Inactivation of Single-Chain Urokinase (Pro-urokinase) by Thrombin and Thrombin-like Enzymes: Relevance of the Findings to the Interpretation of Fibrin-Binding Experiments

By Victor Gurewich and Ralph Pannell

Whereas crude bovine thrombin activated single-chain urokinase-type plasminogen activator (scu-PA), otherwise called pro-urokinase (pro-UK), purified human thrombin converted pro-UK (scu-PA) to a two-chain form that had no amidolytic activity. The two chains (M, ~ 33,000 and 22,000) were disulfide linked and resistant to subsequent activation by plasmin. By contrast, thrombin did not inactivate tissue plasminogen activator or two-chain urokinase. The enzyme from snake venom Agkistrodon contortrix, relatively specific for fibrinopeptide B, had an effect similar to thrombin, whereas the enzyme from Agkistrodon rhodostoma (ancrod), specific for fibrinopeptide A, did not.

When pro-UK (scu-PA) was present during thrombin clotting of fibrinogen, degradation of 125I-pro-UK (scu-PA) in the clot supernatant was seen, whereas virtually full recovery (95%) of radioactivity was found. A loss of latent amidolytic activity in the clot supernatant was also found, the extent of which could be correlated with the degree of degradation of the radiolabeled probe. It was concluded that thrombin inactivation of pro-UK (scu-PA) accounts for the loss of amidolytic activity in the clot supernatant, which has been attributed to fibrin binding. Further confirmation was obtained from experiments in which ancrud was used as the clotting agent. Full recovery of both radioactivity and latent amidolytic activity of pro-UK (scu-PA) in the supernatant was obtained under these conditions. These findings indicate that thrombin may introduce an artifact in the results of certain experiments designed to study the fibrin affinity or fibrinolytic effect of pro-UK (scu-PA).

W E HAVE PREVIOUSLY REPORTED that, in contrast to the commonly used crude bovine thrombin preparation, Thrombostat (Parke Davis, Avon, CT), purified human thrombin did not activate single-chain urokinase-type plasminogen activator (scu-PA), otherwise called pro-urokinase (pro-UK). A contaminant in the crude bovine thrombin was considered responsible for its effect on pro-UK (scu-PA).

Recently Conforti et al reported that treatment of pro-UK (scu-PA) with purified thrombin inhibited the plasmin activity of pro-UK (scu-PA). On more systematic analysis we have found that purified human thrombin and one thrombin-like enzyme from snake venom degrade and inactivate pro-UK (scu-PA) but not tissue plasminogen activator (t-PA) or two-chain urokinase (TC-UK). The relevance of these findings to the interpretation of certain pro-UK/fibrin binding and clot lysis experiments is presented.

EXPERIMENTAL PROCEDURES

Materials. Pro-urokinase (99% single chain), purified from the culture medium of a transformed human kidney cell line was obtained from Collaborative Research, Lexington, MA. High mol wt TC-UK, originating from Green Cross (Osaka, Japan), was obtained as a gift from Dr Bleakley Chandler. Tissue plasminogen activator, predominantly single chain, was obtained from Integrated Genetics (Framingham, MA). Fibrinogen and amidolytic substrate (S-2444) were obtained from Kabi Vitrum (Stockholm, Sweden).

Highly purified human thrombin (1623 NIH U/mg) was a gift of Dr John Fenton to Dr Robert Weinstein of this department. Ancrod (from Agkistrodon rhodostoma venom) was obtained from Sigma (St. Louis). The thrombin inhibitor N-α-(2-naphthylsulfonyl-glycyl)-D.L-amidophenylalaninepiperidide (NAPAP) and the thrombin-like enzyme Contortrix, from the venom of Agkistrodon contortrix, were obtained from American Diagnostica (Greenwich, CT). Phenylalanyl-prolyl-arginyl-chloromethylketone (PPAcK) and bovine serum albumin, "purified" grade, were obtained from Calbiochem (San Diego, CA).

Plasminogen-free fibrinogen was prepared from Kabi fibrinogen by DFP (5 mmol/L) treatment followed by chromatography on Lysine-Sepharose™ equilibrated with 20 mmol/L trisodium citrate, 0.15 mol/L NaCl, and, finally, dialysis against 25 mmol/L HEPES, pH 7.4, 0.15 mol/L NaCl. The preparation was >99% clottable protein.

