Alpha-1-antitrypsin–Pittsburgh

A Potent Inhibitor of Human Plasma Factor XIa, Kallikrein, and Factor XII_f

Cheryl F. Scott, Robin W. Carrell, Charles B. Glaser, Friedrich Kueppers, Jessica H. Lewis, and Robert W. Colman

Thrombosis Research Center, and the Hematology/Oncology and Pulmonary Disease Sections of the Department of Medicine, Temple University Health Sciences Center, Philadelphia, Pennsylvania 19140; Medical Research Institute of San Francisco, at Pacific Medical Center, San Francisco, California 94115; Clinical Biochemistry, Department of Pathology, Christchurch Hospital, Christchurch, New Zealand; and Department of Medicine, University of Pittsburgh, and Central Blood Bank Pittsburgh, Pennsylvania 15261

Abstract

Alpha-1-antitrypsin–Pittsburgh is a human variant that resulted from a point mutation in the plasma protease inhibitor, α_1-antitrypsin (358 Met → Arg). This defect in the α_1-antitrypsin molecule causes it to have greatly diminished anti-elastase activity but markedly increased antithrombin activity. In this report, we demonstrate that this variant protein also has greatly increased inhibitory activity towards the arginine-specific enzymes of the contact system of plasma proteolysis (Factor XIa, kallikrein, and Factor XII_f), in contrast to normal α_1-antitrypsin, which has modest to no inhibitory activity towards these enzymes. We determined the second-order-inactivation rate constant (k'') of purified, human Factor XIa by purified α_1-antitrypsin–Pittsburgh and found it to be 5.1×10^5 M^{-1} s^{-1} (23°C), which is a 7,700-fold increase over the k'' for Factor XIa by its major inhibitor, normal purified α_1-antitrypsin (i.e., 6.6×10^1 M^{-1} s^{-1}). Human plasma kallikrein, which is poorly inhibited by α_1-antitrypsin ($k'' = 4.2$ M^{-1} s^{-1}), exhibited a k'' for α_1-antitrypsin–Pittsburgh of 8.9×10^4 M^{-1} s^{-1} (a 21,000-fold increase), making it a more efficient inhibitor than either of the naturally occurring major inhibitors of kallikrein ($C\bar{1}$-inhibitor and α_2-macroglobulin). Factor XII_f, which is not inhibited by normal α_1-antitrypsin, displayed a k'' for α_1-antitrypsin–Pittsburgh of 2.5×10^4 M^{-1} s^{-1}. This enhanced inhibitory activity is similar to the effect of α_1-antitrypsin–Pittsburgh that has been reported for thrombin. In addition to its potential as an anticoagulant, this recently cloned protein may prove to be clinically valuable in the management of septic shock, hereditary angioedema, or other syndromes involving activation of the surface-mediated plasma proteolytic system.

Introduction

Blood coagulation is a multifaceted process involving zymogen and co-factor activation as well as enzyme inhibition, with many steps of this integrated series of reactions occurring on the surface of platelets. Interference with certain critical steps in the "cascade" can lead to either the pathologic processes of thrombosis or hemorrhage. The most influential inhibitor in the blood coagulation cascade is antithrombin III, which is the major inhibitor of thrombin (1, 2), the enzyme responsible for fibrin formation as well as platelet aggregation (3). It has been demonstrated that a level of antithrombin III equal to at least 80% of normal is necessary to maintain hemostasis (4), and a level of 30–40% of normal results in a predisposition to early venous thromboembolic disease. The most abundant protease inhibitor in plasma, however, is α_1-antitrypsin (α_1 protease inhibitor) (α_1-AT),[1] which exerts little influence on the coagulation cascade, except on Factor XIa of the contact system, where it is the major inhibitor of that enzyme (5), accounting for at least 50% of its inhibition (6) when the value of 25 μM is used for the concentration of α_1-AT in normal plasma (7).

