Proteolytic Cleavage of Single-chain Prourokinase Induces Conformational Change Which Follows Activation of the Zymogen and Reduction of Its High Affinity for Fibrin*

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A plasminogen activator secreted from human kidney cells was highly purified by affinity chromatography on an anti-urokinase IgG-Sepharose column. The purified plasminogen activator was inactive and had a single-chain structure and a Mₐ of 50,000. It not only did not incorporate diisopropyl fluorophosphate, which reacts with active site serine residue in urokinase, but also did not bind to p-aminobenzamidine-immobilized CH-Sepharose, to which urokinase binds. The purified plasminogen activator was converted to the single-chain form by plasmin. The decrease in affinity for fibrin upon activation of single-chain pro-urokinase, which occurs during the conversion of single-chain pro-urokinase to the two-chain form by plasmin, was quenched completely by anti-urokinase IgG, which reacts with active site serine residue in urokinase. The results indicate that the plasminogen activator is an inactive proenzyme form of human urokinase. Therefore, the plasminogen activator was termed single-chain pro-urokinase. The cleavage of single-chain pro-urokinase by plasmin induced conformational change which followed the generation of reactive serine residue at active site, the increase enzyme activity and the reduction of its high affinity for fibrin. These findings suggest that conformational change occurs in both regions responsible for enzyme activity and affinity for fibrin upon activation of single-chain pro-urokinase.

EXPERIMENTAL PROCEDURES

Materials—CNBr-activated Sepharose 4B and CM-Sepharose C-50 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). L-Pyroglutamylglycyl-L-arginine-p-nitroanilide (S-2444) and Glu-

Plasminogen activators convert inactive proenzyme plasminogen to the active enzyme, plasmin (1). They have been classified as two immunologically distinct groups; urokinase-type and tissue-type plasminogen activators (t-PA)² (2). Urokinase isolated from human urine has been used clinically in thrombolytic therapy. Thrombolyis by urokinase is associated with systemic plasminogen activation, the degradation of fibrinogen and the coagulation proteins in circulating blood (3, 4). In contrast, t-PA produced by a human melanoma cell line has been shown to be a more specific and effective thrombolytic agent than urokinase because of its high affinity for fibrin and its thrombus-localized stimulation of plasminogen activator activity without the activation of systemic plasminogen and the degradation of plasma proteins (5–7).

The plasminogen activator produced by human kidney cells in culture has been reported to be identical with the urinary urokinase (8). But Bernik (9) has suggested the existence of a plasminogen activator as a proenzyme, whose activity could be enhanced by trypsin. Furthermore, Skriver et al. (10) recently purified the plasminogen activator released as an inactive proenzyme from murine cells transformed by sarcoma virus.

We also have succeeded in purification of an inactive proenzyme form of human urokinase, single-chain pro-urokinase. In the present study, characterization of single-chain pro-urokinase was investigated and it is suggested that changes in conformation, enzyme activity and affinity for fibrin take place during the conversion of single-chain pro-urokinase to the two-chain form by plasmin.

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The abbreviations used are: t-PA, tissue plasminogen activator; DFP, diisopropyl fluorophosphate; SDS, sodium dodecyl sulfate.

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bovine fibrinogen (Miles Laboratories, Inc.) was used and it contained plasminogen and a trace amount of plasmin that had no effect on fibrin lysis. Urinary urokinase was used as a standard in both assays.  

Plasminogen Cleavage Assay—Glu-plasminogen (250 µg/ml) containing aprotinin (1000 kallikrein-inactivating units/ml) was incubated in 0.1 M Tris-HCl containing 0.025% Triton X-100, pH 7.5, for 2 h at 37°C with single-chain pro-urokinase or the activated single-chain pro-urokinase (50 IU/ml) which had been preincubated with plasmin (0.4 µg/ml) for 1 h at 37°C. The reaction was stopped by boiling the mixture in 1% SDS and 1% β-mercaptoethanol and the samples were subjected to SDS-polyacrylamide gel electrophoresis on 10% gel.  

Inhibition of Fibrinolytic Activity by Antibodies—Single-chain pro-urokinase (100 IU/ml) was incubated with anti-urokinase IgG or anti-PA Ig (0.0122–200 µg/ml) for 2 h at 20°C in P/NaCl containing 0.2% bovine serum albumin. The residual activity was determined by the fibrin plate method.  

