The distinguishing characteristic of vampire bat (Desmodus rotundus) salivary plasminogen activators (DSPAs) is their strict requirement for fibrin as a cofactor. DSPAs consist of structural modules known from urokinase (u-PA) and tissue-type plasminogen activator (t-PA) such as finger (F), epidermal growth factor (E), kringle (K), and protease (P), combining to four genetically and biochemically distinct isoenzymes, exhibiting the formulas FEKP (DSPAa1 and a2) and EKP and KP (DSPAb and DSPAγ). Only DSPAa1 and a2 bind to fibrin. All DSPAs are single-chain molecules, displaying substantial amidolytic activity. In a plasminogen activation assay, all four DSPAs are almost inactive in the absence of fibrin but strongly stimulated by fibrin addition. The catalytic efficiency (kcat/Km) of DSPAa1 increases 105-fold, whereas the corresponding value of t-PA is only 550. The ratio of the bimolecular rate constants of plasminogen activation in the presence of fibrin versus fibrinogen (fibrin selectivity) of DSPAa1, a2, β, γ, and t-PA was found to be 13,000, 6500, 250, 90, and 72, respectively. Whereas all DSPAs are therefore more fibrin dependent and fibrin selective than t-PA, the extent depends on the respective presence of the various domains. The introduction of a plasmin-sensitive cleavage site in a position akin to the one in t-PA partially obliterates fibrin cofactor requirement. Fibrin dependence and fibrin selectivity of DSPAs are accordingly mediated by fibrin binding, which involves the F domain, as yet undefined determinants within the K and P domains, and by the absence of a plasmin-sensitive activation site. These findings transcend the current understanding of fibrin-mediated stimulation of plasminogen activation: in addition to fibrin binding, specific protein-protein interactions come into play, which stabilize the enzyme in its active conformation.

Plasminogen activators (PAs),1 such as t-PA and u-PA, are highly specific serine proteases, which catalyze the hydrolysis of the Arg560-Val561 peptide bond of Glu-plasminogen. The activation product, plasmin, is a potent protease, which digests fibrin and several extracellular matrix proteins. Plasmin also processes the single-chain precursors of t-PA and u-PA to the more active two-chain forms. In contrast to u-PA, the rate of plasminogen activation by t-PA increases by 2-3 orders of magnitude in the presence of fibrin or fibrinogen degradation products (Camidó et al., 1971; Hoylaerts et al., 1982; Ranby, 1982; Bergum and Gardell, 1992). Both t-PA and its substrate, Glu-plasminogen, bind to fibrin, forming a ternary complex that facilitates the conversion of Glu-plasminogen (Hoylaerts et al., 1982; Ranby, 1982; Fears, 1989). t-PA consists of several structural motifs known by structural homology from other proteins: an N-terminal fibronectin-like finger (F), an epidermal growth factor (E), two kringles (K1 and K2), and a serine protease domain (P) (Penna et al., 1983; Pathy, 1990). Several authors suggested the F domain and the lysine-binding site (LBS) of the K2 domain to be the major contributors to t-PA fibrin affinity and to the observed fibrin-mediated enhancement of plasminogen activation (van Zonneveld et al., 1986; Verheijen et al., 1986; de Munk et al., 1989). Recent results, however, indicate that t-PA interacts with fibrin via a binding region that comprises surface areas of other structural modules as well, including the protease domain (Bennett et al., 1991).

In recent years, thrombolytic treatment with t-PA has emerged as state of the art therapy of acute myocardial infarction (Topol, 1991; Collen and Lijnen, 1991). However, when administered in therapeutic doses, t-PA, due to its limited fibrin selectivity, causes plasminemia that may contribute to bleeding complications (Rao et al., 1988; Arnold et al., 1989). Therefore, considerable efforts have been devoted to the design of new variants of t-PA exhibiting improved fibrin selectivity (Higgins and Bennett, 1990; Lijnen and Collen, 1991). Recently, a novel mutan t-PA called TNK, which is more fibrin selective than t-PA, has been characterized (Kaye et al., 1994; Collen et al., 1994).

