Synthetic Substrates for Human Factor VIIa and Factor VIIa–Tissue Factor†

Saulius Butenas, Natalie Ribarik, and Kenneth G. Mann*

Department of Biochemistry, Health Science Complex, University of Vermont, Burlington, Vermont 05405

Received February 11, 1993; Revised Manuscript Received April 16, 1993

ABSTRACT: A series of 100 tripeptide fluorogenic substrates has been synthesized. These substrates contain Arg in the P_1 position, various amino acids in the P_2 and P_3 positions, and different 6-amino-1-naphthalenesulfonamides (ANSN) as the detecting group (P′). The 38 compounds possessing the highest initial rates of factor VIIa hydrolysis were evaluated for substrate kinetic parameters in the presence and absence of tissue factor (TF) and by factor Xa. Most of these substrates had a higher k_{cat}/K_M (k_{eff}) value for the factor VIIa–TF complex than for factor Xa. Substitution of different amino acids in the P_2 position showed that substrates with bulkier amino acids such as Leu, Pro, and Val have higher values for K_M and k_{cat} than those with smaller amino acids (Gly or Ser). The highest second-order rate constants were found for substrates with Val or Pro in the P_2 position. A decrease or increase in volume of the P_2 substituent (Gly, Ser, or Leu) resulted in a decrease in this constant. Substrates with the highest k_{eff} values have Phe in the P_3 position. As the hydrophobicity and volume of the amino acid in the P_3 position decreased, the k_{eff} was reduced. The efficiency of substrates for hydrolysis by factor VIIa was enhanced by an increase of hydrophobicity in the P′ structure. TF enhanced the amidolytic activity of the "family" of 38 substrates with ANSN in the P′ position on an average of 58-fold.

Factor VII circulates as a single-chain zymogen in blood plasma at a concentration of 10 nM (Bajaj et al., 1981). The active form of factor VII, factor VIIa, is a two-chain serine protease which initiates the extrinsic pathway of the coagulation cascade when complexed to its cofactor, tissue factor (TF). A number of recent studies concerning factor VIIa have utilized naturally occurring substrates, such as factor IX and factor X, to determine the potential activity of this protease (Seligsohn et al., 1978; Masys et al, 1982; Sakai et al., 1989; Mertens et al., 1990; Wildgoose et al., 1990; Lawson & Mann, 1991; Paborsky et al., 1991; Ruf & Edgington, 1991; Walsh & Geczy, 1991). Unfortunately, difficulties arise when one attempts to analyze these data, due to the inherent effect of phospholipids on the ability of factor IX and factor X to be activated by the factor VIIa–TF complex (Krishnaswamy et al., 1990; Ruf et al., 1991a) as well as the possible effect of feedback proteolysis of formed enzymes, as seen specifically with factor IXa (Lawson & Mann, 1991).

A limited number of studies involving factor VIIa activity have employed synthetic, low molecular weight substrates. Generally, these studies have made use of peptides containing a 4-nitroaniline leaving group and have shown characteristically low rates of hydrolysis of the substrate by factor VIIa. Therefore, the concentration of factor VIIa used in these experiments had to be significantly higher than physiological levels to permit hydrolysis of the substrate by the protease to be accurately monitored (Bjoern et al., 1991; Pedersen et al., 1991; Ruf et al., 1991a,b), even in the presence of TF (Bjoern et al., 1991; Ruf et al., 1991a,b).

Arginine esters (Zur & Nemerson, 1978; Higashi et al., 1992) and their thio analogs (Kam et al., 1990) have also been used as substrates for factor VIIa, as well as for the factor VIIa–TF complex. These compounds, although much more sensitive to hydrolysis by factor VIIa than the *p*-nitroanilides mentioned above, are subject to significant spontaneous hydrolysis in the absence of the enzyme (Higashi et al., 1992; Kam et al., 1990). In addition, separation of the formed leaving group from the initial substrate may also be required (Higashi et al., 1992). Furthermore, there is little agreement concerning the effect of tissue factor on the esterolytic activity of factor VIIa with these substrates (Zur & Nemerson, 1978; Higashi et al., 1992).

Our laboratory recently reported a new fluorogenic substrate (m-LGR-nds) for factor VIIa possessing a substituted 6-amino-1-naphthalenesulfonamide (ANSN) detecting group (Lawson et al., 1992). This substrate enabled us to quantitatively evaluate factor VIIa activity at nanomolar concentrations of this enzyme.

On the basis of our success with m-LGR-nds, a series of approximately 100 peptide substrates with similar leaving groups has been synthesized. The cleavage of these substrates by factor VIIa or the factor VIIa–TF complex has been tested. After this initial screening, the kinetic constants of hydrolysis for 26 of these substrates were evaluated, and the dependence of their specificity on substitution at positions P_2 and P_3 on the peptide chain has been estimated.[1]

In a previous publication, we evaluated a group of substrates containing only one amino acid residue, arginine, in the P_1 position and different substituents in the sulfonamide moiety of ANSN (P′). We examined the specificity of these substrates with six enzymes involved in coagulation and fibrinolysis (Butenas et al., 1992). No detectable hydrolysis of these compounds by factor VIIa was observed. The addition of two amino acid residues at the P_2 and P_3 positions increased the susceptibility of the substrate to be hydrolyzed by factor VIIa and the factor VIIa–TF complex. The dependence of factor VIIa and factor VIIa–TF complex activity on the P′ structure of the substrates was estimated using 12 compounds of structure D-FPR-ANSNR_1R_2.

† This work was supported by National Institutes of Health Grants R01 HL4697 and HL46703 and by the Department of Biochemistry, University of Vermont. This work was presented at the American Heart Association Meeting, New Orleans, November, 1992.

* Author to whom correspondence should be addressed.

[1] The nomenclature used to identify individual amino acid residues of the substrate (P_1, P_2, P′, etc.) is that of Schechter and Berger (1967).

0006-2960/93/0432-6531$04.00/0

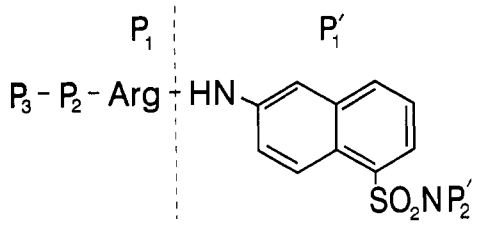

FIGURE 1: Structure of 6-(peptidylamino)-1-naphthalenesulfonamide substrates. P_2 and P_3 are amino acids, and P_2' are substituents in the sulfonamide moiety.

MATERIALS AND METHODS

Recombinant human coagulation factor VIIa was purchased from NOVO Pharmaceuticals. Bovine and human factor Xa were prepared by previously described methods (Krishnaswamy et al., 1987). Recombinant human tissue factor was provided as a gift from Dr. Shu-Len Liu (Hyland Division, Baxter Healthcare Corp.). The concentrations of proteins used in this study were calculated using the following molecular weights and extinction coefficients ($E^{0.1\%}_{280}$): human factor VIIa, 50 000 and 1.39 (Bajaj et al., 1981); bovine factor Xa, 45 300 and 1.24 (Fujikawa & Davie, 1976); human factor Xa, 45 000 and 1.16 (Di Scipio et al., 1977). The concentration of tissue factor was determined by amino acid analysis. The fluorogenic substrates were prepared as previously described (Butenas et al., 1992).