Affinity of Single-chain Pro-urokinase for p-Aminobenzamidine-immobilized CH-Sepharose 4B—Single-chain pro-urokinase (1 x 10^9 IU) or urokinase (1 x 10^8 IU) dissolved in 0.4 M NaCl, 0.1 M sodium phosphate buffer, pH 7.0, was applied to a 1.5 x 10 cm column of p-aminobenzamidine CH-Sepharose 4B. The column was washed with the same buffer and bound material was eluted with 0.4 M NaCl, 0.1 M sodium acetate buffer, pH 4.0 (14, 15).  

PHIDFP Labeling—Single-chain pro-urokinase and urokinase were labeled with [3H]DFP by the method of Vetterlein et al. (16) with slight modification. Samples (10 or 100 µg) were allowed to react with [3H]DFP (20 µCi) in 1 ml of P/NaCl, pH 7.4, containing 0.001% Tween 80 at room temperature for 16 h, and dialyzed against the same buffer at 4°C. The amount of radioactivity incorporated in protein was determined in a liquid scintillation counter.  

Binding to Fibrin Clot—Various amounts of single-chain pro-urokinase or urokinase were added to a reaction mixture containing 2 mg/ml of plasminogen-free fibrinogen, which had been purified by affinity chromatography on lysine-Sepharose (17), 0.1 M NaCl, 0.05 M Tris-HCl, 0.001% Tween 80, 0.2% bovine serum albumin, pH 7.2. The samples were clotted by adding thrombin (1 NIH units/ml, final concentration), after which they were incubated for 15 min at 37°C. The supernatant was separated by centrifugation (7, 18). The plasminogen activator activity in the supernatants was determined by the fibrin plate method. The extent of adsorption was calculated as the difference between the initial activity of the plasminogen activator and that present in the supernatant (7).  

Circular Dichroism—Circular dichroism (CD) measurement was achieved with a JASCO automatic recording spectropolarimeter, model J-20. SC spectra of single-chain pro-urokinase and urokinase were measured in 0.15 M NaCl, 0.01 M Tris-HCl, pH 7.3. CD spectrum of plasmin-treated single-chain pro-urokinase was measured after single-chain pro-urokinase had been incubated with plasmin (0.4 µg/ml) for 1 h at 37°C.  

SDS-Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed using the method of Laemmli (19). Molecular weight was determined by using phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), and trypsin inhibitor (20,100) as standards.

RESULTS

Purification of Plasminogen Activator—We observed that human kidney cells can be cultured for up to 2 months in serum-free medium supplemented with 0.1% human serum albumin with intervals of 2 or 3 days between medium changes and that under these conditions they maintain a steady release of plasminogen activator. The purified plasminogen activator in culture fluid was purified by ion-exchange and immunoaffinity chromatographies. The purified plasminogen activator showed only one single Coomassie Blue-stainable band with a Mr of 50,000 on SDS-polyacrylamide gel electrophoresis on 10% gel under reducing condition as well as nonreducing condition (Fig. 1), which indicates that this plasminogen activator has a single-chain structure.  

Stimulation of Plasminogen Activator Activity by Plasmin—The plasminogen activator activity can be determined by its amidolytic effect on the substrate S-2444 which is a chromogenic substrate for urokinase (20). The activity of purified plasminogen activator derived from the serum-free culture fluid could be increased 300-fold by pretreatment with catalytic amounts of plasmin which little effected on plasmin. After that present in the supernatant (7).

Effect of preincubation with plasmin on urokinase activity of single-chain pro-urokinase

<table>
<thead>
<tr>
<th>Plasminogen activator</th>
<th>Preincubation</th>
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<tr>
<td>µg/ml</td>
<td>µg/ml</td>
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<tr>
<td>Urokinase</td>
<td>plasmin</td>
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<td>32.0</td>
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*Urokinase activity.

### TABLE I

<table>
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<th>Plasminogen activator</th>
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*Urokinase activity.

Fig. 1. SDS-polyacrylamide gel electrophoresis on 10% gel of plasminogen activator from serum-free culture fluid. The protein bands were stained with Coomassie Blue. The lanes shown are: A, nonreduced CM-Sepharose eluate; B, nonreduced anti-urokinase IgG-Sepharose eluate; C, reduced CM-Sepharose eluate; D, reduced anti-urokinase IgG-Sepharose eluate.