We and others (Gardell et al., 1989; Krätzschmar et al., 1991) have previously reported the cloning, expression, and characterization of plasminogen activators derived from the saliva of vampire bats. A total of four different Desmodus rotundus salivary plasminogen activators (DSPAs), which we named DSPAa1, a2, β, and γ have been cloned, expressed, and characterized. DSPAa1 and a2 encompass an F, E, K, and P domain, while DSPAβ lacks the finger module and DSPAγ contains only a K and a P domain. Apart from these differences, DSPAs are very similar (88.7–99.5% amino acid sequence identity; Krätzschmar et al., 1991)). The amino acid sequence of human t-PA is similar related (72.3% (DSPAa1) and 74.2% (DSPAa2) identity; Krätzschmar et al., 1991)). Like u-PA, all DSPAs only contain a single K domain rather than two, as is the case for t-PA. The K module of DSPAs is more similar to the K1 domain of t-PA and does not exhibit an LBS. Furthermore, a plasmin-sensitive activation site, present in the N-terminal region of the t-PA protease domain is absent in DSPAs. Therefore, DSPAs activate plasminogen as single chain molecules (Gardell et al., 1989; Krätzschmar et al., 1991).

Functionally, DSPAs differ from t-PA by their strict require-
ment for a fibrin cofactor. This was studied in great detail for Bat-PA (equivalent to DSPA a2) by Bergum and Gardell (1992) and has been reported for DSPA a1 and DSPA a2 (Schleuning et al., 1992). When compared to t-PA, Bat-PA and DSPA a1 demonstrated an equal or even higher thrombolytic potency in several animal models of arterial thrombosis (Gardell et al., 1991; Mellor et al., 1992; Witt et al., 1992, 1994; Muschick et al., 1993). Importantly, while being equally effective as t-PA, fibrinogen degradation or α2-antiplasmin consumption were considerably lower with Bat-PA and DSPA a1 (Gardell et al., 1991; Mellor et al., 1992; Witt et al., 1992, 1994; Muschick et al., 1993).

The present study evaluates the fibrin selectivities of the recombinant forms of all naturally occurring DSPAs and compares these data with those obtained for t-PA. Furthermore, we present data that suggest a molecular mechanism for the unique fibrin specificity of DSPAs.

MATERIALS AND METHODS

Mutagenesis—Oligonucleotide-directed mutagenesis was performed as described by Lewis and Thompson (1990) using the Promega mutagenesis kit. DSPA a1 cDNA (Krätzschmar et al., 1991) was subcloned into the EcoRI-HindIII sites of the pSELECT-1 phagemid polylinker. The plasmid-sensitive site was introduced annealing the following oligonucleotide: 5'-CAGCCTCGGATTAAAAAGGAGGACT-3'. T-PA cDNA (Waller and Schleuning, 1985) was ligated into the HindIII site of the same vector. The plasmid-sensitive site was inactivated by hybridizing the following oligonucleotide: 5'-CCTACATTTCACAGCACAGGGGCTC-3'. Sequence alterations were verified by DNA sequencing (Sanger et al., 1977).

Purified Proteins and Substrates—Recombinant DSPAs were produced in BHK cells transfected with pMSVPEH expression vectors (Arlett et al., 1988; Wirth et al., 1991) harboring the cDNAs encoding wild-type DSPAs a1, a2, β, and γ (Krätzschmar et al., 1991), the mutated DSPA a1 cDNA, or the mutated T-PA cDNA (Krätzschmar et al., 1991). The recombinant plasminogen activator DSPA a1 was purified from cell culture supernatants by affinity chromatography on immobilized Erythrina trypsin inhibitor (Heussen et al., 1984), which was purchased from Erytech Services (PTY Ltd., Arcadia, South Africa). Recombinant t-PA (Actilyse®) was obtained from Dr. Karl Thomae (Heussen et al., 1984), which was originally written by Munson and Rodbard (1980) and modified by G. A. McPherson (V 2.0), which were obtained from Elsevier-Biosoft (Cambridge, United Kingdom).

Kinetics of Plasminogen Activation—All kinetics were measured spectrophotometrically at ambient temperature using a Bio-Rad microplate reader (model 3550) that was coupled to a Macintosh IIci. Kinetics of plasminogen activation were performed using the coupled enzymatic assay outlined by Nieuwenhuizen et al. (1985) with slight modifications. Briefly, individual assay samples encompassed the following ingredients: 0.5 μg/ml plasminogen activator, 100 μM fibrinogen (ogen) where stated (0.13 units/ml of human thrombin in case of fibrin), 0.05–8 μM Glu-plasminogen activator, 1 ml FIB-PLA buffer. Individual assays contained 20 ml EACA and, for some assays, in addition 100 μg/ml fibrin. All assays were done in triplicates for each plasminogen concentration and were repeated at least 3-fold. To correct for turbidity due to the presence of fibrin, ΔAbs = A 405min was monitored. Although FlavipregPli hydrolysis by thrombin was not significant and there was no detectable autodigestion, a blank (without plasminogen activator) was determined for each plasminogen concentration in duplicate. This control value was subtracted, and the resulting value was converted to [pNAA] using appropriate standard curves. The acceleration of pNA generation (d[pNAA]dt-t), which is directly proportional to the velocity of plasminogen activation, was determined by nonlinear regression analysis of 2nd order polynomial fits of [pNAA] versus time. It was then plotted against the concentration of plasminogen, and kinetic parameters kcat, Km, kcat/Km were calculated by nonlinear regression of data points according to the Michaelis-Menten equation. Computing was carried out on a Macintosh IIci using Kaleidagraph and Microplate Manager software. Kinetic constants of FlavipregPli hydrolysis by plasmin were determined under the aforementioned conditions and verified the assumption that kcat[(pil)] = [FlavipregPli] (Drapier et al., 1979) (data not shown).

