Augmented intrinsic activity of Factor VIIa by replacement of residues 305, 314, 337 and 374: evidence of two unique mutational mechanisms of activity enhancement

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Coagulation Factor VIIa (FVIIa) lacks the ability to spontaneously complete the conversion to a fully active enzyme after specific cleavage of an internal peptide bond (Arg^{152}–Ile^{153}) in the zymogen. Recently, several variants of FVIIa with enhanced intrinsic activity have been constructed. The *in vitro* characterization of these variants has shed light on molecular determinants that put restrictions on FVIIa in favour of a zymogen-like conformation and warrants continued efforts. Here we describe a new FVIIa variant with high intrinsic activity containing the mutations $Leu^{305} \rightarrow Val$, $Ser^{314} \rightarrow Glu$, $Lys^{337} \rightarrow Ala$, and $Phe^{374} \rightarrow Tyr$. The variant, called $FVIIa_{VEAY}$, processes a tripeptidyl substrate very efficiently because of an unprecedented, 5.5-fold lowering of the K_m value. Together with a 4-fold higher substrate turnover rate this gives the variant a catalytic efficiency 22 times that of wild-type FVIIa, which is reflected in a considerably enhanced susceptibility to inhibition by antithrombin and other inhibitors. For instance, the affinity of $FVIIa_{VEAY}$ for the S1 probe and inhibitor *p*-aminobenzamidine is represented by an 8-fold lower K_i value compared with that of FVIIa. Activation of Factor X in solution occurs about 10 times faster with $FVIIa_{VEAY}$ than with FVIIa, due virtually exclusively to an increased k_{cat} value. The high activity of $FVIIa_{VEAY}$ is not accompanied by an increased burial of the N-terminus of the protease domain. A comparison of the kinetic parameters and molecular properties of $FVIIa_{VEAY}$ with those of the previously described mutant V158D/E296V/M298Q-FVIIa ($FVIIa_{IIa}$), and the locations of the substitutions in the two variants, reveals what appear to be two profoundly different structural mechanisms dictating improvements in enzymic performance.

Key words: Factor VIIa mutant, intrinsic activity, superactivity, zymogenicity.

INTRODUCTION

The blood coagulation cascade is a crucial component of the system designed to prevent excessive blood loss following vascular injury. Coagulation is triggered when blood [i.e. zymogen Factor VII (FVII) and activated Factor VII (FVIIa)] contacts the newly exposed subendothelial tissue factor (TF) [1]. The ensuing Factor Xa (FXa)-mediated feedback activation of FVII [2,3] and autoactivation by FVIIa · TF of FVII bound to neighbouring TF molecules [4–6] boost the initiation step. The FVIIa · TF complex generates Factor IXa (FIXa) and FXa, which, via a platelet-dependent propagation step, leads to fibrin deposition [7]. The inherent zymogen-like behaviour of free FVIIa assures a timely appearance of this procoagulant defence mechanism.

The structures of FVII [8], free FVIIa [9–12] and TF-bound FVIIa [13,14] have been solved during the past few years. Based on these structures, regulatory mechanisms behind the zymogen-like property of free FVIIa and its stimulation by TF have been proposed. These hypotheses try to account for the unfavourable equilibrium resulting in an incomplete spontaneous conversion of FVII to an active enzyme upon cleavage of the Arg^{152} (15)-Ile^{153} (16) peptide bond (where chymotrypsinogen numbering is shown in parentheses), as well as accommodating the stimulatory effect of TF association. Certain residues in FVIIa interacting with TF [13], especially Met^{306} (164) [15,16], have been directly shown to mediate the cofactor-induced allosteric stimulation. However, all the determinants of a zymogen-like conformation of free FVIIa, i.e. the residues responsible for favouring an enzymically latent form, have most certainly not been pinpointed and, consequently, the full set of conformational changes representing the zymogen-like to enzyme transition remains largely elusive. The successful generation of FVIIa variants with increased intrinsic activity, using site-directed mutagenesis [17–21] and loop grafting [22,23], has shed some light on this molecular mystery.

