The Mechanism of Plasminogen Activation and Fibrin Dissolution by Single Chain Urokinase-Type Plasminogen Activator in a Plasma Milieu In Vitro

By H.R. Lijnen, B. Van Hoef, F. De Cock, and D. Collen

The relative contribution of several mechanisms to plasminogen activation and fibrin dissolution by urokinase-type plasminogen activator (u-PA) in vitro was quantitated. The activation of plasminogen by recombinant single chain u-PA (rscu-PA), by its two chain derivative (rtcu-PA) and by a plasmin-resistant mutant, rscu-PA-Glu158, obeys Michaelis-Menten kinetics with catalytic efficiencies of 0.00064, 0.046, and 0.00006 L/μmol·s for native plasminogen (Glu-plasminogen) and of 0.0061, 1.21, and 0.0004 L/μmol·s for partially degraded plasminogen (Lys-plasminogen). In a purified system consisting of a fibrin clot submersed in a plasminogen solution, the equi-effective doses (50% lysis in one hour) for rscu-PA, rtcu-PA, and rscu-PA-Glu158 were 16, 6.5, and 32,000 ng/mL for Glu-plasminogen and two- to fourfold lower for Lys-plasminogen. In a plasma milieu, 50% lysis in two hours was obtained for a plasma clot with 2.1 μg/mL rscu-PA, 0.5 μg/mL rtcu-PA, and >200 μg/mL rscu-PA-Glu158 and for a purified fibrin clot with 1.3 μg/mL rscu-PA and 0.27 μg/mL rtcu-PA. After predigestion of a purified fibrin clot with plasmin, the apparent potency of rscu-PA and rtcu-PA increased by 40% and 20%, respectively. In conclusion, rscu-PA has an intrinsic plasminogen activating potential that is only about 1% of that of rtcu-PA and that is 13 times higher than that of rscu-PA-Glu158. Conformational transition of Glu-plasminogen to Lys-plasminogen enhances its sensitivity to activation by all u-PA moieties ten- to 20-fold. Predigestion of fibrin clots with associated increased binding of plasminogen results in a minor apparent increase of the fibrinolytic potency of rscu-PA and rtcu-PA. The relative fibrinolytic potency of rtcu-PA is two to three orders of magnitude higher than that of rscu-PA-Glu158 but only two- to five-fold higher than that of rscu-PA, both in purified systems and in a plasma milieu. These results indicate that conversion of rscu-PA to rtcu-PA constitutes the primary mechanism of fibrin dissolution.

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MATERIALS AND METHODS

Proteins and Reagents

rscu-PA and rscu-PA-Glu158 (obtained by site-directed mutagenesis of Lys158 to Glu) were prepared and characterized as described elsewhere. Before use, these proteins were chromatographed on benzamidine-Sepharose in order to remove traces of two chain derivatives. Protein concentrations were determined by amino acid analysis or with an ELISA specific for u-PA. rtcu-PA was prepared by treatment of rscu-PA with plasmin and rscu-PA-Glu158 by treatment of rscu-PA-Glu158 with Endoproteinase Glu-C, followed by chromatography on benzamidine-Sepharose, as de-
concentration, at different generated plasmin measured with 5-2251 (final concentration, 0.3 mmol/L) after tration of 5 x 10 mol/L each. Under these conditions rscu-PA is 37°C, followed by inhibition of plasmin and rtcu-PA by addition of Kinetics of the Activation of Plasminogen at 37°C in 0.05 mol/L Tris-HCl buffer, pH 7.4, containing 0.01% Tween 80 and 0.038 mol/L NaCl. Purified 125I-labeled fibrin clots were then incubated at 37°C in 0.05 mol/L Tris-HCl buffer, pH 7.4, containing 0.01% Tween 80, 0.038 mol/L NaCl, 1 KIU/mL aprotinin and 1.5 μmol/L Glu-plasminogen or Lys-plasminogen, and lysis of fibrin clots by rscu-PA, rtcu-PA, rscu-PA-Glu158, or rtcu-PA-Glu158 was monitored for one hour at 15-minute intervals and quantitated by the release of radioactivity from the clot into the surrounding liquid. The concentration of u-PA yielding 50% lysis in one hour was determined from plots of the extent of clot lysis (in percent) against the concentration of the plasminogen activators.

