THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 43, Issue of October 27, pp. 25596–25603, 1995

Printed in U.S.A.

Structural Features Mediating Fibrin Selectivity of Vampire Bat Plasminogen Activators*

(Received for publication, May 22, 1995, and in revised form, August 18, 1995)

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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 (DSPAα1 and α2) and EKP and KP (DSPAβ and DSPAγ). Only DSPAα1 and α2 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 (k_{cat}/K_m) of DSPAα1 increases 10^5-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 DSPAα1, α2, 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 Arg^{560}-Val^{561} 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 fibrin(ogen) degradation products (Camiolo *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) (Pennica *et al.*, 1983; Patthy, 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 mutein of t-PA called TNK, which is more fibrin selective than t-PA, has been characterized (Keyt *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 DSPAα1, α2, β, and γ have been cloned, expressed, and characterized. DSPAα1 and α2 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 similarly related (72.3% (DSPAα1) and 74.2% (DSPAα2) 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-

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[1] The abbreviations used are: PA, plasminogen activator; EACA, ϵ-amino caproic acid; PAGE, polyacrylamide gel electrophoresis; DSPA, *D. rotundus* salivary plasminogen activator; LBS, lysine binding site; *p*NA, *p*-nitroaniline; tcDSPAα1, two-chain DSPAα1; tct-PA, two-chain t-PA.

ment for a fibrin cofactor. This was studied in great detail for Bat-PA (equivalent to DSPAα2) by Bergum and Gardell (1992) and has been reported for DSPAα1 as well (Schleuning *et al.*, 1992). When compared to t-PA, Bat-PA and DSPAα1 demonstrated an equal or even higher thrombolytic potency in several animal models of arterial thrombosis (Gardell *et al.*, 1991; Mellot *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 DSPAα1 (Gardell *et al.*, 1991; Mellot *et al.*, 1992; Witt *et al.*, 1992; 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 selectivity of DSPAs.

MATERIALS AND METHODS

Mutagenesis—Oligonucleotide-directed mutagenesis was performed as described by Lewis and Thompson (1990) using the Promega mutagenesis kit. DSPAα1 cDNA (Krätzschmar *et al.*, 1991) was subcloned into the *Eco*RI-*Hin*dIII sites of the pSELECT-1 phagemid polylinker. The plasmin-sensitive site was introduced annealing the following oligonucleotide: 5′-CAGCCTCGCATTAAAGGAGGACTC-3′. T-PA cDNA (Waller and Schleuning, 1985) was ligated into the *Hin*dIII site of the same vector. The plasmin-sensitive site was inactivated by hybridizing the following oligonucleotide: 5′-CCTCAGTTTCACAGCACAGGAGGGCTC-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 pMPSVEH expression vectors (Artelt *et al.*, 1988; Wirth *et al.*, 1991) harboring the cDNAs encoding wild-type DSPAs α1, α2, β, and γ (Krätzschmar *et al.*, 1991), the mutated DSPAα1 cDNA, or the mutated t-PA cDNA (Krätzschmar *et al.*, 1992). The secreted recombinant plasminogen activators were 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 (GmbH, Biberach, Germany). Human Glu-plasminogen, received from Chromogenix (Sweden), was liberated from contaminating lysine by gel filtration using PD-10 columns. Plasminogen-free human fibrinogen was purchased from Calbiochem, and human thrombin was ordered from Sigma. The chromogenic substrates S-2765 (*N*-α-Cbo-D-Arg-Gly-Arg-*p*-nitroanilide-dihydrochloride) and FlavigenPli (D-But-CHT-Lys-*p*-nitroanilide-dihydrochloride) were obtained from Chromogenix and Biopool, respectively. The two-chain form of the mutein [H189R,S190I,T191K] DSPAα1 and of t-PA was prepared by treatment with plasmin immobilized to Sepharose according to Higgins and Vehar (1987). Protein concentrations were determined spectrophotometrically at 280 nm, using extinction coefficients (cm/mg) for 1 mg/ml solutions at 280 nm of 1.70 and 1.62 for Glu-plasminogen (Robbins and Summaria, 1970) and fibrinogen (Blombäck, 1958), respectively. The extinction coeffients of 1.71, 1.65, 1.69, 1.68, and 1.81 for DSPAα1, DSPAα2, DSPAβ, DSPAγ, and t-PA, respectively, were calculated employing the program PeptideSort of the Wisconsin Sequence Analysis Package™ (Devereux *et al.*, 1984). The integrity of Glu-plasminogen was verified by N-terminal sequence analysis.