Radiolabeling. Pro-UK (scu-PA) and TC-UK were labeled with 125I via the lactoperoxidase reaction using immobilized lactoperoxidase/glucose oxidase (Enzymobeads, BioRad Laboratories) as previously described. The resulting specific activities were approximately 30 μCi/μg. Labeling was accompanied by some loss of enzymatic activity, but the labeled pro-UK could be fully cleaved by plasmin at a rate comparable to unleabeled pro-UK (scu-PA).

Amidolytic assay of pro-UK (scu-PA) or TC-UK. Amidolytic activity was measured with Kabi substrate S-2444 (0.75 mmol/L) at 37 °C. The reaction buffer was 0.1 mol/L Tris-HCl (pH 8.8), 0.1 mol/L NaCl and 0.1 mg/mL BSA. One enzyme unit (EU) was defined as the amount of enzyme producing a change in absorbance (405 nm) of 1 absorbance unit per minute. Activation of pro-UK (scu-PA) (0.45 μmol/L) to TC-UK was accomplished by incubation (37 °C) with plasmin (0.125 μmol/L) for 30 to 45 minutes. The amidolytic activity (latent activity) in the mixture was measured with the addition of aprotinin (200 KIU/mL) to the reaction buffer.

Treatment with thrombin or thrombin-like enzymes. The latent amidolytic activity of pro-UK (scu-PA) was measured at intervals after incubation (37 °C) at a concentration of 5,000 IU/mL (50 μg/mL) with thrombin (0.01 to 10 NIH U/mL). Similar experiments were carried out with TC-UK (5,000 IU/mL) and with t-PA (50 μg/mL).

The effect of the thrombin-like enzymes from Agkistrodon

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venoms on the amidolytic activity of pro-UK (scu-PA) were also examined in a similar manner: ancrod (specific for fibrinopeptide A), 5 5 U/mL; and Contortrix (relatively specific for fibrinopeptide B), 6, 200 mU/mL (chromogenic assay units as defined by the supplier). These concentrations gave clotting times of five to ten minutes under the conditions used in the fibrin clot-binding experiments.

The effect of thrombin inhibitors on the degradation of pro-UK (scu-PA) by thrombin was tested. Thrombin (10 NIH U/mL) was pretreated with PPAck (20 μmol/L) for one hour at 37 °C. The competitive inhibitor NAPAP was present at 10 μmol/L during the thrombin treatment of pro-UK (scu-PA). Aprotinin was tested at 500 KIU/mL present during the thrombin treatment.

The fate of pro-UK (scu-PA) during fibrin clot-binding experiments. Fibrin-clot-binding experiments were performed by an extension of the basic method described by Kasai et al. 7 In brief, pro-UK (scu-PA) or TC-UK (30 to 45 IU/mL) were added to a reaction mixture of plasminogen-free fibrinogen (2 mg/mL), 0.15 mol/L NaCl, 25 mmol/L HEPES (pH 7.4), 0.01% Tween 80, and 0.2% bovine serum albumin. The mixture was clotted with thrombin (1 NIH U/mL, final concentration) or ancrod (5 U/mL) and incubated (37 °C) for 15 minutes. The tubes were chilled on ice, vortexed, and centrifuged. The plasminogen activator activity was measured in the supernatant and compared with the activity in tubes without fibrinogen and tubes without thrombin. The average results from five experiments were calculated.

Additional experiments were performed with radiolabeled pro-UK (scu-PA) or TC-UK added to the above mixture. The supernatant and control tubes were analyzed by autoradiography of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as well as for radioactivity.

RESULTS

Thrombin inactivation of the latent activity of pro-UK (scu-PA). When pro-UK (scu-PA) was incubated with purified human thrombin, there was a time and thrombin concentration-dependent loss of the latent UK amidolytic activity (generated by subsequent plasmin treatment of the solution) as shown (Fig 1). Pro-UK (scu-PA) was completely protected by NAPAP when it was included in the incubation mixture; there was only 28% loss of activity when PPAck was used; and aprotinin had no effect on the reaction.

The amidolytic activities of TC-UK and of t-PA were unaffected by incubation with thrombin (10 NIH U/mL for 30 minutes).

