Macrophage elastase (ME) was originally named when metal-dependent elastolytic activity was detected in conditioned media of murine macrophages. Subsequent cDNA cloning of the mouse and human enzyme demonstrated that ME is a distinct member of the matrix metalloproteinase family. To date, the catalytic parameters that describe the hydrolysis of elastin by ME have not been quantified and its activity against other matrix proteins have not been described. In this report, we have examined the action of purified recombinant human ME (rHME), produced in Escherichia coli, on elastin and other extracellular matrix proteins. On a molar basis, rHME is approximately 30% as active as human leukocyte elastase in solubilizing elastin. rHME also efficiently degrades α1-antitrypsin (α1-AT), the primary physiological inhibitor of human leukocyte elastase. In addition, rHME efficiently degrades fibronectin, laminin, entactin, type IV collagen, chondroitin sulfate, and heparan sulfate. These results suggest that HME may be required for macrophages to penetrate basement membranes and remodel injured tissue during inflammation. Moreover, abnormal expression of HME may contribute to destructive processes such as pulmonary emphysema and vascular aneurysm formation. To further understand the specificity of HME, the initial cleavage sites in α1-AT have been determined. In addition, the hydrolysis of a series of synthetic peptides with different P1 residues has been determined. rHME can accept large and small amino acids at the P1 site, but has a preference for leucine.

Macrophage elastase (MMP-12) shares many properties with other members of the matrix metalloproteinase (MMP)gene family, yet it is unique in several ways. Like other MMPs, metalloelastase requires zinc for catalytic activity, is inhibited by the tissue inhibitors of metalloproteinases (TIMPs), and has common structural domains with other MMPs (1, 2). Human macrophage elastase (HME) is most closely related to collagenase-1 (MMP-1) and stromelysin-1 (MMP-3), being 49% identical to each at the amino acid level (3). Moreover, the gene for macrophage elastase, composed of a common 10-exon, 9-intron structure, is on human chromosome 11q22.2-22.3 with at least six other MMPs (4). Despite these similarities, HME possesses certain distinct biological and biochemical properties. Expression appears to be largely restricted to tissue macrophages (4). Upon activation, it not only cleaves its 8-kDa N-terminal domain, but also has a unique propensity to autolytically release its 23-kDa C-terminal domain resulting in a mature active 22-kDa proteinase (3–5).

Macrophage elastase shares its elastolytic activity (6, 7) with only a few MMPs, including the gelatinases (MMP-2 and MMP-9) and, to a lesser extent, matrilysin (8, 9). However, despite this characteristic activity, the relative capacity of metalloelastase (human or mouse) to degrade elastin has never been quantified. In addition, the catalytic capacity of metalloelastase against other extracellular matrix components has never been described. We have recently generated mice that lack the capacity to produce macrophage elastase by gene-targeting. Macrophages from these mice not only lost 95% of their elastolytic capacity, but were also unable to penetrate a synthetic basement membrane (Matrigel) (10). The purpose of this study was to define the capacity of HME to degrade extracellular matrix collagen and characterize its substrate specificity. Knowledge of the catalytic properties of macrophage elastase will help define potential roles for this enzyme in biologic processes associated with macrophage activation and metalloelastase expression in vivo. Current evidence suggests that these processes may include pathologic conditions such as atherosclerosis, tumor invasion/angiogenesis, cerebrovascular disease, and pulmonary emphysema, which currently represent the four leading causes of death in the United States.

**MATERIALS AND METHODS**

**Reagents—**4-Aminophenylmercuric acetate and heparin-agarose were obtained from Sigma. SP-Sepharose and Sepharyl S-200 were obtained from Pharmacia (Uppsala, Sweden). All other chemicals were reagent grade. Bovine ligament elastin and HLE were obtained from Elastin Products (Owensville, MO). Human α1-AT was obtained from Genesis Research Products (Athens, GA). Fibronectin, laminin, type IV collagen, chondroitin sulfate, and heparan sulfate were obtained from Collaborative Research Products (Bedford, MA). Entactin was a generous gift of Dr. Robert Senior and 92-kDa gelatinase and interstitial collag enase were kindly provided by Dr. Howard Wegla, both of the Washington University School of Medicine, St. Louis, MO.