Radioiodination of PAs—The DSPA variants were labeled by the iodogen method according to the manufacturer's protocol (Pierce, no. 28600) and exhibited specific activities of 40–80 KBq/μg protein. In brief, DSPAs (0.2 mg/ml) were labeled with [^{125}I]iodine in 50 mM HEPES, pH 7.5, 0.1 M NaCl in iodogen-coated tubes containing 3.7 MBq sodium [^{125}I]iodide. Following incubation for 15 min at 4 °C, the reactants were separated by gel filtration using a PD-10 column (Pharmacia Biotech Inc.). Protein concentration of labeled DSPAs was determined by a microtiter plate version of the method described by Bearden (1978).

Fibrin Binding—The binding of DSPAs to forming fibrin clots was studied as a competition assay using a slightly modified version of the method described by Rijken *et al.* (1982). In brief, human fibrinogen (plasminogen-free) (120 μg/ml (294 nM clottable protein), final concentration) was mixed with a constant amount of ^{125}I-labeled DSPAs (5–10 nM, approximately 130,000 cpm), various amounts of unlabeled DSPAs (0–5 μM, final concentration), and human thrombin (0.1 NIH units/ml, final concentration). The total volume was 0.1 ml, and the buffer was composed of 25 mM Tris, 40 mM NaCl, 0.5 mM $CaCl_2$, pH 8.0, containing 0.01% Tween 20. The mixture was incubated for 1 h at 37 °C, and the clots were compacted by centrifugation in a Heraeus Biofuge 15 at 15,000 rpm for 15 min. Unbound PA was directly quantified by γ counting of an aliquot of the supernatant in a Canberra Packard Cobra II γ counter. The amount of specifically bound DSPA was calculated as the difference between the total amount of DSPA and that determined in the supernatant following background correction of nonspecific binding (maximum 4% of total cpm).

The dissociation constant for the binding of DSPAs to fibrin and the numbers of binding sites were calculated by nonlinear regression analysis of the data according to the Scatchard equation employing the programs EBDA (equilibrium binding data analysis) and LIGAND, 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 nM plasminogen activator, 100 μg/ml fibrin(ogen) where stated (0.13 units/ml of human thrombin in case of fibrin), 0.05–8 μM Glu-plasminogen, and 1 mM FlavigenPli in a total volume of 0.15 ml of PCLA buffer (Jones and Meunier, 1990). When plasminogen activation was analyzed in the presence of ϵ-amino caproic acid (EACA), individual assays contained 20 mM 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, $\Delta A_{405} - A_{490}$/min was monitored. Although FlavigenPli hydrolysis by thrombin was not significant and there was no detectable autohydrolysis, 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 [*p*NA] using appropriate standard curves. The acceleration of *p*NA generation (d^2[pNA]*dt^{-2}), which is directly proportional to the velocity of plasminogen activation, was determined by nonlinear regression analysis of 2nd order polynomial plots of [*p*NA] *versus* time. It was then plotted against the concentration of plasminogen, and kinetic parameters k_{cat}, K_m, and k_{cat}/K_m 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 FlavigenPli hydrolysis by plasmin were determined under the aforementioned conditions and verified the assumption that K_m(pli) $\ll$ [FlavigenPli] (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 nM plasminogen activator, 100 μg/ml fibrin(ogen), and 0.02–4 mM S-2765 in PCLA buffer. Individual assays performed as triplicates were repeated 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, $\Delta A_{405} - A_{490}$/min was calculated and converted to [*p*NA]. In this case velocities, calculated from linear plots of [*p*NA] *versus t*, were plotted *versus* concentration of S-2765 and analyzed by nonlinear regression to obtain kinetic parameters K_m and k_{cat}.

