Interaction of Mouse Macrophage Elastase with Native and Oxidized Human α_1-Proteinase Inhibitor

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Abstract

Native and oxidized α_1-proteinase inhibitor (α_1-PI) were compared as substrates for the metalloproteinase macrophage elastase. At substrate concentrations at which native α_1-PI was readily degraded by macrophage elastase, oxidized α_1-PI was hardly degraded at all. Incubation of macrophage elastase with oxidized α_1-PI before the addition of native α_1-PI showed that oxidized α_1-PI was not an inhibitor of macrophage elastase. Competition experiments with up to twofold excess oxidized α_1-PI did not interfere with the degradation of native α_1-PI by macrophage elastase. Sequence analysis of amino acids in degraded native α_1-PI showed that macrophage elastase attacked a single peptide bond between Pro-357 and Met-358, the latter representing the P_1 reactive-site residue of α_1-PI. In oxidized α_1-PI, Met-358 was converted to methionine sulfoxide and macrophage elastase hydrolyzed the bond between Phe-352 and Leu-353. These data suggest that methionine may be the primary cleavage site for macrophage elastase and not leucine, as previously thought.

Introduction

The extracellular elastin matrix is protected from degradation by granulocyte elastase by a critical balance of this serine proteinase (1–3) and its major inhibitor, α_1-proteinase inhibitor (α_1-PI)[1] (4). It has been suggested that a disruption of this balance in favor of the proteinase will predispose an individual to degenerative lung diseases (4–6). Previous studies on the interaction of inflammatory macrophages with α_1-PI have shown that macrophage elastase, a secreted metalloproteinase, can disrupt the balance between granulocyte elastase and α_1-PI by proteolytic inactivation of α_1-PI (7, 8). Proteolysis by macrophage elastase reduces the molecular weight of α_1-PI by 4,000, without the formation of a stable macrophage elastase–α_1-PI inhibitory complex.

Oxidation of the reactive-site methionine residue is an important means of inactivating α_1-PI (9) because the oxidized form of α_1-PI is slow in forming an inhibitory complex with granulocyte elastase. As a result, oxidized α_1-PI becomes susceptible to proteolysis by granulocyte elastase and by other serine proteinases it would normally inhibit.

In this study we determined the relative susceptibility of native and oxidized α_1-PI to degradation by macrophage elastase. Because the proteolysis of native α_1-PI by macrophage elastase may be limited to one or a very few sites, we also identified the cleavage sites.

Methods

Macrophage elastase. Mouse macrophage elastase was purified from culture medium conditioned by thioglycollate-elicited mouse peritoneal macrophages (10). Elastinolytic activity was determined using an insoluble [^{3}H]elastin substrate (10).

α_1-PI. Human α_1-PI was either used directly as purchased from Calbiochem-Behring Corp. (San Diego, CA) or repurified after purchase from Sigma Chemical Co. (St. Louis, MO). The principal contaminant was albumin. Repurification was achieved by fractionation on a Cibracon Blue-CL-6-Sepharose column equilibrated with a buffer consisting of 50 mM Tris-HCl, pH 8.5, and 0.01% NaN_3 (11).

Iodination. Homogeneous α_1-PI was radioiodinated by the method of Bolton and Hunter (12). In this method the lysine residues are labeled, thus avoiding the inactivation of α_1-PI by oxidation of methionine that would result from use of the chloramine-T iodination procedure. ^{125}I-Labeled Bolton-Hunter reagent was purchased from New England Nuclear (Boston, MA). The specific activity of ^{125}I-labeled α_1-PI varied from 0.1 to 0.2 μCi/nmol.

Cell-free oxidation of α_1-PI. Oxidized α_1-PI was prepared by mixing native α_1-PI with a 20-fold molar excess of *N*-chlorosuccinimide (Sigma Chemical Co.). The mixture was incubated at ambient temperature for 20 min, and the reaction was stopped by the addition of 120 mol of methionine per mole of α_1-PI. Sham-oxidized α_1-PI was prepared similarly except that the *N*-chlorosuccinimide and methionine were combined and then added to α_1-PI before the 20-min incubation. The inhibitory capacity of the oxidized α_1-PI was measured by determining the activity of pancreatic elastase remaining after incubation for 15 min with the oxidized α_1-PI. Activity was determined by measuring the rate of cleavage of succinyl-trialanyl-paranitroanalide (13).

