Analysis of Tissue Plasminogen Activator Specificity Using Peptidyl Fluorogenic Substrates†

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ABSTRACT: A series of 54 fluorogenic substrates have been synthesized and evaluated for tissue-type plasminogen activator (tPA) hydrolysis in an attempt to create efficient sensitive substrates for tPA and to investigate substrate structure−efficiency correlations. All substrates contain the 6-amino-1-naphthalenesulfonamide (ANSN) leaving group, Arg in the P_1 position, various amino acids in the P_2 and P_3 positions, and various substituents in the sulfonamide moiety of the leaving group (P′ position). The majority of substrates have relatively low K_M values (<100 μM), reaching as low as 2.6 μM, and reasonably high k_{cat} values (up to 3.6 s^{-1}). These substrates have higher affinity, higher hydrolysis rates, and higher efficiency for two-chain tPA than for the single-chain form of this enzyme. Analysis of the P_3 structure influence on substrate efficiency demonstrates that compounds which contain D-isomers of N-blocked bulky amino acids, such as Phe, Leu, and Val, in this position are more efficient for tPA than substrates with N-unblocked small amino acids (Ser or Pro) in the P_3 position. The second-order rate constants and k_{cat} values for substrate hydrolysis increase with decreases in the P_2 amino acid hydrophobicity in the following manner: Leu < Val and Gly < Ser < Pro. Substrates which contain an ANSN leaving group had a higher affinity for tPA than substrates with *p*-nitroaniline or 7-amino-4-methylcoumarin leaving groups. Analyses of substrate hydrolysis dependence on the substrate P′ structure show that the k_{cat} and the second-order rate constants increased with an increase in the size of monoalkyl substituent in the sulfonamide moiety, whereas substrates which contain either glycine methyl ester or a dialkyl group displayed the lowest efficiency for tPA. The substrate Boc-(*p*-F)Phe-Pro-Arg-ANSNHC_2H_5 allowed quantitation of tPA at a concentration as low as 1 pM, a concentration significantly lower than the plasma concentration of this protein. Evaluation of the activation of single-chain tPA by factor Xa demonstrates that *prothrombinase* is approximately 3-fold more efficient in activating sc-tPA than factor Xa alone, increasing the initial rate of activation from 0.0055 nM/s per 1 nM of factor Xa to 0.017 nM/s per 1 nM.

Tissue-type plasminogen activator (tPA) is a serine protease which is produced by many cell lines (Booyse et al., 1981; Goldsmith et al., 1981; Dano et al., 1985) in a single-chain form (sc-tPA) (Rijken & Groeneveld, 1986) which can be converted rapidly to a two-chain form (tc-tPA) (Wallen et al., 1983; Bachmann & Kruithof, 1984; Rijken & Groeneveld, 1986; Andreasen et al., 1991). The amidolytic activity and efficiency toward various ligands by these two forms has been an object of substantial discussion (Rijken & Collen, 1981; Ranby, 1982; Rijken et al., 1982; Ichinose et al., 1984; Andreasen et al., 1984; Astedt et al., 1985; Kruithof et al., 1986; Tate et al., 1987; Petersen et al., 1988; Petersen, L., et al., 1990; Lijnen et al., 1992). Several assay methods have been described for tPA evaluation at its physiological concentration, including fibrinolytic, immunoradiometric, zymographic, and ^{125}I-fibrin microtiter well assays (Astrup & Stage, 1952; Astrup & Mullertz, 1952; Albrechtsen, 1957; Bachmann, 1987). The most popular appears to be the indirect chromogenic assay based on plasminogen activation and subsequent *p*-nitroanilide substrate hydrolysis by plasmin (Collen, 1980; Shimada et al., 1981; Ranby et al., 1982; Gyzander et al., 1984; Andreasen et al., 1991; Krishnamurti et al., 1996). The sensitivity of this method can be enhanced by monitoring the assay in the presence of fibrin fragments due to increased plasminogen activation rate (Nieuwenhuizen et al., 1983; Yonekawa et al., 1992). This method, however, has significant shortcomings, such as feedback conversion of sc-tPA to tc-tPA by plasmin, conversion of Glu-plasminogen to Lys-plasminogen, etc. (Gyzander et al., 1984; Verheijen et al., 1985; Andreasen et al., 1991). For the evaluation of tPA at concentrations significantly higher than physiological, rapid and simple methods of direct chromogenic or fluorogenic assays have been used, employing low molecular weight synthetic substrates (Rijken et al., 1982; Wallen et al., 1982; Ichinose et al., 1984; Astedt et al., 1985; Tate et al., 1987; Petersen et al., 1988; Petersen, L., et al., 1990; Boose et al., 1989; Andreasen et al., 1991; Husain, 1991; Lijnen et al., 1992; Rydzewski & Castellino, 1993; Nieuwenhuizen et al., 1977, 1978; Petersen & Swenson, 1986). Substrate S-2288 (D−Ile−Pro−Arg−pNA) is the most commonly used in such assays. However, there are no systematic studies reported analyzing the correlation between synthetic substrate structure and substrate efficiency for tPA.

In previous publications we have described synthetic substrates for serine proteases containing fluorescent 6-amino-1-naphthalenesulfonamide (ANSN) detecting groups. We have also established substrate structure−efficiency correlations for blood coagulation factors VIIa, Xa, thrombin,

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Table 1: Physicochemical Constants for Peptidyl-ANSN

no.	substrate	MW	% yield	R_f[a]	$[\alpha]^{22}$ [b]
9	Boc-L-FPR-ANSNHC$_2$H$_5$	750.9	78	0.79	−39.4
10	L-FPR-ANSNHC$_2$H$_5$, 2HCl	723.7	95	0.51	−49.7
17	Boc-L-FLR-ANSNHC$_3$H$_7$	781.1	79	0.62	−12.8
19	Boc-D-VGR-ANSNHC$_4$H$_9$	690.8	84	0.57	−20.3
20	D-VGR-ANSNHC$_4$H$_9$, 2HCl	663.6	87	0.33	−39.2
21	Boc-L-VGR-ANSNHC$_4$H$_9$	690.8	74	0.77	−33.3
22	L-VGR-ANSNHC$_4$H$_9$, 2HCl	663.6	91	0.51	−9.0
29	Boc-D-PGR-ANSNH(*i*-C$_3$H$_7$)	674.7	81	0.59	−11.9
30	D-PGR-ANSNH(*i*-C$_3$H$_7$), 2HCl	647.6	78	0.53	−6.2
31	Boc-D-PPR-ANSNH(*i*-C$_3$H$_7$)	715.0	72	0.63	−35.0
32	D-PPR-ANSNH(*i*-C$_3$H$_7$), 2HCl	687.8	92	0.53	−37.8
33	Boc-D-SPR-ANSNHCH$_2$C$_6$H$_5$	752.9	73	0.52	−31.9
34	D-SPR-ANSNHCH$_2$C$_6$H$_5$, 2CF$_3$COOH	880.8	65	0.40	−28.4
35	Boc-L-SPR-ANSNHCH$_2$C$_6$H$_5$	752.9	86	0.55	−32.7
36	L-SPR-ANSNHCH$_2$C$_6$H$_5$, 2HCl	725.7	59	0.33	−31.8
37	Boc-L-(OBz)SPR-ANSNHCH$_2$C$_6$H$_5$	843.1	71	0.72	−43.9
38	L-(OBz)SPR-ANSNHCH$_2$C$_6$H$_5$, 2CF$_3$COOH	971.0	82	0.45	−47.4
39	(*Z*)-L-(Boc)EGR-ANSNHC$_3$H$_7$	796.9	85	0.68	−17.6
40	(*Z*)-L-EGR-ANSNHC$_3$H$_7$, HCl	733.2	93	0.75	−15.0
41	L-EGR-ANSNHC$_3$H$_7$, 2HBr	724.6	89	0.41	−9.7
42	(*Z*)-L-(Boc)EPR-ANSNHC$_3$H$_7$	837.0	88	0.79	−25.1
43	(*Z*)-L-EPR-ANSNHC$_3$H$_7$, HCl	773.4	97	0.65	−29.8
44	L-EPR-ANSNHC$_3$H$_7$, 2HBr	764.7	73	0.23	−20.9

[a] R_f indicates the mobility of compounds in thin layer chromatography using a selected mixture of solvents (1-Butanol−acetic acid−water 4:1:2). [b] $[\alpha]^{22}$ indicates specific optical activity in a given solvent and at a defined % concentration of a given compound (c1; DMSO).

urokinase-type plasminogen activator, and activated protein C (Butenas et al., 1992, 1993, 1995). In the present study we evaluate the efficiency of 54 fluorogenic substrates for tPA. The dependence of substrate efficiency for sc-tPA and tc-tPA on substituents in substrate P and P′ (Schechter & Berger; 1967) positions was also established.