A patient has been described, whose plasma contained, along with normal α_1-AT, a variant of α_1-AT, designated antithrombin–Pittsburgh (8), and later α_1-antitrypsin–Pittsburgh (AT-P) (9). This variant protein was found to result from a point mutation at position 358 in the reactive center of the molecule that substituted arginine for methionine (9), allowing the protein to function as a potent thrombin inhibitor, while greatly diminishing its anti-elastase activity (10). Since α_1-AT is an acute-phase reactant that can increase as much as fourfold during periods of stress (11), it is not surprising that the concentration of the mutant protein, AT-P, in the patient's plasma was elevated after minor trauma (9). The elevated level of AT-P in the patient resulted in numerous severe bleeding episodes, ultimately leading to his death (9). The total antitrypsin content in the patient's plasma (normal α_1-AT = 25 μM) ranged from ~9.2 μM, in the quiescent phase of the disorder, to ~40 μM, in the acute phase (9). AT-P was 4,000-fold more efficient as an inhibitor of thrombin than normal α_1-AT (9), and 40-fold more efficient than the major thrombin inhibitor, antithrombin III, in the absence of heparin (10), thus, offering a possible explanation for the marked decrease in coagulability of the patient's blood.

Since the enzymes of the contact phase of plasma proteolysis (Factors XIa, activated Factor XII, and kallikrein) cleave primarily bonds containing arginine in the P_1 position (12–14), we hypothesized that since the variant protein now contained arginine in its P_1 position, that it might function as an efficient inhibitor of these enzymes. In this paper, we present evidence that AT-P is a more potent inhibitor than any of the naturally occurring plasma protease inhibitors of all of the contact enzymes (Factor XIa, kallikrein, and Factor XII_f), especially Factor XIa, where it displayed a greater affinity than that reported for thrombin. Therefore, the presence of this mutant protein would result in greatly increased total plasma inhibitory capacity towards each contact enzyme, and would dramatically inhibit the contact system of plasma proteolysis.

Methods

Materials. ⟨Glu-Pro-Arg-*p*-nitroanilide (S-2366) was a generous gift of KabiVitrum AB, Molndal, Sweden. Pro-Phe-Arg-*p*-nitroanilide (S-2302)

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1. *Abbreviations used in this paper:* α_1 = AT, α_1 = antitrypsin (α_1 protease inhibitor); AT-P, α_1-antitrypsin–Pittsburgh; k'', second-order rate constant; PEG, polyethylene glycol.

and antithrombin III were purchased from Helena Laboratories, Beaumont, TX. Polyethylene glycol (PEG) (PEG 8000) was obtained from Sigma Chemical Co., St. Louis, MO. BCA protein determination reagent was obtained from Pierce Chemical Co., Rockford, IL. All other reagents were the best grade available.

Purified proteins. Factor XI, kallikrein, and Factor XII_f (the catalytic subunit of activated Factor XII) were prepared from purified zymogens, as previously described (6, 15, 16). Radial immunodiffusion, to measure α_1-antitrypsin antigen, was performed according to the method of Mancini et al. (17), using monospecific antiserum (7), assigning a value of 25 μM to normal, pooled plasma (1.36 mg/ml α_1-AT) (7).

The protein determinations of purified α_1-AT and AT-P were performed with the BCA protein determination reagent.

α_1 Protease inhibitor (α_1-AT) was prepared by the reductive-salting method of Glaser et al. (18), with the following modifications: (*a*) Plasma from normal (MM-type) individuals was used as the starting material instead of Cohn fraction IV-I. (*b*) In the DEAE-cellulose step, a gradient of 10 mM sodium phosphate, pH 7.6, to 10 mM sodium phosphate, pH 6.0, containing 0.2 M NaCl was used. (*c*) A further purification step was carried out on QAE-Sephadex at pH 8.6 using a linear salt gradient in 50 mM Tris-HCl, pH 8.6, from 0.14 M NaCl to 0.2 M NaCl. The resulting protein, a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, was stored in 100-μl aliquots at −70°C as a stock solution of 3.3 mg/ml (60 μM), as determined by radial immunodiffusion (17) and 3.1 mg/ml, as determined by the BCA assay, in 20 mM Tris-Cl, pH 7.4, containing 0.15 M NaCl and 0.1% PEG.

α_1-Antitrypsin-Pittsburgh (AT-P) was isolated from the patient's plasma, as described by Carrell et al. (19). The concentration of the stock solution was 1.25 mg/ml (23 μM), as determined by radial immunodiffusion (17), and 1.4 mg/ml, as determined by the BCA assay, in 20 mM Tris-Cl, pH 7.4, containing 0.15 M NaCl and 0.1% PEG. Aliquots of 100 μl were stored at −70°C. The good agreement between the two methods of protein determination indicates that the antigenicity of the variant antitrypsin is identical to that of normal α_1-AT.

