

## Recombinant $\alpha_1$ -Antitrypsin Pittsburgh (Met<sup>358</sup> → Arg) Is a Potent Inhibitor of Plasma Kallikrein and Activated Factor XII Fragment

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### Abstract

In normal plasma, the serine protease inhibitor  $\alpha_1$ -antitrypsin ( $\alpha_1$ -AT) plays little or no role in the control of plasma kallikrein or activated Factor XII fragment (Factor XII<sub>f</sub>), this function being performed by C1-inhibitor. Recently, an  $\alpha_1$ -AT variant was described with a Met → Arg mutation at the reactive center P<sub>1</sub> residue (position 358) which altered the specificity of inhibition from the Met- or Val-specific protease neutrophil elastase to thrombin, an Arg-specific protease. We have now examined the inhibition of plasma kallikrein and Factor XII<sub>f</sub>, both Arg-specific enzymes, with recombinant  $\alpha_1$ -AT(Met<sup>358</sup> → Arg) produced by an *Escherichia coli* strain carrying a mutated human  $\alpha_1$ -AT gene. The engineered protein was a very efficient inhibitor of both enzymes. It was more effective than C1-inhibitor by a factor of 4.1 for kallikrein and 11.5 for Factor XII<sub>f</sub>. These results suggest that recombinant  $\alpha_1$ -AT(Met<sup>358</sup> → Arg) has therapeutic potential for disease states where activation of the plasma kinin-forming system is observed, for example in hereditary angioedema or septic shock.

### Introduction

$\alpha_1$ -Antitrypsin ( $\alpha_1$ -AT)<sup>1</sup> belongs to a family of serine protease inhibitors that includes antithrombin III,  $\alpha_2$ -antiplasmin, and C1-inhibitor (1). These molecules possess a single and inhibitor-specific reactive site peptide bond that is formed between adjacent amino acid residues termed P<sub>1</sub> and P<sub>1</sub>' (1). The reactivity of these inhibitors with proteolytic enzymes depends heavily upon the nature of the residue at position P<sub>1</sub>, the central position of the reactive center (1). For example, when the P<sub>1</sub> Met of  $\alpha_1$ -AT is replaced by Arg, the resulting  $\alpha_1$ -AT(Met<sup>358</sup> → Arg) or  $\alpha_1$ -AT (Pittsburgh) efficiently inhibits thrombin, but loses the neutrophil elastase-inactivating capacity of normal  $\alpha_1$ -AT (2). Moreover, a recent report indicates that natural  $\alpha_1$ -AT(Met<sup>358</sup> → Arg) is efficient in inhibiting other Arg-specific proteases, including plasma kallikrein and activated Factor XII fragment (Factor XII<sub>f</sub>) (3).

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1. Abbreviations used in this paper:  $\alpha_1$ -AT,  $\alpha_1$ -antitrypsin; Factor XII<sub>f</sub>, Factor XII fragment; r, recombinant; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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$\alpha_1$ -AT complementary DNA has already been cloned and expressed in *Escherichia coli* (4). Furthermore, site-directed mutagenesis of the cloned  $\alpha_1$ -AT cDNA has allowed the synthesis of recombinant (r)  $\alpha_1$ -AT variants with specific mutations at the P<sub>1</sub> residue, such as the Pittsburgh mutant analogue r $\alpha_1$ -AT(Met<sup>358</sup> → Arg) and the oxidation-resistant variant r $\alpha_1$ -AT(Met<sup>358</sup> → Val) (5). In the present report, we have examined the reactivity of r $\alpha_1$ -AT and two variant forms with plasma kallikrein and Factor XII<sub>f</sub>.

### Methods

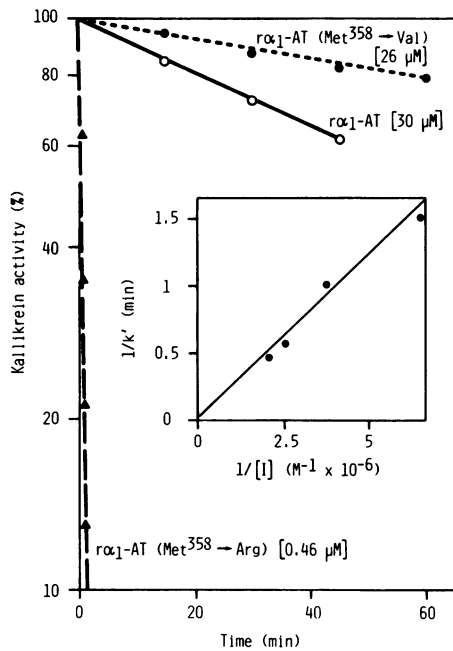
**Proteins.** Plasma kallikrein (6) and Factor XII<sub>f</sub> (7) were prepared as indicated. r $\alpha_1$ -AT, r $\alpha_1$ -AT(Met<sup>358</sup> → Arg), and r $\alpha_1$ -AT(Met<sup>358</sup> → Val) were purified using standard techniques (manuscript in preparation) from the *E. coli* strains previously described (5).