EXPERIMENTAL PROCEDURES

Materials. Fluorogenic substrates were synthesized and characterized as described previously (Butenas et al., 1992, 1993, 1995). The physicochemical constants of new substrates are provided in Table 1. Phosphatidylserine (PS) and phosphatidylcholine (PC) were purchased from Sigma. Phospholipid vesicles (PCPS) composed of 75% PC and 25% PS were prepared as described (Higgins & Mann, 1983).

Recombinant tPA was purchased from Genentech and contained 80% sc-tPA and 20% tc-tPA. Human factor Xa was provided as a gift from Haematologic Technologies Inc. Human factor V was isolated and activated to factor Va by the methods described previously (Katzmann et al., 1981; Nesheim & Mann, 1979). The plasmin inhibitor trasylol was purchased from Boehringer Mannheim. The factor Xa inhibitor TAP (tick anticoagulant protein) was provided as a gift from Sriram Krishnaswamy.

Methods. The separation of two-chain and single-chain tPA was accomplished as described previously (Husain,

Table 2: Substrate Hydrolysis by tPA

no.	substrate	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	initial rate of hydrolysis (M/s/M × 10^{-3})
1	Boc-D-LGR-ANSNHC$_6$H$_{11}$	15	0.097	6300	3.2
2	D-LGR-ANSNHC$_6$H$_{11}$	41	0.057	1400	1.1
3	Boc-D-LSR-ANSNHC$_3$H$_7$	360	0.53	1500	4.3
4	D-LSR-ANSNHC$_3$H$_7$	69	0.089	1300	4.2
5	Boc-D-LPR-ANSNHC$_3$H$_7$	66	0.54	8200	18.5
6	D-LPR-ANSNHC$_3$H$_7$	98	0.31	3200	7.1
7	Boc-D-FPR-ANSNHC$_6$H$_{11}$	47	0.15	3200	9.0
8	D-FPR-ANSNHC$_6$H$_{11}$	36	0.074	2100	5.3
9	Boc-L-FPR-ANSNHC$_2$H$_5$	47	0.011	240	SH[a]
10	L-FPR-ANSNHC$_2$H$_5$	970	0.040	40	SH
11	Boc-(*p*-F)FPR-ANSNHC$_2$H$_5$	71	1.03	14 600	103
12	(*p*-F)FPR-ANSNHC$_2$H$_5$	390	1.47	3800	9.0
13	Boc-D-FVR-ANSNHC$_2$H$_5$	91	0.49	5400	5.8
14	D-FVR-ANSNHC$_2$H$_5$	16	0.044	2700	1.7
15	Boc-D-FLR-ANSNHC$_3$H$_7$	30	0.10	3300	3.1
16	D-FLR-ANSNHC$_3$H$_7$	7.4	0.021	2900	1.6
17	Boc-L-FLR-ANSNHC$_3$H$_7$	ND[b]	ND	ND	SH
18	L-FLR-ANSNHC$_3$H$_7$	ND	ND	ND	SH
19	Boc-D-VGR-ANSNHC$_4$H$_9$	140	0.34	2400	8.4
20	D-VGR-ANSNHC$_4$H$_9$	58	0.082	1400	5.4
21	Boc-L-VGR-ANSNHC$_4$H$_9$	100	0.084	830	1.0
22	L-VGR-ANSNHC$_4$H$_9$	460	0.080	180	0.6
23	Boc-D-VSR-ANSNH(i-C$_3$H$_7$)	ND	ND	ND	2.5
24	D-VSR-ANSNH(i-C$_3$H$_7$)	ND	ND	ND	1.5
25	Boc-D-VPR-ANSNHC$_4$H$_9$	26	0.25	9500	12.1
26	D-VPR-ANSNHC$_4$H$_9$	110	0.71	6400	7.5
27	Boc-D-VLR-ANSNHC$_4$H$_9$	ND	ND	ND	1.0
28	D-VLR-ANSNHC$_4$H$_9$	ND	ND	ND	1.0
29	Boc-D-PGR-ANSNH(i-C$_3$H$_7$)	ND	ND	ND	0.3
30	D-PGR-ANSNH(i-C$_3$H$_7$)	ND	ND	ND	0.3
31	Boc-D-PPR-ANSNH(i-C$_3$H$_7$)	ND	ND	ND	0.2
32	D-PPR-ANSNH(i-C$_3$H$_7$)	ND	ND	ND	0.3
33	Boc-D-SPR-ANSNHCH$_2$C$_6$H$_5$	ND	ND	ND	2.5
34	D-SPR-ANSNHCH$_2$C$_6$H$_5$	ND	ND	ND	0.7
35	Boc-L-SPR-ANSNHCH$_2$C$_6$H$_5$	ND	ND	ND	0.3
36	L-SPR-ANSNHCH$_2$C$_6$H$_5$	ND	ND	ND	0.3
37	Boc-L-(OBz)SPR-ANSNHCH$_2$C$_6$H$_5$	ND	ND	ND	0.6
38	L-(OBz)SPR-ANSNHCH$_2$C$_6$H$_5$	ND	ND	ND	0.8
39	(*Z*)-L-(Boc)EGR-ANSNHC$_3$H$_7$	ND	ND	ND	1.0
40	(*Z*)-L-EGR-ANSNHC$_3$H$_7$	ND	ND	ND	SH
41	L-EGR-ANSNHC$_3$H$_7$	ND	ND	ND	2.1
42	(*Z*)-L-(Boc)EPR-ANSNHC$_3$H$_7$	ND	ND	ND	0.6
43	(*Z*)-L-EPR-ANSNHC$_3$H$_7$	ND	ND	ND	0.4
44	L-EPR-ANSNHC$_3$H$_7$	ND	ND	ND	1.2

[a] Slow hydrolysis. [b] Not detected.

