The Kinetics of Plasminogen Activation by Thrombin-Cleaved Pro-Urokinase and Promotion of its Activity by Fibrin Fragment E-2 and by Tissue Plasminogen Activator

By Jian-ning Liu and Victor Gurewich

Thrombin hydrolyzes the Arg156-Phe157 bond in pro-urokinase (pro-UK), two residues from the activation site, generating a two-chain form (thromb-UK) believed to have little activity and that is resistant to plasmin activation. The kinetic constants for thromb-UK against synthetic substrate (S2444) were found to be essentially identical to pro-UK. Against native plasminogen, thromb-UK had a lower Michaelis constant ($K_m$) and a higher (2-fold) catalytic efficiency. However, this difference with pro-UK was nullified by carboxypeptidase B (CpB) treatment of thromb-UK to remove the C-terminal arginine on the A-chain. Plasminogen activation by thromb-UK was substantially promoted by fibrin fragment E-2 but not by other fibrin derivatives, a phenomenon previously observed with pro-UK. Similarly, clot lysis by thromb-UK was promoted by tissue plasminogen activator because their combined effect was synergistic. Fibrinogenolysis in plasma occurred at 80-fold the concentration of thromb-UK as pro-UK, reflecting the 90-fold greater plasmin resistance of thromb-UK. Addition of a CpB inhibitor to the plasma enhanced fibrinogenolysis by thromb-UK and pro-UK by $\approx 16\%$, consistent with the promotion of both forms by certain C-terminal lysines. In conclusion, CpB-thromb-UK corresponds functionally to a plasmin resistant form of pro-UK, indicating that the catalytic site of the single-chain pro-UK is unaffected by thrombin cleavage. The effect of CpB indicates that the C-terminal Arg of thromb-UK slightly enhances its affinity for plasminogen. Thromb-UK has potential plasminogen-activating activity at surfaces where C-terminal lysines, functionally comparable to fragment E-2, are found.

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Materials and Methods

Materials

Pro-UK, purified from Escherichia coli, was obtained from Farmitalia Carlo Erba (Milan, Italy). It contained less than 1% two-chain UK and was further purified by treatment with disopropylfluorophosphate (DFP) as previously described. A plasin-insensitive mutant form of recombinant pro-UK (r-pro-UK), constructed by site-directed mutagenesis of Lys-158 $\rightarrow$ Ala-158 (A-pro-UK) from
Chinese hamster ovary cells, was obtained from Collaborative Research Inc (Bedford, MA). Two-chain, molecular weight (Mr) 54,000 UK was obtained from Green Cross (Osaka, Japan). Single-chain t-PA was obtained from Genentech (San Francisco, CA). The concentration of UK or pro-UK was standardized with the International Reference Preparation (National Institute for Biological Standards and Controls). For this purpose, the pro-UK was first converted to UK by incubation with plasmin as previously described. The concentrations of the A-pro-UK and thromb-UK were determined from Chinese hamster ovary cells, was obtained from Collaborative Research Inc. The preparation of thromb-UK was then treated with 5.0 mmol/L DFP at 4°C overnight to irreversibly inactivate thrombin and trace UK in the preparation followed by concentration and exhaustive dialysis against 0.02 mol/L NaAc, 0.15 mol/L NaCl, and 0.01% Tween 80 (pH 4.8). The final preparation was examined by 10% SDS-PAGE by the method of Laemmli.

CpB Treatment of Thromb-UK (CpB-Thromb-UK)

Thromb-UK (75 μmol/L) was dissolved in 0.05 mol/L sodium phosphate, 0.15 mol/L NaCl, and 0.01 mol/L ZnCl₂ (pH 7.4), and incubated (37°C) with immobilized CpB (32 mg/mL, including the bead weight) for 6 hours to remove the A-chain C-terminal Arginine residue. The reaction was stopped by removing the immobilized CpB by centrifugation. A small portion of sample was precipitated with 10% trichloroacetic acid (TCA), and its supernatant was subjected to amino acid analysis, which showed that more than 90% of the C-terminal Arg was removed by the CpB treatment. Finally, CpB-thromb-UK was exhaustively dialyzed against 0.02 mol/L NaAc, 0.15 mol/L NaCl, and 0.01% Tween 80 (pH 4.8).