At least three regions of the FVIIa protease domain are functionally important, i.e. the cofactor binding site, the catalytic site region, and the macromolecular substrate exosite (binding site distant from the active site spanning the region from the activation pocket to the calcium site). Allosteric changes are transmitted between these sites (reviewed in [24]), all communication going through the activation domain. The 305–321 (163–170I) helix-loop region is located between the cofactor binding site and the active site, and is therefore hypothesized to be important for the allostery and zymogenicity of FVIIa. In addition, the 313–321 (170A–170I) loop forms part of the S3 subsite. We constructed a new FVIIa variant (where L305V etc. represents the substitution of leucine by valine etc.), L305V/S314E/K337A/F374Y-FVIIa ($FVIIa_{VEAY}$), based on the idea that to properly orient and stabilize

Abbreviations used: Arg^{152} (15) etc., specific amino acid residues with chymotrypsinogen numbering in parentheses; AT, antithrombin; FFR-ck, D-Phe-Phe-Arg-chloromethyl ketone; FIX, coagulation Factor IX; FIXa, activated FIX; FVII, coagulation Factor VII; FVIIa, activated FVII; L305V etc., the substitution of leucine by valine etc.; $FVIIa_{VEAY}$, L305V/S314E/K337A/F374Y-FVIIa; $FVIIa_{IIa}$, V158D/E296V/M298Q-FVIIa; FX, coagulation Factor X; FXa, activated FX; PABA, *p*-aminobenzamidine; SPR, surface plasmon resonance; TF, tissue factor; sTF, soluble TF (residues 1–219).

Chymotrypsinogen numbering (in parentheses): Arg^{152} (15); Ile^{153} (16); Val^{154} (17); Val^{158} (21); Glu^{296} (154); Met^{298} (156); Leu^{305} (163); Met^{306} (164); Thr^{307} (165); Cys^{310} (168); Gln^{312} (170); Gln^{313} (170A); Ser^{314} (170B); Pro^{321} (170I); Cys^{329} (182); Lys^{337} (188); Asp^{343} (194); His^{373} (224); Phe^{374} (225).

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	158	296	298	305	314	337	374
Human FVIIa	--	V	---	E	M	---	L	---	S	---	K	---	F
Bovine FVIIa	--	V	---	K	M	---	L	---	S	---	K	---	F
Mouse FVIIa	--	V	---	E	M	---	L	---	A	---	K	---	I
Rabbit FVIIa	--	V	---	E	M	---	L	---	S	---	K	---	V
Human thrombin	--	D	---	V	Q	---	V	-------	G	---	Y		
Human Factor IXa	--	D	---	V	Q	---	V	-------	R	---	Y		
Human Factor Xa	--	E	---	R	K	---	V	-------	E	---	Y		
Human act. protein C	--	M	---	V	N	---	V	-------	Q	---	Y		

Figure 1 Sequence alignment of FVIIa and other human vitamin K-dependent enzymes emphasizing residues found to regulate the intrinsic activity of FVIIa

The numbers refer to the positions in FVIIa, and gaps in the sequences are indicated by three or more horizontal lines.

the helix-loop region requires linking it more tightly to the body of the protease domain, i.e. to strengthen the hydrophobic and hydrogen bond interactions with the remaining part of the protease domain. A sequence alignment, highlighting positions in FVIIa apparently involved in the zymogenicity regulation as well as the positions mutated in $FVIIa_{VEAY}$, is shown in Figure 1. A more rigid (shorter) loop has been proven to increase the intrinsic activity of FVIIa [22,23], but we believed that our approach would also be successful in maintaining the parent substrate specificity. In this study, we provide a detailed characterization of $FVIIa_{VEAY}$ and compare its properties with those of the previously described V158D/E296V/M298Q-FVIIa ($FVIIa_{IIa}$) [19]. Both mutants possess dramatically improved intrinsic enzymic activity, but their different profiles strongly suggest that the mechanisms responsible for the enhancements are unique.

EXPERIMENTAL

Reagents and standard methods

Recombinant wild-type human FVIIa and soluble TF (sTF) were prepared as described previously [25,26]. The concentrations of FVIIa and sTF were determined by ELISA and by absorbance measurement at 280 nm (using an absorption coefficient of 1.5 for a 1 mg/ml solution of sTF), respectively. Factor X (FX) and FXa were from Enzyme Research Laboratories (South Bend, IN, U.S.A.). Antithrombin (AT) was from Haematologic Technologies (Essex Junction, VT, U.S.A.), standard heparin was from Leo Pharmaceutical (Ballerup, Denmark), *p*-aminobenzamidine (PABA) was from Sigma, D-Phe-Phe-Arg-chloromethyl ketone (FFR-ck) was from Calbiochem (La Jolla, CA, U.S.A.), potassium cyanate and zinc chloride were from Merck, and the chromogenic substrates S-2288 (D-Ile-Pro-Arg-*p*-nitroanilide), S-2238 (D-Phe-Pip-Arg-*p*-nitroanilide; Pip is pipecolic acid), S-2366 (pyroGlu-Pro-Arg-*p*-nitroanilide), and S-2765 (benzyloxycarbonyl-D-Arg-Gly-Arg-*p*-nitroanilide) were from Chromogenix (Mölndal, Sweden). SDS/PAGE was run on 4–12 % NuPAGE Novex Bis-Tris gels (Invitrogen, Carlsbad, CA, U.S.A.).