Table 3: Substrate Hydrolysis by Two-Chain (tc) and Single-Chain (sc) tPA

		tc-tPA			sc-tPA			ratio (tc/sc)		
no.	substrate	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
1	Boc-D-LGR-ANSNHC$_6$H$_{11}$	8.5	0.040	4700	23	0.012	510	0.4	3.3	9.2
2	D-LGR-ANSNHC$_6$H$_{11}$	11	0.034	3000	11	0.0084	710	1.0	4.0	4.2
3	Boc-D-LSR-ANSNHC$_3$H$_7$	13	0.097	7300	20	0.022	1100	0.6	4.4	6.6
4	D-LSR-ANSNHC$_3$H$_7$	17	0.10	6200	14	0.019	1300	1.2	5.3	4.8
5	Boc-D-LPR-ANSNHC$_3$H$_7$	8.8	0.20	23 200	15	0.038	2500	0.6	5.3	9.3
6	D-LPR-ANSNHC$_3$H$_7$	12	0.17	14 200	40	0.061	1500	0.3	2.8	9.5
7	Boc-D-FPR-ANSNHC$_6$H$_{11}$	2.6	0.017	6400	ND[a]	ND	ND			
8	D-FPR-ANSNHC$_6$H$_{11}$	77	0.51	6600	5.7	0.016	2800	13	32	2.4
11	Boc-(*p*-F)FPR-ANSNHC$_2$H$_5$	6.6	0.44	66 500	16	0.11	7100	0.4	4.0	9.4
12	(*p*-F)FPR-ANSNHC$_2$H$_5$	11	0.37	32 800	26	0.099	3700	0.4	3.7	8.9
13	Boc-D-FVR-ANSNHC$_2$H$_5$	32	0.15	4800	12	0.019	1700	2.7	7.9	2.8
14	D-FVR-ANSNHC$_2$H$_5$	7.8	0.025	3200	7.3	0.011	1500	1.1	2.3	2.1
15	Boc-D-FLR-ANSNHC$_3$H$_7$	13	0.047	3600	17	0.010	600	0.8	4.7	6.0
16	D-FLR-ANSNHC$_3$H$_7$	10	0.018	1900	ND	ND	ND			
19	Boc-D-VGR-ANSNHC$_4$H$_9$	14	0.18	12 800	19	0.036	1900	0.7	5.0	6.7
20	D-VGR-ANSNHC$_4$H$_9$	33	0.19	5800	22	0.021	950	1.5	9.0	6.1
25	Boc-D-VPR-ANSNHC$_4$H$_9$	15	0.25	16 500	29	0.065	2300	0.5	3.8	7.2
26	D-VPR-ANSNHC$_4$H$_9$	8.1	0.16	20 200	25	0.059	2300	0.3	2.7	8.8

[a] Not determined.

1991) with several modifications. After the benzamidine−Sepharose column was eluted with 1 M NH_4HCO_3 containing 0.1 M arginine (pH 8), the column was washed with 300 mL of 1 M NaCl to remove ammonium bicarbonate from the column. The elution of tc-tPA with 0.1 M sodium acetate (pH 4.0) was started only after this procedure. All separation procedures were accomplished at 4 °C.

Fluorogenic substrate assays were conducted in 20 mM Tris, 150 mM NaCl, pH 7.4 (TBS) at 25 °C. For all assays, substrates were initially dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 10 mM. This stock solution was then diluted in TBS to a working concentration prior to all assays. tPA at 1 nM final concentration for tc-tPA and at 10 nM final concentration for sc-tPA or for the initial mixture was added to the solution of substrate for the analysis of kinetic constants. The initial rates of substrate hydrolyses were evaluated at 10 nM final tPA concentration and at 1 μM substrate concentration. The reactions were continued until 0.1 μM detecting group concentration was reached. The final volume for all reactions was 1.2 mL. The rate of substrate hydrolysis was evaluated as reported previously (Butenas et al., 1992) using a Perkin-Elmer fluorescence spectrophotometer, Model MPF-44A, equipped with a chart recorder. Kinetic constants for substrate hydrolysis were determined using the nonlinear least-squares fitting program ENZFITTER (Elsevier-BIOSOFT, Cambridge, U.K.).

For tPA quantitative evaluation, varying concentrations of tc-tPA (0−200 pM) were added to TBS containing 30 μM substrate 11 (Table 3), and enzyme/substrate solutions were incubated in polyethylene MiniSorb tubes for 60 min at 25 °C. In a control tube, enzyme was absent. After incubation, solutions were transferred into cuvettes and the relative fluorescence was estimated at 470 nm emission wavelength and 352 nm excitation wavelength using an SLM 8000 spectrophotometer. Relative fluorescence of the control sample was subtracted from the fluorescence of samples containing the enzyme, and the standard line was plotted using the least-squares fitting program ENZFITTER (Elsevier-BIOSOFT).

Sc-tPA Activation by Factor Xa. Factor Xa at a final 40 nM concentration was added to 2 μM sc-tPA in 20 mM HEPES, 150 mM NaCl, pH 7.4 (HBS) containing 5 μg/mL of trasylol. The activation mixture was kept at 37 °C. Six microliter aliquots of activation mixture were removed at 0, 5, 15, 30, 60, 120, 180, and 240 min time points and added to 1194 μL of HBS which contained 5 μg/mL of trasylol, 20 nM TAP, and 100 μM substrate 20 (Table 3). The rate of substrate hydrolysis was evaluated as described above. Similar activation experiments were accomplished in the presence of 20 μM PCPS and 5 mM $CaCl_2$ as well as in the presence of 20 μM PCPS, 5 mM $CaCl_2$, and 80 nM factor Va.

RESULTS

Separation of sc-tPA and tc-tPA. Attempts to separate sc-tPA and tc-tPA from the commercial mixture of these two forms by a previously described method (Husain, 1991) were unsuccessful. Sodium bicarbonate decomposed spontaneously on the benzamidine−Sepharose column at pH 8.0 and room temperature. This caused the evolution of gaseous ammonia and carbon dioxide, and the resin bed became heterogeneous. This process was significantly exacerbated when the acidic sodium acetate solution was added to the resin containing ammonium bicarbonate. To overcome this complication the separation procedure was modified. All the procedures were performed at 4 °C, and after the elution of sc-tPA, the column was extensively washed with 1 M NaCl to remove the ammonium bicarbonate. The sodium acetate solution was then added. These modifications allowed for the effective separation of sc-tPA and tc-tPA. The final products are shown in Figure 1, lanes 3 (sc-tPA) and 4 (tc-tPA).

Utility of Substrates for the Evaluation of tPA Amidolytic Activity. The initial rates for the hydrolysis of 39 substrates by tPA are presented in Table 2. All substrates tested in this study were hydrolyzed by both forms of tPA at detectable rates. The kinetic constants for the hydrolysis of 22 substrates by tPA are listed in this table as well. The kinetic constants for the hydrolysis of 27 substrates by tc-tPA and of 25 substrates by sc-tPA are presented in Tables 3 and 4. Michaelis−Menten constants (K_M) for substrate hydrolysis

Table 4: Kinetic Constants for the Hydrolysis of D-FPR-ANSNR$_1$R$_2$[a]

	substrate		tc-tPA			sc-tPA			ratio (tc-tPA/sc-tPA)		
no.	R_1	R_2	K_M	k_{cat}	k_{cat}/K_M	K_M	k_{cat}	k_{cat}/K_M	K_M	k_{cat}	k_{cat}/K_M
45	H	*i*-C_3H_7	74	0.20	2700	330	0.29	880	0.22	0.69	3.1
46	H	*n*-C_4H_9	48	0.47	9800	120	0.33	2800	0.40	1.4	3.5
47	H	*t*-C_4H_9	84	0.94	11 200	79	0.25	3200	1.1	3.8	3.5
48	H	*n*-C_6H_{13}	95	3.6	37 900	99	1.1	11 100	0.96	3.3	3.4
49	H	$C_6H_5CH_2$	25	0.54	21 600	63	0.45	7100	0.40	1.2	3.0
50	H	$C_2H_4OCH_3$	140	0.66	4700	180	0.27	1500	0.78	2.4	3.1
51	H	CH_2COOCH_3	29	0.15	5200	63	0.083	1300	0.46	1.8	4.0
8	H	c-C_6H_{11}	88	0.32	3600	150	0.15	1000	0.59	2.1	3.6
52	$(CH_2)_6$		96	0.22	2300	250	0.13	520	0.38	1.7	4.4
53	C_2H_5	C_2H_5	120	0.14	1200	320	0.090	280	0.37	1.6	4.3

[a] K_M, μM; k_{cat}, s^{-1}; k_{cat}/K_M, M^{-1} s^{-1}.

