

# Alteration of the Substrate and Inhibitor Specificities of Blood Coagulation Factor VIIa: Importance of Amino Acid Residue K<sup>192</sup> †

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**ABSTRACT:** Initiation of blood coagulation occurs when the plasma serine protease factor VIIa (fVIIa) binds to its cell-surface receptor/cofactor, tissue factor (TF). This binding interaction mediates a large enhancement in both the proteolytic activity and the amidolytic activity (hydrolysis of small peptidyl-amide substrates) of fVIIa. This necessitates local changes in the catalytic center of fVIIa of which little is understood. Studies with thrombin and activated protein C have demonstrated that residue E<sup>192</sup> (chymotrypsinogen numbering system) near the active site of these proteases is an important determinant for substrate and inhibitor specificity. By homology, residue 192 in fVIIa is K, bringing into question the potential role of this residue in fVIIa. We have prepared two mutants of fVIIa in which K<sup>192</sup> has been replaced by either Q (as in factors IX and X) or E (as in thrombin). Both mutants were found to be defective in clotting: fVIIK192Q was 44% active, while fVIIK192E was completely ineffective. This defect was attributable to proportional decreases in specificity constants for activation of factor X. Although both mutant enzymes were catalytically competent with respect to amidolytic activity, the selectivity of fVIIaK192E was greatly altered. Inhibition of both mutants by the TF pathway inhibitor (TFPI) and bovine pancreatic trypsin inhibitor (BPTI) was also drastically altered. Neither mutant was inhibited by TFPI, while fVIIaK192Q was inhibited by BPTI better than wild-type fVIIa. In contrast, fVIIaK192E was poorly inhibited by BPTI and made more refractory to inhibition when bound to TF. These results suggest a potential role for K<sup>192</sup> in governing the substrate and inhibitor specificities of fVIIa.

It is generally accepted that under normal hemostatic conditions the blood coagulation enzyme cascade is triggered when damage to vascular endothelium exposes cellular tissue factor (TF),<sup>1</sup> an integral membrane protein and the receptor/cofactor for the plasma serine protease fVIIa [reviewed by Carson and Brozna (1993)]. Since fVIIa in the absence of TF is a relatively inefficient protease, the fVIIa·TF complex can be considered a two-subunit enzyme where TF acts as the regulatory subunit and fVIIa acts as the catalytic subunit. Upon binding to TF, the enzymatic activity of fVIIa is dramatically enhanced, and it is the fVIIa·TF complex which subsequently catalyzes the activation of fVII, fIX, and fX, thus simultaneously amplifying and propagating the coagulation response.

As implied by the original proposals of an enzyme cascade for describing blood coagulation (Davie & Ratnoff, 1964; Macfarlane, 1964), the amplificative nature of linked enzyme

reactions essentially requires that the proteases involved be highly specific to prevent unwanted activation and enable precise control of the system. It is not surprising, therefore, that the blood-clotting serine proteases all share the property of exhibiting extremely narrow substrate (and inhibitor) specificity. However, unlike the digestive enzymes trypsin and chymotrypsin—the classical representatives of the serine protease family of enzymes—whose specificities and mechanisms of action have been intensively studied, the molecular bases of the specificities of the larger and more complex blood-clotting serine proteases remain largely unresolved.

The ability of TF to affect the kinetics of substrate hydrolysis catalyzed by fVIIa is well documented (Zur & Nemerson, 1980; Bach et al., 1986; Bom & Bertina, 1990; Bom et al., 1990) and generally involves a large increase in the specificity constant ( $k_{cat}/K_m$ ) for substrate hydrolysis. While the Ca<sup>2+</sup>-binding  $\gamma$ -carboxyglutamic acid-rich domain of fVIIa has been suggested to be important for recognition of natural substrates (Martin et al., 1993), there are suggestions in the literature that TF may also play a role in direct macromolecular substrate recognition in the fVIIa·TF complex (Ruf et al., 1992). However, this has yet to be conclusively demonstrated. Unlike other cofactor-regulated enzymes in blood coagulation, fVIIa shows enhanced activity upon binding to TF which is not limited to hydrolysis of native macromolecular protein substrates but also includes hydrolysis of small synthetic peptidyl-amide substrates (Ruf et al., 1991). The enhancement observed with amide substrates appears to be substrate-dependent and can take the form of a decrease in  $K_m$ , an increase in  $k_{cat}$ , or both (Higashi et al., 1992; Butenas et al., 1993, 1994; Neuenschwander et al., 1993). Thus, binding of fVIIa to TF seems

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<sup>1</sup> Abbreviations: TF, tissue factor; TF/PCPS, TF relipidated in phospholipid vesicles composed of 80% phosphatidylcholine (PC) and 20% phosphatidylserine (PS); sTF, soluble TF<sup>1-219</sup>—the extracellular domain of TF; fVII, blood-clotting factor VII (single chain); fVIIa, activated fVII (two chain); fVIIK192Q and fVIIK192E, mutated fVII where amino acid residue K<sup>192</sup> has been changed to Q or E; fVIIaK192Q and fVIIaK192E, activated fVIIK192Q and fVIIK192E; fIX, blood-clotting factor IX; fX, blood-clotting factor X; fXa, activated fX; BPTI, bovine pancreatic trypsin inhibitor; TFPI, TF pathway inhibitor; PCR, polymerase chain reaction. Standard single-letter amino acid abbreviations are used throughout.

to induce additional local changes in the catalytic center of fVIIa which affect substrate recognition and/or hydrolysis.

