Proteolytic activation of tissue plasminogen activator by plasma and tissue enzymes

Akitada Ichinose, Walter Kisiel and Kazuo Fujikawa*

Department of Biochemistry, University of Washington, Seattle, WA 98195, USA

Received 13 August 1984

Tissue kallikrein and factor Xa were found to activate tissue plasminogen activator (t-PA) at a rate comparable with that of plasmin. During the activation reaction, the single-chain molecule was converted into a two-chain form. A slight t-PA activating activity was also found in plasma kallikrein. Other activated coagulation factors, factor XIIa, factor XIa, factor IXa, factor VIIa, thrombin and activated protein C had no effect on t-PA activation. t-PA was also activated by a tissue kallikrein-like enzyme that was isolated from the culture medium of melanoma cells. These results indicate that tissue kallikrein and factor Xa may participate in the extrinsic pathway of human fibrinolysis.

Fibrinolysis *t-PA* *Tissue kallikrein* *Coagulation factor*

1. INTRODUCTION

Tissue plasminogen activator (t-PA) is widely distributed in tissues [1] and plays an important role in fibrinolysis, converting plasminogen to plasmin. Plasmin in turn is responsible for fibrin clot dissolution. t-PA is released into the circulating blood from the vascular endothelial cells [2], although at an extremely low concentration [3].

The incorporation of diisopropylfluorophosphate into an active form of t-PA [4,5] and the primary structure of t-PA predicted from its c-DNA sequence [6] revealed that this protein is a typical serine protease. t-PA isolated from tissues [5,7] or cell culture [4] is an active enzyme that is composed of two polypeptide chains linked by a disulfide bond. t-PA isolated from the blood vessel perfusate is present in a zymogen form as a single polypeptide chain [8]. t-PA, however, was readily converted into the two-chain form by a protease(s) present in the perfusate and this conversion was prevented by the addition of protease inhibitors [9].

These findings suggest that an enzyme(s) present in tissue may be responsible for the conversion of the zymogen of t-PA into the active form. Furthermore, there may be a plasma enzyme(s) that activates t-PA following its release into blood. Thus far, plasmin and trypsin have been shown to activate the zymogen of t-PA in vitro. In the presence of plasmin, a single internal peptide bond in t-PA is cleaved to form the two-chain molecule [7,10]. Plasmin, however, must first be generated before it can participate in this reaction. This suggests that some other plasma serine protease such as a coagulation factor may trigger the activation of t-PA.

We describe the results of the activation of t-PA by both tissue kallikrein and several blood coagulation enzymes. A part of this study was presented in [11].

Abbreviations: t-PA, tissue plasminogen activator; -*p*-NA, -*p*-nitro-anilide; SBTI, soybean trypsin inhibitor; LBTI, lima bean trypsin inhibitor; HMW-kininogen, high molecular mass kininogen; TBS, 50 mM Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl. (Here, t-PA and vascular plasminogen activator are considered to be identical proteins. Thus, both proteins are referred to as t-PA.)

* To whom correspondence should be addressed

Published by Elsevier Science Publishers B.V.

2. MATERIALS AND METHODS

Serum-free conditioned medium of melanoma cells (Bowes) was kindly provided by Charles Hart of ZymoGenetics, Seattle, WA. t-PA was purified in the presence of 20 KIU units/ml of aprotinin by the same method as described in [4]. The final preparation of t-PA was concentrated by dialysis first against 40% polyethylene glycol-20 000 and subsequently against 50 mM Tris–HCl buffer (pH 7.5) containing 1 M NaCl and 0.01% Tween-80. Homogeneous human coagulation factors, factor XIIa [12], factor XI [13], prekallikrein [14], factor VIIa [15], thrombin [16], activated protein C [17], antithrombin-III [18] and factor X [19] were purified according to methods published from this laboratory. Factor XI and prekallikrein were activated by factor XIIa. Factor X was activated by the factor X-activating enzyme from Russell's viper venom. Factor IXa [20], HMW-kininogen [21], tissue factor [22] were prepared by published methods. Porcine pancreatic kallikrein (200 units/mg) was purchased from Calbiochem and further purified at 4°C as follows: 5 mg of tissue kallikrein was dissolved in 2 ml of TBS and passed through a LBTI–Sepharose column (1.5 × 6 cm) to remove any contaminating trypsin. The unadsorbed fraction was then applied to an aprotinin–Sepharose column (1.5 × 8 cm) equilibrated with TBS. Protein was eluted with 0.1 M Tris–HCl (pH 7.5) containing 1 M NaCl and 0.5 M benzamidine. Following concentration of the eluate, the sample was applied to a Sephadex G-150 column (0.9 × 50 cm) equilibrated with TBS. The active fractions, as determined by assay with Val-Leu-Arg-*p*-NA, were pooled and concentrated to 0.5 ml by a Micro-ProDiCon concentrator. Monospecific antisera against human factor X was raised in rabbits by the standard procedure [23]. Affinity-purified anti-factor X was prepared as described [24].

