THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1986 by The American Society of Biological Chemists, Inc.

Vol. 261, No. 7, Issue of March 5, pp. 3098-3102, 1986
Printed in U.S.A.

Isolation and Functional Characterization of the Heavy and Light Chains of Human Tissue-type Plasminogen Activator*

(Received for publication, June 4, 1985)

Dingeman C. Rijken and Eleonore Groeneveld

From the TNO Gaubius Institute for Cardiovascular Research, Herenstraat 5d, 2313 AD Leiden, The Netherlands

Two-chain tissue-type plasminogen activator (t-PA), which consists of a heavy chain ($M_r \simeq 38,000$) and a light chain ($M_r \simeq 31,000$) connected by a disulfide bridge, was reduced with 2-mercaptoethanol and then air-reoxidized at a low protein concentration and carboxamidomethylated. The two chains were separated by means of zinc chelate-agarose, which was found to bind the light chain selectively. The light chain was fully active on the tripeptide substrate H-D-isoleucyl-L-prolyl-L-arginine *p*-nitroanilide (S-2288) and partially active on plasminogen. The plasminogen activator activity of the light chain was, in contrast to that of two-chain t-PA, not stimulated by fibrin or fibrinogen fragments. Fibrin-agarose chromatography of radiolabeled chains showed that only the heavy chain bound to fibrin. These results indicate that the active site-containing light chain in t-PA needs the heavy chain for fibrin stimulation of its plasminogen activator activity.

Plasminogen activators convert plasminogen into plasmin and are involved in fibrinolysis and other physiological processes where extracellular proteolysis takes place. They belong to the group of serine proteinases and have recently been reviewed in Refs. 1 and 2. Human t-PA,[1] as purified from blood vessel perfusates (3), uterine tissue (4, 5), or melanoma cell culture fluid (6–8), has a molecular weight of approximately 70,000 and consists of one polypeptide chain. During isolation procedures (4–6) and fibrinolysis (9), this form is easily converted into a two-chain form, consisting of a heavy chain of approximately 38,000 and a light chain of approximately 31,000, which are connected by a disulfide bridge. The light chain contains the active site (4) and is homologous with other serine proteinases (7, 10). The heavy chain is proposed to contain two kringle structures (10), a finger domain and a growth factor domain (11).

t-PA is a poor plasminogen activator in the absence of fibrin. However, in the presence of a clot, both t-PA and plasminogen bind to fibrin and form, probably, a cyclic ternary complex in which plasminogen is efficiently activated (12–16). This property makes t-PA clot-specific and useful as a thrombolytic agent (17). Recombinant DNA technology is applied for large scale production of t-PA (10) and mutagenesis of the gene may lead to t-PA mutants with even more favorable properties. A better understanding of the structure-function relationship of t-PA is required for the latter approach.

In the present study, a method for the isolation of functionally active polypeptide chains of t-PA was developed and properties involved in fibrinolysis were assigned to the chains.

MATERIALS AND METHODS

Proteins and Materials—Human two-chain t-PA was purified from (Bowes) melanoma cell culture fluid (6, 18). Variant I with three *N*-linked carbohydrate antennas and variant II with two *N*-linked antennas were separated by lysine-agarose chromatography (19) and variant I was used in all experiments. Bovine serum albumin was from Roth (Karlsruhe, FRG), Sephacryl S-200, Sephadex G-25, and lysine-agarose were obtained from Pharmacia. Zinc chelate-agarose was prepared as described previously (4). Fibrin-agarose was prepared by coupling 5 mg of plasminogen-free bovine fibrinogen (Poviet, Boxtel, The Netherlands) per ml of CNBr-activated Sepharose (Pharmacia), followed by successive treatments with thrombin and diisopropyl fluorophosphate. 2-Mercaptoethanol was purchased from Merck, iodoacetamide from BDH, H-D-isoleucyl-L-prolyl-L-arginine *p*-nitroanilide (S-2288) from Kabi, ^{125}I from Amersham, and Iodogen from Pierce.

