Reactivity of α1-Antitrypsin Mutants against Proteolytic Enzymes of the Kallikrein-Kinin, Complement, and Fibrinolytic Systems*

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Increased extracellular proteolysis because of unregulated activation of blood coagulation, complement, and fibrinolysis is observed in thrombosis, shock, and inflammation. In the present study, we have examined whether the plasma kallikrein-kinin system, the classical pathway of complement, and the fibrinolytic system could be inhibited by α1-antitrypsin reactive site mutants. Wild-type α1-antitrypsin contains a Met residue at P1 (position 358), the central position of the reactive center. It did not inhibit plasma kallikrein, β-factor XIIa, plasmin, tissue-type plasminogen activator (t-PA), or urokinase. In contrast, these serine proteases were inhibited by α1-antitrypsin Arg358. For the inhibition of Cls, a double mutant having Arg358 and a Pro → Ala mutation at P2 (position 357) was required. This double modification was made because C1-inhibitor, the natural inhibitor of Cls, has Arg and Ala residues at positions P1 and P2. Plasminogen activator inhibitor 1, the natural inhibitor of t-PA, also has Arg and Ala residues at positions P1 and P2. In a purified system, α1-antitrypsin Ala357-Arg358 was 150-fold less efficient against Cls than C1-inhibitor and 27,000-fold less efficient against t-PA than plasminogen activator inhibitor 1. In plasma, 2.3 μM α1-antitrypsin Ala357-Arg358 reduced by 65% the formation of a complex between kallikrein and C1-inhibitor following activation of the intrinsic pathway of blood coagulation by kaolin. Furthermore, after supplementation by 2.0 μM α1-antitrypsin Ala357-Arg358, zymographic analysis showed that the majority of the free t-PA of normal plasma formed a bimolecular complex with the double mutant. In contrast, 3.4 μM α1-antitrypsin Ala357-Arg358 did not prevent the activation of the classical pathway of complement observed when normal serum is supplemented with anti-C1-inhibitor F(ab')2 fragment. These results demonstrate that α1-antitrypsin Ala357-Arg358 has therapeutic potential for disorders with unregulated activation of the intrinsic pathway of blood coagulation and the fibrinolytic system; however, the double mutant is not an efficient inhibitor for the classical pathway of complement.

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may have a place in the management of disease states (hereditary angioedema, septic shock) where unregulated activation of the plasma kallikrein–kinin system is observed. Wistar rats pretreated with the double mutant (with a dose that did not prolong the thrombin time) were partially protected from the arterial hypotension induced by the administration of β-factor XIIa (19).

The study reported here was undertaken to delineate further the inhibitory characteristics of the mutated α1-antitrypsins and assess whether they could antagonize proteolytic activities of human plasma or serum. Using purified systems, we examined their reactivity against serine proteases of the complement and fibrinolytic systems, including C1s, plasmin, tissue-type plasminogen activator (t-PA)1 and urokinase. In addition, plasma or serum samples were supplemented with the mutants, and we evaluated the effect of this treatment on the activity of the intrinsic pathway of blood coagulation, the classical pathway of complement, and the fibrinolytic system.

**EXPERIMENTAL PROCEDURES**

**Materials**—The chromogenic substrates H-D-Val-Leu-Lys-p-nitroanilide (S-2251), H-D-Val-Leu-Arg-p-nitroanilide (S-2288), H-D-Pro-Phe-p-nitroanilide (S-2302), pyro-Glu-Gly-Arg-p-nitroanilide (S-2254), and Me-CO-Lys(t-Cbo)-Gly-Arg-p-nitroanilide (Spectrozyme Cl-E, American Diagnostica, Greenwich, CT) was a gift from Dr. Richard Hart. Chemical reagents were obtained from Sigma and electrophoretic reagents were from Bio-Rad.