D-Val-Gly-Arg-*p*-NA (S-2322) was obtained through the courtesy of Dr P. Friberger, Kabi, Molndal, Sweden. D-Val-Leu-Arg-*p*-NA (S-2266) was purchased from Helena Lab. *p*-Aminobenzamidine–Sepharose with a spacer of ϵ-aminocaproic acid [11] were prepared by the published methods. SBTI (70 mg), LBTI (100 mg) and aprotinin (25 mg) were bound to 3 g activated CH–Sepharose according to Pharmacia's instruction.

The activation of t-PA was determined by measuring the amidolytic activity of the two-chain form of t-PA with Val-Gly-Arg-*p*-NA. Single-chain t-PA (3 μg) in 15 μl of 50 mM Tris–HCl (pH 7.5) containing 1 mM NaCl and 0.01% Tween-80 was added to a catalytic amount of enzyme in 85 μl of 50 mM Tris–HCl, pH 7.5. At appropriate periods of incubation at 37°C, 10 μl of aprotinin (at a final concentration of 5 μg/ml) and 800 μl of 1 mM Val-Gly-Arg-*p*-NA were added and incubated at 37°C. The initial rate of *p*-nitroaniline release was determined and the enzyme activity was expressed as $\Delta A_{405\ nm}$/Min per μg of protein. Aliquots (35 μl) were also withdrawn at the same times and mixed with 3 μl of 1 M Tris–HCl (pH 6.9) containing 2% SDS and 5% bromophenol blue, heated at 80°C for 20 min with or without 2% 2-mercaptoethanol and subjected to SDS–slab electrophoresis. SDS–slab gel electrophoresis was performed in 10% polyacrylamide gel as described by Laemmli. Protein bands were visualized by silver staining. t-PA was iodinated by the method of [25] using Iodo-Beads (Pierce). Protein concentration of t-PA was determined by the method of Lowry and the published $E^{1\%}_{280\ nm}$ values [12–21] were used for determination of the other protein concentrations.

3. RESULTS

3.1. *Activation of t-PA by tissue kallikrein*

The two-chain of t-PA is the principal form of the protein purified from the culture medium of melanoma cells. The addition of aprotinin to the culture medium increased the amount of the single-chain t-PA with a concomitant decrease of the two-chain t-PA [4]. This suggests that plasmin, tissue kallikrein or some other trypsin-like enzymes sensitive to aprotinin, may cause the formation of the two-chain molecule by limited proteolysis.

Accordingly, a purified preparation of porcine pancreatic kallikrein (tissue kallikrein) was examined for its ability to activate t-PA. The single-chain of t-PA was incubated with tissue kallikrein at a substrate/enzyme weight ratio of 50/1. The amidolytic activity of t-PA increased gradually and reached a maximum in approx. 2 h (fig. 1A). At this time, a 10-fold increase of enzyme activity was observed over the zero-time control. The activation by tissue kallikrein was completely inhibited