Isolation of Heavy and Light Chain of t-PA—Two-chain t-PA, variant I (0.99 mg) in 44 ml of 50 mM Tris-HCl buffer, pH 8.6, containing 1 M NaCl, 10 μM EDTA, and 0.01% Tween 80 was reduced with 0.62 ml of 2-mercaptoethanol (0.2 M final concentration) for 1 h at room temperature under a nitrogen atmosphere. Reduced t-PA was diluted with 1200 ml of 50 mM Tris-HCl buffer, pH 8.6, containing 1 M NaCl, 0.1 μM $CuSO_4$, and 0.01% Tween 80 and reoxidized during dialysis against 10 liters of the dilution buffer for 6 h and once more for 16 h at room temperature. Residual —SH groups were blocked by the addition of 12 ml of 100 mM iodoacetamide and incubation for 1 h at room temperature in the dark.

The procedure to separate the light and heavy chains was continued at 4 °C. After adjusting the pH to 7.5, the protein solution was dialyzed against 10 liters of 50 mM Tris-HCl buffer, pH 7.5, containing 1 M NaCl and 0.01% Tween 80 and then applied to a 10-ml zinc chelate-agarose column at a flow rate of 20 ml/h. The breakthrough (afterwards identified as the heavy chain) was concentrated by dialysis against solid polyethylene glycol 20,000, then dialyzed against 10 mM phosphate buffer, pH 8.0, containing 0.15 M KSCN and 0.01% Tween 80 and applied to a 4-ml lysine-agarose column. The bound protein was eluted with 0.2 M arginine in the column buffer, concentrated with polyethylene glycol 20,000, and gel-filtered on Sephacryl S-200 (1.5 × 100 cm, flow rate 4.6 ml/h, fractions of 2.3 ml) in 10 mM phosphate buffer, pH 7.5, containing 1.6 M KSCN and 0.01% Tween 80. Protein which bound to zinc chelate-agarose (afterwards identified as the light chain) was eluted with 50 mM imidazole in the column buffer, concentrated, and gel-filtered as described for the heavy chain.

Ten-μg portions of heavy and light chain were radiolabeled with 0.5 mCi of ^{125}I using the Iodogen method (20). The labeled proteins (1.2×10^6 cpm/μg of protein) were centrifuged through a 2-ml Sephadex G-25 (coarse) column to remove the excess of free ^{125}I and then gel-filtered on Sephacryl S-200 (1.5 × 40 cm) in 50 mM Tris-

* This work was supported by Praeventiefonds Grant 28-813. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are: t-PA, tissue-type plasminogen activator; Lys-plasminogen, partially degraded plasminogen with NH_2-terminal lysine; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; S-2288, H-D-isoleucyl-L-prolyl-L-arginine *p*-nitroanilide; S-2251, H-D-valyl-L-leucyl-L-lysine *p*-nitroanilide.

HCl buffer, pH 8.0, containing 1 M NaCl and 0.01% Tween 80 to remove aggregated protein.

Assay of Plasminogen Activator Activity—A spectrophotometric assay was used as described by Verheijen *et al.* (21): t-PA or its light chain was incubated in 250 μl of 0.13 μM Lys-plasminogen, 0.3 mM H-D-valyl-L-leucyl-L-lysine *p*-nitroanilide (S-2251), and, if indicated, 0.12 mg/ml CNBr-digested fibrinogen as a stimulator in microtitration plates at 25 °C. The change in absorbance at 405 nm was measured. Reconstitution experiments were performed by adding increasing amounts of heavy chain (0.8–53 nM, final concentration) to 8 nM light chain in the assay mixture, with and without stimulator.

Assay of Amidolytic Activity—t-PA or its light chain was incubated in 200 μl of 0.6 mM H-D-isoleucyl-L-prolyl-L-arginine *p*-nitroanilide (S-2288) in microtitration plates at 25 °C, according to the manufacturer's instructions. For kinetic analyses, the substrate concentration was varied from 0.05 to 1.5 mM and the data were plotted as Lineweaver-Burk plots to calculate K_m and k_{cat}.

