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Proteolytic inactivation of human α_1 antitrypsin by human stromelysin

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α_1Antitrypsin (α_1AT) is the main physiological inhibitor of neutrophil elastase, a serine protease which has been implicated in tissue degradation at inflammatory sites. We report here that the connective tissue metalloproteinase, stromelysin, cleaved α_1AT (54 kDa), producing fragments of approximately 50 kDa and 4 kDa, as shown by gel electrophoresis. The cleavage of α_1AT was accompanied by inactivation of its elastase inhibitory capacity. Isolation of the 4 kDa fragment by reversed-phase HPLC, followed by N-terminal amino acid sequencing, demonstrated that the cleavage of α_1AT occurred at the Pro^{357}-Met^{358} (P_2-P_1) peptide bond, one peptide bond to the N-terminal side of the inhibitory site. We suggest that stromelysin may potentiate the activity of neutrophil elastase by proteolytically inactivating α_1AT.

α_1 Antitrypsin; Proteolytic inactivation; Stromelysin; Neutrophil elastase; Inflammation; Human

1. INTRODUCTION

Human plasma α_1 antitrypsin (α_1AT) inhibits the tissue-degrading activity of elastase, a serine protease released by infiltrating neutrophils at sites of inflammation, by forming a stable 1:1 complex (reviewed in [1]). It has been suggested that in certain inflammatory conditions, such as pulmonary emphysema and rheumatoid arthritis, tissue destruction is caused by an α_1AT-elastase imbalance, since inflammatory fluids taken from patients with these diseases contain a significant proportion of α_1AT which is inactive as an inhibitor (reviewed in [2]).

This inactivation has been attributed to the oxidation of a critical reactive centre methionine residue (at position 358), mediated by the neutrophil myeloperoxidase-Cl^-/H_2O_2 system [2]. Recently, however, it has been shown that human neutrophil collagenase, a metalloproteinase, is capable of inactivating human α_1AT by catalytic cleavage at the Phe^{352}–Leu^{353} peptide bond [3,4]. It was therefore suggested that collagenase may contribute to the persisting activity of neutrophil elastase.

Human stromelysin is a connective tissue metalloproteinase released by cells such as human lung fibroblasts, rabbit alveolar macrophages and human synovial fibroblasts in response to inflammatory mediators such as interleukin-1 and tumour necrosis factor-α (reviewed in [5]). Stromelysin has been shown to cleave α_1AT [6], although no detailed studies were undertaken. In particular, it is not known whether the cleavage of α_1AT is accompanied by inactivation and the position of the peptide bond hydrolysis has not been ascertained. Here we report that recombinant human stromelysin has the capacity to inactivate human plasma α_1AT by cleaving the polypeptide chain near to the reactive site at the Pro^{357}–Met^{358} peptide bond.

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Abbreviations: α_1AT, α_1-antitrypsin; APMA, 4-aminophenylmercuric acetate

2. MATERIALS AND METHODS

2.1. *Activation of latent stromelysin and assay of activity*

Human recombinant prostromelysin was prepared as described earlier [7]. We are grateful to Dr A. Docherty and colleagues at Celltech (Slough, UK) for expression of the recombinant protein. Prostromelysin was activated by incubating with 4-aminophenylmercuric acetate (APMA; 1–2 mM) for 2–4 h at 37°C. Conversion of prostromelysin to stromelysin was confirmed by 10% SDS-PAGE with silver staining and activity assays [7]. Although trypsin is a more efficient activator of prostromelysin than APMA [7], trypsin was found to interfere in the assay of elastase inhibitory capacity.

Activated stromelysin was assayed using [^{14}C]acetylated β-casein at 37°C for 1 h or 4 h [7]. One unit of stromelysin degrades 1 μg of β-casein per min at 37°C.

2.2. *Incubation of α_1AT with stromelysin*

Human plasma α_1AT (Novabiochem, Nottingham, UK) was incubated at 37°C with APMA-activated stromelysin in 100 mM Tris-HCl, containing 30 mM $CaCl_2$, pH 7.6. The concentration of stromelysin and the incubation times were varied as indicated.