Determination of inhibitory activity of purified plasma protease inhibitors of the contact enzymes. Purified enzyme was incubated (separately) with each inhibitor in the appropriate buffer (20–22) containing 0.1% PEG at 23°C. At various times, a portion was removed and assayed for residual amidolytic (enzymatic) activity at 37°C. Factor XIa activity was determined using the synthetic substrate S-2366 (20); kallikrein was assayed with S-2302 (21); and Factor XII_f was measured with S-2302 (22), as previously described. All enzyme assays were performed using a Gilford 210 recording spectrophotometer with a thermostated chamber (37°C).

Calculation of second-order rate constants (k"). The second-order rate law is as follows (23): $1/(a-b)[\ln(b/a)(a-x)/(b-x)] = k''t$, where a = initial inhibitor concentration, b = initial enzyme concentration, x = product concentration at time "t" of the reaction, and k'' is the apparent second-order rate constant. When the left side of each equation is plotted versus time, the slope = k''.

Results

Isoelectric focusing of purified α_1-AT and AT-P. Purified α_1-AT (type M) and purified AT-P were run under the conditions in reference 7. Each band in the AT-P preparation migrated more cathodally than its normal counterpart, indicating that the purified preparation did not contain the M-subtype (data not shown).

Effect of AT-P on plasma Factor XIa. When purified Factor XIa (0.035 μM) was incubated with AT-P (0.035–0.068 μM), at 23°C, a rapid concentration-dependent loss of amidolytic activity resulted. Since the inhibition was too rapid to perform kinetic analyses under pseudo first-order conditions (>10-fold excess of inhibitor), we employed second-order conditions (inhibitor/enzyme ratios = 1.0–1.9). From the data, we calculated a k'' for the inactivation of Factor XIa by AT-P of 5.1×10^5 M^{-1} s^{-1}. This contrasts with the k'' for the inactivation of Factor XIa by normal α_1-AT, which was 6.6×10^1 M^{-1} s^{-1}, in agreement with previous results (6), and normal antithrombin III, which was 1.7×10^2 M^{-1} s^{-1}. Therefore, AT-P is 7,700-fold more potent than normal α_1-AT, and 3,000-fold more potent than antithrombin III towards inactivation of Factor XIa.

Effect of AT-P on plasma kallikrein. Kallikrein (0.24 μM) was incubated with AT-P under second-order conditions (0.25–0.35 μM) at 23°C. The rapid inactivation yielded a $k'' = 8.9 \times 10^4$ M^{-1} s^{-1}, whereas normal α_1-AT is a weak inactivator, under the same conditions ($k'' = 4.2$ M^{-1} s^{-1}) (24), and antithrombin III has moderate inhibitory activity towards kallikrein ($k'' = 3 \times 10^2$ M^{-1} s^{-1}) (24). Therefore, AT-P is also an efficient inhibitor of plasma kallikrein.

Effect of AT-P on the inactivation rate of Factor XII_f. The k'' for the inactivation of Factor XII_f by AT-P, determined with 0.4 μM Factor XII_f and AT-P equalling 0.4–1.15 μM (second-order conditions) at 23°C, was found to be 2.5×10^4 M^{-1} s^{-1}. This was very striking, since normal α_1-AT does not detectably inactivate Factor XII_f (16). Furthermore, AT-P was calculated to be 470-fold more potent than antithrombin III in the inactivation of Factor XII_f (k'' for antithrombin III is 5.3×10^1 M^{-1} s^{-1}) (16).

Comparison of second-order-inactivation rate constants of AT-P with normal plasma protease inhibitors (in purified systems). The second-order inactivation rate constants for the contact enzymes and thrombin for four normal plasma protease inhibitors and AT-P are presented in Table I (the k'' is a measurement of inhibitor efficiency or affinity, independent of its concentration). None of the normal plasma protease inhibitors are very potent for Factor XIa, but the mutant inhibitor, AT-P, is an excellent inhibitor. The two major inhibitors of kallikrein, C1-inhibitor and α_2-macroglobulin, are moderately good inhibitors; however, AT-P is 5–7-fold more potent. This contrasts with normal α_1-AT, which is a very weak kallikrein inhibitor. C1-inhibitor is the only plasma protease inhibitor that efficiently inactivates Factor XII_f (16); however, AT-P appears to be eight-fold more potent. AT-P is 40-fold more potent than the major thrombin inhibitor, antithrombin III, 620-fold more potent than α_2-macroglobulin, and 6,000-fold more potent for the inactivation of thrombin than normal α_1-AT. Furthermore, from these data we conclude that AT-P is a more potent anti-Factor XIa than it is an antithrombin.

