Lorand, L., & Conrad, S. M. (1984) *Mol. Cell. Biochem. 58*, 9-35.
Lorand, L., Urayama, T., De Kiewiet, J. W. C., & Nossel, H. L. (1969) *J. Clin. Invest. 48*, 1954-1064.
Lorand, L., Dailey, J. E., & Turner, P. M. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1057-1059.
Lowe, M. E., & Ashwell, G. (1982) *Arch. Biochem. Biophys. 216*, 704-710.
Miller, G. L. (1959) *Anal. Chem. 31*, 964.
Molnar, J., Gelder, F. B., Lai, M. Z., Siefring, G. E., Jr., Credo, R. B., & Lorand, L. (1979) *Biochemistry 18*, 3909-3916.
Mosesson, M. W., & Amrani, D. L. (1980) *Blood 56*, 145-158.
Putnam, F. W., Ed. (1975) *The Plasma Proteins* Vol. 2, pp 2-50, Academic, New York.
Saba, T. M., Blumenstock, F. A., Weber, P., & Kaplan, J. E. (1978) *Ann. N.Y. Acad. Sci. 312*, 43-55.
Salonen, E.-M., Vartio, T., Hedman, K., & Vaheri, A. (1984) *J. Biol. Chem. 259*, 1496-1501.
Turner, P. M., & Lorand, L. (1988) *FASEB J. 2*, A1783 (Abstr. 8635).
Upchurch, H. F., Conway, E., Patterson, M. K., Jr., Birkbichler, P. J., & Maxwell, M. D. (1987) *In Vitro Cell. Dev. Biol. 23*, 795-800.
Vuento, M., & Vaheri, A. (1979) *Biochem. J. 183*, 331-337.
Yamada, K. M. (1983) *Annu. Rev. Biochem. 52*, 761-799.

The Single-Chain Form of Tissue-Type Plasminogen Activator Has Catalytic Activity: Studies with a Mutant Enzyme That Lacks the Cleavage Site†

Jeri Ann Boose,‡ Esa Kuismanen,§ Robert Gerard,∥,⊥ Joe Sambrook,∥ and Mary-Jane Gething*,‡,∥

Howard Hughes Medical Institute, Department of Biochemistry, and Department of Internal Medicine, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75235, and Department of Biochemistry, University of Helsinki, Unioninkatu 35, 00170 Helsinki, Finland

Received February 29, 1988; Revised Manuscript Received August 15, 1988

ABSTRACT: Tissue-type plasminogen activator (t-PA), the serine protease responsible for catalyzing the production of plasmin from plasminogen at the site of blood clots, is synthesized as a single-chain polypeptide precursor. Proteolytic cleavage at the C-terminal side of Arg_{275} generates a two-chain form of the enzyme whose subunits are held together by a single disulfide bond. We have measured the activities of both forms of the wild-type enzyme, as well as that of a mutant enzyme ($Arg_{275} \rightarrow Gly$), created by oligonucleotide-directed mutagenesis, that cannot be cleaved into a two-chain form. Both types of single-chain t-PAs are enzymatically active and exhibit identical V_{max} and K_m values when assayed with synthetic peptide substrates, indicating that the single amino acid change had no effect on the amidolytic activity of the enzyme. However, cleavage of wild-type t-PA into the two-chain form results in increased activity both on a peptide substrate and on the natural substrates Lys- and Glu-plasminogen in the absence or presence of stimulation by soluble fibrin. The enhanced activity is due to a 3-5-fold increase in the V_{max} of the cleaved enzyme, rather than to any change in the K_m values for the various substrates. During incubation with plasminogen, the single-chain form of wild-type t-PA is converted to the two-chain form by plasmin generated during the reaction. This conversion, from the less active to the more active form of the enzyme, results in a reaction that displays biphasic kinetics. Both the single-chain, cleavage-minus t-PA and the two-chain, wild-type t-PA are stimulated by soluble fibrin, although the single-chain form requires higher levels of fibrin to achieve maximum activity. Finally, both forms of the enzyme can be inhibited to the same extent by the serpin plasminogen activator inhibitor 1 (PAI-1).

Tissue-type plasminogen activator (t-PA), a serine protease that plays a key role in fibrinolysis, converts the zymogen plasminogen to its active form, plasmin, by the specific cleavage of a single peptide bond. Plasmin is a serine protease of broad specificity that degrades the fibrin network of blood clots [reviewed in Collen (1980) and Dano et al. (1985)]. The rate of activation of plasminogen by t-PA increases dramatically in the presence of fibrin (Camiolo et al., 1971), so that high concentrations of plasmin are generated on the surface of a clot (Bergmann et al., 1983; Hoylaerts et al., 1982). This property has led to the clinical application of t-PA as a thrombolytic agent (Matsuo et al., 1981; Collen & Linjen, 1984; Collen, 1987), and the enzyme has become a major focus of scientific attention.

Several groups have cloned cDNA copies of the mRNA coding for t-PA (Pennica et al., 1983; Edlund et al., 1983; Fisher et al., 1985; Kaufman et al., 1985; Harris et al., 1986; Sambrook et al., 1986). Sequencing of these cDNAs and genomic clones has shown that the precursor form of t-PA is 562 amino acids in length (Pennica et al., 1983; Edlund et al., 1983; Pohl et al., 1984; Fisher et al., 1985; Friezner-Degan et al., 1986; Harris et al., 1986). Removal of a hydrophobic signal sequence and a hydrophilic pro sequence from the precursor generates the mature polypeptide of 527 amino acids,

† This work was supported in part by grants from the American Cancer Society (M.J.G.), the Bugher Fund of the American Heart Association (J.S.), and the Texas Affiliate of the American Heart Association (R.G.). E.K. was supported by an EMBO fellowship.

* Address correspondence to this author at the Howard Hughes Medical Institute, University of Texas Southwestern Medical Center.
‡ Howard Hughes Medical Institute, University of Texas Southwestern Medical Center.
§ University of Helsinki.
∥ Department of Biochemistry, University of Texas Southwestern Medical Center.
⊥ Department of Internal Medicine, University of Texas Southwestern Medical Center.

0006-2960/89/0428-0635$01.50/0

which contains 4 potential glycosylation sites, 3 of which appear to be used (Pohl et al., 1984, 1987; Vehar et al., 1984). t-PA is synthesized and secreted from cells as a single-chain protein (M_r ~66 000). However, after exposure to proteases such as plasmin or trypsin, t-PA is cleaved on the C-terminal side of Arg_{275} (Pohl et al., 1984; Ichinose et al., 1984) to generate the two-chain form of the enzyme, whose subunits are linked by a single disulfide bond (Wallen et al., 1982; Rijken & Collen, 1981). The smaller of the two subunits [the light (L) chain of 252 amino acids; M_r ~33 000] is derived from the C-terminal region of the molecule and carries a catalytic site composed of the characteristic triad His_{322}, Asp_{371}, and Ser_{478}. The amino acid sequences surrounding these residues are highly homologous to the corresponding sequences of other serine proteases (Pennica et al., 1983; Ny et al., 1984; Wallen et al., 1983; Dano et al., 1985). The larger of the 2 subunits [the heavy (H) chain of 275 amino acids; M_r ~35 000] contains several domains that are homologous to structures found in other plasma proteins including 2 kringle domains, an epidermal growth factor like domain, and a fibronectin fingerlike domain (Patthy, 1985).

