Activation of Plasminogen by Single-Chain Urokinase or by Two-Chain Urokinase—A Demonstration That Single-Chain Urokinase Has a Low Catalytic Activity (Pro-Urokinase)

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Single-chain urokinase (SC-UK) has an intrinsic amidolytic activity, as measured with synthetic substrate (Kabi S-2444; pyro-Glu-Gly-Arg-pNitroanalide), which was found to be 0.1% to 0.2% that of its plasmin-activated derivative, two-chain UK (TC-UK). A study of the reaction of SC-UK with plasminogen is complicated by the effect of the reaction product, plasmin, on both reactants. The resultant generation of TC-UK and Lys-plasminogen produces secondary reactions which greatly augment plasminogen activation. To confine enzymatic activity to the primary reaction, after pretreatment to eliminate trace TC-UK contaminants, SC-UK was incubated with Glu- or Lys-plasminogen in the presence of aprotinin (500 KIU/mL) to inhibit generated plasmin and dansyl-glutamyl-glycyl-arginyl-chloromethylketone (20 μmol/L), which irreversibly inhibited TC-UK but not SC-UK. Analysis by reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed a plasminogen-activating activity for SC-UK that was ~0.4% that of TC-UK. Both SC-UK and TC-UK preferentially activated Lys-plasminogen over Glu-plasminogen. Similarly, Glu-plasminogen activation was augmented by lysine or soluble fibrin. The ratio of the reaction rates of SC-UK and TC-UK were comparable for Glu- and Lys-plasminogen. It is concluded that there is a major difference in the catalytic activities of SC-UK and TC-UK against plasminogen that is comparable to that against synthetic substrate.

Materials and Methods

Materials. Single-chain UK (>99% single chain; mol wt 55,000), purified from the culture medium of a human kidney tumor cell line, was provided by Collaborative Research (Lexington, MA). Two-chain UK (mol wt 55,000) was a product of Green Cross (Osaka, Japan). The WHO International Reference Preparation of UK, 66/46 (IRP-UK) was used as the activity standard (obtained from the National Institute for Biological Standards and Control, London). Amidolytic substrate (S-2444) was obtained from Kabi Vitrum (Stockholm, Sweden), aprotinin from FBA Pharmaceuticals (New York), dns-GGA-ck from Calbiochem (San Diego), DFP from Sigma Chemicals (St Louis), and “soluble” fibrin (Desafib) from American Diagnostics (Greenwich, CT).

Glu-plasminogen was purified from DFP-treated fresh plasma essentially by the method of Castellino and Powell. Lys-plasminogen was prepared from purified Glu-plasminogen by the method of Lucas and co-workers. Plasmin was prepared by activation of plasminogen (2.5 μmol/L) at pH 8.8 with either an equal volume of a 10% suspension of immobilized UK (Affi-Gel 15; Bio Rad, Richmond, CA) or an equal volume of Streptokinase (10,000 IU/mL) for 30 minutes at 37°C. Amidolytic activity of SC-UK or TC-UK. Amidolytic activity was measured with Kabi substrate S-2444 (pyro-Glu-Gly-Arg-pNitroanalide) (0.75 mmol/L) at 37°C. The reaction buffer was 0.1 mol/L of Tris-HCl (pH 8.8), 0.1 mol/L of NaCl, and 0.1 mg/mL of bovine serum albumin (BSA). One enzyme unit (EU) was defined as...
the amount of enzyme producing a change in absorbance (405 nmol) of 1 absorbance unit each minute. When assayed in this system, the IRP-UK had an activity of 275 EU/100,000 IU.

The activity of SC-UK was expressed in latent international units per milliliter representing the activity after plasmin activation. Activation of SC-UK to TC-UK was accomplished by incubation with an equal volume of plasmin (0.25 μmol/mL) for 30 to 45 minutes (37°C). The amidolytic assay of the resultant TC-UK was performed in the presence of aprotinin (25 to 50 KIU/mL) to eliminate further plasmin action. The activated form of SC-UK was found to be comparable to TC-UK (~275 EU/mg).