**Proteins**—Plasma kallikrein (in the α-form), C1s, β-factor XIIa (factor XII fragment or Hageman factor fragment), plasminogen, and macromolecular Cl were prepared from normal plasma or serum using published procedures (21-25). Low molecular weight urokinase was obtained from Sigma and recombinant t-PA (single-chain) from Genentech. Plasminogen was activated into plasmin with urokinase. Purified C1-inhibitor was kindly provided by Dr. E. P. Faques, Behring, Marburg, West Germany. Recombinant plasminogen activator inhibitor 1 (PAI-1) was a gift from Dr. Tom Reilly, DuPont. Recombinant α1-antitrypsin variants were prepared essentially as described (10, 11, 19) except that the DNA sequence encoding the NH2-terminal amino acid residues of the mature α1-antitrypsin (Glu-Asp-Pro-Glu-Gly) was deleted from the expression plasmid using standard techniques. The NH2-terminal sequence of each of the Escherichia coli-derived variants was thus Met-Asp-Ala-Ala-... The truncated variants displayed inhibition kinetics identical to the full-length forms with neutrophil elastase, α-thrombin and plasma kallikrein for α1-antitrypsin Leu265, α1-antitrypsin Arg285, and the double mutant α1-antitrypsin Ala265-Arg285, respectively. The reactive site sequences of the various serpins employed in this study are shown in Table I. SDS-PAGE analysis indicated they were quantitatively found in the form of a covalent bimolecular complex, when incubated with a molar excess of the appropriate enzyme or serpin.

**Electrophoretic Studies**—SDS-PAGE, unreduced, was performed using vertical slab gels and a Bio-Rad Protean II system by the method of Laemmli (28). The acrylamide concentration in stacking gel was 4%; the concentration present in the separating gels was 10%. For Coomassie Blue-stained gels, samples were incubated for 5 min in a boiling water bath before electrophoresis.

**Plasma and Serum Preparation**—Citrated plasma and serum were prepared using blood obtained from healthy volunteers. For the preparation of plasma, blood was collected in plastic bags containing citrate/phosphate/dextrose/adenine as an anticoagulant (CPDA-1; Baxter Travenol Laboratories). Citrated blood was centrifuged at 2,000 × g for 10 min at 4 °C, and plasma samples were kept at −70 °C. For the preparation of serum, blood was collected in glass tubes, and it was allowed to clot for 6 h at room temperature. Serum was then processed as described for plasma. Plasma was depleted of C1-inhibitor (<1%) by adsorption to immobilized rabbit anti-C1-inhibitor IgG.

**Formation of the Kallikrein-C1-inhibitor Complex in Plasma**—Plasma was activated with kaolin using the following protocol. A mixture containing 5 μl of plasma and 5 μl of the substance to be tested was incubated at room temperature with 5 μl of kaolin suspension (0.1–4 μm particles) at various concentrations. At various times, 10-μl aliquots were transferred to 0.77 ml of 10 mM phosphate buffer, pH 7.2, containing 148 mM NaCl, 4.2 mM KCl, 0.2 mg/ml soybean trypsin inhibitor, 2 mM diisopropyl fluorophosphate, 2 mM benzamidine, and 0.05% Tween 20. After centrifugation at 15,000 × g for 5 min, supernatants were evaluated for concentrations of the kallikrein-

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1 The abbreviations used are: t-PA, tissue-type plasminogen activator; PAI-1, plasminogen activator inhibitor 1; S-2251, H-D-Val-Leu-Lys-p-nitroanilide; S-2288, H-D-Val-Leu-Arg-p-nitroanilide; S-2302, H-D-Pro-Phe-p-nitroanilide; S-2444, pyro-Glu-Gly-Arg-p-nitroanilide; C1-E, Me-CO-Lys(t-Cbo)-Gly-Arg-p-nitroanilide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

C1 inhibitor complex using a modification of a previously described double antibody enzyme-linked immunosorbent assay (29). The complex formed by kallikrein and C1-inhibitor was captured with mAb 4C3, a monoclonal antibody that detects a neoepitope expressed by C1-inhibitor when its reactive center loop is cleaved (30, 31); the complex was revealed using alkaline phosphatase-labeled anti-kallikrein rabbit IgG and p-nitrophenyl phosphate as the substrate.