Enzyme Immunoassays for Heavy and Light Chain—Specific antibodies against the heavy and light chains were isolated by passing 5 mg of total IgG fraction of a goat anti-t-PA antiserum through a 1-ml heavy chain-agarose column (34 μg of heavy chain coupled) and a 1-ml light chain-agarose column (83 μg of diisopropyl fluorophosphate-treated light chain coupled) in series. The antibodies bound to each column were eluted with 0.1 M glycine buffer, pH 2.2, and used in a sandwich enzyme immunoassay, as described previously for t-PA (22).

Miscellaneous Procedures—SDS-PAGE was performed according to Laemmli (23). Proteins were stained with Coomassie Brilliant Blue R-250. Calibration proteins were obtained from Pharmacia. Amino acid analyses were carried out on a Beckman Multichrom amino acid analyzer after hydrolysis of the protein samples with 6 M HCl *in vacuo* at 110 °C for 24 h. Determination of the concentration of t-PA and its chains was based on amino acid analysis using the complete amino acid composition as published by Pennica *et al.* (10).

RESULTS

Reduction and Carboxamidomethylation of Two-chain t-PA—In order to establish minimal conditions for reduction of the single interchain disulfide bridge of two-chain t-PA, t-PA samples were reduced with increasing 2-mercaptoethanol concentrations (0–200 mM) and then treated with iodoacetamide (Fig. 1). SDS-PAGE under nonreducing conditions showed that the two chains remained connected up to 12.5 mM 2-mercaptoethanol. About 25 and 100% of the interchain disulfide bridges were broken at 50 and 200 mM, respectively (not shown). However, both the plasminogen activator activity and the amidolytic activity of t-PA were completely destroyed after treatment with 200 mM 2-mercaptoethanol (Fig. 1), indicating that under these conditions no functionally active light chain could be prepared.

Isolation of Functionally Active Heavy and Light Chain of t-PA—Pilot experiments had shown that the amidolytic activity of reduced t-PA could be recovered by carrying out a reoxidation step before treating the material with iodoacetamide. These observations formed the basis of an isolation procedure for functionally active chains of t-PA (summarized in Table I). In a typical experiment, approximately 1 mg of two-chain t-PA was reduced with 200 mM 2-mercaptoethanol, then reoxidized at a low protein concentration, and finally treated with iodoacetamide. The amidolytic activity on S-2288 of this preparation was 84% of that of the starting material (Table I).

Zinc chelate-agarose chromatography was used to separate the light and heavy chain. The amidolytic activity completely bound to the column and was eluted with an imidazole-containing buffer. Gel filtration on Sephacryl S-200 of zinc chelate-agarose eluate revealed a single protein peak with an apparent molecular weight of 30,000 (Fig. 2, *left*). This peak coincided with the amidolytic activity peak and was considered to be the monomeric form of the active site-containing light chain of t-PA.

The breakthrough of the zinc chelate-agarose column did not contain amidolytic activity on S-2288 (Table I). The protein was concentrated, adsorbed to a lysine-agarose col-

TABLE I

Separation of the heavy and light chains of two-chain t-PA

t-PA was reduced with 0.2 M 2-mercaptoethanol for 1 h at room temperature and subsequently diluted and air-reoxidized during removal of 2-mercaptoethanol by dialysis against buffer containing copper ions (see "Materials and Methods"). The amidolytic activity was determined with S-2288.

