Kinetics of Inhibition of Human Plasma Kallikrein by a Site-Specific Modified Inhibitor Arg^{15}-Aprotinin: Evaluation Using a Microplate System and Comparison With Other Proteases

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Human plasma kallikrein, a product of contact-activated plasma proteolysis, is moderately inhibited by aprotinin, a small polypeptide from bovine lung that has been used as an experimental drug in human disease states. Aprotinin has a Lys residue in the P1 (reactive center) position occupying residue 15. Since kallikrein is an arginine-directed serine protease, we hypothesized that an altered form of aprotinin, Arg^{15}-aprotinin, might be a better inhibitor. Kinetic evaluations were performed in 96-well microplates. We found that the K_{i} (loose or Michaelis-Menten complex) was unchanged by the modification. However, the association rate constant was increased from 1.14 \times 10^{4} (mol/L)^{-1} \text{ s}^{-1} to 1.5 \times 10^{5} (mol/L)^{-1} \text{ s}^{-1}, thus indicating that the inhibition rate was increased 14-fold for the modified protein. The K_{i} (at equilibrium) was decreased from 5.2 \times 10^{-7} mol/L to 1.5 \times 10^{-8} mol/L after substituting Arg for Lys in the P1 position. Therefore, the modified inhibitor binds to plasma kallikrein more tightly than the natural protein. We also investigated the effect of Arg^{15}-aprotinin on tissue kallikrein, plasmin, factor Xla, factor Xla, and thrombin and found that the K_{i} slightly decreased from 5.1 \times 10^{-7} mol/L to 1.2 \times 10^{-7} mol/L for tissue kallikrein and slightly decreased from 2 \times 10^{-6} mol/L to 1 \times 10^{-6} mol/L for plasmin. Arg^{15}-aprotinin did not inhibit thrombin or factor Xla, even though both enzymes are arginine-directed serine proteases. However, factor Xla, although it was not inhibited by aprotinin, had a K_{i} of 3.4 \times 10^{-8} mol/L for Arg^{15}-aprotinin. Therefore, Arg^{15}-aprotinin is a more effective inhibitor of plasma kallikrein as well as factor Xla but shows minimal preference for plasmin and tissue kallikrein. This study also indicates that it is possible and practical to perform kinetic analyses directly in microplates.

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MATERIALS AND METHODS

Human plasma kallikrein, plasmin, H-D-Pro-Phe-Arg-pNA (S-2302), H-D-Val-Leu-Lys-pNA (S-2251), H-D-Phe-Pip-Arg-pNA (S-2238), and H-D-Val-Leu-Arg-pNA (S-2266) were generously furnished by KabiVitrum, Malmö, Sweden, and its US distributor, Helena Laboratories, Beaumont, TX. Human thrombin (2,900 U/mg) was a generous gift of Dr John W. Fenton II, Albany, NY. Microplates utilized in this study were Falcon, tissue culture-treated plates (#3075) purchased from Fisher Scientific Company, King of Prussia, PA. The microplate reader was a Bio-Rad model 2550 EIA reader purchased from Bio-Rad, Inc, Richmond, CA. Polyethylene glycol (PEG 8000) was purchased from Sigma Chemical Co, St Louis. All other reagents were the best grade available.

Human plasma kallikrein. Obtained from KabiVitrum, human plasma kallikrein is purified 200-fold from euglobulin-free plasma and chromatographed on QAE-Sephadex, Sephадex G-150, and Sephadex G-100 according to the method of Gallimore et al. The specific activity is 1.2 Chromozyme-PK U/mg and contains IgG as the only protein contaminant.

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**Human urinary kallikrein.** A generous gift of Dr Julie Chao (Charleston, SC), human urinary kallikrein was purified to homogeneity according to the method of Shimamoto et al. The specific activity was 74 EU/mg using the substrate Tos-Arg-O-[3H]-Me.

**Human plasmin.** Human plasmin (KabiVitrum) is generated from plasminogen that is prepared according to the method of Deutsch and Mertz and activated via a matrix-bound activator. The specific activity is 15 casein units (CU)/mg.

**Human factor XIIa.** Human factor XIIa was prepared as previously detailed from our laboratory. The specific activity was 62 U/mg where 1 unit is the amount in 1 mL normal plasma.