Studies on Complement Components in Serum—The activation status of C1 in serum was determined by measuring levels of functional C4 using the one-step hemolytic assay of Gaither et al. (32). Fresh C4-deficient serum was obtained from a guinea pig colony maintained at the University of Geneva. Normal human serum (2 μl) was supplemented with the compound to test. This mixture was incubated at 37 °C with anti-C1-inhibitor F(ab')2 fragment in a final volume of 100 μl. Anti-C1-inhibitor F(ab')2 fragment was used at a concentration (7 mg/ml serum) that had been shown to completely inhibit the function of endogenous C1-inhibitor (Table III). The buffer employed was barbital-buffered saline (2.5 mM), pH 7.4, supplemented with 0.15 mM CaCl₂ and 0.5 mM MgCl₂. F(ab')2 fragment of preimmune or anti-C1-inhibitor rabbit IgG was prepared by pepsin digestion, Sephacryl S-300 gel filtration, and removal of residual intact IgG by protein A-Sepharose chromatography. For studies examining the inactivation of C1, activated macromolecular C1t was incubated with the agent under investigation; at various times, C1-residual activity was evaluated by measuring its capacity to consume hemolytic C4 in normal human serum. C1t employed for these studies was obtained by activation of C1 for 30 min at 37 °C in half-strength barbital buffer.

Zymographic Analysis—Plasma was incubated for 30 min at room temperature with buffer or the serpin under investigation, and then electrophoresed (without boiling) on 7.5% acrylamide-SDS gels as described above. After electrophoresis, the gels were washed with 2.5% (v/v) Triton X-100 in water and placed on a plasminogen-rich fibrin-agarose underlay (33). Underlays contained 0.75% (w/v) fibrinogen (Sigma), 0.04 unit/ml bovine thrombin (Sigma), 0.05% Triton X-100, and 0.02% sodium azide in 50 mM sodium barbital, pH 7.8, supplemented with 93 mM sodium chloride, 1.7 mM calcium chloride, and 0.7 mM magnesium chloride. After overnight incubation in a humid chamber, underlays were washed with 0.1 M sodium phosphate buffer, pH 7.4, dried in an oven at 80 °C, and stained with Coomassie Blue.

RESULTS

Reaction of α1-Antitrypsin Mutants with Serine Proteases: SDS-PAGE Studies—The capacity of α1-antitrypsin mutants to form 1:1 stoichiometric covalent complexes with proteolytic enzymes was examined with purified proteins. Complex formation was not detectable following incubation of α1-antitrypsin Leu82 with kallikrein, β-factor XIIa, C1t, plasmin, t-PA, or urokinase (Fig. 2, lanes a–f). In contrast, covalent bimolecular enzyme-serpin complexes were formed following incubation of α1-antitrypsin Arg58 with kallikrein (Fig. 3, lane a), β-factor XIIa (lane b), C1t (lane c), plasmin (lane d), t-PA (lane e), or urokinase (lane f). Each lane contained ~20 μg of protein. α1-Antitrypsin Arg58 was incubated with the various enzymes in 20 mM sodium phosphate buffer, pH 7.4, containing 150 mM sodium chloride for 60 min at 38 °C before electrophoresis. Coomassie Blue R-250 was used for protein staining. Left margin numbers are M, × 10⁻².

Inhibition of Serine Proteases by Serpins: Kinetic Constants—The results of the electrophoretic studies described in the preceding paragraph were extended by determining rate constants for the inhibition of the various enzymes by α1-antitrypsin mutants (Table II). Kinetically, α1-antitrypsin Met58 or Leu62 did not react in a detectable fashion with any of the six serine proteases under investigation. In contrast, enzyme inhibition was observed with α1-antitrypsin Arg58 and α1-antitrypsin Ala12-Arg58. Previous studies had shown that these two α1-antitrypsin mutants reacted more rapidly than C1-inhibitor with kallikrein and β-factor XIIa (12, 19).
Results of the present investigation showed that C1s was inhibited by α1-antitrypsin Ala357-Arg358 (but not by α1-antitrypsin Arg358); however, for the inhibition of Cls, the double mutant was ~150 times less efficient than Cl-inhibitor. Additional experiments indicated that α1-antitrypsin Arg358 and α1-antitrypsin Ala357,Arg358 also inhibited plasmin, t-PA, and urokinase (plasmin more rapidly than urokinase, and urokinase more rapidly than t-PA). However, the rate constants seen with the α1-antitrypsin mutants were much slower than the values observed when the fibrinolytic system enzymes were activated in the absence of α1-antitrypsin Arg358 or A1a357-Arg358 (Fig. 5B, squares). The presence of an equimolar (relative to Cl-inhibitor) concentration of Arg358 mutant reduced the level of the kallikrein-Cl-inhibitor complex formed 3 min after kaolin activation of plasma by 53% (Fig. 5B, triangles), whereas a reduction of 65% was observed with an equimolar level of α1-antitrypsin Ala357,Arg358 (Fig. 5B, squares).