Step	Volume	Protein	Amidolytic activity
	ml	*μg*	%
Starting two-chain t-PA	44	990	100
Reduced t-PA	45		<1
Reoxidized t-PA	1260		84
Zinc chelate-agarose eluate	7		70
Sephacryl S-200	14	473	72
Zinc chelate-agarose breakthrough	1260		<1
Lysine-agarose eluate	8		<1
Sephacryl S-200	14	186	<1

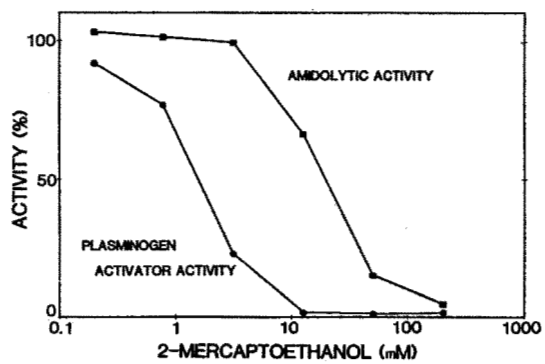

FIG. 1. **Reduction and carboxamidomethylation of two-chain t-PA.** Protein samples of 30 μg/ml in 0.5 M Tris-HCl buffer, pH 8.6, containing 0.3 M NaCl, 5 mM EDTA, and 0.01% Tween 80 were reduced with 2-mercaptoethanol at various concentrations (0–2000 mM) for 1 h at room temperature. The samples were then treated with iodoacetamide (at the same 2-mercaptoethanol concentrations as used for reduction) for ½ h at room temperature in the dark and dialyzed in order to determine plasminogen activator activities in the presence of fibrinogen fragments (●), amidolytic activities on S-2288 (■), and chain cleavage with SDS-PAGE (not shown).

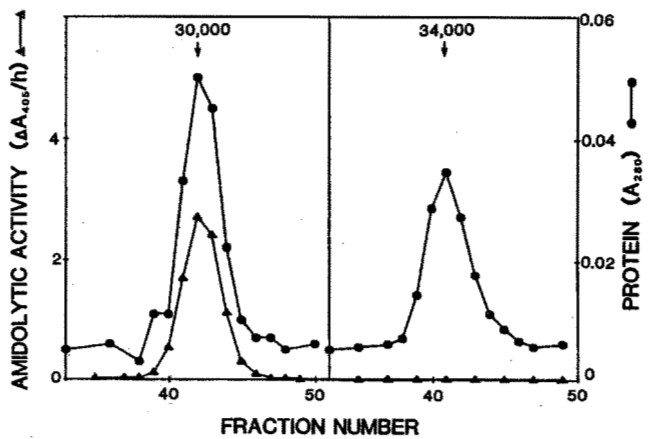

FIG. 2. **Gel filtration on Sephacryl S-200 of the isolated light (*left*) and heavy (*right*) chains of t-PA (see "Materials and Methods").** Both gel filtrations resulted in single protein peaks at 30,000 and 34,000, respectively, indicating the monomeric forms of the chains. Only the light chain showed amidolytic activity on S-2288 (5-μl aliquots assayed).

umn, desorbed with arginine, and gel-filtered on Sephacryl S-200. Fig. 2 (*right*) showed a single protein peak with an apparent molecular weight of 34,000, which was considered to be the monomeric form of the heavy chain of t-PA.

Fig. 3 shows t-PA and its isolated heavy and light chains after SDS-PAGE. The apparent molecular weights were approximately 70,000 for t-PA and between 30,000 and 40,000 for the chains, in agreement with the gel filtration experiments (Fig. 2) and the literature (6, 7). Both t-PA and the heavy chain were a little heterogeneous, possibly due to proteolytic degradation in the starting material.

The amino acid compositions of the light and heavy chain preparations (not shown) were similar to the theoretical compositions as derived from the primary structure of t-PA (10). This is illustrated with the amino acids which are rather unevenly distributed over the two chains: the ratio between the number of residues per 100 residues in the heavy chain preparation and that number in the light chain preparation was 1.2 for Ser (theoretically 1.5), 1.6 for Tyr (theoretically 1.9), 0.8 for Val (theoretically 0.7), 0.7 for Ile (theoretically 0.6), 0.7 for Leu (theoretically 0.5), and 0.6 for His (theoretically 0.6) (the theoretical ratios were calculated from Ref. 10 in the same way as the experimental ratios, that means Cys, Met, Pro, and Trp excluded; Asp and Asn as well as Gln and Glu taken together).