Factor VIIa assays were conducted in 20 mM HEPES, 150 mM NaCl, and 5 mM $CaCl_2$ (pH 7.4; HBS) at 37 °C. Factor VIIa (20 nM) was preincubated for 5 min at 37 °C in HBS, followed by the addition of a solution of substrate. In examining the activity of the factor VIIa–TF complex, factor VIIa (5 nM) and TF (10 nM) were preincubated for 10 min at 37 °C in HBS, followed by the addition of a solution of substrate. Factor Xa assays were conducted in 20 mM Tris and 150 mM NaCl (pH 7.4; TBS) at 22 °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 either HBS or TBS, as appropriate, to a working concentration prior to all assays. The final volume for all reactions was 1.2 mL.

The rate of substrate hydrolysis was evaluated as the change in fluorescence over time, corresponding to the generation of the particular ANSN. Fluorescence was monitored using a Perkin-Elmer Model MPF-44B fluorescence spectrophotometer, equipped with a standard chart recorder. Fluorophore was detected using an excitation wavelength of 352 nm and an emission wavelength of 470 nm. Light-scattering artifacts were minimized with a 399-nm cutoff filter in the emission light beam. Kinetic constants of substrate hydrolysis were determined using the nonlinear least-squares fitting program ENZFITTER (Elsevier-BIOSOFT, Cambridge, UK). This program was used to calculate the kinetic constants of substrate hydrolysis (k_{cat} and K_M) by iteratively fitting initial rates of substrate hydrolysis to the Michaelis–Menten equation.

To evaluate the influence of the P_2 structure on kinetic constants of enzymatic substrate hydrolysis, the K_M, k_{cat}, and k_{eff} values of the groups of substrates with the same P_3 structure were compared (substrates 1, 5, 19; 7, 17, 24; 9, 13, 22; 2, 6, 20; 8, 18, 25; and 10, 14, 23; Table II). To evaluate the influence of the P_3 structure, the groups of substrates with the same P_2 structure were compared as above (1, 7, 9; 13, 19; 17, 22; 2, 8, 10; 14, 20; and 18, 23; Table II). To counterbalance the influence of different substituents in the sulfonamide moiety (P_2' structure; Figure 1) of the substrates presented in Table II, kinetic constants of these compounds were normalized to the substrate with a cyclohexyl group at P_2' (substrate 1). To accomplish this normalization, the data presented in Table IV were used. The experimentally determined constant for substrate 1 (K_M or k_{cat}) was divided by the corresponding experimental constant of the substrate with the P_2' structure being analyzed, and a correction coefficient was obtained. The corresponding experimental constants for the substrate, found in Table II (possessing the same P_2' structure as the compound in Table IV), were multiplied by this coefficient, and a normalized value of the constant was obtained.

Stopped-flow experiments were conducted as previously described (Krishnaswamy et al., 1988) using 20 nM factor VIIa, 30 nM TF, and 200 nM substrate m-LGR-nds in HBS.

Table I: Physicochemical Constants for Peptidyl-ANSN

	substrates	MW	% yield	R_f[a]	$[\alpha]^{22}$[b]
1	D-FPR-ANSNH(c-C_6H_{11})·2HCl	777.81	79	0.52	−66.8
2	Boc-D-FPR-ANSNH(c-C_6H_{11})	805.01	87	0.64	−28.6
3	L-(*p*-F)FPR-ANSNHC_2H_5·2HCl	741.72	89	0.60	−72.8
4	Boc-L-(*p*-F)FPR-ANSNHC_2H_5	768.92	90	0.74	−41.6
5	D-FVR-ANSNHC_2H_5·2HCl	725.72	92	0.57	−35.8
6	Boc-D-FVR-ANSNHC_2H_5	752.92	77	0.82	−18.6
7	D-LPR-ANSNHC_3H_7·2HCl	721.74	93	0.50	−50.1
8	Boc-D-LPR-ANSNHC_3H_7	748.94	82	0.68	−35.5
9	D-VPR-ANSNHC_4H_9·2HCl	703.73	79	0.53	−56.8
10	Boc-D-VPR-ANSNHC_4H_9	730.93	96	0.72	−49.8
11	L-VPR-ANSNHC_4H_9·2HCl	703.73	90	0.51	−49.1
12	Boc-L-VPR-ANSNHC_4H_9	730.93	83	0.75	−46.5
13	D-VLR-ANSNHC_4H_9·2HCl	719.78	85	0.54	−33.3
14	Boc-D-VLR-ANSNHC_4H_9	746.98	98	0.82	−24.6
15	L-VLR-ANSNHC_4H_9·2HCl	719.78	88	0.53	−3.3
16	Boc-L-VLR-ANSNHC_4H_9	746.98	87	0.84	−20.9
17	D-LSR-ANSNHC_3H_7·2CF_3COOH	848.84	83	0.52	−10.6
18	Boc-D-LSR-ANSNHC_3H_7	720.92	76	0.72	−7.8
19	D-FLR-ANSNHC_3H_7·2HCl	753.82	92	0.59	−26.5
20	Boc-D-FLR-ANSNHC_3H_7	781.02	82	0.86	−26.6
21	L-FLR-ANSNHC_3H_7·2HCl	753.82	89	0.60	−12.5
22	D-VSR-ANSNH(*i*-C_3H_7)·2CF_3COOH	834.78	83	0.51	−28.3
23	Boc-D-VSR-ANSNH(*i*-C_3H_7)	706.86	78	0.75	−17.3
24	D-LGR-ANSNH(c-C_6H_{11})·2HCl	703.68	74	0.42	−27.0
25	Boc-D-LGR-ANSNH(c-C_6H_{11})	730.88	85	0.65	−16.4
26	D-PFR-ANSNH(*i*-C_3H_7)·2HCl	737.75	85	0.49	+26.6
27	Mes-D-LGR-ANSN(C_2H_5)$_2$	682.80	87	0.62	−12.9

[a] 1-Butanol–acetic acid–water, 4:1:2 (BAW 412). [b] c = 1, DMSO.

RESULTS

Physicochemical Characterization of the Synthesized Substrates. The physicochemical characteristics for the series of peptide substrates tested are listed in Tables I and III. These substrates were synthesized with relatively high yields as crude products (75–95%) and also as final products (50–80%) after a Sephadex LH-20 column purification. The substrates with an unblocked amino group in the P_3 position have solubilities in aqueous buffer within the millimolar range. Only substrate 31, with a highly hydrophobic linear hexyl group in the sulfonamide moiety, has sub-millimolar solubility. The substrates with blocked N-terminal amino acids have solubilities within the sub-millimolar range as well. All substrates show relative fluorescent intensities at 470 nm of less than 0.1% of that of the respective free fluorophore. Continuous excitation of any of these compounds at 352 nm for a period of 30 min at 37 °C resulted in no significant increase in fluorescence. Nonenzymatic hydrolysis rates were less than 0.5% after a 6-month storage period of the substrates in HBS at 4 °C.

