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### Research Article

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## Protection by Recombinant $\alpha_1$ -Antitrypsin Ala<sup>357</sup> Arg<sup>358</sup> against Arterial Hypotension Induced by Factor XII Fragment

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### Abstract

The specificity of serpin superfamily protease inhibitors such as  $\alpha_1$ -antitrypsin or C $\bar{I}$  inhibitor is determined by the amino acid residues of the inhibitor reactive center. To obtain an inhibitor that would be specific for the plasma kallikrein-kinin system enzymes, we have constructed an antitrypsin mutant having Arg at the reactive center P<sub>1</sub> residue (position 358) and Ala at residue P<sub>2</sub> (position 357). These modifications were made because C $\bar{I}$  inhibitor, the major natural inhibitor of kallikrein and Factor XIIa, contains Arg at P<sub>1</sub> and Ala at P<sub>2</sub>. In vitro, the novel inhibitor,  $\alpha_1$ -antitrypsin Ala<sup>357</sup> Arg<sup>358</sup>, was more efficient than C $\bar{I}$  inhibitor for inhibiting kallikrein. Furthermore, Wistar rats pretreated with  $\alpha_1$ -antitrypsin Ala<sup>357</sup> Arg<sup>358</sup> were partially protected from the circulatory collapse caused by the administration of  $\beta$ -Factor XIIa.

### Introduction

The plasma kinin-forming system comprises the serine protease zymogens Factor XII and prekallikrein and the nonenzymatic cofactor high-molecular-weight kininogen (1). Upon activation, Factor XII is proteolytically converted into  $\alpha$ - and  $\beta$ -Factor XIIa (2), and prekallikrein into  $\alpha$ - and  $\beta$ -kallikrein (3). When zymogen activation takes place on a negatively charged surface, high-molecular-weight kininogen functions as a cofactor because it increases the rate of reciprocal activation of Factor XII by kallikrein and of prekallikrein by Factor XIIa (4). High-molecular-weight kininogen also serves as a substrate for  $\alpha$ - and  $\beta$ -kallikrein and for Factor XIIa (5-7). At an early stage of high-molecular-weight kininogen proteolytic cleavage, kinins such as the nonapeptide bradykinin are released (5-7). These peptides are potent inflammatory mediators that enhance vascular permeability and cause arterial vasodilation and venous constriction (8).

Septic shock and angioedema attacks are associated with activation of the plasma kinin-forming system (1). However, it

is not known whether this mechanism induces the symptoms observed in these disease states or whether activation of this pathway merely represents an accompanying phenomenon. This question could be addressed by examining the influence of a specific and efficient inhibitor of Factor XIIa and kallikrein on the clinical course of these pathological conditions. Furthermore, if a favorable effect is detectable, the availability of such an agent would represent a significant step toward the development of new therapeutic strategies.

In normal plasma,  $\alpha$ - and  $\beta$ -Factor XIIa as well as kallikrein are predominantly controlled by the serine protease inhibitor C $\bar{I}$  inhibitor (9), a molecule whose existence was first recognized by Ratnoff and Lepow (10). This inhibitor is an  $\alpha_2$ -glycoprotein that belongs to the serpin superfamily (11, 12), a recently identified class of related proteins that also includes  $\alpha_1$ -antitrypsin and antithrombin III. The nature of the amino acid residue located at position P<sub>1</sub> of the reactive center peptide bond plays an important part in dictating the specificity of serpins. Studies with natural or recombinant  $\alpha_1$ -antitrypsin mutants have shown that a Met  $\rightarrow$  Arg mutation at P<sub>1</sub> (position 358) changes the specificity of  $\alpha_1$ -antitrypsin from neutrophil elastase to the Arg-specific proteases thrombin, kallikrein,  $\beta$ -Factor XIIa, Factor XIa, Factor Xa, and plasmin (13-18). To obtain an inhibitor with narrower specificity, i.e. a molecule that would be more specific for kallikrein and Factor XIIa, we have now produced by oligonucleotide-directed mutagenesis of cloned  $\alpha_1$ -antitrypsin complementary DNA, an antitrypsin mutant with Met  $\rightarrow$  Arg at P<sub>1</sub> and Pro  $\rightarrow$  Ala at P<sub>2</sub>. The modification at P<sub>2</sub> was made because C $\bar{I}$  inhibitor, which inhibits Factor XIIa and kallikrein, also has Ala at P<sub>2</sub> (11, 12). The novel inhibitor,  $\alpha_1$ -antitrypsin Ala<sup>357</sup> Arg<sup>358</sup>, was then studied for in vitro reactivity with  $\beta$ -kallikrein,  $\beta$ -Factor XIIa, and thrombin. Thereafter, we have examined whether the in vivo administration of this mutated  $\alpha_1$ -antitrypsin molecule had an effect on the thrombin time of Wistar rats or influenced the kinin-dependent circulatory collapse induced in these animals by the administration of  $\beta$ -Factor XIIa (or Factor XII active fragment), a  $M_r$  28,000 serine protease which is a potent liquid-phase activator of prekallikrein but exhibits only minimal clot-promoting activity.