Effect of ancrad or contortrix venom on the latent amidolytic activity of pro-UK (scu-PA). The latent amidolytic activity of pro-UK (scu-PA) was not affected by incubation (37 °C) with ancrad (5 U/mL) for 30 minutes. By contrast, Contortrix venom (200 MU/mL) induced a progressive loss of the latent amidolytic activity of pro-UK (scu-PA), which was comparable to that induced by thrombin. The venom had no effect on the amidolytic activities of TC-UK or t-PA.

Thrombin degradation of pro-UK (scu-PA). A mixture of pro-UK (scu-PA) (5,000 IU or 50 μg/mL) and thrombin (1 NIH U/mL) sampled at 10, 20, 30, 45 and 60 minutes for SDS-PAGE under reducing conditions, showed progressive degradation of single chain pro-UK (scu-PA) to two chains corresponding to about M, 33,000 and 22,000. The two chains appeared to migrate just slightly behind the heavy and light chains of the TC-UK marker. A relatively faint higher molecular weight contaminant is also seen (Fig 2).

When the appearance of thrombin-modified pro-UK (scu-PA) under reducing and nonreducing conditions was compared (Fig 3), the thrombin-modified pro-UK (scu-PA) migrated with a M, of 55,000 under nonreducing conditions (lane 2), indicating that the thrombin cleavage peptides were disulfide linked. The human thrombin appeared as a major and minor band (lane 3) and the pro-UK (scu-PA) as a single band under reducing conditions (lane 1). The higher mol wt contaminant seen in Figure 2 is again seen in the pro-UK-thrombin mixture under reducing conditions (lane 2, Fig 3).

Inactivation and degradation of pro-UK (scu-PA) during fibrin clotting. In the tubes containing buffer, thrombin, and pro-UK (scu-PA), a 49% (mean) loss from control tubes (without thrombin) of the latent amidolytic activity of pro-UK (scu-PA) was found, whereas a 27% (mean) loss was...
found in the fibrin clot supernatant. No significant change in the amidolytic activity of TC-UK was observed either in buffer or in the fibrin clot supernatant. A 95% recovery of radioactivity from $^{125}$I-pro-UK (scu-PA) and complete recovery of $^{125}$I-TC-UK in the clot supernatant were obtained (Table 1). Autoradiography of SDS-PAGE of incubation mixtures from this experiment showed degradation of $^{125}$I-pro-UK (scu-PA) during incubation with thrombin both with and without fibrinogen. Loss of the M, 55,000-band compared with baseline and appearance of a M, 33,000-band was seen (Fig 4). The degradation was greater in buffer (lanes 2 and 3) than in the fibrin clot supernatant (lanes 5 and 6). Samples from two experiments with pro-UK are shown to demonstrate the reproducibility of the findings (lanes 2, 3, 5, and 6). There was no change in the appearance of TC-UK (lanes 1 and 4) (Fig 4).

When ancrod (5 U/mL) was used instead of thrombin, there was no loss of either amidolytic activity or radioactivity in the fibrin clot supernatant.

<table>
<thead>
<tr>
<th>Table 1. Amidolytic Activity and Radioactivity After 15 Minutes Incubation (37 °C) with Thrombin (1 NIH U/mL) or after Thrombin Clotting</th>
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<tr>
<td>Pro-UK</td>
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<tr>
<td>Amidolytic Activity</td>
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<tr>
<td>EU/mL</td>
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<tr>
<td>Control</td>
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<tr>
<td>(100%)</td>
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<tr>
<td>Thrombin + Buffer</td>
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<tr>
<td>(51%)</td>
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<td>Clot Supernatant</td>
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DISCUSSION

This study demonstrates that the latent activity of pro-UK (scu-PA) but not the activities of TC-UK or of single chain t-PA, is inactivated progressively by physiologic concentrations of thrombin. Thrombin converted pro-UK (scu-PA) to a two-chain form that had no amidolytic activity. The two chains generated by thrombin migrated on SDS-PAGE in positions comparable to those of the light and heavy chains of TC-UK. This molecule was not susceptible to activation by plasmin. Conversely, TC-UK was not inactivated by thrombin. The thrombin cleavage site in pro-UK (scu-PA) was close to the plasmin cleavage site within the disulfide-linked domain of the molecule. Inactivation of pro-UK (scu-PA), comparable to that induced by thrombin, was also seen with the thrombin-like snake venom enzyme from Agkistrodon contortrix, relatively specific for fibrinopeptide B, but not with the one from Agkistrodon rhodostoma (ancrod), specific for fibrinopeptide A.