Kinetic Analysis of the Hydrolysis of S2444 and the Activation of Glu-Plasminogen

S2444 hydrolysis. UK (4.0 nmol/L) or 1.0 μmol/L of pro-UK, A-pro-UK, thromb-UK, or CpB-thromb-UK was incubated with a range of concentrations (0.03, 0.06, 0.12, 0.18, 0.24, 0.3, 0.6, 1.2, 1.8, and 2.4 mmol/L) of S2444 in 0.05 mol/L sodium phosphate, 0.15 mol/L NaCl, 0.2% bovine serum albumin (BSA), and 0.01% Tween 80 (pH 7.8) at room temperature. The reaction rate was measured by the linear optical density (OD) increase with time at 410 nm against a reference wavelength of 490 nm (410/490 nm) on a microtiter plate reader. The Michaelis constant (Kₘ) and catalytic rate constant (kcat) were determined from Lineweaver-Burk plots with a computerized program (Enzfitter; Elsevier-Biosoft, Cambridge, UK).

Glu-plasminogen activation. Time-absorbance curves of Glu-plasminogen activation were obtained by measuring the OD increase of the reaction mixture with time at the selected wave length 410 nm and reference wave length 490 nm (410/490 nm) on a microtiter plate reader (Dynatech MR 9000; Dynatech Laboratories, Inc, Alexandria, VA). The reaction mixture contained S2251 (1.5 mmol/L), Glu-plasminogen (1.0, 1.5, 2.5, 3.5, 4.5, 5.5, 7.5, and 10.0 μmol/L), and UK (0.2 nmol/L) or A-pro-UK, thromb-UK, and CpB-thromb-UK (5.0 mmol/L). The reagents were made up in 0.05 mol/L sodium phosphate, 0.15 mol/L NaCl, 0.2% BSA, 0.01% Tween-80, pH 7.8, and incubated at room temperature. The measurement of Glu-plasminogen activation by UK, A-pro-UK, thromb-UK, or CpB-thromb-UK by chromogenic substrate S2251 can be described by the following two reactions because they were plasmin resistant:

\[
\text{Plasminogen} + \text{UK} \rightarrow \text{Plasmin}
\]

<table>
<thead>
<tr>
<th>Reaction</th>
<th>OD Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>UK</td>
<td>2.0</td>
</tr>
<tr>
<td>A-pro-UK</td>
<td>1.5</td>
</tr>
<tr>
<td>thromb-UK</td>
<td>1.0</td>
</tr>
<tr>
<td>CpB-thromb-UK</td>
<td>0.5</td>
</tr>
</tbody>
</table>

The time-absorbance curves of these reactions were plotted from the assay described above. The reaction rate was then determined directly by curve fitting using the integrated rate equation [1], as previously described.

Preparation of Thromb-UK

Pro-UK (1.0 mg/mL) was incubated with 15 μg/mL purified human thrombin in 0.05 mol/L Tris-HCl, 0.038 mol/L NaCl, and 0.01% Tween 80 buffer, pH 7.4, at 37°C for 20 hours. The reaction was terminated by the addition of 1.5-fold excess of hirudin. The active two-chain UK contaminant in the preparation was removed by passage through an affinity column of p-aminobenzamidine-sepharose. The preparation of thromb-UK was then treated with 5.0 mmol/L DFP at 4°C overnight to irreversibly inactivate thrombin and trace UK in the preparation followed by concentration and exhaustive dialysis against 0.02 mol/L NaAc, 0.15 mol/L NaCl, and 0.01% Tween 80 (pH 4.8). The final preparation was examined by 10% SDS-PAGE by the method of Laemmli.
A(t) = V_i \cdot t^2 + B

Where $V_i$ is the reaction rate and $B$ the background (tu absorbance).