Application of specific enzyme immunoassays, using immunoadsorbed antibodies against the light and heavy chain preparations, could not exactly establish the extent of cross-contamination, but indicated upper limits. Fig. 4 (*left*) demonstrates that 460 ng/ml heavy chain preparation contained less than 1.9 ng/ml (0.4%) light chain and Fig. 4 (*right*) demonstrates that 1360 ng/ml light chain preparation contained less than 1.9 ng/ml (0.14%) heavy chain.

Functional Properties of the Isolated Heavy and Light Chain of t-PA—During the isolation procedure, it had already been observed that the light chain had amidolytic activity on S-2288. Kinetic analyses (see "Materials and Methods") showed that the kinetic parameters of the light chain (K_m = 0.50 mM, k_{cat} = 2.8 s^{-1}) were very similar to those of two-chain t-PA (K_m = 0.43 mM, k_{cat} = 2.7 s^{-1}).

The plasminogen activator activity of the light chain was determined in a spectrophotometric assay, consisting of a mixture of lys-plasminogen, the plasmin substrate S-2251 and, if indicated, CNBr-digested fibrinogen as stimulator of the plasminogen activation. Table II shows that the light chain had 23% of the activity of t-PA when measured in the absence of stimulator. CNBr-digested fibrinogen stimulated t-PA activity 50-fold, in correspondence with previous work (15, 16). However, the activity of the light chain was not enhanced at all (a stimulation factor smaller than 1.0 can be ascribed to the inhibitory effect of the fibrinogen fragments on plasmin activity). Essentially the same results were obtained if fibrin monomers (24) instead of fibrinogen fragments were used as stimulator (not shown). These results indicated that the light chain has a very low activity on plasminogen, primarily because its activity is not enhanced by fibrin. The enhancement was not restored by the addition of 0.8–53 nM heavy chain to 8 nM light chain.

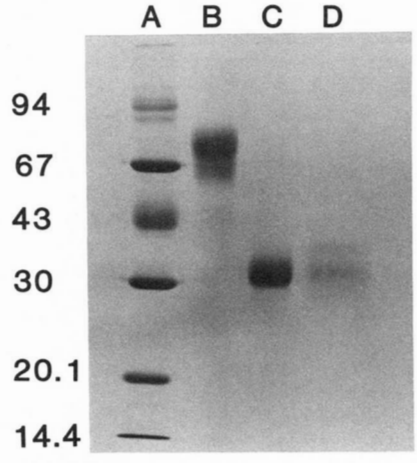

FIG. 3. **SDS-PAGE of two-chain t-PA and its isolated chains.** Amounts of 15 μg of t-PA (*lane B*), of the light chain (*lane C*), and of the heavy chain (*lane D*) were applied onto a 12% polyacrylamide gel under nonreducing conditions. *Lane A* contained a mixture of protein markers: phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α-lactalbumin. *Numbers* on the *left* represent molecular weights (× 10^3).