RESULTS

DSPAs α1, α2, 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 α1, α2, 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, DSPAα1 and α2, exhibited affinity to fibrin, whereas DSPAs β and γ did not (Schleun-

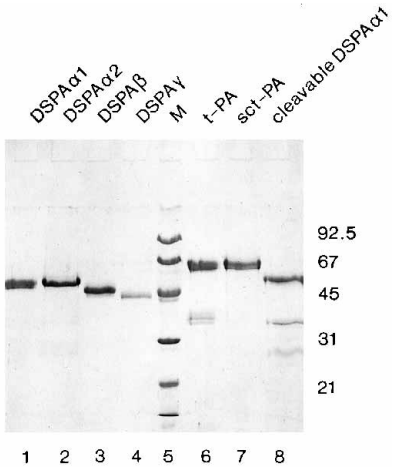

FIG. 1. **SDS-PAGE of purified recombinant DSPAs and t-PA.** Prior to electrophoresis on a SDS-gel containing 12.5% polyacrylamide (Laemmli 1970), all samples were reduced by the addition of dithiothreitol (12.5 mM). Approximately 3 μg of each protein was loaded. The gel was stained with Coomassie Brilliant 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*, [R275H,I276S,K277T] t-PA lacking the plasmin-sensitive site; *lane 8*, [H189R,S190I,T191K] DSPAα1 containing a plasmin-sensitive site.

ing *et al.*, 1992). Using ^{125}I-iodinated recombinant DSPAs, we assessed their affinity for forming fibrin clots *in vitro*. Binding of both DSPAs α1 and α2 was saturable and exhibited similar characteristics (Fig. 2). A Scatchard analysis of the interaction between forming fibrin clots and DSPAα1 or α2 revealed virtually identical K_d values of 154 ± 43 nM and 131 ± 15 nM, and similar molar binding ratios of 0.48 ± 0.08 and 0.61 ± 0.14, respectively (Fig. 2). Interaction with fibrin does not involve a lysine binding site because it was not impaired by the presence of lysine or EACA even in a molar excess of several orders of magnitude (data not shown). Using the protocol outlined under "Materials and Methods," we were unable to detect measurable binding to fibrin of DSPAs β and γ (data not shown).

Glu-Plasminogen Activation by DSPAs and by t-PA in the Absence and Presence of a Fibrin(ogen) Cofactor—In the absence of a fibrin(ogen) cofactor, DSPAs α1, α2, β, and γ exhibited similar, but very low bimolecular rate constants ranging from 4.4 to 9.8 M^{-1} s^{-1} (Table I). The corresponding K_m values were approximately 10 μM or higher, and their k_{cat} values were in the range of 1.0 * 10^{-4} s^{-1}, indicating that DSPAs hardly showed affinity for the substrate and were virtually unable to activate Glu-plasminogen.

In the presence of fibrin, however, the catalytic efficiency of DSPAs was augmented by several orders of magnitude. The steepest increase by a factor of 10^5, resulting in a k_{cat}/K_m value of 684,000 M^{-1} s^{-1}, was observed for DSPAα1. The bimolecular rate constant of DSPAα2 was raised to 517,000 M^{-1} s^{-1}, corresponding to a 53,000-fold enhancement (Table I). DSPAs β and γ exhibited considerably smaller catalytic efficiencies of 9900 M^{-1} s^{-1} and 3510 M^{-1} s^{-1}, reflecting a 1650- and 800-fold increase in catalytic activity, respectively. This demonstrates that binding of the DSPAs to fibrin significantly contributes to enhanced plasminogen activation. For DSPAα1 and α2, the fibrin-mediated enhancement of catalytic efficiency resulted from both a moderate decrease (25- and 34-fold) in K_m and a concomitant profound increase (4300- and 1550-fold) in k_{cat}, respectively. Interestingly, despite their high degree of homology (89% identity in amino acid sequence) (Krätzschmar *et al.*, 1991), the unstimulated activity of DSPAα2 was slightly higher than that of DSPAα1, which, however, was more active in the