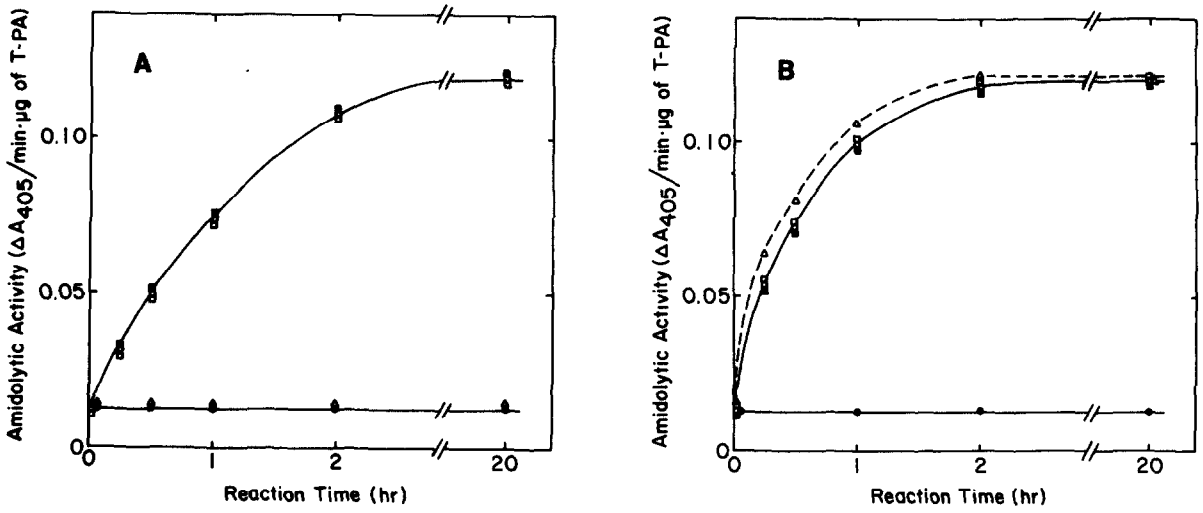

Fig. 1. The time curve for the activation of t-PA by tissue kallikrein (A) and factor Xa (B). (A) The single-chain of t-PA (3 μg) was incubated with 26 ng of tissue kallikrein and amidolytic activity was measured as described in section 2. The complete reaction, ○; with 5 μg/ml aprotinin, ●; with 5 μg/ml SBTI, □; with 5 μg/ml LBTI, ■; t-PA alone, △. (B) t-PA (3 μg) was incubated with 60 ng of factor Xa in the same condition as described in (A). The complete reaction, ○; the reaction with 5 μg/ml antithrombin-III/heparin complex, ●; with 5 μg/ml SBTI or LBTI, □; with 5 μg/ml aprotinin, ■; with 2 mM $CaCl_2$, △.

by aprotinin, but not by SBTI or LBTI. These data are consistent with the sensitivity of tissue kallikrein to these 3 protein inhibitors [26].

The molecular changes of t-PA associated with its activation were examined by SDS-slab gel electrophoresis. In reduced gels, the starting material showed one major doublet corresponding to M_r 64000 along with two minor bands of M_r 36000 and 34000 (fig. 2A). The two minor bands reflect the presence of the two-chain form of t-PA in the starting material. During the activation by tissue kallikrein, the intensity of the M_r 64000 band decreased, while the bands of M_r 36000 and 34000 increased. After 2 h, the single-chain molecule was completely converted to the two-chain form (fig. 2A). To ascertain the proportional relationship between the formation of the two-chain form and the generation of amidolytic activity, following experiments were performed. The radio-labelled single-chain t-PA was activated under the same condition as in fig. 1. Amidolytic activities were measured with the samples taken at the various times and aliquots from the same samples were also run on SDS- gels. The gels were sliced and radioactivity of the bands corresponding to M_r 64000 and 34000- 36000 were measured. The generation of enzyme activities were proportional to the amount of two-chain formed throughout the activation (fig. 3).

In non-reduced gels, the doublet of M_r 64000 remained constant throughout the reaction, indicating that the two-chains were linked by a disulfide bond(s). Additional cleavage of t-PA was not observed, even after prolonged incubation for 20 h. The effect of SBTI, LBTI, antithrombin III/heparin complex and hirudin on the cleavage of the t-PA molecule by tissue kallikrein were also examined by SDS-slab gel electrophoresis. Complete inhibition of the cleavage of t-PA was observed in the presence of aprotinin, while the other inhibitors had no effect on the activation reaction (not shown).