*SDS-PAGE.* SDS-PAGE was performed by the method of Laemmli9 using a 10% polyacrylamide gel. Samples were prepared with 10 mmol/L of dithiothreitol and stained with Coomassie Brilliant Blue G.

*DFP treatment.* Single-chain UK and plasminogen were treated with 1 mmol/L of DFP in 0.1 mol/L of sodium phosphate (pH 7.2), and 0.1 mol/L of NaCl. Incubation was carried out on ice for 18 hours. Under these conditions, TC-UK was totally inactivated, but full recovery of the latent activity of SC-UK was obtained when assayed after decay of the DFP. For preparative use, the treated product was dialyzed against 10 mmol/L of sodium acetate (pH 4.8) and 0.15 mol/L of NaCl and stored frozen.

*Stability of SC-UK to dns-GGA-ck.* At a concentration of 20 μmol/L of dns-GGA-ck, complete and irreversible inhibition of TC-UK was obtained within 30 minutes at 37°C or at room temperature. To test the stability of SC-UK to this inhibitor, SC-UK (0.1 mg/mL) was incubated with 100 μmol/L of dns-GGA-ck for 2 hours at 37°C in 0.2 mol/L of HEPES (pH 7.4), 0.3 mol/L of NaCl, 0.1% Triton X-100 (Sigma, St Louis). The solution was then diluted to 0.025 mg/mL of SC-UK with a buffer of 0.2 mol/L of Tris (pH 8.8), 0.3 mol/L of NaCl and 0.2 mg/mL of BSA, and was allowed to incubate further at room temperature for 16 hours. Under the latter condition, dns-GGA-ck has been found to decay completely. The treated SC-UK and a control sample treated without dns-GGA-ck were then activated with plasmin and assayed with S-2444.

*Evaluation of the influence of hydrolyzed dns-GGA-ck on the intrinsic activity of SC-UK.* To test the effect of the tripeptide of dns-GGA-ck as a potential competitive inhibitor independent of noncompetitive inhibition by the reactive species, a hydrolysate-inactivated sample of the inhibitor was prepared and assayed. Dns-GGA-ck was inactivated by hydrolysis by dilution in 0.1 mol/L of Tris (pH 8.8), 0.3 mol/L of NaCl and 0.2 mg/mL of BSA, and storage at room temperature for 3 days. The resulting product was assayed as a potential competitive inhibitor against both TC-UK and the inherent activity of SC-UK, using the amidolytic assay. The substrate tripeptide is identical with the tripeptide of dns-GGA-ck. The substrate was at an assay concentration of 0.75 mmol/L; the hydrolyzed inhibitor was tested up to 50 μmol/L. No significant inhibition was observed with either SC-UK or TC-UK.

*Plasminogen degradation by SC-UK or TC-UK.* Glu-plasminogen or Lys-plasminogen (0.23 mg/mL) and either SC-UK (5,000, 10,000, or 20,000 IU/mL) or TC-UK (5, 10, or 20 IU/mL) were incubated (37°C) in a reaction medium comprising 0.2 mol/L of HEPES-HCl (pH 7.4), 0.3 mol/L of NaCl, 0.01% Triton X-100, and aprotinin (500 KIU/mL). SC-UK was pretreated with the dns-GGA-ck (80 μmol/L at room temperature for 30 minutes in the reaction buffer) prior to dilution into the assay to eliminate residual TC-UK activity, and dns-GGA-ck (20 μmol/L) was present in the reaction mixture for SC-UK (but not for TC-UK). At 1 and 2 hours, reactions were stopped by dilution of aliquots with SDS sample buffer (reducing).

To test the effect of occupancy of the lysine binding sites of Glu-plasminogen on its susceptibility to SC-UK, the reaction was run as described above for 2 hours but with the addition of either 0.25 mg/mL of soluble fibrin or 25 mg/mL of lysine. A concentration of 5,000 IU/mL SC-UK or 25 IU/mL of TC-UK was used.