In human plasma. 125I-fibrin labeled plasma clots were prepared as described above, following addition to normal human plasma of 500,000 cpm/mL of 125I-labeled fibrinogen, and coagulation with CaCl2 (final concentration, 25 mmol/L), and thrombin (final concentration, 2 NIH U/mL). Lysis of 125I-fibrin labeled plasma clots by rscu-PA (final concentration, 0 to 5.0 μg/mL), rtcu-PA (final concentration, 0 to 2.0 μg/mL), rscu-PA-Glu158 (final concentration, 0 to 200 μg/mL), or rtcu-PA-Glu158 (final concentration, 0 to 1.0 μg/mL) was measured over four hours as previously described, using normal human plasma or plasminogen-depleted plasma reconstituted with 1.5 μmol/L Glu-plasminogen or Lys-plasminogen. The concentration of the u-PA moiety, required to obtain 50% clot lysis in two hours, was determined from plots of percent lysis vs the concentration of the u-PA moiety.

Predigestion of fibrin clots. Purified 125I-labeled fibrin clots were prepared as described above (125 μL volume with 375 μg fibrinogen), and incubated in 1 mL 0.05 mol/L Tris-HCl buffer, pH 7.4, containing 0.01% Tween 80 and 0.038 mol/L NaCl. The clots were predigested with human plasmin (0.6 μg/mL, final concentration) for 30 minutes at 37°C. The extent of fibrin digestion was calculated from the radioactivity released from the clot, corrected for the background radioactivity. Plasmin was neutralized by incubation of the clot in 1 mL normal human plasma for 30 minutes, at 37°C, which abolished further release of radioactivity. Generation of new COOH-terminal amino acids following plasmin predigestion of fibrin clots was determined as follows. Ten unlabeled fibrin clots (with or without predigestion) were suspended in 1.5 mL 0.2 mol/L N-ethylmorpholine-acetate buffer, pH 8.0, and treated with 5.0 U carboxypeptidase B (specific activity 150 U/mg; Boehringer Mannheim, FRG) for one hour at room temperature. Samples of the supernatant (300 μL) were removed before and 15 to 60 minutes after addition of enzyme, heated at 100°C for five minutes and treated with cold 20% CCl4/C0OH with addition of norleucine as internal standard to calculate the recovery of amino acids. Samples were dried and used for amino acid analysis (Beckman 119 CL amino acid analyzer).

Lysis of normal or predigested clots following addition of rscu-PA (range, 0 to 2.0 μg/mL), rtcu-PA (range, 0 to 1.6 μg/mL), or t-PA (range, 0 to 0.80 μg/mL) was then monitored over four hours as described. The concentration of plasminogen activator required to obtain 50% clot lysis in two hours was determined from plots of percent lysis at two hours vs the concentration of plasminogen activator. The effect of predigestion on lysis of fibrin clots by these equi-effective concentrations of the enzymes was then investigated using intact and predigested clots.

Binding of plasminogen to normal or predigested purified unlabeled fibrin clots was determined by incubation for one hour at 37°C in normal human plasma containing traces of 125I-labeled Glu-plasminogen. Bound plasminogen was quantitated from the radioactivity associated with the clots after extensive washing in buffer.

Purified 125I-labeled fibrin clots, with or without predigestion,
incubated for one hour at 37°C in normal human plasma, were transferred to buffer and lysis induced by rscu-PA (range, 20 to 1,280 ng/mL), rtcu-PA (range, 10 to 1,280 ng/mL), or t-PA (range, 0.8 to 800 ng/mL) was monitored over one hour. The concentrations of enzyme required either for 5% or for 50% clot lysis in one hour were determined from plots of percent lysis after one hour vs. the concentration of plasminogen activator. Subsequently, intact or predigested fibrin clots, obtained as described above, were incubated in buffer with concentrations of enzyme producing 5% to 50% lysis in one hour, and lysis was monitored for 60 minutes.