3.2. *Isolation of tissue kallikrein-like protease from the culture medium of melanoma cells and its action on t-PA*

One liter of the serum-free culture medium (without aprotinin) was dialyzed extensively overnight at 4°C against 50 mM Tris-HCl buffer (pH 7.5) containing 0.01% Tween-80. The dialyzed

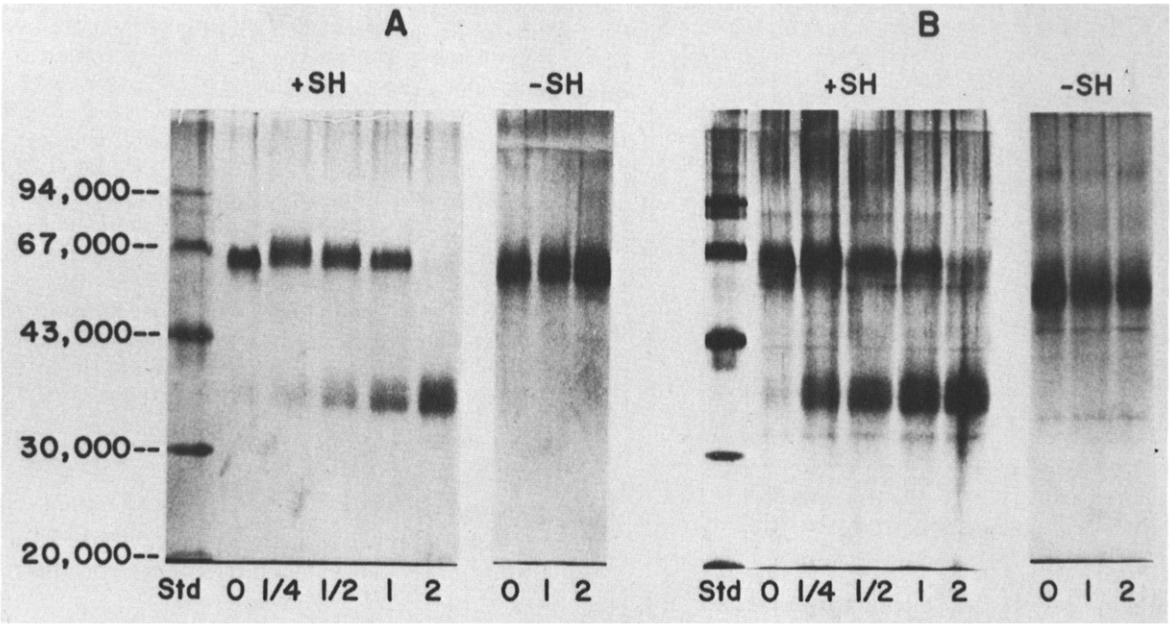

Fig. 2. SDS-slab gel electrophoresis of the activation products of t-PA by tissue kallikrein and factor Xa. The same reaction mixtures containing 1 μg of t-PA as described in the legend of fig.1 were subjected to gel electrophoresis. Numbers at the bottom of the gels represent the reaction time in hours.

sample was clarified by centrifugation and applied to three different specific affinity columns including lysine-Sepharose, SBTI-Sepharose and aprotinin-Sepharose. Enzyme activity in the dialyzed sample failed to bind either lysine-Sepharose or SBTI-Sepharose. This suggests that the enzyme present in the melanoma culture medium is neither plasmin nor trypsin. The culture medium was then applied to an aprotinin-Sepharose column (2.0×5 cm) previously equilibrated with TBS. The column was eluted with 0.5 M benzamidine in TBS, dialyzed and concentrated against TBS to a final vol. of 0.5 ml. As shown in table 1, amidolytic activity was detected in this fraction when it was assayed with D-Val-Leu-Arg-*p*-NA. Furthermore, this activity was very sensitive to aprotinin, but not to SBTI or LBTI. This inhibitor sensitivity is identical with that of porcine pancreatic kallikrein (see fig. 1A).

Since a limited amount of the tissue kallikrein-like activity was obtained, the activation of t-PA by this enzyme was examined only after 20 h incubation. The single-chain of t-PA was incubated with 20 μl of the sample of tissue kallikrein-like enzyme that was equivalent to approximately 10% of the amidolytic activity of porcine kallikrein used in the experiment shown in fig. 1A. The generation of amidolytic activity of t-PA was observed. About 70% of the single-chain t-PA was converted into the two-chain form. This reaction was completely inhibited by aprotinin, but not by LBTI (table 1). These results indicate that the melanoma cells secrete tissue kallikrein or a tissue kallikrein-like protease into the culture medium, and this enzyme is responsible for the formation of the two-chain form of t-PA.

