Pro-Urokinase: A Study of Its Stability in Plasma and of a Mechanism for Its Selective Fibrinolytic Effect

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Highly purified pro-urolase (pro-UK) or single-chain urokinase-type plasminogen activator (scu-PA) was treated with diisopropylfluorophosphate (1 mmol/L) to eliminate traces of two-chain UK activity. This preparation was found to retain a low activity against a chromogenic substrate (S2444), equivalent to 0.1% to 0.5% of the activity of its plasmin-activated derivative. Evidence is presented that the intrinsic activity of pro-UK (scu-PA) was sufficient to activate plasminogen on a fibrin plate or in buffer and was far more reactive against Lys-plasminogen than against Glu-plasminogen. The relative resistance of Glu-plasminogen to activation was overcome by the addition of lysine (25 mmol/L) to the reaction mixture. By contrast, in plasma, pro-UK (scu-PA) was stable and non-reactive for >72 hours when incubated (37 °C). Pro-UK (scu-PA) did not form sodium dodaclyt sulfate-stable inhibitor complexes, whereas complexation occurred rapidly with UK. Only at high concentrations of pro-UK (scu-PA) (>250 IU/mL) did plasminogen activation in plasma occur. The relative inertness of pro-UK (scu-PA) in plasma, in contrast to its low-grade enzymatic activity in buffer, was attributed to the effect of inhibitors. The addition of EDTA or the removal of divalent cations by dialysis was associated with a lower threshold for nonspecific plasminogen activation by pro-UK (scu-PA) in plasma. Replacement of Ca++ but not other cations restored baseline conditions. In the presence of a clot, fibrin-selective plasminogen activation and clot lysis were triggered. Lysis was accompanied by <10% conversion of pro-UK (scu-PA) to two-chain UK, suggesting that the intrinsic activity of pro-UK (scu-PA) itself may have been responsible for fibrinolysis, although a contribution by the small amount of UK generated could not be excluded. Similarly, pro-UK (scu-PA) supported clot lysis for several days in the same plasma before the effect dissipated as a result of degradation to UK. When Glu-plasminogen in plasma was replaced by Lys-plasminogen, or when lysine was added to normal plasma, nonspecific plasminogen activation and fibrinogenolysis occurred. It was concluded that the fibrin specificity of pro-UK (scu-PA) can be explained by its selective activation of fibrin-bound plasminogen and is due to the latter’s Lys-plasminogen-like conformation. The free plasminogen in plasma is spared due to its Glu-form as well as to the stability of pro-UK (scu-PA) in the presence of plasma inhibitors and Ca++. This mechanism appears not to require fibrin binding, and clot lysis is accompanied by little activation of pro-UK (scu-PA) to two-chain UK.

A SINGLE-CHAIN FORM of ~55,000-dalton urokinase (UK) was first purified and identified by Husain et al in our laboratory. It was isolated from urine by affinity chromatography on fibrin/Celite that did not bind other forms of UK. Subsequent studies of a single-chain UK plasminogen activator (scu-PA) purified from the fluid of a transformed human kidney cell culture indicated that it was a latent form activatable by plasmin correlating with conversion to two-chain HMW-UK. Nonetheless, the single-chain UK showed a low inherent amidolytic activity. Wun et al and Nielsen et al purified scu-PA from human carcinoma cells in culture, and from its characteristics, concluded that scu-PA was a proenzyme. A latent, trypsin-activatable UK activity was originally detected in kidney cell culture medium by Bernik and by Nolan et al.

The fibrinolytic properties of pro-UK (scu-PA) were compared with UK in vitro and in laboratory animals. A superior fibrin specificity and lytic efficacy were demonstrated, but the mode of action of pro-UK (scu-PA) was not established. Unlike tissue plasminogen activator (t-PA), fibrin clot binding of pro-UK (scu-PA) in plasma was not found, in contrast to fibrin/Celite binding in urine, indicating that its fibrin-selective proteolytic effect could not be explained on this basis. The present study was undertaken to investigate alternative mechanisms to fibrin binding to explain the fibrinolytic properties of pro-UK (scu-PA).

The findings indicated that under the experimental conditions, fibrin-selective clot lysis was primarily due to a selective activation of fibrin-bound plasminogen by the intrinsic activity of pro-UK (scu-PA) itself. The effect of inhibitors and Ca++ served to prevent the activation of free Glu-plasminogen by pro-UK (scu-PA) in the ambient plasma.

MATERIALS AND METHODS

Pro-urolase (scu-PA) was purified from the spent tissue culture supernatant of a transformed human kidney cell line by Collaborative Research, Inc (Lexington, Mass) as previously described. Two-chain UK (55,000-dalton) was a gift from Serono Laboratories (Randolph, Mass). For some experiments, UK was prepared by plasmin conversion of pro-UK (scu-PA) (see later). Antisera to urinary and to tissue culture UK were obtained from Collaborative Research.