Mutagenesis and protein expression and isolation

Four different plasmids were employed as templates for site-directed mutagenesis using the QuikChange kit (Stratagene, La Jolla, CA, U.S.A.); the wild-type human FVII expression plasmid pLN174 [27] and plasmids containing the cDNA for L305V-FVIIa [18], K337A-FVIIa [19], and L305V/K337A-FVIIa [19] respectively. The following primers were used to introduce the S314E and F374Y mutations (only sense primers given) with base substitutions in italics and the altered codons underlined: S314E, 5′-GCCTGCAGCAG*GAA*ACGGAAGGTGGGAGACT-CC-3′; F374Y, 5′-CGCAACCGTGGGCCAC*TAT*GGGGTGT-ACACC-3′. The construction of $FVIIa_{IIa}$ has been described previously [19]. Plasmids were prepared using the plasmid DNA midi kit and QIAfilter (Qiagen, Valencia, CA, U.S.A.). The entire cDNA encoding $FVIIa_{VEAY}$ was verified by sequencing to confirm that the primary structure only contained the desired four mutations. Baby hamster kidney cell transfection and selection, mutant expression, purification, concentration, and autoactivation were carried out as described previously [16,19].

Surface plasmon resonance (SPR) measurements

Immobilization of sTF (780 resonance units) on a CM5 sensor chip in a Biacore 1000 instrument (Biacore AB, Uppsala, Sweden) was carried out using amine coupling chemistry as described previously [28]. The FVIIa variants were injected at concentrations of 40 nM and 100 nM in 50 mM Hepes, pH 7.4, containing 0.1 M NaCl, 5 mM $CaCl_2$ and 0.02 % Tween 80, at a flow rate of 5 μl/min. The binding and dissociation phases lasted for 7 min and 10 min, respectively, and the temperature was 25 °C. The sTF-coated surface was regenerated between runs with a pulse of 50 mM EDTA, pH 7.5. The kinetic parameters (k_{on} and k_{off}) were calculated using Biaevaluation 2.1 supplied by the manufacturer.

FVIIa activity assays

All assays were run in 50 mM Hepes, pH 7.4, containing 0.1 M NaCl, 5 mM $CaCl_2$, and 1 mg/ml BSA (assay buffer) and monitored at 405 nm in a kinetic microplate reader (SpectraMax 340; Molecular Devices Corp., Sunnyvale, CA, U.S.A.). Initially, the amidolytic activity of the free mutants at a fixed concentration of substrate (1 mM S-2288) was measured using 100 nM wild-type or mutant FVIIa. This assay was also performed in the absence of Ca^{2+} (5 mM EDTA instead of $CaCl_2$ in the buffer). The k_{cat} and K_m values for S-2288 hydrolysis were determined for the FVIIa variants alone (using 100 nM wild-type FVIIa, 10 nM $FVIIa_{VEAY}$, and 25 nM $FVIIa_{IIa}$) and for the FVIIa variants (10 nM) in the presence of 200 nM sTF at substrate concentrations ranging from 0.2–10 mM. The substrate specificity of $FVIIa_{VEAY}$ was studied by incubating 10 nM mutant with S-2288, S-2765, S-2238, and S-2366, all at a concentration of 1 mM, and comparing the rate of hydrolysis with that catalysed by wild-type FVIIa. For comparison, the rates of hydrolysis catalysed by 3 nM thrombin were measured in the same manner.

The kinetic parameters of FX activation were determined by incubating 50 nM FVIIa, 5 nM $FVIIa_{IIa}$, 10 nM $FVIIa_{VEAY}$, and 5 nM FVIIa variant plus 200 nM sTF with 0.1–6.4 μM FX in 50 μl of assay buffer for 20 min at 22 °C. Assay buffer (150 μl) containing 20 mM EDTA (no $CaCl_2$) was added to virtually stop FX activation, followed by the addition of 50 μl of 2.5 mM S-2765. The rate of S-2765 hydrolysis was monitored for 5 min, during which the absorbance increase was linear. The background amidolytic activity of FVIIa or FVIIa–sTF and of the FX preparation used were subtracted, and the amount of FXa generated was estimated from a standard curve (0.5–10 nM).

