THE STOICHIOMETRY OF INHIBITION OF HUMAN PANCREATIC ELASTASE 2 BY HUMAN ALPHA$_1$-ANTITRYPSIN [+]

Michael Tyndall, Corey Largman, James W. Brodrick, and Michael C. Geokas

Department of Medicine, Martinez Veterans Administration Hospital, Martinez, California 94553 and the Department of Medicine, UC-Davis School of Medicine, Davis, California 95616

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Summary: A 1:1 stoichiometry of inhibition of human pancreatic elastase 2 by human α_1-antitrypsin has been determined. The molar binding ratio was calculated using the results of a titration curve for elastase 2 inhibition by α_1-antitrypsin, an experimentally determined concentration of active sites in human elastase 2, and an extinction coefficient calculated from ultracentrifugation studies using interference optics.

INTRODUCTION

Elastase is unique among the pancreatic serine endopeptidases in its ability to hydrolyze elastin (1). For this reason, pancreatic elastase has been suggested as a cause of tissue lesions in the lung (2). Lesions resembling pulmonary emphysema have been experimentally induced by administration of porcine pancreatic elastase to either hamsters or dogs (3). We have previously reported on the isolation, purification, and characterization of two human pancreatic elastases (4). We have also demonstrated that the major form of this enzyme (elastase 2) is present in sera of normal individuals in easily detectable concentrations, as measured by radioimmunoassay (5). Elucidation of the interaction of this enzyme with the protease inhibitors present in human blood serum is necessary in order to formulate possible mechanisms of pancreatic elastase-mediated tissue injury.

Abbreviations: ^{3}H-DIFP, ^{3}H-diisopropylfluorophosphate;TAME, tosyl-L-arginine methyl ester; RBB-Hide, Remazol Brilliant Blue dyed hide powder; and p-NPGB, p-nitrophenyl p'-guanidinobenzoate HCl.

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Human α_1-antitrypsin is a glycoprotein which has been shown to be responsible for approximately 90% of the trypsin inhibitory capacity of plasma (6). The inhibitor exhibits a broad specificity and is present in high concentration in normal human serum. A genetically determined severe α_1-antitrypsin deficient state has been described which is associated with premature development of pulmonary emphysema (7).

Johnson et al (8) have presented evidence for a 2:1 stoichiometry of binding of porcine trypsin by α_1-antitrypsin. These results are in marked contrast to previous reports, which indicated a 1:1 molar complex between α_1-antitrypsin and various serine proteases (9). We therefore undertook a careful determination of the stoichiometry of binding of human pancreatic elastase 2 to human α_1-antitrypsin, the results of which are presented in this report.

MATERIALS AND METHODS

^{3}H-diisopropylfluorophosphate (^{3}H-DIFP) was obtained from Amersham Searle, Inc. Bovine trypsin employed in the determination of inhibitory capacity of α_1-antitrypsin was obtained from Worthington Biochemical Co. Fresh human plasma was obtained from the Martinez Veterans Administration Hospital.

Human α_1-antitrypsin was prepared by the method of Pannell et al (11). This procedure included passage of fresh human plasma through a blue-dextran conjugated-Sepharose 4B column in order to remove serum albumin. Material eluting from this column was subjected to ammonium sulfate precipitation followed by DEAE-cellulose chromatography at pH 8.8 and at pH 6.5. The resulting α_1-antitrypsin migrated as a single band in discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis and inhibited 60.3 units of tryptic activity per mg of α_1-antitrypsin. Tryptic activity was determined by measuring the hydrolysis of TAME. A molecular weight of 53,000 daltons and an extinction coefficient of $\varepsilon^{280}_{1\%} = 5.30$ (11) were employed in the calculation of binding ratios.

Human pancreatic elastase 2 was purified to homogeneity by the previously described procedure (4). The enzyme migrated as a single band in analytical polyacrylamide gel electrophoresis at pH 4.5 and 2.3 and in discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A molecular weight of 25,000 daltons (4) was used for calculations of molar binding ratios. An extinction coefficient for elastase 2 of $\varepsilon^{280}_{1\%} = 24.9$ was determined using interference optics as described by Babul and Stellwagen (12).

Active Site Titration of Elastase 2. Determination of the active site concentration of the elastase 2 solution employed for molar binding ratios was performed by labeling the enzyme with ^{3}H-DIFP as described by Robinson et al (13). The specific activity of the ^{3}H-DIFP employed was determined by labeling an aliquot of a solution of bovine trypsin in which the concentration of active

sites was independently determined by titration with p-NPGB using the method of Chase and Shaw (14). To a 1 ml solution of elastase 2 (0.2 mg) in 0.05 M Tris·HCl (pH 8.0) were added 5 µl of ^{3}H-DIFP (10^5 cpm) in dry isopropanol. The reaction mixture was incubated for 1.5 h at 37° and then applied to a 1.2 x 10 cm column of Sephadex G-25 equilibrated with the reaction buffer. The ^{3}H-DIP-elastase 2 was eluted as a symmetrical peak and the ratio of cpm per mg of protein was determined for each of the peak fractions. A parallel reaction was performed with 0.3 ml bovine trypsin (0.43 mg, 1.75×10^{-8} moles/mg, determined with p-NPGB) and treated in a similar manner. Using the specific activity of ^{3}H-DIFP obtained from the concentration of active sites of the trypsin solution, the concentration of active sites in elastase 2 was calculated to be 1.60×10^{-5}M. This value corresponded to an approximately 99% active solution of elastase 2, based upon an extinction coefficient of 2.49 $(mg/ml)^{-1}$ and a molecular weight of 25,000 daltons (4).

