Rapid Publication

Recombinant α₁-Antitrypsin Pittsburgh (Met$^{358}$ → 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 α₁-antitrypsin (α₁-AT) plays little or no role in the control of plasma kallikrein or activated Factor XII fragment (Factor XIIIf), this function being performed by Cl-inhibitor. Recently, an α₁-AT variant was described with a Met → Arg mutation at the reactive center P₁ 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 XIIIf, both Arg-specific enzymes, with recombinant α₁-AT(Met$^{358}$ → Arg) produced by an Escherichia coli strain carrying a mutated human α₁-AT gene. The engineered protein was a very efficient inhibitor of both enzymes. It was more effective than Cl-inhibitor by a factor of 4.1 for kallikrein and 11.5 for Factor XIIIf. These results suggest that recombinant α₁-AT(Met$^{358}$ → 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

α₁-Antitrypsin (α₁-AT)$^1$ belongs to a family of serine protease inhibitors that includes antithrombin III, α₂-antiplasmin, and Cl-inhibitor (1). These molecules possess a single and inhibitor-specific reactive site peptide bond that is formed between adjacent amino acid residues termed P₁ and Pᵢ (1). The reactivity of these inhibitors with proteolytic enzymes depends heavily upon the nature of the residue at position P₁, the central position of the reactive center (1). For example, when the P₁ Met of α₁-AT is replaced by Arg, the resulting α₁-AT(Met$^{358}$ → Arg) or α₁-AT (Pittsburgh) efficiently inhibits thrombin, but loses the neutrophil elastase-inactivating capacity of normal α₁-AT (2). Moreover, a recent report indicates that natural α₁-AT(Met$^{358}$ → Arg) is efficient in inhibiting other Arg-specific proteases, including plasma kallikrein and activated Factor XII fragment (Factor XIIIf) (3).

α₁-AT complementary DNA has already been cloned and expressed in Escherichia coli (4). Furthermore, site-directed mutagenesis of the cloned α₁-AT CDNA has allowed the synthesis of recombinant (r) α₁-AT variants with specific mutations at the P₁ residue, such as the Pittsburgh mutant analogue rα₁-AT(Met$^{358}$ → Arg) and the oxidation-resistant variant rα₁-AT(Met$^{358}$ → Val) (5). In the present report, we have examined the reactivity of rα₁-AT and two variant forms with plasma kallikrein and Factor XIIIf.

Methods

Proteins. Plasma kallikrein (6) and Factor XIIIf (7) were prepared as indicated. rα₁-AT, rα₁-AT(Met$^{358}$ → Arg), and rα₁-AT(Met$^{358}$ → Val) were purified using standard techniques (manuscript in preparation) from the E. coli strains previously described (5).

Kinetic studies. Kallikrein or Factor XIIIf were preincubated at 23°C with the various forms of rα₁-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 μl of the solution to be tested was added to 300 μl 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 μmol/min with 1 mg kallikrein (8) and 17.8 μmol/min with 1 mg Factor XIIIf (7). Pseudo-first-order (k') and second-order (k") rate constants for the reaction between kallikrein or Factor XIIIf and the various rα₁-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 rα₁-AT, rα₁-AT(Met$^{358}$ → Arg), and rα₁-AT(Met$^{358}$ → Val) followed pseudo-first-order kinetics when these inhibitors were in a 7–380-fold molar excess (Fig. 1). rα₁-AT(Met$^{358}$ → Arg) was dramatically more efficient in inactivating kallikrein than was rα₁-AT or rα₁-AT(Met$^{358}$ → Val). When rα₁-AT(Met$^{358}$ → Arg) was present at a concentration of 0.46 μM, 50% of the kallikrein activity was lost within 0.33 min (k' = 2.1 min⁻¹; Fig. 1, triangles). In contrast, only 38% of kallikrein was inactivated at 45 min with 30 μM rα₁-AT (k" = 0.01 min⁻¹ and k'" ≈ k'/[I] = 3.4 × 10⁻² M⁻¹ min⁻¹; Fig. 1, open circles). Furthermore, 26 μM rα₁-AT(Met$^{358}$ → Val) was required to obtain a 20% reduction of kallikrein activity at 60 min (k" = 0.004 min⁻¹ and k'" ≈ k'/[I] = 1.4 × 10⁻² M⁻¹ min⁻¹; Fig. 1, closed...
circles). The reaction between kallikrein and rα1-AT(Met358 → Arg) was then examined using additional concentrations of this inhibitor; a double-reciprocal plot of k' vs. the rα1-AT(Met358 → Arg) concentration (Fig. 1, inset) indicated that this reaction had a k' of 4.17 × 10^6 M⁻¹ min⁻¹. Subsequent experiments showed that rα1-AT(Met358 → Arg) also rapidly inactivated Factor XII: a k' value of 1.21 min⁻¹ was determined with an inhibitor concentration of 0.57 μM (Fig. 2, triangles), while k' was 2.13 × 10^6 M⁻¹ min⁻¹ (Fig. 2, inset). However, no reaction was detectable between Factor XIIIf and rα1-AT or rα1-AT(Met358 → Val) (Fig. 2, open and closed circles).