**Aprotinin.** Aprotinin was purified from bovine lung as described by Fritz et al.

**Arg^{13}-aprotinin (semisynthetic).** The procedure for replacement of Lys by Arg in position 15 of aprotinin is described in detail elsewhere. Briefly, it consists of the following steps: (1) selective reduction of the Cys^{14}-Cys^{19} disulfide bridge with sodium borohydride; (2) tryptic cleavage of the reactive-site peptide bond Lys^{14}-Ala^{12}; (3) air reoxidation of the half-cystine residues to yield modified aprotinin; (4) removal of Lys^{14} by carboxypeptidase B; (5) coupling of arginine to Cys^{14} in a reverse reaction catalyzed by carboxypeptidase B to yield modified Arg^{13}-aprotinin in minute amounts; (6) trapping of this product by complex formation with kallikrein from porcine pancreas, thus shifting the carboxypeptidase-catalyzed reaction towards synthesis; and (7) kinetic control dissociation of the complex to yield single-chain Arg^{13}-aprotinin as the main product.

The semisynthetic inhibitor was purified by gel filtration and cation-exchange chromatography. It was characterized by amino acid analysis, automated Edman degradation, x-ray crystal structure analysis, inhibition studies with various serine proteinases, polycrystalline gel electrophoresis, and high-pressure liquid cation-exchange chromatography. The Arg^{13}-aprotinin preparation was found to be homogeneous according to all these criteria applied.

**Microassay for plasma kallikrein.** Plasma kallikrein was reconstituted with 0.2% PEG to prevent nonspecific adsorption of proteases. The total volume of enzyme plus buffer and/or inhibitor was routinely 100 μL. Fifty microliters of 2 mmol/L S-2302 was added to the enzyme mixture at room temperature, and hydrolysis was allowed to proceed for 15 to 20 minutes. The hydrolysis reaction was quenched with 50 μL of 33 mmol/L S-2302 in the presence (O) and absence (△) of 0.25 mmol/L aprotinin. At the times indicated, the reaction was quenched with acetic acid (see Materials and Methods).

**Kinetic analysis of inhibition.** The equation used was adapted from that of Fritz and Wunderer for a reversible tight binding inhibitor:

\[
E + I \rightleftharpoons L \rightleftharpoons C
\]

where E = k_{1} + L = k_{-1} + k_{2}I,

K_{i} = k_{i} / k_{-i},

K_{i} = k_{i} / k_{-i} / k_{2}.

Since the formation of L is a fast second-order reaction, k_{i} is assumed to be rate determining for the formation of a stable complex and is approximately equal to the k_{max} for the complex.

**Plasma kallikrein—determination of K_{i}.** Kallikrein was incubated with various concentrations of aprotinin or Arg^{13}-aprotinin for 30 minutes prior to the addition of S-2302. The hydrolysis of the substrate by the residual enzyme activity proceeded for 20 minutes before termination by acetic acid.

**Human thrombin—determination of K_{i}.** Twenty microliters of human thrombin (80 mmol/L) was incubated with either aprotinin, Arg^{13}-aprotinin, or buffer A in a final volume of 120 μL for 30 minutes prior to the addition of 60 μL of 0.75 mmol/L S-2238. Hydrolysis proceeded for three minutes prior to the addition of 50 μL of 50% acetic acid.
Human plasmin—determination of $K_i$. Fifty microliters of human plasmin (0.125 CU/mL) was incubated with either aprotinin, Arg<sup>15</sup>-aprotinin, or buffer (50 mmol/L Tris-Cl, pH 7.4, containing 0.1% PEG) in microplates (final volume, 100 µL) for 30 minutes prior to the addition of 50 µL of 2 mmol/L S-2251. Hydrolysis proceeded for 20 minutes prior to the addition of 50 µL of 50% acetic acid.

Human urinary kallikrein—determination of $K_i$. Fifty microliters of human urinary kallikrein (10 µg/mL) was incubated with aprotinin, Arg<sup>15</sup>-aprotinin, or buffer A in a final volume of 100 µL for 30 minutes prior to the addition of 50 µL of 0.8 mmol/L S-2266. Hydrolysis proceeded for 20 minutes prior to the addition of 50 µL of 50% acetic acid.

