Elastin Degradation by Matrix Metalloproteinases

CLEAVAGE SITE SPECIFICITY AND MECHANISMS OF ELASTOLYSIS

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Robert P. Mecham‡§, Thomas J. Broekelmann‡, Catherine J. Fliszar¶, Steven D. Shapiro¶, Howard G. Welgus¶, and Robert M. Senior¶

From the ‡Division of Cell Biology and Physiology and the ¶Pulmonary and Critical Care Medicine and §Dermatology Divisions, Department of Medicine, Washington University School of Medicine, St. Louis, Missouri 63110

Insoluble elastin was used as a substrate to characterize the peptide bond specificities of human (HME) and mouse macrophage elastase (MME) and to compare these enzymes with other mammalian metalloproteinases and serine elastases. New amino termini detected by protein sequence analysis in insoluble elastin following proteolytic digestion reveal the P′ residues in the carboxyl-terminal direction from the scissile bond. The relative proportion of each amino acid in this position reflects the proteolytic preference of the elastolytic enzyme. The predominant amino acids detected by protein sequence analysis following cleavage of insoluble elastin with HME, MME, and 92-kDa gelatinase were Leu, Ile, Ala, Gly, and Val. HME and MME were similar in their substrate specificity and showed a stronger preference for Leu/Ile than did the 92-kDa enzyme. Fibroblast collagenase showed no activity toward elastin. The amino acid residues detected in insoluble elastin following hydrolysis with porcine pancreatic elastase and human neutrophil elastase were predominantly Gly and Ala, with lesser amounts of Val, Phe, Ile, and Leu. There were interesting specificity differences between the two enzymes, however. For both the serine and matrix metalloproteinases, catalysis of peptide bond cleavage in insoluble elastin was characterized by temperature effects and water requirements typical of common enzyme-catalyzed reactions, even those involving soluble substrates. In contrast to what has been observed for collagen, the energy requirements for elastolysis were not extraordinary, consistent with cleavage sites in elastin being readily accessible to enzymatic attack.

Elastin is the extracellular matrix protein that imparts elastic recoil to tissues. Its cross-linked nature and extreme hydrophobicity make it one of the most stable proteins in the body (1–3). A contributing factor to elastin’s longevity is its relative resistance to proteolysis by all but a limited number of proteinases that are capable of degrading the mature, insoluble proteinase under physiological conditions. These enzymes, referred to as elastases, have a wide distribution in nature and are found in animals as well as in plants and bacteria (4, 5). Elastases are heterogeneous with differing substrate specificities and catalytic mechanisms. In fact, enzymes with elastolytic activity can be found in most of the major proteolytic families, including serine, thiol, aspartic, and metallo enzymes (4). Despite the differences in catalytic mechanisms, all of these elastases share a common specificity for cleaving peptide bonds associated with hydrophobic or aromatic amino acids.

The most widely studied elastases, human neutrophil elastase (HLE)1 and pancreatic elastase (PPE), belong to the serine proteinase family of enzymes. Neutrophil elastase is found in the azurophil granules of polymorphonuclear leukocytes and is essential for phagocytosis and defense against infection. Pancreatic elastase is stored as an inactive zymogen in the pancreas and is secreted into the intestine where it is activated by other digestive enzymes. Both enzymes cleave peptide bonds on the carboxyl-terminal side of amino acids with a small aliphatic side chain, although HLE has a preference for amino acids with longer aliphatic chains at this position (6).