Fig. 2. SDS-polyacrylamide gel electrophoresis showing cleavage of Glu-plasminogen by single-chain pro-urokinase pretreated with plasmin. Glu-plasminogen (A) was incubated with plasmin (B), single-chain pro-urokinase (C), or single-chain pro-urokinase pretreated with plasmin (D).
Activation of Single-chain Pro-urokinase

stimulation with plasmin, the specific activity of the plasminogen activator (1.5 × 10^5 IU/mg of protein) was found to be similar to that of urokinase (1.6 × 10^5 IU/mg of protein). Similarly, the plasminogen activator pretreated with catalytic amount of plasmin converted Glu-plasminogen, which is a natural substrate for urokinase and possesses a single-chain structure, into plasmin with a two-chain structure. However, the plasminogen activator could not induce the conversion (Fig. 2). Furthermore, the potential activity of the plasminogen activator was quenched completely by anti-urokinase IgG, but not by anti-t-PA Ig (Fig. 3).

Incorporation of $[^{3}H]$DFP into single-chain pro-urokinase

<table>
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<th>Plasminogen activator</th>
<th>Incorporated $[^{3}H]$DFP</th>
<th>Incorporated $[^{3}H]$DFP/Plasminogen activator</th>
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</thead>
<tbody>
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<td>Single-chain pro-urokinase</td>
<td>0.001</td>
<td>0.005</td>
</tr>
<tr>
<td>2</td>
<td>0.011</td>
<td>0.006</td>
</tr>
<tr>
<td>Urokinase</td>
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<td>0.170</td>
</tr>
<tr>
<td>2</td>
<td>0.405</td>
<td>0.203</td>
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</table>

The plasminogen activator was cleaved into characteristic urokinase A ($M_r = 20,000$) and B ($M_r = 33,000$) chains upon incubation with plasmin (Fig. 4).

These results indicate that the plasminogen activator secreted from human kidney cells in serum-free medium is an inactive proenzyme form of human urokinase. Therefore, this plasminogen activator was termed single-chain pro-urokinase.

*Fig. 3.* Inhibition of fibrinolytic activity by anti-urokinase IgG and anti-t-PA Ig. Single-chain pro-urokinase (100 IU/ml) was incubated with anti-urokinase IgG or anti-t-PA Ig (0.0122–200 pg/ml) for 2 h at 20 °C in P/NaCl containing 0.2% bovine serum albumin. The residual activity was measured by the fibrin plate method.

*Fig. 4.* Time course of conversion of single-chain pro-urokinase into the two-chain form by plasmin. Single-chain pro-urokinase (240 pg/ml) was incubated with plasmin (0.4 pg/ml) at 37 °C in 0.01 M Tris-HCl, 0.15 M NaCl, pH 7.3. At the indicated times, the reaction was stopped by the addition of aprotinin (1000 kallikrein-inactivating units/ml) and boiling in 1% SDS without (A) or with (B) 1% β-mercaptoethanol. The samples were subjected to SDS-polyacrylamide gel electrophoresis on 10% gel.

*Fig. 5.* Binding of single-chain pro-urokinase to fibrin clots. Single-chain pro-urokinase (●) or urokinase (○) were added to a reaction mixture containing 2 mg/ml of fibrinogen. The sample was clotted with thrombin then incubated for 15 min at 37 °C. The clot and supernatant were separated by centrifugation. The extent of binding was determined as the difference between the initial activity of the plasminogen activator and the activity present in the supernatant.

The plasminogen activator was cleaved into characteristic urokinase A ($M_r = 20,000$) and B ($M_r = 33,000$) chains upon incubation with plasmin (Fig. 4).

These results indicate that the plasminogen activator secreted from human kidney cells in serum-free medium is an inactive proenzyme form of human urokinase. Therefore, this plasminogen activator was termed single-chain pro-urokinase.

Incorporation of $[^{3}H]$DFP into Active Sites in Single-chain Pro-urokinase and Urokinase Molecules—DFP is a specific reagent for serine residue (21) and reacts with active site serine residue in urokinase forming a stable modified enzyme. Urokinase incorporated 34-fold more $[^{3}H]$DFP than single-chain pro-urokinase (Table II). This value is almost consistent with that of stimulation of the plasminogen activator activity (Table I). It seems that the cleavage of single-chain pro-urokinase by plasmin makes unreactive serine residue of the molecule highly reactive.