**Plasminogen Activation by rscu-PA, rtcu-PA, rscu-PA-Glu'58, and rtcu-PA-Glu'58 in the Absence of Fibrin**

In purified systems. Activation of Glu-plasminogen or Lys-plasminogen (final concentration, 1.5 μmol/L) at 37°C in 0.05 mol/L Tris-HCl buffer, pH 7.4, containing 0.01% Tween 80 and 0.038 mol/L NaCl, by rscu-PA (seven concentrations; range, 0 to 0.5 μg/mL for Glu-plasminogen and 0 to 0.1 μg/mL for Lys-plasminogen) or rtcu-PA (seven concentrations; range, 0 to 0.5 μg/mL for Glu-plasminogen and 0 to 0.5 μg/mL for Lys-plasminogen), was monitored during one hour by measuring residual plasminogen, after conversion to plasminogen-streptokinase complex. At 15-minute intervals, 5 μL samples were removed from the incubation mixtures, incubated for ten minutes at 37°C in 300 μL 0.05 mol/L Tris-HCl buffer, pH 7.4, containing 0.038 mol/L NaCl and 0.01% Tween 80, and 1,000 IU of streptokinase. Under these conditions, plasmin is rapidly and quantitatively complexed by α2-antiplasmin. The plasminogen-streptokinase complex, which is not inhibited by α2-antiplasmin, was measured with S-2251 (final concentration, 0.6 mmol/L). The data were plotted as percent plasminogen activation vs. time.

In addition, activation of Glu-plasminogen or Lys-plasminogen (final concentration, 1.5 μmol/L) by rscu-PA-Glu'58 (seven concentrations; range, 0 to 200 μg/mL) for both Glu-plasminogen or Lys-plasminogen) or rtcu-PA-Glu'58 (seven concentrations; range, 0 to 1.0 μg/mL for Glu-plasminogen and 0 to 0.13 μg/mL for Lys-plasminogen) was monitored as described above. The concentrations of u-PA moieties required to obtain 50% plasminogen activation in one hour was then determined graphically from plots of plasminogen activation vs. the concentration of plasminogen activator.

In plasma. Activation of plasminogen at 37°C in normal human plasma or in plasminogen-depleted plasma reconstituted with Glu-plasminogen or Lys-plasminogen, by rscu-PA (seven concentrations; range, 0 to 5.0 μg/mL) or rtcu-PA (seven concentrations; range, 0 to 2.0 μg/mL), was monitored over four hours at hourly intervals, as described above. Activation of plasminogen by rscu-PA-Glu'58 in normal human plasma or in plasminogen-depleted plasma reconstituted with Glu-plasminogen or Lys-plasminogen (seven concentrations; range, 0 to 200 μg/mL) or by rtcu-PA-Glu'58 (seven concentrations; range, 0 to 1.0 μg/mL) was also evaluated as described above. The concentration of u-PA required to obtain 50% plasminogen activation in two hours was determined graphically from dose-response curves of plasminogen activation vs. u-PA concentration.

**Plasminogen Activation by rscu-PA, rtcu-PA, rscu-PA-Glu'58, and rtcu-PA-Glu'58 in Fibrin Clot Lysis Systems**

Plasminogen activation during fibrin clot lysis with these u-PA moieties was monitored both in purified systems and in human plasma by quantitation of residual plasminogen as plasminogen-streptokinase complex. In addition, the extent of plasminogen activation caused by u-PA concentrations that induced 50% clot lysis in one hour in purified systems or in two hours in plasma was determined both in the presence and in the absence of clots.

**Plasminogen Activation With rscu-PA in the Presence of α2-Antiplasmin**

Activation of Glu-plasminogen by rscu-PA was measured in the presence and the absence of α2-antiplasmin. Therefore, Glu-plasminogen (1.5 μmol/L) was added to incubation mixtures (0.05 mol/L Tris-HCl buffer, pH 7.4, containing 0.038 mol/L NaCl and 0.01% Tween 80 at 37°C), containing α2-antiplasmin (0 to 7.5 μmol/L). The mixture was activated with rscu-PA (230 ng/mL) and residual plasminogen was measured as plasminogen-streptokinase complex.

Control experiments on the prevention of conversion of scu-PA to tcu-PA in this experimental system were performed by preincubation of Glu-plasminogen (final concentration, 2.5 μg/mL) at 4°C for 20 hours with α2-antiplasmin (final concentration, 1.6 μmol/L) before addition of Glu-plasminogen (final concentration, 1.5 μmol/L). Under the latter conditions, added rtcu-PA up to 20% of the concentration of rscu-PA, was completely inhibited (as measured either with S-2444 or via plasminogen activation in the same system), while rscu-PA incubated in buffer fully retained its plasminogen activating activity.

In addition, the absence of free plasmin in the incubation mixtures containing α2-antiplasmin was confirmed by addition of S-2251 (final concentration, 1 mmol/L) after tenfold dilution of samples (detection limit for plasmin <0.1% of the plasminogen concentration).