3.3. *Activation of t-PA activated coagulation factors*

Various activated coagulation factors were incubated with single-chain t-PA at an enzyme to substrate weight ratio of 1:50. The contact factors, factor XIIa, factor XIa and kallikrein, were studied in the presence or absence of cofactors, such as HMW-kininogen and kaolin. Effect of phospholipid, $CaCl_2$ and tissue factor were also examined with the vitamin K-dependent proteases, such as factor VIIa, factor IXa, factor Xa, and ac-

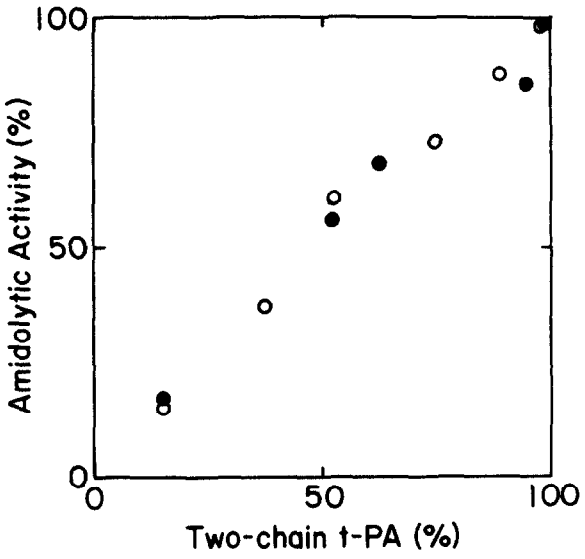

Fig. 3. The proportional relationship between the formation of the two-chain t-PA and the generation of its amidolytic activity. Single-chain t-PA was activated under essentially the same condition as described in fig.1 except that 0.15 μg of ^{125}I-t-PA (3.6×10^5 cpm/μg) was added to the reaction mixture. At 0, 1/4, 1/2, 1, 2 and 4 h, aliquots were taken and amidolytic activities were measured as described in section 2. Other aliquots were run on the reduced 7.5% SDS-gels. The gels were sliced after staining and radioactivity of the bands corresponding to M_r 64000 and 34000–36000 were measured. The enzyme activity generated and the amount of two-chain formed were expressed as percent of the maximum (4 h). Activation by tissue kallikrein, ○ and by factor Xa, ●.

tivated protein C. These cofactors are essential for maximum activity of these enzymes in the coagulation cascade [27].

Table 2 shows the results of the activation of t-PA by several coagulation factors and tissue enzymes. Factor Xa was found to readily activate t-PA and this reaction leveled off at about 2 h incubation (fig. 1B). The activation of t-PA by factor Xa was not observed in the presence of antithrombin III/heparin complex. LBTI or aprotinin

Table 1

Enzyme activity of tissue kallikrein-like protease from the culture medium of melanoma cells

Reactions	Enzyme activity		
	Reaction without inhibitor	Reaction with aprotinin	Reaction with LBTI
Amidolytic act.[a]	0.440	0.071	0.441
Activating act.[b] for T-PA	0.100	0.018	0.093

[a] Tissue kallikrein-like protease isolated from the culture medium of melanoma cells, 20 μl, was incubated for 2 h with 200 μl of 1 mM D-Val-Leu-Arg-p-NA and 80 μl of TBS in the presence or absence of 5 μg/ml of the inhibitor. The absorbance was measured after the addition of 700 μl of 7% acetic acid. Enzyme activity was expressed as $\Delta A_{405\ nm}$/20 h per 20 μl

[b] 3 μg t-PA was incubated with 20 μl of tissue kallikrein-like protease for 20 h in the presence or absence of 5 μg/ml of the inhibitors and t-PA was assayed in the same conditions as described in section 2. Enzyme activity was expressed as $\Delta A_{405\ nm}$/min per μg of t-PA

Table 2

Activation of t-PA by tissue and plasma enzymes

Activators	Amidolytic activity of t-PA ($\Delta A_{405\ nm}$/min per μg of t-PA)	
	30-min incubation	2-h incubation
Tissue enzymes		
Pancreatic kallikrein	0.050	0.108
Bovine trypsin	0.099	0.122
Human plasma enzymes		
Kallikrein	–	0.040
Factor XIIa	–	0.024
Factor XIa	–	0.014
Factor IXa	–	0.012
Factor VIIa	–	0.013
Factor Xa	0.071	0.118
α-Thrombin	–	0.012
Activated protein C	–	0.014
Plasmin	0.095	0.122
Without activator	0.012	0.012