Plasma serine proteases are normally secreted in a proenzyme or zymogen form. These catalytically inactive forms are later activated by proteolytic cleavage at a specific site [reviewed by Neurath and Walsh (1976)]. While the enzymatic activity of the two-chain form of t-PA is undisputed, there has been extensive debate about the activity of the single-chain form of the enzyme. Andreasen et al. (1984), using t-PA purified from human melanoma cells, reported that only the two-chain form of the molecule is able to activate plasminogen in an indirect chromogenic assay and concluded that single-chain t-PA is a true zymogen. Using a similar experimental approach, Ichinose et al. (1984) reached the same conclusion. By contrast, Rijken et al. (1982) reported that the single- and two-chain forms of human melanoma t-PA exhibit equal fibrinolytic activity in a fibrin clot lysis assay. However, other investigators, using single- and two-chain t-PA from both human and porcine sources, have concluded that the single-chain form of t-PA is 2–15-fold less active in hydrolyzing low molecular weight chromogenic substrates and in activating plasminogen (Wallen et al., 1981, 1982, 1983; Ranby, 1982; Ranby et al., 1982, 1983), or in reacting with inhibitors or ligands that bind to the active site (Wallen et al., 1981; Higgins & Lamb, 1986; Ranby et al., 1982). Using recombinant human t-PA, Tate et al. (1987) found that single-chain t-PA is significantly less active than the two-chain form in hydrolyzing chromogenic substrates or plasminogen, except in the presence of physiological levels of fibrinogen, when the two forms of the enzyme appear to have equivalent activities.

A possible explanation for these conflicting results is that preparations of single-chain t-PA may be partially or wholly converted to two-chain t-PA by contaminating proteases, or by plasmin generated during the activity assay itself. In this paper, we have determined the catalytic activity of a single-chain form of human t-PA that cannot be proteolytically processed to the two-chain form. We have used an SV40 vector system to express a cDNA encoding wild-type human t-PA, as well as a mutant t-PA cDNA which has been altered at the H-chain/L-chain cleavage site by employing oligonucleotide-directed mutagenesis to change Arg_{275} to a glycine residue. We have used several different assays to compare the enzyme activities of the single-chain and two-chain forms of the wild-type protein with that of the single-chain form of the cleavage-minus mutant and have employed gel electrophoresis of radiolabeled t-PAs to confirm the molecular form of the enzymes before and during the activity assays. We conclude that single-chain forms of t-PA are, indeed, catalytically active and responsive to stimulation by soluble fibrin or inhibition by PAI-1. Cleavage of the enzyme has no effect on its affinity for its substrates. Both the increased activity of two-chain t-PA on a peptide substrate and the enhanced plasminogen activation in the presence of soluble fibrin are due to an increase in the V_{max} of the enzyme.

Experimental Procedures

Reagents. The chromogenic substrates Spectrozyme t-PA (methylsulfonyl-D-cyclohexyltyrosyl-glycyl-arginine-*p*-nitroaniline acetate) and Spectrozyme PL (H-D-norleucyl-hexahydrotyrosyl-lysine-*p*-nitroanilide diacetate salt), Glu-plasminogen, soluble fibrin (DESAFIB), and a goat anti-human melanoma t-PA polyclonal antiserum were purchased from American Diagnostica Inc., Greenwich, CT. Lys-plasminogen was from Calbiochem, San Diego, CA. Plasmin, aprotinin, and soybean trypsin inhibitor were obtained from the Sigma Chemical Co. (St. Louis, MO). Recombinant wild-type t-PA (entirely in the two-chain form) immunopurified following expression from Chinese hamster ovary cells (Kaufman et al., 1985) was a kind gift of The Wellcome Foundation Ltd. (Beckenham, U.K.).

Recombinant DNA Techniques. Buffers and reaction conditions for restriction enzymes, T4 DNA ligase, and the Klenow fragment of DNA polymerase I were those listed by the commercial source, New England Biolabs (Beverly, MA). Isolation of DNA fragments, preparation of plasmid DNAs, and other standard recombinant DNA techniques were carried out as described [see Sambrook et al. (1989) and other references cited therein]. Transformation of *Escherichia coli* DH1 or TG1 cells was done by the method of Hanahan (1983).

Site-Specific Mutagenesis of t-PA cDNA Using a Mismatched Oligonucleotide. The cloning of a full-length human t-PA cDNA, beginning with polyadenylated mRNA purified from the Bowes melanoma cell line, has been described elsewhere (Sambrook et al., 1986). The wild-type t-PA sequences were excised from plasmid pSV/t-PA35 (Sambrook et al., 1986) by digestion with *Kpn*I and *Bam*HI restriction endonucleases and inserted between the *Kpn*I and *Bam*HI sites of the double-stranded (ds) replicative form of M13mp19 phage DNA. An 18-mer oligonucleotide that corresponded to sequences encoding amino acids spanning the t-PA cleavage site, except for the required mismatch designed to alter Arg_{275} to Gly (see Figure 1), was used to carry out oligonucleotide-directed mutagenesis. Procedures for the identification and characterization of the desired mutants have been described in detail (Zoller & Smith, 1985; Doyle et al., 1985). Once the desired base substitution had been confirmed, the ds replicative forms of two independently derived mutant phage DNAs were prepared and purified by centrifugation on CsCl gradients. The *Kpn*I–*Bam*HI DNA fragments encoding the mutant t-PA were isolated and used to replace the equivalent wild-type sequences in the SV/t-PA35 recombinant expression vector (Sambrook et al., 1986).

Generation of SV40-HA Recombinant Virus Stocks and Infection of CV-1 Cells. SV40/t-PA recombinant genomes containing the wild-type and mutant forms of the t-PA gene were transfected into CV-1 cells using DEAE-dextran and chloroquine as previously described (Doyle et al., 1985; Sambrook et al., 1986). High-titer virus stocks were developed by serial passage and used to infect fresh monolayers of CV-1 cells to produce wild-type and mutant t-PA proteins. Con-

ditions for growth and infections of CV-1 cells were as described previously (Doyle et al., 1985).

Assays for t-PA. Infected CV-1 monolayers producing t-PA were washed 3 times at 18-h postinfection with Dulbecco's minimal essential medium (DMEM) lacking serum and then incubated in DMEM for a further 46–48 h before samples of medium were collected and assayed. Medium was also collected from mock-infected cells treated in the same manner. For some experiments, wild-type and cleavage-minus mutant t-PAs were immunopurified as described by Einarrson et al. (1985) using a rabbit anti-human t-PA antibody coupled to Sepharose 4B. The wild-type t-PA remained exclusively in the single-chain form during the purification procedure (L. Roman, unpublished results). A solid-phase radioimmunoassay was performed as previously described (Sambrook et al., 1986) using a goat anti-human t-PA polyclonal antiserum to quantitate the amount of t-PA antigen present in the cell culture medium, which varied between 0.5 and 2.5 μg/mL for both wild-type and cleavage-minus recombinant t-PAs.

Metabolic labeling of infected CV-1 monolayers with a mixture of [^{35}S]methionine and [^{35}S]cysteine (Trans Label, ICN) or [^{35}S]cysteine (NEN, Du Pont) was performed as previously described (Doyle et al., 1985) between 42 and 45-h postinfection. Immunoprecipitation of aliquots of medium and separation of labeled proteins by SDS–PAGE were also performed as described previously (Doyle et al., 1985). Where appropriate, labeled t-PA samples were digested with plasmin at a final concentration of 1 μg/mL on ice for 0–20 min. Proteolysis was quenched at the end of the reaction by the addition of a 10-fold excess of both aprotinin and soybean trypsin inhibitor.

The enzymatic activity of t-PA was measured by using three different assays:

(*1*) *Direct Chromogenic Assay.* This assay employs the chromogenic substrate, methylsulfonyl-D-cyclohexyltyrosyl-glycyl-arginine-*p*-nitroaniline acetate (Spectrozyme t-PA), which is cleaved directly by t-PA at the arginine–*p*-nitroaniline bond. The assay was performed as described by the manufacturer (American Diagnostica Inc.), and reaction progress was monitored against the appropriate blank by measuring the production of free *p*-nitroaniline spectrophotometrically at 405 nm. Assays were incubated at 37 °C in 96-well flat-bottomed microtiter plates, and optical density was measured at 2-h intervals with a Bio-tek microplate autoreader over an 8-h period. Under the conditions used, the assay was linear with time. Each test reaction mixture contained 2.5 ng of the t-PA sample to be tested in a final volume of 200 μL. Appropriately diluted samples of medium from mock-infected cells were analyzed as control samples, and the OD values obtained (<0.01 unit) were subtracted from the corresponding test values.