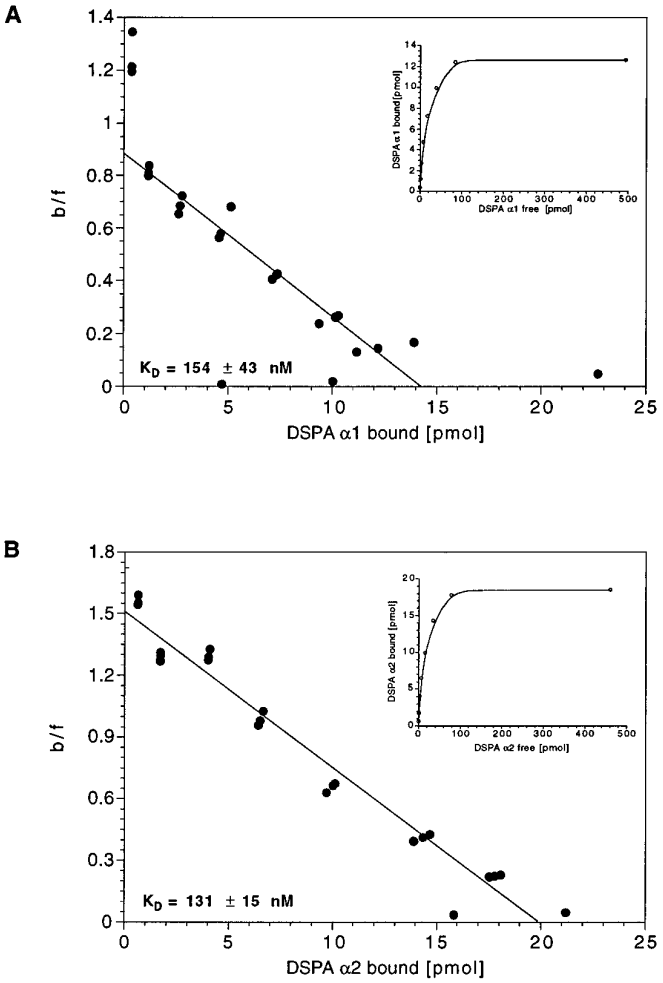

FIG. 2. **Scatchard analysis of the interaction between DSPAα1 or DSPAα2 and forming fibrin clots.** *A*, Scatchard analysis of fibrin binding data obtained for ^{125}I-DSPAα1. The plot is based on a model involving one type of binding site. *Inset*, equilibrium binding of ^{125}I-DSPAα1 to fibrin. Binding was determined at variable concentrations of unlabeled DSPAα1 as described under "Materials and Methods." Each point is represented by the results of three independent determinations. The average standard error for the equilibrium binding curve was approximately 7%. Specific binding was calculated by subtracting nonspecific binding (*e.g.* in the presence of excess unlabeled DSPAα1) from total binding. Nonspecific binding was 4% of total cpm. *B*, Scatchard plot of fibrin binding data of ^{125}I-DSPAα2. The plot is based on a model involving a sole type of binding site. *Inset*, equilibrium binding of ^{125}I-DSPAα2 to fibrin. Binding was determined as described under "Materials and Methods." The results of three independent experiments are displayed. The average standard error for the equilibrium binding curve was about 6%. Specific binding was calculated by subtracting nonspecific binding (*e.g.* in the presence of excess unlabeled DSPAα2) from total binding. Nonspecific binding was 6% of total cpm.

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 k_{cat} value of DSPAα1 rather than a more pronounced decrease in its K_m (Table I). The reduction in K_m and in particular the increase in k_{cat} 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 k_{cat}/K_m 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

TABLE I

Kinetic parameters of Glu-plasminogen activation by DSPAs and t-PA (influence of fibrinogen or fibrin)

Kinetics of Glu-plasminogen activation were measured as outlined under "Materials and Methods." The kinetic parameters were calculated from nonlinear regression analysis of the Michaelis-Menten graph plotting the acceleration of FlavigenPli hydrolysis *versus* plasminogen concentration. The stimulation factor observed in the presence of fibrinogen or fibrin is given in the second column from the right. It was calculated as the ratio of the bimolecular rate constants in the presence of fibrinogen or fibrin over those in their absence. The right column presents a measure of the fibrin selectivity of the plasminogen activators indicated as activity ratio (Fbn/Fbg). It was calculated as the quotient of the catalytic efficiencies in the presence of fibrin and fibrinogen.