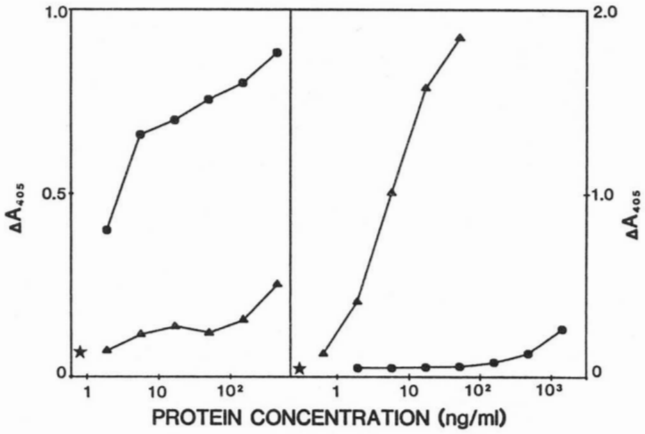

FIG. 4. **Cross-contamination of light (●) and heavy (▲) chain preparations of t-PA, determined with specific enzyme immunoassays for light (*left*) and heavy (*right*) chain.** Various dilutions of the chain preparations were incubated in the wells of microtitration plates coated with rabbit antibodies against t-PA. The wells were then successively incubated with specific goat antibodies against the light or heavy chain preparations, with rabbit anti-goat IgG antibodies conjugated with alkaline phosphatase and with *p*-nitrophenylphosphate. The color production was measured at 405 nm after 20 min. The *stars* indicate the background of the assays, obtained when buffer was used as a sample.

TABLE II

Plasminogen activator activity of t-PA and its light chain in the absence and presence of CNBr-digested fibrinogen (CNBr-Fbg)

The activities were measured in microtitration plates at 25 °C under standard assay conditions (using a mixture of lys-plasminogen, S-2251 and, if indicated, CNBr-Fbg, see "Materials and Methods") and expressed as a change in absorbance at 405 nm per time squared (h^2) and per concentration of activator (μM).

	Plasminogen activator activity		Stimulation factor
	−CNBr-Fbg	+CNBr-Fbg	
	$\Delta A/h^2/\mu M$	$\Delta A/h^2/\mu M$	
Two-chain t-PA	115 (100%)	5770 (4017%)	50
Light chain	26 (23%)	8.3 (7%)	0.32

The fibrin-binding properties of the light and heavy chain of t-PA were studied on fibrin-agarose columns, under conditions in which intact t-PA bound completely (not shown). Fig. 5 demonstrates that ^{125}I-labeled light chain had hardly any affinity for the column; only a small fraction (10% of the radioactivity) bound and was eluted with 1.6 M KSCN. In contrast, the main part of ^{125}I-labeled heavy chain bound to fibrin-agarose and could be eluted afterwards. Only small portions showed no affinity (fractions 2–4, 19% of the radioactivity) or a weak affinity (fractions 5–8, 17% of the radio-

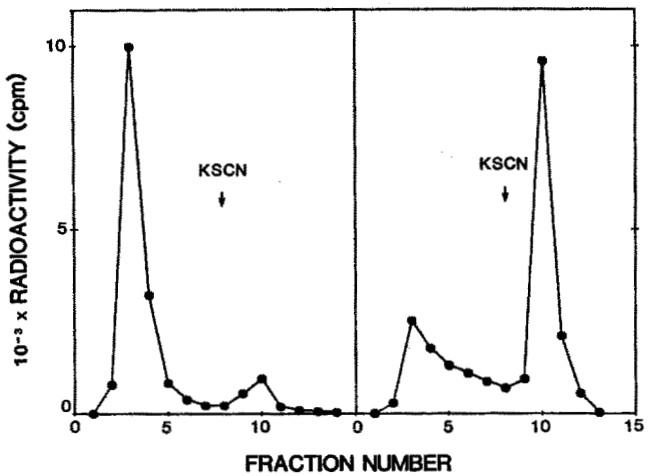

FIG. 5. **Chromatography of light (*left*) and heavy (*right*) chains of t-PA on fibrin-agarose columns.** Samples of 0.5 ml of ^{125}I-labeled chains (2.10^4 cpm) in 50 mM Tris-HCl buffer, pH 7.5, containing 0.25 M NaCl, 0.01% Tween 80, and 1 mg/ml albumin were applied to 2-ml fibrin-agarose columns at room temperature (flow rate, 4 ml/h; fraction volume, 1 ml). The columns were washed with 6 ml of buffer and with 1.6 M KSCN in the aforementioned Tris-HCl buffer to elute bound radioactivity. Total recoveries of applied radioactivity were 93–98%.

activity) for the column, which may be due to destruction during the labeling procedure.