Table I. Comparison of Second-order Inactivation Rate Constants (23°C)

	(k'', M^{-1} s^{-1})			
	Factor XIa	Kallikrein	Factor XII_f	Thrombin
C1-INH	250 (6)	17,000 (24)	3,000 (16)	0
α_2M	0 (6)	11,500 (24)	0 (16)	500 (37)
AT-III	170 (6)	300 (24)	53 (16)	7,200 (36)
α_1-AT	66 (6)	4.2 (24)	0 (16)	48 (27)
AT-P	510,000	89,000	25,000	310,000 (10)

Inactivation rate constants, determined for Factor XIa and kallikrein by α_1-AT and antithrombin III (AT-III), agreed well with our previous determinations (6, 24). Therefore, we are presenting a comparison of the inactivation rate constants of the enzymes by AT-P with the previously published data. Numbers in parentheses denote references. C1-INH, C1-esterase inhibitor; α_2M, α_2 macroglobulin.

Comparison of theoretical half-lives of each enzyme with each inhibitor at normal plasma concentration and AT-P plasma concentration. When the value of k'' is multiplied by its concentration in plasma, (7, 25, 26), the constant, k' (pseudo–first-order reaction rate constant), can be calculated, which is an estimate measure of the total inhibitory capacity of that inhibitor at plasma concentration. The reciprocal of k' (pseudo–first-order reaction rate constant) would then be the more familiar half-life ($t_{1/2}$) of the enzyme at plasma concentrations (Table II) of each inhibitor. The $t_{1/2}$ of Factor XIa, in the presence of plasma concentrations of its most effective inhibitor, α_1-AT, is 600 s (6), compared with 0.21 s by AT-P at its concentration in the plasma of the patient during the quiescent phase, (9.2 μM [9]), and 0.05 s during the acute phase (40 μM [9]). This increase in inhibitor activity represents a decrease of 2,800–12,000-fold in the $t_{1/2}$ of Factor XIa in AT-P plasma. Similarly, the $t_{1/2}$ of kallikrein in the presence of plasma C$\bar{1}$-inhibitor concentrations is 35 s (24), and α_2-macroglobulin is 25 s (24), whereas AT-P at the concentration in the plasma of the patient would result in a $t_{1/2}$ for kallikrein of 1.22 s in the quiescent phase, and 0.28 s in the acute phase, which is an average decrease of 25- to 100-fold in the $t_{1/2}$, as compared with the two naturally occurring inhibitors. For Factor XII_f, the major inhibitor, C$\bar{1}$ inhibitor, yields at $t_{1/2}$ of 190 s (16), compared with 4.3 s for AT-P at the concentration in the patient's plasma during the quiescent phase, and 1.0 s during the acute phase, which is a decrease of 44–190-fold in the $t_{1/2}$. Therefore, the total plasma inhibitory activity in the AT-P patient's plasma was at least 1–4 orders of magnitude greater than the inhibitory activity of the naturally occurring contact enzyme inhibitors in normal plasma. Furthermore, the inhibition of the contact enzymes by AT-P was comparable with or greater than the inhibition of thrombin by AT-P (157- to 687-fold decrease of $t_{1/2}$ as compared with antithrombin III).