Enzyme	Cofactor	K_m	$k_{cat} * 10^3$	k_{cat}/K_m	Stimulation factor	Ratio Fbn/Fbg
		μM	s^{-1}	$M^{-1} s^{-1}$		
DSPAα1	None	9.5 ± 4.6	0.06 ± 0.02	6.7 ± 3.9	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 ± 0.022	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
sct-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	68.2 ± 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 DSPAα1	None	7.6 ± 0.3	0.32 ± 0.02	135 ± 130	1	
	Fbg	16.3 ± 2.7	8.4 ± 1.5	516 ± 126	4	
	Fbn	0.37 ± 0.06	209 ± 72	565,000 ± 215,000	4180	1100

higher than that in the presence of fibrinogen. The respective values for DSPAs β and γ were 235 and 90 (Table I). DSPAα1 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 fibrin(ogen) cofactor, t-PA was 260-fold more efficient in activating Glu-plasminogen than DSPAα1. 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 10^5-fold for DSPAα1. In the presence of fibrinogen, the catalytic efficiency of t-PA was increased to 13,600 M^{-1} s^{-1}, which was 260- and 170-fold higher than the respective values measured for DSPAα1 and α2 (Table I). Fibrin increased the catalytic efficiency of t-PA by only 72-fold over that in the presence of fibrinogen, meaning that DSPAα1 was about 180-fold more fibrin selective than t-PA. DSPAα2 exhibited a 90-fold higher fibrin selectivity than t-PA, and even the finger-deficient variant DSPAβ 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.

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; Boose *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 to 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] DSPAα1) as well as plasmin-insensitive sct-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] DSPAα1 contained about 10% two-chain material (*lanes 6* and *8*, Fig. 1) as verified by Western analysis (data not shown). Whereas the DSPAα1 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 DSPAα1 mutein exhibited a bimolecular rate constant of 135 M^{-1} s^{-1}, which reflected a 20-fold increase over that of wild-type DSPAα1. The catalytic efficiency of sct-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 k_{cat}/K_m value of plasmin-sensitive DSPAα1 mutein to 516 M^{-1} s^{-1}, which in comparison to the wild type, corresponded to a 10-fold increase. The respective value (638 M^{-1} s^{-1}) of sct-PA was 20-fold decreased. In the presence of fibrin, however, both muteins displayed catalytic efficiencies (DSPAα1 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 DSPAα1 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, DSPAα1 and DSPAα2 were still about 16- and 8-fold more fibrin selective than uncleavable sct-PA, and plasmin-sensitive DSPAα1 was 15-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 to 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

absence or presence of a fibrin cofactor. Fibrin increased the k_{cat}/K_m of DSPAα1 and α2 equally by about 14-fold over that in its absence, while fibrinogen led only to a marginal enhancement of less than 2-fold (Table II). The fibrin-mediated promotion of their bimolecular rate constant was due to a 5-fold drop in K_m and a 2–3-fold increase in k_{cat}. Although fibrin and fibrinogen exerted quantitatively similar effects, their absolute catalytic efficiency of S-2765 hydrolysis was different. Independent of the presence or absence of a fibrin cofactor, DSPAα2 was always 2-fold more efficient in hydrolyzing S2765 than DSPAα1. This elevated activity was brought about by a higher k_{cat} value. As expected, because they do not bind to fibrin, an appreciable fibrin-mediated enhancement of S-2765 hydrolysis by DSPAs β and γ did not occur (Table II).

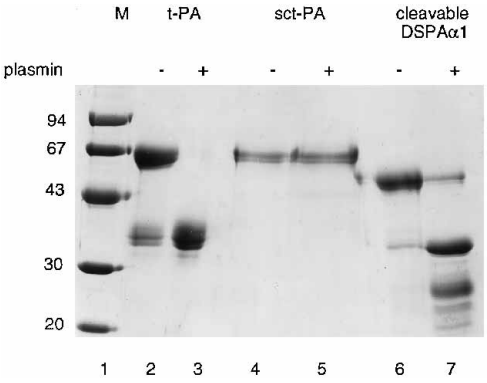

FIG. 3. **Plasmin-mediated conversion of [R275H,I276S,K277T] t-PA and [H189R,S190I,T191K] DSPAα1.** Recombinant proteins were prepared as described under "Materials and Methods." Approximately 5 μg each of t-PA, [R275H,I276S,K277T] t-PA, and [H189R,S190I,T191K] DSPAα1 were incubated for 30 min at 37 °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.