Recent studies with the related proteases thrombin and activated protein C have revealed that amino acid residue 192 (E<sup>192</sup>)<sup>2</sup> is important for conferring limited substrate and inhibitor specificity to these enzymes (Le Bonniec & Esmon, 1991; Rezaie & Esmon, 1993; Guinto et al., 1994). Crystal structures of inhibited thrombin molecules have positioned this residue at the entrance to the active site cleft (Stubbs & Bode, 1993), where its effect on specificity is presumably maximized. By homology, fIX and fX both contain Q at this position—a rather conservative substitution—which coincides with residue 192 in trypsin. In sharp contrast, residue 192 in fVIIa is K. The reversed charge and the presumed similar positioning of this residue in fVIIa bring into question the potential role of K<sup>192</sup> in the observed specificities of fVIIa and in the enhancement in activity observed upon binding to TF.

We report here studies performed with two recombinant mutant fVIIa molecules in which K<sup>192</sup> has been substituted with either Q or E. We found that residue 192 of fVIIa, similar to thrombin and activated protein C, was important in the substrate and inhibitor specificity of this enzyme. The results suggest that TF binding may affect movement of residue 192 in fVIIa, resulting in an altered cofactor effect of TF. Thus, K<sup>192</sup> may contribute at least in part to the increase in the specificity constant observed with various substrates when fVIIa is bound to TF.

## EXPERIMENTAL PROCEDURES

**Materials and Reagents.** Chromozym t-PA substrate (MeSO<sub>2</sub>-D-phenylalanyl-glycylarginyl-p-nitroanilide acetate) was from Boehringer-Mannheim Corp. (Indianapolis, IN), CBS 34.47 substrate [(D-cyclohexylglycyl)(L-α-(aminobutyryl)arginyl)-p-nitroanilide hydrochloride] was from Diagnostica Stago (Asnières, France), BPTI was from Worthington Biochemical Corp. (Freehold, NJ), and recombinant full-length TFPI was a gift of Dr. George Broze from Washington University in St. Louis, MO.

**Construction and Expression of Recombinant fVII Mutants.** Constructs for expressing fVII K<sup>192</sup> mutants were made by PCR-based site-directed mutagenesis (Horton et al., 1990) using pJH47, the wild-type fVII cDNA (ATCC 59790; American Type Culture Collection, Rockville, MD) subcloned into the mammalian expression vector pcDNAI (Invitrogen, San Diego, CA) (Neuenschwander & Morrissey, 1992). The PCR mutation primer pairs were 5'-CTC CTG CCA GGG GGA CAG TGG AG-3'/5'-TCC CCC TGG CAG GAG TCC TTG CT-3' and 5'-CTC CTG CGA GGG GGA CAG TGG AG-3'/5'-TCC CCC TCG CAG GAG TCC TTG CT-3'. The mutated codon (codon 341) is underscored and results in a single base change in each case: AAG → CAG for the K → Q mutation and AAG → GAG for the K → E mutation. First-round PCR amplifications were performed with pJH47 by pairing each of the four mutation primers with the appropriate reverse primer (T7 or SP6 promoter primer; Promega). PCRs were hot-started using AmpliMax

(Perkin-Elmer Cetus) and cycled 35 times through the following conditions: 1 min at 95 °C, 1 min at 50 °C, and 1.5 min at 72 °C. All ramp times were set at 0.5 min.

Second-round PCR amplifications of the mutated DNA fragments were done by mixing matched pairs of purified first-round PCR products in the presence of both the T7 and SP6 promoter primers. These mixtures were then cycled through the following conditions: 1 min at 95 °C, 1 min at 45 °C, and 1.5 min at 72 °C (15 cycles) followed by 1 min at 95 °C, 1 min at 50 °C, and 1.5 min at 72 °C (35 cycles). The PCR products were purified and shuttled as *KspI/KpnI* fragments (~400 bp) directly into pJH59, which is the wild-type fVII coding sequence subcloned from pJH47 into pGEM-7Zf(+) (Promega) between the *BamHI* and *SphI* sites. The resulting fVII constructs (pJH60, coding for fVIIK192Q, and pJH61, coding for fVIIK192E) were verified by DNA sequencing before being subcloned into pcDNAI between the *BamHI* and *SphI* sites to produce pJH64 and pJH65, respectively. These constructs were transfected into human 293 cells (ATCC CRL 1573) as previously described for wild-type fVII (Neuenschwander & Morrissey, 1992).

**Protein Purifications.** Recombinant human wild-type fVII and human fVII mutants were isolated from human 293 cell supernatant concentrates using a Ca<sup>2+</sup>-dependent monoclonal antibody as previously described (Neuenschwander & Morrissey, 1992). Analysis by SDS-PAGE (not shown) revealed that the recombinant fVII preparations were solely in the zymogen (single-chain) form. Quantitative conversion of fVII preparations to the two-chain enzyme form was accomplished by incubation for 30 min at 37 °C with 0.1 vol of a 50% bead suspension of fXa-agarose [bovine fXa coupled to Affigel-10 (Biorad) at a density of 5 mg/mL] in the presence of 1 mM CaCl<sub>2</sub>. The reaction was terminated by the addition of EDTA to 2 mM and removal of the agarose beads by centrifugal filtration (UltraFree-MC filter unit; Millipore Corp., Bedford, MA).