Kinetics of S-2765 Hydrolysis—Kinetics of S-2765 hydrolysis were performed similarly. Assay volume was 0.15 ml containing 10 μM plasminogen activator, 100 μg/ml fibrinogen (ogen), and 0.02–4 μM S-2765 in PCOM buffer. Individual assay samples were performed as triplicates and verified at least three times. Hydrolysis of S-2765 by thrombin was not detectable under these conditions. Omitting the plasminogen activator, blanks carried out in duplicates were determined for every concentration of S-2765. As described above, ΔAbs = A 405min was calculated and converted to [pNAA]. In this case velocities, calculated from linear plots of [pNAA] versus t, were plotted versus concentration of S-2765 and analyzed by nonlinear regression to obtain kinetic parameters kcat and km.

RESULTS

DSPAs a1, a2, β, and γ were expressed in BHK cells as described (Krätzschmar et al., 1992). Recombinant proteins were purified to homogeneity from cell culture supernatants by affinity chromatography on immobilized Erythrina trypsin inhibitor (Heussen et al., 1984). As judged from SDS-PAGE analysis, preparations of recombinant DSPAs a1, a2, β, and γ were homogeneous, and the proteins displayed an apparent molecular mass of 52, 52, 46, and 44 kDa, respectively (Fig. 1, lanes 1–4).

DSPA Affinity for Fibrin—Investigating the fibrin affinity of DSPAs isolated from bat saliva, we had previously observed that only the two full-length variants, DSPA a1 and a2, exhibited affinity to fibrin, whereas DSPAs β and γ did not (Schleun-
Fibrin Cofactor Requirements of Vampire Bat Plasminogen Activators

Prior to electrophoresis on a SDS-gel containing 12.5% polyacrylamide (Laemmli 1970), all samples were reduced by the addition of dithiothreitol (12.5 mM). Approximately 3 μg of each protein was loaded. The gel was stained with Coomassie Brilliant Blue (G250). Proteins were produced as outlined under “Materials and Methods.” Lane 1, rDSPAα1; lane 2, rDSPAα2; lane 3, rDSPAβ; lane 4, rDSPAγ; lane 5, marker proteins (M) whose molecular mass is indicated on the right; lane 6, rt-PA (Actilyse®); lane 7, [R279H,J276S,K277T] t-PA lacking the plasin-sensitive site; lane 8, [H189R,S190I,T191K] DSPAα1 containing a plasin-sensitive site.

In the presence of fibrin, however, the catalytic efficiency of DSPAs was augmented by several orders of magnitude. The corresponding kcat values were approximately 7% higher in the presence of fibrin (Table I). The 2-fold higher fibrin-mediated enhancement of the catalytic efficiency of DSPAα1 was due to a steeper increment in the kcat value of DSPAα1 rather than a more pronounced decrease in its Km value. The reduction in Kcat and in particular the increase in kcat was significantly smaller for DSPAs β and γ (Table I).

In comparison to the absence of a cofactor, fibrinogen promoted the catalytic efficiency of DSPAs by 7-9-fold, resulting in kcat/Km values ranging from 39 to 79 M-1 s-1, which were several orders of magnitude smaller than those observed in the presence of fibrin (Table I). The ratio of catalytic efficiencies in the presence of fibrin to the corresponding values in the presence of fibrinogen, which serves as a measure of “fibrin selectivity”, amounted to 12,900 for DSPAα1. The bimolecular rate constant of DSPAα2 in the presence of fibrin was 6550-fold smaller than that of DSPAα1, which, however, was more active in the presence of fibrin (Table I). The 2-fold higher fibrin-mediated enhancement of the catalytic efficiency of DSPAα1 was due to a steeper increment in the kcat value of DSPAα1 rather than a more pronounced decrease in its Km value. The reduction in Kcat and in particular the increase in kcat was significantly smaller for DSPAs β and γ (Table I).
higher than that in the presence of fibrinogen. The respective values for DSPAs β and γ were 235 and 90 (Table I). DSPA1 therefore exhibited the highest fibrin selectivity, and this was mainly attributable to its superior stimulation by fibrin.