Utility of Peptide Substrates for the Evaluation of Factor Xa, Factor VIIa, and Factor VIIa–TF Activity. Kinetic constants for the hydrolysis of 27 substrates by factor Xa,

factor VIIa, and the factor VIIa–TF complex are listed in Table II. Additionally, kinetic constants for the hydrolysis of 11 other substrates by factor VIIa and factor VIIa–TF are listed in Table IV. A majority of the substrates presented in Table II are considerably more specific for the factor VIIa–TF complex than for factor Xa. In general, these substrates are poor substrates for factor Xa. The only exception is substrate 27. As described in our previous publications (Lawson et al., 1992; Butenas et al., 1992), it has a second-order rate constant of 291 000 M^{-1} s^{-1}. We established that the compounds with the highest efficiencies for factor Xa in this "family" of substrates have substrates with glycine in the P_2 position (S. Butenas and K. G. Mann, unpublished data). This conclusion coincides with that for *p*-nitroanilide substrates (Lottenberg et al., 1986). The catalytic constants vary over a wide range, 0.008–36.4 s^{-1}; however, substrates possessing a D-amino acid in the P_3 position have higher catalytic constants than their L counterparts (substrates 9–16 and 19–21; Table II). Values for Michaelis–Menten constants vary from 6 μM for substrate 20 to 1063 μM for substrate 23. The k_{cat} has a larger influence on the second-order rate constant than the K_M has for the various substrates, resulting in higher second-order rate constants for D isomers than for their corresponding L counterparts.

The catalytic constants for factor VIIa alone vary over a wide range, 0.0009–0.16 s^{-1} (substrates 25, 38, and 31 respectively). In the presence of TF, k_{cat} varies from 0.23 s^{-1} (substrate 21) to 13.86 s^{-1} (substrate 34). Michaelis–Menten constants for factor VIIa vary from 7 μM (substrate 2) to 1.3 mM (substrate 34). The presence of TF positively influences the rate of substrate hydrolysis as compared to that of factor VIIa alone. The addition of tissue factor causes an increase in the catalytic constants for all substrates presented in Tables II and IV. The lowest ratio of k_{cat} for the factor VIIa–TF complex vs factor VIIa alone is 24 (substrate 30), and the highest is 485 (substrate 23). Tissue factor appears to affect K_M minimally, primarily influencing k_{cat}.The ratio of K_M values for the factor VIIa–TF complex vs factor VIIa alone varies from 0.39 for substrate 35 to as much as 12.2 for substrate 23. The effect of tissue factor on the catalytic constant directly coincides with the effect seen for the corresponding Michaelis–Menten constant (Figure 2). The presence of tissue factor elevates the amidolytic efficiency (k_{eff}) of factor VIIa for this family of substrates on the average of 58-fold.

Influence of the P_2 Amino Acid on the Substrate Kinetic Constants. The kinetic constants for substrates with Gly, Ser, Pro, Val, or Leu in the P_2 position were evaluated. We found that the highest K_M values in the groups with identical P_3 and P′ (normalized data) have substrates with proline or leucine in the P_2 position. The substrates with serine or glycine in the P_2 position have lower K_M values. This observation is valid for substrate hydrolysis by factor VIIa as well as by the factor VIIa–TF complex when the P_3 amino acid is N-unblocked (Table V). For substrates with an N-blocked amino function in the P_3 position, the influence of the P_2 amino acid on K_M is not straightforward. However, the lowest values for K_M correspond to substrates with glycine in this position.

The dependence of catalytic constants on the P_2 amino acid essentially mirrors that of the Michaelis–Menten constants. Substrates with proline or leucine in this position have higher k_{cat} values than their analogs with glycine or serine in this position. However, in the case of second-order rate constants, this relationship changes slightly. The highest second-order rate constants have substrates with valine in the P_2 position, with slightly lower values for the proline- or leucine-substituted analogs. The lowest k_{eff} was calculated for those with serine or glycine in the P_2 position (Figure 3A,B) for factor VIIa as well as for the factor VIIa–TF complex. This relationship does not depend on the presence or absence of an *N-tert*-butoxycarbonyl (Boc) blocking group at the P_3 amino acid.

Influence of the P_3 Structure on the Substrate Kinetic Constants. The kinetic constants for substrates possessing Leu, Val, or Phe in the P_3 position were evaluated. In the groups of substrates with the same P_2 and P′ (normalized) structures, the highest Michaelis–Menten constants as well as the highest kinetic constants have substrates with leucine in the P_3 position (Table V). This relationship does not depend on the presence or absence of tissue factor or Boc blocking group. However, the substrates with phenylalanine in this position have the highest k_{eff} values (Figure 3C,D).

The kinetic constants of substrate hydrolysis by factor VIIa depend not only on the amino acid used in the P_3 position but also on the stereoisomer of this amino acid. The Boc blocking group influences these constants as well. For hydrolysis by factor VIIa alone, a higher K_M as well as k_{cat} is observed when a D-amino acid is in the P_3 position as compared with the corresponding L-amino acid at this position (substrates 9–16). Values for K_M increase for both isomers when the Boc blocking group is eliminated from this position. The unblocking of the P_3 position results in higher catalytic constants as well. To summarize, the k_{eff} is generally higher for D isomers than for their L analogs, and this difference is enhanced when the P_3 position is unblocked.

Influence of the Detecting Group Structure (P′) on Substrate Specificity. Twelve compounds with a D-FPR peptide chain and different substituents in the sulfonamide moiety were synthesized in order to evaluate the influence of P′ on substrate specificity (Table IV).

The catalytic constants for factor VIIa vary from 0.0009 s^{-1} for substrate 38, with glycine methyl ester in the detecting group, to 0.34 s^{-1} for substrate 34, with a detecting group containing a branched isopropyl group. Likewise, the Michaelis–Menten constants vary from 30 μM for substrate 38 to 1300 μM for substrate 34. The second-order rate constant increases as the length of the alkyl chain in the sulfonamide moiety increases, reaching a maximum with *n*-hexyl in this position (substrate 31; Figure 4A). Substrate 32, with an aromatic benzyl ring in the P′ structure, also has a relatively high k_{eff}. The least promising substrates are those with either a glycyl methyl ester (substrate 38) or a *tert*-butyl group (substrate 37) in the sulfonamide moiety. Branching of the detecting group appears to decrease effectiveness, as can be seen by comparing the kinetic constants of a substrate containing an *n*-butyl-substituted detecting group (substrate 30) versus its *tert*-butyl-substituted counterpart (substrate 37) or *n*-propyl (substrate 29) versus isopropyl (substrate 34). These observations concerning the influences of P′ on values for k_{eff} are applicable to substrate hydrolysis by factor VIIa alone as well as by the factor VIIa–TF complex (Figure 4A,B). As observed for factor VIIa alone, the catalytic and Michaelis–Menten constants for the factor VIIa–TF complex vary over a wide range. The lowest catalytic constant, 0.34 s^{-1}, corresponds to the substrate with a carboxy-protected glycine in the detecting group (38). This substrate does have a relatively high Michaelis–Menten constant of 0.303 mM, but this constant is significantly larger for substrates 28 or 34 which contain ethyl- or isopropyl-substituted sulfonamides (0.793 or 0.921 mM, respectively). Substrates hydrolyzed by the lowest K_M have *n*-propyl (29) or benzyl (32) substituents