In the absence of a fibrin(ogen) 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 tct-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 mutein exhibited a 4-fold higher catalytic efficiency in the absence of a fibrin(ogen) cofactor (Table II).

Influence of EACA on Plasminogen Activation by DSPAs and t-PA—The influence of plasminogen conformation on the stimulation of plasminogen activation by DSPAs and t-PA was addressed using EACA. Upon occupying plasminogen's LBS, EACA mediates the conversion of plasminogen's compact structure into a more extended and open form (Mangel *et al.*, 1990; Ponting *et al.*, 1992). Since plasminogen binds to fibrin via its LBS, it is thought that the flexible conformation of the EACA-complexed protein mimics that of fibrin-bound plasminogen. In the presence of EACA, conversion of plasminogen by all plasminogen activators tested was enhanced by 4–9-fold (Table III), in line with results published previously for t-PA (Urano *et al.*, 1988). Since EACA did not influence the rate of FlavigenPli hydrolysis by plasmin (data not shown), the increase in the bimolecular rate constant was most likely due to a change in plasminogen conformation, rendering it a more favorable substrate.

Since alterations in plasminogen conformation only accounted for a small portion of fibrin's stimulatory effect, it is

TABLE II

Kinetic parameters of S-2765 hydrolysis by DSPAs and t-PA in the presence or absence of a fibrin(ogen) 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 *p*NA generation *versus* concentration of S-2765. The stimulation factor observed in the presence of a fibrin(ogen) cofactor is presented in the second column from the right. It was calculated as the ratio of the bimolecular rate constants 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	k_{cat}	k_{cat}/K_m	Stimulation factor	Ratio Fbn/Fbg
		mM	s^{-1}	$M^{-1}\ s^{-1}$		
DSPAα1	None	0.908 ± 0.063	5.5 ± 0.2	6060 ± 480	1	
	Fbg	0.66 ± 0.11	7.5 ± 0.6	11,360 ± 2100	1.9	
	Fbn	0.183 ± 0.017	15.4 ± 0.5	84,150 ± 8280	13.9	7.4
DSPAα2	None	1.24 ± 0.07	16.4 ± 0.5	13,230 ± 850	1	
	Fbg	0.93 ± 0.15	21.4 ± 1.9	23,010 ± 4240	1.7	
	Fbn	0.209 ± 0.012	35.2 ± 0.7	168,420 ± 10,230	12.7	7.3
DSPAβ	None	0.99 ± 0.11	6.5 ± 0.4	6570 ± 830	1	
	Fbg	1.05 ± 0.024	8.8 ± 0.1	8380 ± 220	1.2	
	Fbn	0.70 ± 0.09	7.1 ± 0.4	10,140 ± 1420	1.5	1.2
DSPAγ	None	1.1 ± 0.1	4.0 ± 0.2	3640 ± 380	1	
	Fbg	0.87 ± 0.02	5.6 ± 0.1	6440 ± 190	1.8	
	Fbn	0.69 ± 0.08	5.7 ± 0.3	8260 ± 840	2.3	1.3
t-PA	None	0.389 ± 0.007	13.7 ± 0.1	35,220 ± 690	1	
	Fbg	0.253 ± 0.008	15.2 ± 0.2	60,080 ± 2060	1.7	
	Fbn	0.13 ± 0.011	18.4 ± 0.4	141,540 ± 11,300	4.0	2.4
tct-PA	None	0.164 ± 0.013	13.5 ± 0.3	82,320 ± 6730	1.0	
	Fbg	0.156 ± 0.004	13.3 ± 0.4	85,260 ± 3370	1.04	
	Fbn	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	
	Fbg	0.4 ± 0.02	10.9 ± 0.2	27,260 ± 2850	1.1	
	Fbn	0.321 ± 0.013	20.9 ± 0.4	65,110 ± 2920	2.6	2.4