Final purification of the enzyme forms was accomplished by Mono-Q ion-exchange chromatography (FPLC; Pharmacia LKB Biotechnology Inc.) as follows. Activation mixtures were concentrated 10-fold (Centricon-30; Amicon Inc., Beverly, MA) and diluted 1:1 with 20 mM Tris-HCl, pH 7.5, containing 100 mM NaCl and 2 mM EDTA before loading onto a 2-mL Mono-Q column at ambient temperature. The column was washed extensively in the same buffer before being developed with a linear 25-mL NaCl gradient (0.1–0.55 M). The fVIIa protein typically eluted as a single peak at 0.33 M NaCl. In some instances an additional minor peak was observed at roughly 0.39 M NaCl and pooled separately. Under the conditions used, fXa eluted at >0.5 M NaCl (not shown). Protein pools were dialyzed exhaustively into 20 mM Hepes-NaOH, pH 7.5, containing 100 mM NaCl and 0.002% NaN<sub>3</sub> and stored in small aliquots at –80 °C. Recombinant zymogen fVII proteins were purified in the same way but omitting the activation step.

Analysis of the γ-carboxyglutamic acid content (Smalley & Preusch, 1988) of the various fVII and fVIIa preparations established that all recombinant fVII proteins were fully carboxylated: 9.9–10.4 mol of γ-carboxyglutamic acid/mol of protein as compared to 10 mol/mol for plasma-derived fVIIa (Neuenschwander & Morrissey, 1992). Additional analysis of selected preparations by Mono-Q chromatography using a CaCl<sub>2</sub> gradient (0–20 mM) showed a single peak eluting at 1–2 mM CaCl<sub>2</sub> (not shown), consistent with a

<sup>2</sup> The amino acid sequence numbering used for thrombin, activated protein C, and fVIIa is according to the chymotrypsinogen numbering system, as previously suggested by Bode et al. (1989) for numbering residues of serine proteases sharing topological homology with chymotrypsin.

homogenous preparation of  $\gamma$ -carboxyglutamic acid-containing material.

Human fIX and fX were purified from plasma as described (Thompson, 1977; Le Bonniec et al., 1992). Recombinant sTF was made in *Escherichia coli* and purified as previously described (Rezaie et al., 1992). Human full-length TF was purified as described (Morrissey et al., 1988) from brain acetone powder which was prepared as described (Broze et al., 1985). Purified TF was relipidated into phospholipid vesicles composed of 80% PC and 20% PS using the method of Mimms et al. (1981). The concentration of available TF in relipidated preparations was determined as previously described (Neuenschwander & Morrissey, 1994). Concentrations of sTF and fVII proteins were determined using the following published extinction coefficients ( $\epsilon_{280}^{1\%}$ ) and  $M_r$  values: sTF, 14.8 and 26 800 (Rezaie et al., 1992); fVII, 13.9 and 50 000 (Bajaj et al., 1981).

**Enzymatic Activity Assays.** Amidolytic activities of wild-type fVIIa and fVIIa mutants were examined using the chromogenic substrates chromozym t-PA and CBS 34.47 in the absence or presence of sTF as previously described (Neuenschwander et al., 1993). Assays for measuring the activation of fX were as previously described (Neuenschwander & Morrissey, 1994) and assays of fIX activation essentially as described (Fiore et al., 1992) but using 20 mM Hepes-NaOH, pH 7.5, containing 100 mM NaCl, 0.002% NaN<sub>3</sub>, and 0.1% bovine serum albumin as the first-stage buffer. Reaction conditions for all assays are given in the figure legends. The clotting activity of various fVII proteins was determined in plastic tubes by a standard one-stage fVII clotting assay using immuno-depleted fVII-deficient plasma (Diagnostica Stago) and rabbit brain thromboplastin (Sigma). In short, 0.1 mL of fVII-deficient plasma was incubated at 37 °C with 0.1 mL of fVII zymogen at the indicated concentration and 0.1 mL of thromboplastin reagent for 30 s. Clotting was initiated by a 0.1-mL addition of prewarmed 33 mM CaCl<sub>2</sub>, and the time to clot formation was determined by visual inspection using the manual tilt-tube method.

**Enzymatic Inhibition Assays.** Reversible competitive inhibition of fVIIa proteins was examined using the chromogenic substrate CBS 34.47 (1 mM final) and either TFPI (0–830 nM final) or BPTI (0–231  $\mu$ M final) essentially as described (Neuenschwander et al., 1993) for measuring the amidolytic activity of fVIIa in the absence of inhibitors but with the following modifications. Enzymes (50 nM in the absence of sTF or 5 nM in the presence of 200 nM sTF) were preincubated for 5 min at ambient temperature in buffer containing 5 mM CaCl<sub>2</sub>. Substrate and inhibitor were then added *en masse*, and the activity of the mixture was monitored at 405 nm. The observed activity profile typically showed a nonlinear phase of substrate hydrolysis (presteady state) followed by a linear phase of hydrolysis representative of the steady state (Longstaff et al., 1990). Thus, enzymatic rates at equilibrium ( $v_s$ ) were obtained by linear regression of the latter portion of each curve. Under the conditions used, less than 10% substrate depletion was observed over the course of each assay. Values of the inhibition constant,  $K_i$ , can be estimated from plots of  $v_s$  versus inhibitor concentration ( $I$ ) by fitting with eq 1 (Cha, 1975). Equation 1 describes simple reversible competitive inhibition where  $v_o$  is the steady-state rate in the absence of inhibitor,  $S$  is the experimental substrate concentration, and  $K_m$  is the Michaelis

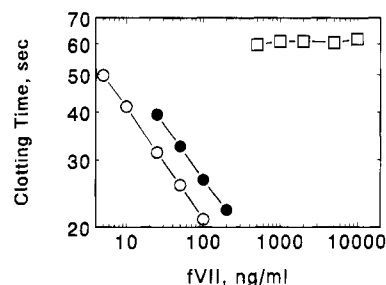


FIGURE 1: Single-stage clotting assay of wild-type fVII and fVII K<sup>192</sup> mutants. Immuno-depleted fVII-deficient plasma was supplemented with the indicated amount of zymogen fVII, and the clotting time was measured as described under Experimental Procedures: (○) recombinant wild-type fVII, (●) fVIIK192Q, and (□) fVIIK192E.