Human fibrinogen and amidolytic substrates (S2251 and S2444) were obtained from Kabi (Stockholm); a crude bovine thrombin "thrombostat" preparation was obtained from Parke-Davis (Morris Plains, NJ) and highly purified human thrombin, from Collaborative Research. Streptokinase was a product of Hoechst-Roussell (Sumersville, N.J.).

Aprotinin (Trasylol) was obtained from FBA Pharmaceuticals (New York). Thrombolastin, diisopropylfluorophosphate (DFP), Tris [Tris(hydroxymethyl) aminomethane] and HEPES (N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid) were obtained from Sigma Chemical Co (St Louis). Bovine serum albumin (BSA), "purified," was obtained from Calbiochem (La Jolla, Calif).

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Fresh frozen citrated plasma was obtained from St. Elizabeth's Hospital Blood Bank. Three units of this stock plasma were pooled and stored in aliquots at −20°C. Fresh plasma was prepared from the blood of volunteers collected into citrate, and serum from the same donors was prepared from fresh blood collected in glass and incubated (37°C) for two hours. Plasma or serum was dialyzed (72 hours) against 25 mmol/L HEPES-buffered saline at pH 7.4 to remove divalent cations and chelators.

The integrity of the native form of plasminogen in the stock plasma was ascertained after isolation of the plasminogen (see later) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 5% gels alongside Glu- and Lys-plasminogen.

Glu-plasminogen was purified on Lysine-Sepharose essentially by the method of Castellino and Powell28 from DFP-treated fresh plasma. Lys-plasminogen was prepared from purified Glu-plasminogen by the method of Lucas et al.11

Plasmin was prepared by activation of plasminogen (2.5 μmol/L) at pH 8.8 with an equal volume of a 10% suspension of immobilized UK (Affi-Gel 15; Bio-Rad, Richmond, Calif) for 30 minutes at 37°C.

Plasminogen activator activity was measured on a standard fibrin plate made with human fibrinogen2 against the World Health Organization International Reference Preparation (IRP) of UK, 66/46 (National Institute of Biological Standards and Control, London). Assignment of unitage (in International Units [IU]) for pro-UK (scu-PA) solutions was based on latent activity, after activation with plasmin and dilution to eliminate significant plasmin (see later).

UK amidolytic activity was measured with Kabi substrate S2444 at 37°C. The reaction buffer was 0.1 mol/L Tris-HCl (pH 8.8, 0.1 mol/L NaCl) 0.1 mg/mL BSA, with or without aprotinin (200 KIU/mL); and the substrate was 0.75 mmol/L. One enzyme unit (EU) was defined as the amount of enzyme producing a change in absorbance (405 nm) of 1 absorbance unit per minute in 0.4 mL of reaction mixture stopped with 0.4 mL of saline/5% acetic acid. Aprotinin was added (100 KIU/mL) to inhibit plasmin in some samples. When assayed in this system, the IRP-UK had an activity of about 275 EU/100,000 IU.

Plasmin was defined as activity against Kabi substrate S-2251 and assayed in a reaction buffer of 0.1 mol/L Tris-HCl (pH 7.4), 0.1 mol/L NaCl, and 0.1 mg/mL BSA. Plasmin was activated for assay by preincubation with streptokinase (5,000 U/mL) for 40 minutes at 37°C.

To measure latent activity, pro-UK (scu-PA) (12,500 IU/mL) was incubated with plasmin (0.1 μmol/L) for 30 to 45 minutes at 37°C and then diluted for assay on fibrin plate or assayed for amidolytic activity with the addition of aprotinin (200 KIU/mL) to the reaction buffer. Pro-UK (scu-PA) in plasma was measured with S2444 after secondary activation using streptokinase. The concentration was calculated by subtracting the UK activity present without activation.

SDS-PAGE was performed by the method of Laemmli13 using a 12.5% polyacrylamide gel. Samples were prepared either with or without 10 mmol/L dithiothreitol (DTT). Gels were either stained with silver16 or dried for autoradiography against Kodak X-Omat R film.

Pro-UK (scu-PA) was labeled with 125I by way of the lactoperoxidase reaction, using immobilized lactoperoxidase-glucose oxidase (Enzymobeads, Bio-Rad). Fifty micrograms of pro-UK in 70 μL of 0.2 mol/L sodium phosphate (pH 7.2) was reacted with 50 μL Enzymobeads suspension, 10 μL (2 mCi) Na125I (New England Nuclear, Boston), and 25 μL of 1% d-glucose for 15 minutes at room temperature. Free iodine was separated on a column of Sephadex G-10 (Pharmacia Fine Chemicals, Piscataway, N J) in 10 mmol/L sodium acetate (pH 4.8), 0.1 mol/L NaCl, 0.1% Triton X-100 (Sigma, St Louis). The resulting specific activity of the pro-UK (scu-PA) was approximately 30 Ci/μg. Labeling was accompanied by some conversion to UK and some loss of enzymatic activity, but the product could be fully cleaved by plasmin at a rate comparable to unlabeled pro-UK (scu-PA). Radiola beled UK was made by plasmin conversion of 125I-pro-UK (scu-PA).