(*2*) *Indirect Chromogenic Assay.* In this assay, the release of free *p*-nitroaniline from the chromogenic substrate H-D-norleucyl-hexahydrotyrosyl-lysine-*p*-nitroanilide diacetate salt (Spectrozyme PL) is catalyzed by plasmin and measured spectrophotometrically (OD 405_{nm}). The reaction mixture (100 μL) contained 150–200 pg of the t-PA sample to be tested, Spectrozyme PL (0.4 mM), either Glu-plasminogen (1.0 μM) or Lys-plasminogen (0.1 μM), and soluble fibrin (DESAFIB, 25 μg/mL) in a buffer containing 50 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 1 mM EDTA, and 0.01% Tween-80. Assays were performed at 37 °C in a 96-well flat-bottomed microtiter plates, and OD_{405nm} was measured against the appropriate blank with a Bio-tek microplate autoreader over a 2-h period. Aliquots of buffer or appropriately diluted samples of medium from mock-infected cells were analyzed as control samples, and the OD values obtained (<0.01 unit) were subtracted from the corresponding test values. Under the conditions used in this standard assay, both single- and two-chain forms of the enzyme are maximally stimulated by soluble fibrin. The inclusion of soluble fibrin enhanced the activity of wild-type, two-chain t-PA approximately 20-fold, in agreement with the observations of others using the DESAFIB preparation (Karlan et al., 1987). This level of stimulation is similar to that achieved in assays employing nonpolymerized fibrinogen (Tate et al., 1987) but is significantly less than that observed using polymerized fibrin (Ranby, 1982).

(*3*) *Fibrin Zymography.* This assay was performed essentially as described by Granelli-Piperno and Reich (1978). Briefly, fibrin agar indicator gels were prepared at 42 °C by mixing a 2% agarose solution with prewarmed phosphate-buffered saline (PBS, Gibco) containing plasminogen (19.5 μg/mL). Fibrinogen (10 mg/mL) in PBS (42 °C) was added; the solution was mixed rapidly and poured onto a 5.5 cm × 8.0 cm plastic surface. After samples of conditioned medium were fractionated by SDS–PAGE, the polyacrylamide gels were soaked in a 2.5% Triton X-100 solution for 1 h to remove the SDS and placed on the surface of the fibrin agar indicator gel. The indicator gel was developed at 37 °C in a humidified chamber for 6–16 h, stained with a 0.01% solution of amido black in 70% methanol/10% acetic acid, destained, and then photographed. The light areas of the gel correspond to zones of lysis resulting from the interaction of t-PA with the plasminogen present in the indicator gel. Indicator gels without plasminogen contained no lytic zones (not shown). This method not only reveals free t-PA but also t-PA/t-PA inhibitor complexes (Stalder et al., 1985; Kruithof et al., 1984; Granelli-Piperno & Reich, 1978).

PAI-1. Plasminogen activator inhibitor 1 (PAI-1) was obtained from the conditioned medium of primary endothelial cell cultures derived from human umbilical veins and partially purified by concanavalin A–Sepharose affinity chromatography (Van Mourik et al., 1984), activated by guanidine hydrochloride treatment (Hekman & Loskutoff, 1985), and further purified by Sephacryl S200 gel filtration.

Results

Construction of the Cleavage-Minus t-PA Mutant. The amino acid sequence surrounding the H-chain/L-chain cleavage site of wild-type t-PA (Pennica et al., 1983; Pohl et al., 1984) is shown in Figure 1, together with the corresponding sequence in the cleavage-minus t-PA mutant. The arginine residue at position 275 in wild-type t-PA is substituted by a glycine residue in the mutant protein. To generate this amino acid change, a single nucleotide substitution was introduced into the wild-type t-PA gene by site-directed mutagenesis using the mismatched oligonucleotide shown in Figure 1. Following confirmation of the nucleotide alteration by DNA sequence analysis (data not shown), the DNA fragment encoding the mutant, cleavage-minus t-PA was isolated and inserted into an SV40 recombinant viral vector, replacing the wild-type t-PA sequences (Sambrook et al., 1986). High-titer stocks of recombinant viruses encoding the wild-type and cleavage-minus t-PAs were developed as described previously (Doyle et al., 1985; Sambrook et al., 1986).

The Cleavage-Minus t-PA Mutant Is Resistant to Cleavage by Plasmin. CV-1 monolayers infected with the recombinant SV40 virus stocks secrete wild-type or mutant t-PAs into the culture medium from about 36–72-h postinfection. Samples of medium obtained from CV-1 monolayers radiolabeled with

Cleavage site of wild-type t-PA	-Gln-Pro-Gln-Phe-Arg-Ile-Lys-Gly- (275 276)
Corresponding coding sequence	-CAG-CCT-CAG-TTT-CGC-ATC-AAA-GGA-
Mutagenic oligonucleotide	5' G-CCT-CAG-TTT-GGC-ATC-AA 3'
Sequence of mutant t-PA	-Gln-Pro-Gln-Phe-Gly-Ile-Lys-Gly- (275 276)

FIGURE 1: Oligonucleotide-directed mutagenesis of the sequence encoding the cleavage site of t-PA. The amino acid sequence surrounding the cleavage site (arrow) of wild type t-PA is shown together with the corresponding nucleotide sequences. The equivalent sequences of the cleavage-minus t-PA mutant are also shown. The synthetic oligonucleotide primer used for oligonucleotide-directed mutagenesis is complementary to the wild-type sequence except for a single mismatch which results in the conversion of the CGC codon specifying arginine-275 in the wild-type sequence to GGC specifying glycine in the mutant.

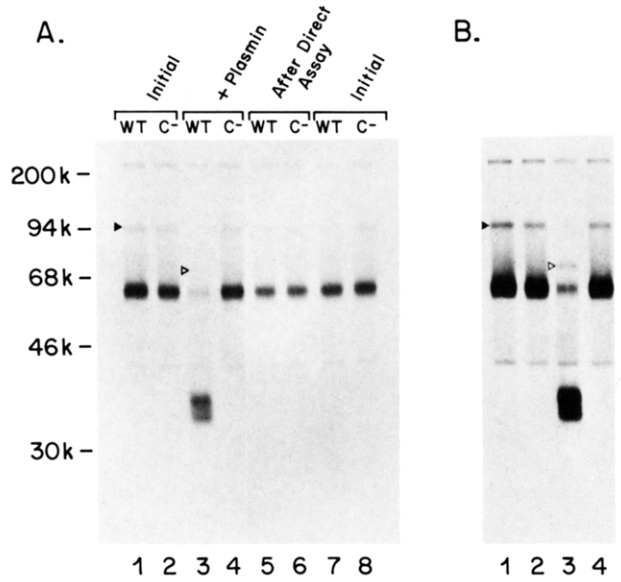

FIGURE 2: Sensitivity of wild-type and mutant t-PAs to cleavage by plasmin. CV1 cell monolayers, infected with SV40/t-PA recombinant viruses for 42 h, were labeled with [^{35}S]methionine for 3 h (150 μCi/60-mm dish of infected cells), and the supernatant media were collected. The labeled t-PA polypeptides were immunoprecipitated either immediately or after being assayed, and then separated by SDS–PAGE under reducing conditions (see Experimental Procedures). (Panel A) Lanes 1, 2, 7, and 8 show wild-type (WT) and cleavage-minus (C^-) t-PAs as they are secreted from CV1 cells. Lanes 3 and 4 show WT and C^- t-PAs following digestion with 1 μg/mL plasmin on ice for 20 min (see Experimental Procedures). Lanes 5 and 6 show WT and C^- t-PAs at the completion (8 h) of the direct chromogenic assay (see Experimental Procedures and Figure 3). (Panel B) A longer exposure of lanes 1–4 is shown with arrowheads indicating that t-PA/PAI-1 complexes.

