One-chain Urokinase-type Plasminogen Activator from Human Sarcoma Cells Is a Proenzyme with Little or No Intrinsic Activity*

(Received for publication, December 7, 1987)

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We have compared the plasminogen activating capacity of one- and two-chain urokinase-type plasminogen activator (u-PA). In a 125I-plasminogen conversion assay in the presence of high amounts of a plasmin inhibitor, one-chain u-PA pretreated with diisopropyl fluorophosphate had no detectable activity, the detection limit corresponding to the activity of a 400-fold lower amount of two-chain u-PA. In coupled assays in which generated plasmin was measured with a synthetic substrate, activity was clearly observed with the one-chain preparation, but the initial rate of plasminogen activation was lower than that of a 250-fold smaller concentration of two-chain u-PA. The coupled assays for one-chain u-PA are self-activating because plasmin catalyzes conversion of one- to two-chain u-PA, and it is not possible to decide whether the low activity of one-chain u-PA observed with this type of assay is intrinsic or due to contaminations. On the basis of these findings and a discussion of previous studies, it is concluded that one-chain u-PA has a variety of properties similar to the one-chain proenzyme forms of other serine proteases and that it should, therefore, be considered as a genuine proenzyme form of u-PA.

In all mammalian species so far investigated at least two pathways leading to plasminogen activation are known involving different plasminogen activators, the urokinase-type (u-PA), which among other functions is involved in tissue degradation and cell migration under normal and pathological conditions including cancer, and the tissue-type (t-PA), which is a key enzyme in thrombolysis (for reviews, see Refs. 1–8).

We have previously reported that u-PA is released from cultured murine (9) and human (10) cells as a one-chain proenzyme with little or no plasminogen-activating capacity as determined in a fibrinolytic assay and that this proenzyme, by limited proteolysis with catalytic amounts of plasmin, can be converted to its active two-chain counterpart. Similar findings have been reported by other groups (11–13). The zymogen nature of one-chain u-PA is further supported by studies showing that it has little or no reactivity with macro-molecular plasminogen activator inhibitors (11, 14–16) and the active-site reagent diisopropyl fluorophosphate (9, 10, 12, 17, 18) and that it has essentially no amidolytic activity with synthetic substrates (11, 13, 17–24). These results suggest that extracellular activation of pro-u-PA constitutes an important step in the regulation of the u-PA pathway of plasminogen activation (6, 25).

However, in contrast to the above findings, other investigators have reported that one-chain u-PA, both from natural sources and produced by recombinant techniques, has considerable intrinsic plasminogen-activating activity (but no amidolytic activity) (19–24, 26, 27). In one study, the activity of recombinant one-chain u-PA with plasminogen was found to be even higher than that of two-chain u-PA (26). We have, therefore, reinvestigated the kinetic properties of our one-chain u-PA preparations.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Bovine pancreatic trypsin inhibitor (BPTI; Kunitz inhibitor; Trasylo) was a kind gift from Bayer AG (Wuppertal, West Germany); fetal bovine serum and Dulbecco’s modified Eagle’s medium were obtained from Gibco; protein A-Sepharose and cyanogen bromide-activated Sepharose 4B were from Pharmacia LKB Biotechnology Inc.; L-kojiglutamyl-glycyl-t-arginine-p-nitroanilide [(Glu-Gly-Arg-pNA; S2444)] and H-o-valyl-t-phenyl-t-lysine-p-nitroanilide [(Val-Phe-Lys-pNA; S2390)] from Kabi; and Na125I (14.7 mCi/μg iodine) and [3H]DFP (4 Ci/mmol) from Amersham Corp.

Cell Culture—A cell line derived from a human fibrosarcoma (HT-1080) was a kind gift from Dr. A. Vaheri (Helsinki, Finland). The cell line was maintained as a monolayer culture and grown to confluency using Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 0.1 mg/liter BPTI. Culture fluid for purification of plasminogen activator was prepared under serum-free conditions as previously described (10), except that all buffers were supplemented with 0.1 mg/liter BPTI.

u-PA Preparations—One-chain u-PA was purified from conditioned cell culture media by a single pass of conditioned HT-1080 cell culture media over a Sepharose 4B column coupled with monoclonal mouse IgG anti-u-PA clone 2, essentially as described (10, 28); u-PA retained on the column after washing was eluted with 3 M NaSCN in 0.1 M Tris-HCl, pH 7.8, 0.1% Triton X-100. NH4SCN was removed by gel filtration on Sephadex G-25. One-chain u-PA (1 μM) was converted to two-chain u-PA by incubation at 37 °C for 30 min with 0.01 μM plasmin (10).