When pro-UK (scu-PA) was incubated with thrombin plus fibrinogen, there was a loss of latent activity in the clot supernatant relative to a buffer control without thrombin. But this loss of activity in the supernatant, previously attributed to fibrin binding, could be correlated with the degree of degradation seen by SDS-PAGE autoradiography. Further evidence that the pro-UK (scu-PA) did not bind to fibrin under these conditions was the full recovery of both counts from $^{125}$I-pro-UK (scu-PA) and plasmin-activatable amidolytic activity in the supernatant when ancrod was used instead of thrombin to clot fibrinogen. Thus in this experimental model, thrombin inactivation of pro-UK (scu-PA) introduces an artifact that may masquerade as fibrin binding.

Conversely, when fibrin affinity is determined by the recovery of UK-antigen after clotting, degradation of pro-UK (scu-PA) would obscure fibrin binding under experimental conditions in which the fibrin binding of pro-UK itself may occur. The sensitivity of pro-UK (scu-PA) to degrada-
tion by thrombin may also lead to an underestimation of its contribution to lysis when this is studied by measuring spontaneous fibrinolysis after thrombin clotting.10

The addition of fibrinogen attenuated the inactivation and degradation of pro-UK (scu-PA) by thrombin. This was observed in the fibrin clot-binding experimental model in which the extent of degradation found in the clot supernatant was about 45% less than that in the buffer control. This effect may be explained by competition of the substrate fibrinogen (6 μmol/L) with the substrate pro-UK (scu-PA) (9 nmol/L) for the active site of thrombin.

During the finalization of this manuscript, the cleavage of pro-UK (scu-PA) by thrombin at the Arg 156-Phe 157 bond, two residues from the activation site, was identified by Ichinose et al. 11 In view of this, it is noteworthy that cleavage at this site renders the Lys 158-Ile 159 bond not susceptible to activation by plasmin. Perhaps not surprisingly, cleavage within the disulfide-linked domain of pro-UK causes a major conformational change in this region, causing the susceptible bonds for plasmin and respectively for thrombin to become nonrecognizable.

This effect of thrombin explains an earlier observation that pro-UK (scu-PA), in contrast to UK or streptokinase, induces little endolysis when incorporated into a forming clot (unpublished), whereas pro-UK (scu-PA) induces exolysis of a preformed clot incubated in plasma very effectively. 1 Physiologically the inactivation of pro-UK (scu-PA) by thrombin may serve to maintain the integrity of a fresh clot by inhibiting endolysis. Tissue plasminogen activator and TC-UK, though resistant to thrombin, are inhibited by plasminogen activator inhibitor-1,12 whereas pro-UK is not.13 Therefore a separate mechanism appears to exist in forming clot to inactivate pro-UK (scu-PA) and to prevent the generation of TC-UK.

These findings are relevant to the interpretation of certain published studies but do not resolve the controversy regarding the fibrin binding of pro-UK (scu-PA). They only suggest that pro-UK (scu-PA) does not bind to fibrin under the present experimental conditions. Since a single chain form of UK was first identified in urine and isolated because of its specific binding to fibrin/Celite,14-16 a certain fibrin affinity for pro-UK (scu-PA) has been assumed. However, this property of pro-UK appears to be conditional, as suggested by the observation of Husain 17 that Zn2+ is a requirement. We have recently confirmed this Zn2+ dependence using fibrin-lined tubes pretreated with an antithrombin. Binding of pro-UK (scu-PA) to fibrin/Celite was also confirmed in this study and was shown to be potentiated by Zn2+, in contrast to TC-UK, which did not bind and was unaffected by Zn2+.18

In conclusion, the sensitivity of pro-UK (scu-PA) to degradation by thrombin is relevant to the interpretation of some experimental findings and probably is involved in modulating the biologic function of pro-UK (scu-PA).

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Inactivation of single-chain urokinase (pro-urokinase) by thrombin and thrombin-like enzymes: relevance of the findings to the interpretation of fibrin-binding experiments

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