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Research Article

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Recombinant α_1-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 α_1-antitrypsin (α_1-AT) plays little or no role in the control of plasma kallikrein or activated Factor XII fragment (Factor XIIf), this function being performed by Cl-inhibitor. Recently, an α_1-AT variant was described with a Met → Arg mutation at the reactive center P_1 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 XIIf, both Arg-specific enzymes, with recombinant α_1-AT(Met^{358} → Arg) produced by an *Escherichia coli* strain carrying a mutated human α_1-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 XIIf. These results suggest that recombinant α_1-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

α_1-Antitrypsin (α_1-AT)[1] belongs to a family of serine protease inhibitors that includes antithrombin III, α_2-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 P_1 and P_1' (1). The reactivity of these inhibitors with proteolytic enzymes depends heavily upon the nature of the residue at position P_1, the central position of the reactive center (1). For example, when the P_1 Met of α_1-AT is replaced by Arg, the resulting α_1-AT(Met^{358} → Arg) or α_1-AT (Pittsburgh) efficiently inhibits thrombin, but loses the neutrophil elastase-inactivating capacity of normal α_1-AT (2). Moreover, a recent report indicates that natural α_1-AT(Met^{358} → Arg) is efficient in inhibiting other Arg-specific proteases, including plasma kallikrein and activated Factor XII fragment (Factor XIIf) (3).

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1. *Abbreviations used in this paper:* α_1-AT, α_1-antitrypsin; Factor XIIf, Factor XII fragment; r, recombinant; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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α_1-AT complementary DNA has already been cloned and expressed in *Escherichia coli* (4). Furthermore, site-directed mutagenesis of the cloned α_1-AT cDNA has allowed the synthesis of recombinant (r) α_1-AT variants with specific mutations at the P_1 residue, such as the Pittsburgh mutant analogue rα_1-AT(Met^{358} → Arg) and the oxidation-resistant variant rα_1-AT(Met^{358} → Val) (5). In the present report, we have examined the reactivity of rα_1-AT and two variant forms with plasma kallikrein and Factor XIIf.

Methods

Proteins. Plasma kallikrein (6) and Factor XIIf (7) were prepared as indicated. rα_1-AT, rα_1-AT(Met^{358} → Arg), and rα_1-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 XIIf were preincubated at 23°C with the various forms of rα_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 μl of the solution to be tested was added to 330 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 XIIf (7). Pseudo–first-order (k') and second-order (k'') rate constants for the reaction between kallikrein or Factor XIIf and the various rα_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%. Nonreducing conditions were used.

Results

The inactivation of kallikrein amidolytic activity by various concentrations of rα_1-AT, rα_1-AT(Met^{358} → Arg), and rα_1-AT(Met^{358} → Val) followed pseudo–first-order kinetics when these inhibitors were in a 7–380-fold molar excess (Fig. 1). rα_1-AT(Met^{358} → Arg) was dramatically more efficient in inactivating kallikrein than was rα_1-AT or rα_1-AT(Met^{358} → Val). When rα_1-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 rα_1-AT ($k' = 0.01$ min^{-1} and $k'' \simeq k'/[I] = 3.4 \times 10^2$ M^{-1} min^{-1}; Fig. 1, open circles). Furthermore, 26 μM rα_1-AT(Met^{358} → Val) was required to obtain a 20% reduction of kallikrein activity at 60 min ($k' = 0.004$ min^{-1} and $k'' \simeq k'/[I] = 1.4 \times 10^2$ M^{-1} min^{-1}; Fig. 1, closed

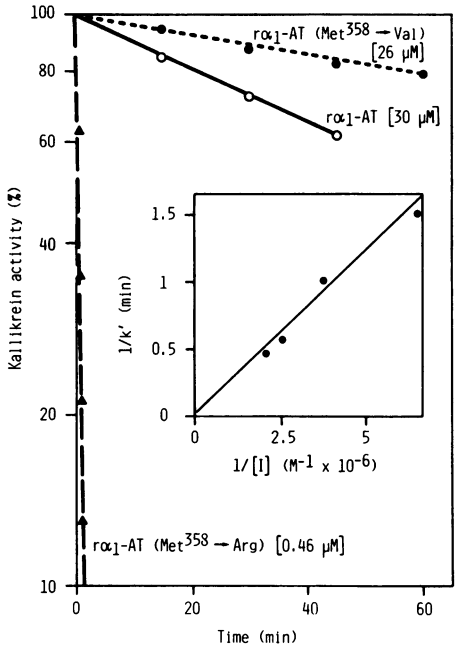

Figure 1. Kinetics of inactivation of kallikrein amidolytic activity by rα$_1$-AT, rα$_1$-AT(Met^{358} → Arg), and rα$_1$-AT(Met^{358} → Val). Kallikrein (final concentration, 0.03 μM) was incubated with the various inhibitors and then assayed at various times for residual amidolytic activity. The inset shows a double reciprocal plot of k' and the concentration of rα$_1$-AT(Met^{358} → Arg) [I]. The line drawn is a least-squares fit of the experimental points (r = 0.98). The equation of the line is y = $0.24x + 0.01$.