DFP Treatment of u-PA—Purified one-chain u-PA (0.5-2 μM) was incubated with 1 mM DFP for 2 h at 37 °C followed by further addition of 1 mM DFP and incubation for 2 h at 37 °C. The DFP-treated one-chain u-PA preparation was then passed over a 1-mL Sepharose column coupled with immobilized mouse monoclonal IgG anti-u-PA (1 mg/mL gel). The column was washed and eluted as described above. DFP-treated one-chain u-PA was stored at −80 °C. Labeling of one- and two-chain u-PA with [3H]DFP was done as described (29).
Proenzyme to Urokinase-type Plasminogen Activator

Plasminogen—Human Glu'-plasminogen was prepared from fresh human male plasma by a modification (30) of the method of Deutsch and Mertz (31). Plasmin was generated from plasminogen by incubation with urokinase in 50% glycerol (30). Lys'-plasminogen was prepared by incubation of Glu'-plasminogen (0.82 mg/ml) in min-light chain were calculated after correction for background radioactivity obtained by electrophoresis of the 125I-Glu'-plasminogen proenzyme by the use of a combination of monoclonal and polyclonal antibodies, as described (32). The concentration of Glu'- and Lys'-plasminogen was measured by spectrophotometry at 280 nm (E_{280}^\text{1%} = 16.8). SDS-PAGE was performed in a stacking system of slab gels, as described (33).

Protein Concentrations—Concentrations of u-PA were measured by an enzyme-linked immunosorbent assay for human u-PA and its proenzyme by the use of a combination of monoclonal and polyclonal antibodies, as described (32). The concentration of Glu'- and Lys'-plasminogen was measured by spectrophotometry at 280 nm (E_{280}^\text{1%} = 16.8). SDS-PAGE was performed in a stacking system of slab gels, as described (33).

**Assay I.** 125I-Glu'-Plasminogen Conversion—The principles of this assay have been described (30, 33). Human Glu'-plasminogen was labeled with 125I by the procedure of Helmkamp et al. (34) using a 4-fold molar excess of ICI relative to plasminogen. A specific activity of 0.5 μCi/μg plasminogen was obtained. For measurements of plasminogen activation, one- or two-chain u-PA (0.1–40 nM) was incubated with 0.01 μM 125I-plasminogen, 0.24 μM unlabeled plasminogen, and 1.3 mg/ml BPTI in 500 μl of 0.05 M Tris-HCl, pH 7.4, 0.04 M NaCl, 0.01% Tween 80. At various time intervals, aliquots of 50 μl were removed, and the reaction was stopped by addition of 50 μl of 0.08 M Tris-HCl, pH 6.8, 10% SDS. Unlabeled Glu'-plasminogen (5 μg) and plasmin (10 μg) were added as markers. In some experiments, one-chain u-PA was preincubated for 1 h with 1 μg/ml monoclonal mouse anti-human u-PA clone 2 IgG (28). Percentage conversion of 125I-plasminogen to 125I-plasmin was determined by SDS-PAGE. Under reducing conditions, followed by staining of the gel, sectioning of each lane into 1-mm slices, and determination of the radioactivity in each slice by γ spectrometry. The percentages of the total radioactivity that represented Glu'-plasminogen, plasmin-heavy chain, and plasmin-light chain were calculated after correction for background radioactivity obtained by electrophoresis of the 125I-Glu'-plasminogen preparation alone.

**Assay II.** Activation of Plasminogen in the Presence of Val-Phe-Lys-pNA—In principle, this assay was carried out as previously described (35–37). The reaction was performed at 37 °C in 0.04 M NaCl, 0.05 M Tris-HCl, pH 7.4, 0.01% Tween 80. The reaction mixture to which one- or two-chain u-PA was added contained 0.3 μM Glu'- or Lys'-plasminogen and 1 mM Val-Phe-Lys-pNA. The plasmin generated cleaves the peptidyl nitroanilide substrate, and the progress curve in formation of pNA is followed by spectrophotometry at 405 nm. During the assay, most of the plasmin generated is complexed with the peptidyl substrate (S); however, a fraction of the plasmin (P_li) given by Equation 1

\[
[P_{li}]/[P_{li}]_{0} = K_{r}/(K_{r} + [S])
\]

is free to react with one-chain u-PA. Here, K_{r} is the Michaelis constant for Val-Phe-Lys-pNA. Since K_{r} = 0.04 mM and [S] = 1 mM, a value of 1/26 is obtained for [P_{li}]/[P_{li}]_{0}.