evident that the major contribution to fibrin stimulation is mediated by the ternary complex formation and/or the interaction of DSPAα1 and α2 with fibrin (Hoylaerts *et al.*, 1982; Wu *et al.*, 1990). To discriminate between these two mechanisms, we have measured enzyme kinetics in the presence of both fibrin and EACA. Under these conditions, only the plasminogen activator is able to bind to fibrin, whereas binding of plasminogen to fibrin is inhibited (Lucas *et al.*, 1983; Nesheim *et al.*, 1990). Binding of t-PA is solely mediated by the finger domain because kringle 2-dependent binding of t-PA to fibrin is inhibited (van Zonneveld *et al.*, 1986; Nesheim *et al.*, 1990).

By comparison to their catalytic efficiencies in the presence of fibrin alone, the additional presence of EACA diminished the catalytic activities of DSPAs α1, α2, tc DSPAα1, and t-PA by 90–24-fold (Table IV). As expected, this decrease was entirely due to an increase in the apparent K_m, since the k_{cat} values were similar to that obtained in the presence of fibrin alone (Tables I and IV). Hence, the dissociation of plasminogen from the fibrin template caused similar decrements, within the same order of magnitude, of plasminogen activator catalytic efficiencies. However, a comparison of the bimolecular rate constants in the presence of fibrin and EACA to those in the presence of EACA alone revealed striking differences between DSPAs and t-PA. The addition of fibrin to the reaction mixture that already contained EACA raised DSPAα1's k_{cat}/K_m from 26 to 7790 M^{-1} s^{-1} (about 300-fold), whereas that of t-PA was promoted by only 6-fold from 7480 to 41,270 M^{-1} s^{-1} (Tables III and IV). In accordance with our earlier observation, the activity of DSPAα1 was 2-fold more highly promoted by the addition of fibrin than that of DSPAα2 (Table IV). In the presence of fibrin and EACA, the catalytic efficiency of the plasmin-sensitive DSPAα1 mutein was enhanced to 11,020 M^{-1} s^{-1}, which represented an only 12-fold increase over that, 890 M^{-1} s^{-1}, in the presence of EACA alone.

TABLE III

Catalytic efficiency of plasminogen activation by DSPAs and t-PA in the presence of EACA

Kinetics of plasminogen activation were measured as described under "Materials and Methods." Kinetic parameters were calculated as outlined in the legend to Table I. The stimulation factor represents the quotient of the bimolecular rate constant in the presence over that in the absence (Table I) of 20 mM EACA.

Plasminogen activator	k_{cat}/K_m	Stimulation factor
	M^{-1} s^{-1}	
DSPAα1	26 ± 23	3.9
DSPAα2	39 ± 11	4.0
DSPAβ	33 ± 30	5.5
DSPAγ	26 ± 9	5.9
tc DSPAα1	890 ± 250	6.6
t-PA	7480 ± 5980	4.3
sct-PA	320 ± 190	9.4

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 DSPAα1 or α2 and fibrin is also demonstrated by the enhancement of S-2765 hydrolysis. Upon binding to fibrin, the catalytic activities of DSPAα1 and α2 were raised by about 1 order of magnitude, whereas those of DSPAs 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 K_d values of DSPAα1 and DSPAα2 (Fig. 2) are within the range of values published for t-PA (0.13–0.58 μM) (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 K_d of 0.13 μM and a molar binding ration of 0.6, values that are almost identical to those of DSPAα1 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 DSPAα1, DSPAα2, 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 DSPAα1 and DSPAβ, the extent of fibrin stimulation differs by a factor of 62 (Table I). These data

TABLE IV

Kinetic parameters of plasminogen activation by fibrin-binding DSPAs and t-PA in the presence of both fibrin and EACA

Kinetics of plasminogen activation were measured as described under "Materials and Methods," and apparent kinetic parameters were calculated from nonlinear regression analysis of the Michaelis-Menten graph plotting the acceleration of FlavigenPli hydrolysis *versus* the concentration of plasminogen. The ratio of the bimolecular rate constant in the presence of both fibrin and 20 mM EACA over that in the presence of 20 mM EACA alone is given in the penultimate column to the right. The final column depicts the ratio of the catalytic activity in the presence of fibrin over that in the presence of both fibrin and 20 mM EACA.