Bacterial elastases belonging to the family of metalloproteinases have been known for many years (5). These proteinases require Zn2+ atoms for activity and in contrast to the serine proteinases cleave peptide bonds on the amino-terminal side of the amino acid that determines specificity. Elastases from Bacillus thermoproteolyticus (thermolysin) and Streptomyces frae- diae are four and eight times more active than PPE, respectively, making them some of the most potent elastolytic proteinases reported (5). Recently, metalloproteinases secreted by mammalian cells have been shown to have elastolytic activity (7–9). Like their bacterial counterparts, these proteinases contain Zn2+ atoms and express primary substrate specificity through the S3′–P1′ interaction.2

Members of the matrix metalloproteinase (MMP) family can be grouped into six categories based on similarities in their domain organization and protein substrate specificities. These groups include 1) the collagenases, 2) the gelatinases or “type IV collagenases,” which include two distinct enzymes of 72 kDa (MMP 2) and 92 kDa (MMP 9), 3) thestromelysins, 4) matrilysin, 5) metalloelastase, and 6) the mT-MMPs containing a membrane-spanning segment in their hemopexin-like domains conferring cell surface localization. Of the known MMPs, the 92- and 72-kDa gelatinases, mouse and human macrophage metalloelastases (HME and MME), and matrilysin degrade insoluble elastin. Matrilysin and the autoprocessed macrophage elastases possess only a catalytic domain, which must

1 The abbreviations used are: HLE, human neutrophil elastase; PPE, porcine pancreatic elastase; MME, mouse macrophage elastase; HME, human macrophage elastase; MMP, matrix metalloproteinase; DNFB, 2,4-dinitrofluorobenzene.
2 Using the nomenclature of Schechter and Berger (31), subsites adjacent to the scissile peptide bond are labeled P1, . . . Pn in the amino-terminal direction and Pn′, . . . P1′ in the carboxyl-terminal direction.
also convey binding and catalytic specificity for the elastin substrate. Binding specificity of 92- and 72-kDa gelatinases for elastin, however, resides in the three fibronectin type II-like repeats inserted in tandem in their catalytic domains.

To learn more about the mechanism of elastin degradation by MMPs, we developed a novel assay to detect protease cleavage sites in insoluble elastin. Traditionally, elastolytic enzymes have been detected and characterized through their ability to release soluble peptides from insoluble elastin or to cleave synthetic peptides of known sequence. Several assay methods focusing on the solubilized peptides have been described, but these lack the ability to provide detailed information about the specificity of peptide bond hydrolysis. By detecting cleavage sites directly in the insoluble protein, we have been able to determine peptide bond and subsite preferences of the elastolytic enzymes and detect differences in specificity that would not be evident using assay techniques involving synthetic substrates or solubilized forms of the protein. Our results demonstrate that HME and MME have an overall preference for amino acids with large aliphatic side chains in the P1 position. There are also interesting differences between these enzymes and the 92-kDa gelatinase and serine elastases.

**MATERIALS AND METHODS**

**Preparation of Elastin and Blocking with DNFB**—Insoluble elastin was purified from bovine ligamentum nuchae using the hot alkali technique of Lansing et al. (12) and was shown by amino acid analysis to be free of microfibrillar protein and other contaminants. Radiolabeled elastin was prepared using sodium [3H]borohydride (13). To block amino groups generated by random peptide bond cleavage during purification, insoluble elastin was washed twice using microcentrifugation and left on a rotator table for 1.5 h at room temperature. Insoluble elastin was washed twice using microcentrifugation and the pellet washed twice with water and twice with acetonitrile. The pellet was then suspended and dried onto a bioprene-treated glass fiber and left on a rotator table for 1.5 h at room temperature. Insoluble elastin in the sample was pelleted by centrifugation, the supernatant was discarded, and the pellet was resuspended in 5 ml of phosphate buffer, and 400 μl of DNFB was added. After vortexing, the tube was covered with foil and left on a rotator table for 1.5 h at room temperature. Insoluble elastin in the sample was pelleted by centrifugation, the supernatant was discarded, and the pellet was resuspended and dried onto a bioprene-treated glass fiber filter and sequenced using the normal liquid phase sequencing cycles.