Elastase 2 Activity Measurements. Previous work (4) has shown that RBB-hide is a good general proteolytic substrate for elastase 2. This substrate was prepared as described previously (15) and employed at a final concentration of 10 mg/ml in assay buffer consisting of 0.1 M Tris·HCl (pH 8.2) containing 25 mM $CaCl_2$, 1 mM Triton X-100.

Inhibition Assays. Incubation of elastase 2 with α_1-antitrypsin and the subsequent determinations of proteolytic activity were designed to maintain a 2 ml total assay volume containing 0.32 µg of elastase 2. Elastase 2 (0.32 µg in 10 µl) in 10 mM sodium phosphate (pH 6.5) was incubated in duplicate with 0 to 1.48 µg of α_1-antitrypsin in a total volume of 2.0 ml of assay buffer. After a 45 min incubation at 23°, the contents of each tube was carefully transferred to an assay tube containing 10 mg of RBB-hide. The RBB-hide assay mixture was incubated for 30 min at 37°. The assay tubes were then placed in ice water, centrifuged at 6,000 rpm for 10 min at 4°, and finally kept on ice for a minimal period until the absorbance of each assay mixture at 595 nm could be determined. The inhibitor-enzyme ratios at complete inhibition were determined by extrapolating the linear portion of the dose-response curve for inhibition as described by Laskowski and Sealock (16). In all cases, the linear part of the inhibition curve extended to 85% inhibition or more.

RESULTS AND DISCUSSION

A molar extinction coefficient for human pancreatic elastase 2 of $\varepsilon^{280}_{1\%} = 24.9$ was determined by ultracentrifugation studies. This value is higher than the extinction coefficient of $\varepsilon^{280}_{1\%} = 20.2$ reported for porcine elastase (17). This finding probably reflects a greater content of tryptophan in the human enzyme than is found in the porcine enzyme (17).

Initial inhibition experiments were performed with concentrated incubation mixtures which were diluted approximately ten-fold for assay of elastolytic activity. Although these data could be extrapolated to a binding ratio of approximately 1:1, the curve was non-linear at low inhibitor concentrations. In order to minimize possible effects produced by dilution of the enzyme

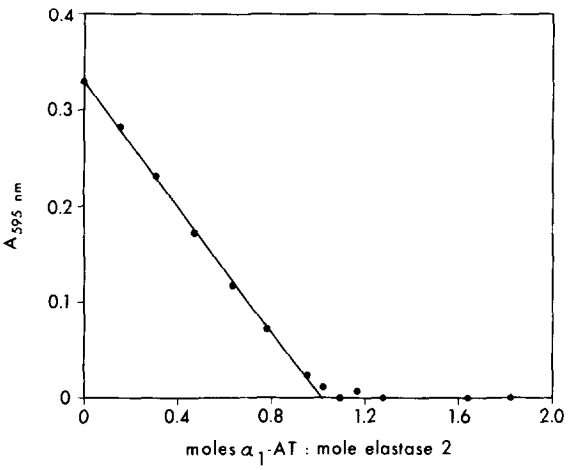

Figure 1. TITRATION OF HUMAN ELASTASE 2 WITH HUMAN α_1-ANTITRYPSIN. A constant amount of elastase 2 was incubated with varying amounts of α_1-antitrypsin (0-1.8 fold molar excess) in a constant volume of 2 ml of assay buffer as described in Methods. Following a 45 min incubation period, the mixtures were assayed for proteolytic activity on RBB-hide as described in Methods.

inhibitor incubation mixtures for assay, experiments were performed in which the enzyme and inhibitor were incubated under assay conditions. When such an experiment was performed using a 45 min incubation period, a linear inhibition curve of elastase 2 by α_1-antitrypsin was obtained for the entire range of inhibitor employed, as shown in Figure 1. Data from this curve was combined with the extinction coefficient and active site titration values determined for elastase 2 to calculate a binding ratio of 0.98 mole of elastase 2 per mole of α_1-antitrypsin. When this experiment was performed using 15 min incubation periods, the resulting curve was non-linear, indicating that the reaction of enzyme with the inhibitor was not complete.

The results of these experiments provide evidence for a 1:1 complex for pancreatic elastase 2 and α_1-antitrypsin. This result contradicts the quantitative data reported by Johnson *et al* (8) who found that α_1-antitrypsin bound two moles of porcine trypsin. The same group also reported a two to one binding for human trypsin as well as bovine chymotrypsin to α_1-antitrypsin (18). Recently, this group has suggested that leukocytic elastase is bound by

α_1-antitrypsin in a 1:1 complex (19). These authors also retracted their earlier results with porcine trypsin, but no data were presented to demonstrate a 1:1 complex for either enzyme. On the other hand, Kress and Laskowski (9) have reported a 1:1 binding of bovine trypsin by α_1-antitrypsin. Moroi and Yamasaki have isolated a complex of bovine trypsin and human α_1-antitrypsin and have calculated a 1:1 molar binding ratio on the basis of amino acid composition data (10).

In contrast to previous work, the present study employed highly purified, essentially 100 per cent active elastase 2 and human α_1-antitrypsin. Data were obtained which demonstrate a 1:1 binding of enzyme by the inhibitor. This result suggests that the concentration of α_1-antitrypsin in human blood plasma is probably twice that suggested by Johnson et al (8).

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