Proteolysis of α_1-PI by macrophage elastase. Reaction mixtures containing macrophage elastase and either native or oxidized α_1-PI (1:100 wt/wt) were incubated at 37°C in a buffer consisting of 50 mM Tris-HCl, 50 mM NaCl, and 10 mM $CaCl_2$, pH 8.0, for 1–15 h before the addition of sufficient EDTA to give a final concentration of 20 mM. Inhibition studies were carried out by preincubating macrophage elastase with up to a tenfold molar excess of oxidized α_1-PI for 30 min. Radiolabeled native α_1-PI was then added to determine if macrophage elastase could still cleave it. In competition studies, the concentration of ^{125}I-labeled native α_1-PI was held constant while the concentration of nonradiolabeled oxidized α_1-PI was increased. The ratio of native to oxidized α_1-PI varied from 1:0.1 to 1:2. Sufficient amounts of proteolyzed α_1-PI for sequence analysis were obtained by incubating either native or oxidized α_1-PI at 53 μM with 0.13 μM macrophage elastase at 37°C. Additional α_1-PI was added every 30 min for the next 2 h, giving a final concentration of 65 μM. The final ratio of enzyme to substrate was 1:500 (mol/mol). By this strategy we maintained an optimal cleavage rate of α_1-PI without increasing the concentration of macrophage elastase.

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1. *Abbreviations used in this paper:* α_1-PI, α_1-proteinase inhibitor; PTH, parathyroid hormone.

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After incubation, the final mixture was diluted to 5 ml with water, dialyzed against water, and lyophilized before sequence analysis.

Electrophoresis. Sodium dodecyl sulfate (SDS)–polyacrylamide gradient gel electrophoresis was performed according to the method of Laemmli (14) as modified (7). After electrophoresis the protein bands were stained with 0.05% (vol/vol) Coomassie Blue R250 in methanol. Radiolabeled proteins were located by autoradiography on Kodak X-Omat R x-ray film (15).

Sequence analysis. Proteolyzed samples of native and oxidized α_1-PI (28–30 nmol) were sequenced with a Beckman 890C spinning-cup sequenator. Usually, ten cycles were carried out for each sample. The individual fractions were converted to stable parathyroid hormone (PTH) amino acids manually (16), and 5% of each fraction was analyzed for 15 min isocratically on a DuPont Zorbax-ODS high-pressure liquid chromatography column in 6 mM NaOAc, pH 4.5, with 42% acetonitrile. The PTH amino acids were detected at 254 nm, except for PTH-Ser and PTH-Thr, which were detected at 313 nm. Residues that could not be unequivocally identified were confirmed by back-hydrolysis of the PTH derivative (17), followed by amino acid analysis. The following protein samples were analyzed by this procedure: (*a*) native α_1-PI; (*b*) native α_1-PI cleaved by macrophage elastase; (*c*) oxidized α_1-PI; and (*d*) oxidized α_1-PI cleaved by macrophage elastase. Samples (*a*) and (*d*) were prepared twice with different ratios of proteinase to substrate, and each sample was analyzed separately.

Results

Comparison of native and oxidized α_1-PI as substrates for macrophage elastase. Because our previous studies were limited to studying the interaction of mouse peritoneal macrophage elastase and rabbit alveolar macrophages with native α_1-PI (7, 8), we have investigated the interaction of purified mouse peritoneal macrophage elastase with oxidized α_1-PI. To determine their relative sensitivity to proteolysis by macrophage elastase, we carried out separate reactions with native and oxidized α_1-PI. In each case the ratio of macrophage elastase to α_1-PI was 1:40 (mol/mol). Under these conditions, native α_1-PI was readily degraded, whereas oxidized α_1-PI was degraded only slightly (Fig. 1). Reaction mixtures containing sham-oxidized α_1-PI appeared to be identical to those containing native α_1-PI. Therefore, oxidized α_1-PI was a comparatively poor substrate for macrophage elastase.