### Methods

**Proteins.** Plasma kallikrein in the  $\beta$  form (19) and  $\beta$ -Factor XIIa (20) were purified as previously described. Human thrombin (T 6759; 3,000 NIH [National Institutes of Health] U/mg) was purchased from Sigma Chemical Co., St. Louis, MO.  $\alpha_1$ -Antitrypsin Arg<sup>358</sup> and  $\alpha_1$ -antitrypsin Val<sup>358</sup> were prepared from *E. coli* strains previously described (16). For

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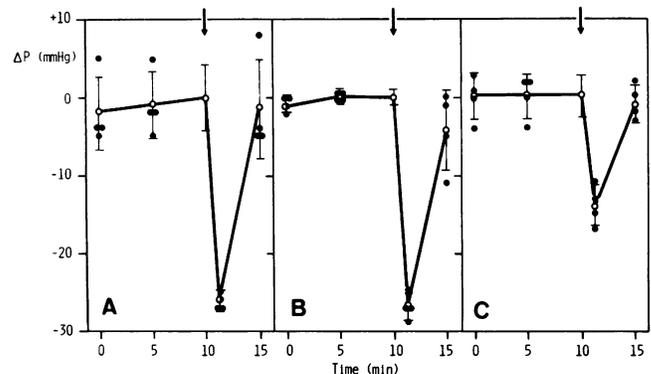
the production of  $\alpha_1$ -antitrypsin Ala<sup>357</sup> Arg<sup>358</sup>, the  $\alpha_1$ -antitrypsin coding sequence was modified by cassette mutagenesis (17) to generate Ala and Arg codons at positions 357 and 358. These modifications were verified by DNA sequencing. The double mutant was then synthesized and purified from *E. coli* cultures using the methods described for the other  $\alpha_1$ -antitrypsin variants (16, 17).

**Kinetic studies.** Kallikrein,  $\beta$ -Factor XIIa, or thrombin were preincubated at room temperature with  $\alpha_1$ -antitrypsin Arg<sup>358</sup> or  $\alpha_1$ -antitrypsin Ala<sup>357</sup> Arg<sup>358</sup>. At various times, aliquots were assayed for residual kallikrein or  $\beta$ -Factor XIIa amidolytic activity using the chromogenic substrate H-D-Pro-Phe-Arg-*p*-nitroanilide (S-2302), whereas residual thrombin activity was assessed on H-D-Phe-Pip-Arg-*p*-nitroanilide (S-2238). S-2302 and S-2238 were obtained from Kabi Diagnostica, Stockholm, Sweden. With S-2302, a 0.6 mM solution was prepared in 85 mM sodium phosphate buffer, pH 7.6, containing 127 mM NaCl. With S-2238, a 0.12 mM solution was prepared in 97 mM sodium phosphate buffer, pH 7.6, containing 145 mM NaCl. 10  $\mu$ l of the solution to be tested was added to 330  $\mu$ l of substrate at 37°C, and the absorbance change at 405 nm was continuously recorded with a Cary 210 spectrophotometer (Varian Associates, Inc., Palo Alto, CA). Under these conditions, the hydrolysis rate of S-2302 was 82  $\mu$ mol/min with 1 mg of kallikrein and 18  $\mu$ mol/min with 1 mg of  $\beta$ -Factor XIIa, whereas 1 mg of thrombin hydrolyzed S-2238 at a rate of 201  $\mu$ mol/min. Second-order reaction rate constants were derived from pseudo-first-order plots (21) or directly calculated using an integrated form of the second-order equation (22).