constant for substrate hydrolysis.

$$v_s = v_o \frac{K_i(1 + S/K_m)}{I + K_i(1 + S/K_m)} \quad (1)$$

In order to allow direct comparison of data obtained in the absence and presence of sTF, eq 1 was modified by factoring with  $100/v_o$  to yield percentages of initial activity. It should be noted that due to the inherent error in experimental determinations of  $v_o$ , the resulting right-side term ( $100v_o/v_o$ , the percentage of the initial activity in the absence of inhibitor) was allowed to float in regression procedures and not fixed at 100. All regression procedures were done using the Marquardt (1963) algorithm as described by Bevington (1969) on data analysis software written and provided by Dr. Jolyon Jesty of the State University of New York at Stony Brook.

## RESULTS

**Expression and Initial Characterization of fVIIK192Q and fVIIK192E.** Recombinant wild-type fVII, fVIIK192Q, and fVIIK192E were expressed in human 293 cells at levels of 0.5–0.8  $\mu$ g/mL, and isolated from roller-bottle cell supernatants as single-chain zymogens as described under Experimental Procedures. Analysis of the clotting activity of the purified zymogens showed that fVIIK192Q retained 44% clotting activity compared to recombinant wild-type fVII while fVIIK192E was completely ineffective in promoting clotting, even at levels of up to 10  $\mu$ g/mL (Figure 1). The low clotting activity of these proteins was not due to a defect in their ability to be proteolytically activated since both were rapidly and efficiently converted to the two-chain enzyme form by fXa (data not shown). In addition, the lack of activity of fVIIK192E was presumably not due to an inability to bind to TF since low levels of this mutant protein (>0.5  $\mu$ g/mL) effectively prolonged the fVII clotting time in normal human plasma, suggesting that it can compete with native fVIIa for binding to TF in the assay (not shown).

**Proteolytic Activation of fIX and fX.** The basis of the low clotting activities observed with fVIIK192Q and fVIIK192E was investigated by examining the ability of the purified two-chain forms of each mutant to activate fIX and fX in a purified system (Figure 2). Determination of initial rates of fIX activation (Figure 2A) indicated that fVIIaK192Q had 15% activity compared to wild-type fVIIa and fVIIaK192E had only 2% activity. Similar results were obtained for fX activation (Figure 2B) where fVIIaK192Q had 16% activity and fVIIaK192E had 0.4% activity. Examination of the

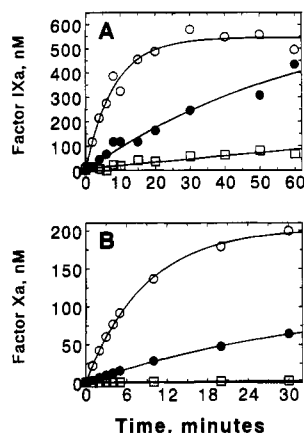


FIGURE 2: Activation of native macromolecular substrates by wild-type fVIIa and fVIIa K<sup>192</sup> mutants in a purified system. (A) Activation of fIX. Assays were performed as described under Experimental Procedures using 500 nM fIX, 1 nM TF/PCPS, 5 mM CaCl<sub>2</sub>, and 500 pM recombinant wild-type fVIIa (○), fVIIaK192Q (●), or fVIIaK192E (□). (B) Activation of fX. Assays were performed as described under Experimental Procedures using 250 nM fX, 1 nM TF/PCPS, 5 mM CaCl<sub>2</sub>, and 100 pM recombinant wild-type fVIIa (○), fVIIaK192Q (●), or fVIIaK192E (□).

Table 1: Specificity Constants for fX Activation<sup>a</sup>

enzyme	$k_{cat}/K_m$ (M <sup>-1</sup> s <sup>-1</sup> )	
	-TF/PCPS	+TF/PCPS (×10 <sup>-7</sup> )
wild-type fVIIa	1.3	7.0
fVIIaK192Q	0.31	2.2
fVIIaK192E	0.026	0.043

<sup>a</sup> Values of  $k_{cat}/K_m$  were determined directly by the method of Crompton and Waley (1986) as previously described (Neuenschwander & Morrissey, 1994).

specificity constants for fX activation by fVIIaK192Q and fVIIaK192E in the absence or presence of TF/PCPS (Table 1) revealed that TF/PCPS was able to enhance the specificity of both fVIIa mutants, despite the generally lower values obtained with the mutants compared to wild-type fVIIa. Thus, the low activities observed in clotting assays can be largely attributed to the reduced ability of fVIIaK192Q and fVIIaK192E to cleave fIX and fX.

**Amidolytic Activities of fVIIaK192Q and fVIIaK192E.** It is known that TF can enhance the activity of fVIIa toward small peptidyl chromogenic substrates (Ruf et al., 1991). Thus, the activities of fVIIaK192Q and fVIIaK192E were examined using the chromogenic substrates chromozym t-PA and CBS 34.47 which have been previously demonstrated to be nearly equivalent choice substrates for fVIIa (Neuenschwander et al., 1993). Kinetic parameters of substrate hydrolysis were determined for both fVIIa mutants in the absence and presence of sTF and compared to those obtained using wild-type fVIIa under the same conditions.