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2.3. Electrophoresis

SDS-PAGE (10–20% gradient polyacrylamide mini gels) and transblotting to nitrocellulose were performed as described previously [8]. Immunostaining was with sheep anti-human α_1AT antibody (Unipath, Bedford, UK) and peroxidase-conjugated rabbit anti-sheep immunoglobulin (Dako, High Wycombe, UK). Bands were visualised by developing with diaminobenzidine tetrahydrochloride.

2.4. Assay of elastase inhibitory capacity of α_1AT

Aliquots were taken from the stromelysin digests, at the times given, and assayed for elastase inhibitory capacity by a kinetic spectrophotometric method [9]. The digest (10 μl) was added to 965 μl of 0.2 M Tris-HCl buffer (pH 8.0), containing 0.1% Triton X-100, in a reaction cuvette. Porcine pancreatic elastase (14 μl, final concentration 14 nM; Sigma, Poole, UK) was added to this solution and the mixture was incubated at 37°C for 15 min. The synthetic substrate *N*-succinyl-Ala-Ala-Ala *p*-nitroanilide (Sigma) was added (11 μl; final concentration 1.2 mM) and the residual elastase activity was measured (change in A_{410} per min).

2.5. Purification and N-terminal sequencing of low-M_r cleavage fragment

Fragments resulting from the digestion of α_1AT by stromelysin were separated on a reversed-phase PLRP-S 300 Å 8 μm column (250×4 mm; Polymer Laboratories Ltd, Church Stretton, Shrops., UK) with an acetonitrile gradient (0–100%; flow rate 1 ml/min) in 0.1% trifluoroacetic acid. Peaks were monitored at 214 nm and aliquots from each were dried under vacuum and subjected to 10–20% SDS-PAGE with silver staining. The low-M_r fragment purified and identified in this fashion was subjected to N-terminal amino acid sequencing (6 cycles) by the automated gas-phase method, using an Applied Biosystems model 477A sequencer (Dr L. Packman, Cambridge University Protein Sequencing Facility).

3. RESULTS AND DISCUSSION

The stromelysin-catalysed hydrolysis of a single peptide bond within the polypeptide chain of α_1AT was demonstrated by gel electrophoresis, reversed-phase HPLC and N-terminal sequencing. A parallel inactivation of the elastase inhibitory activity of α_1AT was also shown.

On incubation of human plasma α_1AT with APMA-activated recombinant human stromelysin, intact α_1AT (approximately 54 kDa) was cleaved to give a new band of approximately 50 kDa; the time course of this cleavage, analysed by SDS-PAGE and immunoblotting is shown in Fig. 1A. The corresponding low-M_r cleavage fragment (approximately 4 kDa) was not detected by immunoblotting under the conditions used, but was visible on silver-stained gels (not shown) when higher concentrations of sample were applied. No other bands were detected.

When the α_1AT-degrading activity of the above stromelysin preparation was tested at a recombinant protein/α_1AT ratio of 1:10 (w/w), less than 50% of the α_1AT was cleaved after 4 h (Fig. 1B). This activity is lower than that of rabbit-synovial stromelysin: at an enzyme/α_1AT ratio of 1:200 (w/w), approximately 50% cleavage of α_1AT was obtained after 4 h [6]. It is known [7] that the casein-degrading activity of human stromelysin (both natural and recombinant) is about ten times lower than that of the rabbit enzyme. The reasons for this are unclear, but activation of purified human prostromelysin with APMA does not appear to give complete conversion to the active form [7]. In the study by Chin et al. [6] mentioned above, a crude isolate of stromelysin from rabbit synovial fibroblasts was activated with APMA to allow the isolation of all active enzymes in the final purification steps. Furthermore, when we activated a second batch of prostromelysin in an identical fashion to the first, a higher activity was obtained: almost all of the α_1AT was cleaved in 4 h at a 1:10 (w/w) ratio (Fig. 1B). For com-