**Animal studies.** Male Wistar rats (Madorin, Fullinsdorf, Switzerland), weighing 232–271 g, were used for this study. For the experiments designed to evaluate the anticoagulant action of  $\alpha_1$ -antitrypsin mutants, the animals were anesthetized with ether during the entire experiment and they were not exposed to exogenous heparin. After cannulation of the right femoral vein with a PE-10 catheter, the animals received a bolus injection of the agent under investigation. 5 min later, 2 ml of blood were obtained by cannulation of the right external iliac artery with a PE-50 catheter. Blood samples were immediately transferred into plastic tubes containing 0.1 vol of 0.13 M sodium citrate, and plasma samples were prepared by centrifugation at 3,000 *g* for 15 min. The thrombin time was the coagulation time obtained on addition of 1 vol of thrombin at 12 U/ml (Topostasin, Hoffman La Roche, Basel, Switzerland) to 1 vol of plasma. Under these conditions, the thrombin time of normal rat plasma was of 26–29 s, whereas values ranging from 12.5 to 13.0 s were observed with normal human plasma. For the experiments designed to evaluate influence of  $\alpha_1$ -antitrypsin mutants on  $\beta$ -Factor XIIa-induced arterial hypotension, the following protocol was employed. Under light ether anesthesia, the right external iliac artery of the animals was cannulated with a PE-50 catheter and the right femoral vein with a PE-10 catheter. All catheters contained a heparinized 5% dextrose solution. The rats were then placed in a plastic tube for the restriction of their movements, where they were left to recover from anesthesia. Arterial pressure and heart rate were monitored using a pressure transducer (Statham, Hato Rey, PR) connected to an electrogalvanometer (Philips 2000, Eindhoven, Netherlands), and recorded on a light-sensitive oscillograph (Mannarp 150, Electronic Institute, London, UK). The study was initiated 90–120 min after the end of anesthesia, when blood pressure and heart rate were stable and normal. Over a period of 5 min, the animals were pretreated by a continuous intravenous perfusion of the agent under investigation. 5 min after the end of the pretreatment period, a bolus intravenous injection of  $\beta$ -Factor XIIa (1.0  $\mu$ g) was administered. Blood pressure and heart rate were continuously recorded during 15 min, from the start of the pretreatment injection (Fig. 1, time = 0) until 5 min after the bolus administration of  $\beta$ -Factor XIIa (Fig. 1, time = 15 min). Blood pressure changes were expressed as means  $\pm$  SD, and groups were compared using one-way analysis of variance and Student's *t* tests.

## Results

**Interaction of blood proteolytic enzymes with  $\alpha_1$ -antitrypsin mutants: *in vitro* studies.** The inactivation of kallikrein,  $\beta$ -Factor XIIa, and thrombin by  $\alpha_1$ -antitrypsin Ala<sup>357</sup> Arg<sup>358</sup>, and  $\alpha_1$ -an-



**Figure 1.** Hypotensive reaction to  $\beta$ -Factor XIIa. Male Wistar rats were pretreated during 5 min (from time = 0 to time = 5 min) with saline (A), 0.7 mg of  $\alpha_1$ -antitrypsin Val<sup>358</sup> (B), and 0.7 mg of  $\alpha_1$ -antitrypsin Ala<sup>357</sup> Arg<sup>358</sup> (C). 5 min later (at time = 10 min), the animals received 1.0  $\mu$ g of  $\beta$ -Factor XIIa (vertical arrows). Each group was composed of four animals. Blood pressure changes (mmHg, mean  $\pm$  SD) are relative to the values measured at time = 10 min, before administration of  $\beta$ -Factor XIIa. SD values at time = 10 min indicate the dispersion of actual blood pressure values. The actual values (mmHg, mean  $\pm$  SD) at 10 min were: 128  $\pm$  6 (A), 127  $\pm$  6 (B), and 122  $\pm$  10 (C). (Open circles) Mean values; (closed circles) individual values.

titrypsin Arg<sup>358</sup> was studied using enzyme/inhibitor molar ratios ranging from 1:1 to 1:20.  $\alpha_1$ -Antitrypsin Ala<sup>357</sup> Arg<sup>358</sup> inactivated kallikrein with a second-order rate constant of  $3.6 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ , a value that was 5.2 times greater than the value observed with  $\alpha_1$ -antitrypsin Arg<sup>358</sup> and 21.2 times greater than the value determined with C $\bar{I}$  inhibitor (Table I). However,  $\alpha_1$ -antitrypsin Ala<sup>357</sup> Arg<sup>358</sup> was less efficient than  $\alpha_1$ -antitrypsin Arg<sup>358</sup> for the inactivation of  $\beta$ -Factor XIIa (3.8 times) as well as of thrombin (4.9 times) (Table I).