**Bacterial Expression and Purification of Recombinant HME—**Full-length HME cDNA was ligated into an NdeI/BamHI cassette into the pET 5b vector which permitted translation in the proper reading frame beginning with the HME initiation methionine. pET 5b alone (control plasmid) and pET 5b/HME were transformed into E. coli strain BL21(DE3) (Novagen Inc., Madison, WI). Single colonies of E. coli (+/−

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rHME) from Lennox Broth (LB)/agar plates with 20 μg/ml ampicillin were grown to log phase in 1 liter of LB media (with ampicillin) in a shaking incubator at 37 °C. To induce T7 RNA polymerase and drive high level expression of rHME, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.4 mM. Cells were maintained in cultured broth for an additional 4 h. Cell pellets were resuspended in 50 mM Tris, pH 8.0, with 10 mM CaCl2 and 150 mM NaCl and lyonized by sonication. After centrifugation, the rHME was localized in the pellet. It was solubilized with 40 ml of 8 M urea containing 50 mM Tris, pH 8.0, with 10 mM CaCl2 and 30 mM NaCl continuously stirred for 2 h at 4 °C. Following centrifugation, the supernatant containing soluble rHME was collected.

The extracts from several 1-liter preparations were dialyzed against 3.5 liters urea in the Tris-containing solution and the lysates were applied sequentially to SP-Sepharose ion exchange chromatography, high resolution Sephacryl S-200 column gel filtration (column 2.5 × 90 cm, bed volume 400 ml, flow rate 30 ml/h), and hiragigin-agarose affinity ion exchange chromatography. Following these purification procedures, polyacrylamide gel electrophoresis (SDS-PAGE) and both Coomassie and silver staining demonstrated rHME without any visible contaminating proteins. The identity of this protein as HME was confirmed by Western blotting. N-terminal amino acid sequence analysis of the final product was determined by Edman degradation.

The bichinchoninic acid protein assay (Pierce Chemical Co.) and a TIMP inhibition assay were used to determine the concentration of rHME. The latter method involved preincubation of known concentrations of TIMP with fixed amounts of HME followed by addition of Ac-Pro-Leu-Gly-S-Leu-Gly-OEt. Hydrolysis of this thiopeptolide substrate (Bachem Bioscence, King of Prussia, PA) by metalloproteinases was determined as described previously (11). The concentration of rHME was further confirmed by pulse liquid sequencing performed on an Applied Biosystems model 473A sequencer equipped with the model 610A analysis software. The initial yield of the protein was extrapolated from the repetitive yield calculations for the first 10 cycles of sequencing. The average repetitive yield during the run was 96.4%. Comparison of the TIMP inhibition assay and pulse liquid sequencing results demonstrated that greater than 90% of the purified rHME was catalytically active. Control plasmids subjected to the same purification scheme were catalytically inactive against all substrates tested.

The catalytic domain of matrilysin was expressed and purified to homogeneity in bacteria using the same techniques described for metalloelastase. Matrilysin expressed in bacteria had equal catalytic activity to matrilysin expressed in eukaryotic cells (12).

Degradation of Basement Membrane Components by MMPs—To quantitatively compare the degradative capacity of rHME to either matrilysin or 92-kDa gelatinase, the basement membrane components fibronectin, laminin, entactin, chondroitin sulfate, heparan sulfate, and 460 nM for type IV collagen; 23 and 230 nM for all other substrates) were used as substrates. Specifically, fibronectin, laminin, entactin, chondroitin sulfate, heparan sulfate, and 6% laminin slab gels were stained with 1% Coomassie Brilliant Blue. For entactin, duration of incubation was reduced to 15 min. Products resolved with polyacrylamide gel electrophoresis. Specifically, degradation was interpolated linearly to estimate the concentration of both metalloproteinases was incubated with substrates as otherwise described above. For entactin, duration of incubation was reduced to 15 min and reaction temperature was reduced to 25 °C. Samples were resolved by SDS-polyacrylamide gel electrophoresis and stained with 1% Coomassie Brilliant Blue.

Elastin degradation was quantified by measuring solubilization of insoluble 3H-elastin. Bovine ligament elastin (Elastin Products, Owensville, MO) was radiolabeled with 3H-sodium borohydride (DuPont NEN), as described previously (5, 9).