The single-chain of t-PA (3 μg) was activated by the enzymes at an enzyme/substrate weight ratio of 1/50 under the same conditions as described in the legend of fig. 1B. t-PA activity was assayed with D-Val-Gly-Arg-p-NA as described in section 2. –, not determined.

had no detectable effect on the activation reaction, while SBTI had a slight inhibitory effect. During the activation of t-PA by factor Xa, single-chain t-PA was completely converted into the two-chain form in 2 h, in parallel with the generation of its amidolytic activity (fig. 2A and 3). This conversion was also completely inhibited by a complex of antithrombin III/ heparin and anti-human factor Xa, but not by hirudin, SBTI or LBTI. These results exclude a possible activation by contaminating enzyme in the factor Xa preparation. $CaCl_2$ at 2 mM slightly stimulated the activating activity of factor Xa (fig. 1B), but phospholipid had no effect even in the presence of $CaCl_2$. After a 2 h incubation period, a small amount of t-PA amidolytic activity was obtained by plasma kallikrein. The ability of other coagulation factors including thrombin, factor VIIa, factor IXa, factor XIa, factor XIIa and activated protein C to activate t-PA was negligible, even in the presence of their cofactors (table 2).

4. DISCUSSION

The experiments presented here demonstrate that factor Xa readily converts single-chain t-PA to its two-chain form with a concomitant increase in t-PA amidolytic activity. Other activated coagulation factors including thrombin, factor VIIa, factor IXa, factor XIa, factor XIIa and activated protein C failed to activate t-PA. The efficiency of t-PA activation by factor Xa was similar to that observed for plasmin and tissue kallikrein. The activation of t-PA by factor Xa was not appreciably affected by the presence of either phospholipid, $CaCl_2$ or both. This represents the first known instance where factor Xa proteolysis is unaffected by these cofactors. Whether the activation of t-PA by factor Xa is a physiologically meaningful reaction remains to be determined.

Like t-PA, tissue kallikrein is distributed in various tissues. A trace amount of tissue kallikrein is also present in plasma [28]. The best known function of tissue kallikrein is the generation of the vasodilator peptide, kallidin, from low-molecular-mass kininogen or HMW-kininogen. The present study reveals that the activation of t-PA is another possible biological function of this protease.

The t-PA preparations isolated from uterus [5], heart [7] and melanoma cells [4] in the absence of aprotinin are principally in the two-chain form. Thus, it was of interest to identify and isolate the aprotinin-sensitive protease present in the culture medium responsible for the conversion of single-chain t-PA to the two-chain form. We have isolated a tissue kallikrein-like enzyme from the culture medium that converts single-chain t-PA to its two-chain form in the test tube. The inhibitor sensitivity and substrate specificity of the melanoma tissue kallikrein were identical to that observed for the pancreatic kallikrein used here to activate t-PA. Thus, it is reasonable to conclude that the tissue kallikrein isolated from the medium of melanoma cells represents a major, if not sole, activator of single-chain t-PA secreted by the melanoma cells.

Although protease inhibitors are added during the purification procedures, single-chain t-PA free of the two-chain form has not been obtained from the culture of melanoma cells. Authors in [29] and [30] reported that a portion of the amidolytic activity or plasminogen-activating activity found in their single-chain preparation was due to the intrinsic enzyme activity of single-chain form. Recently, controversial results were reported in [31] that the entire amidolytic or plasminogen activating activity of the single-chain t-PA preparation was accounted for by the contaminating two-chain form.

The results presented in fig. 3 are similar to [31] and show that the enzyme activity due to the single-chain form in our starting material is certainly lower than the 10–12% that was obtained by amidolytic activity. This indicates that our single-chain preparation was contaminated by the two-chain form and this two-chain form contributes to most enzyme activity in the starting materials. This contamination of two-chain form was detected on the gels by sensitive silver staining (fig.2). Thus, the increase of enzyme activity of single-chain t-PA during the activation could be considerably greater than 7- to 10-fold described here. If the single-chain has no enzyme activity, as claimed in [31], an activation of t-PA by an enzyme such as tissue kallikrein or factor Xa is necessary for the initiation of fibrinolysis.

ACKNOWLEDGEMENTS

The authors gratefully thank Dr E.W. Davie for his many helpful discussions and encouragement

throughout the present study. The skillful technical assistance of Lee Hendrickson is gratefully acknowledged. This study was supported in part by a National Institute of Health grant, HL 16919 and an American Heart Association grant, 80-681.