**Kinetic studies.** Kallikrein or Factor XII<sub>f</sub> were preincubated at 23°C with the various forms of r $\alpha_1$ -AT and assayed at various times for residual amidolytic activity using the chromogenic substrate H-D-Pro-Phe-Arg-p-nitroanilide (S-2302) (Kabi Diagnostica, Stockholm, Sweden). A 0.6-mM solution of the substrate was prepared in 85 mM sodium phosphate buffer, pH 7.6, containing 127 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., Instrument Group, Palo Alto, CA). Under these conditions, the hydrolysis rate of S-2302 was 82  $\mu$ mol/min with 1 mg kallikrein (8) and 17.8  $\mu$ mol/min with 1 mg Factor XII<sub>f</sub> (7). Pseudo-first-order ( $k'$ ) and second-order ( $k''$ ) rate constants for the reaction between kallikrein or Factor XII<sub>f</sub> and the various r $\alpha_1$ -AT species were determined according to Kitz and Wilson (9).

**Electrophoretic studies.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10) was performed using vertical slab gels. The concentration of acrylamide in the separating gel was 10%. Non-reducing conditions were used.

### Results

The inactivation of kallikrein amidolytic activity by various concentrations of r $\alpha_1$ -AT, r $\alpha_1$ -AT(Met<sup>358</sup> → Arg), and r $\alpha_1$ -AT(Met<sup>358</sup> → Val) followed pseudo-first-order kinetics when these inhibitors were in a 7-380-fold molar excess (Fig. 1). r $\alpha_1$ -AT(Met<sup>358</sup> → Arg) was dramatically more efficient in inactivating kallikrein than was r $\alpha_1$ -AT or r $\alpha_1$ -AT(Met<sup>358</sup> → Val). When r $\alpha_1$ -AT(Met<sup>358</sup> → Arg) was present at a concentration of 0.46  $\mu$ M, 50% of the kallikrein activity was lost within 0.33 min ( $k' = 2.1 \text{ min}^{-1}$ ; Fig. 1, triangles). In contrast, only 38% of kallikrein was inactivated at 45 min with 30  $\mu$ M r $\alpha_1$ -AT ( $k' = 0.01 \text{ min}^{-1}$  and  $k'' \approx k'/[I] = 3.4 \times 10^2 \text{ M}^{-1} \text{ min}^{-1}$ ; Fig. 1, open circles). Furthermore, 26  $\mu$ M r $\alpha_1$ -AT(Met<sup>358</sup> → Val) was required to obtain a 20% reduction of kallikrein activity at 60 min ( $k' = 0.004 \text{ min}^{-1}$  and  $k'' \approx k'/[I] = 1.4 \times 10^2 \text{ M}^{-1} \text{ min}^{-1}$ ; Fig. 1, closed



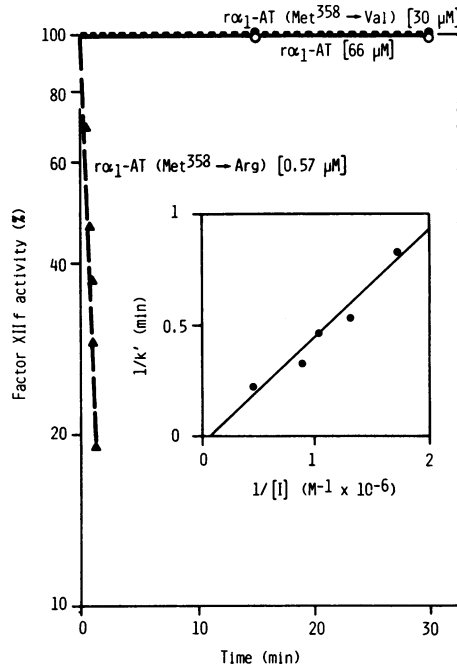
**Figure 1.** Kinetics of inactivation of kallikrein amidolytic activity by  $r\alpha_1$ -AT,  $r\alpha_1$ -AT(Met<sup>358</sup> → Arg), and  $r\alpha_1$ -AT(Met<sup>358</sup> → Val). Kallikrein (final concentration, 0.03  $\mu$ M) was incubated with the various inhibitors and then assayed at various times for residual amidolytic activity. The inset shows a double reciprocal plot of  $k'$  and the concentration of  $r\alpha_1$ -AT(Met<sup>358</sup> → Arg) [I]. The line drawn is a least-squares fit of the experimental points ( $r = 0.98$ ). The equation of the line is  $y = 0.24x + 0.01$ .