**Anticoagulant effect of  $\alpha_1$ -antitrypsin mutants.** Anesthetized male Wistar rats weighing 232–271 g received an intravenous bolus of  $\alpha_1$ -antitrypsin Arg<sup>358</sup> or  $\alpha_1$ -antitrypsin Ala<sup>357</sup> Arg<sup>358</sup>. 5 min later, the anticoagulant effect of these treatments was assessed using the thrombin time. Control values were obtained by studying animals treated with  $\alpha_1$ -antitrypsin Val<sup>358</sup>, an oxidation-resistant variant that possesses the same inhibitory spectrum as  $\alpha_1$ -antitrypsin Met<sup>358</sup> and therefore does not exhibit anticoagulant activity (16). Two animals treated with 0.7 mg of  $\alpha_1$ -antitrypsin Arg<sup>358</sup> had a prolonged thrombin time, respectively by a factor of 2.3 and 1.4, whereas a 1.1-fold prolongation was detectable in the animal that received half this dose (Table II). In contrast, rats treated with 0.7 mg of  $\alpha_1$ -antitrypsin Ala<sup>357</sup>

**Table I.** Kinetic Constants for Inactivation of Kallikrein,  $\beta$ -Factor XIIa, and Thrombin by Recombinant  $\alpha_1$ -Antitrypsin Mutants and Natural C $\bar{I}$  Inhibitor

	Plasma kallikrein	$\beta$ -Factor XIIa	Thrombin
$\alpha_1$ -Antitrypsin Ala <sup>357</sup> Arg <sup>358</sup>	$3.6 \times 10^5$	$9.2 \times 10^3$	$7.3 \times 10^4$
$\alpha_1$ -Antitrypsin Arg <sup>358</sup>	$6.9 \times 10^{4*}$	$3.5 \times 10^{4*}$	$3.6 \times 10^5$
C $\bar{I}$ inhibitor	$1.7 \times 10^{4\dagger}$	$3.1 \times 10^{3\ddagger}$	ND

Values are in  $\text{M}^{-1} \text{ s}^{-1}$ .

\* Reference 18.

† Reference 23.

‡ Reference 20.

Arg<sup>358</sup> had thrombin time values similar to those observed in controls (Table II).

**Effect of  $\alpha_1$ -antitrypsin mutants on  $\beta$ -Factor XIIa-induced arterial hypotension.** Conscious normotensive male Wistar rats weighing 241–268 g were pretreated intravenously during 5 min with either saline (Fig. 1 A), 0.7 mg of  $\alpha_1$ -antitrypsin Val<sup>358</sup> (Fig. 1 B), or 0.7 mg of  $\alpha_1$ -antitrypsin Ala<sup>357</sup> Arg<sup>358</sup> (Fig. 1 C). 5 min after the end of pretreatment, the animals received an intravenous bolus of 1.0  $\mu$ g of  $\beta$ -Factor XIIa (Fig. 1, vertical arrows). Nearly identical peak arterial blood pressure reductions were recorded in rats pretreated with saline ( $-26 \pm 1$  mmHg,  $n = 4$ ; Fig. 1 A) or  $\alpha_1$ -antitrypsin Val<sup>358</sup> ( $-27 \pm 2$  mmHg,  $n = 4$ ; Fig. 1 B), whereas significantly smaller blood pressure decreases were observed in animals pretreated with  $\alpha_1$ -antitrypsin Ala<sup>357</sup> Arg<sup>358</sup> ( $-14 \pm 3$  mmHg,  $n = 4$ ,  $P < 0.01$ ; Fig. 1 C).

## Discussion

$\alpha_1$ -Antitrypsin Ala<sup>357</sup> Arg<sup>358</sup> differs from  $\alpha_1$ -antitrypsin Arg<sup>358</sup> in possessing Ala instead of Pro at position P<sub>2</sub> of the reactive center. This additional mutation modifies the relative inhibition of kallikrein and thrombin (Table I).  $\alpha_1$ -Antitrypsin Ala<sup>357</sup> Arg<sup>358</sup> is a better inhibitor of kallikrein than of thrombin (4.9-fold), in contrast to  $\alpha_1$ -antitrypsin Arg<sup>358</sup>, which is more efficient against thrombin than against kallikrein (5.2-fold). Thus, it can be calculated that at inhibitor concentrations causing identical rate of kallikrein inhibition,  $\alpha_1$ -antitrypsin Ala<sup>357</sup> Arg<sup>358</sup> will induce 25 times less inhibition of thrombin than  $\alpha_1$ -antitrypsin Arg<sup>358</sup>. Another characteristic of  $\alpha_1$ -antitrypsin Ala<sup>357</sup> Arg<sup>358</sup> is that the mutated recombinant protein is more than one order of magnitude more efficient in inhibiting kallikrein and  $\beta$ -Factor XIIa than C $\bar{I}$  inhibitor, an unexpected finding since C $\bar{I}$  inhibitor is the most potent natural inhibitor of these enzymes (9). Furthermore, because C $\bar{I}$  inhibitor and  $\alpha_1$ -antitrypsin Ala<sup>357</sup> Arg<sup>358</sup> have the same P<sub>1</sub> and P<sub>2</sub> residues, our results demonstrate that additional factors other than the residues located in the immediate vicinity of the reactive site contribute to the reactivity of protease inhibitors of the serpin superfamily.