Immunoprecipitations were performed by the method of Kessler15 using Staphylococcus aureus cells obtained from Calbiochem (Pan sorbin). Samples containing an estimated 200 to 500 ng UK were precipitated with 10 μL of antiserum against urinary UK. The immunosorption buffer was 0.1 mol/L Tris (pH 7.2), 0.1 mol/L NaCl, 0.1% Triton X-100. After electrophoresis and autoradiography, the distribution of counts in bands was determined by counting cut sections of the dried gel. The percentage of conversion was calculated and corrected for the two-chain UK contaminant in the probe. Some degradation of the single-chain UK invariably occurred during radiolabeling.

Enzyme treatment of pro-UK (scu-PA). Pro-UK (scu-PA) was activated and converted to its two-chain derivative by treatment with plasmin. Twenty-five microliters of pro-UK (scu-PA) (0.9 μmol/L in 0.1 mol/L Tris, pH 7.4, 0.3 mol/L NaCl) was mixed with 25 μL of plasmin (0.25 μmol/L in the same buffer) and reacted for 45 minutes at 37°C.

Conversion to UK was evaluated by amidolytic assay and by reducing SDS-PAGE at time points as well as by fibrin plate assay (after 1,000-fold dilution that eliminates any plasmin effect on the assay as determined by control).

Fibrinogen was determined as thrombin-clottable protein.

Analysis of DFP-treated pro-UK (scu-PA). Pro-UK (scu-PA), UK, and plasminogen were treated with 0.01 to 1.0 mmol/L DFP in 1.0 mol/L sodium phosphate (pH 7.2), 0.1 mol/L NaCl, 0.5 mg/mL BSA. Incubation was carried out on ice for 18 hours. For analytical inhibition studies, treated samples were diluted tenfold with 0.1 mol/L Tris (pH 8.8), 0.2 mg/mL BSA, 0.1 mol/L NaCl, and then further incubated and assayed daily until the decay of DFP was complete. For preparative use, the treated product was dialyzed against 10 mmol/L sodium acetate (pH 4.8), 0.15 mol/L NaCl, and stored frozen.

To investigate whether pro-UK (scu-PA) had any enzymatic activity in and of itself, it was first treated with DFP (1 mmol/L) and then assayed with the chromogenic substrate S2444. Assays were done both before and after DFP had substantially decayed in solution. The DFP-treated pro-UK (scu-PA) was also assayed on a standard fibrin plate and in buffer.

Clot lysis induced by pro-UK (scu-PA) or activated pro-UK (scu-PA). 125I-Labeled clots were prepared from stock plasma. Plasma (0.5 mL) plus 1.5 μCi 125I-fibrinogen (Ibrin, human fibrinogen; Amersham Corp, Arlington Heights, Ill) was clotted with thromboplastin and 10 mmol/L CaCl2. The clots were formed in 5-mm (inside diameter) glass tubes, incubated 30 minutes (25°C), and kept refrigerated overnight as previously described.

These clots were incubated (37°C) in 3 mL of stock plasma in the presence of added pro-UK (scu-PA) or plasmin-activated pro-UK. At intervals, samples of the incubation medium were removed for measurement of radioactivity and enzymatic activity against chromogenic substrate (S2251). For this, plasma samples were diluted 1:200 and kept on ice. Fibrinogen concentrations were determined after six hours' incubation, on 1-mL aliquots of the lysis supernatant with 2,000 units of aprotinin added.

Effect of Lys-plasminogen vs Glu-plasminogen. To evaluate the reaction of pro-UK (scu-PA) with Glu- or Lys-plasminogen, each of the reactants was first treated with DFP to eliminate traces of the activated species. The reaction mixture comprised 0.1 μmol/L pro-UK (scu-PA) and 0.1 μmol/L Glu- or Lys-plasminogen in 0.1 mmol/L HEPES (pH 7.4), 0.15 mol/L NaCl, 0.25 mg/mL BSA,
0.75 mmol/L plasmin substrate S2251 + 25 mmol/L lysine. Reaction mixtures of 100 µL were incubated at room temperature in microtiter plate wells and monitored repeatedly using a Titertek Multiscan microplate reader (Flow Laboratories, Dublin, Va).

Plasminogen-depleted plasma was reconstituted with either Lys- or Glu-plasminogen (0.2 mg/mL). Aliquots (3 mL each) were paired (3 mL each), and standard radiolabeled clots were added to one tube of each pair. Pro-UK (scu-PA) (150 IU/mL) and a radiolabeled probe were added to all four tubes. Clot lysis was followed, and plasminogen was measured at zero time and at completion of clot lysis. At the same time points, samples were obtained for autoradiography of the probe after immunoprecipitation.