circles). The reaction between kallikrein and  $r\alpha_1$ -AT(Met<sup>358</sup> → Arg) was then examined using additional concentrations of this inhibitor; a double-reciprocal plot of  $k'$  vs. the  $r\alpha_1$ -AT(Met<sup>358</sup> → Arg) concentration (Fig. 1, inset) indicated that this reaction had a  $k''$  of  $4.17 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$ . Subsequent experiments showed that  $r\alpha_1$ -AT(Met<sup>358</sup> → Arg) also rapidly inactivated Factor XIIIf: a  $k'$  value of  $1.21 \text{ min}^{-1}$  was determined with an inhibitor concentration of  $0.57 \mu\text{M}$  (Fig. 2, triangles), while  $k''$  was  $2.13 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$  (Fig. 2, inset). However, no reaction was detectable between Factor XIIIf and  $r\alpha_1$ -AT or  $r\alpha_1$ -AT(Met<sup>358</sup> → Val) (Fig. 2, open and closed circles).

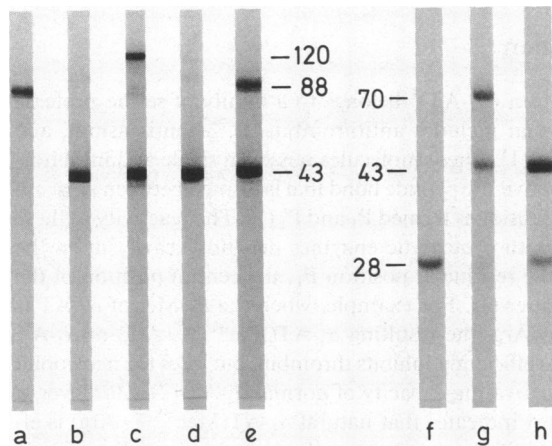
The reaction between kallikrein (relative molecular weight [ $M_r$ ]  $M_r$  88,000 and 85,000; Fig. 3, lane a) or Factor XIIIf ( $M_r$  28,000; Fig. 3, lane f) and either  $r\alpha_1$ -AT(Met<sup>358</sup> → Arg) ( $M_r$  43,000; Fig. 3, lane b) or  $r\alpha_1$ -AT ( $M_r$  43,000; Fig. 3, lane d) was then analyzed by SDS-PAGE. Whereas the incubation of kallikrein with  $r\alpha_1$ -AT(Met<sup>358</sup> → Arg) resulted, within 5 min, in the formation of a complex stable in sodium dodecyl sulfate with an apparent  $M_r$  of 120,000 (Fig. 3, lane c), no such complex was formed using  $r\alpha_1$ -AT under the same conditions (Fig. 3, lane e). A similar observation was made with Factor XIIIf, which formed a complex with an apparent  $M_r$  of 70,000 when incubated for 1 min with  $r\alpha_1$ -AT(Met<sup>358</sup> → Arg) (Fig. 3, lane g), but not when incubated for 10 min with  $r\alpha_1$ -AT (Fig. 3, lane h).

## Discussion

The results presented here demonstrate that  $r\alpha_1$ -AT(Met<sup>358</sup> → Arg) is a very efficient inhibitor of both plasma kallikrein and



**Figure 2.** Kinetics of inactivation of Factor XIIIf amidolytic activity by  $r\alpha_1$ -AT,  $r\alpha_1$ -AT(Met<sup>358</sup> → Arg), and  $r\alpha_1$ -AT(Met<sup>358</sup> → Val). Factor XIIIf (final concentration, 0.11  $\mu$ M) was incubated with the various inhibitors and then assayed at various times for residual amidolytic activity. The inset shows a double-reciprocal plot of  $k'$  and the concentration of  $r\alpha_1$ -AT(Met<sup>358</sup> → Arg) [I]. The line drawn is a least-square fit of the experimental points ( $r = 0.97$ ). The equation of the line is  $y = 0.47x - 0.03$ .