The data summarized in Table I also depict how the kinetic parameters of DSPAs compare to those obtained for t-PA. In the absence of a fibrinogen cofactor, t-PA was 260-fold more efficient in activating Glu-plasminogen than DSPA1. In the presence of fibrin, however, both plasminogen activators were similarly effective (Table I). The enhancement of the bimolecular rate constant of t-PA in the presence of fibrin was only 550-fold as compared to 105-fold for DSPA1. In the presence of fibrinogen, the catalytic efficiency of t-PA was increased to 13,600 M⁻¹ s⁻¹, which at 260- and 170-fold higher than the respective values measured for DSPA1 and α2 (Table I). Fibrinogen increased the catalytic efficiency of t-PA by only 72-fold over that in the presence of fibrinogen, meaning that DSPA1 was about 180-fold more fibrin selective than t-PA. DSPA2 exhibited a 90-fold higher fibrin selectivity than t-PA, and even the finger-deficient variant DSPAγ was still 3-fold more fibrin selective. The latter strongly indicates that fibrin selectivity is not merely a consequence of the plasminogen activator’s affinity for fibrin.

### Table I

| Enzyme    | Cofactor | Kₘ (µM) | kcat × 10³ (s⁻¹) | kcat/Kₘ (s⁻¹) | Stimulation factor | Ratio Fbn/Fbg |
|-----------|----------|---------|-----------------|---------------|-------------------|---------------|
| DSPAα1    | None     | 9.5 ± 4.6 | 0.06 ± 0.02     | 6.7 ± 3.9     | 1                 | 1             |
|           | Fbg      | 16.3 ± 7.5 | 0.86 ± 0.23     | 53 ± 28       | 8                 |               |
|           | Fbn      | 0.38 ± 0.04 | 260 ± 70       | 684,000 ± 198,000 | 102,100 | 12,900 |
| DSPAα2    | None     | 12.3 ± 9.6 | 0.12 ± 0.03     | 9.8 ± 8.0     | 1                 |               |
|           | Fbg      | 20.3 ± 6.8 | 1.6 ± 0.44      | 79 ± 34       | 8                 |               |
|           | Fbn      | 0.36 ± 0.05 | 186 ± 62       | 517,000 ± 187,000 | 52,700 | 6550 |
| DSPAβ     | None     | 14.9 ± 9.5 | 0.13 ± 0.05     | 6.0 ± 5.0     | 1                 |               |
|           | Fbg      | 9.3 ± 1.0  | 0.39 ± 0.04     | 42 ± 7        | 7                 |               |
|           | Fbn      | 0.95 ± 0.09 | 9.4 ± 0.9      | 9900 ± 1300   | 1650 | 235 |
| DSPAγ     | None     | 12.1 ± 5.6 | 0.054 ± 0.02    | 4.4 ± 2.8     | 1                 |               |
|           | Fbg      | 6.7 ± 0.8  | 0.26 ± 0.03     | 39.0 ± 7      | 9                 |               |
|           | Fbn      | 1.1 ± 0.2  | 3.9 ± 0.6       | 3510 ± 850    | 800 | 90 |
| sc-PA     | None     | 17.7 ± 5.5 | 0.6 ± 0.17      | 34 ± 14       | 1                 |               |
|           | Fbg      | 2.4 ± 0.3  | 1.5 ± 0.1       | 638 ± 92      | 19               |               |
|           | Fbn      | 0.13 ± 0.01 | 682 ± 5.9    | 525,000 ± 61,000 | 15,480 | 820 |
| t-PA      | None     | 5.2 ± 1.4  | 9.1 ± 1.5       | 1760 ± 450    | 1                 |               |
|           | Fbg      | 4.6 ± 0.6  | 62.6 ± 6.2      | 13,600 ± 2230 | 8                 |               |
|           | Fbn      | 0.21 ± 0.06 | 207 ± 55       | 972,000 ± 382,000 | 550 | 72 |
| Cleavable | None     | 7.6 ± 0.3  | 0.32 ± 0.02     | 135 ± 130     | 1                 |               |
| DSPAα1    | Fbg      | 16.3 ± 2.7 | 8.4 ± 1.5       | 516 ± 126     | 1                 |               |
|           | Fbn      | 0.37 ± 0.06 | 209 ± 72       | 565,000 ± 215,000 | 4180 | 1100 |