Inhibition of FVIIa by PABA, AT and Zn^{2+}

The K_i for inhibition by PABA was determined. FVIIa (100 nM), $FVIIa_{VEAY}$ (10 nM) and $FVIIa_{IIa}$ (25 nM) alone, and

FVIIa variant (10 nM) together with sTF (200 nM) were incubated with various concentrations of PABA (10–1280 μM) in assay buffer for 5 min. The substrate S-2288 (final concentration 1 mM) was then added to measure the residual activity. Because of competitive binding of PABA and S-2288, the apparent K_i values were calculated from the PABA IC_{50}, taking into account the concentration of S-2288 ([S]) and the K_m for this substrate using the equation $K_i = IC_{50}/(1 + [S]/K_m)$. The inhibitory effect of 100 μg/ml AT in the presence of 1 unit/ml heparin was studied, as described previously [19], using the same enzyme concentrations. The effect of 0.1 mM $ZnCl_2$ on the amidolytic activity of the free forms of the three FVIIa variants was analysed in assay buffer at the same enzyme concentrations.

Carbamylation of FVIIa

$FVIIa_{VEAY}$ (100 nM), $FVIIa_{IIa}$ (250 nM), wild-type FVIIa (1 μM) and 100 nM FVIIa variant in the presence of 2 μM sTF were incubated with 0.2 M KOCN in assay buffer without albumin. At different time points, samples were withdrawn and diluted 10-fold in assay buffer containing 1 mM S-2288 to measure the residual amidolytic activity [19].

RESULTS

Enzymic activity of $FVIIa_{VEAY}$

When the amidolytic activity of $FVIIa_{VEAY}$ was measured at a fixed concentration of peptidyl substrate (1 mM S-2288), an approximately 20-fold enhancement, compared with wild-type FVIIa, was observed. When the mutations L305V, S314E, K337A, and F374Y were introduced individually into FVIIa, the three first gave an activity that was 2.5–4-fold higher than that of FVIIa, whereas F374Y-FVIIa exhibited unaltered activity (results not shown). In addition, a number of different residues have been tried at these positions. For instance, in position 305, Leu has also been replaced by Ala, Thr, and Ile. The first two substitutions resulted in decreased activity, while Ile enhanced the activity of FVIIa to approximately the same extent as Val (results not shown). In position 314, Met was introduced with the intention of improving local hydrophobic interactions, with a reduction in activity as a consequence. The four substitutions in $FVIIa_{VEAY}$ were combined in a number of double and triple mutants, but in no case did we obtain an activity close to that of $FVIIa_{VEAY}$ (results not shown). This strongly suggests that all four mutations are required for the most dramatic increase in activity to occur and that the nature of the introduced side chain is important. Some of the mutants were part of earlier studies (L305V-FVIIa, K337A-FVIIa, and L305V/K337A-FVIIa) and the results obtained with these variants agreed with previous findings [18,19]. We also found that F374Y-FVIIa, in complex with sTF, only possessed approx. 45 % of the amidolytic activity of FVIIa · sTF and approx. 50 % of the specific clotting activity of FVIIa in a TF-initiated assay (results not shown). This is in accordance with results previously obtained with F374P-FVIIa [18,29], supporting the pivotal role of residue 374 (225) in the communication between the region in FVIIa interacting with TF and the active site.