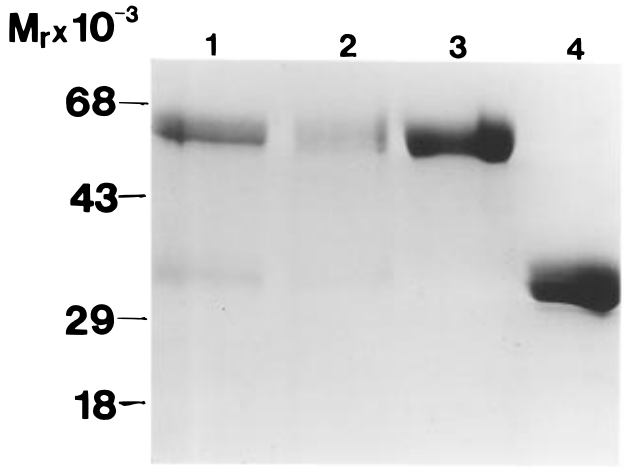

FIGURE 1: Separation of two-chain and single-chain tPA. Separation was accomplished as described in Experimental Procedures. The samples were analyzed by a 5−15% (linear gradient) SDS−PAGE gel under reducing conditions. Proteins were visualized by Coomassie Blue staining. Lane 1, initial mixture of sc- and tc-tPA; lane 2, flow-through fractions; lane 3, sc-tPA; lane 4, tc-tPA.

by tc-tPA vary from 2.6 μM (substrate 7) to 140 μM (substrate 50). K_M for sc-tPA vary from 7.3 μM (substrate 14) to 330 μM (substrate 45). Eighteen of 25 substrates presented in Tables 3 and 4 display a higher K_M for the single-chain form of tPA than for the two-chain enzyme. The values of catalytic constants (k_{cat}) for two-chain tPA vary from 0.017 s^{-1} (substrate 7) to 3.6 s^{-1} (substrate 48). The maximal rate of substrate hydrolysis by single-chain tPA is lower than that by two-chain enzyme, and the k_{cat} for substrate hydrolysis by this form varies from 0.0084 s^{-1} (substrate 2) to 1.1 s^{-1} (substrate 48). The second-order rate constants for this group of fluorogenic substrates are of moderate value and are 2−9-fold higher for tc-tPA than for sc-tPA. Thus, substrate 11 which has the highest second-order rate constant for tc-tPA (66 500 M^{-1} s^{-1}) is 9.4-fold less efficient for the single-chain form for which the second-order rate constant is 7100 M^{-1} s^{-1}. The lowest ratio of 2.1 for the second-order rate constants is observed for substrate 14.

The Influence of the P_3 Structure on Substrate Efficiency. The kinetic constants for substrates with N-blocked and unblocked D-Val, D-Leu, and D-Phe in the P_3 position were evaluated (Tables 2 and 3). The initial rates of hydrolysis for these compounds, their L-counterparts and for substrates with D-Pro, D- and L-Ser and L-Glu in the P_3 position were initially evaluated employing a mixture of sc- and tc-tPA (Table 2). Data presented in Tables 2 and 3 demonstrate that substrates which contain a *tert*-butyloxycarbonyl (Boc) protecting group at the N-terminal (P_3) amino acid were hydrolyzed with a higher efficiency than their analogs with an unblocked amino acid in the P_3 position. N-blocked substrates have higher second-order rate constants and initial rates of hydrolysis. These increased k_{cat}/K_M were mostly associated with an elevated k_{cat} for substrates with the N-blocked amino acid in the P_3 position. This observation was not found for substrates which contain glutamic acid in the P_3 position. In the latter case substrates with unblocked both α-amino and γ-carboxylic functions were hydrolyzed by tPA at higher rates (compounds 41 and 44; initial rates of hydrolysis = 0.0021 and 0.0012 M/s/M, respectively) than substrates in which both functional groups were blocked (compounds 39 and 42; initial rates of hydrolysis = 0.0010 and 0.0006 M/s/M, respectively) or which contain a blocked amino group in the P_3 glutamic acid (compounds 40 and 43; initial rates of hydrolysis undetectable and 0.0004 M/s/M, respectively). Introduction of a Boc-blocking group into the side-chain of glutamic acid increases the initial rate of hydrolysis for substrates which contain a protected α-amino function (compare compounds 39 and 42 with compounds 40 and 43, respectively).

Analyses of the initial rates of substrate hydrolysis demonstrate that substrates which contain the D-amino acid isomer in the P_3 position are hydrolyzed by tPA faster than compounds which contain L-isomers in this position (Table 2). In fact, the relatively slow hydrolysis rates for substrates with L-amino acids in the P_3 position did not permit the determination of kinetic constants for these compounds.

To compare the influence of the P_3 amino acid on substrate kinetic constants and/or the initial rates of hydrolysis, the compounds were analyzed in groups containing substrates with the same amino acids in the P_1 and P_2 positions and various amino acids in the P_3 position. Thus, in one group, substrates 1, 19, and 29 are analyzed which contain Gly in the P_2 position and the N-blocked P_3 amino acid; another group contained substrates 2, 20, and 30 with Gly in the P_2 position and the N-unblocked P_3 amino acid. In the next two groups substrates 5, 7, 25, 31, and 33 and 6, 8, 26, 32, and 34 are presented which contain Pro in the P_2 position and N-blocked or an N-unblocked amino acid in the P_3 position, respectively. The data of Table 2 suggest that substrates with bulkier amino acids in the P_3 position (Val, Leu, Phe, Glu) have higher efficiency for tPA than analogs which contain small amino acids (Ser or Pro) in this position. The maximum efficiency was observed for substrates which contain Val (compounds 25, 26) or Leu (compound 1) (Table

2) at P_3. The efficiency of all substrates with Pro or Ser in the P_3 position is relatively low. The significance of Val or Leu in the P_3 position is valid for tc-tPA as well as for sc-tPA with the highest efficiency in the groups observed for substrates 19, 20, 26 (Val), and 5 (Leu) (Table 3); however, the effect is less significant for sc-tPA. Substrates with Val in the P_3 position display elevated K_M and k_{cat}. The influence of the P_3 residue on k_{cat} is more significant than on K_M and is accompanied by increased second-order rate constants for substrates with Val in the P_3 position.

The importance of the P_3 structure on substrate efficiency is obvious for substrate 11 with *p*-fluorine-substituted phenylalanine in this position. This substrate was the most efficient among all presented in this study, despite the unfavorable L-isomer of the amino acid in the P_3. This substitution increases the second-order rate constant over 60-fold (compare with substrate 9) due to significant increase (almost by 2 orders of magnitude) in k_{cat} (Table 2). Surprisingly, substitution of L-(*p*-F)Phe in substrate 11 with the D-isomer did not further influence the substrates efficiency for tPA (data not shown).

Influence of P_2 Substitution on Substrate Efficiency. The hydrolysis data for substrates which contain the same amino acids in the P_1 and P_3 positions and various substituents in the P_2 position clearly demonstrate that the highest catalytic efficiency for both tc-tPA and sc-tPA was obtained for substrates with proline in the P_2 position (compounds 5, 6, 7, 8, 25, and 26) (Table 3). An increase in the second-order rate constants of substrate hydrolysis caused by the amino acid substituent at P_2 follows the pattern Leu < Val and Gly < Ser < Pro. A similar dependence on the P_2 amino acid is observed for k_{cat} values. In contrast, any changes in K_M values are independent of the P_2 structure. The above observations are valid for both forms of tPA.