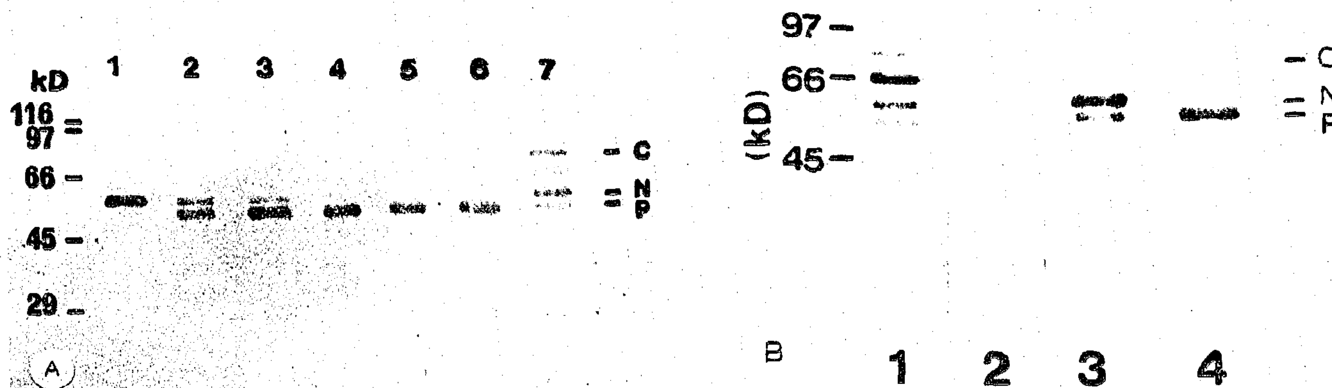

Fig. 1. Cleavage of α_1AT by stromelysin. (A) Time course. α_1AT (28.8 μg) was mixed with APMA-activated stromelysin (17.20 units; specific activity 665 units/mg) and incubated at 37°C. A control sample contained α_1AT alone. Aliquots were removed after various time intervals, added to SDS sample buffer and boiled for 5 min. Samples were then analysed by 10–20% gradient SDS-PAGE followed by immunoblotting with an anti-human α_1AT antibody (see section 2.3). (Lane 1) α_1AT incubated alone. (Lanes 2–6) α_1AT incubated with stromelysin for: (2) 15 min; (3) 30 min; (4) 90 min; (5) 180 min; (6) 360 min. (Lane 7) α_1AT incubated with an equimolar concentration of human neutrophil elastase (Novabiochem) for 5 min at 37°C. (B) Comparison of two stromelysin batches. (Lane 1) α_1AT incubated with an equimolar concentration of human neutrophil elastase for 5 min at 37°C; (lane 2) α_1AT alone incubated for 4 h at 37°C; (lanes 3 and 4) α_1AT incubated with two different batches of APMA-activated stromelysin at a recombinant protein/α_1AT ratio of 1:10 (w/w) for 4 h at 37°C: (lane 3) batch 1 stromelysin (665 units/mg); (lane 4) batch 2 stromelysin (856 units/mg). Numbers to the side of each panel indicate the positions of molecular mass marker proteins, whilst letters indicate: C, α_1AT-elastase complex (83 kDa); N, native α_1AT (54 kDa); P, high-M_r cleavage fragment of α_1AT (50 kDa).

parison, when human neutrophil collagenase was incubated with α_1AT at a ratio of 1:200 (w/w), 50% of the α_1AT was cleaved after 6 h [4].

The cleavage of α_1AT by stromelysin was accompanied by a loss of the elastase inhibitory capacity of α_1AT. The time course of the proteolytic inactivation of α_1AT in the presence of various concentrations of stromelysin, determined by kinetic spectrophotometric analysis, is shown in Fig. 2. The rate of α_1AT inactivation was dependent on the concentration of stromelysin.

When the low-M_r fragment, isolated by reversed-phase HPLC (Fig. 3) was subjected to six cycles of N-terminal sequencing, the sequence obtained was Met-Ser-Ile-Pro-Pro-Glu. This indicates that cleavage occurred at the Pro^{357}-Met^{358} (P_2-P_1) peptide bond, one peptide bond to the N-terminal side of the reactive centre. The reactive centre of α_1AT is situated within an exposed loop region (residues 348–363), which is known to be protease-sensitive [1].