Infusion of certain plasma protein fraction lots has induced circulatory collapse in surgical patients. Because the preparations involved contained  $\beta$ -Factor XIIa (24), it was proposed that the mechanism responsible for the hypotensive reaction included prekallikrein activation by  $\beta$ -Factor XIIa, followed by high-molecular-weight kininogen cleavage by kallikrein leading to the release of bradykinin. Subsequent studies showed that arterial hypotension is also observed when purified and catalytically active  $\beta$ -Factor XIIa is employed instead of plasma protein fraction (25, 26). In these experiments, the hypotensive response is me-

diated by bradykinin/kinin molecules. Indeed, more severe hypotension is observed in rats treated with captopril (25, 26), a kininase II inhibitor that causes decreased bradykinin catabolism. Moreover, animals treated with the bradykinin competitive inhibitor B4162 are partly protected from the hypotensive reaction to  $\beta$ -Factor XIIa (27). A similar observation is now made in animals pretreated with  $\alpha_1$ -antitrypsin Ala<sup>357</sup> Arg<sup>358</sup> (Fig. 1 C), thereby demonstrating that the double mutant can prevent a well documented kinin-mediated reaction. The effect of  $\alpha_1$ -antitrypsin Ala<sup>357</sup> Arg<sup>358</sup> is related to its specific and efficient inactivation of the proteolytic enzymes of the kallikrein-kinin system. Indeed,  $\alpha_1$ -antitrypsin Val<sup>358</sup>, which is active against neutrophil elastase but not against  $\beta$ -Factor XIIa or kallikrein (18), did not prevent the hypotensive response to  $\beta$ -Factor XIIa (Fig. 1 B). Furthermore, because  $\alpha_1$ -antitrypsin Ala<sup>357</sup> Arg<sup>358</sup> prevents the kinin-dependent consequences of prekallikrein and Factor XII activation, it is likely that this inhibitor will also inhibit the kinin-independent manifestations that are observed when these enzymes are activated. These reactions include activation of the blood coagulation and fibrinolytic pathways and of blood neutrophils (1).

In vivo manifestation of the specificity of  $\alpha_1$ -antitrypsin Ala<sup>357</sup> Arg<sup>358</sup> and further indication of its clinical potential was obtained by studying the anticoagulant properties of the double mutant. With a dose of 0.7 mg of  $\alpha_1$ -antitrypsin Ala<sup>357</sup> Arg<sup>358</sup>, which reduced the hypotensive reaction to  $\beta$ -Factor XIIa (Fig. 1 C), no prolongation of the thrombin time was detectable, whereas with the same dose of  $\alpha_1$ -antitrypsin Arg<sup>358</sup>, the thrombin time was prolonged significantly (Table II). Therefore, in vivo,  $\alpha_1$ -antitrypsin Ala<sup>357</sup> Arg<sup>358</sup> can efficiently inhibit the enzymes of the plasma kinin-forming system without causing increased inhibition of thrombin, an observation that confirms and amplifies the results obtained by our kinetic studies in purified systems.

Further studies are now required to establish the inhibitory activity of  $\alpha_1$ -antitrypsin Ala<sup>357</sup> Arg<sup>358</sup> toward other Arg-specific proteases of the coagulation, complement, and fibrinolytic systems. These studies will determine whether  $\alpha_1$ -antitrypsin Ala<sup>357</sup> Arg<sup>358</sup> is sufficiently specific for the enzymes of the plasma kinin-forming system or whether additional mutants should be constructed. If new molecules must be created, the detailed sequence informations now available for most plasma protease inhibitors of the serpin superfamily should permit the generation of a new set of molecules with the desired specificity.

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Table II. In Vivo Anticoagulant Effect of  $\alpha_1$ -Antitrypsin Mutants

$\alpha_1$ -Antitrypsin	Dose	Thrombin time*
	mg	s
Val <sup>358</sup>	0.7	28.1 and 29.3
Arg <sup>358</sup>	0.7	64.5 and 40.5
	0.35	31.3
Ala <sup>357</sup> Arg <sup>358</sup>	0.7	28.5 and 29.3

\* Values are average of duplicate determinations and there is one value per animal.

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