Table II: Kinetic Constants[a] for the Substrate Hydrolyses

substrates	enzymes			rel. specif. FVIIa–TF		initial rate of hydrolysis[d] (nM/s)
	FVIIa[b]	FVIIa–TF[b]	FXa[c]	FVIIa	FXa	
1. D-FPR-ANSNH(c-C_6H_{11})						1.55
K_M	152/152	329/329	150	2.2	2.2	
k_{cat}	0.054/0.054	8.37/8.37	0.32	155	26.2	
k_{cat}/K_M	355/355	25400/25400	2130	71.5	11.9	
2. Boc-D-FPR-ANSNH(c-C_6H_{11})						0.30
K_M	7/7	78/78	99	10.9	0.8	
k_{cat}	0.0033/0.0033	1.18/1.18	0.11	358	10.7	
k_{cat}/K_M	451/451	15100/15100	1110	33.5	13.6	
3. (*p*-F)FPR-ANSNHC_2H_5						0.29
K_M	185/33	95/39	95	0.5	1.0	
k_{cat}	0.017/0.007	0.60/0.66	0.25	35	2.4	
k_{cat}/K_M	93/201	6300/16300	2610	67.7	2.4	
4. Boc(*p*-F)FPR-ANSNHC_2H_5						0.28
K_M	50/9	217/89	500[e]	4.4	0.4	
k_{cat}	0.008/0.003	0.88/0.9	1.30	110	0.7	
k_{cat}/K_M	160/346	4100/10600	2610	25.6	1.6	
5. D-FVR-ANSNHC_2H_5						1.19
K_M	73/13	230/94	20	3.1	11.7	
k_{cat}	0.017/0.007	2.39/2.63	0.018	141	132.7	
k_{cat}/K_M	234/505	10400/2690	888	44.4	11.7	
6. Boc-D-FVR-ANSNHC_2H_5						1.86
K_M	44/8	71/29	20[e]	1.6	3.6	
k_{cat}	0.018/0.007	0.84/0.92	0.052	47	16.2	
k_{cat}/K_M	406/877	11800/30600	2600	29.1	4.5	
7. D-LPR-ANSNHC_3H_7						0.98
K_M	301/692	301/1625	171	1.0	1.8	
k_{cat}	0.069/0.14	4.50/27.0	3.29	65	1.4	
k_{cat}/K_M	231/203	14900/16400	19200	64.5	0.8	
8. Boc-D-LPR-ANSNHC_3H_7						0.51
K_M	29/67	81/435	5764	2.8	0.01	
k_{cat}	0.011/0.023	0.45/2.70	114.00	41	0.04	
k_{cat}/K_M	385/339	5500/6000	20000	14.3	0.3	
9. D-VPR-ANSNHC_4H_9						0.87
K_M	89/73	52/166	164	0.6	0.3	
k_{cat}	0.019/0.009	0.76/2.36	3.32	40	0.2	
k_{cat}/K_M	220/136	14400/13800	20200	65.5	0.7	
10. Boc-D-VPR-ANSNHC_4H_9						0.49
K_M	73/60	102/326	660	1.4	0.2	
k_{cat}	0.0086/0.004	0.89/2.76	10.10	103	0.09	
k_{cat}/K_M	117/73	8700/8400	15300	74.4	0.6	
11. L-VPR-ANSNHC_4H_9						0.06
K_M	60/49	342/1094	90[e]	5.7	3.8	
k_{cat}	0.0018/0.0015	0.33/1.02	0.030	183	11	
k_{cat}/K_M	30/19	1000/1000	334	33.3	2.9	
12. Boc-L-VPR-ANSNHC_4H_9						0.33
K_M	41/34	142/454	145	3.5	1.0	
k_{cat}	0.0038/0.0019	0.59/1.83	0.068	155	8.7	
k_{cat}/K_M	91/56	4200/4000	471	46.2	8.9	
13. D-VLR-ANSNHC_4H_9						0.65
K_M	63/52	162/518	33[e]	2.6	4.9	
k_{cat}	0.0110/0.005	2.20/6.82	0.084	200	26.2	
k_{cat}/K_M	171/106	13600/13100	2530	79.5	5.3	
14. Boc-D-VLR-ANSNHC_4H_9						0.48
K_M	42/34	171/547	19[e]	4.1	9.2	
k_{cat}	0.0066/0.003	1.56/4.84	0.055	236	28.4	
k_{cat}/K_M	159/99	9100/8700	2970	57.2	3.1	
15. L-VLR-ANSNHC_4H_9						0.51
K_M	49/40	70/224	53[e]	1.4	1.3	
k_{cat}	0.0045/0.0022	0.31/0.96	0.015	69	20.7	
k_{cat}/K_M	91/56	4400/4200	280	48.4	15.7	
16. Boc-L-VLR-ANSNHC_4H_9						0.58
K_M	19/16	43/138	8[e]	2.3	5.4	
k_{cat}	0.0040/0.0020	0.35/1.08	0.014	88	25.0	
k_{cat}/K_M	215/133	8300/8000	1760	38.6	4.7	
17. D-LSR-ANSNHC_3H_7						0.64
K_M	67/154	75/405	104	1.1	0.7	
k_{cat}	0.0076/0.016	0.50/3.00	1.14	66	0.4	
k_{cat}/K_M	113/99	6600/7300	11000	58.4	0.6	
18. Boc-D-LSR-ANSNHC_3H_7						0.23
K_M	29/67	111/599	344[e]	3.8	0.3	
k_{cat}	0.0034/0.007	0.41/2.46	2.95	121	0.1	
k_{cat}/K_M	118/104	3700/4100	8580	31.4	0.4	
19. D-FLR-ANSNHC_3H_7						0.55
K_M	41/94	54/292	91	1.3	0.6	
k_{cat}	0.0080/0.017	0.74/4.44	0.031	93	23.9	
k_{cat}/K_M	194/171	13700/15100	336	70.6	40.8	