**Figure 3.** Lane a, SDS-PAGE (10%) of plasma kallikrein; lane b,  $r\alpha_1$ -AT(Met<sup>358</sup> → Arg); lane c, the mixture resulting from a 5-min incubation of kallikrein with a molar excess of  $r\alpha_1$ -AT(Met<sup>358</sup> → Arg); lane d,  $r\alpha_1$ -AT; lane e, the mixture resulting from a 5-min incubation of kallikrein with a molar excess of  $r\alpha_1$ -AT; lane f, Factor XIIIf; lane g, the mixture resulting from a 1-min incubation of Factor XIIIf with a molar excess of  $r\alpha_1$ -AT(Met<sup>358</sup> → Arg); and lane h, the mixture resulting from the incubation of Factor XIIIf with a molar excess of  $r\alpha_1$ -AT. Kallikrein and Factor XIIIf were incubated with  $r\alpha_1$ -AT(Met<sup>358</sup> → Arg) or  $r\alpha_1$ -AT at 23°C and the reactions were stopped at the indicated times by adding 0.5 vol of 0.2 M Tris-HCl, pH 6.8, containing 50% glycerol, 5% SDS, and 0.004% bromophenol blue and placing the reaction vessels in a boiling water bath for 5 min. Each lane contained  $\sim 10 \mu\text{g}$  of protein, which was stained using Coomassie Blue. Center numbers are  $M_r \times 10^{-3}$ .

Factor XIIIf. Kinetically, the second-order rate constant,  $k''$ , for the reaction between kallikrein and this inhibitor, was 17,000 times greater than the  $k''$  calculated for the reaction with natural  $\alpha_1$ -AT (11),  $r\alpha_1$ -AT, or  $r\alpha_1$ -AT(Met<sup>358</sup> → Val) (Fig. 1). In addition, a  $k''$  value of  $2.13 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$  was found for the reaction between  $r\alpha_1$ -AT(Met<sup>358</sup> → Arg) and Factor XIIIf. Since this serine protease did not react at a detectable rate with natural  $\alpha_1$ -AT (7),  $r\alpha_1$ -AT, or  $r\alpha_1$ -AT(Met<sup>358</sup> → Val) (Fig. 2), this observation emphasizes the critical role of Arg at the P<sub>1</sub> position for the inactivation of Factor XIIIf.

Plasma protease inhibitors of the  $\alpha_1$ -AT family react with their target enzymes to form stable and apparently covalent enzyme-inhibitor complexes (1). When the products of the inactivation of kallikrein or Factor XIIIf by  $r\alpha_1$ -AT(Met<sup>358</sup> → Arg) were analyzed by SDS-PAGE, new species with  $M_r$  of 120,000 and 70,000 were formed (Fig. 3, lanes *c* and *g*). Because the relative molecular weight of the new components are in reasonable agreement with the sum of the relative molecular weight of the parent molecules, our present results indicate that the reaction of kallikrein or Factor XIIIf with  $r\alpha_1$ -AT(Met<sup>358</sup> → Arg) leads to the formation of 1:1 stoichiometric complexes.

The predominant inhibitor in normal plasma of kallikrein and Factor XIIIf is C1-inhibitor (7, 12). In purified systems, this inhibitor reacts with kallikrein and Factor XIIIf with  $k''$  values of 1.02 and  $0.19 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$ , respectively (7, 13). Thus, on a molar basis,  $r\alpha_1$ -AT(Met<sup>358</sup> → Arg) was more efficient than C1-inhibitor with both kallikrein (4.1-fold) and Factor XIIIf (11.5-fold). This suggests that  $r\alpha_1$ -AT(Met<sup>358</sup> → Arg) could be useful for the management of disease states associated with unregulated activation of prekallikrein and Factor XII, such as hereditary angioedema attacks, septic shock, and the adult respiratory distress syndrome (14–16). The only patient described with the  $\alpha_1$ -AT (Pittsburgh) variant died at age 14 after an intermittent but lifelong hemorrhagic diathesis (2, 17). When the patient was bleeding, the plasma concentration of  $\alpha_1$ -AT(Met<sup>358</sup> → Arg) was  $\sim 40 \mu\text{M}$ , which fell by a factor of 3.6 during the quiescent stage (2), suggesting that adequate hemostasis can be achieved with around  $10 \mu\text{M}$   $r\alpha_1$ -AT(Met<sup>358</sup> → Arg). With this level of synthetic inhibitor, Factor XIIIf could be effectively blocked, since its half-time would be reduced to 0.03 min, i.e., 57 times less than the 1.7 min calculated with C1-inhibitor at normal plasma concentration [ $2.2 \mu\text{M}$ ] (18).

$r\alpha_1$ -AT(Met<sup>358</sup> → Arg) is an efficient inhibitor of several Arg-specific proteases of biological importance, including thrombin, plasma kallikrein, and Factor XIIIf. By further manipulations of the  $\alpha_1$ -AT reactive center, it should be possible to design additional new inhibitors of therapeutic value that act on a narrower range of enzymes, which, for example, inhibit kallikrein and not thrombin.

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