DISCUSSION

In the present study, functionally active light and heavy chains from two-chain t-PA were isolated and characterized. Isolation procedures for functionally active chains of other disulfide-bridged multichain proteinases often make use of mild reduction with 2-mercaptoethanol or dithiothreitol (*e.g.* plasmin (25), urokinase (26), kallikrein (27), coagulation factor XI (28)). However, under the experimental conditions of the present study, the enzymatic activities of t-PA were destroyed before the interchain disulfide bridge was broken, indicating that this bridge is relatively resistant to reduction and that the t-PA chains cannot be isolated in a functional form in this manner.

In another approach, t-PA was reduced first and then air-reoxidized. This resulted in the recovery of the amidolytic activity. In a preliminary report, we performed the reoxidation at a protein concentration of 100 μg/ml and in the absence of copper ions (29). The main part of the light chains was recovered as monomers, whereas the heavy chains were recovered as high molecular weight aggregates. In the present study, reoxidation was performed at a low protein concentration (0.8 μg/ml) to minimize the chance of interchain disulfide bridges and in the presence of 0.1 μM copper ions. This resulted in monomeric forms of both light and heavy chains.

The two chains were separated with zinc chelate-agarose, which appeared to bind the light chain specifically. Gel filtration of the light chain resulted in a single protein peak and this step is, therefore, not absolutely required in the isolation procedure. The heavy chain in the breakthrough of the zinc chelate-agarose column could not be gel-filtered, as the Tween 80 concentration became too high after concentration. Use of 0.001% instead of 0.01% Tween 80 in the dilution buffer may eliminate this problem. Removal of the bulk of Tween 80 was achieved with lysine-agarose (this column material apparently does not bind t-PA through the active site in the light chain but through the heavy chain). Subsequent gel filtration resulted again in a single protein peak, which contained, however, just 186 μg of protein (36% recovery). An explanation for the low recovery might be that the heavy chain preparation bound only partially to lysine-agarose, for instance as a consequence of partially incorrect reoxidation of disulfide bridges.

The amidolytic activity of the light chain was very similar to that of the parent two-chain t-PA, indicating that the active site of the light chain was fully intact. The plasminogen activator activity of the light chain *versus* plasminogen as determined under standard assay conditions without fibrinogen degradation products as stimulator was only 23% of that activity for two-chain t-PA. This may indicate that essential parts of the light chain outside the active site were not completely and correctly reoxidized and/or that the heavy chain is, to some extent, required for interaction with plasminogen. The major difference between the light chain and t-PA is that the activity on plasminogen of the former is not stimulated by fibrinogen degradation products or by fibrin. This indicates that the light chain has a very low fibrinolytic activity and thus is not suitable as a thrombolytic agent. The heavy chain is, most probably, required for stimulation by fibrin. This could not be proven by reconstitution experiments but was strongly supported by fibrin binding studies, which showed that the heavy chain and not the light chain bound to fibrin-agarose. The latter finding is in line with the suggestion that the finger domain of the heavy chain, like those in fibronectin, is involved in fibrin binding (11) as well as with another suggestion that the two-kringle structures of the heavy chain, like the kringles in plasminogen, are responsible for the fibrin binding (1).

Localization of other functional properties of t-PA on its chains is in progress.

Acknowledgment—Dr. E. Klasen, Sylvius Laboratory, State University of Leiden, is acknowledged for the performance of the amino acid analyses.