**RESULTS AND DISCUSSION**

**Energy Requirements and Deuterium Effects Characterizing Elastin Degradation by Metalloproteinases versus Neutrophil Elastase**—To gain insights into biochemical mechanisms of elastolysis by metalloproteinases, we compared several catalytic parameters with those exhibited by the well characterized serine protease, neutrophil elastase. Insoluble elastin is hydrophobic, extensively cross-linked, and highly resistant to proteolytic degradation, properties similar to another extracellular matrix molecule, fibrillar collagen. Collagenases are responsible for the degradation of fibrillar collagen, and we have previously studied the kinetics of this biologic process for collagenase-1 (18, 20). As summarized in Table I, the degradation of type I collagen fibrils is temperature-dependent, with an activation energy of 100,000 cal/mol (18). However, the energy requirements for collagen degradation are reduced by a factor of two if the native collagen is in solution form (monomeric) and by a factor of ten if the collagen is denatured (gelatin). Likewise, the deuterium isotope effect, a measure of the dependence of catalysis upon proton transfer, also varies strongly with the state of substrate organization, with kH/kD values ranging from 9.0 for fibrillar collagen to 1.0 for denatured gelatin α chains.

Due to the highly insoluble nature of elastin, we were interested in whether the degradation of elastin by matrix metalloproteinases showed marked dependence upon energy and water transfer like that found with type I collagen. Arrhenius plots describing the degradation of insoluble elastin by neutrophil elastase, the 92-kDa gelatinase, and matrilysin are shown in Fig. 1. All three enzymes showed a linear dependence on temperature, with activation energies in the range of 8,412–

### Table I

| Enzyme        | Substrate                        | E_A  | k_H/k_D |
|---------------|----------------------------------|------|---------|
| 92-kDa gelatinase | Insoluble elastin                | 11,047 | 2.3     |
| Matrilysin    | Insoluble elastin                | 8,412  | 2.0     |
| Neutrophil elastase | Insoluble elastin                | 13,646 | 1.7     |
| Collagenase   | Type I collagen (Fibrillar)      | 101,050 | 9.0    |
| Collagenase   | Type I collagen (Solution Monomers) | 49,200 | 2.0     |
| Collagenase   | Type I gelatin                   | 13,200 | 1.0     |
| Matrilysin    | Entactin                         | 10,060 | ND      |

Values for activation energies (E_A) and deuterium isotope effect (k_H/k_D) observed in elastin and collagen degradation.

Values for activation energy (E_A) and initial velocities were calculated as described under “Materials and Methods.”
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13,646 cal/mol (Table I), values similar to that of most enzyme-catalyzed reactions. Catalytic rates changed 2-fold per 10 °C, rather than the 3-fold per 2 °C exhibited for the degradation of type I collagen fibrils by interstitial collagenase. Deuterium solvent kinetic isotope effect values (kD/kH) for elastin degradation by matrilysin, 92-kDa gelatinase, and neutrophil elastase ranged from 1.7 to 2.3 (Table I), values again typical for most enzyme-catalyzed reactions. Therefore, although elastin is highly insoluble and extensively cross-linked, we found that the activation energies involved in elastolysis by matrilysin and 92-kDa gelatinase were much lower than the energies previously observed for degradation of triple helical collagen by interstitial collagenase-1. Similar results regarding elastin degradation were found with neutrophil elastase, a serine peptidase. Deuterium isotope effects were also minimal for elastin degradation compared with those of collagen.

