Plasminogen Activation with Single-chain Urokinase-type Plasminogen Activator (scu-PA)

STUDIES WITH ACTIVE SITE MUTAGENIZED PLASMINOGEN (Ser740→Ala) AND PLASMIN-RESISTANT scu-PA (Lys158→Glu)*

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The mechanism of the activation of plasminogen by single-chain urokinase-type plasminogen activator (single-chain u-PA, scu-PA) was studied using rscu-PA-Glu158, a recombinant plasmin-resistant mutant of human scu-PA obtained by site-specific mutagenesis of Lys158 to Glu, and rPlg-Ala740, a recombinant human plasminogen in which the catalytic site is destroyed by mutagenesis of the active-site Ser740 to Ala. Conversion of 125I-labeled single-chain plasminogen to two-chain plasmin was quantitated on reduced sodium dodecyl sulfate-gel electrophoresis combined with autoradiography and radioisotope counting of gel bands. The catalytic efficiencies of both rscu-PA-Glu158 and rscu-PA for the activation of rPlg-Ala740 and of natural plasminogen were comparable and were 250-500-fold lower than that of recombinant two-chain u-PA (tcu-PA) for rscu-PA-Glu158 and 100-200-fold lower for rscu-PA. Pretreatment of rscu-PA-Glu158 or rscu-PA with excess α2-antiplasmin, which efficiently neutralizes all contaminating rtcu-PA, did not significantly reduce the catalytic efficiency of these single-chain moieties, indicating that they have a low but significant intrinsic plasminogen activating potential. The low intrinsic catalytic efficiency of rscu-PA for the conversion of plasminogen to plasmin may be sufficient to generate trace amounts of plasmin, which may regulate plasminogen activation by converting poorly active rscu-PA to very active rtcu-PA.

Urokinase-type plasminogen activator (u-PA)† can be obtained as a single-chain molecule (single-chain u-PA, scu-PA) or as a two-chain proteolytic derivative (tcu-PA, urokinase) following cleavage of the Lys158-Ile159 peptide bond with plasmin or kallikrein. scu-PA has a very low reactivity toward low M, synthetic substrates or active site inhibitors that are very reactive toward tcu-PA (for references, see Ref. 1).

Plasminogen, the substrate of u-PA, is a single-chain glycoprotein with an M, of 92,000. It is converted to the two-chain serine protease plasmin by specific cleavage of the Arg560-Val561 peptide bond; the heavy (NH2-terminal) chain of M, 60,000 is connected with the light (COOH-terminal) chain of M, 25,000 through two disulfide bonds. The active center of the enzyme, located in the light chain, is composed of His602, Asp545, and Ser740. Native plasminogen has NH2-terminal glutamic acid (Glu-plasminogen), but is easily converted to modified forms with NH2-terminal lysine, valine, or methionine (commonly designated Lys-plasminogen), by plasmic cleavage of the Arg56-Met568, Lys57-Lys71, or Lys72, Val78 peptide bonds. Activation of Glu-plasminogen by tcu-PA obeys Michaelis-Menten kinetics, with reported kinetic parameters between 1.4 and 200 µM for the Michaelis constant (Km) and between 0.26 and 1.5 s⁻¹ for the catalytic rate constant (kcat). Lys-plasminogen is activated 3-10-fold more efficiently than Glu-plasminogen by all known plasminogen activators (for references, see Ref. 2).

Estimations of the catalytic efficiency of scu-PA for the activation of plasminogen have varied widely. Initially we reported that scu-PA has a catalytic efficiency similar to that of tcu-PA (3). Several groups have reported a lower but significant catalytic efficiency ranging between 0.4 and 6% of that of tcu-PA (4, 5) while other authors have reported that scu-PA is a genuine proenzyme without enzymatic activity (6-8).

We have proposed that in mixtures of plasminogen and scu-PA the conversion of plasminogen to plasmin and of scu-PA to tcu-PA can quantitatively be described by a sequence of three reactions each of which obeys Michaelis-Menten kinetics (3, 9). In the first reaction, scu-PA directly activates plasminogen to plasmin, then plasmin converts scu-PA to tcu-PA, and finally residual plasminogen is activated by tcu-PA. This mechanism attributes some intrinsic plasminogen activating potential to scu-PA, which initially was found to be very high (3) but which was subsequently shown to be of the order of 1% of that of tcu-PA (9, 10).