**Effect of Plasma on Clot Lysis by u-PA**

Clot lysis in purified systems with Glu-plasminogen (1.5 μmol/L) using concentrations of scu-PA (20 ng/mL) or rtcu-PA (10 ng/mL) yielding about 50% clot lysis in one hour was monitored in the absence or the presence of increasing amounts of plasma depleted in both plasminogen and α2-antiplasmin (0% to 40%, vol/vol) or of this depleted plasma reconstituted with purified α2-antiplasmin to a concentration of 1 μmol/L. For each amount of added plasma, the apparent residual concentration of rscu-PA or rtcu-PA was expressed as percent of the total concentration, using a calibration curve constructed in the absence of depleted plasma.

Plasma depleted both in plasminogen and α2-antiplasmin was passed three times over a column of insolubilized rscu-PA (0.5 mL plasma/mg rscu-PA). After each chromatography, the column was eluted with 1.6 mol/L KSCN and the pooled eluates were dialyzed against 0.05 mol/L Tris-HCl buffer, pH 7.4, containing 0.038 mol/L NaCl and 0.01% Tween 80. The effect of the plasmas and the eluate on clot lysis in purified systems was then monitored as described above.

**RESULTS**

**Kinetics of the Activation of Plasminogen (Glu-plasminogen or Lys-plasminogen) by rscu-PA, rtcu-PA, rscu-PA-Glu'58, and rtcu-PA-Glu'58**

Activation of both Glu-plasminogen and Lys-plasminogen by all u-PA moieties studied obeyed Michaelis-Menten kinetics as evidenced by linear double reciprocal plots of the initial activation rate v the plasminogen concentration (not shown). The kinetic constants, obtained by linear regression analysis (r > 0.995 in all cases), are summarized in Table 1. Activation of both Glu-plasminogen and Lys-plasminogen by...
MECHANISM OF ACTION OF scu-PA

rscu-PA-Glu158 occurs with lower affinity (higher $K_m$) than observed with rscu-PA, resulting in a 13- to 15-fold lower catalytic efficiency ($k_2/K_m$). The catalytic efficiency of rscu-PA and rscu-PA-Glu158 for the activation of Lys-plasminogen is about tenfold higher than that of Glu-plasminogen. This is apparently due to an increase in $k_2$ for rscu-PA and to a decrease in $K_m$ for rscu-PA-Glu158. Activation of plasminogen by the two chain u-PA moieties occurs with a catalytic efficiency that is two to three orders of magnitude higher than that with their single chain precursors: for Glu-plasminogen the ratio is 70-fold for the wild-type forms and 850-fold for the Glu158 mutants; for Lys-plasminogen corresponding values are 200-fold for wild-type and 1,650-fold for the Glu-mutants. The catalytic efficiencies for the activation of Lys-plasminogen are 10- to 25-fold higher than those for Glu-plasminogen, mainly as a result of increased affinities (lower $K_m$).

Pretreatment of rscu-PA-Glu158 with plasmin to convert potential traces of contaminating rscu-PA to rtcu-PA, followed by addition of excess plasmin and tscu-PA inhibitor, did not significantly affect the activation rate of Glu-plasminogen ($k_2/K_m = 3.6 \times 10^{-5}$ or $3.1 \times 10^{-5}$ L/µmol·s before or after plasmin treatment).

**Fibrinolytic Potency of rscu-PA, rtcu-PA, rscu-PA-Glu158, and rtcu-PA-Glu158.**

In purified systems. Addition of u-PA moieties to labeled fibrin clots caused a time- and dose-dependent lysis as quantitated by the release of $^{125}$I-labeled degradation products. Sigmoidal curves similar to those described in detail elsewhere were obtained. Fifty percent lysis in one hour of a purified labeled fibrin clot immersed in a Glu-plasminogen solution was obtained with 6.5 to 7.0 ng/mL of rtcu-PA and rscu-PA-Glu158, while this required 16 ng/mL rscu-PA and 32,000 ng/mL rscu-PA-Glu158 (Table 2). Two- to fourfold lower concentrations were required for equivalent clot lysis using a Lys-plasminogen solution.

In human plasma. Fifty percent lysis in two hours of a labeled plasma clot immersed in normal plasma was obtained with approximately 0.5 µg/mL of rtcu-PA and rscu-PA-Glu158, with 2.1 µg/mL rscu-PA but with up to 200 µg/mL rscu-PA-Glu158. Similar concentrations were required for lysis in plasminogen depleted plasma reconstituted with Glu-plasminogen whereas two- to four-fold lower concentrations were required for depleted plasma reconstituted with Lys-plasminogen (Table 2).