RESULTS

Expression and Purification of Recombinant HME—rHME was expressed in Escherichia coli and, after cell lysis, solubilized in urea, dialyzed, and subsequently subjected to SP-Sepharose ion exchange chromatography, Sephacryl S-200 column gel filtration, and heparin-agarose affinity ion exchange chromatography (Fig. 1). After the first chromatographic step, all of the rHME migrated with the apparent molecular mass of 22 kDa corresponding to the mature processed form. At each purification step, equal amounts of protein were incubated with insoluble 3H-elastin. The specific activity increased greater than 250-fold after the final purification step (Table 1). N-terminal sequence analysis was performed on the final material to confirm its purity and identify the N terminus of the active form of recombinant HME. A single Phe-Arg-Glu sequence was identified that corresponds to cleavage at the Hsp90-Phe bond, just C-terminal to the conserved cysteine switch motif. It should be noted, however, that the N terminus of mature native HME has not been identified to date. Finally, this expression and purification procedure gave ~500 μg of purified rHME from 1 liter of E. coli.

Degradation of Basement Membrane Components by HME—The basement membrane proteins fibronectin, entactin, laminin, chondroitin sulfate, and heparan sulfate were incubated with rHME or matrilysin at 37 °C as described under “Materials and Methods.” Native and pepsinized type IV collagen was also incubated with rHME or 92-kDa gelatinase under similar conditions at 25 °C. A lower incubation temperature was se-
HME and Elastolysis

Selected to avoid conditions that would favor denaturation of the collagen molecule in solution. Cleaved products were resolved by SDS-polyacrylamide gel electrophoresis and stained with 1% Coomassie Brilliant Blue (Fig. 2). The amounts of rHME or matrilysin required to produce 50% cleavage of fibronectin, entactin, laminin, and chondroitin sulfate are summarized in Table II. rHME and matrilysin both effectively cleave entactin, laminin, and chondroitin sulfate. rHME and matrilysin both effectively cleave entactin.

**TABLE I**

| Chromatographic step | Yield (%) | Specific activity |
|----------------------|-----------|------------------|
| Crude lysate (urea)  | 100       | 1                |
| SP-Sepharose         | 38        | 44               |
| Gel filtration       | 30        | 170              |
| Heparin-agarose      | 18        | 265              |

Incubation of rHME with insoluble elastin was extended for up to 96 h and catalysis continued to proceed in a linear manner (data not shown), demonstrating that the 22 kDa species is stable under the conditions tested.

**Elastolytic Activity of rHME**—The elastolytic activity of rHME was measured by quantifying solubilization of 3H-elastin as measured by the release of 3H into the supernatant. Activity is expressed as micrograms of elastin degraded. Because elastin is an insoluble substrate, classic Michaelis-Menten kinetics could not be applied in comparing rHME and HLE. Under conditions of substrate excess with small amounts of rHME or HLE, the amount of elastin degraded was linearly related to enzyme concentration (Fig. 3). The specific activity of HME as calculated from the first three data points is 33 μg of elastin degraded/mg of enzyme/min. The specific activity of rHME against elastin was one-third that of HLE (Fig. 4). Incubation of rHME with insoluble elastin was extended for up to 96 h and catalysis continued to proceed in a linear manner (data not shown), demonstrating that the 22 kDa species is stable under the conditions tested.

**Comparison of efficiencies of basement membrane component cleavage by HME and matrilysin**

Varying concentrations of both metalloproteinases were incubated with substrate as described under “Materials and Methods.” Degradation was interpolated linearly to estimate the concentration of metalloproteinase required to produce 50% substrate cleavage.

**TABLE II**

| Substrate      | Enzyme required for 50% conversion (pmol) |
|----------------|-----------------------------------------|
|                | HME          | Matrilysin                     |
| Fibronectin    | 0.32         | 1.8                            |
| Entactin       | 3.6          | 2.7                            |
| Laminin        | 3.6          | 22.7                           |
| Chondroitin sulfate | 1.6      | 2.5                            |

Incubation of α1-AT by rHME abolished its activity is expressed as micrograms of elastin degraded. Because elastin is an insoluble substrate, classic Michaelis-Menten kinetics could not be applied in comparing rHME and HLE. Under conditions of substrate excess with small amounts of rHME or HLE, the amount of elastin degraded was linearly related to enzyme concentration (Fig. 3). The specific activity of HME as calculated from the first three data points is 33 μg of elastin degraded/mg of enzyme/min. The specific activity of rHME against elastin was one-third that of HLE (Fig. 4). Incubation of rHME with insoluble elastin was extended for up to 96 h and catalysis continued to proceed in a linear manner (data not shown), demonstrating that the 22 kDa species is stable under the conditions tested.

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**Comparison of efficiencies of basement membrane component cleavage by HME and matrilysin**

Varying concentrations of both metalloproteinases were incubated with substrate as described under “Materials and Methods.” Degradation was interpolated linearly to estimate the concentration of metalloproteinase required to produce 50% substrate cleavage.