Clot lysis with 150 IU/mL pro-UK (scu-PA) was performed in stock plasma to which lysine (0 to 25 mmol/mL) was added.

Effect of fibrin on the activation of plasminogen and pro-UK (scu-PA) in plasma. Pro-UK (scu-PA) (200 to 1,000 IU/mL) was incubated in the presence or absence of a standard plasma clot in fresh plasma, serum, or plasminogen-depleted plasma. Clot lysis, UK (S-2444), and plasmin (S-22511) generation were monitored by periodic sampling of the plasma.

Fate of 125I-pro-UK (scu-PA) during clot lysis. 125I-pro-UK (scu-PA) and pro-UK (scu-PA) (100 IU/mL) were added to fresh plasma containing a plasma clot, and the mixture was incubated (37°C). At intervals, the plasma was sampled until clot lysis was complete. The plasma samples were analyzed by SDS-PAGE under reducing conditions and by autoradiography after immunoprecipitation. Conversion was quantitated by cutting bands from the dried gel and counting them. A 125I-fibrinogen-labeled clot was incubated in parallel and the fractional lysis rate plotted from it.

The conversion of pro-UK (scu-PA) to UK during clot lysis was also studied by measuring the generation of activity against chromogenic substrate S2444 during serial lysis of five and ten clots over a period of five days, incubated in the same plasma. Amidolytic assays for pro-UK (scu-PA) and UK were performed after seven hours' incubation of the plasma mixture. The clots for these experiments were made from fibrinogen (1.5 mg) clotted in the presence of 125I-fibrinogen (1 µCi) with Ca++ and thrombin. Two clots were incubated (37°C) daily in plasma (5 mL) containing pro-UK (scu-PA) (100 IU/mL). Urokinase, pro-UK (scu-PA), and plasminogen were measured in the plasma at baseline and after completion of clot lysis. At the end of each day, the plasma mixture was then refrigerated (4°C) overnight. The next morning, two new clots were added and the mixture incubated and assays repeated six hours later. This procedure was repeated for five successive days. Bacterial growth was suppressed by antibiotics.

The experiment was repeated with one clot added daily to evaluate the effect of fibrin surface area on the activation of plasminogen and pro-UK (scu-PA).

Effect of divalent cations on clot lysis and nonselective plasmin generation by pro-UK (scu-PA). Pro-UK (scu-PA) (100 IU/mL) or UK (300 IU/mL) were incubated (37°C) in the following media: stock plasma or serum containing EDTA in a range of concentrations (0.5 to 20.0 mmol/L), serum or stock plasma dialyzed to remove cations and citrate, dialyzed plasma reconstituted with divalent cations: Ca++ (20 µmol/L), Zn++ (50 µmol/L), Mg++ (1 mmol/L), Mn+++ (1.5 µmol/L) or dialyzed serum reconstituted with Ca++ (5 mmol/L) plus or minus the other cations.

Clot lysis, UK, plasmin, and plasminogen were measured. Clots in dialyzed media were made up from dialyzed plasma clotted with thrombin and Ca++. Degradation of 125I-pro-UK (scu-PA) during clotting. 125I-Pro-UK (scu-PA) (100,000 cpm, ~1 IU) was added to 0.3 mL of freshly drawn human plasma (or buffer) with or without the addition of various inducers of clotting: thromboplastin (Sigma), thrombin (Parke-Davis, Thrombostop), or kaolin. The mixtures were incubated for one hour at 37°C. Then the clots were centrifuged and the supernatants sampled. Samples of 100 µL were mixed with 25 µL of aprotinin (finally, 1,000 KIU/mL) and 125 µL of 2x immunoblotting buffer, applied to a 0.2-mL column of protein A-Sepharose (Sigma) to remove endogenous immunoglobulins, and washed through with 1 mL of 1x immunoblotting buffer to provide the sample for immunoprecipitation and electrophoresis under reducing conditions. The extent of pro-UK (scu-PA) degradation was analyzed by autoradiography.

RESULTS

Enzymatic activation and conversion to UK. Treatment of the pro-UK (scu-PA) with plasmin increased its activity against the amidolytic substrate at a rate dependent on the plasmin concentration. At 0.1 µmol/L plasmin, pro-UK (scu-PA) (0.5 µmol/L) was 93% activated in 15 minutes at 37°C and was fully activated after one hour. At 0.1 µmol/L plasmin, activation was slow but was extrapolated to completion in about 18 hours (Fig 1). Activation was correlated with conversion to the two chains of UK as determined by reducing SDS-PAGE (Fig 2).

Reaction with DFP. The latent activity of pro-UK (scu-PA) and the expressed activity of UK showed markedly different susceptibilities to inhibition by DFP. When pro-UK (scu-PA) was treated with 1.0 mmol/L DFP, the plasmin-activatable amidolytic activity could be completely recovered after decay of the DFP. On the other hand, UK activity was completely inhibited at 0.02 mmol/L DFP and retained only 5% activity at 0.01 mmol/L DFP.