Fibrin Stimulation and Fibrin Selectivity Depend on the Presence of a Plasmin-sensitive Site—Several studies indicated that the abolition of the t-PA plasmin-sensitive site led to an improved fibrin selectivity of the t-PA molecule, which was mainly due to a reduced activity in the absence of a stimulator (Petersen et al., 1988; Boisse et al., 1989; Higgins et al., 1990; Paoni et al., 1993). Since the protease domains of DSPAs do not contain a plasmin-sensitive cleavage site, we wanted to estimate the contribution of fibrin stimulation and selectivity of this structurally distinct feature. To allow for a direct comparison in our experimental systems, cDNAs encoding plasmin-sensitive DSPAα1 ([H189R,S190I,T191K] DSPA1) as well as plasmin-insensitive sc-PA ([R275H,I276S,K277T] t-PA) were constructed and expressed as outlined under "Materials and Methods." Homogeneity of affinity-purified muteins was verified by SDS-PAGE (lanes 6–8, Fig. 1). Similar to t-PA, the preparation of [H189R,S190I,T191K] DSPA1 contained about 10% two-chain material (lanes 6 and 8, Fig. 1) as verified by Western analysis (data not shown). Whereas the DSPA1 mutein was easily converted to its two-chain form by treatment with plasmin, [R275H,I276S,K277T] t-PA remained single chain (Fig. 3).

In the absence of a stimulator, the DSPA1 mutein exhibited a bimolecular rate constant of 135 M⁻¹ s⁻¹, which reflected a 20-fold increase over that of wild-type DSPA1. The catalytic efficiency of sc-PA was reduced, in comparison to t-PA, by 50-fold to 34 M⁻¹ s⁻¹ (Table I), which is in good agreement to the activity decrease observed previously (Andreasen et al., 1991; Petersen et al., 1988; Tate et al., 1987). Fibrinogen raised the kcat/Kₘ value of plasmin-sensitive DSPA1 mutein to 51 s⁻¹, which in comparison to the wild type, corresponded to a 10-fold increase. The respective value (638 M⁻¹ s⁻¹) of sc-PA was 20-fold decreased. In the presence of fibrin, however, both muteins displayed catalytic efficiencies (DSPA1 mutein, 565,000 M⁻¹ s⁻¹; and t-PA mutein, 525,000 M⁻¹ s⁻¹) that were similar to the respective wild-type proteins (Table I). In comparison to fibrinogen, fibrin promoted the catalytic efficiency of plasmin-sensitive DSPA1 by 1100-fold, which was 12-fold less than that of the uncleavable wild-type enzyme. In case of t-PA, the absence of the cleavage site resulted in an 11-fold increase of its fibrin selectivity. Importantly, DSPA1 and DSPA2 were still about 16- and 8-fold more fibrin selective than t-PA (Table I), implying that other features apart from the lack of the plasmin-sensitive cleavage site must contribute to the superior fibrin selectivity of DSPAs.

Hydrolysis of S-2765 by DSPAs and t-PA in the Presence and Absence of Fibrin or Fibrinogen—The contribution of fibrin stimulation of the direct interaction between the plasminogen activators and fibrin was assessed by monitoring PA-catalyzed hydrolysis of S-2765, a small chromogenic substrate, in the
Fibrin Cofactor Requirements of Vampire Bat Plasminogen Activators

The kinetics of S-2765 hydrolysis were measured as described under "Materials and Methods." The kinetic parameters were derived from nonlinear regression analysis of Michaelis-Menten plots depicting velocity of pNA generation versus concentration of S-2765. The stimulation factor observed in the presence of a fibrinogen cofactor is presented in the second column from the right. It was calculated as the ratio of the bimolecular rate constant in the presence of fibrinogen or fibrin to those in their absence. The fibrin selectivity given in the right column was calculated as the ratio of activities (fibrin/fibrinogen).