**Assay III.** Plasmin-catalyzed Activation of One-chain u-PA—The reaction was measured at 57 °C in 0.05 M Tris-HCl, pH 8.1, 0.05% Triton X-100, 0.125% gelatin. One-chain u-PA (55 nM) was incubated with plasmin at various fixed concentrations. Aliquots of 100 μl were withdrawn at various time intervals and added to 400 μl of reaction buffer containing 1.54 μM BPTI and 0.4 mM C1 and 0.4 mM Glu-Gly-Arg-pNA. This mixture was incubated for 1 h; the reaction was stopped by addition of 20 μl of 1 M HCl, and the absorbance was measured at 405 nm. Provided plasmin-catalyzed conversion of one-chain u-PA can be described as a simple first-order reaction, the change in activity as a result of its conversion is described by Equation 2 (38),

\[
\nu_{s} - \nu = \exp(-kt)
\]

which relates the activity (\nu), at time t and after full conversion of one-chain to two-chain u-PA (\nu_{s}), to the apparent first-order constant, k. The second-order constant k_{cat}/K_{m} for the reaction between plasmin and one-chain u-PA is derived from Equation 3.

\[
k = \frac{k_{cat}}{K_{m}} \text{ [Pli]}
\]

**Assay IV.** Activation of Plasminogen in the Presence of Val-Phe-Lys-pNA and BPTI—This reaction was monitored at 25 °C in 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.4, 0.01% Tween 80 (37). The reaction mixture, to which one- or two-chain u-PA was added, contained 0.2 μM Lys'-plasminogen and various fixed concentrations of Val-Phe-Lys-pNA (S) and BPTI (I), as indicated in each experiment. The assay was performed in the wells of a microtiter plate, and the absorbance was followed at 405 nm. With two-chain u-PA, a linear progress curve in the concentration of pNA was observed. The slope of the curve, d[pNA]/dt, is related to the plasminogen activation rate, \nu_{s}, by Equation 4 (37),

\[
\nu_{s} = k_{k_{e}} / k_{k} \frac{[I] \cdot d[pNA]}{[S]} dt
\]

where k_{e} and K_{e} are the kinetic constants (k_{cat} and K_{m}) for plasmin-catalyzed cleavage of Val-Phe-Arg-pNA.

**THEORY**

It is assumed that plasminogen and one-chain u-PA have negligible catalytic activity compared to that of plasmin and two-chain u-PA, respectively. The activation of a mixture containing one-chain u-PA (pro-u-PA), plasminogen (Plg), and a plasmin inhibitor (I) in which the reaction is initiated by trace amounts of two-chain u-PA (Y) is then described by the following model.

\[
\text{Plg} \xrightarrow{k_{1}} X \xrightarrow{k_{I}} Y \xrightarrow{k_{2}} \text{pro-u-PA}
\]

The generation of plasmin (X) is described by Equation 5,

\[
\frac{dx}{dt} = k_{1}y - k_{2}x
\]

where under initial conditions (when only a small fraction of plasminogen is activated and only a small fraction of the inhibitor is complexed to plasmin) \( k_{1} \approx k_{1}[\text{Plg}], k_{1} \approx k_{1}[I] \).

Similarly, the generation of two-chain u-PA (Y) is described by Equation 6,

\[
\frac{dy}{dt} = k_{2}x
\]

where initially (when only a small fraction of pro-u-PA is activated) \( k_{2} \approx k_{0}[\text{pro-u-PA}] \).