**RESULTS**

*Effect of inhibitors on the amidolytic activities of SC-UK and TC-UK.* Preparations of SC-UK were found to have a low but variable specific activity against the synthetic substrate S-2444 as expressed by the ratio of activities before and after plasmin treatment. The variability in this measurement could be attributed to even very small contamination with TC-UK. When preparations were pretreated with DFP (1 mmol/L), sufficient to abolish the activity of the contaminant, a more consistent result was obtained, which showed that SC-UK had an inhibitor resistant activity corresponding to 0.1% to 0.2% of its latent activity as previously observed.15

A similar result was obtained with the more specific inhibitor dns-GGA-ck. Treatment with 20 μmol/L of dns-GGA-ck completely inactivated TC-UK, whereas after provision for decay of the inhibitor by hydrolysis (described in the Methods section), the plasmin activatable activity of SC-UK was at least 97% recoverable.

*Effects of inhibitors on the reaction of SC-UK with plasminogen.* In mixtures of SC-UK with plasminogen (whether Glu or Lys), there was rapid activation of the plasminogen and conversion of SC-UK to the two chains of TC-UK, indicating that TC-UK was being generated and possibly was dominating the reaction. When aprotinin (100 KIU/mL) and dns-GGA-ck (20 μmol/L) were included in the reaction mixture, the activation of plasminogen was greatly decreased even though electrophoretic analysis revealed that significant traces of SC-UK were still being converted to TC-UK. When the concentration of aprotinin was increased to 500 KIU/mL, however, the secondary reaction of SC-UK activation appeared to be prevented.

*Comparison of the activation of Glu-plasminogen by SC-UK and TC-UK.* Under these conditions limiting the secondary reactions, the activity of SC-UK was quantitatively compared with that of TC-UK. With both forms of UK, there was a concentration- and time-dependent generation of the heavy (mol wt 67,000) and light (mol wt 25,000) chains of plasmin (Figs 1A and B). Densitometric scanning of the gels showed that the plasmin produced by 10,000 IU/mL of SC-UK in 1 hour was approximately equal to that which occurred with 20 IU/mL of UC in 2 hours. Thus, a ratio of ~250 was calculated, ie, SC-UK was 0.4% as active as TC-UK against plasminogen. This ratio was comparable to its activity against synthetic substrate (0.1% to 0.2%).

*Comparison of the activations of Lys-plasminogen and Glu-plasminogen.* The Lys-plasminogen was substituted for Glu-plasminogen in the above reaction mixtures, the rate of conversion by both activators was increased, as shown in Figs 2A and B, representing 1 and 2 hours of incubation, respectively. A similar differential between the activities of TC-UK and SC-UK was seen with Lys- as with Glu-plasminogen (5,000 IU/mL of SC-UK × 1 hour was comparable to 10 IU/mL of TC-UK × 2 hours, or a ratio of ~250).
Effect of lysine and of soluble fibrin on Glu-plasminogen activation. When lysine or soluble fibrin was used as a modifier of Glu-plasminogen, the observed increased susceptibility of Lys-plasminogen to activation was found to extend to this conformationally modified form. Quantitation by densitometric scanning of the gel shown in Fig 3 showed that whereas 5,000 IU/mL of SC-UK activated <15% of Glu-plasminogen in 2 hours, the inclusion of 25 mmol/L of lysine enhanced activation to ~45%. Soluble fibrin at 0.25 mg/mL gave a significant but smaller enhancement to ~30% conversion.

DISCUSSION

When plasminogen activation was measured under conditions designed to isolate the reaction inherent to SC-UK, a consistent magnitude of discrepancy was observed between SC-UK and TC-UK in their abilities to activate either Glu-plasminogen or Lys-plasminogen. The activity of SC-UK was ~0.4% that of TC-UK, a differential comparable to their respective rates of hydrolysis of synthetic substrate S-2444. These findings are compatible with other zymogenic characteristics of SC-UK, such as its relative resistance to
PLASMINOGEN ACTIVATION BY SC-UK

![Image of a gel electrophoresis diagram showing SC-UK and TC-UK bands.](image-url)