[^{35}S]methionine from 42–45-h postinfection were immunoprecipitated using an anti-t-PA polyclonal antiserum. Figure 2A shows an analysis of these immunoprecipitates by SDS–PAGE under reducing conditions. CV-1 cells expressing either wild-type (lanes 1 and 7) or cleavage-minus (lanes 2 and 8) t-PA secrete a protein of M_r ~66 000 that is specifically precipitated by the anti-t-PA antiserum. Furthermore, because the gel was run under reducing conditions and because labeled material is not observed in the M_r 30 000–35 000 region of these lanes, the results, in agreement with previous findings (Sambrook et al., 1986), indicate that infected CV-1 cells secrete recombinant t-PA in the single-chain precursor form. Treatment of the recombinant wild-type t-PA with plasmin produces the two-chain form of the enzyme, which migrates under reducing conditions as three bands of M_r 33 000–35 000 (lane 3) as a result of incomplete glycosylation of the heavy chain of a proportion of the t-PA molecules (Sambrook et al., 1986; Pohl et al., 1987). However, plasmin has no effect on the cleavage-minus t-PA (lane 4).

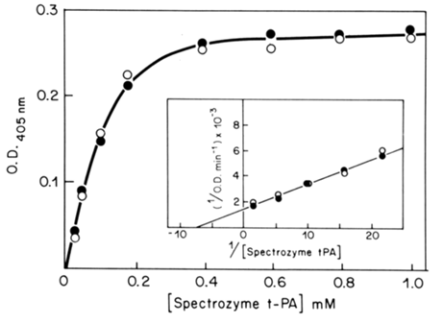

FIGURE 3: Comparison of the activities of wild-type and cleavage-minus t-PAs using the direct chromogenic assay. Wild-type (●) and cleavage-minus (○) t-PAs were assayed by using varying concentrations of chromogenic substrate (Spectrozyme t-PA) as described under Experimental Procedures. The data are presented in both Michaelis–Menten and Lineweaver–Burk (inset) formats.

Wild-Type and Cleavage-Minus t-PAs Exhibit Identical Activities in the Direct Chromogenic Assay. A direct chromogenic assay was used to study the enzymatic activities of antigenically equal amounts of wild-type and cleavage-minus t-PAs. This assay employs the low molecular weight chromogenic substrate Spectrozyme t-PA, which is cleaved directly by the enzyme to yield free *p*-nitroaniline, which can be measured spectrophotometrically. Wild-type and cleavage-minus t-PAs secreted from CV-1 cells behave indistinguishably in this assay, exhibiting identical K_m values of 0.14 mM and V_{max} values of 5.7×10^{-4} ODU/min.

To determine the molecular forms (single-chain versus two-chain) of the wild-type and mutant t-PA molecules that were present in the reaction mixture at the completion of the direct assay, t-PAs labeled with [^{35}S]methionine were incubated with Spectrozyme t-PA under conditions identical with those employed in the experiment described in Figure 3, then immunoprecipitated, and examined by SDS–PAGE under reducing conditions. The results, shown in Figure 2, demonstrate that both wild-type t-PA (lane 5) and cleavage-minus t-PA (lane 6) remained in the single-chain form for the duration of the assay. The fact that the single-chain forms of both wild-type and cleavage-minus t-PAs exhibit equal activities in the direct assay indicates that the single amino acid substitution ($Arg_{275} \rightarrow Gly$) that prevents cleavage has not affected the catalytic activity of the light chain of the mutant enzyme.

To compare the properties of single-chain and two-chain forms of t-PA in the direct chromogenic assay, we determined the kinetic constants for an antigenically equal amount of wild-type t-PA that had been immunopurified and shown to be entirely in the two-chain form. We obtained values for K_m and V_{max} of 0.11 mM and 1.6×10^{-3} ODU/min, respectively. Therefore, there is little or no difference between the K_m values of the single-chain and two-chain enzymes. However, cleavage of t-PA results in an approximately 3-fold increase in the V_{max} of the reaction using the Spectrozyme t-PA substrate.

Wild-Type and Cleavage-Minus t-PAs Exhibit Different Levels of Activity in the Indirect Chromogenic Assay. During the indirect chromogenic assay, t-PA converts plasminogen to plasmin which, in turn, cleaves the chromogenic substrate (see Experimental Procedures) so that free *p*-nitroaniline is released. Two forms of plasminogen, which differ in their kinetics of activation by t-PA (Wallen, 1980), can be used as substrate for this assay. Glu-plasminogen (K_m = 0.16 μM), which has glutamic acid as its N-terminal residue, is the native, precursor form of plasminogen in blood. Limited cleavage of

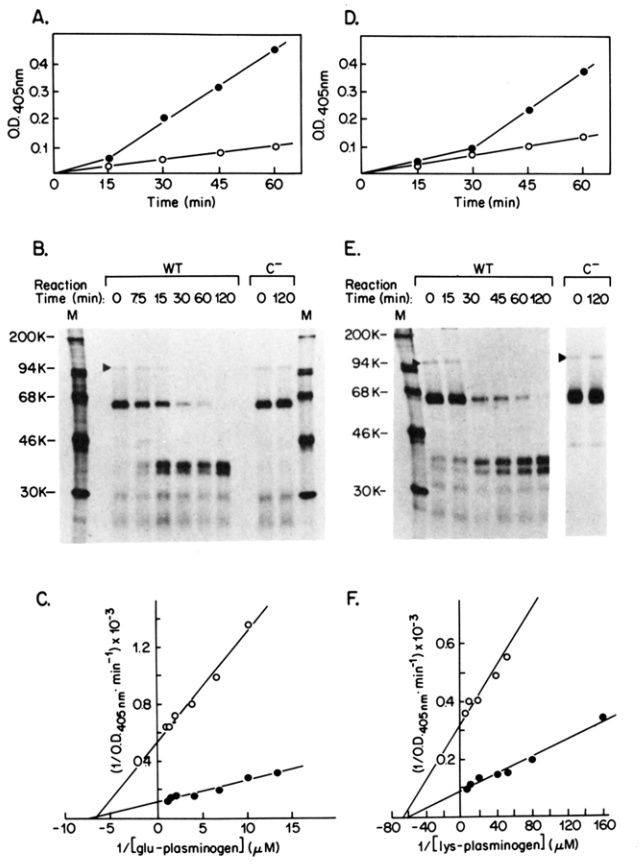

FIGURE 4: Comparison of the activities of wild-type and cleavage-minus t-PAs using the indirect chromogenic assay. Wild-type and cleavage-minus t-PAs were assayed as described under Experimental Procedures using either Glu-plasminogen (panels A–C) or Lys-plasminogen (panels D–F) as substrate. Panels A and C show the progress of the reaction with time using saturating concentrations of Glu-plasminogen (1.0 μM) or Lys-plasminogen (0.1 μM). Panels B and E show autoradiographs of SDS–polyacrylamide gels of the t-PA species immunoprecipitated from the reaction mixtures at different times after initiation of the reaction. Panels C and F show Lineweaver–Burk analyses for the wild-type enzyme in the two-chain form and the cleavage-minus enzyme in the single-chain form. Wild-type t-PA (●) (WT); cleavage-minus t-PA (○) (C^-).

Glu-plasminogen by plasmin removes a 76-residue peptide from the N-terminus to generate Lys-plasminogen (K_m = 0.02 μM) (Violand, 1976). The activity of the wild-type enzyme can be stimulated by 1–3 orders of magnitude by the addition of various forms of fibrin to the assay mixture (Hoylaerts et al., 1982; Ranby, 1982; Suenson et al., 1984; Suenson & Petersen, 1986).

The indirect chromogenic assay was used to monitor the progress of the reaction catalyzed by antigenically equal amounts of the wild-type and cleavage-minus t-PAs in the presence of 25 μg/mL soluble fibrin preparation DESAFIB using both forms of plasminogen as substrates. During a 1-h incubation period, the reaction catalyzed by the cleavage-minus enzyme progressed at an apparently linear rate whether Glu-plasminogen (Figure 4A) or Lys-plasminogen (Figure 4D) was used as substrate. By contrast, the reaction catalyzed by wild-type t-PA exhibited a lag phase of approximately 15 min using Glu-plasminogen and of approximately 30 min using Lys-plasminogen, followed by a second phase in which the reaction rate was increased 3–4-fold. In both cases, the initial reaction rates were similar to the constant reaction rates obtained using the cleavage-minus t-PA.