$FVIIa_{VEAY}$ was subjected to an extensive characterization and its properties were compared with those of an FVIIa variant with entirely different substitutions but a similar large increase in intrinsic activity, $FVIIa_{IIa}$ [19]. The two variants exhibit different Ca^{2+} requirements for the expression of amidolytic activity. $FVIIa_{VEAY}$, and all the above mentioned subvariants, lost > 90 % of the amidolytic activity when Ca^{2+} was omitted from the assay buffer, whereas $FVIIa_{IIa}$ retained approximately two-thirds of its activity [19]. Moreover, the inhibitory effect of Zn^{2+} was less pronounced with $FVIIa_{IIa}$ than with $FVIIa_{VEAY}$. The loss of activity caused by the presence of 0.1 mM Zn^{2+} was 55 % and 85 %, respectively. Wild-type FVIIa lost 90 % of its activity under these conditions. The finding that a reduced requirement for Ca^{2+} seems to go hand-in-hand with a reduced sensitivity to Zn^{2+} supports our earlier observation that there is at least partial competition between Ca^{2+} and Zn^{2+} in binding to FVIIa [30]. Like $FVIIa_{IIa}$, $FVIIa_{VEAY}$ appears to retain its substrate specificity, as judged by the similar relative increase in amidolytic activity using four different chromogenic substrates. The rate of hydrolysis catalysed by $FVIIa_{VEAY}$ was increased 22-fold (S-2288), 27-fold (S-2366), and 34-fold (S-2238 and S-2765), respectively, compared with that of FVIIa. Because two of the mutations in $FVIIa_{VEAY}$ were substitutions of the residue occupying the corresponding position in thrombin for that in FVIIa, it was of interest to check whether $FVIIa_{VEAY}$ had acquired a more thrombin-like substrate specificity. The rate of hydrolysis by thrombin was 16–178 times faster than that by FVIIa depending on which of the four substrates we used. This demonstrates that $FVIIa_{VEAY}$ retains the specificity of wild-type FVIIa and does not resemble thrombin, an expected finding considering that the substitutions are not located in the vicinity of the active site. The kinetic parameters of S-2288 hydrolysis for the FVIIa variants are shown in Table 1. $FVIIa_{VEAY}$ has a 4-fold higher V_{max} value than FVIIa, indistinguishable from that of $FVIIa_{IIa}$. Saturation with sTF increases V_{max} for the two mutants by a factor of 2.2 to 3, whereas FVIIa displays a 9.7-fold enhancement, resulting in similar V_{max} values for all three FVIIa forms when bound to sTF (Table 1). The affinity for S-2288 is much improved in $FVIIa_{VEAY}$ as manifested in a 5.5-fold lower K_m value. The reduction in K_m is more moderate for $FVIIa_{IIa}$. After binding to sTF, both FVIIa and $FVIIa_{IIa}$ exhibit higher (and indistinguishable) affinity for the substrate. The K_m value of $FVIIa_{VEAY}$ is decreased only 2-fold on complex formation with sTF, but enough to make it persistently lower than that of FVIIa · sTF.

Table 1 Chromogenic substrate hydrolysis and inhibition by PABA

Values are means ± S.D. ($n = 3$).

Enzyme	K_m (mM)	V_{max} (m*A*/min)*	Relative catalytic efficiency†	K_i (μM)
FVIIa	10.4 ± 0.9	7.7 ± 1.1	1	1410 ± 190
$FVIIa_{VEAY}$	1.9 ± 0.2	31.1 ± 2.0	22	177 ± 25
$FVIIa_{IIa}$	6.7 ± 0.2	31.8 ± 1.9	6.4	226 ± 34
FVIIa · sTF	1.6 ± 0.1	74.7 ± 4.0	63	77 ± 12
$FVIIa_{VEAY}$ · sTF	0.85 ± 0.06	92.9 ± 11.4	147	38 ± 5
$FVIIa_{IIa}$ · sTF	1.7 ± 0.1	71.5 ± 3.3	57	50 ± 5

* Values normalized to an enzyme concentration of 10 nM.
† Efficiency compared with that of free wild-type FVIIa (which is given the arbitrary value of 1).

The ability of $FVIIa_{VEAY}$ to activate FX is considerably improved, albeit not to the same extent as that of $FVIIa_{IIa}$ (Table 2). The catalytic turnover rate is increased 8 times compared with that of FVIIa. The proteolytic activity of FVIIa and $FVIIa_{VEAY}$ are stimulated by sTF to different extents, the $FVIIa_{VEAY}$ · sTF and FVIIa · sTF complexes being indistinguishable as catalysts of FX activation. $FVIIa_{IIa}$ is at least 3 to 4 times more active than $FVIIa_{VEAY}$ in both the free and TF-bound forms. The effects of the mutations in $FVIIa_{VEAY}$ and $FVIIa_{IIa}$ on the K_m for FX are small compared with the effects on k_{cat}.

Inhibitor susceptibility of $FVIIa_{VEAY}$

$FVIIa_{VEAY}$ (and $FVIIa_{IIa}$) displayed an increased affinity for the S1 subsite-recognizing inhibitor PABA. We obtained a K_i value of

Table 2 Kinetic parameters of FX activation

Values are means ± S.D. ($n = 4$).

Enzyme	K_m (μM)	$10^{-3} \times k_{cat}$ (s^{-1})	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
FVIIa	4.4 ± 0.9	0.15 ± 0.02	34
$FVIIa_{VEAY}$	2.7 ± 0.4	1.2 ± 0.1	440
$FVIIa_{IIa}$	1.8 ± 0.2	5.3 ± 0.3	2900
FVIIa · sTF	3.0 ± 0.2	7.7 ± 0.2	2600
$FVIIa_{VEAY}$ · sTF	2.1 ± 0.3	7.0 ± 0.4	3300
$FVIIa_{IIa}$ · sTF	2.1 ± 0.2	24 ± 2	11400