Differentiation of Equation 6 and insertion in Equation 5 yields Equation 7.

\[
\frac{d^{2}y}{dt^{2}} + k_{3} \frac{dy}{dt} - k_{4}[k_{1}y] = 0
\]

The solution to this second-order differential equation is given by Equation 8,

\[
y = C_{1} \exp(\lambda_{1}t) + C_{2} \exp(\lambda_{2}t)
\]

where \( C_{1} \) and \( C_{2} \) are integration constants and the exponential constants \( \lambda_{1} \) and \( \lambda_{2} \) are found by solving the characteristic equation (Equation 9),

\[
\lambda^{2} + k_{1}\lambda - k_{0}[k_{1}] = 0
\]

which yields Equation 10.

\[\lambda_{1} = 0.5 (\sqrt{\lambda_{0}^{2} + 4 k_{1}[k_{1}]}) \] (10a)

\[\lambda_{2} = 0.5 (-\sqrt{\lambda_{0}^{2} + 4 k_{1}[k_{1}]} \] (10b)

This model also describes the progress curves for two-chain
u-PA generation (Equation 8) in the absence of plasmin inhibitor. In this case, $k_1 = 0$ is inserted in Equation 10, which reduces to Equation 11.

$$
\lambda_1 = \sqrt{k_1 k_2} \\
\lambda_2 = -\sqrt{k_1 k_2}
$$

(11a) (11b)

$C_1$ and $C_2$ can be determined by insertion of appropriate initial conditions for $t = 0$. In any case, after a transient phase, the term containing the positive value, $\exp(\lambda_1 t)$, predominates and determines the doubling time for generation of $Y$.

Generation of $X$ is determined by an equation similar to Equation 8, which is obtained by insertion in Equation 6. The doubling time in $X$ is also determined mainly by the term $\exp(\lambda_1 t)$.

**RESULTS**

**Preparation and Characterization of One-chain u-PA**—One-chain u-PA was purified by affinity chromatography with monoclonal antibodies from culture fluid of HT-1080 cells which had been maintained in serum-free BPTI-containing medium for between 5 and 28 days. The preparations were pure one-chain u-PA with no detectable impurities as revealed by SDS-PAGE under reducing as well as nonreducing conditions (detection limit 5%). When such preparations were exposed to $[^3H]$DFP (Fig. 1), no labeled material was incorporated into intact one-chain u-PA. This is in contrast to a sample of the same amount of two-chain u-PA analyzed in parallel. Here, extensive labeling was found, with an electrophoretic mobility under reducing conditions that corresponded to that of the B chain ($M_r \approx 32,000$ (10, 39)). A very small peak with an identical electrophoretic mobility was observed with the one-chain u-PA preparation (not visible in Fig. 1). This peak constituted $\approx 0.1\%$ of the radioactivity incorporated into the two-chain u-PA preparation analyzed in parallel, and it may represent a contamination of the one-chain u-PA preparation with two-chain u-PA.

$^{125}$I-Plasminogen Conversion in the Presence of BPTI—Initially the activity of the one-chain u-PA preparation was studied with the $^{125}$I-plasminogen conversion assay in which the conversion to plasmin is monitored by SDS-PAGE under reducing conditions (30, 33). This assay can be performed in the presence of high concentrations of BPTI, which do not inhibit u-PA (30, 33) but inhibit plasmin-catalyzed conversion of one- to two-chain u-PA (9, 10). Incubation of $^{125}$I-Glu$^\text{BPTI}$-plasminogen with 40 nM one-chain u-PA for 15 min caused no detectable conversion, whereas a considerable conversion occurred by incubation for 15 min with the same amount of two-chain u-PA (Fig. 2, a-c). After one hour’s incubation with the one-chain u-PA preparation, a slight conversion of the plasminogen was observed (Fig. 2d). This conversion could be inhibited completely by either monoclonal antibodies previously found to inhibit u-PA activity (28) or by pretreatment of the one-chain u-PA preparation with DFP (Fig. 2, e-f), indicating that it was due to contaminating two-chain u-PA. A comparison between the results obtained with the two-chain u-PA and the DFP-treated one-chain u-PA preparations (Fig. 2g) showed that two-chain u-PA under these conditions had a plasminogen conversion activity distinctly higher than that of a 400-fold greater concentration of one-chain u-PA.