**Predigestion of fibrin clots.** Incubation of purified fibrin clots with plasmin as described under Materials and Methods resulted in 6.9 ± 1.6% (n = 48) predigestion of the clots, as determined from the release of radioactivity. COOH-terminal amino acid sequence analysis revealed a maximal release of lys in amounts of 0.160 ± 0.02 nmol (mean ± SD; n = 3) after 30 to 60 minutes (above the background of nondigested clots) per predigested fibrin clot containing 375 µg fibrinogen (1.1 nmol). On addition of the plasminogen activators, dose- and time-dependent lysis of the fibrin clots was obtained (not shown). Plots of percent lysis after two hours vs the concentration of plasminogen activator (not shown) revealed that equi-effective concentrations (50% lysis of normal clots in two hours) were 1,300 ng/mL for rscu-PA, 270 ng/mL for rtcu-PA, and 90 ng/mL for t-PA. Lysis after two hours in normal human plasma of normal or predigested clots with rscu-PA (1,300 ng/mL), rtcu-PA (270 ng/mL), or single-chain t-PA (90 ng/mL) showed an increase of lysis after predigestion from 56 ± 9% to 85 ± 7% for rscu-PA (n = 10; $P < .001$), from 56 ± 11% to 66 ± 13% for rtcu-PA (n = 10; $P = .079$), and from 50 ± 5% to 57 ± 6% for rt-PA (n = 9; $P = .021$). These increases correspond to an apparent increase of the enzyme concentrations from 1.3 to 1.8 µg/mL for rscu-PA, from 0.28 to 0.33 µg/mL for rtcu-PA, and from 0.9 to 1.1 µg/mL for rt-PA.

Binding of plasminogen to normal or predigested purified fibrin clots was, respectively, 0.97 ± 0.22% (n = 6) and 2.7 ± 0.25% (n = 6) as determined after a one-hour incubation period at 37°C in human plasma. For normal fibrin clots, incubated in normal plasma for one hour and then transferred to buffer, 50% lysis in one hour was obtained with 130 ng/mL, 70 ng/mL, or 120 ng/mL for rscu-PA, rtcu-PA, or t-PA, respectively.

Table 1. Kinetic Constants for the Activation of Glu-plasminogen or Lys-plasminogen by Different u-PA Moieties

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<th>Glu-plasminogen</th>
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<td></td>
<td>$K_m$ (µmol/L)</td>
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<td>rscu-PA</td>
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Table 2. Concentration (ng/mL) of u-PA Required to Obtain 50% Clot Lysis

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<td>rtcu-PA-Glu158</td>
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<td>&gt;200,000</td>
</tr>
<tr>
<td>rtcu-PA-Glu158</td>
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<td>300</td>
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*Pluripotent labeled fibrin clots in purified systems, with $1.5$ µmol/L Glu-plasminogen or Lys-plasminogen, within one hour.

†Pluripotent clots labeled with $125$I-fibrin in normal human plasma or in plasminogen-depleted plasma reconstituted with either Glu-plasminogen or Lys-plasminogen, within two hours.
obtained with 65 ng/mL in the presence of Lys-plasminogen, clots before incubation for one hour in normal human plasma, subsequent lysis in buffer induced after one hour by rscu-PA (130 ng/mL), rtcu-PA (70 ng/mL), or rt-PA (120 ng/mL) increased from 46 ± 16% to 80 ± 12% for rscu-PA (n = 10; P < .001), from 39 ± 15% to 73 ± 13% for rtcu-PA (n = 10; P < .001), or from 43 ± 3% to 75 ± 7% for rt-PA (n = 10; P < .001). At lower concentrations of rscu-PA, rtcu-PA, or rt-PA, yielding 5% to 10% lysis of intact fibrin clots in one hour, predigestion of the clots also resulted in comparable relative increases of lysis with all three enzymes (not shown).

Plasminogen Activation by rscu-PA, rtcu-PA, rscu-PA-Glu'58, and rtcu-PA-Glu'58 in the Absence of Fibrin

In purified systems. In the absence of fibrin, Glu-plasminogen or Lys-plasminogen (1.5 µmol/L) in buffer were activated in a concentration-dependent manner by rscu-PA, rtcu-PA, rscu-PA-Glu'58, or rtcu-PA-Glu'58 (not shown). The concentrations of rtcu-PA required to obtain 50% plasminogen activation in one hour, were about twofold lower than those of rscu-PA (Table 3). Concentrations of rscu-PA-Glu'58 yielding 50% plasminogen activation in one hour are comparable with those of rscu-PA and rtcu-PA (Table 3). With rscu-PA-Glu'58, 50% activation was obtained with 65 µg/mL in the presence of Lys-plasminogen, whereas with Glu-plasminogen, 50% activation was not reached with 200 µg/mL. Fifty percent activation of Lys-plasminogen was obtained with 10- to 15-fold lower concentrations for all u-PA moieties, as compared with Glu-plasminogen.