To determine whether the change in reaction rates observed in Figure 4A,D corresponded to the conversion of the single-chain form of the wild-type enzyme to the two-chain form by plasmin generated during the assay, t-PAs labeled with [^{35}S]cysteine were assayed for different lengths of time, immunoprecipitated, and then examined by SDS–PAGE under reducing conditions. Figure 4B shows the results of this analysis when Glu-plasminogen was used as the substrate. At the start of the reaction (lane 1), wild-type t-PA was present exclusively as the single-chain, 66-kDa form. However, as the reaction progressed, the wild-type t-PA was converted to the two-chain form, with a time course corresponding to that of the increase in the activity of the enzyme (Figure 4A). One hour after the initiation of the reaction, essentially all of the wild-type t-PA was present in the two-chain form. By contrast, cleavage-minus t-PA remained in the single-chain form throughout the course of the 2-h assay. Similar results were obtained when Lys-plasminogen was used as the substrate for the assay (Figure 4E), although conversion of the wild-type t-PA to the two chain molecular form occurred more slowly, a result that is in agreement with the longer lag period seen in Figure 4D. Conversion of wild-type t-PA to the two-chain form was complete by 2 h after initiation of the reaction. Once again, the cleavage-minus t-PA remained in the single-chain form throughout the 2-h assay period. Taken together, these data indicate that early in the assay, when both wild-type and cleavage-minus t-PAs are in the single-chain form, the activities of the two enzymes are very similar if not identical. Since the same result was obtained using the direct chromogenic assay (Figure 3), we can conclude that the one-chain form of t-PA is active both on a low molecular weight peptide substrate and on its physiological substrate, plasminogen, and that the substitution of a single amino acid at the cleavage site of t-PA has little or no effect on the catalytic activity of the enzyme.

However, the two-chain form of t-PA is more enzymatically active than the single-chain enzyme. The results shown in Figure 4C,F were obtained under conditions where wild-type t-PA had been completely converted to the two-chain form by plasmin generated during the assay. Although the single- and two-chain forms of the enzyme exhibited very similar affinities for the plasminogen substrates, the V_{max} values were approximately 3–5-fold higher for two-chain t-PA (Figure 4C,F). Thus, using Glu-plasminogen as substrate (Figure 4C), both two-chain wild-type t-PA and single-chain cleavage-minus t-PA exhibited apparent Michaelis–Menten kinetics with K_m values of 0.13 and 0.14 μM, respectively. However, the V_{max} of the two-chain wild-type protein was 4.6-fold greater than that of cleavage-minus t-PA. A similar result was observed when Lys-plasminogen was used as substrate (Figure 4F). In this case, the K_m values for the wild-type and cleavage-minus t-PAs were essentially identical (0.017 and 0.016 μM, respectively), but the V_{max} for wild-type t-PA was 3.5-fold greater than that of cleavage-minus t-PA. Very similar kinetic parameters were obtained using immunopurified, single-chain forms of the wild-type and cleavage-minus t-PAs secreted from infected CV-1 cells (results not shown). Furthermore, we demonstrated that the kinetic parameters displayed by the wild-type enzyme secreted from CV-1 cells, following its conversion to the two-chain form during the assay, were identical with those of an immunopurified two-chain t-PA synthesized in Chinese hamster ovary cells. The K_m values obtained for the two-chain t-PAs with Glu- or Lys-plasminogen substrates in the presence of fibrin correspond closely to previously published values (Holyaerts et al., 1982) and reflect the fact that Lys-plasminogen is the better substrate for t-PA.

Both Wild-Type and Cleavage-Minus t-PAs Are Stimulated by Fibrin. Plasminogen activation by the wild-type and

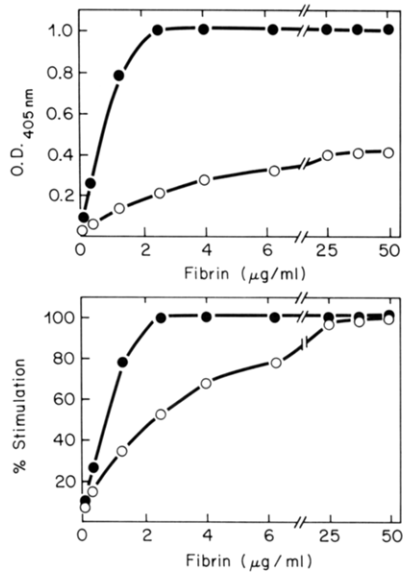

FIGURE 5: Effect of soluble fibrin on the activities of wild-type and cleavage-minus t-PAs. Wild-type and cleavage-minus t-PAs were assayed as described under Experimental Procedures using Lys-plasminogen as substrate in the presence of varying concentrations of DESAFIB. The incubation period for the assay was 2 h. Wild-type t-PA (●); cleavage-minus t-PA (○).

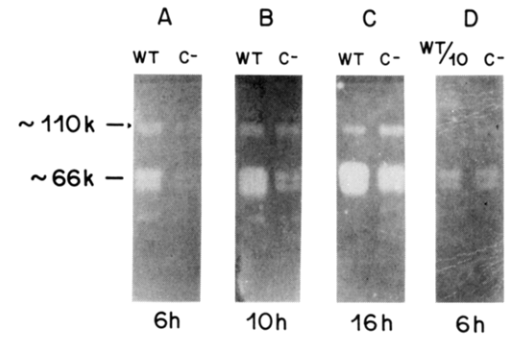

FIGURE 6: Analysis of the activity of wild-type and cleavage-minus t-PAs by fibrin zymography. Antigenically equal amounts of wild-type (WT) and cleavage-minus (C^-) t-PAs were separated on acrylamide gels and then processed by fibrin zymography as described under Experimental Procedures. Stained indicator gels developed for 6–16 h at 37 °C are shown in panels A–D. In panel D, the amount of wild-type t-PA antigen tested was 10 times less than that of the cleavage-minus mutant. The arrow indicates the t-PA/PAI-1 lytic zone.

cleavage-minus t-PAs was compared over a broad range of concentrations of soluble fibrin using the indirect chromogenic assay. Both wild-type and cleavage-minus t-PAs were activated by DESAFIB (Figure 5), although maximum stimulation of the single-chain, cleavage-minus enzyme required significantly higher concentrations of soluble fibrin (>25 μg/mL) than was needed to fully activate the two-chain, wild-type enzyme (2.5 μg/mL). At the saturating concentration of DESAFIB, the maximal activity of the single-chain enzyme corresponded to ~40% of the activity of the two-chain enzyme assayed under the same conditions.

In the absence of fibrin, both the wild-type and cleavage-minus enzymes displayed low levels of activity on the substrate, Lys-plasminogen (Figure 5). Under this condition, only a small percentage of the wild-type t-PA molecules were converted to the two-chain form during the assay, due to the low level of generation of plasmin. However, using purified, two-chain t-PA, we have demonstrated that in the absence of fibrin, cleavage of t-PA results in an approximately 3-fold increase in the efficiency of Lys-plasminogen activation (results not shown). This degree of activation is consistent with that obtained with the chromogenic substrate (see above), although it may represent an underestimate due to the low levels of activity of both forms of the enzyme in the absence of fibrin.