**TABLE II**

| Substrate      | Enzyme required for 50% conversion (pmol) |
|----------------|-----------------------------------------|
|                | HME          | Matrilysin                     |
| Fibronectin    | 0.32         | 1.8                            |
| Entactin       | 3.6          | 2.7                            |
| Laminin        | 3.6          | 22.7                           |
| Chondroitin sulfate | 1.6      | 2.5                            |

Purification of recombinant HME expressed in E. coli

| Chromatographic step | Yield (%) | Specific activity |
|----------------------|-----------|------------------|
| Crude lysate (urea)  | 100       | 1                |
| SP-Sepharose         | 38        | 44               |
| Gel filtration       | 30        | 170              |
| Heparin-agarose      | 18        | 265              |
analyzed by SDS-PAGE (Fig. 6). Cleavage products with molecular masses of 50, 29, 25, and 4 kDa were identified, corresponding to two scissions of intact \( \alpha_1 \)-AT to yield 50 and 4 kDa or 29 and 25 kDa products, respectively. N-terminal amino acid sequence analysis identified the two major cleavage sites: Phe\(^{352}\)-Leu\(^{353}\), generating the 50- and 4-kDa degradation products and Glu\(^{199}\)-Val\(^{200}\), generating the 29- and 25-kDa fragments. As noted above, however, upon prolonged incubation the only stable degradation product was the 50-kDa species, corresponding to cleavage at Phe\(^{352}\)-Leu\(^{353}\) within the reactive loop.

**Peptide Substrate Specificity of Cleavage by HME**—The most important subsite in determining MMP substrate specificity is \( P' \). Thus, the relative rates of hydrolysis of nine octapeptides differing only in the residues in subsite \( P' \) have been quantified for HME (Table III). The preference follows the order: Leu > Ala > Lys > Phe > Tyr > Trp > Arg > Ser > Glu. In general, HME tolerates a variety of large and small residues at the \( P' \) position. This is consistent with the expectation that HME should have a deep \( S' \) pocket based on its predicted amino acid sequence (see "Discussion").

**DISCUSSION**

Elastin is a highly cross-linked and hydrophobic insoluble extracellular matrix protein that imparts elastic recoil to a...
the variety of tissues including musculoskeletal ligaments, arterial vessels, and lung parenchyma (18). These properties contribute to its extreme stability and resistance to proteolysis by all but a limited number of proteinases. When compared in parallel assays, rHME has approximately one-third the elastolytic capacity of HLE. A similar difference in elastolytic capability between recombinant 92-kDa gelatinase and HLE has been observed (9). It is probable, however, that under most circumstances HLE remains within the neutrophil, degrading internalized foreign material, and that only inadvertent release of HLE from the cell, during “frustrated phagocytosis” or death of the short-lived neutrophil, may cause tissue destruction. On the other hand, in response to mediators of inflammation, MMPs, including HME, are characteristically secreted into the extracellular space where they modulate matrix remodeling. With sustained accumulation of macrophages and neutrophils, e.g. in chronic inflammation of the lung induced by cigarette smoking, HME and HLE expression may be poorly regulated with consequent elastin destruction, ultimately producing the distinctive changes of pulmonary emphysema.

Several circulating and locally secreted inhibitors of HLE have been identified. The primary physiological inhibitor is α1-AT which forms a stable complex with the enzyme (19). A variety of MMPs are capable of degrading α1-AT (12, 15, 16) and abolishing its ability to inhibit HLE. MMPs, therefore, may significantly modulate the physiological role of α1-AT. We now show that HME, in particular, is an order of magnitude more active than matrilysin, previously shown to be the most potent MMP capable of degrading α1-AT (12). In addition, two initial cleavages were identified, rather than the single degradation cleavages were identified, rather than the single degradation sites of inflammation. HLE, conversely, can cleave and inactivation may be further augmented by inactivating the primary inhibitor of HLE and by attracting additional neutrophils to the site of inflammation. HLE, therefore, possesses a deep pocket which can accommodate Arg at P9 position and, in fact, rHME cleaved the reactive loop of α1-AT at Phe352, Leu353. Of interest, HME is the only MMP known that can accommodate Arg at P1 position. This finding could help in the design and synthesis of a selective HME inhibitor. This may be useful in conditions with aberrant inflammatory responses which may lead to macrophage-mediated tissue destruction.

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Hydrolysis of a Broad Spectrum of Extracellular Matrix Proteins by Human Macrophage Elastase
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