Curve fitting was performed on early time points, before depletion of substrates became limiting or significant UK was generated from the activation of thromb-UK and CpB-thromb-UK, using the Enz-fitter program. The $K_M$ and $k_{cat}$ were determined from Lineweaver-Burk plots with the same computer program.

A-pro-UK was used as a surrogate for pro-UK to avoid interference by UK generation during the reaction. No UK generation from thromb-UK was detectable in these experiments as determined by synthetic substrate (S2444).

**Promotion of Plasminogen Activation by Fibrin Fragment E-2 and Other Fibrin Derivatives**

The promotion by fibrinogen, FCB-2, Desafib, D-dimer, and fragment E-2 on Glu-plasminogen activation by thromb-UK, CpB-thromb-UK, and A-pro-UK was determined by measuring the OD increase with time in the reaction mixture at 410 nm against a reference wavelength of 490 nm (410/490 nm) on the microtiter plate reader. The reaction mixture contained 1.5 mmol/L of u-PA (thromb-UK, CpB-thromb-UK, or A-pro-UK) in 0.05 mol/L sodium phosphate, 0.15 mol/L NaCl, 0.2% BSA, 0.01% Tween 80, pH 7.8, at room temperature. The rate of the reaction was obtained directly by curve fitting using the integrated rate equation [1]. The $K_M$ and $k_{cat}$ were determined from Lineweaver-Burk plots with the computer program Enzfitter.

**Plasmin Sensitivity of Thromb-UK—A Kinetic Study of Thromb-UK Activation by Lys-Plasmin**

A range of concentrations of pro-UK or thromb-UK (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 2.5, 3.5, and 5.0 μmol/L) was incubated with Lys-plasmin (0.1 mmol/L) in the presence of 1.2 mmol/L S2444 in 0.05 mol/L Tris-HCl, 0.15 mol/L NaCl, 0.01 mol/L CaCl$_2$, and 0.01% Tween 80, pH 7.4, at room temperature over time. The same rate of concentrations of pro-UK or thromb-UK without plasmin was incubated with S2444 as control. The 0.1 mmol/L plasmin had no direct effect on S2444 hydrolysis in the experimental condition. The amount of UK generated was measured by the OD increase with time at 410 nm against a reference wavelength of 490 nm (410/490 nm) on a microtiter plate reader (MR 5000; Dynatech Laboratories). Because pro-UK or thromb-UK activation by plasmin can be described as a two-order reaction by the following two reactions,

\[
\text{r-pro-UK or thromb-UK} \rightarrow \text{UK}
\]

the reaction rates can be determined directly by curve fitting using the integrated rate equation [1]. Curve fitting was performed on early time points after subtracting minor OD contribution from pro-UK or thromb-UK alone, and before depletion of substrates became significant. The $K_M$ and $k_{cat}$ were determined from Lineweaver-Burk plots with the computer program Enzfitter. Because generation of the N-terminal Ile$_{59}$ during thromb-UK activation by plasmin was previously established, N-terminal analysis of thromb-UK was not repeated.

In the above sections, each experiment was performed in triplicate. At the end of the reaction, less than 5% Glu-plasminogen had been consumed and Lys-plasminogen was undetectable by acid urea gel electrophoresis.

**Plasma Fibrinogenolytic Activity**

Thromb-UK (0 to 100 μg/mL) or pro-UK (0 to 10 μg/mL) was incubated in 1.0 mL of pooled bank plasma at 37°C for 6, 16, and 24 hours in the presence and absence of CpB (0.95 μmol/L), after which 0.2 mL of aprotinin (10,000 KIU/mL) was added and the plasma fibrinogen remaining was measured by the thrombin-clotting method.

