Biochimica et Biophysica Acta, 391 (1975) 193—200

BBA 67468

THE INTERACTION OF α-1-ANTITRYPSIN WITH CHYMOTRYPSIN, TRYPSIN AND ELASTASE

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(Received October 3rd, 1974)

Summary

The mode of inhibition by α-1-antitrypsin of chymotrypsin, trypsin and pancreatic elastase was examined by a kinetic method. All three enzymes were completely bound to α-1-antitrypsin; therefore, the dissociation constant of the enzyme-inhibitor complex was too low to measure with these methods. The dissociation constants for the three enzyme-inhibitor complexes were estimated to be $< 5 \cdot 10^{-9}$ M. However, α-1-antitrypsin could be specifically displaced from Sepharose-bound elastase with an irreversible inhibitor of that enzyme.

Additional experiments showed that dioxane, 20% (v/v), blocked 100% of the inhibition of elastase, 16% of the inhibition of trypsin, and 0% of the inhibition of chymotrypsin. The effect was reversed by diluting the dioxane to 1% (v/v).

These findings indicated that α-1-antitrypsin was tightly bound to the three enzymes studied but did not allow discrimination as to the nature of the inhibition. However, the competitive mode of inhibition was suggested by the displacement of α-1-antitrypsin from elastase by an irreversible inhibitor of the enzyme that bound covalently at the enzyme-active site. The variable susceptibility of the enzyme to blockage of the inhibition by low concentrations of *p*-dioxane suggested that hydrophobic bonds may be important in the interaction with α-1-antitrypsin.

Introduction

α-1-Antitrypsin is an α-globulin in human serum. The protein is a glycoprotein with a molecular weight of 52 000 that inhibits many proteolytic enzymes [1]. In persons who have a genetically determined deficiency of this

Abbreviation: $PhMeSO_2F$, phenylmethane sulfonyl fluoride.

enzyme inhibitor emphysema develops at an early age. An understanding of the mode of action of the inhibitor may suggest methods of developing drugs that may replace the missing inhibitor.

Few studies of the mode of inhibition of α-1-antitrypsin have been presented. In one previous report [2] a hypothesis for the mechanism of action of α-1-antitrypsin was proposed: α-1-antitrypsin has two overlapping inhibitor sites; one site contains an arginyl residue that binds to proteases that cleave peptides at arginyl residues, and the other site contains an aromatic amino acid or leucyl residue that binds to proteases that cleave peptides at one of these two types of residues. In addition, there is an inhibitory site that inactivates some aspect of the active site of serine proteases. Other studies have suggested that the enzyme activity can be blocked by maleylation and have therefore suggested that there is a lysine at the trypsin inhibitory site [3].

The present study examines the mode of inhibition by α-1-antitrypsin of three of the enzymes with which it reacts rapidly: chymotrypsin, trypsin and elastase. In addition, further quantitative differences in the interaction of α-1-antitrypsin with these enzymes can be demonstrated at low concentrations of dioxane where the enzymes retain their activity.

Methods

Enzyme assays

Three times crystallized bovine α-chymotrypsin and chromatographically purified porcine pancreatic elastase were obtained from Worthington Biochemicals (Freehold, N.J.). Porcine trypsin (three times crystallized, Miles Research Laboratories, Kankakee, Ill.) was used in order to preclude the initial instability demonstrated by bovine trypsin [4].

Enzyme reaction rates were measured with a radiometer pH stat (Copenhagen, Denmark) including a TTT-2 pH meter and titrator, SBR3 recorder, and an ABU 13, 0.25-ml autoburette. The temperature was controlled with a water jacket around the cuvette. The titrant was 0.1 M NaOH. Substrates used to measure catalytic rates were *N*-acetyl-L-tyrosine ethyl ester (Calbiochemicals, San Diego, Calif.), *N*-acetyl-L-alanyl-L-alanyl-L-alanine methyl ester (Cyclo Chemical Co., Los Angeles, Calif.) and *p*-tosyl-L-arginine methyl ester (Calbiochemicals) for chymotrypsin, elastase and trypsin, respectively. The *N*-acetyl-L-alanyl-L-alanyl-L-alanine methyl ester and *p*-tosyl-L-arginine methyl ester were dissolved in Tris · HCl buffer 0.01 M, pH 7.75, with 0.1 M KCl and 0.05 M $CaCl_2$. The *N*-acetyl-L-tyrosine ethyl ester was dissolved in the same buffer with 5% *N,N*-dimethyl formamide. Preliminary experiments showed that full inhibition of enzymes was achieved in less than 1 min. However, enzyme-inhibitor solutions were preincubated at 26° C for 10 min. The substrates in 2 ml of buffer were allowed to equilibrate at pH 7.8 at 25° C. Then the equilibrated enzyme-inhibitor solutions were added in 0.1-ml aliquots, and the reaction rate recorded for 3 to 5 min.