| Enzyme | Cofactor | $K_m$ (mM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$) | Stimulation factor | Ratio Fbn/Fbg |
|--------|----------|------------|----------------------|--------------------------|--------------------|--------------|
| DSPAα1 | None     | 0.908 ± 0.063 | 5.5 ± 0.2 | 6060 ± 480 | 1 | 1 |
| DSPAα1 | Fbg      | 0.66 ± 0.11 | 7.5 ± 0.6 | 11,360 ± 2100 | 1.9 | 1.9 |
| DSPAα1 | Fbn      | 0.183 ± 0.017 | 15.4 ± 0.5 | 84,150 ± 8280 | 13.9 | 13.9 |
| DSPAα2 | None     | 1.24 ± 0.07 | 16.4 ± 0.5 | 13,230 ± 850 | 1 | 1 |
| DSPAα2 | Fbg      | 0.93 ± 0.15 | 21.4 ± 1.9 | 23,010 ± 4240 | 1.7 | 1.7 |
| DSPAα2 | Fbn      | 0.209 ± 0.012 | 35.2 ± 0.7 | 168,420 ± 10,230 | 12.7 | 12.7 |
| DSPAγ  | None     | 0.59 ± 0.11 | 6.5 ± 0.4 | 6570 ± 830 | 1 | 1 |
| DSPAγ  | Fbg      | 1.05 ± 0.024 | 8.8 ± 0.1 | 8380 ± 220 | 1.2 | 1.2 |
| DSPAγ  | Fbn      | 0.70 ± 0.09 | 7.1 ± 0.4 | 10,140 ± 1420 | 1.5 | 1.5 |
| DSPAγ  | None     | 1.1 ± 0.1 | 4.0 ± 0.2 | 3640 ± 380 | 1 | 1 |
| t-PA   | None     | 0.69 ± 0.08 | 5.7 ± 0.3 | 8260 ± 840 | 2.3 | 2.3 |
| t-PA   | Fbg      | 0.389 ± 0.007 | 13.7 ± 0.1 | 35,220 ± 690 | 1 | 1 |
| t-PA   | Fbn      | 0.253 ± 0.009 | 15.2 ± 0.2 | 60,080 ± 2060 | 1.7 | 1.7 |
| t-PA   | Fcn      | 0.13 ± 0.011 | 38.4 ± 0.4 | 141,540 ± 11,300 | 4.0 | 4.0 |
| tc t-PA| None     | 0.164 ± 0.013 | 13.5 ± 0.3 | 82,320 ± 6730 | 1.0 | 1.0 |
| tc t-PA| Fbg      | 0.156 ± 0.004 | 13.3 ± 0.4 | 85,260 ± 3370 | 1.04 | 1.04 |
| tc t-PA| Fcn      | 0.125 ± 0.016 | 14.4 ± 0.5 | 115,200 ± 15,300 | 1.4 | 1.4 |
| tc DSPAα1 | None     | 0.39 ± 0.02 | 9.6 ± 0.2 | 24,680 ± 1230 | 1.1 | 1.1 |
| tc DSPAα1 | Fbg   | 0.4 ± 0.02 | 10.9 ± 0.2 | 27,260 ± 2850 | 1.1 | 1.1 |
| tc DSPAα1 | Fcn   | 0.321 ± 0.013 | 20.9 ± 0.4 | 65,110 ± 2920 | 2.6 | 2.6 |

In the absence of a fibrinogen cofactor, DSPAα1 and α2 exhibited 17 and 37.5%, respectively, of t-PA catalytic efficiency. While fibrinogen had only a very small effect on the $k_{cat}/K_m$ of t-PA, fibrin promoted its bimolecular rate constant by 4-fold. In the presence of the latter the activity of DSPAα2 was equivalent to that of t-PA, whereas S-2765 hydrolysis by DSPAα1 was 2-fold less efficient (Table II).

As observed for t-PA and DSPAα1, the extent of fibrin-mediated stimulation of S-2765 hydrolysis was dependent on whether they occurred in their single or two-chain forms. The single chain forms of DSPAα1 and t-PA were more highly stimulated than their two-chain counterparts (Table II). The intrinsic activity of tc t-PA was not significantly stimulated by fibrin, while tc DSPAα1’s catalytic efficiency was still enhanced (2.6-fold), albeit 5-fold less than that of the single chain molecule. This effect was attributed to an increased $k_{cat}$, which was not observed for t-PA. By comparison to the wild-type molecule, the tc DSPAα1 mutant exhibited a 4-fold higher catalytic efficiency in the absence of a fibrinogen cofactor (Table II).

**Fig. 3.** Plasmin-mediated conversion of [R275H, I276S, K277T] t-PA and [H189R, S190I, T191K] DSPAα1. Recombinant proteins were prepared as described under "Materials and Methods." Approximately 5 μg each of t-PA, [R275H, I276S, K277T] t-PA, and [H189R, S190I, T191K] DSPAα1 were incubated for 30 min at 37°C in the absence (lanes 2, 4, and 6) or presence of Sepharose-immobilized plasmin (lanes 3, 5, and 7). The samples were analyzed by SDS-PAGE as outlined in the legend to Fig. 2. Lane 1, marker proteins (M) whose molecular mass is indicated on the left; Lanes 2, 4, and 6, t-PA, [R275H, I276S, K277T] t-PA, and [H189R, S190I, T191K] DSPAα1, respectively, incubated in the absence of plasmin, or as shown in lanes 3, 5, and 7, treated with plasmin.