*p*-Aminobenzamidine-binding Sites Present in Single-chain Pro-urokinase and Urokinase—Urokinase binds to *p*-aminobenzamidine via its side-chain binding pocket present in active center (14, 15). Urokinase or single-chain pro-urokinase dissolved in 0.4 M NaCl, 0.1 M sodium phosphate buffer, pH
by adding thrombin. The affinity of single-chain pro-urokinase and urokinase. CD spectra of single-chain pro-urokinase and urokinase. CD spectra of plasmin-treated single-chain pro-urokinase (C) was measured after single-chain pro-urokinase had been incubated with plasmin (0.4 µg/ml) for 1 h at 37 °C. 7.0, was applied to the p-aminobenzamidine CH-Sepharose. Only 2% of the original activity of urokinase passed the column and the adsorbed activity was eluted by changing the buffer to 0.4 M NaCl, 0.1 M sodium acetate buffer, pH 4.0. The recovery of activity from the column was 80% of the original activity.

On the other hand, 98% of the original activity of single-chain pro-urokinase passed the column and only 0.1% of the original activity was eluted. These results indicate that p-aminobenzamidine-binding site present in active center are not functional in single-chain pro-urokinase.

Binding of Single-chain Pro-urokinase to Fibrin Clots—The fibrin-binding activities of single-chain pro-urokinase and urokinase were compared. Fibrinogen was mixed with single-chain pro-urokinase or urokinase, and fibrin clot was formed by adding thrombin. The affinity of single-chain pro-urokinase for fibrin was much higher than that of urokinase (Fig. 5).

Conformational Change upon Activation of Single-chain Pro-urokinase—Conformational change upon activation of single-chain pro-urokinase was investigated by CD. The CD spectra between 195 and 240 nm showed significant difference between single-chain pro-urokinase and urokinase (Fig. 6). On the other hand, the CD spectrum of the plasmin-treated single-chain pro-urokinase was almost identical with that of urokinase (Fig. 6). The change in CD spectra indicates the reduction of α helical and β contents upon the activation of single-chain pro-urokinase. Similar change in CD spectra was also observed upon activation of chymotrypsiaogen (22). It is therefore suggested that the secondary structures of single-chain pro-urokinase and urokinase are different and that the difference is due to conformational change upon the cleavage of single-chain pro-urokinase by plasmin.

**DISCUSSION**

The plasminogen activator produced by human kidney cells in culture has been reported that it was an active two-chain form with a Mₐ of 50,000 (23, 24), which was identical with urokinase produced in human urine (8). In those studies, the cells were maintained in serum-supplemented medium or serum-free medium for a long period of time (5-6 weeks) without medium change.

We have recently succeeded in cultivation of human kidney cells for up to 2 months in serum-free medium supplemented with 0.1% human serum albumin with intervals for 2 or 3 days between medium changes and in maintenance of a steady release of plasminogen activator under these conditions. The plasminogen activator in culture fluid was highly purified by affinity chromatography on an anti-urokinase IgG-Sepharose column. The plasminogen activator was inactive and had a single-chain structure and a Mₐ of 50,000. It was converted to the active two-chain form with the same Mₐ, by catalytic amounts of plasmin and its activity was increased 300-fold. The potential activity was quenched completely by anti-urokinase IgG, but not by anti-t-PA Ig. Furthermore, the plasminogen activator not only did not incorporate DFP, which reacts with active site serine residue in urokinase, but also did not bind to p-aminobenzamidine immobilized CH-Sepharose, to which urokinase binds via its side-chain binding pocket present in active center. These results indicate that the plasminogen activator is an inactive proenzyme form of human urokinase. Therefore, it was termed single-chain pro-urokinase.

The affinity of single-chain pro-urokinase for fibrin was much higher than that of urokinase. Plasminogen (25) and t-PA (26) have kringle domains that participate in their affinity for fibrin. Urokinase recently has been shown to have a kringle domain that has an extensive homology with the plasminogen and t-PA kringles (27). In confirmation of previous reports (28, 29) we found a low affinity of urokinase for fibrin in the study reported here. These results suggest that the kringle structure of single-chain pro-urokinase takes a functionally active form which is changed to the less active form during the conversion of single-chain pro-urokinase to the two-chain form.

The cleavage of single-chain pro-urokinase by plasmin induced conformational change which followed the generation of reactive serine residue at active site, the increase in enzyme activity, and the reduction of its high affinity for fibrin. These findings suggest that the cleavage of single-chain pro-urokinase induces conformational change in both regions responsible for enzyme activity and affinity for fibrin upon activation of single-chain pro-urokinase.

In vitro thrombolytic studies disclosed that single-chain pro-urokinase is a more specific and effective thrombolytic agent than urokinase and its thrombolytic properties are similar to those of t-PA (30). Single-chain pro-urokinase with its inactive proenzyme form and its high affinity for fibrin might be more advantageous than urokinase in thrombolytic therapy.

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**REFERENCES**

Activation of Single-chain Pro-urokinase