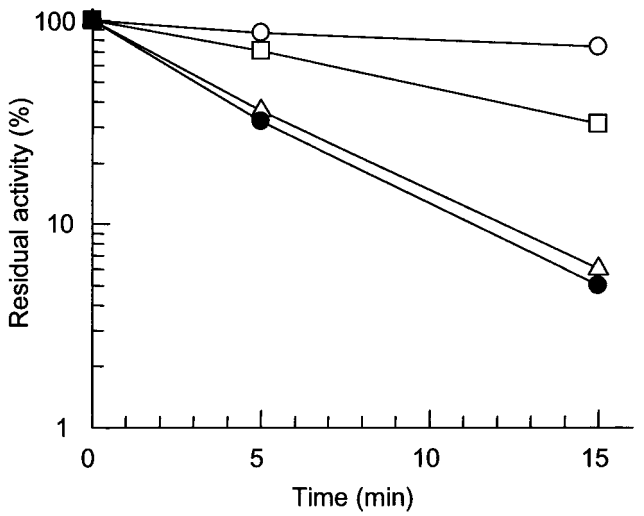

Figure 2 Inhibition of FVIIa variants by AT/heparin

The residual activity of FVIIa (○), $FVIIa_{VEAY}$ (△), $FVIIa_{IIa}$ (□), and FVIIa · sTF (●) after the indicated incubation times with AT is shown. The curves show the results of a representative experiment ($n = 3$).

approx. 200 μM, about 7 times lower than that of FVIIa (Table 1). After complex formation with sTF, the affinity for PABA increased further, with a K_i value significantly below 100 μM for $FVIIa_{VEAY}$ (and for $FVIIa_{IIa}$ and wild-type FVIIa). A more mature active site could also be inferred from inhibition studies using AT in the presence of heparin (Figure 2). $FVIIa_{VEAY}$ was inhibited by AT at a rate virtually identical to that of FVIIa in complex with sTF. FVIIa was inhibited very slowly, and the activity of $FVIIa_{IIa}$ disappeared at an intermediate rate, in agreement with earlier findings [19]. Supportive results were obtained in an experiment using 1 μM FFR-ck as the inhibitor, in which $FVIIa_{VEAY}$ and FVIIa · sTF retained about 10 % of the amidolytic activity after a 1 min incubation and $FVIIa_{IIa}$ and FVIIa retained 20 % and 85 % of the activity respectively (results not shown). To facilitate comparison with previously described mutants [18], L305V-FVIIa retained 45 % of its activity in this assay.

Accessibility of Ile^{153} in $FVIIa_{VEAY}$

The rate of carbamylation of the N-terminus of $FVIIa_{VEAY}$ was found to be similar to that seen with FVIIa. In only a few cases, the most striking being $FVIIa_{IIa}$ [19] and a FVIIa variant with a different substitution pattern in the same positions [21], have constructed superactive FVIIa variants been shown to possess a significantly more buried N-terminus of the protease domain than does wild-type FVIIa. This strongly indicates that a dramatic increase in intrinsic activity can be achieved both without ($FVIIa_{VEAY}$) and with ($FVIIa_{IIa}$) an accompanying burial of the amino group of Ile^{153} (16).

Table 3 Binding of FVIIa variants to sTF

FVIIa variant	$10^5 \times k_{on}$ ($M^{-1} \cdot s^{-1}$)	$10^{-3} \times k_{off}$ (s^{-1})	K_d (nM)
FVIIa	3.3 ± 0.4	1.6 ± 0.3	4.8
$FVIIa_{VEAY}$	3.8 ± 0.2	2.8 ± 0.1	7.4
$FVIIa_{IIa}$	3.6 ± 0.7	0.65 ± 0.12	1.8
FFR-FVIIa	4.5 ± 0.2	0.42 ± 0.09	0.9
FFR-$FVIIa_{VEAY}$	4.6 ± 0.4	2.1 ± 0.1	4.6
FFR-$FVIIa_{IIa}$	4.8 ± 0.3	0.42 ± 0.05	0.9