Human factor XIa—determination of $K_i$. Fifty microliters of human factor XIa (0.04 U/mL) was incubated with aprotinin, Arg<sup>15</sup>-aprotinin, or buffer A in a final volume of 100 µL for 30 minutes. Fifty microliters of S-2302 (2 mmol/L) was added, and hydrolysis proceeded for ten minutes prior to quenching with 50 µL of 50% acetic acid.

Human factor XIIa—determination of $K_i$. Fifty microliters of human factor XIIa (0.02 U/mL) was incubated with either aprotinin, Arg<sup>15</sup>-aprotinin, or buffer A in a final volume of 100 µL for 30 minutes. Fifty microliters of S-2302 (2 mmol/L) was added, and hydrolysis proceeded for 90 minutes prior to the addition of 50 µL of 50% acetic acid.

RESULTS

Determination of $K_i$ of plasma kallikrein with aprotinin and Arg<sup>15</sup>-aprotinin. Kallikrein was added to various concentrations of either aprotinin (Fig 2, left) or Arg<sup>15</sup>-aprotinin (Fig 2, right) together with S-2302 and was assayed as in Materials and Methods. Inhibition was competitive and reversible for both inhibitors, similar to the action of aprotinin on other enzymes. From the data in Fig 2, the $K_m$ and the $K_m$ (app) were plotted against the inhibitor concentration to determine the $K_i$ (loose Michaelis-Menten inhibition constant), which was calculated to be $1.02 \times 10^{-7}$ mol/L, for both inhibitors (Fig 3).

![Fig 2. Determination of $K_i$ and $K_{app}$ for plasma kallikrein with aprotinin and Arg<sup>15</sup>-aprotinin. Kallikrein (3 nmol/L) was incubated with aprotinin (A) or Arg<sup>15</sup>-aprotinin (B) ranging from 64 to 192 nmol/L in the presence of S-2302 ranging from 0.133 to 0.66 mmol/L. The hydrolysis of the substrate proceeded for 20 minutes before quenching with acetic acid. •, 0; O, 64 nmol/L aprotinin or Arg<sup>15</sup>-aprotinin; ×, 128 nmol/L aprotinin or Arg<sup>15</sup>-aprotinin; □, 192 nmol/L aprotinin or Arg<sup>15</sup>-aprotinin.](image-url)

Determination of $k_2$ ($k_{assoc}$) of the complex of plasma kallikrein with aprotinin and Arg<sup>15</sup>-aprotinin. Kallikrein was incubated with an equimolar concentration of Arg<sup>15</sup>-aprotinin (Fig 4) for various times prior to addition of S-2302. The association rate constant ($k_2$) for Arg<sup>15</sup>-aprotinin was found to be $1.5 \times 10^5$ (mol/L)<sup>-1</sup>s<sup>-1</sup>. Kallikrein (20 nmol/L) was incubated with aprotinin ranging in concentration from 150 nmol/L to 600 nmol/L. At various times, a portion was transferred to a microplate containing S-2302, and the hydrolysis proceeded as in Materials and Methods. By either a first-order plot or calculation by the second-order rate equation, the $k_{assoc}$ was calculated to be $1.14 \times 10^2$ (mol/L)<sup>-1</sup>s<sup>-1</sup> (data not shown). Therefore, the
association rate for kallikrein and the modified inhibitor is ninefold greater than that for the natural inhibitor.

**Determination of the equilibrium constant (K,) of plasma kallikrein and aprotinin or Arg15-aprotinin.** The K, for kallikrein and aprotinin was calculated to be $3.2 \times 10^{-7}$ mol/L (Fig 5A) and $1.5 \times 10^{-8}$ mol/L for kallikrein and Arg15-aprotinin (Fig 5B). Therefore, the affinity of Arg15-aprotinin for plasma kallikrein is 20-fold tighter than for the unmodified inhibitor.

**Calculation of the k, for kallikrein and aprotinin or Arg15-aprotinin.** The k, was calculated by the following equation:

$$k_2 = K_1 k_3.$$

The k, for kallikrein and aprotinin was calculated to be $3.6 \times 10^{-3} \text{s}^{-1}$ and $2.5 \times 10^{-3} \text{s}^{-1}$ for kallikrein and Arg15-aprotinin. The half-life of the complex ($t_{1/2} = 0.693/k_2$) would therefore be $3.2$ minutes for kallikrein inhibited by aprotinin and $4.6$ minutes for kallikrein inhibited by Arg15-aprotinin.