The active sites of trypsin and chymotrypsin were titrated with *p*-nitrophenyl-*p*-guanido benzoate · HCl (Nutritional Biochemical Co., Cleveland, Ohio) by the method of Chase and Shaw [5]. Concentrations of trypsin and chymotrypsin are related in terms of active site concentrations, and concentra-

tion of elastase is determined by absorbance at 280 nm using specific extinction coefficient $E^{1\%}_{280\ nm} = 22.2$ [6].

Elastase affinity column

Twice crystallized elastase was purified by the method described by Shotten [6] and coupled to CNBr-activated Sepharose [7,8]. Freshly drawn plasma was passed over the elastase column slowly for 60 min. The Sepharose was washed with a concentration gradient of NaCl (0—1 M) and a pH gradient (pH 7—10) and equilibrated with Tris buffer (0.1 M, pH 7.5). The Sepharose was removed from the column and incubated with continuous stirring at 4° C for 4-h periods with freshly prepared phenylmethane sulfonyl fluoride ($PhMeSO_2F$), $1 \cdot 10^{-3}$ M, in Tris buffer, 0.1 M, pH 7.5, in 5% isopropyl alcohol. Double immunodiffusion analysis was performed on the concentrated eluate by the method of Ouchterlony [9] and immunoelectrophoresis was performed by the method of Scheidegger [10].

Purification of α-1-antitrypsin

α-1-Antitrypsin was purified by a method developed in our laboratory and previously reported [11]. The purified inhibitor was immunologically monospecific and had a molecular weight of 52 000. Between 80 and 100% of the protein was active in trypsin inhibition. It inhibited equimolar amounts of chymotrypsin and trypsin and the phenotype [11] was unchanged from normal serum. α-1-Antitrypsin concentration was determined by assuming an equimolar inactivation of trypsin and chymotrypsin by the inhibitor. Further documentation of the purity of the protein was presented elsewhere [11].

Kinetic tests

The mode of inhibition of each enzyme was examined according to the method of Straus and Goldstein [12,13] as modified by Henderson [14]. In Straus and Goldstein's formulation [12,13], zone behavior or tightness of binding can be determined by a linear function described by the equation:

$$I/i = K_I \cdot 1/(1 - i) + E_0 \tag{1}$$

where I = total inhibitor concentration, $i = 1 - (V'/V)$ is the fractional extent of inhibition, K_I = dissociation constant of the enzyme-inhibitor complex, V' = maximal velocity of the inhibited reaction, V = maximal velocity of the uninhibited reaction and E_0 = the initial enzyme concentration. While Straus and Goldstein's formulations [12,13] dealt specifically with noncompetitive and competitive situations, Henderson [14] showed that the approach could be generalized and he derived an equation that was independent of the mode of action of the inhibitor:

$$\frac{I_t}{i} = E_0 + \frac{D}{\sum \frac{N_i}{K_I}} \cdot \frac{v_0}{v_i} \tag{2}$$

where N_i represents the distribution of the enzyme in the form that combines

with the inhibitor [15]. If the concentrations of enzyme and substrate(s) are kept constant, then this equation predicts that measurements of v_0 and v_i at increasing concentrations of inhibitor, dose-response measurements, should give linear plots, and that in most cases the plots permit the determination of the mode of inhibition. However, an important limiting case was pointed out by Goldstein [13], when $E_0/K_I > 100$ Eqn 2 becomes:

$$I = E_0 \left(1 - \frac{v_i}{v_0}\right) \tag{3}$$

In this case, virtually all of the inhibitor is bound to the enzyme. For this case it is not possible to obtain changes in slope for mechanistic studies. Because all of the inhibitors had dissociation constants that were too low to measure ($E_0/K_I > 100$), additional investigations of the mode of inhibition could not be investigated further by the more recent methods developed by Henderson [14].