When pro-UK (scu-PA) was treated with 1 mmol/L DFP, 50 times the concentration needed to inactivate UK, a small amidolytic activity persisted even in the presence of inhibitory levels of DFP. This amidolytic activity was equivalent to about 0.1% to 0.5% of the latent activity after plasmin activation.

When the purified zymogens, pro-UK (scu-PA) and plasminogen, were incubated together in buffer, activation of
plasminogen could be observed after a lag phase that varied with the concentration of the reactants and with the form of plasminogen used (see later).

Fibrin plate assay. The spotting of DFP-treated pro-UK (scu-PA) onto a standard fibrin plate always generated some fibrinolytic activity. On our standard plates made up from human fibrinogen (plasminogen concentration ~0.01 mg/mL final concentration), the specific activity of DFP-treated pro-UK (scu-PA) was about 25,000 IU/mL. Because plasmin is generated in this indirect assay, pro-UK (scu-PA) activation by plasmin cannot be avoided during the course of the assay, resulting in accelerated fibrinolysis due to UK. Untreated pro-UK (scu-PA) preparations (90% to 97% zymogen) had specific activities ranging from 40,000 to 60,000 IU/mg, the higher value being obtained with preparations having a higher percentage of UK contamination. An important additional variable influencing the final result is the plasminogen concentration in the plate, as previously described. Higher specific activities are obtained with higher plasminogen concentrations, presumably due to more rapid activation of pro-UK (scu-PA).

After full activation of pro-UK (scu-PA) with plasmin, described earlier, a specific activity of approximately 100,000 IU/mg was obtained. The latter represents the only way by which the fibrin plate assay of pro-UK (scu-PA) can be made subject to standardizable interpretation.

Clot lysis in plasma by pro-UK (scu-PA) compared with its activated form. A marked difference in clot lysis by pro-UK (scu-PA) and its plasmin-activated derivative was found. At identical concentrations (65 IU/mL), pro-UK (scu-PA) after a lag phase caused accelerated and complete clot lysis, whereas its derivative induced more rapid early lysis that failed to go to completion. Fibrinogen degradation (30%) and detectable plasmin elaboration in the plasma accompanied the latter but was not seen with pro-UK (scu-PA) (Fig 3).

Stability of 125I-pro-UK (scu-PA) in plasma. Autoradiography of 125I-pro-UK (scu-PA) (~1 nmol/L) incubated (37 °C) for four days in plasma or serum showed no change in its appearance from baseline. By contrast, incubation of 125I UK (~1 nmol/L) (activated pro-UK) (scu-PA) resulted in the formation of higher molecular weight bands representing complexes with plasma protease inhibitors. The failure of UK to react completely reflects the incomplete stability of the enzymatic activity to labeling (Fig 4). Stability of the single-chain form of pro-UK (scu-PA) was also shown in buffer (0.1 mol/L HEPES, pH 7.2) over four days at 37 °C.

Stability of pro-UK (scu-PA) during clot lysis in plasma. Examination of autoradiographs of reduced SDS-gels of 125I-pro-UK (scu-PA) incubated in plasma during lysis of
a single clot by pro-UK (scu-PA) (150 IU/mL) showed no apparent conversion of the pro-UK (scu-PA) probe. Some contamination by two-chain UK is seen at baseline (B.L.) due to degradation during radiolabeling (Fig 5).

More precise measurements showed only slight conversion. Counting of the sliced gels at $t_0$ gave counts corresponding to 73% and 27% for single- and two-chain UK, respectively. In the control with no clot, after seven hours' incubation, there was no change in this composition of the probe. However, in the presence of a clot, almost 10% conversion accompanied complete clot lysis.

When serial clot lysis with pro-UK (scu-PA) (100 IU/mL) was performed with two clots added daily to the same plasma, there was no significant conversion of pro-UK (scu-PA) to UK by amidolytic assay (S2444 before and after SK activation) until after the second day, at which time 6 mg of fibrin had lysed. Thereafter, a gradual conversion to UK was seen that did not go to completion, even though plasmin elaboration was undoubtedly potentiated by fibrin degradation products. By the end of the fifth day, 20% of the pro-UK (scu-PA) still remained. The total enzyme expression (pro-UK [scu-PA] and UK) was increased after the third day, probably due to some consumption of inhibitors. In the control tubes containing no clots, far less activation of pro-UK (scu-PA) was seen, with >60% remaining at the end of day 5 (Fig 6). When only a single clot was added instead of two clots, the conversion to UK was about half as rapid.

No significant plasmin generation or plasminogen consumption in the plasma during serial clot lysis with two clots was seen until days 4 and 5, when the losses were 40% and 50% of baseline values, respectively. Because fibrin degradation products potentiate plasmin elaboration in this assay, these results may underestimate plasminogen losses. In the control tubes without clots, plasminogen concentrations remained unchanged for five days.