REFERENCES

1. Bachmann, F., and Kruithof, E. K. O. (1984) *Semin. Thromb. Hemostasis* **10,** 6–17
2. Cederholm-Williams, S. A. (1984) *BioEssays* **1,** 168–173
3. Binder, B. R., Spragg, J., and Austen, K. F. (1979) *J. Biol. Chem.* **254,** 1998–2003
4. Rijken, D. C., Wijngaards, G., Zaal-de Jong, M., and Welbergen, J. (1979) *Biochim. Biophys. Acta* **580,** 140–153
5. Wallén, P., Rånby, M., Bergsdorf, N., and Kok, P. (1981) *Prog. Fibrinolysis* **5,** 16–23
6. Rijken, D. C., and Collen, D. (1981) *J. Biol. Chem.* **256,** 7035–7041
7. Wallén, P., Pohl, G., Bergsdorf, N., Rånby, M., Ny, T., and Jörnvall, H. (1983) *Eur. J. Biochem.* **132,** 681–686
8. Nielsen, L. S., Hansen, J. G., Andreasen, P. A., Skriver, L., Danø, K., and Zeuthen, J. (1983) *EMBO (Eur. Mol. Biol. Organ.)* J. **2,** 115–119
9. Rijken, D. C., Hoylaerts, M., and Collen, D. (1982) *J. Biol. Chem.* **257,** 2920–2925
10. Pennica, D., Holmes, W. E., Kohr, W. J., Harkins, R. N., Vehar, G. A., Ward, C. A., Bennett, W. F., Yelverton, E., Seeburg, P. H., Heyneker, H. L., Goeddel, D. V., and Collen, D. (1983) *Nature* **301,** 214–221
11. Bányai, L., Váradi, A., and Patthy, L. (1983) *FEBS Lett.* **163,** 37–41
12. Wallén, P. (1977) in *Thrombosis and Urokinase* (Paoletti, R., and Sherry, S., eds) pp. 91–102, Academic Press, New York
13. Hoylaerts, M., Rijken, D. C., Lijnen, H. R., and Collen, D. (1982) *J. Biol. Chem.* **257,** 2912–2919
14. Rånby, M. (1982) *Biochim. Biophys. Acta* **704,** 461–469
15. Verheijen, J. H., Nieuwenhuizen, W., and Wijngaards, G. (1982) *Thromb. Res.* **27,** 377–385
16. Nieuwenhuizen, W., Verheijen, J. H., Vermond, A., and Chang, G. T. G. (1983) *Biochim. Biophys. Acta* **755,** 531–533
17. Collen, D., and Lijnen, H. R. (1984) *Arteriosclerosis* **4,** 579–585

18. Kluft, C., Van Wezel, A. L., Van der Velden, C. A. M., Emeis, J. J., Verheijen, J. H., and Wijngaards, G. (1983) in *Advances in Biotechnological Processes* (Mizrahi, A., and Van Wezel, A. L., eds) Vol. 2, pp. 97–110, Alan R. Liss, Inc., New York
19. Bennett, W. F. (1983) *Thromb. Haemostasis* **50,** 106 (Abstr. 315)
20. Fraker, P. J., and Speck, J. C. (1978) *Biochem. Biophys. Res. Commun.* **80,** 849–857
21. Verheijen, J. H., Mullaart, E., Chang, G. T. G., Kluft, C., and Wijngaards, G. (1982) *Thromb. Haemostasis* **48,** 266–269
22. Rijken, D. C., Van Hinsbergh, V. W. M., and Sens, E. H. C. (1984) *Thromb. Res.* **33,** 145–153
23. Laemmli, U. K. (1970) *Nature* **227,** 680–685
24. Rånby, M., and Wallén, P. (1981) *Prog. Fibrinolysis* **5,** 232–235
25. Robbins, K. C., Summaria, L., Hsieh, B., and Shah, R. J. (1967) *J. Biol. Chem.* **242,** 2333–2342
26. Sumi, H., and Robbins, K. C. (1983) *J. Biol. Chem.* **258,** 8014–8019
27. Van der Graaf, F., Tans, G., Bouma, B. N., and Griffin, J. H. (1982) *J. Biol. Chem.* **257,** 14300–14305
28. van der Graaf, F., Greengard, J. S., Bouma, B. N., Kerbiriou, D. M., and Griffin, J. H. (1983) *J. Biol. Chem.* **258,** 9669–9675
29. Rijken, D. C. (1984) *Haemostasis* **14,** 14 (Abstr. 18)