Table II (Continued)

substrates	enzymes			rel. specif. FVIIa–TF		initial rate of hydrolysis[d] (nM/s)
	FVIIa[b]	FVIIa–TF[b]	FXa[c]	FVIIa	FXa	
20. Boc-D-FLR-ANSNHC$_3$H$_7$						1.19
K_M	12/28	39/211	6[e]	3.2	6.5	
k_{cat}	0.0043/0.009	0.48/2.88	0.028	112	17.1	
k_{cat}/K_M	357/314	12400/13600	4730	34.7	2.6	
21. L-FLR-ANSNHC$_3$H$_7$						0.31
K_M	11/25	133/718	13[e]	11.9	10.1	
k_{cat}	0.0013/0.0027	0.23/1.38	0.008	177	28.8	
k_{cat}/K_M	114/100	1700/1900	610	14.9	2.8	
22. D-VSR-ANSNH(i-C$_3$H$_7$)						0.37
K_M	42/5	63/23	204	1.5	0.3	
k_{cat}	0.0027/0.0004	0.44/0.26	1.13	163	0.4	
k_{cat}/K_M	64/88	7000/11800	5540	109.4	1.3	
23. Boc-D-VSR-ANSNH(i-C$_3$H$_7$)						0.13
K_M	29/4	360/130	1063	12.2	0.3	
k_{cat}	0.0013/0002	0.63/0.38	6.50	485	0.1	
k_{cat}/K_M	44/60	1700/2900	6110	38.6	0.3	
24. D-LGR-ANSNH(c-C$_6$H$_{11}$)						0.20
K_M	220/220	85/85	122[e]	0.4	0.7	
k_{cat}	0.0088/0.009	0.36/0.36	3.87	41	0.09	
k_{cat}/K_M	40/40	4300/4300	31700	107.5	0.1	
25. Boc-D-LGR-ANSNH(c-C$_6$H$_{11}$)						0.13
K_M	26/26	158/158	46[e]	6.1	3.4	
k_{cat}	0.0009/0.0009	0.24/0.24	2.17	267	0.1	
k_{cat}/K_M	34/34	1500/1500	7200	44.1	0.2	
26. D-PFR-ANSNH(i-C$_3$H$_7$)						0.19
K_M	125/287	57/308	298	0.5	0.2	
k_{cat}	0.0078/0.016	0.24/1.44	17.20	31	0.01	
k_{cat}/K_M	62/85	4300/7300	57700	69.4	0.1	
27. Mes-D-LGR-ANSN(C_2H_5)$_2$						0.18
K_M	179/106	198/594	125	1.1	1.6	
k_{cat}	0.0066/0.0073	0.79/4.58	36.40	120	0.02	
k_{cat}/K_M	37/71	4000/7800	291000	108	0.01	

[a] Calculated/normalized for P_2' influence; K_M is in μM, k_{cat} in s^{-1}, and k_{cat}/K_M in M^{-1} s^{-1}. [b] HBS pH 7.4, 37 °C, [Ca^{2+}] 5 mM. [c] TBS pH 7.4, 22 °C. [d] HBS pH 7.4, 37 °C, [Ca^{2+}] = 2 mM, [S] = 50 μM, [FVIIa] = 5 nM, and [TF] = 10 nM. [e] Bovine Xa.

Table III: Physicochemical Constants for D-FPR-ANSNR$_1$R$_2$·2HCl

substrate	R_1	R_2	MW	% yield[a]	R_f[b]	$[\alpha]^{22}$ [c]
28	H	C_2H_5	723.71	63	0.61	−74.5
29	H	n-C_3H_7	737.74	66	0.58	−74.6
30	H	n-C_4H_9	751.76	67	0.58	−58.3
31	H	n-C_6H_{13}	779.81	82	0.52	−81.4
32	H	$CH_2C_6H_5$	785.78	55	0.58	−69.5
33	$(CH_2)_6$		777.80	53	0.53	−97.7
34	H	i-C_3H_7	737.74	64	0.48	−83.8
35	H	$C_2H_4OCH_3$	753.74	57	0.46	−94.7
36	C_2H_5	C_2H_5	751.76	49	0.51	−82.7
37	H	t-C_4H_9	751.76	80	0.48	−82.0
38	H	CH_2COOCH_3	767.73	54	0.54	−84.0

[a] After purification on Sephadex LH-20. [b] BAW 412. [c] c = 1, DMSO.

(61 and 56 μM, respectively) in the sulfonamide moiety. The substrates with the highest k_{cat} values, 13.86 or 8.37 s^{-1}, have isopropyl or cyclohexyl in the P′ position, respectively.

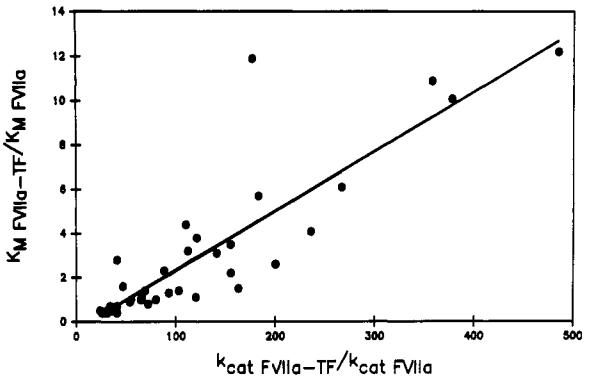

FIGURE 2: Influence of TF on the substrate kinetic constants. The k_{cat} of substrate hydrolysis by factor VIIa in the presence of TF was divided by the corresponding k_{cat} of substrate hydrolysis by factor VIIa in the absence of TF. The K_M ratios for these substrates were calculated in the same manner. Data for all 38 substrates presented in Tables II and IV have been used.

DISCUSSION

Prior to the publication by Lawson et al. (1992), the only compounds that had been described as substrates for blood-clotting enzyme factor VIIa were p-nitrobenzyl esters of N^{α}-blocked arginine (Zur & Nemerson, 1978), thiobenzyl esters of arginine or lysine, or peptides with a carboxy-terminal arginine (Kam et al., 1990). However, these substrates are not widely used due to their inherent shortcomings (Kam et al., 1990; Higashi et al., 1992) and/or because they become impractical due to the complicated manipulations required for their use (Higashi et al., 1992). p-Nitroanilide chromogenic substrates such as Spectrozyme FXa, S-2288, S-2238, and S-2266 (Ruf et al., 1991a,b; Bjoern et al., 1991; Pedersen et al., 1991; Krishnaswamy, 1992; Shigematsu et al., 1992) have found more frequent reference in the literature, but these compounds have low specificities for factor VIIa alone in comparison with the sensitivity of the detecting method and require the presence of tissue factor, an increased concentration of enzyme, or a prolonged time of assay to be useful. Substrates with a 4-methylcoumaryl-7-amino (MCA) detecting group would be effective in factor VIIa assays (Shigematsu et al., 1992) if the contradiction between high Michaelis–Menten constants and low solubility in water for this type of substrates could be justified.