p-Dioxane susceptibility

The ability of α-1-antitrypsin to inhibit chymotrypsin, trypsin and elastase was examined in the presence of low concentrations of *p*-dioxane (spectral grade, Matheson, Coleman and Bell Manufacturing, Norwood, Ohio). Buffer concentrations were adjusted to the same concentrations as the solutions without *p*-dioxane. Enzyme reaction rates were determined at each concentration of *p*-dioxane in the presence and in the absence of inhibitor. The amount of inhibition in various concentrations of *p*-dioxane was compared with the amount of inhibition in solutions without *p*-dioxane. *p*-Dioxane by itself does not reduce enzyme activity at the concentrations employed.

Results

Mode of inhibition by kinetic analysis

Zone-behavior analysis of α-1-antitrypsin in the presence of chymotrypsin, trypsin and elastase showed that the plot of I/i against $1/(1 - i)$ was linear, that the slope was not significantly different from zero, and that the intercept at the I/i axis was approximately equal to E_0 (E_0 = 0.27 μM for trypsin and chymotrypsin and 50 μM for elastase (Fig. 1A–C). In all three cases the inhibitor existed in Zone C and therefore K_I was very small.

Additional attempts to demonstrate competition of substrate with inhibitor, using elastase in 8% (v/v) *p*-dioxane or in solutions with no *p*-dioxane and substrate levels up to 400 mM, failed. This concentration of substrate represented its maximal solubility. The substrates of chymotrypsin and trypsin are not as soluble as the substrate of elastase, and maximal concentrations failed to compete with these enzymes as well.

Competition of $PhMeSO_2F$ with α-1-antitrypsin

After fresh plasma was passed over the elastase affinity column and the column was washed with separate concentration gradients of NaCl up to 1 M and pH gradients up to pH 10, the column was equilibrated with Tris buffer,

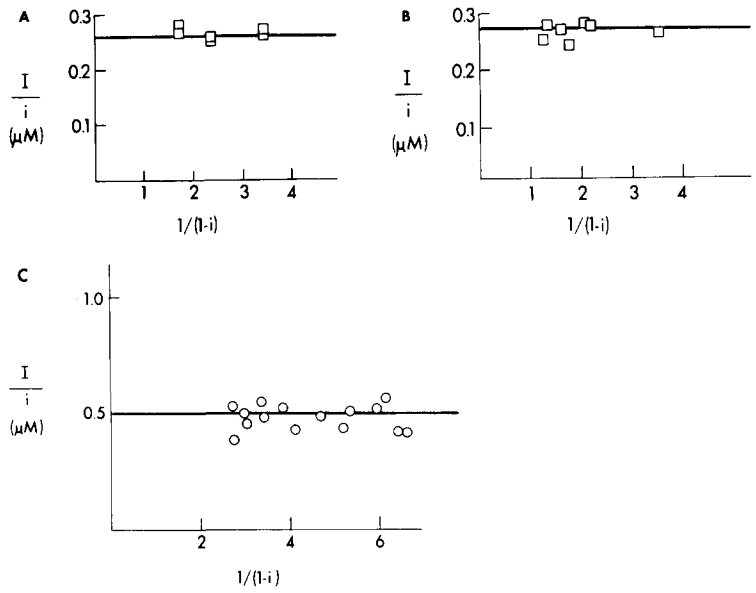

Fig. 1. Zone behavior of α-1-antitrypsin. (A) Chymotrypsin. E_0 = 0.27 μM and S_0 varied from 2 to 10 mM. (B) Trypsin. E_0 = 0.27 μM and S_0 varied from 2.5 to 20.0 mM. (C) Elastase. E_0 = 0.5 μM and S_0 varied from 1.125 to 80 mM. The slope (K_I) is not significantly different from zero and the I/i intercept $\cong E_0$; therefore, Zone C behavior was demonstrated with all three enzymes. S_0 = initial substrate concn.