$FVIIa_{VEAY}$ binding to sTF

SPR was employed to assess the effects of the mutations in $FVIIa_{VEAY}$ on sTF binding, and to ensure that the functional characterization of $FVIIa_{VEAY}$ in the presence of the cofactor was performed at appropriate protein concentrations. The affinity of $FVIIa_{VEAY}$ for sTF was found to be slightly lower than that of wild-type FVIIa (Table 3). In contrast, $FVIIa_{IIa}$ bound sTF with increased affinity as shown previously [19]. The kinetic parameters of wild-type FVIIa binding to sTF and the resulting binding constant were in good agreement with those obtained in numerous earlier studies of the interaction between FVIIa and TF apoprotein by SPR [16,21,27]. Differences in binding kinetics were found exclusively in the dissociation phase, with $FVIIa_{VEAY}$ leaving sTF faster and $FVIIa_{IIa}$ slower respectively than does FVIIa. The mutation responsible for the smaller binding energy involved in the $FVIIa_{VEAY}$ · sTF interaction is not clear. However, we have previously shown that the L305V mutation has a slightly negative impact on sTF binding [18] and that the K337A mutation has an opposite effect of approximately the same magnitude [19]. Hence, one or both of the S314E and F374Y mutations are most likely to be responsible for a reduction in affinity for sTF, but it is also possible that the effect of a particular mutation differs with the environment. After incorporation of FFR-ck, FVIIa bound sTF with higher affinity as seen previously [31]. The binding energies for the interactions between FVIIa and $FVIIa_{IIa}$ respectively and sTF became indistinguishable after inhibitor binding and the binding energy gap between FVIIa and $FVIIa_{VEAY}$ increased. This is because the rate of dissociation of the $FVIIa_{VEAY}$ · sTF complex was not reduced to the same level upon FFR-ck incorporation, whereas the association of all three FVIIa variants with sTF was approximately equally affected. The reason for the relatively small effect of active site occupancy on the affinity of $FVIIa_{VEAY}$ for sTF is obscure. The active site of F374Y-FVIIa is clearly not properly influenced by sTF binding (suboptimal amidolytic activity of the complex), and the same applies for F374P-FVIIa [29]. Nevertheless, F374P-FVIIa inhibited with FFR-ck has been shown to have the same affinity for TF as wild-type FFR-FVIIa, indicating a functioning allostery in the reverse direction [29]. L305V-FVIIa, S314E-FVIIa, and K337A-FVIIa in complex with sTF had (at least) normal activity.

DISCUSSION

Recently several engineered variants of FVIIa with increased intrinsic enzymic activity have been reported [17–23]. The