Discussion

The variant protein, AT-P, has been described as antitrypsin that mutated to "antithrombin" (9). However, kinetic analyses have revealed that AT-P is actually 40-fold more potent than naturally occurring antithrombin III for the inactivation of thrombin (11), as well as 300–3,000-fold more potent than antithrombin III for the inactivation of the enzymes of the contact-activated plasma protease system—a pathway that is involved in coagulation, fibrinolysis, and the inflammatory response. The most profound effect of AT-P is on the enzyme, Factor XIa—the only contact enzyme whose deficiency can lead to a hemorrhagic disorder. The major plasma protease inhibitor of Factor XIa is α_1-AT (5), and accounts for 51% (6) of the total plasma inhibitory activity when calculated using 25 μM (7) as the concentration of α_1-AT in plasma. However, under normal circumstances, this inhibition is slow, when compared with the inhibition of other plasma proteases by their major inhibitors. For example, α_1-AT is also the major inhibitor of human neutrophil elastase, with a k'' of 6.5×10^7 M^{-1} s^{-1} (27), compared with the k'' for Factor XIa of 6.6×10^1 M^{-1} s^{-1} (5). It is interesting to note that the mutant form of α_1-AT, AT-P, has decreased inhibitory activity towards human neutrophil elastase ($k'' = 2.2 \times 10^3$ M^{-1} s^{-1}) (10), while it has increased inhibitory activity towards Factor XIa ($k'' = 5.1 \times 10^5$ M^{-1} s^{-1}). This functional transformation is a consequence of a single substitution in the reactive center of the inhibitor molecule. Since Factor XIa prefers arginine bonds (12) in the P_1 position, it was inhibited more efficiently by the mutant protein than by normal α_1-AT, in contrast to human neutrophil elastase, which exhibits a preference for methionine and valine residues (28), and is, therefore, inhibited more efficiently by normal α_1-AT. Kallikrein and Factor XII_f also cleave at arginyl residues (13, 14), which explains their preference for AT-P over normal α_1-AT. The importance of the P_1 residue, which contributes to the carboxyl moiety of the cleaved peptide bond, has been emphasized recently for the plasma proteinase inhibitors of serine proteases (10, 29).

When present at plasma concentrations (as in the patient with AT-P), the inhibitor not only controls thrombin activity, but to an even greater extent, Factor XIa (Tables I and II). Although Factor XI-deficient individuals usually have mild bleeding episodes, as compared with patients who are severely deficient in Factor VIII or Factor IX, severe bleeding has been reported in a Factor XI-deficient individual who developed a potent antibody to Factor XI (30). This patient, early in life, bled excessively after surgery and also, as an adult, had several episodes of spontaneous intramuscular hematomas. It is possible that an abnormal inhibitor of a coagulation factor would result in a different clinical picture from a simple deficiency of the factor. If this is true, then AT-P also may have been responsible for a "pseudo-Factor XI deficiency" that could have further contributed to the hemostatic defect in that patient.

The fact that AT-P is a more efficient inhibitor of the contact proteases than any of their naturally occurring plasma protease inhibitors (Table I), suggests the possibility for its clinical use. The combination of the recombinant DNA technologies of cloning in bacteria (31) and site-directed mutagenesis in yeast (32, 33) that have allowed substitution of valine in the P_1 position of α_1-antitrypsin to produce an elastase inhibitor that is resistant to oxidation, have also allowed replacement of methionine with arginine to produce an inhibitor of coagulation proteinases that is identical to AT-P (33a). Therefore, in gram-negative sepsis, where activation of contact proteases and consumption of plasma protease inhibitors occurs (34), or during contact activation in the absence of C$\bar{1}$-inhibitor (hereditary angioedema) (35), infusion of genetically engineered AT-P may increase the survival rate of these critically ill patients by rapidly inhibiting the proteases of the contact system, while concurrently blocking thrombin action, which may prevent the development of disseminated

Table II. Calculated Theoretical Half-lives (s) of Enzymes at Plasma Inhibitor Concentrations (23°C)

Inhibitor	Factor XIa	Kallikrein	Factor XII_f	Thrombin
μM				
C$\bar{1}$-INH (1.7)*	2,500	[35]	[190]	0
α_2-M (3.5)*	0	[25]	0	700
AT-III (2.5)*	2,300	1,300	7,500	[55]
α_1-AT (25)‡	[600]	9,500	0	830
AT-P a (9.2)§	0.21	1.22	4.3	0.35
AT-P b (40)§	0.05	0.28	1.0	0.08

* Plasma protease inhibitor concentrations were based on references 25 and 26.
‡ Reference 7.
§ Reference 9; a, quiescent; b, acute.
[ ], Major inhibitor for each enzyme.
$t_{1/2}$, $1/k''$ (I), when I equals inhibitor concentration; α_2M, α_2 macroglobulin; AT-III, antithrombin III.

intravascular coagulation. Experiments are currently underway, with an animal model, to test this hypothesis.

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