0.1 M, pH 7.5. The first aliquot eluted from the column with $PhMeSO_2F$ contained albumin and α-1-antitrypsin; the subsequent two aliquots contained only α-1-antitrypsin. The protein reacted in double immunoprecipitin analysis against antisera to α-1-antitrypsin and gave a single line against antisera to whole serum. There was no reactivity against antisera to orosomucoid, albumin, prealbumin or α-2-macroglobulin. In addition, the protein gave only one band on immunoelectrophoresis against antisera to whole human serum. However, the material had no enzyme inhibitory activity. The material is currently being further characterized in our laboratory. In two subsequent experiments, exhaustive elution of the column before treatment with $PhMeSO_2F$ eliminated the albumin in the early $PhMeSO_2F$ eluate. Control experiments were performed to eliminate the possibility that $PhMeSO_2F$ adversely affected the function of α-1-antitrypsin. A solution of purified α-1-antitrypsin, $1.9 \cdot 10^{-5}$ M in 0.025 M Tris, pH 7.6, was divided into two aliquots. One was made up to 10^{-3} M in $PhMeSO_2F$ by adding $PhMeSO_2F$ in isopropanol to a final isopropanol concentration of 5% (v/v). Only isopropanol was added to the other aliquot. After 4 h at 27° C, the two aliquots were dialyzed exhaustively against five changes of buffer for 4 days and the dialyzed aliquots were assayed for inhibitory activity. The $PhMeSO_2F$ was removed both because of the dialysis and because it spontaneously hydrolyzed by 4 h in aqueous solutions. There was no difference in inhibitory activity between the two aliquots.

Semiquantitative analysis of the elastase activity on the Sepharose was performed. The Sepharose beads coupled to elastase were centrifuged in constant-bore capillary tubes for 3 min in a hematocrit centrifuge. Varied lengths containing packed beads were cut from the capillaries and the contents of the

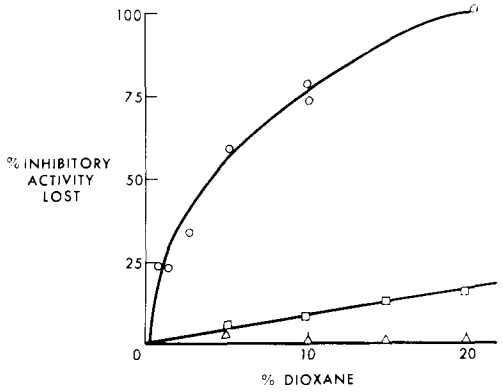

Fig. 2. Effect of *p*-dioxane on inhibition of enzymes by α-1-antitrypsin. In order to determine the fraction of enzyme inhibited, enzyme reaction rates were determined with and without inhibitor at each concentration of *p*-dioxane. Enzyme reaction rates were changed less than 5% in the highest concentrations of *p*-dioxane used. Percent of inhibitory activity lost was determined by calculating the percent of inhibitory activity lost in *p*-dioxane as compared to inhibitory activity in solutions without *p*-dioxane.

measured segment blown into the pH stat cuvette. A plot of the volume of Sepharose, bound to elastase, versus rate of hydrolysis of a 12 mM solution of the elastase substrate was linear with a regression coefficient of 0.93. After the serum was passed through the elastase column, the activity of the elastase on Sepharose beads was reduced by 4 and 10% in two separate experiments. Treatment of elastase-bound Sepharose with $PhMeSO_2F$ as just described resulted in about 30% reduction of enzyme catalytic activity after each treatment.

Sepharose 4B in the absence of elastase did not eliminate the inhibitory activity of purified α-1-antitrypsin. In addition, when serum was passed through a column of Sepharose 4B with no elastase, α-1-antitrypsin could not be eluted with $PhMeSO_2F$.

Enzyme inhibition in the presence of p-dioxane

The inhibition of all three enzymes was studied at various concentrations of *p*-dioxane (Fig. 2). Elastase inhibition was most susceptible to diminution at low concentrations of *p*-dioxane. Trypsin inhibition showed less susceptibility, and chymotrypsin inhibition was unaffected by *p*-dioxane concentrations up to 20% (v/v). In each case the activity of the enzymes varied by $<5\%$ of the values determined in solutions without *p*-dioxane. The effect of *p*-dioxane on inhibition was completely reversible by diluting the solutions of enzyme and inhibitor to 1% (v/v) before titrating the enzyme activity.