The results obtained using chromozym t-PA (Table 2) revealed that while fVIIaK192Q exhibited kinetic properties similar to wild-type fVIIa, fVIIaK192E was significantly less active in both the absence and presence of sTF. In the absence of sTF, the lower activity observed with fVIIaK192E was largely attributable to a reduced  $k_{cat}$  for substrate hydrolysis. However in the presence of sTF, both  $K_m$  and  $k_{cat}$  were adversely effected. More importantly, examination of the ratios of the kinetic parameters in the presence and

Table 2: Kinetic Parameters for Hydrolysis of Substrate Chromozym t-PA<sup>a</sup>

enzyme	$K_m$ (M × 10 <sup>3</sup> )	$k_{cat}$ (s <sup>-1</sup> )	$k_{cat}/K_m$ (M <sup>-1</sup> s <sup>-1</sup> × 10 <sup>-3</sup> )
-sTF			
wild-type fVIIa	3.7 ± 0.57	5.5 ± 0.64	1.5 ± 0.057
fVIIaK192Q	3.5 ± 0.92	4.1 ± 0.92	1.2 ± 0.042
fVIIaK192E	2.6 ± 0.49	0.52 ± 0.13	0.20 ± 0.011
+sTF			
wild-type fVIIa	0.62 ± 0.049	30 ± 4.2	49 ± 11
fVIIaK192Q	0.78 ± 0.064	25 ± 3.1	33 ± 6.7
fVIIaK192E	2.3 ± 0.021	7.9 ± 1.1	3.4 ± 0.49

<sup>a</sup> Determined as previously described (Neuenschwander & Morrissey, 1994). Values are means of at least two determinations ± standard deviations.

Table 3: Kinetic Parameters for Hydrolysis of Substrate CBS 34.47<sup>a</sup>

enzyme	$K_m$ (M × 10 <sup>3</sup> )	$k_{cat}$ (s <sup>-1</sup> )	$k_{cat}/K_m$ (M <sup>-1</sup> s <sup>-1</sup> × 10 <sup>-3</sup> )
-sTF			
wild-type fVIIa	3.0 ± 0.35	3.7 ± 0.042	1.3 ± 0.13
fVIIaK192Q	2.3 ± 0.071	5.0 ± 0.42	2.2 ± 0.14
fVIIaK192E	2.2 ± 0.85	4.7 ± 0.49	2.3 ± 0.64
+sTF			
wild-type fVIIa	0.94 ± 0.16	28 ± 1.9	30 ± 3.1
fVIIaK192Q	0.57 ± 0.089	26 ± 1.7	47 ± 6.8
fVIIaK192E	0.87 ± 0.24	26 ± 1.1	31 ± 8.5

<sup>a</sup> Determined as previously described (Neuenschwander & Morrissey, 1994). Values are means of at least two determinations ± standard deviations.

absence of sTF demonstrates that sTF binding enhanced the  $k_{cat}$  of fVIIaK192E ~3-fold more than that of wild-type fVIIa while having no effect on the  $K_m$ . Thus, the cofactor effect of sTF on fVIIa is altered in the fVIIaK192E mutant with respect to chromozym t-PA hydrolysis.

In contrast to the altered activities toward fIX, fX, and chromozym t-PA, fVIIaK192E was found to be essentially equivalent to wild-type fVIIa when its activity was examined toward the thrombin substrate CBS 34.47 (Table 3). This crucial finding demonstrates the catalytic competence of fVIIaK192E when confronted with the proper substrate. In this case, sTF was able to enhance the activity of fVIIaK192E comparably to that observed with wild-type fVIIa, albeit with still a slight reduction in the fold increase in specificity constant. Thus, these results show that changing the identity of residue 192 can alter the substrate specificity of fVIIa without destroying the intrinsic catalytic competence of the enzyme or the ability of sTF to effect an increase in activity.

**Inhibition by TFPI and BPTI.** Residue 192 has been shown to be important for determining the Kunitz-type inhibitor specificity of both thrombin and activated protein C (Rezaie & Esmon, 1993; Guinto et al., 1994). Thus, the effect of mutating K<sup>192</sup> in fVII was examined with respect to inhibition by either TFPI (the major inhibitor of fVIIa·TF in plasma) or BPTI in the absence and presence of sTF. Although TFPI is a slow tight-binding inhibitor of the fVIIa·TF complex in the presence of fXa (Broze et al., 1988), it has been demonstrated that TFPI can also inhibit the fVIIa·TF complex in the absence of fXa, albeit with a much higher  $K_i$  (Callander et al., 1992). Thus, inhibition by TFPI at various concentrations was examined in the absence of fXa to avert potential complications which may be caused

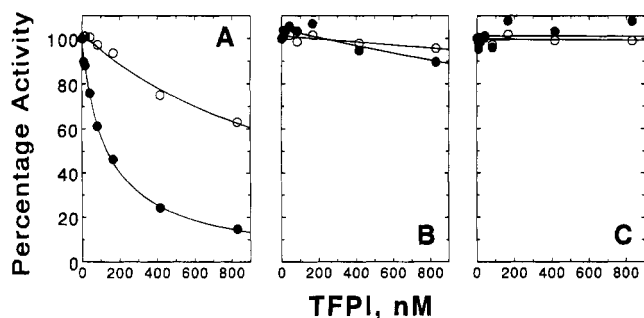


FIGURE 3: Inhibition of amidolytic activity of wild-type fVIIa and fVIIa K<sup>192</sup> mutants by TFPI. Inhibition assays were performed as described under Experimental Procedures in either the absence (○) or presence (●) of sTF. Data were fitted with eq 1 to obtain the indicated estimates of inhibition constants in the absence/presence of sTF. (A) Recombinant wild-type fVIIa:  $K_i \approx 980$  nM/ $K_i = 69$  nM. (B) fVIIaK192Q:  $K_i \sim 10$   $\mu$ M/ $K_i \sim 2$   $\mu$ M. (C) fVIIaK192E:  $K_i$  values for this mutant could not be estimated.