In our previous publications, we presented a new fluorogenic substrate for factor VIIa (Lawson et al., 1992) and data concerning the influence of P′ structure of substrate on the amidolytic activity of several enzymes involved in the blood

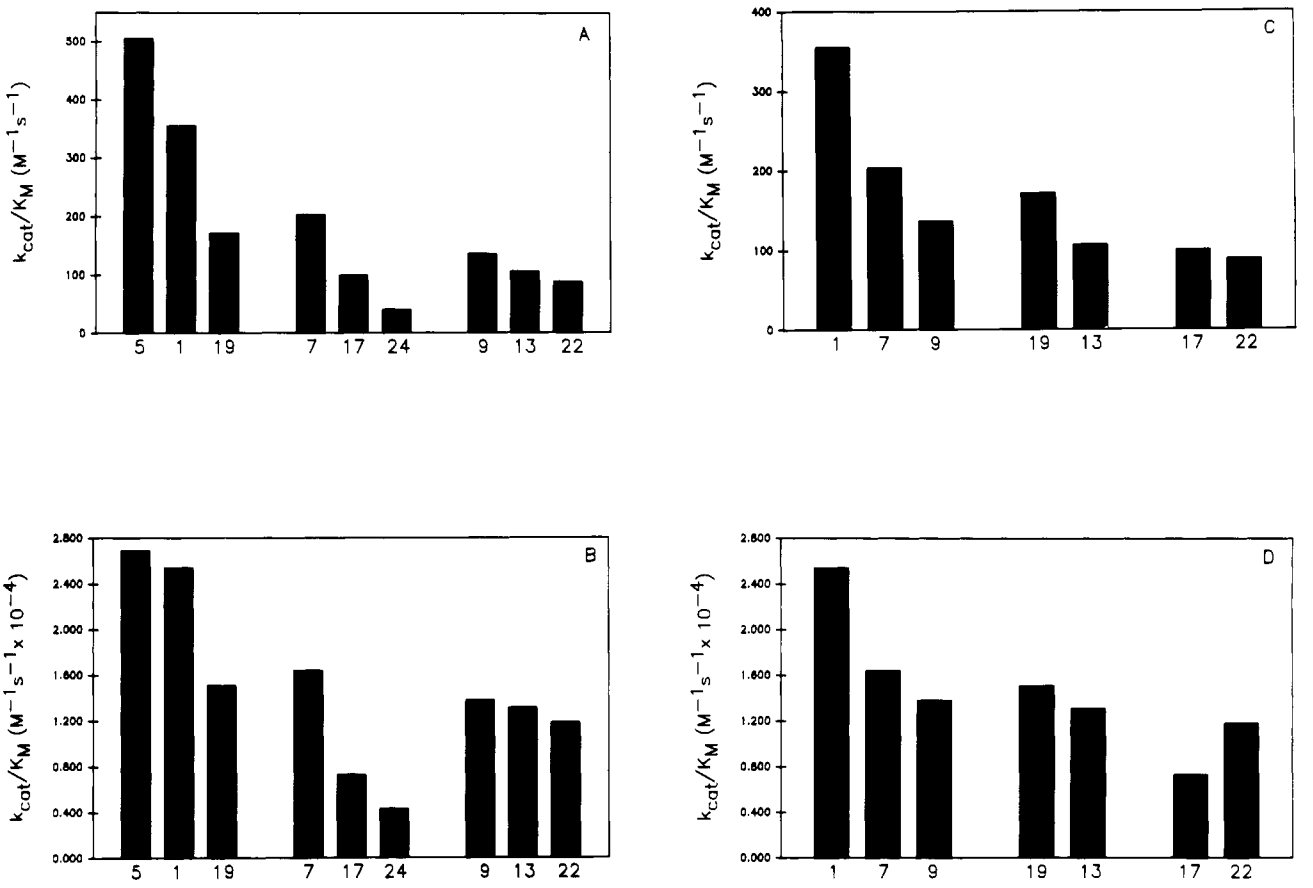

FIGURE 3: Influence of P_2 (A and B) and P_3 (C and D) amino acids on substrate efficiency. Panels A and C: k_{cat}/K_M of substrate hydrolysis by factor VIIa in the absence of TF. Panels B and D: k_{cat}/K_M for substrate hydrolysis by factor VIIa in the presence of TF. The groups of substrates are selected and constants are normalized for P′ influence as described in Materials and Methods.

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

substrate			FVIIa			FVIIa–TF			rel. specif. FVIIa–TF/FVIIa[b]		
	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
28	H	C_2H_5	828	0.14	164	793	7.74	9800	0.96	55	60
29	H	n-C_3H_7	65	0.026	403	61	1.40	23100	0.94	54	57
30	H	n-C_4H_9	186	0.11	573	102	2.69	26400	0.55	24	46
31	H	n-C_6H_{13}	216	0.16	727	146	5.40	36900	0.68	34	51
32	H	$CH_2C_6H_5$	126	0.080	635	56	2.10	36900	0.44	26	58
1	H	c-C_6H_{11}	152	0.054	355	329	8.37	25400	2.16	155	72
33		$(CH_2)_6$	116	0.033	284	118	2.64	22400	1.02	80	79
34	H	i-C_3H_7	1300	0.34	259	921	13.86	15000	0.71	41	58
35	H	$C_2H_4OCH_3$	278	0.057	205	108	1.49	13800	0.39	26	67
36	C_2H_5	C_2H_5	256	0.047	185	110	1.44	13000	0.43	31	70
37	H	t-C_4H_9	109	0.0060	52	91	0.43	4700	0.83	72	90
38	H	CH_2COOCH_3	30	0.0009	30	303	0.34	1100	10.10	378	37

[a] K_M is in μM, k_{cat} in s^{-1}, and k_{cat}/K_M in M^{-1} s^{-1}. [b] Ratio.

coagulation process (Butenas et al., 1992). However, factor VIIa hydrolyzed the (N^{α}-Z)Arg-ANSNs presented in the last publication at nondetectable rates. To establish which amino acids in the P_2 and P_3 positions of the original structure may increase the specificity for hydrolysis by factor VIIa and the factor VIIa–tissue factor complex, approximately 100 substrates possessing different peptide chains were synthesized. These substrates were screened for initial rate of hydrolysis by the factor VIIa–TF complex. After this preliminary screening, 26 substrates were chosen for further kinetic analysis.

In the groups of substrates possessing the same P_1, P_3, and P′ structures, the highest Michaelis–Menten constants have substrates with bulky amino acids in the P_2 position. However, these substrates also have the highest catalytic constants. As the bulkiness and the size of the amino acid in the P_2 position decrease, the K_M and k_{cat} values decrease accordingly. The catalytic constants, however, are influenced more by these substitutions than Michaelis–Menten constants, and this causes higher second-order rate constants for substrates with bulky amino acids in the P_2 position. If we assume that Val, Pro, Leu, Ser, and Gly are substituted at the P_2 position, the values for k_{eff} decrease in the following manner: Val, Pro > Leu > Ser > Gly. The volume of these amino acids decreases in a similar pattern: Leu > Val > Pro > Ser > Gly (Zamyatnin, 1972). The highest second-order rate constants have substrates possessing valine or proline in the P_2 position. Leucine substitution is less promising than its valine or proline counterparts because the relatively high K_M of this substrate is not coupled with as high a k_{cat} value as might be expected. Substitution by serine or glycine at the P_2 position causes a reduction in the second-order rate constant because the