**Plasminogen Activation in the Presence of Synthetic Plasmin Substrates**—The results obtained with the $^{125}$I-plasminogen conversion assay are in apparent conflict with some reports on activity of one-chain u-PA from other sources (19-23 see the Introduction). Those studies suggest that one-chain u-PA has a high ability to activate plasminogen when this is measured in coupled assays where generated plasmin activity is monitored with a chromogenic substrate. We, therefore, also tested the activity of our one-chain u-PA preparation in such a coupled assay, using Val-Phe-Arg-pNA as plasmin substrate (Fig. 3). Under these conditions, considerable plasmin generation was observed with the one-chain u-PA preparation. However, with Glu$^\text{BPTI}$-plasminogen it became detectable only after a pronounced lag time of more than 10 min, in contrast to an immediate activity observed with two-chain u-PA (Fig. 3, b and c). DFP treatment of the one-chain u-PA preparation increased the lag time slightly (Fig. 3d), while the further addition of two-chain u-PA, at a concentration that was 1000-fold lower than that of the one-chain u-PA, decreased the lag time considerably (Fig. 3e). Studies with Lys$^\text{BPTI}$-plasminogen gave essentially similar results, although the lag time observed with the one-chain u-PA preparation was considerably shorter (Fig. 3d’). Full two-chain u-PA activity was recovered after plasmin cleavage of DFP-treated one-chain u-PA (Fig. 3, f, f’). These results confirm the previous findings that plasmin is actually generated in such reaction mixtures containing one-chain u-PA. They also demonstrate that this type of assay is unsuitable for testing whether one-chain u-PA has some intrinsic activity, because it is very sensitive to possible contamination with minute amounts of two-chain u-PA, generating plasmin which in turn may generate more two-chain u-PA.

**Plasmin-catalyzed Activation of One-chain u-PA**—To define the conditions for a coupled u-PA assay in which the side effect produced by the plasmin generated was reduced to an acceptable level, we studied plasmin activation of our one-chain u-PA preparation with the use of an amiodextrin u-PA substrate (Fig. 4). The one-chain u-PA preparation had no measurable amidolytic activity, but it could be converted into an active form by incubation with plasmin. Fig. 4, a-e, shows the time course of this activation at various plasmin concentrations. Replotting of the results in a semilogarithmic plot (Fig. 4, s’-c’) indicated that the activation was a first-order reaction (see Equation 2), and an apparent second-order constant $k_{\text{act}}/K_m = 3.3 \times 10^3$ M$^{-1}$ s$^{-1}$ was calculated (Equation 3). This is somewhat higher than the value of $k_{\text{act}}/K_m = 0.5 \times 10^6$ M$^{-1}$ s$^{-1}$ determined by Collen et al. (26) under slightly different conditions. The activation of one-chain u-PA is thus
FIG. 2. 

**Proenzyme to Urokinase-type Plasminogen Activator**

plasmin activity generated during the coupled Val-Phe-Lys-pNA assay can be obtained by addition of BPTI to the reaction mixture (37, 40). When plasminogen is activated at a constant rate, a steady-state level of free plasmin, controlled by the concentration of the substrate and of BPTI, is rapidly achieved, and the assay provides a linear progress curve, the slope of which is proportional to the plasminogen activation rate and to the ratio between the concentrations of Val-Phe-Lys-pNA and BPTI (see Equation 4). This assay was used to measure Lys*-plasminogen activation with the one- and two-chain u-PA preparations at four BPTI concentrations with up to 6-fold different values (Fig. 5). The Val-Phe-Lys-pNA concentrations were also varied, so that in all four cases the ratio between the concentrations of BPTI and Val-Phe-Lys-pNA was the same. With a constant rate of plasminogen activation, the same rate of nitroaniline production was, therefore, expected in the four experiments. The results obtained with two-chain u-PA were consistent with the predictions (Fig. 6, *curves* a–d); a leveling off that was observed particularly with low peptidyl substrate concentrations can be explained by substrate depletion and/or product inhibition of the amidolytic reaction. In contrast, the progress curves with one-chain u-PA were nonlinear in this assay, the rate of nitroaniline production increasing steadily with time. Furthermore, the nitroaniline production increased strongly with decreasing concentrations of BPTI and Val-Phe-Lys-pNA.

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These findings indicate that the plasminogen activation rate obtained with the one-chain u-PA preparation in the presence of free plasmin increases with time, and they suggest that there is a steady increase in the amount of two-chain u-PA and that this increase depends on the concentration of free plasmin, which is highest in the samples with lowest BPTI concentrations. With the lowest level of free active plasmin (curve d'), an estimate of the maximal value for the initial rate of plasminogen activation is indicated by the dotted line. This corresponds to the activity expected for two-chain u-PA at a concentration \( \approx 250 \)-fold lower than the actual one-chain u-PA concentration.