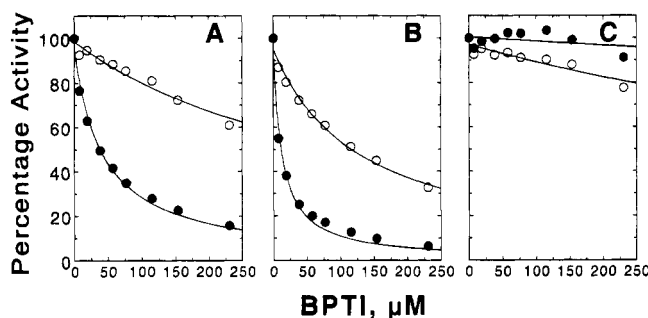


FIGURE 4: Inhibition of amidolytic activity of wild-type fVIIa and fVIIa K<sup>192</sup> mutants by BPTI. Inhibition assays were performed as described under Experimental Procedures in either the absence (○) or presence (●) of sTF. Data were fitted with eq 1 to obtain the indicated estimates of inhibition constants in the absence/presence of sTF. (A) Recombinant wild-type fVIIa:  $K_i \approx 324$   $\mu$ M/ $K_i = 20$   $\mu$ M. (B) fVIIaK192Q:  $K_i = 89$   $\mu$ M/ $K_i = 4.5$   $\mu$ M. (C) fVIIaK192E:  $K_i \sim 0.8$  mM/ $K_i \sim 2.4$  mM.

by fXa activity toward CBS 34.47 substrate in the amidolytic assay of fVIIa. The results are presented in Figure 3 and show that in the absence of sTF, TFPI was able to inhibit wild-type fVIIa with an estimated  $K_i$  of 980 nM. This inhibition was enhanced roughly 14-fold when fVIIa was bound to sTF:  $K_i = 69$  nM. In contrast, the activity of fVIIaK192Q was poorly inhibited by TFPI at levels of up to 800 nM, and fVIIaK192E was completely refractory to inhibition by these levels of TFPI. Nearly identical results were obtained when these mutants were bound to sTF.

A far different pattern emerged when these mutants were examined for inhibition by BPTI (Figure 4). Wild-type fVIIa was inhibited by BPTI with an estimated  $K_i$  of 324  $\mu$ M in the absence of sTF and a  $K_i$  of 20  $\mu$ M in the presence of sTF (16-fold enhancement by sTF). In both cases, inhibition of fVIIaK192Q was found to be enhanced compared to wild-type fVIIa:  $K_i = 89$   $\mu$ M in the absence of sTF, and  $K_i = 4.5$   $\mu$ M in the presence of sTF. This corresponds to a 20-fold enhancement of inhibition by sTF and a 4-fold enhancement overall for fVIIaK192Q compared to wild-type fVIIa. In sharp contrast to these results, fVIIaK192E was very poorly inhibited by BPTI in the absence of sTF (estimated  $K_i$  of  $\sim 0.8$  mM), and this mutant was made even more refractory to inhibition when bound to sTF (estimated  $K_i$  of  $\sim 2.4$  mM).

## DISCUSSION

In an attempt to decipher the local changes which occur in the catalytic center of fVIIa upon TF binding, we have extended to fVIIa the initial observations made by Esmon and colleagues with thrombin and activated protein C (Le Bonniec & Esmon, 1991; Rezaie & Esmon, 1993; Guinto et al., 1994) on the importance of amino acid residue 192 in defining substrate and inhibitor specificities of blood coagulation serine proteases. The role of K<sup>192</sup> in fVIIa seems to be more complex, however, due to the nature of the cofactor effect of TF on the amidolytic activity of fVIIa. Although the K<sup>192</sup>  $\rightarrow$  Q mutation in fVIIa resulted in a lowered clotting activity of this enzyme which can be attributed to a lowered specificity constant for activation of fIX and fX, no significant differences in specificity constants were observed toward the chromogenic substrates chromozym t-PA and CBS 34.47. In fact, no differences were observed between this mutant and wild-type fVIIa with all chromogenic substrates examined.<sup>3</sup> Thus, although contributing to specificity of fVIIa in proteolysis of macromolecular substrates, the presence of a basic amino acid at position 192 per se does not seem to be required in specificity of fVIIa for the tripeptidyl-amide substrates we examined. This could be due to the altered stereochemistry in the synthetic substrates compared to the natural substrates but may also be due to a lack of effect, either direct or indirect, of residue 192 on substrate P<sub>3</sub>–P<sub>1</sub> residues. This would not be completely surprising since residue 192 in thrombin has been suggested to be part of the P<sub>3</sub>' specificity pocket (Stubbs & Bode, 1995), in which case it would not be expected to affect specificity of amide substrates lacking P' residues. However, residue 192 has been shown to be in the vicinity of the P<sub>1</sub> residue in the F-P-R-chloromethyl ketone-inhibited thrombin structure (Bode et al., 1989). Thus, residue 192 in fVIIa may be able to alter amide substrate recognition despite the lack of substrate P' residues, through effects on the substrate P<sub>1</sub> residue.