It is apparent that the catalysis of insoluble elastin is characterized by temperature effects and water requirements typical of most enzyme-catalyzed reactions, even those involving soluble substrates. The degradation of elastin therefore differs from the degradation of fibrillar collagen by interstitial collagenase, which involves enormous dependence upon energy and proton transfer. In this regard, we previously hypothesized that the extremely high values of EA and kD/kH in collagenolysis reflect the extent to which triple helical structure and the aggregation of collagen molecules into the fibril presents a barrier to the transport of the water required for peptide bond hydrolysis. Further implicating such a scenario is the observation that the Gly725-Ile726 and Gly 725-Leu726 peptide bonds cleaved in the insoluble protein as shown schematically in Fig. 1. The amino termini in the insoluble residue exposed as the result of proteolytic digestion represent the P1 peptide bonds cleaved in insoluble elastin by both metalloproteinases and serine proteinases are more accessible to these enzymes, with water transport much less of a barrier than for soluble proteins that gradually wash off the immobile membrane during the sequencing reaction, small particles of elastin can be placed on the membrane and sequenced without losses due to washout. Because elastin is a cross-linked polymer of tropoelastin molecules, sequence analysis of the insoluble protein should theoretically show one sequence, without losses due to washout. Because elastin is a cross-linked polymer of tropoelastin molecules, sequence analysis of the insoluble protein should theoretically show one sequence, consistent with the idea that the cleavage sites in elastin are readily accessible to enzymatic attack.

Despite the seemingly dichotomous thermodynamics of collagen versus elastin degradation, our assays may not measure precisely equivalent catalytic events. Solubilization of highly cross-linked elastin requires at least two proteolytic cleavages, except when peptides are liberated from the amino or carboxyl terminus of tropoelastin chains. The first cleavage would free a polypeptide chain and the second, solubilizing cleavage would release the peptide and thus occur on a less constrained substrate. In contrast, collagen is solubilized by a single %4:14 cleavage through all three constituent α polypeptide chains. Our data do not eliminate the possibility that the initial cleavages in elastin (occurring before any solubilized peptides are released), especially in the vicinity of the desmosine and isodesmosine bridges on cross-linked tropoelastin molecules, would show a large activation energy and marked deuterium isotope effect.

Identification of Proteinase Cleavage Sites in Elastin Using Protein Sequence Analysis—Previous approaches to characterizing the peptide bond specificity of elastolytic proteinases involved the identification of cleavage sites in soluble proteins or oligopeptides of known sequence. Although much has been learned about the catalytic properties of elastolytic enzymes using this approach, the sites of peptide bond hydrolysis in insoluble elastin may not be predicted from these types of studies because of differences in available sequences on the insoluble protein recognized by the proteinase’s catalytic domain or influences of remote site contacts between enzyme and substrate. To more precisely determine proteinase cleavage sites in elastin, we used protein sequencing to identify new amino termini generated by proteinase-induced peptide bond cleavage in the insoluble protein as shown schematically in Fig. 2. The amino termini in the insoluble residue exposed as the result of proteolytic digestion represent the P1 residues in the carboxyl-terminal direction from the scissile bond. The relative proportion of each amino acid in this position reflects the proteolytic preference of the elastolytic enzyme. In some instances, comparison of results from a few rounds of sequencing with the known primary sequence of elastin can indicate the precise peptide bonds that were cleaved by the enzyme.

The insolubility of elastin makes it ideally suited for sequence analysis using gas phase or pulse liquid sequencers. In contrast to soluble proteins that gradually wash off the immobilizing membrane during the sequencing reaction, small particles of elastin can be placed on the membrane and sequenced without losses due to washout. Because elastin is a cross-linked polymer of tropoelastin molecules, sequence analysis of the insoluble protein should theoretically show one sequence, GGVP . . . , beginning at the amino terminus of each component monomeric chain. However, several amino acids (Gly, Ala, Val, Pro, and Leu being the most prevalent) are evident in this first sequencing cycle. This was not unexpected because the elastin used in this study was purified by hot NaOH. Hot NaOH treatment produces random cleavage of a small number of peptide bonds throughout the polymer. As a result, each hydrolyzed peptide bond produces a new amino terminus for sequencing.