Fibrin Zymography of Wild-Type and Cleavage-Minus t-PAs. Wild-type and cleavage-minus t-PAs were also analyzed for their enzymatic activities by fibrin zymography (Granelli-Piperno & Reich, 1978). This procedure utilizes the observation that inactivation of t-PA during SDS–PAGE can be reversed by exchanging SDS for the nonionic detergent Triton X-100. After detergent exchange, t-PA activity can be detected by layering the polyacrylamide gel over an agarose indicator gel containing plasminogen and fibrinogen. During subsequent incubation at 37 °C, t-PA molecules diffuse from the polyacrylamide gel into the indicator gel and activate plasminogen. The plasmin thus formed then degrades the fibrin present in the indicator gel, and zones of lysis appear. Stained indicator gels of wild-type and cleavage-minus t-PAs incubated at 37 °C for 6–16 h are shown in Figure 6. When antigenically equal amounts of wild-type and mutant t-PAs were analyzed by zymography, cleavage-minus t-PA displayed approximately one-tenth the enzymatic activity of wild type t-PA (panels A–C). This result is confirmed by the result shown in panel D; when one-tenth of the amount of the wild-type protein was loaded on the polyacrylamide gel compared to that of the cleavage-minus protein, the intensities of the lytic zones were approximately equal. The most likely explanation for the 10-fold difference between wild-type and cleavage-minus t-PAs is that the plasmin generated during the course of the assay acts not only to degrade the fibrinogen present in the indicator gel but also to cleave single-chain wild-type t-PA to the more active two-chain form. This cleavage is not possible for the mutant t-PA protein.

Wild-Type and Cleavage-Minus t-PAs Are Inhibited to the Same Extent by PAI-1. t-PA is inhibited by a specific plasminogen activator inhibitor (PAI-1) synthesized by endothelial cells [reviewed by Sprengers and Kluft (1987)]. To test the relative sensitivity to PAI-1 of the single- and two-chain forms of t-PA, antigenically equal amounts of the single-chain, wild-type, and cleavage-minus t-PAs secreted from CV-1 cells and the immunopurified two-chain, wild-type t-PA were preincubated with various concentrations of partially purified inhibitor for 1 h at room temperature before measuring their activities using the indirect chromogenic assay. The results, presented in Figure 7, indicate that all three preparations of t-PA could be effectively inhibited by PAI-1. The similar degree of inhibition of the wild-type and cleavage-minus t-PAs secreted from CV-1 cells was not unexpected since both forms of t-PA remain in the single-chain form during preincubation with inhibitor, even though the wild-type enzyme would be converted to the two-chain during the subsequent activity assay. However, the fact that essentially identical results were obtained with the immunopurified, two-chain enzyme indicates that PAI-1 inhibits both single-chain and two-chain t-PAs to the same extent.

PAI-1 interacts with t-PA to form an SDS-stable complex of M_r ~110 000 (Kruithof et al., 1984; Pizzo et al., 1986). Bands of this molecular weight are clearly visible in the autoradiographs shown in Figures 2 and 4B,E (see solid arrowheads). To demonstrate that this species does represent the t-PA/PAI-1 complex, we have observed that the band increases in intensity when t-PA samples are preincubated with increasing concentrations of partially purified PAI-1 (data not shown). The data shown in Figures 2 and 4 indicate that the both the wild-type and cleavage-minus t-PAs bind PAI-1, since bands of M_r ~110 000 corresponding to both wild-type

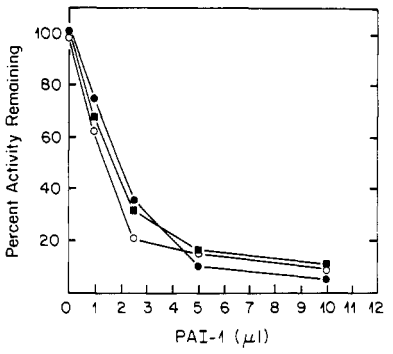

FIGURE 7: Inhibition of wild-type and cleavage-minus t-PAs by PAI-1. Antigenically equal amounts of wild-type and cleavage-minus t-PAs were incubated with various amounts of PAI-1 for 1 h at room temperature prior to analysis by the indirect chromogenic assay using Lys-plasminogen as substrate (see Experimental Procedures). Cleavage-minus t-PA (○); single-chain, wild-type t-PA (●); two-chain, wild-type t-PA (■).

t-PA/PAI-1 and cleavage-minus t-PA/PAI-1 complexes are observed (e.g., Figure 2A,B, lanes 1 and 2). Thus, the single amino acid difference between wild-type and cleavage-minus t-PAs does not prevent the interaction of the enzyme with its inhibitor. Furthermore, the results shown in Figures 2 and 4 demonstrate that plasmin is able to cleave wild-type t-PA even when it is bound to PAI-1 because a band corresponding to the complex between the t-PA light chain and PAI-1 (M_r ~80 000, open arrowhead) is generated following treatment with plasmin (Figure 2A,B, lane 3). No reduction in the apparent molecular weight of the complex is observed after treatment of cleavage-minus t-PA with plasmin (Figure 2A,B, lane 4).

Complex formation between t-PA and PAI-1 can also be observed using zymography (Stalder et al., 1985; Kruithof et al., 1984; Granelli-Piperno & Reich, 1978), apparently because some of the t-PA dissociates from the t-PA/inhibitor complex during detergent exchange and exhibits fibrinolytic activity (Stalder et al., 1985). A zone of lysis in the M_r ~110 000 region of the gel was detected following zymography of both wild-type and cleavage-minus t-PAs (Figure 6, arrows). These zones of lysis represent t-PA released from t-PA/PAI-1 complexes because their intensities increased when t-PA samples were preincubated with partially purified PAI-1 prior to zymography (data not shown). At early time points during zymography (Figure 6, panel A), more lysis was seen at the gel position corresponding to the wild-type t-PA/PAI-1 complex than at the position corresponding to the mutant t-PA/PAI-1 complex, a result consistent with the higher specific activity of the two-chain, wild-type protein. However, when the zymography was continued for longer incubation times, the intensities of the zones of lysis at the positions corresponding to the inhibitor complexes became approximately equal for the wild-type and mutant proteins (Figure 6, panels B and C). It is possible that this phenomenon results from a higher rate of dissociation of the single-chain form of the enzyme from the complex, although direct studies of the binding of inhibitor to the two forms of t-PA will be required to understand this result.

Discussion

Much controversy has surrounded the issue of the level of endogenous activity displayed by single-chain t-PA. We believe that this has resulted from uncertainty about (1) the molecular form of the enzyme added to the assay and (2) the extent of conversion of single-chain t-PA to the two-chain form during the reaction. To avoid these problems, we have constructed and analyzed a mutant form of the enzyme that cannot be cleaved into the two-chain form and have monitored the extent to which the wild-type t-PA is cleaved during the course of enzymatic assays. The data presented in this paper demonstrate that the precursor, single-chain form of t-PA is enzymatically active, and therefore is not a true zymogen. While single-chain t-PA is less active than the two-chain enzyme, it is responsive to stimulation by soluble fibrin and can be inhibited by the serpin PAI-1 to the same extent as the two-chain form of the enzyme.

We examined the properties of two forms of single-chain t-PA following their secretion from monkey CV-1 cells expressing wild-type or mutant t-PA polypeptides. The wild-type t-PA was present in the culture supernatant exclusively in the single-chain form and remained uncleaved throughout amidolytic assays and during immunopurification, although it became converted to the two-chain form during plasminogen activation. Once cleaved to the two-chain form, the enzyme was indistinguishable in its properties from immunopurified, recombinant two-chain t-PA derived from a Chinese hamster ovary cell line (Kaufman et al., 1985). The mutant t-PA, constructed by oligonucleotide-directed mutagenesis, lacks the cleavage site and thus remained in the single-chain form throughout all assay procedures. The enzymatic activities of the single-chain forms of the wild-type and mutant enzymes were identical, proving that the substitution of glycine for arginine at the cleavage site had no effect on the catalytic site of the t-PA molecule.