The fractional clot lysis rate for the first three days (3 mg fibrin per day) remained constant. By day 4, clot lysis was retarded and did not go to completion (Fig 7). On the fifth day, no lysis took place.

**Effect of fibrin clots on the activation of pro-UK (scu-PA) and plasminogen in plasma.** The proteolytic effect of pro-UK (scu-PA) in plasma was invariably clot-related, but fibrin specificity was seen only at concentrations of pro-UK (scu-PA) up to <250 IU/mL. Within this range, clot specificity was not accompanied by detectable plasmin or UK generation.
or by fibrinogen degradation. At higher concentrations (250 to 500 IU/mL), some plasmin generation in the ambient plasma accompanied clot lysis, but this was not detectable in the absence of a clot. At concentrations of 500 to 1,000 IU/mL, plasmin and UK were generated spontaneously and simultaneously after one to three hours' incubation, but their level was considerably potentiated by the addition of a clot. In plasminogen-depleted plasma, no UK generation occurred, indicating that plasmin was responsible for pro-UK (scu-PA) activation under these experimental conditions (Fig 8).

When the experiments were done in serum, the fibrin selectivity of the proteolytic effect of pro-UK (scu-PA) was retained at concentrations that were about 20% to 30% higher than in corresponding citrate plasma.

**Effect of Lys-plasminogen v Glu-plasminogen.** When purified, DFP-treated pro-UK (scu-PA) and plasminogen were mixed in a buffer system in the presence of plasmin substrate (S2251), there was a mutual interaction resulting in the generation of plasmin after a delay.

Lys-plasminogen was considerably more reactive to pro-UK (scu-PA) than was Glu-plasminogen. The latter reaction had a longer delay phase and did not reach linearity after 50 minutes, whereas the reaction with Lys-plasminogen reached linearity within 15 minutes. The linear phase of the curve indicates that the plasminogen has been fully activated. In the presence of lysine (25 mmol/L), an acceleration of the activation of Glu-plasminogen by pro-UK (scu-PA) occurred, resulting in the two substrates reacting equivalently. A slight delay in the activation of Lys-plasminogen was seen due to the inhibitory effect of lysine (Fig 9).

Clot lysis by pro-UK (scu-PA) (200 IU/mL) in plasma reconstituted with Lys-plasminogen started rapidly and then slowed without going to completion, resembling the clot lysis curve of activated pro-UK (scu-PA), shown in Fig 3. Moreover, a clot lysis in the presence of Lys-plasminogen was associated with a loss of 90% of the plasma plasminogen and conversion of most of the pro-UK (scu-PA) to two-chain UK, as shown in autoradiographs of a radiolabeled pro-UK (scu-PA) probe incubated in the same plasma. In the matched plasma containing Glu-plasminogen, clot lysis followed the dynamics characteristic of pro-UK (scu-PA) and was not associated with plasminogen loss or activation of pro-UK (scu-PA) (Fig 10).

In control tubes without clots, pro-UK (scu-PA) in Lys-plasminogen–reconstituted plasma induced comparable plasminogen consumption and two-chain UK generation as with a clot. Therefore, in the presence of Lys-plasminogen, the fibrin clot selectivity of the proteolytic effect of pro-UK (scu-PA) in plasma was lost. A comparable nonspecific...
PRO-UK–FIBRIN SELECTIVE MECHANISM

Fig 10. Plots of clot lysis by pro-UK (scu-PA) (200 IU/mL) in the presence of Glu- (×—×) or Lys- (○—○) plasminogen–reconstituted plasma. Autoradiographs of the radiolabeled pro-UK (scu-PA) probe show its composition at baseline (B.L.) and after five hours' incubation in Glu- (G) or Lys- (L) plasminogen/plasma. The single-chain pro-UK (scu-PA) and the heavy and light chains of UK are the major bands shown. The UK contamination at B.L. is due to degradation of pro-UK (scu-PA) during radiolabeling. The concentration of plasminogen (Plg) expressed as a percentage of B.L. at three hours in Glu- (solid line bar) or Lys- (broken-line bar) plasminogen/plasma is shown.

plasminogen activation by pro-UK (scu-PA) occurred when lysine (1, 5, or 25 mmol/L) was added to normal plasma. At 25 mmol/L lysine, a 95% depletion of plasminogen took place in three hours (similar to that in Fig 10).

Effect of divalent cations on pro-UK (scu-PA)-induced clot lysis and on its selective fibrinolytic effect. Increasing concentrations (1 to 10 mmol/L) of EDTA added to serum caused a concentration-dependent potentiation of clot lysis by a small concentration of pro-UK (scu-PA) (15 IU/mL), with only 10% clot lysis in six hours in the absence of EDTA and 100% clot lysis with 10 mmol/L EDTA. A similar potentiation of clot lysis by pro-UK was found with UK. However, with pro-UK (scu-PA), the addition of EDTA considerably reduced its fibrin specificity. This potentiating effect on clot lysis at the cost of selectivity could also be induced by EDTA (Ethylene glycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, Sigma, St Louis) or high concentrations of citrate.