The effect of the plasminogen concentration on the plasminogen activation reaction is shown in Fig. 6. With two-chain u-PA, the progress curves were linear at all plasminogen concentrations (Fig. 6, curves a-g), and a linear Lineweaver-Burk plot was obtained (insert), from which a \( K_m \approx 10 \) \( \mu M \) for Lys\(^{77}\)-plasminogen was determined. With the one-chain u-PA preparation, nonlinear progress curves were obtained at all plasminogen concentrations (Fig. 6, curves a'-g'), implying a considerable uncertainty in initial rate determinations. These results also imply that, in contrast to the data obtained with two-chain u-PA, a double-reciprocal plot of the one-chain u-PA data would not lead to a meaningful \( K_m \) determination.

**DISCUSSION**

This study clearly shows that one-chain u-PA produced by HT-1080 cells has very little enzyme activity compared with two-chain u-PA, both when it is measured in a \(^{125}\)I-plasminogen conversion assay in the presence of high concentrations of BPTI and when measured in coupled plasminogen activation assays, with or without BPTI present. In the plasminogen conversion assay, DFP-treated one-chain u-PA had no detectable activity, the detection limit corresponding to the activity of a 400-fold lower amount of two-chain u-PA. In the coupled assays, plasmin generation was clearly observed with the one-chain u-PA preparation, but the initial rate of plasminogen activation was lower than that of a 250-fold smaller concentration of two-chain u-PA.

It is not possible to decide whether this low activity is due to intrinsic activity of one-chain u-PA. A mixture of plasminogen and one-chain u-PA is a metastable system that is activated either by trace amounts of plasmin or two-chain u-PA, or by intrinsic activity of plasminogen or one-chain u-PA (see Ref. 41 for a discussion of the equilibrium between inactive and active forms of proenzymes). A kinetic model for the development of active enzymes in such a system predicts...
Exponential progress curves in the concentrations of both activated enzymes (see "Theory"). Addition of a plasmin inhibitor does not alter the self-activating character of the system but increases the doubling time for the generation of active enzymes, assuming $k_1 = 3 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$, $k_2 = 2 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$, according to Equation 11 a doubling time ($\beta/\alpha$) of about 3 min is obtained for a reaction mixture containing 1 mM one-chain u-PA and 0.2 $\mu$M Glu1-plasminogen. By addition of 0.3 $\mu$M BPTI ($k_1 = 5.6 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$), according to Equation 10 the doubling time is increased to about 17 min. The addition of a synthetic plasmin substrate also tends to increase the doubling time due to a decrease in effective enzyme concentration (see Equation 1). With 1 mM Val-Phe-Lys-pNA ($K_m = 0.04 \text{ M}$), this leads to an approximately 5-fold increase, while the presence of 1 mM Val-Leu-Lys-pNA ($K_m = 0.15 \text{ M}$), which has been frequently used in plasminogen activation assays (19-21, 23, 24, 26), increases the doubling time approximately 3-fold. Trace amounts of active enzymes (two-chain u-PA, t-PA, or plasmin) contaminating the purified one-chain u-PA or plasminogen preparations may, therefore, wholly or in part account for the observed activity of one-chain u-PA in the coupled assay. Also contamination with nonenzymatic factors that might bind to and activate one-chain u-PA or plasminogen by mechanisms similar to that of streptokinase activation of plasminogen (43, 44) may have contributed to the measured activity.

The present results confirm and extend our own previous studies showing that purified mouse (9) and human (10) one-chain u-PA preparations have little or no plasminogen-activating capability in a fibrinolytic assay, and they are also in agreement with similar results obtained for human one-chain u-PA by other groups (11-13). This picture of one-chain u-PA as an essentially inactive proenzyme is, however, in contrast to the interpretation reached by some investigators (19-24, 26, 27). Based on kinetic studies with human u-PA obtained from urine (21), cell cultures (22), and produced by recombinant techniques in Escherichia coli (20, 26) and in Chinese hamster ovary cells (24), they concluded that one-chain u-PA has intrinsic plasminogen activation capability. A coupled plasminogen activation assay like the one shown in Fig. 3 was used in all those studies, except that the experiments were performed using a different peptidyl substrate, Val-Leu-Lys-pNA, which has a lower affinity for plasmin. As described above, this results in a higher fraction of free plasmin and leads to an even higher rate of two-chain u-PA generation than encountered under our assay conditions. Our results with plasminogen activation in the presence of one-chain u-PA indicate that due to two-chain generation the progress curves are nonlinear. This prevents a meaningful determination of $k_{cat}$ and $K_m$. From the data presented with u-PA from natural sources in the studies cited above, however, it is not possible to evaluate to what extent the results have been influenced by small amounts of contaminating or generated two-chain u-PA and plasmin, and it cannot be decided whether the conflict between those results and ours is real or only apparent.