The Pro^{357}-Met^{358} cleavage site in α_1AT is also shared by *Pseudomonas aeruginosa* elastase [10] and mouse macrophage elastase [11]. Furthermore, whilst the primary site of α_1AT cleavage by human neutrophil collagenase is Phe^{352}–Leu^{353} (P_7-P_6), a secondary cleavage at Pro^{357}–Met^{358} occurs after prolonged incubation [3]. Mouse macrophage elastase is regarded as the inflammatory cell counterpart to stromelysin, because the two enzymes have similar substrate specificities [5,11]. It has already been suggested that macrophage elastase may contribute to tissue destruction in inflammatory conditions such as emphysema by proteolytically inactivating α_1AT [11]. In the light of our present findings, we suggest that stromelysin may play a similar pathologic role. Consistent with such a role for stromelysin, is the recent observation that this enzyme is present at high concentrations in rheumatoid synovial fluids, being approximately 20-fold more concentrated than collagenase (M. Lark, Merck Sharp & Dohme, Rahway, NJ, manuscript submitted). Within the rheumatoid joint, the negatively charged cartilage surface interferes with the ability of α_1AT to inhibit elastase, since α_1AT carries a net negative charge at neutral pH [12]. Thus, the proteolytic inactivation of α_1AT by stromelysin-releasing cells present within the cartilage or immediately adjacent to the cartilage, may be particularly significant.

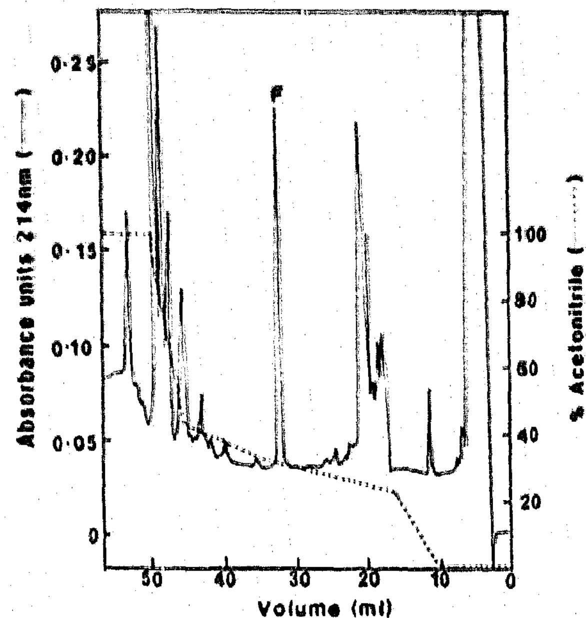

Fig. 3. Reversed-phase HPLC of α_1AT degradation products. APMA-activated stromelysin (24.9 units) was mixed with 100 µg of α_1AT and incubated for 2 h at 37°C. The resulting digest was mixed with guanidine-HCl (final concentration 6 M) before injection onto the column. F, low-M_r cleavage fragment.

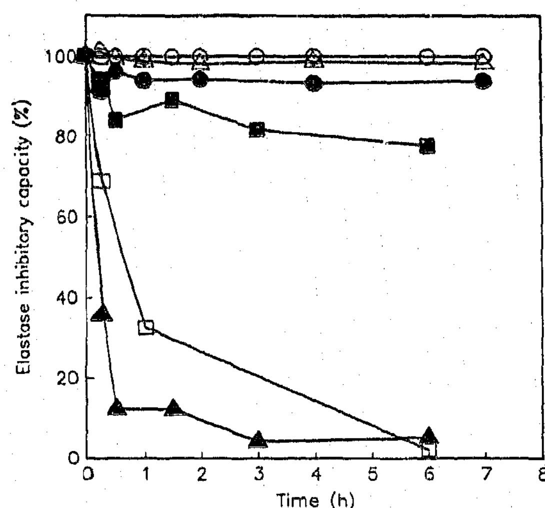

Fig. 2. Time course of inactivation of α_1AT by various amounts of added stromelysin. α_1AT (28.8 µg) was mixed with the following amounts of APMA-activated stromelysin (665 units/mg): 0.34 (△), 1.72 (●), 3.44 (■), 8.60 (□) and 17.20 units (▲). A control sample (○) contained α_1AT alone. The reaction mixtures were incubated at 37°C, aliquots were removed after various time intervals and the elastase inhibitory capacity was measured as described in section 2.4.

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