Similar findings were obtained when divalent cations were removed from plasma or serum by dialysis. Reconstituting the dialyzed serum (or plasma) with divalent cations (M++) other than Ca++ had little effect. When Ca++ (5 mmol/L) was added to dialyzed serum, clot lysis was restored to predialysis kinetics (Fig 11) and nonselective plasminogen activation that occurred in the absence of Ca++ was inhibited and selectivity was restored (Fig 12).

Conversion during clotting. Partial conversion of radio-labeled pro-UK (scu-PA) to two-chain UK was seen during clotting, the extent of which varied with the conditions. Without additions, the clotting of native plasma in uncoated glass tubes caused 37% conversion; with thrombin added, 37%; and with thromboplastin, 41%. When clotting of native plasma was stimulated by kaolin, 60% conversion to UK was seen. The addition of kaolin to citrated plasma containing pro-UK (scu-PA) induced 16% conversion, whereas no conversion was seen in the absence of kaolin under these conditions.

DISCUSSION

The persistence of amidolytic activity in pro-UK (scu-PA), after treatment with sufficient DFP to inactivate trace UK contamination, is consistent with our earlier finding that 14C-DFP could be incorporated into the molecule. A reaction with DFP, indicative of an available active site, has also been found in other zymogens, such as trypsinogen, and is not incompatible with its classification as a proenzyme.

The intrinsic activity of pro-UK (scu-PA) was found to be sufficient to activate its principal substrate in buffer or on a fibrin plate. Pro-UK was far more reactive with the Lys-form of plasminogen than with the Glu-form. Such a difference in reactivity is not unprecedented, as it has also been reported for UK. The relative resistance of Glu-plasminogen to activation by pro-UK (scu-PA) was overcome by the addi-

Fig 11. Effect of divalent cations on clot lysis by pro-UK (scu-PA) (300 IU/mL). Clot lysis plots in dialyzed serum (●—●), dialyzed serum + M++ (○—○), dialyzed serum + M++ + 5 mmol/L Ca++ (△—△), and in control serum (■—■).

Fig 12. Effect of Ca++ on plasmin generation (enzyme units per milliliter) by pro-UK (scu-PA) (300 IU/mL) after incubation of pro-UK (scu-PA) in dialyzed serum (○—○), dialyzed plasma (□—□), dialyzed serum + Ca++ (△—△), control serum (●—●), and control plasma (X—X).
tation of lysine to the reaction mixture. This effect of lysine can be explained by its interaction with a lysine binding site on Glu-plasminogen, which induces a conformational change, making Glu-plasminogen functionally similar to Lys-plasminogen.\(^\text{19,20}\)

On a fibrin plate, the specific activity of pro-UK (scu-PA) is variable and not subject to standardizable interpretation. For DFP-treated pro-UK (scu-PA), the specific activity was equivalent to about 25% of that of UK, and in the presence of 3% to 10% UK contamination, the specific activity was 40% to 60% of UK. The plasminogen concentration also affects the results obtained by this assay.\(^4\) The final result is largely determined by the extent of pro-UK (scu-PA) activation by plasmin. It is therefore recommended that the fibrin plate assay of pro-UK (scu-PA) be used only as a measure of latent units, ie, after its full activation by plasmin, which gave a specific activity of about 100,000 IU/mg.

In plasma, pro-UK (scu-PA) fibrinolytic capacity was stable for several days, as previously shown,\(^4\) and, unlike UK, did not form SDS-stable inhibitor complexes. At concentrations of \(\geq 250\) IU/mL, pro-UK (scu-PA) did not activate native plasminogen, in contrast to the activation seen in a purified system, indicating that its intrinsic activity was neutralized by plasma inhibitors. When lysine was added or when native Glu-plasminogen in plasma was replaced by Lys-plasminogen, plasmin generation by pro-UK (scu-PA) occurred.

The depletion of divalent cations by the addition of EDTA or by dialysis reduced the threshold at which activation of native plasminogen by pro-UK (scu-PA) took place in plasma. This effect was shown to be attributable specifically to \(\text{Ca}^{++}\), as reconstitution of dialyzed serum by \(\text{Ca}^{++}\), and not by other divalent cations, restored baseline conditions.

The addition of a fibrin clot to plasma containing pro-UK (scu-PA) triggered the activation of plasminogen. Plasmin generation was not detectable in the ambient plasma at lower concentrations of pro-UK (scu-PA) (\(\leq 250\) IU/mL). At higher plasma concentrations of pro-UK (scu-PA), the addition of a clot initiated detectable plasmin generation, fibrinogenolysis, and pro-UK (scu-PA) activation. Pro-UK (scu-PA) conversion to UK was not found when plasminogen-depleted plasma was used, indicating that pro-UK (scu-PA) activation was induced by plasmin. At still higher concentrations of pro-UK (scu-PA) (\(>500\) IU/mL), native plasminogen activation and fibrinogenolysis occurred in plasma spontaneously, but these reactions were augmented by the addition of a fibrin clot.