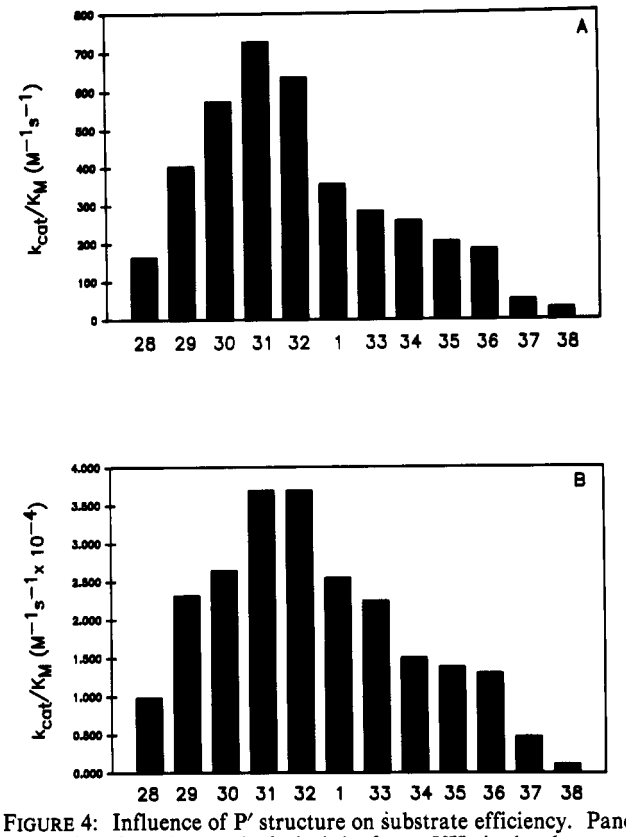

FIGURE 4: Influence of P′ structure on substrate efficiency. Panel A: k_{cat}/K_M for substrate hydrolysis by factor VIIa in the absence of TF. Panel B: k_{cat}/K_M for substrate hydrolysis by factor VIIa in the presence of TF.

relatively low K_M is not counteracted by a large enough k_{cat}. The effect of P_2 structure on substrate kinetic constants is minimized when the amino function of the P_3 substituent is blocked with a bulky Boc protecting group.

It is assumed that factor VIIa hydrolyzes the substrates utilized in this study according to the mechanism suggested for natural protein and chromogenic substrates (Kedzy & Kaiser, 1970):

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} EP_2 + P_1 \xrightarrow{k_3} E + P_2$$

where E and S are the enzyme and substrate, ES represents the enzyme–substrate complex, EP_2 is the acyl enzyme, P_1 is the detecting group, P_2 is the peptide core of the original substrate, and k represents catalytic constants, numbered accordingly. Kinetic constants deduced from this mechanism are as follows:

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \quad \text{and} \quad K_M = \frac{k_3 k_{-1}}{k_1(k_2 + k_3)}$$

Preliminary stopped-flow experiments employing substrate m-LGR-nds have been conducted in an attempt to identify the limiting steps in the mechanism described. A "burst" of detecting group as described by Chase and Shaw (1970) was not observed for either factor VIIa or the factor VIIa–TF complex. Using m-LGR-nds as a representative for all substrates, we can conclude that $k_3 > k_2$ and that the equations presented above can be written in this way:

$$k_{cat} \approx k_2 \quad \text{and} \quad K_M \approx \frac{k_{-1}}{k_1}$$

Bulkier amino acids in the P_2 position most likely cause a higher level of steric hindrance for ES formation (lower k_1). Additionally, the complex may be less stable and its dissociation can be more significant (higher k_{-1}). As a result of these two processes, the Michaelis–Menten constant should increase. However, the binding of substrate with such a bulky amino acid in the P_2 position may cause changes in the substrate structure (i.e., deformation of bonds, including the cleavage bond) and in the same way induce ES complex degradation, leading to EP_2 and P_1 formation (higher k_2).

To evaluate the influence of P_3 on substrate hydrolysis by factor VIIa and the factor VIIa–TF complex, we were able to compare only three amino acids: phenylalanine, leucine, and valine. These amino acids have relatively high hydrophobicities (Nozaki & Tanford, 1971) and volumes (which both decrease in the order written above). The highest Michaelis–Menten and catalytic constants have substrates possessing the intermediary leucine. However, the catalytic constant is influenced by the changes of P_3 amino acids more significantly than the Michaelis–Menten constant. The decreasing series of second-order rate constants, Phe > Leu > Val, mirrors that of decreasing hydrophobicity and volume. Most likely, as the volume of P_3 increases, the corresponding kinetic constants will be influenced in the same manner as described for P_2. The interactions between the hydrophobic P_3 structure and the hydrophilic amino acids that form the active site of factor VIIa may also affect these constants.

Similar results are generated when an L amino acid in the P_3 position is replaced by its D counterpart. In the case of factor VIIa alone, substrates with D-amino acids have higher constants for K_M and k_{cat} than those with the corresponding L-amino acids. The increase in the Michaelis–Menten constants may be caused by poorer recognition of substrate by factor VIIa caused by the presence of the D stereoisomer (lower k_1) and elevated dissociation (higher k_{-1}). However, a D isomer in the P_3 position results in facilitated degradation of the ES complex (higher k_2). The bulky Boc protecting group (which could be considered a P_4 structure) minimizes these effects as well as the differences in the kinetic constants of the two isomers, especially in the presence of tissue factor.

To examine the influence of P′ on substrate efficiency, we decided to separate the invariable part of the detecting group, the 1,6-disubstituted naphthalene ring, from the modifiable part of this group, the amino-linked group of the sulfonamide

Table V: Influence of P_2 and P_3 Amino Acids on Substrate Kinetic Constants

	P_2		P_3	
constants	factor VIIa	factor VIIa–TF	factor VIIa	factor VIIa–TF
P_3 N-unblocked				
K_M	P > L > (V) > G > S	P,L > (V) > S > G	L > F > V	L > F,V
k_{cat}	P > L > (V) > S > G	P,L > (V) > S > G	L > F > V	L > F,V
k_{cat}/K_M	V > P > L > S > G	V,P > L > S > G	F > L > V	F > L,V
P_3 N-blocked				
K_M		L > P,V,S > G	L > V > F	L > V > F
k_{cat}	L,V,P, > S > G	L > P > (V) > S > G	L > V,F	L,V > F
k_{cat}/K_M	V > P,L > S > G	V > P,L > S > G	F > L > V	F > L,V

moiety. These two segments of the P′ structure will be referred to as P_1' and P_2', respectively (Figure 1). From Table IV we can see that there are very wide variations in both experimental kinetic constants (K_M and k_{cat}) caused by changes in the P_2' structure. These variations do not depend on volume or on the hydrophobicity of substituents in this position. For these substrates, changes in K_M directly coincide with changes in k_{cat}. Second-order rate constants show strong dependence on P_2' structure. As the length of the hydrocarbon chain increases and as the hydrophobicity of linear aliphatic substituents increases, there is an increase in substrate efficiency for factor VIIa in both the presence and absence of tissue factor (Figure 4A,B). The substrates with hydrophobic phenyl or cyclohexyl groups also have reasonably high second-order rate constants. On the other hand, substrate 38 with a relatively low hydrophobic glycine methyl ester in the P_2' structure has a second-order rate constant 24-fold lower than substrate 31 with *n*-hexyl in this position. Most likely, this effect is caused by hydrophilic–hydrophobic interactions between amino acids forming the active site of factor VIIa and the P_2' substituent.