The reaction between kallikrein (relative molecular weight (M_r), 88,000 and 85,000; Fig. 3, lane a) or Factor XIIIf (M_r, 28,000; Fig. 3, lane f) and either rα1-AT(Met358 → Arg) (M_r, 43,000; Fig. 3, lane b) or rα1-AT (M_r, 43,000; Fig. 3, lane d) was then analyzed by SDS-PAGE. Whereas the incubation of kallikrein with rα1-AT(Met358 → 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 rα1-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 rα1-AT(Met358 → Arg) (Fig. 3, lane g), but not when incubated for 10 min with rα1-AT (Fig. 3, lane h).

**Discussion**

The results presented here demonstrate that rα1-AT(Met358 → Arg) is a very efficient inhibitor of both plasma kallikrein and Factor XIIIf. 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.

**Figure 1.** Kinetics of inactivation of kallikrein amidolytic activity by rα1-AT, rα1-AT(Met358 → Arg), and rα1-AT(Met358 → Val). Kallikrein (final concentration, 0.03 μM) 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 rα1-AT(Met358 → Arg) [1]. 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.

**Figure 2.** Kinetics of inactivation of Factor XIIIf amidolytic activity by rα1-AT, rα1-AT(Met358 → Arg), and rα1-AT(Met358 → Val). Factor XIIIf (final concentration, 0.11 μM) 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 rα1-AT(Met358 → Arg) [1]. 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, rα1-AT(Met358 → Arg); lane c, the mixture resulting from a 5-min incubation of kallikrein with a molar excess of rα1-AT(Met358 → Arg); lane d, rα1-AT; lane e, the mixture resulting from a 5-min incubation of kallikrein with a molar excess of rα1-AT; lane f, Factor XIIIf; lane g, the mixture resulting from a 1-min incubation of Factor XIIIf with a molar excess of rα1-AT(Met358 → Arg); and lane h, the mixture resulting from the incubation of Factor XIIIf with a molar excess of rα1-AT. Kallikrein and Factor XIIIf were incubated with rα1-AT(Met358 → Arg) or rα1-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 ~10 μg of protein, which was stained using Coomassie Blue. Center numbers are M_r × 10^3.
Factor XIIa. Kinetically, the second-order rate constant, $k^\prime$, for the reaction between kallikrein and this inhibitor, was 17,000 times greater than the $k^\prime$ calculated for the reaction with natural $\alpha_1$-AT (11), $\alpha_1$-AT, or $\alpha_1$-AT(Met$^{358}$ → Arg) (Fig. 1). In addition, a $k^\prime$ value of 2.13 × 10$^6$ M$^{-1}$ min$^{-1}$ was found for the reaction between $\alpha_1$-AT(Met$^{358}$ → Arg) and Factor XIIa. Since this serine protease did not react at a detectable rate with natural $\alpha_1$-AT (7), $\alpha_1$-AT, or $\alpha_1$-AT(Met$^{358}$ → Val) (Fig. 2), this observation emphasizes the critical role of Arg at the P$_1$ position for the inactivation of Factor XIIa.

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 XIIa by $\alpha_1$-AT(Met$^{358}$ → Arg) were analyzed by SDS-PAGE, new species with $M_1$ 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 XIIa with $\alpha_1$-AT(Met$^{358}$ → Arg) leads to the formation of 1:1 stoichiometric complexes.

The predominant inhibitor in normal plasma of kallikrein and Factor XIIa is $\alpha_2$-CI-inhibitor (7, 12). In purified systems, this inhibitor reacts with kallikrein and Factor XIIa with $k^\prime$ values of 1.02 and 0.19 × 10$^6$ M$^{-1}$ min$^{-1}$, respectively (7, 13). Thus, on a molar basis, $\alpha_1$-AT(Met$^{358}$ → Arg) was more efficient than $\alpha_2$-CI-inhibitor with both kallikrein (4.1-fold) and Factor XIIa (11.5-fold). This suggests that $\alpha_1$-AT(Met$^{358}$ → 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$^{358}$ → Arg) was ~40 μM, which fell by a factor of 3.6 during the quiescent stage (2), suggesting that adequate hemostasis can be achieved with around 10 μM $\alpha_1$-AT(Met$^{358}$ → Arg). With this level of synthetic inhibitor, Factor XIIa could be effectively blocked, since its halftime would be reduced to 0.03 min, i.e., 57 times less than the 1.7 min calculated with CI-inhibitor at normal plasma concentration [2.2 μM] (18).

$\alpha_1$-AT(Met$^{358}$ → Arg) is an efficient inhibitor of several Arg-specific proteases of biological importance, including thrombin, plasma kallikrein, and Factor XIIa. 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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References