Several methodological difficulties, however, hamper the quantitative investigation of the activation of plasminogen by scu-PA. These include variability in kinetic properties of scu-PA obtained from different sources (9, 11), generation of more readily activable Lys-plasminogen forms (12), and efficient conversion of scu-PA to tcu-PA by generated plasmin during the activation process (4-11). To investigate the significance of the conversion of scu-PA to tcu-PA for the activation of plasminogen, we have previously constructed plasmin-resist-
ant mutants of scu-PA by site-specific mutagenesis of Lys\(^{128}\) to Glu or to Gly, thereby destroying the plasmin cleavage site for conversion to tcu-PA (13). These mutants still activate plasminogen, albeit with a catalytic efficiency which is at least an order of magnitude lower than that observed with wild-type scu-PA, which suggests that conversion of scu-PA to tcu-PA is not a prerequisite for plasminogen activation.

In the present study we have further improved the methodology for studying the mechanism of plasminogen activation by scu-PA by using a recombinant plasminogen mutant obtained by site-specific mutagenesis of the active site Ser to Ala (rPlg-Ala\(^{740}\)) whereby the catalytic site of plasmin is destroyed (14). The catalytic efficiency of rscu-PA, rscu-PA-Glu\(^{15}\), and rtcu-PA for the conversion of rPlg-Ala\(^{740}\) and of natural plasminogen (nPlg) to their two-chain derivatives was determined by reduced SDS-gel electrophoresis.

**MATERIALS AND METHODS**

Recombinant scu-PA (rscu-PA), prepared by expression of cDNA encoding human scu-PA in *Escherichia coli* was a gift from Grünenthal AG (Aachen, Federal Republic of Germany). Its specific activity associated with the generation of enzymatic activity. This was shown characterizing as described (9, 17). Plasminogen preparations were human plasminogen (nPlg) was purified from human plasma and characterized as described elsewhere (13). Plasmin (2% molar ratio) for 20 min at 37 °C in 0.05 M Tris-HCl buffer, pH 7.4, containing 0.038 M NaCl, 0.01% Tween 80, and 0.21 mg/liter in three preparations from approximately 10-liter batches of cultured medium. Before use, 6-aminohexanoic acid was removed by extensive dialysis against 0.05 M NaCl, 0.01% Tween 80, and 20 μM aprotinin, with rscu-PA (final concentration = 15, 150, or 300 nM), rscu-PA-Glu\(^{15}\) (final concentration = 75, 150, or 300 nM), or rtcu-PA (final concentration = 0.37, 0.75, or 1.50 nM). At different time intervals (0-60 min) samples were removed from the incubation mixtures and reduced immediately at 100 °C for 3 min in the presence of 1% SDS and 1% dithioerythritol. SDS-PAGE was performed on 10-15% polyacrylamide gels after reduction with dithioerythritol (not shown). The specific fibrinolytic activity of rtcu-PA was 180,000 ± 15,000 IU/mg and the specific amidolytic activity was 100,000 ± 17,000 IU/mg (mean ± S.D.; n = 3). rscu-PA-Glu\(^{15}\), obtained by site-specific mutagenesis of Lys\(^{128}\) to Glu, was prepared and characterized as described elsewhere (13). To remove trace amounts of rscu-PA or of active rtcu-PA, the preparation of rscu-PA-Glu\(^{15}\) (final concentration = 14 μM) was treated with plasmin (1% on a molar basis) for 30 min at 37 °C. Plasmin was then neutralized by addition of aprotinin (final concentration = 2 μM) and D-Val-Phe-Lys-CH\(_2\)Cl (final concentration = 10 μM) and rscu-PA by addition of Glu-Gly-Arg-CH\(_2\)Cl (final concentration = 1 μM). Lys-plasmin(ogen) was measured with a specific ELISA, using monoclonal antibody MA-LPm1, which does not react with Glu-plasminogen (24). tcu-PA was quantitated with an ELISA specific for tcu-PA, using MA-12E6A8 which reacts 15,000-fold better with tcu-PA than with scu-PA (31).