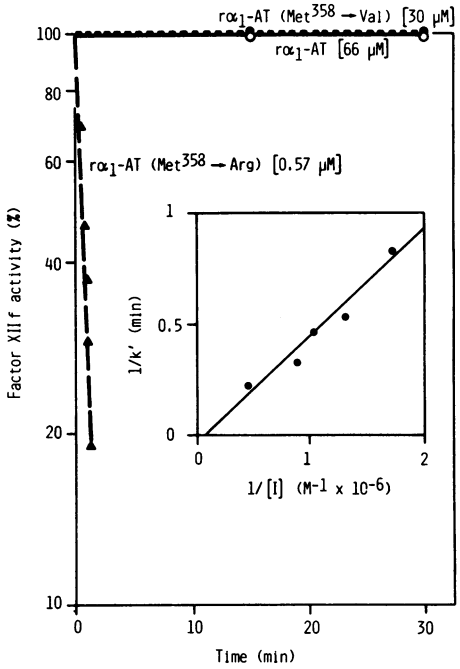

Figure 2. Kinetics of inactivation of Factor XIIf amidolytic activity by rα$_1$-AT, rα$_1$-AT(Met^{358} → Arg), and rα$_1$-AT(Met^{358} → Val). Factor XIIf (final concentration, 0.11 μM) was incubated with the various inhibitors and then assayed at various times for residual amidolytic activity. The inset shows a double-reciprocal plot of k' and the concentration of rα$_1$-AT(Met^{358} → Arg) [I]. The line drawn is a least-square fit of the experimental points (r = 0.97). The equation of the line is y = $0.47x - 0.03$.

circles). The reaction between kallikrein and rα$_1$-AT(Met^{358} → Arg) was then examined using additional concentrations of this inhibitor; a double-reciprocal plot of k' vs. the rα$_1$-AT(Met^{358} → 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 rα$_1$-AT(Met^{358} → Arg) also rapidly inactivated Factor XIIf: 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 XIIf and rα$_1$-AT or rα$_1$-AT(Met^{358} → 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 XIIf (M_r 28,000; Fig. 3, lane *f*) and either rα$_1$-AT(Met^{358} → Arg) (M_r 43,000; Fig. 3, lane *b*) or rα$_1$-AT (M_r 43,000; Fig. 3, lane *d*) was then analyzed by SDS-PAGE. Whereas the incubation of kallikrein with rα$_1$-AT(Met^{358} → 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 rα$_1$-AT under the same conditions (Fig. 3, lane *e*). A similar observation was made with Factor XIIf, which formed a complex with an apparent M_r of 70,000 when incubated for 1 min with rα$_1$-AT(Met^{358} → Arg) (Fig. 3, lane *g*), but not when incubated for 10 min with rα$_1$-AT (Fig. 3, lane *h*).

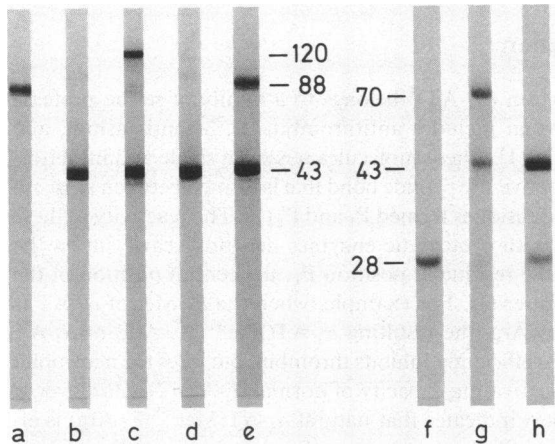

Figure 3. Lane *a*, SDS-PAGE (10%) of plasma kallikrein; lane *b*, rα$_1$-AT(Met^{358} → Arg); lane *c*, the mixture resulting from a 5-min incubation of kallikrein with a molar excess of rα$_1$-AT(Met^{358} → Arg); lane *d*, rα$_1$-AT; lane *e*, the mixture resulting from a 5-min incubation of kallikrein with a molar excess of rα$_1$-AT; lane *f*, Factor XIIf; lane *g*, the mixture resulting from a 1-min incubation of Factor XIIf with a molar excess of rα$_1$-AT(Met^{358} → Arg); and lane *h*, the mixture resulting from the incubation of Factor XIIf with a molar excess of rα$_1$-AT. Kallikrein and Factor XIIf were incubated with rα$_1$-AT(Met^{358} → Arg) or rα$_1$-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 ~10 μg of protein, which was stained using Coomassie Blue. Center numbers are $M_r \times 10^{-3}$.

Discussion

The results presented here demonstrate that rα$_1$-AT(Met^{358} → Arg) is a very efficient inhibitor of both plasma kallikrein and

Factor XIIf. 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 α_1-AT (11), rα_1-AT, or rα_1-AT(Met^{358} → Val) (Fig. 1). In addition, a k'' value of 2.13×10^6 M^{-1} min^{-1} was found for the reaction between rα_1-AT(Met^{358} → Arg) and Factor XIIf. Since this serine protease did not react at a detectable rate with natural α_1-AT (7), rα_1-AT, or rα_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 XIIf.

Plasma protease inhibitors of the α_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 XIIf by rα_1-AT(Met^{358} → 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 XIIf with rα_1-AT(Met^{358} → Arg) leads to the formation of 1:1 stoichiometric complexes.

The predominant inhibitor in normal plasma of kallikrein and Factor XIIf is Cl-inhibitor (7, 12). In purified systems, this inhibitor reacts with kallikrein and Factor XIIf with k'' values of 1.02 and 0.19×10^6 M^{-1} min^{-1}, respectively (7, 13). Thus, on a molar basis, rα_1-AT(Met^{358} → Arg) was more efficient than Cl-inhibitor with both kallikrein (4.1-fold) and Factor XIIf (11.5-fold). This suggests that rα_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 α_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 α_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 rα_1-AT(Met^{358} → Arg). With this level of synthetic inhibitor, Factor XIIf 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 Cl-inhibitor at normal plasma concentration [2.2 μM] (18).

rα_1-AT(Met^{358} → Arg) is an efficient inhibitor of several Arg-specific proteases of biological importance, including thrombin, plasma kallikrein, and Factor XIIf. By further manipulations of the α_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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