Discussion

Previous investigators who have studied the nature of the inhibition by α-1-antitrypsin based on double reciprocal plots of enzyme reaction rate and substrate concentration concluded that α-1-antitrypsin is a non-competitive inhibitor of plasmin [16]; however, the studies did not employ initial reaction rates, and they did not consider the problems of applying the formulations of

Lineweaver and Burk [17] to tightly bound inhibitors. In addition, these and other investigators suggested that α-1-antitrypsin inactivates trypsin and chymotrypsin stoichiometrically; however, the studies were not performed with enzymes that were titrated for the molarity of their active sites [16,18].

In this study some of the kinetic parameters of interaction of α-1-antitrypsin with chymotrypsin, trypsin and elastase were examined. Examination of the mode of inhibition by the methods of Straus and Goldstein [12] showed that chymotrypsin, trypsin and elastase are very tightly bound to the inhibitor. α-1-Antitrypsin was shown to exist in Zone C when trypsin, chymotrypsin or elastase was added; therefore, the K_I was too low to measure by these methods and all of the measurable α-1-antritrypsin was bound to enzyme. In Zone C inhibitors, E_0/K_I is <100; therefore, the K_I for chymotrypsin and trypsin with α-1-antitrypsin was $<2.7 \cdot 10^{-9}$ M and for elastase was $<5.0 \cdot 10^{-9}$ M. In addition, the mechanism of inhibition could not be determined by these kinetic methods.

Because the dissociation constant between enzyme and substrate was too low to demonstrate competition with α-1-antitrypsin for sites on enzymes, the ability of an irreversible inhibitor of one of the enzymes to displace α-1-antitrypsin was studied. The specific displacement of α-1-antitrypsin from Sepharose-bound elastase by an irreversible inhibitor of elastase that binds covalently with the seryl residue at the enzyme catalytic site [19] suggests that α-1-antitrypsin binds to elastase at the enzyme catalytic site. It is not clear why the α-1-antitrypsin did not retain its inhibitory activity. One interesting possibility is that the inhibitor was cleaved at its inhibitory site. Partial proteolysis at the inhibitory site of other inhibitors during affinity chromatography has been described [20]; however, modification by proteolytic cleavage of other peptide bonds has only been described with temporary inhibitors [21,22]. Therefore, the possibility should be explored that the affinity column described herein may be useful in studying the inhibitory site on α-1-antitrypsin.

There are two elastase inhibitors in human serum: α-1-antitrypsin and α-2-macroglobulin [1]. Enzymes bound to α-2-macroglobulin can still catalyze the hydrolysis of small synthetic substrates [23]. Therefore, α-2-macroglobulin probably does not bind to enzymes at the catalytic site. Such a mechanism may account for the failure of $PhMeSO_2F$ to displace α-2-macroglobulin from the elastase column.

Quantitative differences between the interaction of α-1-antitrypsin with these three enzymes was demonstrated by showing a variable susceptibility of the amount of inhibition in low concentrations of *p*-dioxane. Two factors indicate that the *p*-dioxane did not denature either the enzyme or the inhibitor: first the inhibitor maintained full stoichiometric activity for chymotrypsin, and second the effect was reversed by diluting the *p*-dioxane back to a concentration of 1% (v/v). These data suggest that some of the bond energy for enzyme-inhibitor association may be derived from hydrophobic interactions [24].

In conclusion, α-1-antitrypsin is tightly bound to chymotrypsin, trypsin and elastase; however, the inhibitor can be displaced from elastase by an irreversible inhibitor of this enzyme that is covalently bound to the seryl residue at the enzyme catalytic site. Quantitative and perhaps qualitative differences exist

in the manner in which it inhibits these three enzymes, and hydrophobic forces may be important in the interaction of elastase and trypsin with α-1-antitrypsin.

Further characterization of the product of affinity chromatography of α-1-antitrypsin on an enzyme affinity column may provide a means of identifying the enzyme inhibitory sites.

Acknowledgments

This work was supported by the National Heart and Lung Institute Pulmonary SCOR Grant HL 14201(03), NHLI Grant 1-KO4 HL 70601-01, and Council for Tobacco Research, U.S.A. Grant No. 954. I am grateful to Mrs Dagmar Geczy for expert technical assistance.

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