Initial studies of the activity of single-chain t-PA utilized the wild-type enzyme purified from natural sources under conditions that inhibited the generation of the two-chain protein during the isolation procedure (Wallen et al., 1982, 1983; Ranby, 1982; Ranby et al., 1982). More recently, Tate et al. (1987) constructed and expressed a mutant t-PA that differs from ours in the amino acid (Glu) that was substituted at the cleavage site. Their mutant was resistant to cleavage by plasmin but could be cleaved to the two-chain form by *Staphylococcal* V8 protease (Tate et al., 1987). All groups agree that homogeneous preparations of one-chain t-PA display significant levels of enzymatic activity when tested in direct assays using a variety of chromogenic peptide substrates. Nevertheless, the amidolytic efficiencies of single-chain and two-chain t-PA molecules are not equal: in the different studies, a 3–9-fold difference has been observed between the activities of the two forms of the enzyme. We observed that cleavage of t-PA resulted in little or no change in the K_m of the enzyme for the chromogenic peptide substrate, methylsulfonyl-D-cyclohexyltyrosyl-glycyl-arginine-*p*-nitroaniline acetate, so that the increased activity of the two-chain form was entirely due to an increase in V_{max}. By contrast, others have reported a decrease in the K_m for H-D-valyl-glycyl-arginine-*p*-nitroanilide (up to ~2-fold) in addition to an increase in K_{cat} (Ranby et al., 1982). In addition, Tate et al. (1987) reported that the K_m of two-chain t-PA for H-D-isoleucyl-prolyl-arginine-*p*-nitroanilide was 5–6-fold higher than that of the single-chain form, although there was little change in the K_{cat} of the enzyme. It is possible that the use of different peptide substrates explains these variations in the effect of cleavage of the t-PA molecule on the amidolytic process.

It is also generally agreed that single-chain t-PA is active on its natural substrate plasminogen in the absence or presence of stimulation by fibrin (Ranby, 1982; Rijken et al., 1982; Wallen et al., 1982; Tate et al., 1987). However, there have been conflicting reports as to whether the single-chain enzyme is as active as the two-chain form, or significantly less active.

Because of the potential for cleavage of t-PA by the plasmin formed in the reaction, cleavage-site mutants are especially useful for comparing levels of plasminogen activation by single-chain and two-chain t-PAs. Like Tate et al. (1987), we found the single-chain enzyme to be less efficient than the cleaved form in activating plasminogen in the absence of fibrin. We also found that although the soluble fibrin product DESAFIB stimulated plasminogen activation by both molecular species of t-PA, the single-chain forms of both the mutant and the wild-type enzymes were 2.5–5-fold less active than the two-chain enzyme. Similar results were obtained with either Glu-plasminogen or Lys-plasminogen substrates. As is the case with the peptide substrate, we observed that cleavage of t-PA caused no change in the K_m of the enzyme for either form of plasminogen and that the increase in the efficiency of plasminogen activation was entirely due to an increased V_{max} for the reaction. By contrast, Tate et al. (1987) report that the activity of their mutant enzyme equalled that of the wild-type, two-chain t-PA when assayed in the presence of physiological levels of fibrinogen and Glu-plasminogen. We cannot rule out that the difference between our results and those of Tate et al. is due to the substitution of different amino acids for Arg_{275}. However, the possibility remains that their results could be explained by cleavage of the mutant t-PA, perhaps by contaminating glutamic acid proteases, because the molecular form of the enzyme was not monitored during the assay.

We obtained an approximately 20-fold enhancement of plasminogen activation by both the single-chain and two-chain forms of the enzyme using saturating amounts of the soluble fibrin DESAFIB. This level of stimulation is similar to that obtained with two-chain t-PA by others using this fibrin preparation (Karlan et al., 1987) or nonpolymerized fibrinogen (Tate et al., 1987). Significantly greater enhancement (up to 1000-fold) can be obtained by using polymerized fibrin (Ranby, 1982). By examining the effect of altering the concentration of soluble fibrin on the efficiency of plasminogen activation by uncleaved or cleaved t-PA, we found that maximum stimulation of the single-chain enzyme required significantly higher concentrations (>25 μg/mL) of DESAFIB than did the two-chain form, which reached maximum activity in the presence of soluble fibrin at concentrations >2.5 μg/mL. This result indicates that the single-chain t-PA is less able than the two-chain form to adopt its most active conformation following fibrin binding. Tate et al. (1987) have reported that one-chain t-PA bound to fibrin to a greater extent than the two-chain form. Higgins and Vehar (1987) have carried out a detailed study of the interaction of one-chain and two-chain t-PA with intact and plasmin-degraded fibrin and observed that although more one-chain than two-chain t-PA was bound to fibrin, the values for K_d and for the numbers of moles of t-PA bound per mole of fibrin monomer were the same, within experimental error, for the two forms of the enzyme. Nevertheless, our finding that stimulation of the activity of single-chain t-PA required higher levels of fibrin than did that of the cleaved enzyme is not in conflict with the results of the binding studies, since it is accepted that high-affinity binding of t-PA involves the finger domain, while stimulation involves a subsequent interaction with the kringle domains (van Zonneveld et al., 1986; Larsen et al., 1988; Gething et al., 1988).

In this study, we have observed that the single-chain, cleavage-minus t-PA mutant can bind to and be efficiently inhibited by the serpin plasminogen activator inhibitor 1 (PAI-1). Titration of either one-chain or two-chain t-PA with differing amounts of PAI-1 indicated that both forms of the enzyme could be inhibited to the same extent, in agreement with previous studies using wild-type t-PA (Colucci et al., 1986; Jorgensen et al., 1987; Hekman & Loskutoff, 1988). Although we did not compare the initial rates of reaction of the inhibitor with the two forms of the enzyme, the observation by Jorgensen et al. (1987) and Hekman and Loskutoff (1988) that two-chain t-PA reacts approximately 3–4 times faster than the single-chain enzyme with PAI-1, which acts as a suicide pseudosubstrate for t-PA, is consistent with our observations that two-chain t-PA is approximately 3–5-fold more active than the single-chain enzyme on both high and low molecular weight substrates. Other workers have found that two-chain t-PA also reacts faster than the single-chain form with low molecular weight inhibitors such as diisopropyl phosphorofluoridate (Ranby et al., 1982) or dansylpeptidyl chloromethyl ketone (Higgins & Lamb, 1986).

The question remains as to the physiological significance of the cleavage of single-chain t-PA to the two-chain form. t-PA is synthesized and secreted by vascular endothelial cells in the one-chain form (Rijken et al., 1980) and converted to the two-chain form by plasmin at the site of blood clots (Ichinose et al., 1984). Perhaps the purpose of the inherent basal level of activity of single-chain t-PA is to trigger the activation of plasminogen on the clot surface. The plasmin thus formed not only would act to degrade the fibrin network of the clot and expose additional high-affinity binding sites on the clot for t-PA and Glu-plasminogen (Suenson et al., 1984; Higgins & Vehar, 1987) but also would act on single-chain t-PA to generate the more active two-chain form of the enzyme. In addition, the newly formed plasmin would act upon Glu-plasminogen to produce the more effective t-PA substrate Lys-plasminogen. Thus, the in situ generation of plasmin, two-chain t-PA, and Lys-plasminogen would cause amplification of plasminogen activation and result in even greater specificity of t-PA for fibrin clots. In this way, the endogenous activity of single-chain t-PA would act to initiate the sequence of events involved in clot degradation.

Following submission of this paper, Petersen et al. (1988) published a report on studies of a cleavage site mutant identical with the one described in this work. Our results and conclusions are very similar, particularly in respect to the intrinsic activity of single-chain t-PA. These authors found that cleavage of t-PA resulted in an approximately 10-fold increase in the amidolytic activity on a peptide substrate, and an approximately 30-fold increase in plasminogen activation in the absence of fibrin, but only a 2-fold increase in the activity in the presence of polymerized fibrin. As we have also observed, the increase was in each case due to the higher V_{max} of the two-chain enzyme, and there was no difference between the K_ms of the single- and two-chain enzymes for the various substrates.

ACKNOWLEDGMENTS

We thank Laura Roman for assistance with the immunopurification procedure for t-PA, Anna Magee for expert technical support, and Ed Madison for many helpful discussions.

REFERENCES

Andreasen, P. A., Nielsen, L. S., Grondahl-Hansen, J., Skriver, L., Zeuthen, J., Stephens, R. W., & Dano, K. (1984) *EMBO J. 3*, 51–56.

Bergmann, S. R., Fox, K. A. A., Terpogossian, M. M., Sobel, B. E., & Collen, D. (1983) *Science (Washington, D.C.) 220*, 1181–1183.