Before we could use this elastin to detect new sequencing...
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**Fig. 2. Strategy for characterizing proteinase cleavage sites in insoluble elastin.** Free amino groups in insoluble elastin resulting from random peptide bond cleavage occurring during purification with hot alkali were blocked using DNF. DNFB reacts covalently with both primary and secondary amino groups and prevents their subsequent reaction with the sequencing reagent phenylisothiocyanate. The blocked elastin was then incubated with a proteinase, and the new amino termini generated by proteinase-induced peptide bond cleavage were determined by protein sequence analysis. The amino termini exposed as the result of proteolytic digestion represent the P₁ residues in the carboxyl-terminal direction from the scissile bond.

sites produced by proteinase cleavage, it was necessary to eliminate the background signal produced during the purification procedure. This was accomplished by incubating purified insoluble elastin with DNFB. DNFB reacts covalently with both primary and secondary amino groups (21) and prevents their subsequent reaction with the sequencing reagent phenylisothiocyanate. Treatment of purified elastin with DNFB effectively blocked all free amino groups such that no significant signal was detected through multiple sequencing cycles.

**Time Course of Digestion**—To determine the time course for elastolysis, radiolabeled counts released into the supernatant were monitored during the reaction, and the insoluble pellet was taken for sequencing when approximately 1, 2.5, 5, 10, and 50% of the total counts were released. The rationale for selecting these data points was that early digestion times should reflect the primary and preferential cleavage sites in the molecule. Gly, Ala, Val, Ile, and Leu were the predominant amino acids detected at each time point, and there was little change in the relative ratio of these amino acids in the first cycle of sequencing up to 10% hydrolysis. Only at 50% hydrolysis were significant differences noted (not shown).

**Cleavage Sites in Elastin for Different Proteinases**—Figs. 3 and 4 show the amino-terminal amino acids found in insoluble elastin following incubation with two serine elastases (PPE and HLE) and three metalloproteinases (MME, HME, and 92-kDa gelatinase). In all cases, the digestion reaction was allowed to proceed until 10% of the total radiolabeled counts were solubilized from the insoluble elastin substrate. The data are presented as a molar ratio normalized to the number of leucine residues detected in each sequencing reaction. To interpret the substrate specificity of the proteinases, it is important to understand that the amino acid residue being detected in the first cycle of amino-terminal sequencing is the amino acid on the carboxyl-terminal side of the scissile bond or the P₁ amino acid. The second round of sequencing would contain amino acids in the P₂ position, the third cycle would contain the P₃ amino acids, and so forth.

Although it is not possible to directly determine the P₁ amino acid using our sequencing approach, it is possible to make predictions using nearest neighbor analysis. Fig. 5 shows nearest neighbor relationships for bovine tropoelastin indicating the frequency with which amino acid pairs occur in the molecule. Because of the unusual sequence properties of elastin, if the P₁ amino acid is known, then it is possible to use this table to predict the amino acids most likely to be in the P₃ position.

**Porcine Pancreatic and Human Neutrophil Elastase**—The amino acid residues detected in insoluble elastin following hydrolysis with PPE and HLE are shown in Fig. 3. In both cases, Gly and Ala were the predominant amino acids with lesser amounts of Val, Phe, Ile, and Leu. There were some interesting differences between the two enzymes, however. HLE digestion resulted in elevated levels of Gly over Ala, whereas approximately equivalent levels of these amino acids were present in elastin degraded by PPE. Valine levels were higher in elastin digested with PPE compared with that digested with HLE.

Past studies have shown that the primary substrate specificity of PPE and HLE mostly resides with the P₁ residue (4). Thus, the P₁ amino acids determined in our study do not directly indicate the proteolytic preferences for peptide bond cleavage in elastin by these two enzymes. Nearest neighbor analysis, however, indicates that approximately 50% of the Gly residues in elastin are preceded by another Gly, Ala, or Val residue. Likewise, Ala is preceded by Gly 27% of the time and by another Ala 45% of the time. These findings predict that the bonds being cleaved by the elastases are predominantly (Gly/Ala/Val)-Gly and (Gly/Ala)-Ala sequences, in agreement with the known specificity of these proteinases for preferential cleavage of the nonbulky amino acids glycine and alanine in the P₁ position.

