosynthesis of macrophage elastase was demonstrated by incorporation of [³⁵S]methionine in culture. The band on SDS/polyacrylamide gels identified as elastase increased from 0.1% of the total protein in untreated macrophages to 0.5% in

the presence of colchicine, although total incorporation of [35 S]methionine did not change. This effect of colchicine on macrophages is in agreement with the initial observation of the modulation of proteinase secretion by colchicine (Werb & Gordon, 1975a; Gordon & Werb, 1976). The distribution of the three forms of elastase secreted by untreated macrophages and colchicine-treated macrophages was the same, but all forms were present at a lower concentration in the absence of colchicine. Apparently, colchicine does not alter the nature of the secreted elastase, but rather the quantity secreted. It is equally likely that the effect of colchicine on elastase secretion may be mediated by a decrease in the synthesis of an elastase inhibitor rather than by an increase in the synthesis of elastase. The specific mechanism by which colchicine affects secretion of elastase is not known, but it may alter mRNA translation at the ribosomal level (Redman *et al.*, 1978).

The enhancement of pancreatic elastase activity by SDS has been described previously (Kagan *et al.*, 1972; Kagan, 1978) as well as the enhancement of elastase activity from crude macrophage medium (Werb & Gordon, 1975a). These reports implied that the effect of SDS was to enhance the binding of enzyme to substrate. An effect of SDS–elastin possibly unrelated to alteration of the substrate was detected in the first step of purification (Table 1). There was an apparent activation of macrophage elastase by dialysis against NH_4HCO_3 when activity was determined by elastin alone, but there was no activation at this stage when SDS–elastin substrate was used. It would appear that SDS–elastin allows the expression of all available elastase activity, thus masking the effect of any activation or inhibition that might be present. The dissociation of an enzyme–inhibitor complex by SDS was demonstrated with $\alpha_2\text{M}$ –elastase complexes. In that case, human and mouse $\alpha_2\text{M}$ were potent irreversible inhibitors of macrophage elastase when activity was determined with the non-SDS–elastin substrate. It is possible that, in enhancement of the elastinolytic activity of macrophage conditioned medium, SDS was playing a similar role by interfering with an endogenous elastase inhibitor. Whatever the mode of stimulation of elastase activity from crude conditioned medium, these observations indicated that only 10–15% of the secreted elastase was active in the medium.

The three forms of macrophage elastase had the same inhibitor profile. The ineffectiveness of *N*-ethylmaleimide, mersalyl, $\text{iPr}_2\text{P-F}$ and phenylmethanesulphonyl fluoride indicated that macrophage elastase is unlikely to be a thiol or serine proteinase. By using purified elastase, we were unable to confirm a previous report of $\text{iPr}_2\text{P-F}$ inhibition of the macrophage elastase activity from

crude conditioned medium (Werb & Gordon, 1975a) that was based on data obtained from an elastin–agarose plate assay (Schumacher & Schill, 1972; Gordon *et al.*, 1976). Erroneous data may be generated from inhibition studies with enzyme mixtures (Barrett, 1980) such as that present with elastase from crude conditioned medium. Owing to its relative insensitivity, compared with the [^3H]–elastin assay, the elastin–agarose plate assay requires SDS-modified elastin and therefore would be especially inappropriate for inhibitor studies, such as those using $\alpha_2\text{M}$.

Macrophage elastase was not inhibited by $\text{Ac(Ala)}_4\text{CH}_2\text{Cl}$, an active-site-directed inhibitor of granulocyte and pancreatic elastase (Powers & Tuhy, 1973; Ardelt *et al.*, 1976). The macrophage enzyme was completely resistant to α_1 -proteinase inhibitor, the major inhibitor of granulocyte elastase *in vivo*, but extremely sensitive to inhibition by $\alpha_2\text{M}$. Further evidence that the macrophage enzyme is an elastase of distinct catalytic mechanisms includes the inability of the enzyme to degrade the alanine ester substrates frequently used as substrates for granulocyte and pancreatic elastase (Bieth *et al.*, 1974; Gordon & Werb, 1976). Also, the observed prevalence of *N*-terminal leucine residues generated by macrophage elastase suggests that amino acids on the carboxy side of the cleavage sites are recognized.

We have classified macrophage elastase as a metalloproteinase because of its inhibition by 1,10-phenanthroline and EDTA, the re-activation of EDTA inhibition by Zn^{2+} and the ineffectiveness of inhibitors of serine and thiol proteinases (Barrett, 1977, 1980). Although Ca^{2+} was required for elastinolytic activity, and high- Ca^{2+} buffers had a stabilizing effect during purification, Ca^{2+} was not the only metal ion required for elastase activity. Indeed, EDTA (0.1 mM) in the presence of 5 mM- Ca^{2+} was still an effective inhibitor. The well-characterized metalloproteinase, thermolysin, also has both Ca^{2+} and Zn^{2+} requirements (Colman *et al.*, 1972). Inhibition by excess Zn^{2+} has also been reported for other metalloproteinases (Mori-hara *et al.*, 1965; Mori-hara & Tsuzuki, 1975; Kenny, 1977). The lack of inhibition by phosphoramidon indicated that the active site of macrophage elastase is different from that of thermolysin or *Pseudomonas* elastase (Mori-hara & Tsuzuki, 1978; Nishino & Powers, 1980). Phosphoramidon also was not inhibitory for the tissue metalloproteinase, collagenase. The *N*-terminal amino acids revealed by endopeptidase cleavage of insoluble elastin by macrophage elastase and thermolysin were similar in that both preferentially cleaved on the amino side of leucine residues, but differed in that macrophage elastase did not cleave adjacent to valine residues, and also gave larger peptides. Macrophage elastase

also was less effective than pancreatic elastase in degrading elastin to oligopeptides.

Similar inhibition profiles have been observed for tissue proteinases other than macrophage elastase (Werb & Gordon, 1975*b*; Werb *et al.*, 1978). Macrophage elastase can utilize immunoglobulins, fibrinogen and cartilage proteoglycans as substrates (Banda & Werb, 1980*a,b*). The relationship between macrophage elastase and the cartilage proteoglycan-degrading enzyme reported by others (Hauser & Vaes, 1978) is unknown. Macrophage elastase does not activate rabbit fibroblast collagenase and is therefore unlikely to be the collagenase-activating macrophage metalloproteinase (Horwitz *et al.*, 1976).

Macrophage elastase was functionally and catalytically distinct from the serine proteinases, granulocyte and pancreatic elastase. (Because both macrophages and granulocytes are white blood cells, the term leucocyte elastase is ambiguous and should not be used.) Further characterization of the catalytic mechanism, inhibitors, substrates, and interrelationships among forms will be necessary to help define the potential biological functions of macrophage elastase. The specific characteristics of macrophage elastase indicate that it is a significant probe of macrophage-mediated inflammation and connective-tissue degradation in the chronic diseases emphysema and atherosclerosis, which are characterized by elastin degradation.

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