Influence of the P′ Structure on Substrate Hydrolysis by tPA. Ten substrates containing D-FPR tripeptide in the P structure and various substituents in the sulfonamide moiety of the leaving group (P′ structure) were synthesized and evaluated for their hydrolysis by sc-tPA and tc-tPA (Table 4 and Figure 2). We did not observe any significant dependence of the K_M on the substituent in the substrate P′ structure. Compounds 49 and 51, which contain benzyl and glycine methyl ester substituents, respectively, demonstrated the highest affinity (the lowest K_M) for both forms of tPA. Substrates 52 and 53 which contain a completely substituted amino group in the sulfonamide moiety display relatively high K_M (Figure 2, panel A).

In contrast, the catalytic constants (k_{cat}) of substrate hydrolysis demonstrate a distinct dependence upon the structure of sulfonamide substituents, varying by over 20-fold (Figure 2, panel B). In the group of compounds which contain monoalkyl residues in this moiety (substrates 45−48), the k_{cat} increases with increases of substitutent size. Thus, the k_{cat} for compound 45 which contains a propyl group as the sulfonamide substituent is significantly lower for sc-tPA as well as for tc-tPA (0.29 and 0.20 s^{-1}, respectively) than that for substrate 48 which contains a hexyl group in the P′ position (1.1 and 3.6 s^{-1}, respectively). Compound 48 displays the highest k_{cat} among the all substrates evaluated in this study; an observation valid for both forms of tPA. Substrates 49 and 50, which contain benzyl and methoxyethyl residues in the P′ structure, respectively, are hydrolyzed by both forms of tPA at reasonable rates as well, whereas

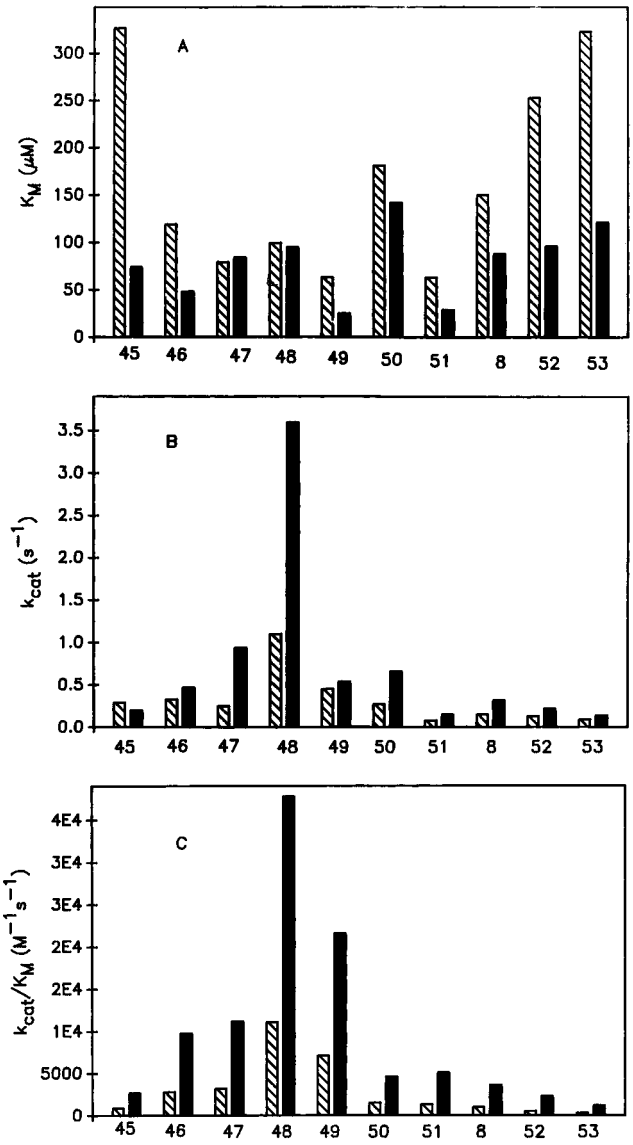

FIGURE 2: Influence of the P′ structure on substrate D-FPR-ANSNR$_1$R$_2$ hydrolysis by sc-tPA (striped bars) and tc-tPA (filled bars). (A) Michaelis-Menten constants; (B) catalytic constants; (C) second-order rate constants.

substrates 51 and 53 with glycine methyl ester and dimethyl group in this position, respectively, display the lowest k_{cat} values among the substrates of Table 4.

The pattern of second-order rate constant dependence upon the substrate P′ structure follows, in general, that demonstrated by the catalytic constants (Figure 2, panels B and C). Thus, in the group of substrates with monoalkyl sulfonamide substituents (compounds 45−48), the second-order rate constants for both forms of tPA increase with the increased size of alkyl residue. This constant is barely influenced by the substitution of the linear *n*-butyl group (substrate 46) by its branched counterpart, the *tert*-butyl group (substrate 47). The second-order rate constant is relatively high for compound 49 due to the relatively low K_M values of this substrate for the hydrolysis by both forms of tPA. The lowest efficiency for tPA was displayed by substrates 52 and 53 which contain completely substituted sulfonamide amino groups.

The data presented in Table 4 clearly demonstrate that relatively moderate changes in the substrate P′ structure may significantly alter the enzymatic hydrolysis of these com-