**Fibrin Clot Lysis**

$^{125}$I-labeled clots prepared from 0.25 mL plasma were prepared as previously described. Clot lysis experiments were performed in 3 mL plasma with a range of concentrations of thromb-UK (10, 20, 30, 40, 70, and 80 μg/mL) or t-PA (5, 10, 30, 50, 75, 100, and 150 ng/mL) and certain combinations of t-PA and thromb-UK. Lysis was quantitated from the release of radioactivity and expressed as a percent of the value at complete lysis against time.

In the above two sections, each experiment was performed in triplicate.

RESULTS

**SDS-PAGE**

Pro-UK, thromb-UK, or CpB-thromb-UK were single, comigrating bands (≈45 Kd) in the unreduced gel. The UK, being glycosylated, migrated slightly higher. After reduction, thromb-UK, CpB-thromb-UK, and UK migrated in the expected two bands, as previously described.

**Kinetic Analysis of the Hydrolysis of S2444 by Thromb-UK, CpB-thromb-UK, Pro-UK, A-pro-UK, and UK**

The $K_M$ of thromb-UK (51 μmol/L) was similar to those of pro-UK or A-pro-UK (56 μmol/L) and CpB-thromb-UK (60 μmol/L), but was about 1.7-fold lower than that of UK (86 μmol/L) (Table 1). In addition, the $k_{cat}$ of thromb-UK was comparable to those of pro-UK, A-pro-UK, and CpB-thromb-UK, but was not 1.341-fold lower than that of UK. Therefore, the catalytic efficiency ($k_{cat}/K_M$) of thromb-UK against S2444 was 0.13% that of UK, comparable to those of pro-UK or A-pro-UK and CpB-thromb-UK, which were 0.1% and 0.11% that of UK, respectively (Table 1).

**Kinetic Analysis of Glu-Plasminogen Activation by Thromb-UK, CpB-thromb-UK, Pro-UK, A-pro-UK, and UK**

By contrast to S2444, the $K_M$ of thromb-UK (5.7 μmol/L) was significantly lower than that of A-pro-UK (8.5 μmol/L) and its catalytic efficiency ($k_{cat}/K_M$) against Glu-plasminogen was twice as high (Table 2). Thromb-UK activity was 1.0% that of UK, whereas it was 0.5% for A-pro-UK. However, after CpB treatment, the CpB-thromb-UK catalytic efficiency was reduced and became comparable to that of A-pro-UK (3.0 and 2.6, respectively; Table 2).

**Promotion of Plasminogen Activation by Fibrin Fragment E-2 and Other Fibrin Analogues**

In the presence of 5.0 μmol/L of fibrinogen, FCB-2, Desafib, or D-dimer, no significant promotion was seen in thromb-UK, CpB-thromb-UK, or A-pro-UK induced plasminogen activation (data not shown). By contrast, in the presence of 5.0 μmol/L of fibrin fragment E-2, a 21.35-, 34.10-, and 38.92-fold promotion (F*) was obtained in plas-
minogen activation by thromb-UK, CpB-thromb-UK, and A-pro-UK, respectively (Table 3). Promotion by fragment E-2 was largely due to a 15 to 20-fold increase in $k_{\text{cat}}$ compared with the value in buffer because the $K_M$ of the reactions was reduced less than twofold by fragment E-2. The $\approx$twofold difference ($F''$) in the catalytic efficiencies ($k_{\text{cat}}/K_M$) of thromb-UK (5.4) and A-pro-UK (2.6) found in buffer was attenuated by the presence of fragment E-2 ($F'''$), which caused the catalytic efficiencies to become more comparable (11.5, 102.3, and 101.2 for thromb-UK, CpB-thromb-UK, and A-pro-UK, respectively; Table 3). This suggests that the C-terminal lysines in fragment E-2 interfered with the interaction between a plasminogen kringle and the C-terminal arginine on the A-chain of thromb-UK, which is believed to account for twofold higher activity of thromb-UK compared with CpB-thromb-UK. The latter had a catalytic efficiency equivalent to that of A-pro-UK in both buffer (2.6 and 3.0) and in the presence of fragment E-2 (101 and 102, respectively; Table 3).