In human plasma. In the absence of fibrin, 50% activation of plasminogen was obtained with 3.7 µg/mL of rscu-PA and with 0.4 µg/mL of rtcu-PA both in normal plasma and in plasminogen-depleted plasma reconstituted with Glu-plasminogen and with seven- to tenfold lower concentration of rscu-PA or rtcu-PA in plasminogen-depleted plasma reconstituted with Lys-plasminogen (Table 3). Equi-effective plasminogen activation (50% within two hours) was obtained with rtcu-PA-Glu'58 at comparable concentrations as with rtcu-PA. A concentration of 200 µg/mL rscu-PA-Glu'58 caused 46% plasminogen activation in two hours in plasma reconstituted with Lys-plasminogen but only 13% or 11% in normal plasma or plasma reconstituted with Glu-plasminogen (not shown).

Plasminogen Activation by rscu-PA, rtcu-PA, rscu-PA-Glu'58, and rtcu-PA-Glu'58 in Fibrin Clot Lysis Systems

In purified systems. In the presence of fibrin, 50% plasminogen activation in one hour was obtained with 85 ng of rscu-PA in a Glu-plasminogen solution and with 20 ng/mL in a Lys-plasminogen solution (Table 3). Equi-effective concentrations (50% plasminogen activation) of rtcu-PA and rtcu-PA-Glu'58 are comparable for both Glu-plasminogen (50 and 68 ng/mL) and Lys-plasminogen (14 and 35 ng/mL), while rscu-PA-Glu'58 is up to 2,000-fold less efficient. Concentrations of rscu-PA or rtcu-PA required for 50% lysis in one hour of fibrin clots immersed in Glu-plasminogen or Lys-plasminogen solutions caused <5% plasminogen activation.

In human plasma. In the presence of a fibrin clot, 50% plasminogen activation in two hours in normal plasma or in plasma-reconstituted plasma de Gulated with Glu-plasminogen is obtained with 3.3 µg/mL of rscu-PA whereas 50% activation in depleted plasma reconstituted with Lys-plasminogen requires sevenfold less rscu-PA. Plasminogen activation with rtcu-PA and rtcu-PA-Glu'58 occurs at three- to tenfold lower concentrations (Table 3). Only 10% to 15% plasminogen activation is obtained with 200 µg/mL rscu-PA-Glu'58 in normal plasma or plasma reconstituted with Glu-plasminogen as compared with 45% in plasma reconstituted with Lys-plasminogen.

The concentrations of rscu-PA required for 50% clot lysis in normal plasma in two hours, cause about 25% plasminogen activation both in the presence or the absence of fibrin (not shown).

<table>
<thead>
<tr>
<th>Table 3. Activation of Glu-plasminogen and Lys-plasminogen by Different u-PA Moieties in a Purified System and in Plasma</th>
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<tbody>
<tr>
<td><strong>Purified Systems</strong></td>
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<tr>
<td>Without fibrin</td>
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<tr>
<td>rscu-PA</td>
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<td>Lys-plg</td>
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Abbreviations: Glu-plg, Glu-plasminogen; Lys-plg, Lys-plasminogen.

The data represent concentration (ng/mL) of u-PA required to obtain 50% plasminogen activation, in purified systems within one hour with 1.5 µmol/L Glu-plasminogen or Lys-plasminogen, or within two hours in normal plasma or plasminogen-depleted plasma reconstituted with either Glu-plasminogen or Lys-plasminogen.
Plasminogen Activation With rscu-PA in the Presence of α₂-Antiplasmin

rscu-PA caused a time- and concentration-dependent activation of Glu-plasminogen both in the absence and the presence of α₂-antiplasmin (Fig 1). In the presence of α₂-antiplasmin (both at 1.5 and 7.5 μmol/L), plasminogen activation was significantly reduced, probably as a consequence of plasmin inhibition and prevention of the conversion of rscu-PA to rtcu-PA. Overnight preincubation at 4°C of rscu-PA with α₂-antiplasmin, before addition of Glu-plasminogen, did not influence subsequent plasminogen activation, whereas such preincubation with rtcu-PA completely abolished its fibrinolytic activity (not shown). Direct measurement of plasmin activity with S-2251 revealed that the plasmin concentration in the incubation mixtures with α₂-antiplasmin remained below the detection limit corresponding to conversion of 0.1% of plasminogen to plasmin.