Potential inhibition of macrophage elastase by oxidized α_1-PI. The inability of macrophage elastase to degrade oxidized α_1-PI could be due to an inhibitory effect of oxidized α_1-PI on macrophage elastase. To examine this possibility, we carried out two experiments. In the first, 2 pmol of macrophage elastase was incubated for 30 min at ambient temperature with up to 20 pmol of oxidized α_1-PI before incubation with 74 pmol of ^{125}I-labeled native α_1-PI. When the reaction mixtures were analyzed by SDS–polyacrylamide gel electrophoresis, there was no inhibition of the proteolysis of native α_1-PI (Fig. 2). To determine if oxidized α_1-PI might act as a competitive inhibitor, increasing amounts of oxidized α_1-PI were mixed with fixed amounts of ^{125}I-labeled native α_1-PI and macrophage elastase. The ratio of native to oxidized α_1-PI varied from 1:0.1 to 1:2. When these reaction mixtures were analyzed by SDS–polyacrylamide gel electrophoresis, there was no interference with the proteolysis of native α_1-PI (Fig. 3). These data suggest that oxidized α_1-PI is not an effective inhibitor of macrophage elastase.

Analysis of the cleavage site in α_1-PI. The limited proteolysis of native α_1-PI by macrophage elastase always resulted in a reduction of the molecular weight of α_1-PI by 4,000 without the formation of any other intermediate-sized fragments. This suggested that macrophage elastase cleaves α_1-PI at only one site. As shown in Table I and Fig. 4, data from sequence analysis indicated two NH_2-terminals. After eliminating the known α_1-PI NH_2-terminal sequence, it is evident that the other sequence must have been generated by cleavage between Pro-357 and Met-358, the reactive-site P_1 residue. According to the known amino acid sequence of α_1-PI, a cleavage at this site would remove a fragment with a molecular weight of 4,200. This compares favorably with our estimate, based on migration in SDS–polyacrylamide gels, of a reduction in molecular weight of 4,000.

In α_1-PI that has been inactivated by oxidation, the reactive-site residue Met-358 is converted to methionine sulfoxide (9). It is likely that methionine sulfoxide interferes with the binding of macrophage elastase to oxidized α_1-PI, and thus retards the cleavage amino to methionine sulfoxide–358. As noted in Fig. 1, there was slight proteolysis of oxidized α_1-PI. By increasing the concentration of oxidized α_1-PI without increasing the concentration of macrophage elastase, a sufficient amount of the oxidized cleavage fragment was accumulated to permit sequence analysis. There was no cleavage at methionine sulfoxide–358. Rather, the bond between Phe-352 and Leu-353 was cleaved (Table I and Fig. 4). Because oxidized α_1-PI is not as readily proteolyzed as native α_1-PI, the cleavage amino to Leu-353 is

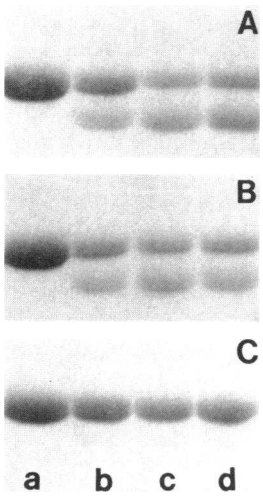

Figure 1. Proteolysis of (*A*) native α_1-PI, (*B*) sham-oxidized α_1-PI, and (*C*) oxidized α_1-PI, analyzed on SDS–polyacrylamide electrophoretic gels. (*a*) α_1-PI incubated for 15 h at 37°C without macrophage elastase; (*b–d*) reaction mixtures containing macrophage elastase and α_1-PI (1:40 mol:mol) incubated at 37°C for (*b*) 1 h, (*c*) 5 h, or (*d*) 15 h.