**Plasmin Sensitivity of Thromb-UK**

Thromb-UK was 90.7-fold less sensitive to plasmin activation than pro-UK (Table 4). This difference was entirely due to a 128-fold lower $k_{\text{cat}}$ because the $K_M$ of thromb-UK (2.98 μmol/L) was, in fact, lower than that of pro-UK (4.22 μmol/L). This higher affinity of thromb-UK for plasmin is consistent with an interaction between the C-terminal arginine of thromb-UK and a plasmin kringle.

**Fibrinogenolysis in Plasma**

Thromb-UK was far more inactive than pro-UK in plasma, as reflected in its ability to induce fibrinogen degradation. The incubation (37°C) of 80 pg/mL thromb-UK induced essentially no fibrinogen degradation in 6 hours. By contrast, 4 µg/mL of pro-UK induced greater than 80% fibrinogen degradation under the same conditions. When incubation was extended to 24 hours, a similar difference between thromb-UK and pro-UK was observed (Fig 1).

Table 1. Catalytic Activities of the u-PAs (UK, A-pro-UK, pro-UK, thromb-UK, and CpB-thromb-UK) Against Synthetic Substrate (S2444)

<table>
<thead>
<tr>
<th></th>
<th>UK</th>
<th>A–pro-UK</th>
<th>Thromb-UK</th>
<th>CpB–thromb-UK</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_M$ (μmol/L)</td>
<td>86 ± 16</td>
<td>56 ± 6</td>
<td>51 ± 10</td>
<td>60 ± 9</td>
</tr>
<tr>
<td>$k_{\text{cat}}$ (10$^6$ A/min · nmol/L)</td>
<td>1.809 ± 71</td>
<td>1.0 ± 0.2</td>
<td>1.2 ± 0.5</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>$k_{\text{cat}}/K_M$</td>
<td>18.71</td>
<td>0.0178</td>
<td>0.0235</td>
<td>0.0200</td>
</tr>
<tr>
<td>$F^*$ (%)</td>
<td>100</td>
<td>0.10</td>
<td>0.13</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Abbreviation: $F^*$, ($k_{\text{cat}}$, uK/$K_M$, uK)/($k_{\text{cat}}$, uK/$K_M$), the catalytic efficiency relative to that of UK.

The presence of CpBI enhanced fibrinogen degradation by both thromb-UK (60 µg/mL) and pro-UK (2 µg/mL) to a similar extent, being 15.6% ± 2.7% and 16.9% ± 4.4%, respectively, compared with controls in the same plasma without CpBI.

**Fibrin Clot Lysis**

The minimum dose required for 100% clot lysis by t-PA alone within 6 hours was 150 ng/mL (Fig 2), whereas for thromb-UK alone it was 80 µg/mL (Fig 3). When combined, 30 ng/mL t-PA plus 30 µg/mL thromb-UK or 20 ng/mL t-PA plus 40 µg/mL thromb-UK induced equivalent clot lysis to that induced by either activator alone (Fig 4). From this, the sum of their fractional doses was calculated to be 0.57 (30/80 + 30/150) and 0.63 (40/80 + 20/150). These findings are consistent with synergy by the criteria of Berenbaum because the sum of the fractional doses was less than 1.23

No significant (<10%) fibrinogen loss occurred in association with fibrinolysis in any of these experiments.