When Glu-plasminogen was replaced by Lys-plasminogen in plasma, the fibrin specificity of clot lysis by pro-UK (scu-PA) was lost. Nonselective plasminogen activation occurred at low concentrations of pro-UK (scu-PA). In contrast to normal plasma, clot lysis was accompanied by a major conversion of pro-UK (scu-PA) to two-chain UK and by fibrinogenolysis. The clot lysis curve in plasma reconstituted with Lys-plasminogen resembled that seen with UK, having a rapid onset and then arresting before lysis was completed. Clot lysis by pro-UK (scu-PA) characteristically starts slowly, accelerates, and goes to completion if sufficient time is given.\(^4\) Nonselective activation of plasminogen by pro-UK (scu-PA), similar to that seen in the presence of Lys-plasminogen, was also seen when lysine was added to normal plasma.

These results in plasma parallel the findings in the purified system of a preferential activation of Lys-plasminogen over Glu-plasminogen or of a “pseudo-Lys” form induced by binding of Glu-plasminogen to a lysine residue. Glu-plasminogen is similarly bound to a lysine residue on fibrin,\(^21\) resulting in a comparable conformational change. The selective activation of fibrin-bound plasminogen by pro-UK (scu-PA) can therefore be explained on this basis. Once plasminogen is activated on the fibrin surface, plasmin will induce modifications in fibrin that expose additional plasminogen binding sites.\(^22\) By this mechanism, more substrate can become available for activation by pro-UK (scu-PA), perhaps explaining the accelerated clot lysis that follows a slow initial phase, characteristic of pro-UK (scu-PA)-induced clot lysis.

Determination of the kinetics of the pro-UK–Glu-plasminogen interaction is complicated by the effect of the reaction product (plasmin) on both “enzyme” (pro-UK) (scu-PA) and substrate (Glu-plasminogen). The result is a generation of UK and Lys-plasminogen that must be scrupulously prevented, since even trace amounts will augment plasmin formation considerably. The problem of maintaining the integrity of the reactants during this reaction has thwarted efforts to determine the true kinetics of pro-UK (scu-PA) itself with undegraded native plasminogen. However, the present findings demonstrate that Glu- and Lys-plasminogen have different reaction susceptibilities to pro-UK (scu-PA).

Fibrin binding of pro-UK (scu-PA) has not been demonstrated in citrate plasma, although it was the property of fibrin affinity that was responsible for the first isolation, purification, and characterization of pro-UK (scu-PA).\(^13\) This discrepancy may have an explanation in the recent finding by Husain in our laboratory that the fibrin affinity of pro-UK (scu-PA) is dependent on \(\text{Zn}^{++}\).\(^20\) In our model system, \(\text{Zn}^{++}\) is largely chelated by citrate, whereas urine, the original source of pro-UK (scu-PA),\(^13\) is relatively rich in \(\text{Zn}^{++}\). In any event, the present findings concern those elements of the mechanism of pro-UK’s fibrin selectivity that are independent of direct fibrin binding.

Little conversion of pro-UK (scu-PA) to UK (\(<10%\)) during clot lysis was demonstrated. Moreover, the fibrinolytic effect was fully preserved for several days during the serial lysis of six clots, showing a remarkable conservation not only of pro-UK (scu-PA), but also of plasminogen. These findings suggest that under the experimental conditions, lysis may have been maintained by pro-UK (scu-PA) itself, although some contribution by its activated derivative cannot be excluded, since generation of a small amount of UK was seen. Under physiologic conditions, at the low concentration at which pro-UK (scu-PA) is found in blood (5 to 10 \(\mu\)g/L),\(^24\) pro-UK (scu-PA) activation is more apt to be a precondition for clot lysis. Stimulation of the contact phase of coagulation was shown to activate pro-UK (scu-PA) and may help to trigger a mutual activation of pro-UK (scu-PA) and plasminogen, as previously suggested.\(^4\) The increased susceptibil-
ity of fibrin-bound plasminogen to activation by pro-UK (scu-PA) or by UK, as well as any local conditions that favor fibrin binding of pro-UK (scu-PA), should help to promote the fibrin surface as a locus of this reaction.

In conclusion, pro-UK (scu-PA), under the experimental conditions, maintained clot lysis that was accompanied by little (<10%) conversion to UK. The fibrin specificity of pro-UK (scu-PA) in plasma was shown to be related to a selective activation of fibrin-bound plasminogen (pseud-Lys), a mechanism that is independent of the fibrin-binding property of pro-UK (scu-PA). The resistance of plasminogen in the ambient plasma to activation is attributed to its Glu-form and the stabilizing effect of plasma inhibitors and Ca++.

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REFERENCES

Pro-urokinase: a study of its stability in plasma and of a mechanism for its selective fibrinolytic effect

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