REFERENCES

[1] Wilson, E.L., Becker, M.L.B., Hoal, E.G. and Dowdle, E.B. (1980) Cancer Res. 40, 933–938.
[2] Aoki, N. (1974) J. Biochem. 75, 731–741.
[3] Rijken, D.C., Juhan-Vague, I., De Cock, F. and Collen, D. (1983) J. Lab. Clin. Med. 101, 274–284.
[4] Rijken, D.C. and Collen, D. (1981) J. Biol. Chem. 256, 7035–7041.
[5] Rijken, D.C., Wijngaards, G., De Jong, M.Z. and Welbergen, J. (1979) Biochim. Biophys. Acta 580, 140–153.
[6] 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.
[7] Wallen, P., Bergsdorf, N. and Ranby, M. (1982) Biochim. Biophys. Acta 719, 318–328.
[8] Binder, B.R., Spragg, J. and Austen, K.F. (1979) J. Biol. Chem. 254, 1998–2003.
[9] Husain, S.S., Lipinski, B. and Gurewich, V. (1981) Proc. Natl. Acad. Sci. USA 78, 4265–4269.
[10] Wallen, P., Pohl, G., Bergsdorf, N., Ranby, M., Ny, T. and Jornvall, H. (1983) Eur. J. Biochem. 132, 681–686.
[11] Ichinose, A., Kisiel, W. and Fujikawa, K. (1984) Fed. Proc. 43, 1957 (Abs.).
[12] Fujikawa, K. and Davie, E.W. (1981) Methods Enzymol. 80, 198–211.
[13] Kurachi, K. and Davie, E.W. (1981) Methods Enzymol. 80, 211–220.
[14] Heimark, R.L. and Davie, E.W. (1981) Methods Enzymol. 80, 157–172.
[15] Hedner, U. and Kisiel, W. (1983) J. Clin. Invest. 71, 1836–1841.
[16] Kisiel, W. (1979) J. Clin. Invest. 64, 761–769.
[17] Canfield, W.M. and Kisiel, W. (1982) J. Clin. Invest. 70, 1260–1272.
[18] Mahoney, W.C., Kurachi, K. and Hermodson, M.A. (1980) Eur. J. Biochem. 105, 545–552.
[19] McMullen, B.A., Fujikawa, K., Kisiel, W., Sasagawa, T., Howald, W.N., Kwa, E.Y. and Weinstein, B. (1983) Biochemistry 22, 2875–2884.
[20] Amphlett, G.W., Kisiel, W. and Castellino, F.J. (1981) Arch. Biochem. Biophys. 208, 576–585.
[21] Kato, H., Nagasawa, S. and Iwanaga, S. (1981) Methods Enzymol. 80, 172–198.
[22] Quick, A.J. (1966) Hemorrhagic Disease and Thrombosis, pp. 438–439, Lea and Febiger, Philadelphia.
[23] Harboe, N. and Ingild, A. (1973) Immunization, isolation of immunoglobulins and estimation of antibody titre, in: A Manual of Quantitative Immunoelectrophoresis (Axelson, N.H. et al. eds) pp. 161–164, Universitetsforlaget, Oslo.
[24] Leytus, S., Chung, D.W., Kisiel, W., Kurachi, K. and Davie, E.W. (1984) Proc. Natl. Acad. Sci. USA 81, 3699–3702.
[25] Markwell, M.A.K. (1982) Anal. Biochem. 125, 427–432.
[26] Chao, J., Woodley, C., Chao, L. and Margolius, H.S. (1983) J. Biol. Chem. 258, 15173–15178.
[27] Davie, E.W., Fujikawa, K., Kurachi, K. and Kisiel, W. (1979) Adv. Enzymol. 48, 277–318.
[28] Geiger, R., Clausnitzer, B., Fink, E. and Fritz, H. (1980) Z. Physiol. Chem. 361, 1795–1803.
[29] Ranby, M., Bergsdorf, N. and Nilsson, T. (1982) Thromb. Res. 27, 175–183.
[30] Rijken, D.C., Hoylaerts, M. and Collen, D. (1982) J. Biol. Chem. 257, 2920–2925.
[31] Andreasen, P.A., Nielsen, L.S., Grondahl-Hansen, J., Skriver, L., Zeuthen, J., Stephens, R.W. and Dano, K. (1984) EMBO J. 3, 51–56.