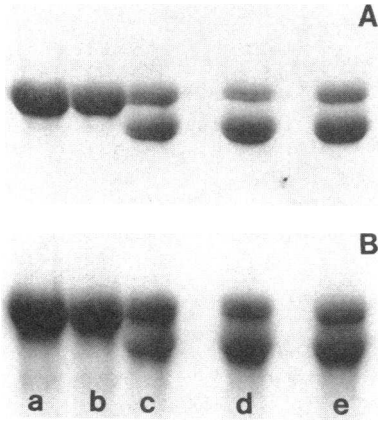

Figure 2. Effect of oxidized α_1-PI on the activity of macrophage elastase. (*A*) Coomassie Blue staining for total protein. (*B*) Autoradiograph of ^{125}I-labeled native α_1-PI only. Various amounts of nonradiolabeled oxidized α_1-PI were incubated for 30 min with macrophage elastase before the addition of 74 pmol of ^{125}I-labeled native α_1-PI, incubation at 37°C for 5 h, and analysis on SDS–polyacrylamide gels. The final ratios of macrophage elastase to oxidized α_1-PI to ^{125}I-labeled native α_1-PI (mole/mole/mole) were (*a*) 0:0:74, (*b*) 0:2:74, (*c*) 2:0:74, (*d*) 2:1:74, and (*e*) 2:20:74.

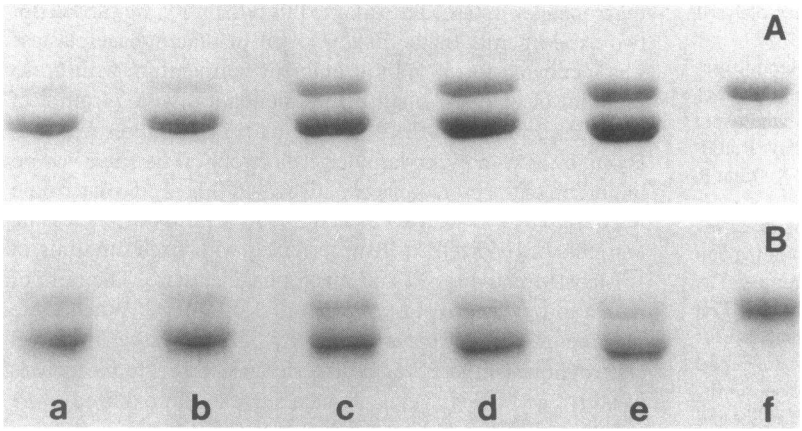

Figure 3. Oxidized α_1-PI as a competitive substrate for macrophage elastase. (*A*) Coomassie Blue staining for total protein. (*B*) Autoradiograph of the ^{125}I-labeled native α_1-PI only. SDS–polyacrylamide gels contained reaction mixtures in which the amounts of macrophage elastase (1 pmol) and native α_1-PI (37 pmol) were held constant and the amount of oxidized α_1-PI was varied from 0 to 74 pmol. The ratios of ^{125}I-labeled native α_1-PI to oxidized α_1-PI in each lane were (*a*) 1:0, (*b*) 1:0.1, (*c*) 1:0.5, (*d*) 1:1, and (*e*) 1:2. (*f*) 37 pmol of native α_1-PI incubated without macrophage elastase.

probably a secondary site that would not normally be cleaved at lower substrate concentrations or when the Met-358 site is open. Cleavage at the Leu-353 site would result in a reduction of the molecular weight of the oxidized α_1-PI by 4,500. It is unlikely that the difference between the 4,200-mol-wt fragment of native α_1-PI and the 4,500-mol-wt fragment of oxidized α_1-PI would be detected on SDS–polyacrylamide electrophoretic gels.

Discussion

The proteolysis of α_1-PI by macrophage elastase is based on the recognition of the reactive-site residue Met-358. That this is the preferred site for macrophage elastase binding in the native molecule explains the poor interaction of macrophage elastase with oxidized α_1-PI. Presumably, the conversion of Met-358 to methionine sulfoxide after oxidation prevents macrophage elastase from binding and cleaving at that site. Our data show that oxidized α_1-PI does not interfere with the proteolysis of native α_1-PI by serving as a competitive inhibitor.

The residue recognized by metalloproteinases contributes the amino group to the peptide bond that is cleaved (10, 18), which is why macrophage elastase, a metalloproteinase, recognizes Met-358 rather than Pro-357. We were surprised that this bond was the primary cleavage site because previous work showed that macrophage elastase cleaved the peptide bond amino to leucine and isoleucine residues (10, 18). However, those studies were done using elastin (10) and insulin B-chain (18) as substrates, neither of which contains methionine residues. The data presented here suggest that leucine may be a secondary cleavage site and that methionine may be the primary site. That macrophage elastase does not degrade elastin as effectively as the serine elastases may be due to the lack of primary cleavage sites (i.e., methionine) for macrophage elastase in elastin.