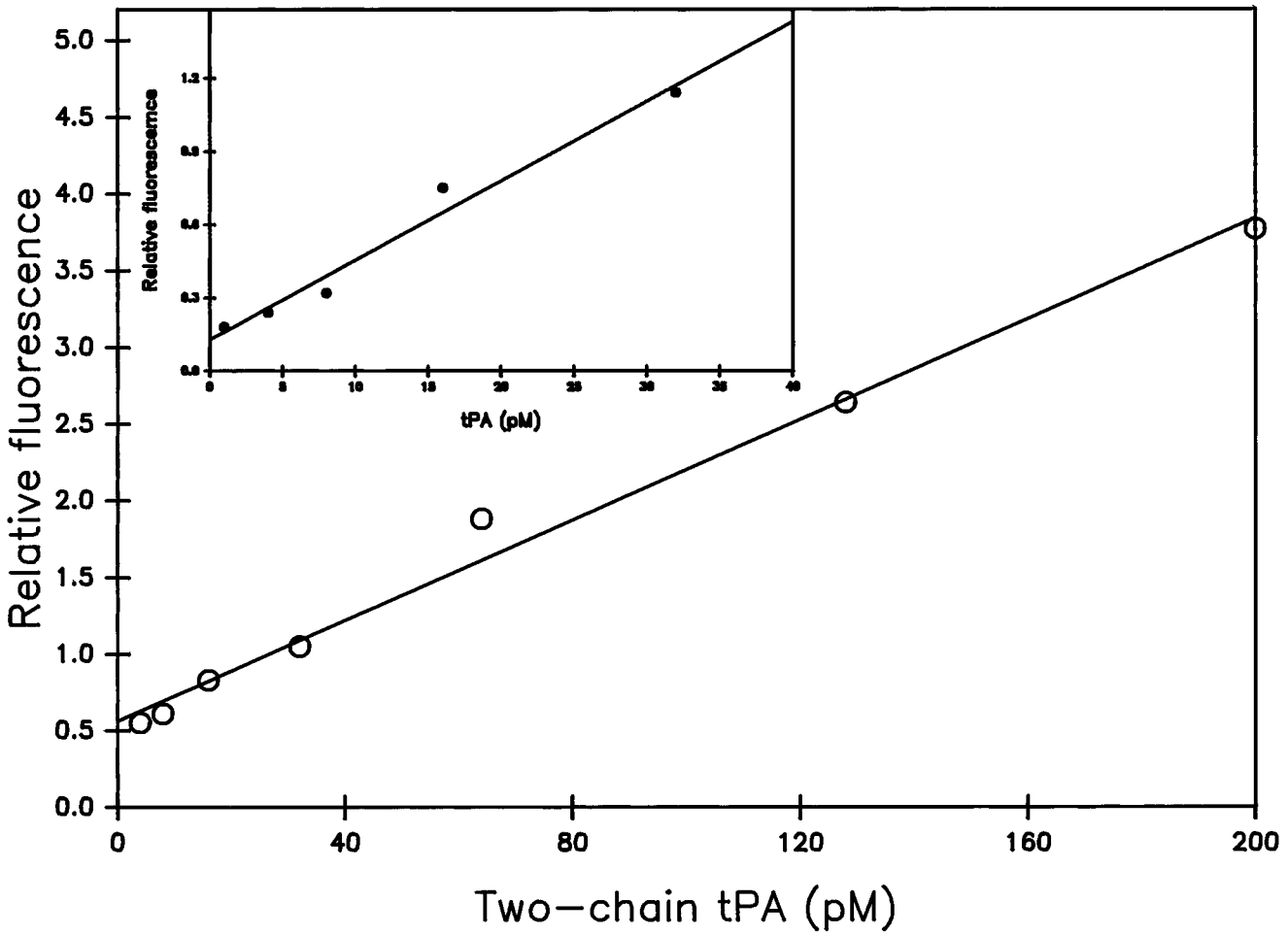

FIGURE 3: Quantitation of low tPA concentrations. Tc-tPA or tPA (inset) at varying concentrations (0−200 pM and 0−40 pM, respectively) was incubated for 60 min at 25 °C in TBS containing 30 μM substrate 11. Relative fluorescence was estimated as described in Experimental Procedures.

pounds by tPA. For tPA, the changes in substrate affinity caused by the modifications in the P′ structure are relatively moderate, and the highest K_M ratio for the sc-tPA is 5.2 (substrates 45 and 49 or 51) and for tc-tPA is 5.6 (substrates 50 and 49). The k_{cat} values are influenced by the substitution in the P′ structure to a significantly higher extent. Thus, the highest ratio of these constants for the tc-tPA reaches as much as 26 (substrates 48 and 53), whereas for the sc-tPA the highest ratio is 13 (substrates 48 and 51). The second-order rate constants are most sensitive to changes in the substrate sulfonamide adduct. Thus, replacement of *n*-hexyl group (substrate 48) by the dimethyl group (substrate 53) decreases the second-order rate constant for sc-tPA 40-fold, whereas for tc-tPA such replacement causes a 32-fold decrease in this constant.

Direct Quantitation of tPA at Low Concentrations. Specific quantitative evaluation of tPA was accomplished employing substrate 11 at 30 μM concentration. Spontaneous hydrolysis of this substrate under the experimental conditions used was undetectable. One hour incubation of enzyme with substrate allowed us to detect tc-tPA at concentrations as low as 1 pM. A linear dependence of relative fluorescence on tc-tPA concentration was observed from 4 to 200 pM enzyme (the highest concentration chosen) (Figure 3). At tc-tPA concentrations below 4 pM, the slope of relative fluorescence dependence on enzyme concentration is steeper than for higher enzyme concentrations.

Activation of Sc-tPA by Factor Xa and by Prothrombinase. Factor Xa activates sc-tPA at 0.0055 nM/s per nM enzyme (nM/s/nM) when enzyme is present at 40 nM and substrate at 2 μM concentration. This activation process is relatively slow and is only 60% complete in 4 h (Figure 4). The presence of Ca^{2+} and PCPS does not influence the initial rate of sc-tPA activation. However, the addition of factor Va, an essential cofactor to factor Xa in forming *prothrombinase*, increases the initial rate of sc-tPA activation approximately 3-fold, i.e., to 0.017 nM/s/nM. We also observed spontaneous sc-tPA proteolysis to the two-chain form in the absence of factor Xa. The rate of autolytic cleavage is not influenced either by PCPS and Ca^{2+} or by factor Va. Approximately 10% of sc-tPA was converted to tc-tPA in 4 h. Gel electrophoresis shows that incubation for 65 h at 37 °C results in 50% proteolysis of sc-tPA (data not shown). This autoproteolysis of sc-tPA also occurred when purified sc-tPA was stored at −20 °C for a longer period of time.

DISCUSSION

The structure of synthetic substrates and their efficiency for various blood derived serine proteases such as plasmin, factor Xa, thrombin, kallikreins and urokinase, factors VIIa, IXa, XIa, and XIIa, and activated protein C were analyzed in several previous publications (Sherry et al., 1966; Morita et al., 1977; Aurell et al., 1978; Claeson et al., 1978;

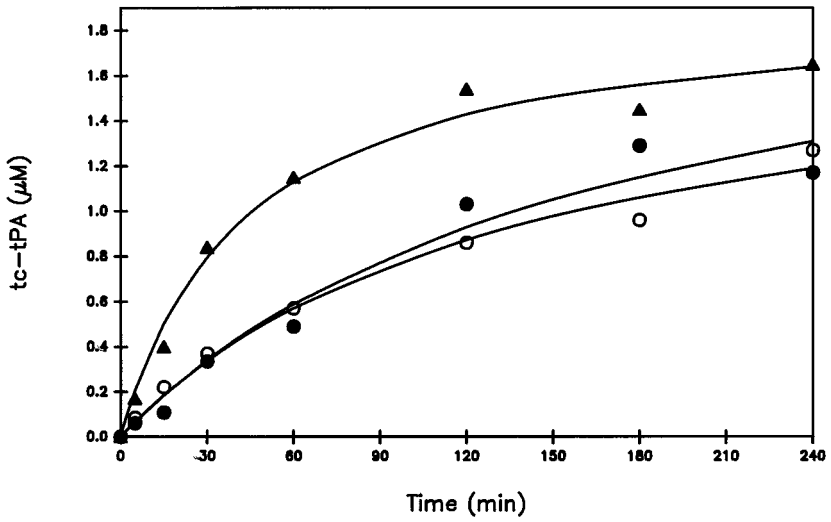

FIGURE 4: Sc-tPA activation by factor Xa. Forty nanomolar factor Xa was added to HBS (open circles); HBS, 5 mM $CaCl_2$, and 20 μM PCPS (filled circles); HBS, 5 mM $CaCl_2$, 20 μM PCPS, and 80 nM factor Va (filled triangles) containing 2 μM sc-tPA. Six microliter aliquots were removed at 0, 5, 15, 30, 60, 120, 180, and 240 min time points and added to 1194 μL HBS containing 5 μg/mL trasylol, 20 nM TAP, and 100 μM substrate 20 (D-VGR-ANSNHC$_4$H$_9$). Tc-tPA generation was estimated as described in Experimental Procedures.

Lonsdale-Eccles et al., 1980; McRae et al., 1981; Lottenberg et al., 1983, 1986; Castillo et al., 1983; Cho et al., 1984; Harnois-Pontoni et al., 1991; Butenas et al., 1992, 1993, 1995). The data concerning the influence of the substrate $P_n-P_1-P_n'$ structure on its efficiency for a given enzyme are controversial (Morita et al., 1977; McRae et al., 1981; Lottenberg et al., 1983; Cho et al., 1984) and do not provide clear answers about the most favorable amino acids at the various P positions (Harnois-Pontoni et al., 1991). The influence of a substrate structure on its efficiency for tPA, to our knowledge, has not been yet established.

The present study uses 54 substrates containing five different amino acids in the P_2 position and six in the P_3 position. The analysis of substrate hydrolysis data shows undoubtedly that substrates containing bulky and relatively hydrophobic amino acids Val, Leu, and Phe (Zamyatnin, 1972) in the P_3 position had higher efficiency for tPA than those containing relatively small and non-hydrophobic substituents (Ser, Pro) in this position. The highest efficiencies (k_{cat}/K_M), however, were obtained with substrates containing Val or Leu in the P_3 position but not with Phe, which has the largest volume and hydrophobicity of the side-chain among amino acids used for the P_3 position. The significant increase in the volume and hydrophobicity of the P_3 amino acid by the Boc protecting group caused an additional increase in substrate efficiency. The significance of a D-amino acid in substrate P_3 position has been discussed previously (Rezaie & Esmon, 1993). This stereoisomer may influence the location of the P_3 residue in the enzyme active site pocket.