### Table II

Kinetic parameters of S-2765 hydrolysis by DSPAs and t-PA in the presence or absence of a fibrinogen cofactor

The kinetics of S-2765 hydrolysis were measured as described under "Materials and Methods." The kinetic parameters were derived from nonlinear regression analysis of Michaelis-Menten plots depicting velocity of pNA generation versus concentration of S-2765. The stimulation factor observed in the presence of a fibrinogen cofactor is presented in the second column from the right. It was calculated as the ratio of the bimolecular rate constant in the presence of fibrinogen or fibrin to those in their absence. The fibrin selectivity given in the right column was calculated as the ratio of activities (fibrin/fibrinogen).


DISCUSSION

There are three plausible mechanisms pertinent to fibrin-mediated stimulation of plasminogen activation, all based on protein-protein interactions: 1) a template-mediated rendezvous mechanism furthering the physical encounter of both enzyme and substrate, 2) the exposure of the activation site of plasminogen, following a conformational change induced by fibrin binding, and (3) a stabilizing effect of fibrin on the active site of plasminogen activators, probably mediated by domain-domain interactions.

We have attempted to attribute the observed stimulatory effects to one or the other of these mechanisms. In contrast to t-PA, the major contribution of fibrin to its overall stimulatory effect on plasminogen conversion by DSPAs α1 and α2 is mediated by its interaction with the plasminogen activator itself (Table IV). The template effect appears to be less important, whereas it is paramount to fibrin-mediated enhancement of plasminogen activation by t-PA (Hoylaerts et al., 1982). Corroborating the results from direct measurements of fibrin binding, an interaction of DSPA α1 or α2 and fibrin is also demonstrated by the enhancement of S-2765 hydrolysis. Upon binding to fibrin, the catalytic activities of DSPA α1 and α2 were raised by about 1 order of magnitude, whereas those of DSPA β and γ were increased only marginally (Table II). Therefore, in case of DSPAβ and γ, the increase in the plasminogen activation rate is most likely due to a conformational change in plasminogen induced by its interaction with partially degraded fibrin (Suenson et al., 1984), although domain-domain interactions occurring within the DSPA molecules might also be involved.

The striking difference between DSPAs and t-PA, as far as fibrin stimulation is concerned, is not a consequence of disparate fibrin affinities. The Kd values of DSPA α1 and DSPA α2 (Fig. 2) are within the range of values published for t-PA (0.13–0.58 mM) (Higgins and Vehar, 1987; Husain et al., 1989; Nesheim et al., 1990; Bergum and Gardell, 1992; Horrevoets et al., 1994). The data are particularly consistent, if only finger-dependent binding of t-PA is analyzed. Under these conditions, Nesheim et al. (1990) measured a Kd of 0.13 μM and a molar binding ratio of 0.6, values that are almost identical to those of DSPA α1 and α2 (Fig. 2). Furthermore, the dependence on the fibrin concentration was very similar for DSPAs and t-PA. Half-maximal velocities were achieved at 25 ± 3, 31 ± 5, and 13 ± 2 μg/ml for DSPA α1, DSPA α2, and t-PA, respectively (data not shown).

All DSPAs exhibited only marginal activity in the absence of a fibrin(ogen) cofactor (Table I). Upon addition of fibrinogen, their second order rate constants increased similarly by roughly 1 order of magnitude, which is in contrast to the markedly diverging stimulatory effect mediated by fibrin. For instance, in case of DSPA α1 and DSPA α2, the extent of fibrin stimulation differs by a factor of 62 (Table I). These data

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**Table III**

| Plasminogen activator | kcat/Km | Stimulation factor |
|-----------------------|---------|--------------------|
|                       | M⁻¹s⁻¹  |                    |
| DSPA α1               | 26 ± 23 | 3.9                |
| DSPA α2               | 39 ± 11 | 4.0                |
| DSPA β                | 33 ± 30 | 5.5                |
| DSPA γ                | 26 ± 9  | 5.9                |
| tc DSPA α1            | 890 ± 250 | 6.6              |
| t-PA                  | 7480 ± 5980 | 4.3             |
| sc t-PA               | 320 ± 190 | 9.4               |