Kinetic studies with unglycosylated recombinant one-chain u-PA produced by expression of the human gene in E. coli have suggested that this has a much higher activity than its glycosylated counterpart from eukaryotic cells (26). A reinvestigation confirmed that the E. coli-derived one-chain u-PA has a higher activity than the one-chain form obtained from cell cultures (23). However, the claim of the original investigation (26), that at low plasminogen concentrations the activity of the one-chain form from E. coli is even higher than that of its two-chain counterpart, could not be sustained (23). At high plasminogen concentrations, the activity of the one-chain u-PA was found to be 0.3% of two-chain u-PA's activity (23). The most serious drawback to the coupled assay as applied to determination of one-chain u-PA enzyme activity was recently counteracted by the study of variants of recombinant one-chain u-PA that are partly resistant to plasmin cleavage (24). In these variants, Lys-158 of the plasmin cleavage site was changed by site-directed mutagenesis. It is interesting to note that the catalytic efficiency defined as an apparent second-order constant ($k_{cat}/K_m$) for these variants was very low ($=1 \times 10^3 \text{ M}^{-1} \text{s}^{-1}$) (24), as compared to $k_{cat}/K_m = 2 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ obtained for two-chain u-PA-catalyzed Glu1-plasminogen activation (26). We interpret these results to mean that the activity of these preparations of variants of recombinant one-chain u-PA was $=200$-fold lower than that of two-chain u-PA and that the results, therefore, are in good agreement with ours.

In summary, conclusive evidence for an intrinsic activity of one-chain u-PA significantly higher than the maximal value measured in the present study has not been reported. At most, this corresponds to 0.4% of the two-chain u-PA activity. In addition, one-chain u-PA has, as discussed in the Introduction, a variety of other characteristics similar to the one-chain proenzyme forms of other serine proteases (for a review, see Ref. 46), which seems to justify its classification as a genuine proenzyme.

Also the one-chain proenzyme form of t-PA has a considerably lower activity than two-chain t-PA, but in this case, pro-t-PA has decisive intrinsic activity with both plasminogen (catalytic efficiency $\approx 3\%$ of that of two-chain t-PA) and peptidyl substrates (40, 47-49), and pro-t-PA also incorporates DFP (47, 50, 51). Recent results (40) suggest that fibrin binding induces an activated state of one-chain t-PA with activities comparable to those of two-chain t-PA. In addition to this difference in proenzyme properties between u-PA and t-PA, they also differ with respect to fibrin binding (52) and binding to a cell surface receptor specific for u-PA (45, 53-55). These differences are likely to be important for an understanding of the exact physiological functions of the two different plasminogen activator systems, which still remains to be elucidated.

Pro-u-PA appears to be the predominant extracellular form of u-PA, not only in cell cultures but also in the intact organism (9-12, 14, 17, 18, 56, 57), and extracellular activation of pro-u-PA may be a crucial step in the physiological regulation of the u-PA pathway of plasminogen activation (6, 25). The plasmin-catalyzed activation of pro-u-PA provides a positive feedback mechanism that accelerates and amplifies the effect of activation of a small amount of pro-u-PA. This amplification may involve factors other than u-PA and plasmin. Enhancement of plasminogen activation is thus also obtained by decreased rates of inactivation of plasmin and two-chain u-PA by their respective inhibitors. It remains to be investigated whether initiation of the u-PA pathway of plasminogen activation physiologically is achieved by amplification of a low pre-existing intrinsic activity of pro-u-PA (see Ref. 41) or whether as yet unknown triggering factors that activate pro-u-PA are involved.

Acknowledgments—The excellent technical assistance of Maj-Britt Hansen, Lis Gericke, Gitte Plesner, Signe Trentembler, and Elke Gottfriedsen is gratefully acknowledged.

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