Our data demonstrate that the variations in substrate efficiency are in strong coincidence with the hydrophobicity of amino acids located in the P_2 position. The hydrophobicity of P_2 position substituents decrease in the following order: Leu > Val > Gly > Ser > (Pro) (Nozaki & Tanford, 1971). Substrates containing Pro in the P_2 position had the highest efficiency for tPA. Increases in the substituents hydrophobicity in the order (Pro) < Ser < Gly were accompanied by associated decreases in substrate efficiency for tPA, i.e., substrates containing Ser in the P_2 position had lower efficiency than those with Pro in this position, and substrates with Gly in the P_2 position were less efficient for sc- and tc-tPA than those with Ser in this position. Substrates containing the highly hydrophobic Val and Leu in the P_2 position also had low efficiencies for tPA. This effect may be caused by the relatively high hydrophilicity of amino acids surrounding the triad which forms the tPA active site.

The data discussed above lead to the conclusion that an efficient substrate for tPA will have a bulky and hydrophobic amino acid in the P_3 position and proline in the P_2 position. This observation indicates that the qualities of the amino acids in the sequences of synthetic substrates significantly differ from those of the natural substrate plasminogen. The latter contains proline in the P_3 structure whereas the P_2 position is occupied by a moderately hydrophobic glycine (Petersen et al., 1990). We tested in our experiments a substrate which possesses Boc-D−Pro−Gly−Arg sequence in the P structure (substrate 29). Analyses of the initial rates of hydrolysis lead to the conclusion that this substrate is 40−60-fold less efficient for tPA than substrates which contain a hydrophobic amino acid in the P_3 position and a proline in the P_2 position. Similarly, Ding et al. (1995) have shown that peptides which contain bulky Phe in the P_3 position are hydrolyzed by tPA at a significantly higher rate than those which repeat the amino acid sequence of the plasmin cleavage site (Madison et al., 1995). The discrepancy between the P sequence of synthetic substrates and that of the cleavage site of plasmin may be caused by various factors including a preferable access of the cleavage site for tPA and the influence of the P′ structure.

We have reported in previous publications that substrates possessing the 6-amino-1-naphthalenesulfonamide leaving groups have higher affinity (lower K_M) for serine proteases than their analogs containing *p*-nitroaniline or 7-amino-4-methylcoumarin leaving groups (Butenas et al., 1992, 1993, 1995). The present data establish the same conclusions for tPA, i.e., the substrates presented in this study have higher affinity for tPA than those with detecting groups mentioned above (Nieuwenhuizen et al., 1977; Andreasen et al., 1991).

Further analyses of a substrate's P′ structure influence on substrate efficiency for tPA were accomplished employing ten substrates which contain the same P structure and various

substituents in the sulfonamide moiety (P′ structure). The data presented in this study demonstrate that substrate hydrolysis by tPA shows the same dependence upon the length of alkyl chain in the leaving group as substrate hydrolysis by thrombin, urokinase-type plasminogen activator (uPA), APC, and factor VIIa (Butenas et al., 1992, 1993, 1995). Substitution of a linear *n*-butyl residue by the branched *tert*-butyl analog, however, unexpectedly increases the efficiency of substrate for the tPA, whereas this substitution in the case of other serine proteases leads to decreases in the efficiency of the substrate. In analogy with uPA, both forms of tPA hydrolyze substrates which contain the cyclohexyl moiety in the leaving group slowly, whereas for thrombin, APC, and factor VIIa substrates containing this substituent are among the most efficient. These minor differences, however, do not exclude tPA from the group of serine proteases which include thrombin, factor VIIa, and uPA, which "prefer" substrates with Pro in the P_2 position and with bulky amino acids in the P_3 position. The rate of substrate hydrolysis by this group of serine proteases increases with increasing length of an alkyl substituent in the leaving group as well. This effect may be caused by increasing steric hindrance occurring between substrate sulfonamide substituent and amino acids of enzyme S_1' or relatively small S_2' cavity (Lamba et al., 1996). As a result, the leaving group which contains a long aliphatic chain may be eliminated from those sites faster than one containing a small substituent. Alternatively, the influence of the structure of the leaving group on substrate turnover rate cannot be excluded.

Hu et al. (1996) have demonstrated in their study that tc-tPA binds reversible inhibitors more efficiently than sc-protease. Data presented in this study demonstrate that tc-tPA binds ANSN-substrates with higher affinity and hydrolyzes them at a higher rate than the sc-counterpart. However, despite differences in substrate hydrolysis rate and affinity for those two enzymatic forms of tPA, modifications in the substrate P and P′ structure do not significantly alter the substrate preference for these two forms of tPA, i.e., all substitutions shift the substrate efficiency in the same direction for both, sc-tPA and tc-tPA.

Reasonably high rates of enzymatic ANSN-substrate hydrolysis, low spontaneous hydrolysis rates, the high yield of fluorescence of the leaving group relative to the uncleaved substrate, and high sensitivity of fluorescent detection method allow the evaluation of low concentrations of tPA in a simple and direct assay. We assume that on the basis of ANSN-substrates a simple and precise single-step method for the detection of plasma concentrations of tPA may be developed in replacement of more complicated two-step methods employing plasminogen (Collen, 1980; Shimada et al., 1981; Ranby et al., 1982; Gyzander et al., 1984; Andreasen et al., 1991; Krishnamurty et al., 1996).

We have developed a direct sc-tPA activation assay based upon the dissimilarity in amidolytic activity of two tPA forms. Ichinose et al. (1984) reported the activation of sc-tPA by factor Xa. They found that factor Xa activates sc-tPA with an efficiency similar to that of plasmin. We expanded these experiments and establish that *prothrombinase*, the enzymatic complex composed of factor Va and factor Xa associated on a membrane surface (Nesheim et al., 1979), is approximately 3-fold more efficient than factor Xa by itself in activating sc-tPA. This observation leads to the potential that in the last steps of the blood coagulation process, when the concentration of the major substrate, prothrombin, is significantly decreased, this enzymatic complex may be responsible for the activation of sc-tPA. Thus, during the early steps of fibrinolysis, *prothrombinase* may be responsible for sc-tPA activation.

In summary, ANSN-substrates allow the direct evaluation of tPA at its plasma concentration. The efficiency and affinity of this class of substrates for tPA are influenced by the hydrophobicity and by the volume of amino acids present in the peptide core of the substrate as well as by the structure of the leaving group. The highest efficiency is displayed by compounds which contain Pro in the P_2 structure and Val or Leu in the P_3 structure and a linear hexyl residue in the sulfonamide moiety.