**DISCUSSION**

In the present study, the catalytic and fibrinolytic properties of thromb-UK were characterized and compared with those of single-chain pro-UK and a plasmin-insensitive Lys158 mutant pro-UK (A–pro-UK), a surrogate for pro-UK. The mutant was used only for those experiments in which UK generation during the reactions would have interfered with the measurements. The findings indicated that two-chain thromb-UK had properties that mimicked those of pro-UK. The catalytic constants of thromb-UK against synthetic substrate were comparable to those of pro-UK or A–pro-UK (Table 1). Against Glu-plasminogen, thromb-UK shared the exceptional property of pro-UK, previously reported,24 of a significantly lower $K_M$ (8.5 μmol/L) than that of the active enzyme, UK (42.5 μmol/L). In fact, thromb-UK had a lower $K_M$ (5.7 μmol/L) than pro-UK, but this increased (to 10.5 μmol/L) after CpB treatment (Table 2). The similarity in the catalytic activities of CpB–thromb-UK and A–pro-UK in-

Table 2. Catalytic Activities of the u-PAs Against Glu-Plasminogen

<table>
<thead>
<tr>
<th></th>
<th>UK</th>
<th>A–pro-UK</th>
<th>Thromb-UK</th>
<th>CpB–thromb-UK</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_M$ (μmol/L)</td>
<td>42.51 ± 2.41</td>
<td>8.60 ± 1.13</td>
<td>5.70 ± 0.90</td>
<td>10.53 ± 3.15</td>
</tr>
<tr>
<td>$k_{\text{cat}}$ (10$^6$ A/min² · nmol/L)</td>
<td>21.340 ± 1,440</td>
<td>22 ± 2</td>
<td>31 ± 3</td>
<td>32 ± 7</td>
</tr>
<tr>
<td>$k_{\text{cat}}/K_M$</td>
<td>502</td>
<td>2.6</td>
<td>5.4</td>
<td>3.0</td>
</tr>
<tr>
<td>$F^*$ (%)</td>
<td>100</td>
<td>0.5</td>
<td>1.0</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Abbreviation: $F^*$, ($k_{\text{cat}}$, uK/$K_M$, uK)/($k_{\text{cat}}$, uK/$K_M$), the catalytic efficiency relative to that of UK.
dicates that the latent catalytic site of pro-UK is unaffected by cleavage of the Arg156-Phe157 bond, by contrast to the major effect that is induced when the Lys158-Ile159 bond, the activation site, is cleaved.

These findings suggest that the amino-terminal Ile 159 on the B-chain of UK is required for the stabilization of the catalytic site and expression of its full activity. Furthermore, cleavage of the Ile159-Ile160 bond by elastase generates a two-chain UK without activity. This further suggests that the full length of the B-chain is needed to form the salt bridge between the N-terminal Ile159 and the active domain Asp354.

The finding that CpB treatment of thromb-UK abrogated its twofold higher activity against plasminogen by lowering the affinity (K_M) is consistent with an interaction between the N-terminal Ile159 and the active domain Asp354. The fragment E-2 induced promotion of native (Glu-) plasminogen activation by CpB-thromb-UK, similar to that of A-pro-UK (39-fold), was found in the present study (Table 3). Promotion by thromb-UK was a little less (21-fold), probably due to its higher baseline activity. Only a single concentration (5.0 μmol/L) of fragment E-2 was used to make it equivalent to that of the other fibrin(ogen) derivatives (FCB-2, Desafib, and D-dimer) that precipitate at higher concentrations. As a result, the promotional factor was less than previously reported for A-pro-UK.12 A 34-fold promotion of plasminogen activation by CpB-thromb-UK and promotion was even greater when the ratio of fragment E-2: Glu-plasminogen was increased.