Camiolo, S. M., Thorsen, S., & Astrup, T. (1971) *Proc. Soc. Exp. Biol. Med. 38*, 277–280.

Collen, D. (1980) *Thromb. Haemostasis 43*, 77–89.

Collen, D. (1987) *J. Am. Coll. Cardiol. 10*, 11B–15B.
Collen, D., & Linjen, H. R. (1984) *Arteriosclerosis (Dallas) 4*, 579–585.
Colucci, M., Paramo, J. A., & Collen, D. (1986) *J. Lab. Clin. Med. 108*, 53–59.
Dano, K., Andreasen, P. A., Grondahl-Hansen, J., Kristensen, R., Neilsen, L. S., & Skriver, L. (1985) *Adv. Cancer Res. 44*, 139–266.
Doyle, C., Sambrook, J., & Gething, M.-J. (1985) *J. Cell Biol. 100*, 704–714.
Edlund, T., Ny, T., Ranby, M., Heden, L.-O., Palm, G., Holmgren, E., & Josephson, S. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 349–352.
Einarrson, M., Brandt, J., & Kaplan, L. (1985) *Biochim. Biophys. Acta 830*, 1–10.
Fisher, R., Waller, E. K., Grossi, G., Thompson, D., Tizard, R., & Schleuning, W.-D. (1985) *J. Biol. Chem. 260*, 11223–11230.
Friezner-Degan, S., Rajput, B., & Reich, E. (1986) *J. Biol. Chem. 261*, 6972–6985.
Gething, M.-J., Adler, B., Boose, J.-A., Gerard, R. D., Madison, E. L., McGookey, D., Meidell, R. S., Roman, L. M., & Sambrook, J. (1988) *EMBO J. 9*, 2731–2740.
Granelli-Piperno, A., & Reich, E. (1978) *J. Exp. Med. 148*, 223–234.
Hanahan, D. (1983) *J. Mol. Biol. 166*, 557–580.
Harris, T. J. R., Patel, T., Marston, F. A. O., Little, S., Emtage, S., Opdenakker, G., Rombauts, W., Billiau, A., & de Somer, P. (1986) *Mol. Biol. Med. 3*, 279–292.
Hekman, C. M., & Loskutoff, D. J. (1985) *J. Biol. Chem. 260*, 11581–11587.
Hekman, C. M., & Loskutoff, D. J. (1988) *Arch. Biochem. Biophys. 262*, 199–210.
Higgins, D. L., & Lamb, M. C. (1986) *Arch. Biochem. Biophys. 249*, 418–426.
Higgins, D. L., & Vehar, G. A. (1987) *Biochemistry 26*, 7786–7791.
Hoylaerts, M., Rijken, D. C., Lijnen, H. R., & Collen, D. (1982) *J. Biol. Chem. 257*, 2912–2919.
Ichinose, A., Kissel, W., & Fugikawa, K. (1984) *FEBS Lett. 175*, 412–418.
Jorgensen, M., Philips, M., Thorsen, S., Selmer, J., & Zeuthen, J. (1987) *Thromb. Haemostasis 58*, 872–878.
Karlan, B. Y., Clark, A. S., & Littlefield, B. A. (1987) *Biochem. Biophys. Res. Commun. 142*, 147–154.
Kaufman, R., Wasley, L. C., Spiliotes, A. J., Gossels, S. D., Lat, S. A., Larsen, G. R., & Kay, R. (1985) *Mol. Cell Biol. 5*, 1750–1759.
Kruithof, E. K. O., Tran-Thang, C., Ransijn, A., & Bachmann, F. (1984) *Blood 64*, 907–913.
Larsen, G. R., Henson, K., & Blue, Y. (1988) *J. Biol. Chem. 263*, 1023–1029.
Matsuo, O., Rijken, D. C., & Collen, D. (1981) *Nature (London) 291*, 590–591.
Neurath, H., & Walsh, K. A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3825–3832.
Ny, T., Elgh, F., & Lund, B. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5355–5389.
Patthy, L. (1985) *Cell (Cambridge, Mass.) 41*, 657–663.
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., & Collen, D. (1983) *Nature (London) 301*, 214–221.
Petersen, L. C., Johannessen, M., Foster, D., Kumar, A., & Mulvihill, E. (1988) *Biochim. Biophys. Acta 952*, 245–254.
Pizzo, S. V., Fuchs, H. E., Doman, K. A., Petruska, D. B., & Berger, H. (1976) *Arch. Intern. Med. 146*, 188–191.
Pohl, G., Kallstrom, M., Bergsdorf, N., Wallen, P., & Jornvall, H. (1984) *Biochemistry 23*, 3701–3707.
Pohl, G., Kenne, L., Nilsson, B., & Einarsson, M. (1987) *Eur. J. Biochem. 170*, 69–75.
Ranby, M. (1982) *Biochim. Biophys. Acta 704*, 461–469.
Ranby, M., Bergsdorf, N., & Nillson, T. (1982) *Thromb. Res. 27*, 175–183.
Ranby, M., Suenson, E., Bergsdorf, N., Norrman, B., & Wallen, P. (1983) *Prog. Fibrinolysis 6*, 182–184.
Rijken, D. C., & Collen, D. (1981) *J. Biol. Chem. 206*, 7035–7041.
Rijken, D. C., Wijngaards, G., & Welliergen, J. (1980) *Thromb. Res. 18*, 815–830.
Rijken, D. C., Hoylaerts, M., & Collen, D. (1982) *J. Biol. Chem. 257*, 2920–2925.
Sambrook, J., Hanahan, D., Rodgers, L., & Gething, M.-J. (1986) *Mol. Biol. Med. 3*, 459–481.
Sambrook, J., Maniatis, T., & Fritsch, E. F. (1989) *Molecular Cloning: A Laboratory Manual* (2nd ed.), Cold Spring Harbor Laboratories, Cold Spring Harbor, NY.
Sprengers, E. D., & Kluft, C. (1987) *Blood 69*, 381–387.
Stalder, M., Hauert, J., Kruithof, E. K. O., & Bachmann, F. (1985) *Br. J. Haematol. 61*, 169–176.
Suenson, E., & Petersen, L. C. (1986) *Biochim. Biophys. Acta 870*, 510–519.
Suenson, E., Lutzen, O., & Thorsen, S. (1984) *Eur. J. Biochem. 140*, 513–519.
Tate, K. M., Higgins, D. L., Holmes, W. E., Winkler, M. E., Heyneker, H. L., & Vehar, G. A. (1987) *Biochemistry 26*, 338–343.
Van Mourik, J. A., Lawrence, D. A., & Loskutoff, D. J. (1984) *J. Biol. Chem. 259*, 14914–14921.
Van Zonneveld, A.-J., Veerman, H., & Pannekoek, H. (1986) *J. Biol. Chem. 261*, 14214–14218.
Vehar, G. A., Kohr, W. J., Bennett, W. F., Pennica, D., Ward, C. A., Harkins, R. N., & Collen, D. (1984) *Bio/Technology 2*, 1051–1057.
Violand, B. N. (1976) *J. Biol. Chem. 251*, 3906–3912.
Wallen, P. (1980) in *Fibrinolysis* (Klein, D. L., & Reddy, K. N. N., Eds.) pp 1–24, CRC Press, Boca Raton, FL.
Wallen, P., Ranby, M., Bergsdorf, N., & Kok, P. (1981) *Prog. Fibrinolysis 5*, 16–23.
Wallen, P., Bergsdorf, N., & Ranby, M. (1982) *Biochim. Biophys. Acta 719*, 318–328.
Wallen, P., Pohl, G., Bergsdorf, N., Ranby, M., Ny, T., & Jornvall, H. (1983) *Eur. J. Biochem. 132*, 681–686.
Zamarron, C., Lijnen, H. R., & Collen, D. (1984) *J. Biol. Chem. 259*, 2080–2083.
Zoller, M., & Smith, M. J. (1985) *DNA 3*, 479–488.