In direct contrast to the K<sup>192</sup>  $\rightarrow$  Q mutation, reversing the charge at position 192 (the fVIIa K<sup>192</sup>  $\rightarrow$  E mutation) resulted in an active enzyme which was completely ineffective as a procoagulant and showed even more exquisite specificity than wild-type fVIIa with respect to hydrolysis of chromogenic substrates. Of all the chromogenic substrates examined,<sup>3</sup> the thrombin substrate CBS 34.47 was the only one found to be comparably hydrolyzed by this mutant and wild-type fVIIa. Although the precise molecular basis of this remains unknown, kinetic parameters obtained with one of the poorer substrates for this mutant (chromozym t-PA) revealed that the ability of TF to lower the  $K_m$  for this substrate is altered in the fVIIaK192E mutant while effects on  $k_{cat}$  are retained. Thus, mutation of K<sup>192</sup>  $\rightarrow$  E likely interferes with the required reorganization of the catalytic center of fVIIa which normally occurs upon TF binding and yields the proper enhancement in substrate recognition. This interference is apparently overcome or ignored with the CBS 34.47 substrate. Since this substrate does not contain an

<sup>3</sup> Other chromogenic substrates examined were chromozym PCa and L-BAPNA (Boehringer-Mannheim); spectrozyme TH and spectrozyme fXa (American Diagnostica); S2366, S2765, S2222, and S2238 (Chromogenix, formerly Kabi); and protein C substrate II (Calbiochem). Relative enzymatic activities were measured as previously described (Neuenschwander et al., 1993).

N-terminal blocking group, we hypothesized that the free N-terminus of this substrate may be interacting with fVIIa in a manner which precludes the inhibitory effect of the K<sup>192</sup> → E mutation. However, examination of the activity of fVIIaK192E toward a derivative of CBS 34.47 in which the N-terminus is blocked with MeSO<sub>2</sub> (SQ263)<sup>4</sup> revealed that fVIIaK192E was still capable of hydrolyzing the blocked substrate as well as the unblocked substrate (not shown). Thus, the differential activity of fVIIaK192E toward CBS 34.47 substrate versus chromozym t-PA and other substrates is due to either beneficial interactions involving E<sup>192</sup> and other sites on CBS 34.47 (directly or indirectly) or specific interactions between CBS 34.47 and regions in fVIIa which are independent of, and unaffected by, E<sup>192</sup>. If the latter is true, the fact that TF enhanced the activity of fVIIaK192E normally with respect to hydrolysis of CBS 34.47 implies that TF binding affects more than one site in the catalytic center of fVIIa and that these sites are used differentially by various small substrates to effect a decrease in *K<sub>m</sub>*.

Examination of the inhibition of both fVIIa K<sup>192</sup> mutants revealed that K<sup>192</sup> in fVIIa is essential for inhibition by TFPI. The finding that fVIIaK192Q is not inhibited by TFPI is consistent with preliminary studies by Wildgoose et al. (1993) but is nonetheless somewhat surprising in that a similar mutation in activated protein C (APC E192Q) makes this enzyme highly susceptible to inhibition by TFPI, evidently via the fXa-binding Kunitz domain of this inhibitor (Rezaie & Esmon, 1993). Thus, either the identity of residue 192 is not the only determinant involved in differential protease recognition of TFPI or the required positioning of residue 192 is not retained in fVIIa. In either case, fVIIa would seem to be different than fXa and activated protein C in that Q at position 192 is not sufficient for conferring fXa-like TFPI specificity.

In contrast to the results with TFPI, K<sup>192</sup> → Q mutation in fVIIa resulted in enhanced inhibition by BPTI, in both the absence and presence of sTF. This is similar to the results obtained by Rezaie and Esmon (1993) with APC E192Q. The disparate results obtained with fVIIaK192Q and TFPI versus fVIIaK192Q and BPTI are somewhat perplexing in that both BPTI and the fVIIa-binding Kunitz domain of TFPI contain identical P<sub>4</sub>-P<sub>1</sub>' residues (G-P-C-K~A). Thus, the differences observed in inhibition of fVIIaK192Q by these two Kunitz-type inhibitors may lay outside these regions or in subtle differences in the conformation of these otherwise identical inhibitor sequences.

Mutation of K<sup>192</sup> → E in fVIIa made fVIIa more resistant to inhibition by BPTI, consistent with the notion that E at position 192 in the blood coagulation serine proteases confers resistance to Kunitz-type inhibitors (Rezaie & Esmon, 1993; Guinto et al., 1994). The observation that fVIIaK192E became even more refractory to BPTI inhibition when bound to sTF directly contrasts with the observed increase in inhibition of wild-type fVIIa and fVIIaK192Q in the presence of sTF. This result suggests that sTF binding to fVIIaK192E may have affected movement of E<sup>192</sup>. In this regard, it is interesting to note the flexibility of residue 192 in thrombin (Brandstetter et al., 1992). It should be noted, however, that even if this is the case with fVIIaK192E, movement of E<sup>192</sup>

can not necessarily be extrapolated to residue 192 in fVIIaK192Q or wild-type fVIIa. Nonetheless, this result is strongly suggestive and spurs the need for further investigation.

In conclusion, we find that residue 192 in fVIIa plays a role in the substrate and inhibitor specificity of this enzyme, as previously observed with other blood coagulation proteases. However, the precise role of K<sup>192</sup> in defining this specificity appears to be different in fVIIa than with thrombin and activated protein C. The potential ability of TF to directly or indirectly reorient K<sup>192</sup> in the active site of fVIIa upon binding may be at least in part responsible for the differences observed and may also contribute in part to the cofactor effect of TF in enhancing the activity of fVIIa.