Effect of Plasma on Clot Lysis by u-PA

Figure 2A shows that lysis by rscu-PA and rtcu-PA of purified fibrin clots immersed in Glu-plasminogen was inhibited in a concentration-dependent way by addition of plasma depleted in both plasminogen and α₂-antiplasmin. When the...
apparent residual enzyme activity was plotted vs the volume
fraction of added plasma (Fig 2B), it appeared that rscu-PA
is inhibited to 50% in the presence of about 2.5% (vol/vol) of
depleted plasma. For rtcu-PA, 50% inhibition required about
40% depleted plasma. When the depleted plasma was reconsti-
tuted with α₂-antiplasmin, clot lysis by both rscu-PA and
rtcu-PA was proportionally reduced (Fig 2A). Recycling of
plasminogen and α₂-antiplasmin depleted plasma on insolub-
ilized rscu-PA did not remove this inhibitory potential
toward rscu-PA, and the pooled eluates of the column did not
inhibit clot lysis by rscu-PA (Fig 3).

DISCUSSION

In the present study, we have reinvestigated the mecha-
nism of plasminogen activation and fibrin dissolution by
rscu-PA, in in vitro systems and with the use of quantitative
dose-effect curves, in order to delineate the relative contribu-
tion of several pathways to the fibrinolytic potency and
fibrin-specificity of rscu-PA. Our results confirm and extend
previous observations from several laboratories that rscu-PA
has some intrinsic plasminogen activating potential,5,9 that
conversion of rscu-PA to rtcu-PA results in a markedly
increased catalytic efficiency,10,15 that conformationally
altered plasminogen (Lys-plasminogen) is more susceptible
to activation than native plasminogen (Glu-plasmino-
gen),11-14 that plasma exerts an inhibitory action on the
fibrinolytic potency of rscu-PA,4 and that predigestion of
fibrin renders it more susceptible to lysis.14

Recombinant scu-PA, expressed in Chinese hamster ovary
cells, appears to have an intrinsic plasminogen activation
potential with a catalytic efficiency that is approximating 1%
of that of its two chain derivative, rtcu-PA. This is confirmed
by the intrinsic enzymatic activity of the cleavage-resistant
rscu-PA-Glu15815 although this mutant has a catalytic effi-
ciency that is about 15-fold lower than that of wild-type
rscu-PA. This intrinsic plasminogen activating activity of
rscu-PA-Glu158 is maintained after removal of potential trace
contamination of rscu-PA and rtcu-PA. In test systems with
plasmin-mediated fibrin clot lysis as end-point, rscu-PA has
a 2,000-fold higher fibrinolytic potency than rscu-PA-Glu158,
but only a 2.5-fold lower potency than rtcu-PA. This indi-
cates that conversion of rscu-PA to rtcu-PA during the
fibrinolytic process contributes a major positive feedback
mechanism for fibrinolysis. Partially degraded plasminogen
(Lys-plasminogen) is ten- to 20-fold more susceptible to
activation by both rscu-PA and rtcu-PA in purified systems
and in plasma. This higher sensitivity of Lys-plasminogen to
activation does not appear to be specific for scu-PA and
tcu-PA, since a similar change in sensitivity has previously
been observed for streptokinase11 and t-PA.13 Furthermore,
in a plasma milieu as well as in purified systems, the
fibrinolytic potency (in terms of clot lysis) of rscu-PA is only
about fourfold higher in the presence of 1.5 μmol/L Lys-
plasminogen as compared with Glu-plasminogen. These
findings would suggest that the marked threshold phenome-
non in the dose-response curves of clot lysis with rscu-PA is
primarily due to conversion of rscu-PA to rtcu-PA during the
fibrinolytic process. The binding of plasminogen to fibrin,
resulting in a conformational transition to a more susceptible
Lys-plasminogen-like form, may thus provide an additional
but quantitatively much less important feedback mechanism
than the conversion of rscu-PA to rtcu-PA.