Activation of Macromolecular Cl—Studies with human serum have shown that the C1s-C1r-C1s catalytic subunit of C1 undergoes rapid autoactivation in the absence of Cl-inhibitor. Kinetic analysis has revealed that the reaction comprises two steps: (a) intramolecular activation of C1 into CT, and (b) intermolecular activation of C1 by CT (42, 43). When Cl-inhibitor is present in the system, autoactivation is slow because CTr and Cfs only express limited proteolytic activity. The following experiment was designed to assess whether α1-antitrypsin Ala357,Arg358 could influence spontaneous C1 activation. Normal serum was supplemented with the agent under investigation and with anti-Cl-inhibitor F(ab')2 fragment; the various mixtures were then incubated at 37°C and evaluated at various times for residual C4 hemolytic activity. With this method, there is a reciprocal relationship between the state of activation of C1 and the levels of C4. As shown in Table III, negligible levels of functional C4 (9% at 30 min) were measured in samples supplemented with the double mutant; in contrast, the addition of exogenous C1-inhibitor efficiently prevented the consumption of C4 (Table III). In another experiment, purified C7 was preincubated with α1-antitrypsin Ala357,Arg358, and the resulting mixture subsequently evaluated for residual C7 activity using functional C4 levels as an indicator (Table IV). As indicated by the previous experiment and by the studies in purified system, α1-antitrypsin Ala357,Arg358 was not an efficient inhibitor of C7. Indeed, after a 15-min preincubation with a 10-
Inhibition of Serine Proteases by α1-Antitrypsin Mutants

**TABLE III**

<table>
<thead>
<tr>
<th>Reactants</th>
<th>Preincubation before addition of serum</th>
<th>C4 activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CI</td>
<td>5 min 15 min 30 min</td>
<td></td>
</tr>
<tr>
<td>CI + α1-antitrypsin Ala357-Arg368 (0.014 μM)</td>
<td>0.04 0.50 0.68</td>
<td></td>
</tr>
<tr>
<td>CI + α1-antitrypsin Ala357-Arg368 (0.14 μM)</td>
<td>0.79 0.83 0.83</td>
<td></td>
</tr>
</tbody>
</table>

* Results are reported as fraction of original C4 activity.

**TABLE IV**

<table>
<thead>
<tr>
<th>Serpin</th>
<th>F(ab')2</th>
<th>C4 activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.0</td>
<td>1.00</td>
</tr>
<tr>
<td>Preimmune</td>
<td>1.0</td>
<td>0.97</td>
</tr>
<tr>
<td>Anti-C1-inhibitor</td>
<td>1.0</td>
<td>0.96</td>
</tr>
</tbody>
</table>

Normal human serum (2 μl) was incubated at 37 °C in barbital-buffered saline with the agent under investigation and preimmune or anti-C1-inhibitor F(ab')2 fragment in a final volume of 100 μl. At the times indicated, C4 levels were determined, using C4-deficient guinea pig serum as the substrate.

Inhibition of t-PA in Plasma—Plasma or its euglobulin fraction contain free and serpin-bound t-PA (44-46), the predominant inhibitor of t-PA in normal plasma being PAI-1 (20). To establish whether the α1-antitrypsin mutants could influence the fibrinolytic activity of normal plasma, we used the electrophoretic-zymographic system described by Gnarra-Piperno and Reich (33). With this method, the molecular mass of free and protease inhibitor-bound PA in complex biological samples can be determined with accuracy (33, 46). In normal plasma, t-PA was present in the form of free enzyme (M, 68,000) as well as complexed to PAI-1 (M, 110,000) (Fig. 6, lane a). A pattern that was not modified after a 30-min incubation with 2.0 μM α1-antitrypsin Leu358 (Fig. 6, lane b). In contrast, incubation of plasma with 2.0 μM α1-antitrypsin Ala357-Arg368 resulted in the emergence of a new lysis zone (M, 99,000) and in the concomitant reduction of free t-PA (M, 68,000) (Fig. 6, lane c). Similarly, incubation of plasma with 4 nm PAI-1 (Fig. 6, lane d) caused a drastic decrease in free t-PA (M, 68,000) and an increase in the lysis band associated with the complex between t-PA and PAI-1 (M, 110,000).