**RESULTS**

In mixtures of rPlg-Ala\(^{740}\) (1.5 μM) and of tcu-PA during the experiment was monitored by ELISA using samples collected into Glu-Gly-Arg-CH\(_2\)Cl (final concentration = 10 μM).

In additional experiments, rscu-PA, rscu-PA-Glu\(^{15}\) (final concentration = 900 nM each), or rtcu-PA (final concentration = 1.5 μM) were preincubated with a,anti-antiplasmin (final concentration = 2 μM) for 2 h at 37 °C before addition of rPlg-Ala\(^{740}\) (final concentration = 1.5 μM) and aprotinin (final concentration = 20 μM).

The catalytic efficiencies (k\(_{cat}\)/K\(_{m}\)) of the three u-PA moieties for the activation of nPlg or rPlg-Ala\(^{740}\) are calculated from the initial rate of plasmin generation, the concentration of plasminogen and u-PA used, using the formula u/

\[ k_{cat}/K_m = \frac{\text{u}}{\left[\text{u-PA}\right]} \times \frac{\text{[Plg]} - \left[\text{Km} + \left[\text{Plg}\right]\right]}{\left[\text{[Plg]} - \left[\text{Km} + \left[\text{Plg}\right]\right]\right]} \]

For rscu-PA and rscu-PA-Glu\(^{15}\), the labeled single-chain material was progressively converted to two-chain material as revealed by reduced SDS-gel electrophoresis and autoradiography (Fig. 1). The amount of two-chain material quantitated by measuring the 125I content of the corresponding bands of the SDS-gel increased linearly with time for all three u PA moieties studied (Fig. 2). A very similar response was obtained using nPlg instead of rPlg-Ala\(^{740}\) (not shown). The catalytic efficiencies (k\(_{cat}\)/K\(_{m}\)) of the three u-PA moieties for the activation of nPlg or rPlg-Ala\(^{740}\) are calculated from the initial rate of plasmin generation, the concentration of plasminogen and u-PA used, using the formula u/

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Monitoring of Lys-plasmin(ogen) levels during incubation confirmed the absence of Lys-forms in the experiments with rPlg-Ala<sup>740</sup> (<0.2% at 60 min with the highest concentration of rscu-PA, rscu-PA-Glu<sup>158</sup> or rtcu-PA used), whereas in the experiments with nPlg the concentration of Lys-forms reached a maximum of 7, 0.8, and 2.5% at 60 min using the highest concentration of rscu-PA, rscu-PA-Glu<sup>158</sup>, and rtcu-PA, respectively. Monitoring of rtcu-PA levels in the experiments with rscu-PA and rscu-PA-Glu<sup>158</sup> confirmed that no active two-chain u-PA was generated during the experiments (<0.5% at 60 min at the highest concentration of u-PA), either with nPlg or with rPlg-Ala<sup>740</sup>. Preincubation of rscu-PA or rscu-PA-Glu<sup>158</sup> with α<sub>2</sub>-antiplasmin, under conditions where up to 0.5% contaminating rtcu-PA is completely neutralized, did not abolish the activation of rPlg-Ala<sup>740</sup> (Fig. 3). The catalytic efficiencies for the activation of rPlg-Ala<sup>740</sup> by rscu-PA or by rscu-PA-Glu<sup>158</sup> in the presence of α<sub>2</sub>-antiplasmin were, respectively, 13 or 27% (mean of two independent experiments) lower than in the absence of α<sub>2</sub>-antiplasmin, whereas rtcu-PA was totally inactivated by preincubation with α<sub>2</sub>-antiplasmin.

**DISCUSSION**

Elucidation of the activation mechanism of plasminogen by scu-PA is complicated by the fact that generated plasmin may convert scu-PA to tcu-PA, which has a much higher catalytic efficiency for plasminogen, or that it may degrade Glu-plasminogen to Lys-plasminogen with a much greater sensitivity to activation. Both phenomena indeed would result in marked acceleration of plasminogen activation during the experiment. We have previously measured plasminogen activation by scu-PA with a coupled substrate assay, in which excess plasmin substrate S-2251 was used to measure plasmin and at the same time to prevent or reduce conversion of scu-PA to tcu-PA (3, 9). In addition, we have used plasmin-resistant mutants of scu-PA (rscu-PA-Glu<sup>158</sup>) which are not converted to active tcu-PA moieties by plasmin (13). These experiments have suggested that scu-PA has a very low, but probably significant plasminogen-activating potential. In the experimental systems used, it was, however, impossible to exclude trace contamination of the reagents with plasmin and/or tcu-PA and to totally prevent conversion of scu-PA to tcu-PA by traces of generated plasmin.