Plasminogen activator	K_m	$k_{cat} * 10^3$	k_{cat}/K_m	Increase *versus* EACA alone	Decrease *versus* fibrin alone
	μM	s^{-1}	M^{-1} s^{-1}		
DSPAα1	44.3 ± 5.8	345 ± 49	7790 ± 1500	300	88
DSPAα2	46.3 ± 7.6	265 ± 45	5720 ± 1350	147	90
tc DSPAα1	17.4 ± 3.8	190 ± 40	11,020 ± 3200	12	51
t-PA	6.3 ± 1.0	260 ± 30	41,270 ± 8100	5.5	24

therefore suggest that the stimulatory effect exerted by fibrinogen is not conferred via the DSPAs but is rather mediated by an interaction between plasminogen and fibrinogen (Lucas *et al.*, 1983).

The ratio of the bimolecular rate constants of plasminogen activation in the presence of fibrin *versus* fibrinogen is defined as fibrin selectivity. Since DSPAα1, α2, and t-PA exhibited very similar bimolecular rate constants in the presence of fibrin, the significant difference in fibrin selectivity of DSPAα1/α2 and t-PA is mainly caused by their unequal catalytic efficiencies in the presence of fibrinogen (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 ϵ-amino caproic 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 intrinsic catalytic activity about 5-fold (Table II). Hence, the lack of a plasmin-sensitive site within the DSPAα1 protease domain contributes significantly to both the impressive stimulation by fibrin and its fibrin selectivity. Abolition of the plasmin-sensitive site of t-PA brings a quantitatively similar effect into bearing, namely a 28-fold increase in fibrin stimulation (Table I). Concomitantly, the fibrin selectivity is improved 11-fold, again quantitatively equal to the effect observed for the cleavable DSPAα1 mutein. The role of the t-PA plasmin cleavage site for fibrin stimulation has been investigated previously, and similar results have been obtained (Tate *et al.*, 1987; Petersen *et al.*, 1988; Higgins *et al.*, 1990). Nienaber *et al.* (1992) suggest that the interaction of the single-chain form of t-PA with fibrin(ogen) induces a conformational change at the active site stabilizing an "active locked" conformation. Since the influence on fibrin stimulation and on fibrin selectivity of a plasmin-sensitive site in the protease domain of DSPAα1 or t-PA is almost identical, it is likely that the stimulation of the DSPAα1 protease domain by fibrin is due to a similar mechanism.

Notably, plasmin-sensitive DSPAα1 was still 15-fold more fibrin selective than t-PA, and DSPAα1 discriminated more than 7-fold better between fibrin and fibrinogen than sct-PA (Table I). This difference is also corroborated by amidolytic data. While fibrin raised the intrinsic catalytic activity of tc DSPAα1 by 2–3-fold over that in the presence of fibrinogen, the k_{cat}/K_m of tct-PA remained essentially unchanged (Table II). Therefore, the heterotropic effect conferred by fibrin does not depend solely on the lack of the plasmin-sensitive cleavage site but involves other, yet unknown, determinants within the molecule.

Only DSPAα2 (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 (Rånby, 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 k_{cat} values for plasmin-mediated hydrolysis of the chromogenic substrate (FlavigenPli *versus* SpectrozymePl). The relative stimulation factors, however, are in good agreement (the Bat-PA k_{cat}/K_m 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 K_m was 0.6 μM and therefore smaller than in the presence of fibrinogen (Bergum and Gardell, 1992). By contrast, DSPAα2 affinity for Glu-plasminogen was very low as indicated by a K_m of 12.3 μM, a value very similar to those determined for DSPAα1 (9.5 μM) and sct-PA (17.7 μM). Also, our unstimulated K_m 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 Rånby (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 heterotropic 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.

Acknowledgments—We are grateful to Drs. Michael McCaman, Linda Cashion, and Thomas Petri for fermentation of recombinant cell cultures. We thank Gisela Hübner-Kosney, Dyana Schwerdt, Dania Schmidt, and Andrea Toben for excellent technical support. We thank Dr. J. Verheijen for critical reading of the manuscript.

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