The fragment E-2 induced promotion of native (Glu-) plasminogen activation by A-pro-UK was shown to give it an activity similar to that of UK, indicating that pro-UK has a potentially optimal catalytic activity without undergoing proteolytic activation. The present study suggests that thromb-UK has a similar potential catalytic activity against plasminogen despite its resistance to activation by plasmin. Because thromb-UK had about 1% the activity of UK against fibrin(ogen) derivatives. Because of the selectivity and magnitude of this promotion, this phenomenon helps to explain the mechanism responsible for the fibrin specificity of pro-UK.12 A 34-fold promotion of plasminogen activation by CpB-thromb-UK, similar to that of A-pro-UK (39-fold), was found in the present study (Table 3). Promotion by thromb-UK was a little less (21-fold), probably due to its higher baseline activity. Only a single concentration (5.0 μmol/L) of fragment E-2 was used to make it equivalent to that of the other fibrin(ogen) derivatives (FCB-2, Desafib, and D-dimer) that precipitate at higher concentrations. As a result, the promotional factor was less than previously reported for A-pro-UK.12 A 34-fold promotion of plasminogen activation by CpB-thromb-UK and promotion was even greater when the ratio of fragment E-2: Glu-plasminogen was increased.

The fragment E-2 induced promotion of native (Glu-) plasminogen activation by A-pro-UK was shown to give it an activity similar to that of UK,13 indicating that pro-UK has a potentially optimal catalytic activity without undergoing proteolytic activation. The present study suggests that thromb-UK has a similar potential catalytic activity against plasminogen despite its resistance to activation by plasmin. Because thromb-UK had about 1% the activity of UK against plasmin.

\[ K_M (\mu M) \]

<table>
<thead>
<tr>
<th></th>
<th>A-pro-UK</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>k_cat (A/min · nmol/L)</td>
<td>4.23 ± 1.12</td>
<td>8.50 ± 1.13</td>
</tr>
<tr>
<td>k_cat/K_M</td>
<td>101.2</td>
<td>2.6</td>
</tr>
<tr>
<td>F*</td>
<td>38.92</td>
<td>1.0</td>
</tr>
<tr>
<td>F**</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>F***</td>
<td>---</td>
<td>2.08</td>
</tr>
</tbody>
</table>

Abbreviations: F*, (k_cat&A-pro-UK)/k_cat&UK; F**, (k_cat&A-pro-UK)/k_cat&A-pro-UK; F***, (k_cat&A-pro-UK)/k_cat&A-pro-UK, the catalytic efficiency relative to that of A-pro-UK in buffer.

### Table 4. Kinetics of Plasmin Activation of Thromb-UK and Pro-UK

<table>
<thead>
<tr>
<th></th>
<th>Thromb-UK</th>
<th>Pro-UK</th>
</tr>
</thead>
<tbody>
<tr>
<td>K_M(μM)</td>
<td>2.98 ± 0.25</td>
<td>4.22 ± 0.94</td>
</tr>
<tr>
<td>k_cat (10^6 A/min · nmol/L)</td>
<td>300.3 ± 16.0</td>
<td>38,600 ± 1,100</td>
</tr>
<tr>
<td>k_cat/K_M</td>
<td>100.8</td>
<td>9,146.9</td>
</tr>
<tr>
<td>F*</td>
<td>1</td>
<td>90.7</td>
</tr>
</tbody>
</table>

Abbreviation: F*, (k_cat&Thromb-UK)/k_cat&Pro-UK; (k_cat&Pro-UK)/k_cat&Thromb-UK, the catalytic efficiency relative to that of thromb-UK.
minogen, a 100-fold promotion by fragment E-2 would give it an activity equivalent to UK (which is not significantly promoted by fragment E-2). Therefore, when one molecule of thromb-UK meets one molecule of fragment E-2-bound Glu-plasminogen, it has the potential to activate this Glu-plasminogen with the same efficiency as UK. At sites where C-terminal lysines corresponding to those in fragment E are found, thromb-UK may be an effective plasminogen activator.

Fragment E is the plasminogen-binding region of fibrin fragment Y and is believed to be present in partially degraded fibrin. In addition, it is possible that certain cell surface lysines that bind plasminogen may correspond to the three C-terminal lysines of fragment E-2. For example, Manchanda and Schwartz showed that plasminogen activation by a plasmin-insensitive Lys158 pro-UK mutant was enhanced 100-fold on a monocyte cell surface. Because the thrombomodulin:thrombin complex and heparin sulfate both accelerate the generation of thromb-UK, the endothelial surface is probably the cell surface where most thromb-UK will be generated.