In the present study, we have measured the catalytic efficiency of scu-PA for plasminogen in a system which is intrin-
Plasminogen Activation with scu-PA

physically devoid of tcu-PA or plasmin activity. This is achieved by using a plasmin-resistant mutant of rscu-PA (rscu-PA-Glu') and an active site mutant of recombinant plasminogen (rPlg-Ala') which is totally inactive after conversion to a two-chain molecule by plasminogen activators (14). Generation of plasmin was quantitated by autoradiography after SDS-PAGE under reducing conditions. In addition, the experiments were performed in the presence of excess aprotinin and the generation of tPA-PA and Lys-plasminogen was monitored with specific ELISAs.

Our results indicate that the catalytic efficiencies (kcat/Km) of both rscu-PA and rscu-PA-Glu' are comparable to the value of ~1% obtained previously in a coupled substrate assay with natural scu-PA obtained from CALU-3 cells (9) or with rscu-PA obtained from Chinese hamster ovary cells (13). In a previous study, the catalytic efficiency of rscu-PA from E. coli, provided by Genentech was, however, found to be comparable to that of rtcu-PA (3); the molecular basis of this discrepancy with rscu-PA from E. coli obtained from an alternative source remains unclear. A catalytic efficiency of rscu-PA for plasminogen of about 0.4% of that of tcu-PA was reported previously by Pannell and Gurewich (4), whereas Ellis et al. (5) found a catalytic efficiency of 4-6% of that of tcu-PA. In contrast, other authors have reported that rscu-PA has no plasminogen-activating potential (6-8).

The 3-fold difference in catalytic efficiency for plasminogen activation of rscu-PA and rscu-PA-Glu' found in the present study contrasts with the 10-fold higher value for rscu-PA as compared to rscu-PA-Glu' obtained previously (13). This may be due to the fact that the previously used coupled substrate assay (3, 9) does not totally prevent conversion of scu-PA to tcu-PA, resulting in overestimation of the intrinsic activity of scu-PA.

The methodology used in the present study allows exclusion of the generation of both tcu-PA and plasmin activity during the experiment and thereby establishes that rscu-PA has a low but significant intrinsic plasminogen activating potential. This may be important for the regulation of the mechanism of plasminogen activation by scu-PA via generation of a small amount of plasmin which in turn converts poorly active scu-PA to very active tcu-PA, resulting in an accelerated activation of plasminogen. This positive feedback mechanism has also been implied in fibrin clot dissolution by scu-PA in human plasma in vitro (10). Furthermore, it was suggested that conversion of scu-PA to tcu-PA by plasmin occurs mainly at the fibrin surface without extensive systemic conversion in the surrounding plasma (31).

It has been demonstrated previously that certain zymogens, such as chymotrypsinogen and trypsinogen, are not entirely enzymatically inactive but have a very low intrinsic activity which, upon activation, increases by several orders of magnitude (25-27). The single-chain form of tissue-type plasminogen activator (u-PA) has significant enzymatic activity toward low molecular weight synthetic substrates (10-15% of that of the two-chain form) (23) whereas its plasminogen activating potential is comparable to that of the two-chain form (29). The structural basis of this high activity as compared to other single-chain forms of serine proteases has not yet been explained. The intrinsic enzymatic activity of scu-PA appears to be intermediate to these extremes.

The mechanism of plasminogen activation in purified systems and of fibrin dissolution in human plasma in vitro presented here may, however, not be identical to the physiological mechanism of action of scu-PA. It was indeed found that plasmin-resistant mutants of scu-PA (i.e. rscu-PA-Glu') have only a 3-5-fold lower in vivo thrombolytic potency as compared to wild-type scu-PA (30), suggesting that for in vivo thrombolysis, conversion of scu-PA to tcu-PA may play a less important role.

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