From the kinetic constants in Table 1, one can calculate
that 50% activation in one hour of 1.5 μmol/L Glu-plasmin-
ogen or Lys-plasminogen would require rtcu-PA concen-
trations of 150 ng/mL and 6.0 ng/mL, respectively, rscu-
PA-Glu158 concentrations of 150 μg/mL and 18 μg/mL,
respectively, and rscu-PA concentrations of, respectively, 12
and 1.2 μg/mL. The values found experimentally for rtcu-
PA agree well with these estimates, for rscu-PA-Glu158 they
are two- to fourfold higher, but for rscu-PA the measured
concentrations are 60- to 80-fold lower, namely 210 ng/mL
for Glu-plasminogen and 15 ng/mL for Lys-plasminogen.
This further indicates that conversion of rscu-PA to rtcu-PA
contributes significantly to the fibrinolytic potency of rscu-
PA, via an increased catalytic efficiency for plasminogen
activation of the two chain derivative. Following predigestion
of a fibrin clot by plasmin, subsequent lysis by rscu-PA, but
also by rtcu-PA and rt-PA is enhanced. This may be
explained by increased binding of plasminogen to newly
exposed COOH-terminal lysine residues on the partially
digested fibrin matrix.24 Enhanced plasmin generation at the
fibrin surface may further accelerate conversion of scu-PA to
tcu-PA, resulting in an even more pronounced feedback
effect of this conversion. This enhanced lysis of predigested
clots thus is observed for all three enzymes evaluated,
although the extent appears to be marginally higher for
rscu-PA than for rtcu-PA and rt-PA. On preincubation of
fibrin clots in human plasma for one hour at 37°C, sufficient
plasminogen is bound to allow subsequent clot lysis by
rscu-PA, rtcu-PA, and rt-PA in buffer. In this setup, a
comparably enhanced lysis of predigested (about 5%) fibrin
clots as compared with normal clots is also observed for
rscu-PA, rtcu-PA, or rt-PA. These results indicate that
partial predigestion of a fibrin clot by plasmin results in
MECHANISM OF ACTION OF scu-PA

additional plasminogen binding and subsequently enhanced lysis by rscu-PA, rtcu-PA, and t-PA. This effect appears not to be specific for rscu-PA.

The role of conversion of scu-PA to tcu-PA is further substantiated by activation experiments of plasminogen in the presence of $\alpha_2$-antiplasmin. Indeed, a significant reduction of plasminogen activation by scu-PA is observed when generated plasmin is efficiently neutralized. In addition, the results of clot lysis experiments (Table 2) show that equipotent effective concentrations of rscu-PA-Glu$^{18}$ are two- to three-orders of magnitude higher as compared with rscu-PA, whereas their catalytic efficiencies differ only by a factor of 15. This again indicates that the plasminogen activating potency of rscu-PA is lower when conversion to rtcu-PA is prevented.

Plasma depleted both in plasminogen and $\alpha_2$-antiplasmin has a higher inhibitory potential on fibrinolysis with rscu-PA than with rtcu-PA. This confirms that plasma contains one or more components that interfere with plasminogen activation by scu-PA.$^4$ Attempts to remove this activity from plasma by repeated chromatography on insolubilized rscu-PA or on an insolubilized plasmin-resistant mutant$^{25}$ (rscu-PA-Pro$^{19}$, results not shown), have, however, failed. This indicates that the inhibitory mechanism for rscu-PA in plasma is complex and suggests that more than one protein or interaction may be involved. The nature of this inhibitory effect in plasma thus remains to be further defined.

In conclusion, our results demonstrate that rscu-PA has some intrinsic plasminogen activating potential, which is, however, only 1% of that of rtcu-PA. Conversion of rscu-PA to rtcu-PA during the fibrinolytic process constitutes the primary positive feedback mechanism, whereas binding of plasminogen to fibrin or predigestion of fibrin may result in relatively minor additional acceleration of the fibrinolytic process.

The mechanism of plasminogen activation and fibrin dissolution with scu-PA in a plasma milieu in vitro, however, may not be identical to its physiological mechanism of action. Indeed, dose-response curves of rscu-PA in plasma clot lysis systems in vitro show a marked threshold phenomenon,$^{18}$ whereas the dose-response curves for thrombolysis in vivo, both in rabbit and dog models are linear.$^{19}$ The relative contribution of conversion of rscu-PA to rtcu-PA to thrombolysis in vivo remains to be further explored.

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The mechanism of plasminogen activation and fibrin dissolution by single chain urokinase-type plasminogen activator in a plasma milieu in vitro

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