It was previously proposed that a corollary to the fragment E-2 promotion of plasminogen activation by pro-UK was synergy with t-PA, because the latter was complementary in its plasminogen activation, being promoted not by fragment E-2 but rather by the fragment D region of fibrin. Therefore, the present finding that plasminogen activation by thromb-UK was promoted only by fragment E-2 and was synergistic in clot lysis with t-PA is in support of this concept. The clot lysis experiments, in fact, paralleled those previously obtained with a Lys158 mutant pro-UK, whose activity was similarly promoted by t-PA. The sum of the fractional concentrations of t-PA and the Lys158 mutant, which were needed to induce a fibrinolytic effect comparable to that of either activator alone, was 0.41 to 0.75. The synergy found in the present study was similar in extent. Thromb-UK induced complete clot lysis within 6 hours at a concentration of 80 μg/mL, more than 100-fold greater than that previously found to be required for pro-UK under similar conditions. However, in the presence of t-PA, thromb-UK was considerably more active, and the sum of their fractional doses (t-PA [20 to 30 ng/mL] and thromb-UK [20 to 30 ng/mL]) was 0.57 to 0.63, indicative of synergy as generally defined.

The finding that thromb-UK was synergistic with t-PA suggests that synergy is related to the mode of action of pro-UK itself, as previously suggested, rather than being due to a potentiation of pro-UK activation by the presence of t-PA. Although UK generation from thromb-UK was not specifically measured in the present study, it was unlikely due to its 90-fold greater resistance to plasmin (Table 4). Moreover, the extent of synergy observed with thromb-UK was comparable to that seen with the plasmin-insensitive Lys158 mutant and was significantly greater than that obtained with pro-UK. Only with the latter is local UK generation known to occur during fibrinolysis. Therefore, in the presence of pro-UK activation, synergy appears to be blunted rather than augmented.

The promotion of thromb-UK activity by a small amount of t-PA may help explain the reported finding that the fibrinolytic activity of thromb-UK was about 50% that of pro-UK in vivo, whereas it was only 2% of pro-UK in vitro in a plasma milieu. In vivo, the endogenous t-PA of the experimental
animal may have replicated the promotional effect of t-PA seen in the present in vitro clot lysis experiments.

Fibrinogenolysis in plasma occurred at \( \approx 80 \)-fold the concentration of thromb-UK as pro-UK, reflecting the \( \approx 90 \)-fold greater plasma resistance of thromb-UK (Table 4). The greater stability of thromb-UK in plasma can be attributed to its plasmin resistance and is consistent with the belief that the nonspecific effects of pro-UK seen during thrombolysis are related to systemic UK generation. In these experiments, fibrinogenolysis by thromb-UK and pro-UK was enhanced by about 16% and 17% in the presence of an inhibitor of CpB, suggesting that plasma CpB was active despite the presence of citrate. The inhibiting effect of plasma CpB was presumably related to removing certain relevant C-terminal arginines or lysines such as the A-chain C-terminal arginine of thromb-UK and the C-terminal lysine from UK. These findings in a plasma milieu therefore appear to mirror the effects of CpB on plasminogen activation by thromb-UK or UK in purified systems.

In conclusion, hydrolysis of the Arg156-Phe157 bond in pro-UK, two residues from the activation site, generates a two-chain derivative, thromb-UK, that has very similar catalytic properties to single-chain pro-UK. This indicates that the N-termi nal isoleucine on the B-chain of UK is important for stabilization of the catalytic site and full expression of activity. Similar to pro-UK, plasminogen activation by thromb-UK was promoted by fragment E-2 and thromb-UK was synergistic with t-PA in fibrinolysis. Therefore, at surfaces where C-terminal lysines functionally comparable to those on fibrin fragment E are found, thromb-UK may have significant plasminogen-activating activity.

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