Other proteinases besides macrophage elastase degrade α_1-PI (19–23). Papain and some serine proteinases cleave the peptide bond between Met-358 and Ser-359 (24). *Pseudomonas aeruginosa* elastase, also a metalloproteinase, cleaves the same bond as macrophage elastase (25), while another metalloproteinase from the venom of *Crotalus adamanteus* recognizes Met-351 and cleaves the Met-351–Ala-350 bond while leaving the Met-358–Pro-357 bond intact (26). That the peptide bonds amino to Met-351 and Met-358 are cleaved by different proteinases is evidence that both sites are normally available for proteolysis. The specificity of macrophage elastase for Met-358 rather than Met-351 may be due to the Ala-Ala-Gly-Ala sequence (residues 347–350) immediately preceding Met-351 (Fig. 4). Because macrophage elastase does not cleave alanine-rich peptide substrates (10, 27), these nonpolar amino acid residues may prevent macrophage elastase from recognizing Met-351.

Tissue metalloproteinases may play an important role in the turnover of connective tissue. Metalloelastase activity has been identified in the secretions of cultured human alveolar macrophages (28) and in human lung lavage fluid (29). In both cases these elastase activities were not inhibited by α_1-PI, and in one study (29) it was suggested that elevated elastase concentrations decreased the concentration of antigenic α_1-PI. The secretion of

Table I. Sequence Analysis of Native and Oxidized α_1-PI before and after Degradation by Macrophage Elastase

	Amino acids encountered			
Step	Native α_1-PI	Oxidized α_1-PI	Native α_1-PI degraded by macrophage elastase	Oxidized α_1-PI degraded by macrophage elastase
1	Glu	Glu	Glu, Met	Glu, Leu
2	Asp	Asp	Asp, Ser	Asp, Glu
3	Pro	Pro	Pro, Ile	Pro, Ala
4	Gln	Gln	Gln, Pro	Gln, Ile
5	Gly	Gly	Gly, Pro	Gly, Pro
6	Asp	Asp	Asp, Glu	Asp, Met
7	Ala	Ala	Ala, Val	Ala, Ser
8	Ala	Ala	Ala, Lys	Ala, Ile
9	Gln	Gln	Gln, Phe	Gln, Pro
10	Lys	Lys	Lys, Asn	Lys, Pro

Using the yields at steps 4 and 9 (Gln) of undegraded native α_1-PI, a low repetitive yield of 89% was calculated.

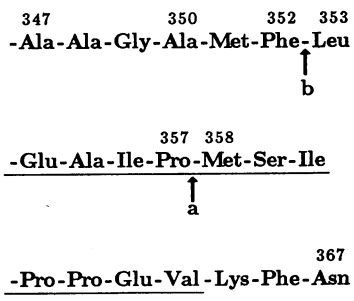

Figure 4. Partial sequence of α_1-PI. The underlined residues constitute the active site of α_1-PI. The peptide bonds cleaved by macrophage elastase are indicated for native α_1-PI (*arrow a*) and oxidized α_1-PI (*arrow b*).

elastase by rabbit alveolar macrophages in culture was correlated with the proteolytic inactivation of α_1-PI (8). It is possible that other tissue metalloproteinases besides macrophage elastase may also degrade and inactivate α_1-PI (30). In certain pathologic conditions, exogenous metalloproteinases can inactivate α_1-PI (20–23) and other proteinase inhibitors (31). Therefore, tissue metalloproteinases and exogenous metalloproteinases must be considered regulatory variables that could disrupt the balance between serine proteinases and proteinase inhibitors in vivo.

More intriguing is the possibility that oxidation of α_1-PI may, in some circumstances, play a protective role by preventing degradation of α_1-PI by metalloproteinases such as macrophage elastase. For example, although proteolysis causes irreversible inactivation of α_1-PI, oxidation can theoretically be reversed by an endogenous reductive mechanism (32). If this were shown to be the case in vivo, then one advantage in having methionine as the reactive-site residue of α_1-PI would be to avoid proteolysis.

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