**Comparison of inhibitor potency for aprotinin and Arg15-aprotinin on other proteases.** The evaluation of the interaction of aprotinin and Arg15-aprotinin on human urinary kallikrein, human plasmin, human factor Xlla, human factor Xla, and human thrombin is presented in Table 1. Arg15-aprotinin was a much more potent inhibitor than aprotinin for plasma kallikrein, but only slightly more potent for plasmin and tissue kallikrein. Thrombin and factor XIa were not inhibited by either aprotinin or by Arg15-aprotinin. Although factor Xla was not inhibited by aprotinin, it was potently inhibited by the modified inhibitor.

**DISCUSSION**

The clinical use of aprotinin has been described for pancreatitis and other pathophysiological conditions with varying degrees of success. Since the K, value for the kallikrein-aprotinin complex ($3.2 \times 10^{-7}$ mol/L) approaches the in vivo concentration of plasma prekallikrein ($4.5 \times 10^{-7}$ mol/L), a considerable molar excess of aprotinin would be needed to inhibit any liberated kallikrein. This study indicates that Arg15-aprotinin is a more efficient inhibitor of kallikrein than aprotinin since there is a 13-fold increase in the rate of association and a 20-fold decrease in the dissociation rate of the complex. These advantages make it more attractive for the clinical inactivation of plasma kallikrein since the rate of inactivation is sixfold greater than that obtainable with plasma concentrations of Cl inhibitor, the major plasma protease inhibitor of plasma kallikrein. However, the reversible nature of the inhibitor may limit its application to acute situations since, with a half-life of the tight enzyme-inhibitor complex equal to 4.6 minutes, continuous infusions of relatively high concentrations would have to be used to produce the tight binding necessary to obviate the presence of low concentrations of this pathologically produced biologically potent enzyme because dilution in the plasma would favor dissociation.

The previous determinations of the K, of aprotinin for human plasma and tissue kallikreins were performed at pH 8.0. Since Engel et al demonstrated that both the k, and the K, are pH dependent, we chose to perform all kinetic analyses under similar conditions of pH and ionic strength in order to achieve a more accurate comparison of the binding
of both aprotinin and Arg^{15}-aprotinin to various plasma proteases. Furthermore, the pH and ionic strength of our buffers closely approximates the conditions in blood. Under these conditions, plasma kallikrein and tissue kallikrein have similar affinities for aprotinin, and the Kᵢ is further lowered for plasma kallikrein by substitution of the arginine residue in position 15. Plasmin, which appears to prefer lysyl residues, was minimally affected by the substitution, similar to the lack of effect of arginine in the P₁ position of the homologous sea anemone inhibitor. The most striking change, however, was with factor X₁α where aprotinin failed to inhibit this enzyme, whereas Arg^{15}-aprotinin had a very high affinity. This finding is similar to the marked increase in factor X₁a inhibition seen with an arginine substitution in the P₁ position of α₁-antitrypsin. Therefore, Arg^{15}-aprotinin is an efficient inhibitor of factor X₁a, an enzyme for which few inhibitors have been described.

The P₁ position of an inhibitor molecule has been postulated to be the most critical determinant of inhibitory specificity. However, if the P₁ residue were the only crucial determinant of inhibitor function, then Arg^{15}-aprotinin should have been an excellent inhibitor of thrombin and factor XIIa since both are arginine-directed serine proteases. Since Arg^{15}-aprotinin did not inhibit either enzyme, it appears that the P₂ as well as other residues are also important contributors to inhibitory function.

The use of the microplate system for formal kinetic analyses can drastically reduce assay time as well as substantially cut the cost of each determination. The system also obviates the need for a thermostated, sophisticated spectrophotometer. Furthermore, microplate assays can be performed virtually anywhere since the only requirement is a small bench area. This new technology should prove useful in any situation where a chromogenic substrate is used.

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Kinetics of inhibition of human plasma kallikrein by a site-specific modified inhibitor Arg15-aprotinin: evaluation using a microplate system and comparison with other proteases

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