Proline residues, which comprise almost 15% of the total amino acid residues in elastin, were remarkably absent in the first two rounds of sequencing of cleavage sites generated by both PPE and HLE. Exclusion of proline at the P₁ subsite is predicted from the three-dimensional structure of PPE (22) and has been confirmed experimentally in previous studies (4). Proline can fit into the S₂ subsite of both enzymes, however, so
its absence in the P2 position of cleavage sites in elastin cannot be due to steric factors. Nearest neighbor analysis shows that proline residues are seldom found after glycine or alanine residues, the most prevalent amino acids found at P1. Thus the absence of proline in P2 is simply a statistical circumstance resulting from its sequence distribution in the elastin molecule.

**Elastolytic Metalloproteinases**—The predominant amino acids found in the P1 position following cleavage of insoluble elastin by the 92-kDa gelatinase, HME, and MME were Ala, Gly, Val, Leu, and Ile (Fig. 4). HME and MME were similar in their substrate specificity and showed a stronger preference for Leu/Ile in the P1 position than did the 92-kDa enzyme. In contrast, amino acids found in the first sequencing cycle for the 92-kDa gelatinase somewhat resembled that of serine elastases with high levels of Ala and Val.

Our findings are essentially in agreement with the known specificity of MMPs for hydrophobic, aliphatic residues in the P1 position (23–25). There are, however, some interesting differences between the 92-kDa gelatinase and the two macrophage elastases that warrant comment. HME and MME were Ala, Gly, Val, Leu, and Ile (Fig. 4). HME and MME were similar in their substrate specificity and showed a stronger preference for Leu/Ile in the P1 position than did the 92-kDa enzyme. In contrast, amino acids found in the first sequencing cycle for the 92-kDa gelatinase somewhat resembled that of serine elastases with high levels of Ala and Val.

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**Fig. 4.** Amino-terminal amino acids found in insoluble elastin following incubation with 92-kDa gelatinase, MME, and HME. Insoluble elastin was sequenced after 10% of the total radiolabeled counts had been released into the medium. Shown is the molar ratio of each amino acid at step 1 of the sequencing reaction normalized so that leucine = 1.

**Fig. 5.** Nearest neighbor analysis of bovine tropoelastin. The middle value in each square represents the number of occurrences of each dipeptide sequence in bovine elastin with amino acids at position n along the top and n + 1 towards the amino-terminus along the left-hand side of the matrix. The number in the bottom right of each square is the percentage of the amino acid at the top of the column that occurs in that particular dipeptide sequence. The number in the top left position of each square is the percentage of the amino acid in the left column that is followed by the amino acid in the top column. For example, there are 83 occurrences of the Gly-Val sequence. 86% of the valine residues in elastin are preceded by glycine, and 35% of the glycine residues in elastin are followed by valine. Values less than 0.5 are rounded to 0%.

Degradation of Elastin by Mammalian Metalloproteinases—The predominant amino acids found in the P1 position following cleavage of insoluble elastin by the 92-kDa gelatinase, HME, and MME were Ala, Gly, Val, Leu, and Ile (Fig. 4). HME and MME were similar in their substrate specificity and showed a stronger preference for Leu/Ile in the P1 position than did the 92-kDa enzyme. In contrast, amino acids found in the first sequencing cycle for the 92-kDa gelatinase somewhat resembled that of serine elastases with high levels of Ala and Val.

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The use of Edman sequencing techniques to determine proteolytic cleavage sites in insoluble elastin represents a novel approach to deciphering the peptide bond specificities of elastolytic proteinases. Because the mechanism of elastin solubilization is not well understood, it is difficult to accurately infer the complete peptide bond specificity of an elastolytic proteinase using soluble proteins or synthetic substrates. Although many of our findings are consistent with results previously reported with synthetic model substrates, we have also found new and interesting aspects of the proteolytic susceptibility of elastin in its mature, insoluble state. Being able to detect and characterize peptide bond cleavage in insoluble elastin provides information of greater potential relevance to degradative diseases involving elastin than would otherwise be available.

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