Fig 3. Reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of mixtures of Glu-plasminogen incubated (37 °C) for 2 hours with single-chain urokinase (SC-UK) (5,000 IU/mL) (1) alone, (2) plus lysine, and (3) plus soluble fibrin; or two-chain urokinase (TC-UK) (25 IU/mL) (1) alone, (2) plus lysine, and (3) plus soluble fibrin; (a) soluble fibrin marker. In lanes 2 and 3, the appearance of the heavy and light chains of plasmin are seen at mol wts 87,000 and 25,000, respectively.

inhibitors like DFP or dns-GGA-ck, and its stability in plasma.9

The observance of such a differential between the reactivities of TC-UK and of SC-UK with the physiological substrate, Glu-plasminogen, is at variance, however, with the report of Collen and colleagues10 in which the activity of SC-UK was stated to have a catalytic efficiency comparable to that of TC-UK. The discrepancy must be evaluated in terms of the methods of analysis used and particularly the measures taken to eliminate side reactions, principally the generation of TC-UK. These authors used the competitive influence of the synthetic plasmin substrate itself (S2251 at 1 mmol/L) to inhibit the action of plasmin on SC-UK at a concentration that is four times the Km, but that occupies plasmin only to the extent of 80% of its Vmax (calculated from Michaelis-Menten equation). Therefore, plasmin inhibition may not have been complete. Moreover, the product of this substrate, bearing a carboxy-terminal lysine residue, has the potential to modify Glu-plasminogen by occupying the lysine binding site which promotes its activatability either by UK16 or by pro-UK (Fig 3). Ultimately, the confirmation of one view or the other must await the application of additional experimental approaches to the problem of isolating the primary reaction.

Evidence for a low inherent activity of SC-UK has also been observed in other systems. Preparations free of TC-UK contamination induce fibrinolysis on a standard fibrin plate and in a model plasma clot lysis system.7,8 We have proposed that this inherent activity of SC-UK may constitute a trigger that leads to plasmin generation on the clot surface primarily by way of the positive feedback of the secondary reaction of TC-UK production.7,17 The side reaction that is triggered by plasmin activation of SC-UK, posing methodologic complications, probably serves as a useful physiologic amplifier, greatly increasing the rate of localized plasmin generation.

Although the intrinsic activity of the SC-UK is not incompatible with its proenzyme designation,18 the magnitude of this activity is relatively large. Therefore, SC-UK may be seen as occurring somewhere along a spectrum defined by an absolutely inactive zymogen at one extreme and the fully activated enzymatic form, here represented by TC-UK, at the other extreme. The appropriate place for SC-UK along this spectrum has been defined here only in qualitative terms. Quantitative appraisal of that position has been thwarted by the methodologic problems of measuring the kinetics of the primary reaction of SC-UK and Glu-plasminogen in functional isolation.

With Lys-plasminogen as substrate, plasmin generation was significantly more rapid with either activator, although the ratio of their reaction rates remained comparable. Similarly, a potentiation of plasmin generation occurred when lysine or soluble fibrin was added to Glu-plasminogen reaction mixtures. This effect of lysine or soluble fibrin is related to the conformational change induced in Glu-plasminogen by occupancy of the fibrin/lysine binding site(s).19 We recently proposed that the preferential activation of fibrin-bound plasminogen is an important element of the mechanism responsible for the fibrin specificity of SC-UK.15 A preferential activation of Lys-plasminogen over Glu-plasminogen has previously been reported for TC-UK.19

In conclusion, under conditions in which the primary reaction inherent to SC-UK was isolated and compared with that of TC-UK, a major difference was consistently observed between their respective activities, whether as hydrolysis of the synthetic substrate S-2444, reaction with inhibitors such as DFP or dns-GGA-ck, or proteolytic activation of either Glu-plasminogen or Lys-plasminogen.

REFERENCES


Activation of plasminogen by single-chain urokinase or by two-chain urokinase--a demonstration that single-chain urokinase has a low catalytic activity (pro-urokinase)

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