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REFERENCES

Albrechtsen, O. K. (1957) *Br. J. Haematol. 3*, 284−291.
Andreasen, P. A., Nielsen, L. S., Grondahl-Hansen, J., Skriver, L., Zeuthen, J., Stephes, R. W., & Dano, K. (1984) *Eur. Mol. Biol. Organ. J. 3*, 51−56.
Andreasen, P. A., Petersen, L. C., & Dano, K. (1991) *Fibrinolysis 5*, 207−215.
Astedt, B., Bladh, B., Christensen, U., & Lecander, I. (1985) *Scand. J. Clin. Lab. Invest. 45*, 429−435.
Astrup, T., & Stage, A. (1952) *Nature 4335*, 929.
Astrup, T., & Mullertz, S. (1952) *Arch. Biochem. Biophys. 40*, 346−356.
Aurell, L., Simonsson, R., Arielly, S., Karlsson, G., Friberger, P., & Claeson, G. (1978) *Haemostasis 7*, 92−94.
Bachmann, F. (1987) Plasminogen activators, in *Hemostasis and thrombosis* (Colman, R. W., Hirsh, J., Marder, V. J., & Salzman, E. W., Eds.) pp 318−339, J. B. Lippincott Company, Philadelphia.
Bachmann, F., & Kruithof, E. K. O. (1984) *Semin. Thromb. Hemostasis 10*, 6−17.
Boose, J. A., Kuismanen, E., Gerard, R., Sambrook, J., & Gething, M.-J. (1989) *Biochemistry 28*, 635−643.
Booyse, F. M., Scheinbuks, J., Radek, J., Osikowicz, G., Feder, S., & Quarfoot, A. J. (1981) *Thromb. Res. 24*, 495−504.
Butenas, S., Orfeo, T., Lawson, J. H., & Mann, K. G. (1992) *Biochemistry 31*, 5399−5411.
Butenas, S., Ribarik, N., & Mann, K. G. (1993) *Biochemistry 32*, 6531−6538.
Butenas, S., Drungilaite, V., & Mann, K. G. (1995) *Anal. Biochem. 225*, 231−241.
Castillo, M. J., Kurachi, K., Nishino, N., Ohkubo, I., & Powers, J. C. (1983) *Biochemistry 22*, 1021−1029.
Cho, K., Tanaka, T., Cook, R. R., Kisiel, W., Fujikawa, K., Kurachi, K., & Powers, J. C. (1984) *Biochemistry 23*, 644−650.
Claeson, G., Aurell, L., Friberger, P., Gustavsson, S., & Karlsson, G. (1978) *Haemostasis 7*, 62−68.
Collen, D. (1980) *Thromb. Haemostasis 43*, 77−89.
Dano, K., Andreasen, P. A., Grondahl-Hansen, J., Kristensen, P., Nielsen, L. S., & Skriver, L. (1985) *Adv. Cancer Res. 44*, 139−266.
Ding, L., Coombs, G. S., Strandberg, L., Navre, M., Corey, D. R., & Madison, E. L. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 7627−7631.
Goldsmith, G. P., Ziats, N. P., & Robertson, A. L. (1981) *Exp. Mol. Pathol. 35*, 257−264.
Gyzander, E., Eriksson, E., & Teger-Nilsson, A. C. (1984) *Thromb. Res. 35*, 547−558.

Harnois-Pontoni, M., Monsigny, M., & Mayer, R. (1991) *Anal. Biochem. 193*, 248−255.
Higgins, D. L., & Mann, K. G. (1983) *J. Biol. Chem. 258*, 6503−6508.
Hu, Ch.-K., Kohnert, U., Sturzebecher, J., Fisher, S., & Llinas, M. (1996) *Biochemistry 35*, 3270−3276.
Husain, S. S. (1991) *Arch. Biochem. Biophys. 285*, 373−376.
Ichinose, A., Kisiel, W., & Fujikawa, K. (1984) *FEBS Lett. 175*, 412−418.
Katzmann, J. A., Nesheim, M. E., Hibbard, L. S., & Mann, K. G. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 162−166.
Krishnamurti, C., Keyt, B., Maglasang, P., & Alving, B. M. (1996) *Blood 87*, 14−19.
Kruithof, E. K. O., Vassalli, J.-D., Schleuning, W.-D., Mattaliano, R. J., & Bachmann, F. (1986) *J. Biol. Chem. 261*, 11207−11213.
Laemmli, U. K. (1970) *Nature 227*, 680−685.
Lamba, D., Bauer, M., Huber, R., Fisher, S., Rudolph, R., Kohnert, U., & Bode, W. (1996) *J. Mol. Biol. 258*, 117−135.
Lijnen, H. R., Webb, P. D., Van Hoef, B., De Cock, F., Stassen, J. M., Prior, S. D., & Collen, D. (1992) *Thromb. Haemostasis 67*, 239−247.
Lonsdale-Eccles, J. D., Hogg, D. H., & Elmore, D. T. (1980) *Biochim. Biophys. Acta 612*, 395−400.
Lottenberg, R., Hall, J. A., Blinder, M., Binder, E. P., & Jackson, C. M. (1983) *Biochim. Biophys. Acta 742*, 539−557.
Lottenberg, R., Hall, J. A., Pautler, E., Zupan, A., Christensen, U., & Jackson, C. M. (1986) *Biochim. Biophys. Acta 874*, 326−336.
Madison, E. L., Coombs, G. S., & Corey, D. R. (1995) *J. Biol. Chem. 270*, 7558−7562.
McRae, B. J., Kurachi, K., Heimark, R. L., Fujikawa, K., Davie, E. W., & Powers, J. C. (1981) *Biochemistry 20*, 7196−7206.
Morita, T., Kato, H., Iwanaga, S., Takada, K., Kimura, T., & Sakakibara, S. (1977) *J. Biochem. 82*, 1495−1498.
Nesheim, M. E., & Mann, K. G. (1979) *J. Biol. Chem. 254*, 1326−1334.
Nieuwenhuizen, W., Wijngaards, G., & Groeneveld, E. (1977) *Anal. Biochem. 83*, 143−148.
Nieuwenhuizen, W., Wijngaards, G., & Groeneveld, E. (1978) *Haemostasis 7*, 146−149.
Nieuwenhuizen, W., Verheijen, J. H., Vermond, A., & Chang, G. T. G. (1983) *Biochim. Biophys. Acta 755*, 531−533.
Nozaki, Y., & Tanford, C. (1971) *J. Biol. Chem. 246*, 2211−2217.
Petersen, L. C., & Suenson, E. (1986) *Biochim. Biophys. Acta 883*, 313−325.
Petersen, L. C., Johannessen, M., Foster, D., Kumar, A., & Mulvihill, E. (1988) *Biochim. Biophys. Acta 952*, 245−254.
Petersen, L. C., Boel, E., Johannessen, M., & Foster, D. (1990) *Biochemistry 29*, 3451−3457.
Petersen, T. E., Martzen, M. R., Ichinose, A., & Davie, E. W. (1990) *J. Biol. Chem. 265*, 6104−6111.
Ranby, M. (1982) *Biochim. Biophys. Acta 704*, 461−469.
Ranby, M., Norrman, B., & Wallen, P. (1982) *Thromb. Res. 27*, 743−749.
Rezaie, A. R., & Esmon, C. T. (1993) *J. Biol. Chem. 268*, 19943−19948.
Rijken, D. C., & Collen, D. (1981) *J. Biol. Chem. 256*, 7035−7041.
Rijken, D. C., & Groeneveld, E. (1986) *J. Biol. Chem. 261*, 3098−3102.
Rijken, D. C., Hoylaerts, M., & Collen, D. (1982) *J. Biol. Chem. 257*, 2920−2925.
Rydzewski, A., & Castellino, F. J. (1993) *Arch. Biochem. Biophys. 300*, 472−482.
Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157−162.
Sherry, S., Alkjaersig, N., & Fletcher, A. P. (1966) *Thromb. Diath. Haemorr. 16*, 18−31.
Shimada, H., Mori, T., Takada, A., Noda, Y., Takai, I., Kohda, H., & Nishimura, T. (1981) *Thromb. Haemostasis. 46*, 507−510.
Tate, K. M., Higgins, D. L., Holmes, W. E., Winkler, M. E., Heyneker, H. L., & Vehar, G. A. (1987) *Biochemistry 26*, 338−343.
Verheijen, J. H., de Jong, Y. F., & Chang, G. T. G. (1985) *Thromb. Res. 39*, 281−288.
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.
Yonekawa, O., Voskuilen, M., & Nieuwenhuizen, W. (1992) *Biochem. J. 283*, 187−191.
Zamyatnin, A. A. (1972) *Prog. Biophys. Mol. Biol. 24*, 107−123.

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