**Table IV**

| Plasminogen activator | K_m | kcat | Increase versus EACA alone |
|-----------------------|-----|------|----------------------------|
|                       | μM  | 10⁻⁵ |                         |
|                       | M⁻¹s⁻¹ |     |                         |
| DSPA α1               | 64.3 ± 5.8 | 345 ± 49 | 7790 ± 1500 | 300 | 88 |
| DSPA α2               | 46.3 ± 7.6 | 265 ± 45 | 5720 ± 1350 | 147 | 90 |
| tc DSPA α1            | 17.4 ± 3.8 | 190 ± 40 | 11,020 ± 3200 | 12 | 51 |
| t-PA                  | 6.3 ± 1.0 | 260 ± 30 | 41,270 ± 6100 | 5.5 | 24 |
Therefore suggest that the stimulatory effect exerted by fibrinogen is not conferred via the DSPAs but is rather mediated by other, yet unknown, determinants within the protease domain of DSPA. The ratio of the bimolecular rate constants of plasminogen activation in the presence of fibrin versus fibrinogen is defined as fibrin selectivity. Since DSPAα1α2 and t-PA exhibited very similar bimolecular rate constants in the presence of fibrin, the significant difference in fibrin selectivity of DSPAα1α2 and t-PA is mainly caused by their unequal catalytic efficiencies in the presence of fibrin (Table I).

To further understand the underlying structure-function relationship, we have analyzed the properties of a mutein of DSPAα1, whose protease domain contained a plasmin-sensitive site (Tables I-IV). In the presence of fibrin, the catalytic efficiency of plasmin-sensitive DSPAα1 was strikingly less (24-fold) increased than that of the wild-type protein (Table I). This decrease in fibrin stimulation was entirely attributable to a diminished stimulation via the plasminogen activator protease domain because upon prevention of the template effect by addition of α-amino capric acid, fibrin stimulated the catalytic efficiency only 12-fold as opposed to 300-fold as observed for DSPAα1 (Table IV). Since the bimolecular rate constants of DSPAα1 and its plasmin-sensitive mutein were almost identical in the presence of fibrin (Table I), the decreased stimulatory effect was a consequence of the mutein’s higher basal activity. Further, the fibrin selectivity of cleavable DSPAα1 was decreased about 12-fold (Table I) and the fibrin-stimulated intrin-

sive rate constant for t-PA is mainly caused by their unequal catalytic efficiencies in the presence of fibrinogen (Table I).

Therefore, the heterotropic effect conferred by fibrin does not involve other, yet unknown, determinants within the protease domain. Abolition of the plasmin-sensitive cleavagesite partially obliterates therequirement for stabilization of a preformed active site. Introduction of a plasmin-sensitive cleavage site partially obliterates the requirement for the fibrin cofactor. Further understanding of the molecular details of this interaction will depend on the results of structural analysis, which is currently underway.

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DSPAα1α2 (Bat-PA) and t-PA have been evaluated in a way comparable to this study by other authors. Pertaining to t-PA, our data are similar to those previously reported (Rânby, 1982; Urano et al., 1988; de Vries et al., 1991; Bergum and Gardell, 1992). Our values were generally higher than those published for Bat-PA (corresponding to DSPAα2) by Bergum and Gardell (1992). This difference can be explained by use of different Km values for plasmin-mediated hydrolysis of the chromogenic substrate (FlavigenPl versus SpectrozymePl). The relative stimulation factors, however, are in good agreement for Bat-PA K intra/km (43500-fold increase in the presence of fibrin, DSPAα2 52,700-fold). Fibrin (fibrin II) stimulated DSPAα2 catalytic efficiency 10,900-fold more than fibrinogen as compared to the ratio of 6550 determined in our system. Our data, however, do not confirm that in the absence of a stimulator the Bat-PA Km was 0.6 μM and therefore smaller than in the presence of fibrinogen (Bergum and Gardell, 1992). By contrast, DSPAα2 affinity for Galu-plasminogen was very low as indicated by a Km of 12.3 μM, a value very similar to those determined for DSPα1 (9.5 μM) and t-PA (17.7 μM). Also, our unstimulated Km value for t-PA, 5.2 μM, was similar to the 6.7 μM reported by Bergum and Gardell (1992) and agreed very well with the values of 7.6 and 9 μM published by Rânby (1982) and Urano et al. (1988), respectively.

In summary, we have provided a biochemical rationale for the striking fibrin selectivity of DSPAs: finger-dependent fibrin binding confers a heterotrophic effect, which is conceivably mediated by domain-domain interactions on the protease domain to stabilize a preformed active site. Introduction of a plasmin-sensitive cleavage site partially obliterates the requirement for the fibrin cofactor. Further understanding of the molecular details of this interaction will depend on the results of structural analysis, which is currently underway.
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