**DISCUSSION**

The availability of mutated serpins might represent a significant step for the development of new therapeutic strategies for clinical conditions such as thrombosis, shock, and inflammation, where unregulated proteolysis is often observed. In previous studies, we showed that α1-antitrypsin Arg358 and α1-antitrypsin Ala357-Arg368 rapidly reacted with plasma kallikrein and β-factor XIIa (12, 19). We have now examined the inhibitory characteristics of these α1-antitrypsin variants against proteolytic enzymes of the complement and fibrinolytic systems. Our present results amplify the conclusion that the amino acid residues located at positions P1 and P2 of the reactive site have an important role in dictating the specificity of inhibition (9-15). Whereas α1-antitrypsin Met368 or Leu368 did not inhibit any of the Arg-specific enzymes under investigation, α1-antitrypsin Arg368 inhibited not only kallikrein and β-factor XIIa, but also plasmin, t-PA, and urokinase (Figs. 2 and 3; Table II). For the inhibition of C1s, a second mutation (Pro → Ala) was required at position P3 (Fig. 4, Table II). Furthermore, comparison of the rate constants obtained with the α1-antitrypsin reactive site mutants and C1-inhibitor or PAI-1 demonstrated that other factors besides the structure of the reactive center are required for efficient enzyme inhibition. α1-Antitrypsin Ala357-Arg368, C1-inhibitor, and PAI-1 contain the same Arg and Ala residues at positions P1 and P2 of the reactive site (Table I). However, the double mutant only weakly inhibited C1s and t-PA, whereas C1-inhibitor did not inhibit t-PA or urokinase, and PAI-1 did not inhibit C1s (Table II). Thus, the presence of secondary sites of interaction is necessary for the rapid reaction between
certain serpins and their target enzymes, a conclusion also supported by the results of a recent study on the reaction of a t-PA mutant with PAI-1 (47). Experiments with serpin deletion mutants or fusion proteins (for example segments of Cl-inhibitor or PAI-1 linked to an α1-antitrypsin backbone with Arg at P1 and Ala at P2) will be required for the positive identification of these secondary sites of interaction.

Previous experiments with conscious normotensive Wistar rats showed that, at a dose which did not cause increased inhibition of thrombin, α1-antitrypsin Ala357-Arg358 protected the animals against the circulatory collapse induced by the administration of β-factor XIIa (19). In the present study, we investigated whether the double mutant could also inhibit proteolytic activities generated in human plasma or serum. To examine this issue, plasma samples were supplemented with α1-antitrypsin Ala357-Arg358 at a concentration equivalent to that employed in the rat experiments described above (19). α1-Antitrypsin Ala357-Arg358 efficiently inhibited the contact activation system of human plasma, since it prevented by 65% the formation of a complex between kallikrein and Cl-inhibitor in kaolin-activated plasma (Fig. 5R). α1-Antitrypsin Ala357-Arg358 also inhibited the fibrinolytic activity present in normal plasma, which can be related to the presence of free t-PA (Fig. 6). These observations strongly suggest that α1-antitrypsin Ala357-Arg358 has therapeutic potential for disorders where activation of the plasma kallikrein-kinin and the fibrinolytic systems is observed, for example in hypotensive septicemia (41). In contrast, the double mutant had no detectable effect on the autoactivation of Cl in serum. Whereas the residual hemolytic activity of C4 after 30 min of incubation was 73% in a serum sample supplemented with 0.76 μM exogenous Cl-inhibitor, C4 was only 9% in serum containing 3.4 μM α1-antitrypsin Ala357-Arg358 (Table III). Therefore, it is unlikely that the mutated serpin could antagonize the unregulated activation of the classical pathway of complement observed in systemic lupus erythematosus and certain forms of arthritis or nephritis (48). Finally, the usefulness of α1-antitrypsin Ala357-Arg358 for the therapy of hereditary angioedema attacks will have to be determined. Patients with hereditary angioedema lack Cl-inhibitor and suffer from recurrent attacks of mucocutaneous swellings and abdominal pain (49). It is not known whether the clinical manifestations of angioedema attacks are caused by products of the classical pathway of complement or the plasma kallikrein-kinin system (50, 51). A positive response to the administration of the double mutant would indicate a major pathogenetic role for factor XII and prekallikrein activation, whereas no response would be expected if complement activation is the central mechanism for the genesis of these attacks.

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