Factor VIIa–tissue factor complex formation dramatically increases the ability of the substrate to be hydrolyzed by factor VIIa. Komiyama and co-workers have shown that the K_M for factor X or factor IX hydrolysis by factor VIIa does not depend on the presence or absence of tissue factor (Komiyama et al., 1990). The data provided in Tables II and IV clearly show that our series of low molecular weight substrates are cleaved by factor VIIa alone, as well as when it is complexed to TF, and that the Michaelis–Menten constants are not significantly influenced by the presence of TF. It has been reported that tissue factor causes conformational changes of factor VIIa (Nemerson, 1988), that these changes accelerate the enzyme–cofactor–substrate complex degradation, and in this way enhance the catalytic constant of substrate hydrolysis.

In spite of very wide variations in all three kinetic constants caused by changes in P_2, P_3, and P_2' structure, the extent of enhancement of factor VIIa amidolytic efficiency by tissue factor for any particular substrate does not depend significantly on substituents in these positions. This cofactor enhances the second-order rate constant on an average of 58-fold (range 37–90).

In summary, the kinetic constants for substrate hydrolysis by factor VIIa are influenced by different amino acids substituted in the peptide core (P_2–P_3) as well as by different substituents attached to the P_1' structure (P_2'). Substrate efficiency for factor VIIa depends on the volume of the substituents in the P structure and on the hydrophobicity of substituents in the P′ structure.

ACKNOWLEDGMENT

We are grateful to Rasa Bytautaite of the Institute of Biochemistry, Vilnius, Lithuania, and to Dr. Vida Drungilaite for their participation in substrate synthesis. We are grateful to Dr. Chun Kung and Eric Hayes for their contributions to the stopped-flow experiments. We are grateful to Dr. Shu-Len Liu of Baxter Healthcare Corp. for providing recombinant human tissue factor.

REFERENCES

Bajaj, S. P., Rapaport, S. I., & Brown, S. F. (1981) *J. Biol. Chem. 256*, 253–259.

Bjoern, S., Foster, D. C., Thim, L., Wiberg, F. C., Christensen, M., Komiyama, Y., Pedersen, A. H., & Kisiel, W. (1991) *J. Biol. Chem. 266*, 11051–11057.

Butenas, S., Orfeo, T., Lawson, J. H., & Mann, K. G. (1992) *Biochemistry 31*, 5399–5411.

Chase, T., & Shaw, E. (1970) *Methods Enzymol. 19*, 20–27.

Di Scipio, R. G., Hermodson, M. A., Yates, S. G., & Davie, E. W. (1977) *Biochemistry 16*, 698–706.

Fujikawa, K., & Davie, E. W. (1976) *Methods Enzymol. 45*, 89–95.

Higashi, Sh., Nishimura, H., Fuji, Sh., Takada, K., & Iwanaga, S. (1992) *J. Biol. Chem. 267*, 17990–17996.

Kam, Ch. M., Vlasuk, G. P., Smith, D. E., Arcuri, K. E., & Powers, J. C. (1990) *Thromb. Haemostasis 64*, 133–137.

Kedzy, F. J., & Kaiser, E. T. (1970) *Methods Enzymol. 19*, 1–20.

Komiyama, Y., Pedersen, A. H., & Kisiel, W. (1990) *Biochemistry 29*, 9418–9425.

Krishnaswamy, S. (1992) *J. Biol. Chem. 267*, 23696–23706.

Krishnaswamy, S., Church, W. R., Nesheim, M. E., & Mann, K. G. (1987) *J. Biol. Chem. 262*, 3291–3299.

Krishnaswamy, S., Jones, K. C., & Mann, K. G. (1988) *J. Biol. Chem. 263*, 3823–3834.

Krishnaswamy, S., Field, K. A., Edgington, T. S., Morrissey, J. H., & Mann, K. G. (1990) *Circulation 82*, III-133 (abstr.).

Lawson, J. H., & Mann, K. G. (1991) *J. Biol. Chem. 266*, 11317–11327.

Lawson, J. H., Butenas, S., & Mann, K. G. (1992) *J. Biol. Chem. 267*, 4834–4843.

Lottenberg, R., Hall, J. A., Pautler, E., Zupan, A., Christensen, U., & Jackson, C. M. (1986) *Biochim. Biophys. Acta 874*, 326–336.

Masys, D. R., Bajaj, S. P., & Rapaport, S. I. (1982) *Blood 60*, 1143–1150.

Mertens, K., Briet, E., & Giles, A. R. (1990) *Thromb. Haemostasis 64*, 138–144.

Nemerson, Y. (1988) *Blood 71*, 1–8.

Nozaki, Y., & Tanford, C. (1971) *J. Biol. Chem. 246*, 2211–2217.

Paborsky, L. R., Caras, I. W., Fisher, K. R., & Gorman, C. M. (1991) *J. Biol. Chem. 266*, 21911–21916.

Pedersen, A. H., Lund-Hansen, T., Komiyama, Y., Petersen, L., Oestergard, P. B., & Kisiel, W. (1991) *Thromb. Haemostasis 65*, 528–534.

Ruf, W., & Edgington, T. S. (1991) *Thromb. Haemostasis 66*, 529–533.

Ruf, W., Rehemtulla, A., & Edgington, T. S. (1991a) *J. Biol. Chem. 266*, 2158–2166.

Ruf, W., Kalnik, M. W., Lund-Hansen, T., & Edgington, T. S. (1991b) *J. Biol. Chem. 266*, 15719–15725.

Sakai, T., Lund-Hansen, T., Paborsky, L., Pedersen, A. H., & Kisiel, W. (1989) *J. Biol. Chem. 264*, 9980–9988.

Sakai, T., Lund-Hansen, T., Thim, L., & Kisiel, W. (1990) *J. Biol. Chem. 265*, 1890–1894.

Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157–162.

Seligsohn, U., Osterud, B., & Rapaport, S. I. (1978) *Blood 52*, 978–988.

Shigematsu, Y., Miyata, T., Higashi, Sh., Miki, T., Sadler, J. E., & Iwanaga, S. (1992) *J. Biol. Chem. 267*, 21329–21337.

Walsh, J. D., & Geczy, C. L. (1991) *Thromb. Haemostasis 66*, 552–558.

Wildgoose, P., Kazim, A. L., & Kisiel, W. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7290–7294.

Zamyatnin, A. A. (1972) *Prog. Biophys. Mol. Biol. 24*, 107–123.

Zur, M., & Nemerson, Y. (1978) *J. Biol. Chem. 253*, 2203–2209