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## REFERENCES

- Bach, R., Gentry, R., & Nemerson, Y. (1986) *Biochemistry* 25, 4007–4020.
- Bajaj, S. P., Rapaport, S. I., & Brown, S. F. (1981) *J. Biol. Chem.* 256, 253–259.
- Bevington, P. R. (1969) in *Data Reduction and Error Analysis for the Physical Sciences*, pp 204–246, McGraw-Hill, New York.
- Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. R., & Hofsteenge, J. (1989) *EMBO J.* 8, 3467–3475.
- Bom, V. J., & Bertina, R. M. (1990) *Biochem. J.* 265, 327–336.
- Bom, V. J., Reinalda-Poot, J. H., Cupers, R., & Bertina, R. M. (1990) *Thromb. Haemost.* 63, 224–230.
- Brandstetter, H., Turk, D., Hoeffken, H. W., Grosse, D., Stürzebecher, J., Martin, P. D., Edwards, B. F. P., & Bode, W. (1992) *J. Mol. Biol.* 226, 1085–1099.
- Broze, G. J., Jr., Leykam, J. E., Schwartz, B. D., & Miletich, J. P. (1985) *J. Biol. Chem.* 260, 10917–10920.
- Broze, G. J., Jr., Warren, L. A., Novotny, W. F., Higuchi, D. A., Girard, J. J., & Miletich, J. P. (1988) *Blood* 71, 335–343.
- Butenas, S., Ribarik, N., & Mann, K. G. (1993) *Biochemistry* 32, 6531–6538.
- Butenas, S., Lawson, J. H., Kalafatis, M., & Mann, K. G. (1994) *Biochemistry* 33, 3449–3456.
- Callander, N. S., Rao, L. V. M., Nordfang, O., Sandset, P. M., Warn-Cramer, B., & Rapaport, S. I. (1992) *J. Biol. Chem.* 267, 876–882.
- Carson, S. D., & Brozna, J. P. (1993) *Blood Coagulation Fibrinolysis* 4, 281–292.
- Cha, S. (1975) *Biochem. Pharmacol.* 24, 2177–2185.
- Crompton, I. E., & Waley, S. G. (1986) *Biochem. J.* 239, 221–224.
- Davie, E. W., & Ratnoff, O. D. (1964) *Science* 145, 1310–1312.
- Fiore, M. M., Neuenschwander, P. F., & Morrissey, J. H. (1992) *Blood* 80, 3127–3134.
- Guinto, E. R., Ye, J., Le, B. B. F., & Esmon, C. T. (1994) *J. Biol. Chem.* 269, 18395–18400.
- Higashi, S., Nishimura, H., Fujii, S., Takada, K., & Iwanaga, S. (1992) *J. Biol. Chem.* 267, 17990–17996.
- Horton, R. M., Cai, Z., Ho, S. N., & Please, L. R. (1990) *BioTechniques* 8, 528–535.

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- Le Bonniec, B. F., & Esmon, C. T. (1991) *Proc. Natl. Acad. Sci. U.S.A.* 88, 7371–7375.
- Le Bonniec, B. F., Guinto, E. R., & Esmon, C. T. (1992) *J. Biol. Chem.* 267, 6970–6976.
- Longstaff, C., Campbell, A. F., & Fersht, A. R. (1990) *Biochemistry* 29, 7339–7347.
- Macfarlane, R. G. (1964) *Nature* 202, 498–499.
- Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math.* 11, 431–441.
- Martin, D. M. A., O'Brien, D. P., Tuddenham, E. G. D., & Byfield, P. G. H. (1993) *Biochemistry* 32, 13949–13955.
- Mimms, L. T., Zampighi, G., Nozaki, Y., Tanford, C., & Reynolds, J. A. (1981) *Biochemistry* 20, 833–840.
- Morrissey, J. H., Revak, D., Tejada, P., Fair, D. S., & Edgington, T. S. (1988) *Thromb. Res.* 50, 481–493.
- Neuenschwander, P. F., & Morrissey, J. H. (1992) *J. Biol. Chem.* 267, 14477–14482.
- Neuenschwander, P. F., & Morrissey, J. H. (1994) *J. Biol. Chem.* 269, 8007–8013 [correction in (1994) *J. Biol. Chem.* 269, 16983].
- Neuenschwander, P. F., Branam, D. E., & Morrissey, J. H. (1993) *Thromb. Haemost.* 70, 970–977.
- Rezaie, A. R., & Esmon, C. T. (1993) *J. Biol. Chem.* 268, 19943–19948.
- Rezaie, A. R., Fiore, M. M., Neuenschwander, P. F., Esmon, C. T., & Morrissey, J. H. (1992) *Protein Expression Purif.* 3, 453–460.
- Ruf, W., Rehemtulla, A., Morrissey, J. H., & Edgington, T. S. (1991) *J. Biol. Chem.* 266, 2158–2166 [correction in (1991) *J. Biol. Chem.* 266, 16256].
- Ruf, W., Miles, D. J., Rehemtulla, A., & Edgington, T. S. (1992) *J. Biol. Chem.* 267, 6375–6381.
- Smalley, D. M., & Preusch, P. C. (1988) *Anal. Biochem.* 172, 241–247.
- Stubbs, M. T., & Bode, W. (1993) *Thromb. Res.* 69, 1–58.
- Stubbs, M. T., & Bode, W. (1995) *Trends Biochem. Sci.* 20, 23–28.
- Thompson, A. R. (1977) *J. Clin. Invest.* 59, 900–910.
- Wildgoose, P., Birktoft, J. J., Foster, D., Worsaae, H., Christensen, P. R., & Petersen, L. C. (1993) *Thromb. Haemost.* 69, 957 (abstract).
- Zur, M., & Nemerson, Y. (1980) *J. Biol. Chem.* 255, 5703–5707.

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