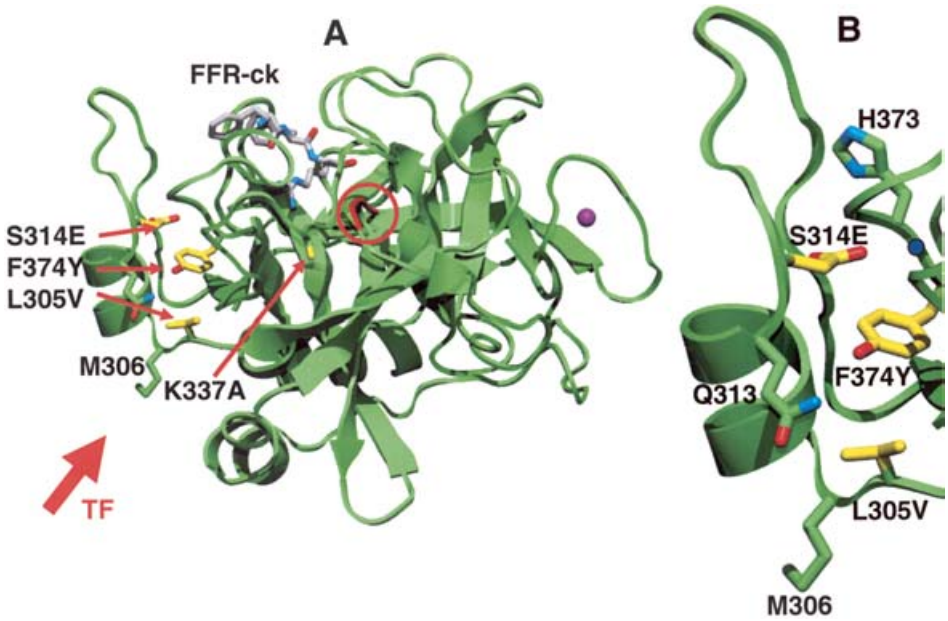

Figure 3 Mutations in FVIIa$_{VEAY}$

(**A**) Protease domain of FVIIa shown as a cartoon drawing in green. The covalently bound inhibitor FFR-ck is shown as a stick model presentation in grey and the calcium ion bound to the protease domain is in magenta. In yellow are the carbon atoms of the four mutated side chains L305V, S314E, K337A and F374Y. The conformations of the mutated side chains have been modelled using rotamer libraries. The activation pocket hosting the N-terminus is marked by a red circle, and the N-terminus is shown inside in red. The red arrow marked 'TF' indicates the direction in which the cofactor tentatively approaches and binds the protease domain. (**B**) Close-up of the region containing the three mutations L305V, S314E and F374Y. The 313–321 (170A–170I) loop is preceded by the 307–312 (165–170) helix which is in turn preceded by Met^{306} (164) responsible for mediating the allosteric effect of TF [15,16]. Apart from a favourable hydrophobic interaction between the aliphatic side chain atoms of Glu^{314} (170B) and the aromatic ring of Tyr^{374} (225), a hydrogen bond interaction between the carboxylate of Glu^{314} and the main chain amide of Tyr^{374} (in blue) may further strengthen the interaction. Alternatively, Glu^{314} might interact electrostatically or via hydrogen bonds with His^{373} (224). A hydrogen bond between the hydroxyl of Tyr^{374} and the side chain amide of Gln^{313} (170A) may tighten the connection of the 307–312 helix to the rest of the protease domain. The structure is from the FVIIa–TF complex ([13]; Protein Database entry code 1dan). The drawings and modelling were made using Quanta 2000 (Accelrys Inc., San Diego, CA, U.S.A.).

modifications of wild-type FVIIa leading to these molecules show that several specific residues and loop segments contribute to the zymogen-like features of FVIIa. Moreover, distinct activity-enhancing mechanisms appear to be put into action in different variants. In the loop variants of FVIIa, a clear effect on either substrate binding or turnover is seen depending on which loop is grafted [22,23]. Similarly, substitutions at selected positions also elicit different, activity-enhancing molecular responses depending on the site of the mutation. Two of the most active FVIIa variants to date, mutated in positions 158 (21), 296 (154), and 298 (156) [19,21] or, as described in the present study, in positions 305 (163), 314 (170B), 337 (188), and 374 (225), appear to be governed by unique and different mechanisms. Differences can be discerned, for instance, regarding the degree of increased affinity for a peptidyl substrate, in the Ca^{2+} requirement, and when looking at the relative increases in amidolytic and proteolytic activities. Another conspicuous and mechanistically interesting observation is the effect (as in FVIIa$_{IIa}$) or lack of effect (as in FVIIa$_{VEAY}$) on the propensity of the N-terminal amino group of Ile^{153} (16) to form a salt bridge with the side chain of Asp^{343} (194). Nevertheless, the activity of both wild-type FVIIa (with a relatively exposed N-terminus) and FVIIa$_{IIa}$ (more buried N-terminus) suffers dramatically when an additional glycine residue is introduced [Val^{154} (17) → Gly] [32] to make the tail more flexible (E. Persson, unpublished work). Thus the salient characteristic of FVIIa$_{VEAY}$ is a high intrinsic amidolytic activity, whereas the prominent feature of FVIIa$_{IIa}$ is a high intrinsic proteolytic activity. In the latter mutant, the locations of the three mutations also lead to effects related to the N-terminus and the Ca^{2+} dependence. How the modifications in FVIIa$_{VEAY}$ and FVIIa$_{IIa}$ relate to a conceivable zymogen-to-enzyme transition mechanism involving β strand reregistration is not known [8].

As mentioned above, carbamylation experiments, measuring the rate of cyanate-induced inhibition of FVIIa resulting from chemical modification of the N-terminal amino group [33], reveal a major difference between FVIIa$_{VEAY}$ and FVIIa$_{IIa}$. A hypothesis has been put forward for how the mutations in FVIIa$_{IIa}$ residing in the vicinity of the activation pocket where the N-terminus inserts exert their stimulatory effect, at least in part, by tethering the N-terminal tail [19]. The mutations in FVIIa$_{VEAY}$ do not clearly affect this motional freedom. Rather, the locations of the mutations in FVIIa$_{VEAY}$ and the higher affinity of this mutant for S-2288 compared with that of FVIIa$_{IIa}$, despite a similar maturation of the S1 site as illustrated by similar K_i values for PABA, suggest that the functional improvement of FVIIa$_{VEAY}$ is mediated by a stabilization of the 313–321 (170A–170I) loop (Figure 3). This loop forms part of the S3 and more distant upstream subsites which are sensed by the tripeptidyl substrate S-2288, utilizing a more extended recognition, but not by the S1-focused inhibitor PABA (Figure 3A). Figure 3(B) shows the region containing the mutations L305V, S314E and F374Y (in yellow). The two latter mutations were introduced with the purpose of improving the contact between the helix-loop region containing residues 307–321 (165–170I), in particular the loop, and the rest of the protease domain. This is achieved by strengthening hydrophobic as well as hydrogen-bond interactions at the interface. We suggest that there is an enhanced hydrophobic interaction between the side chains of Glu^{314} (170B) and Tyr^{374} (225). Hydrogen bonds between the side chain of Glu^{314} (170B) and the main chain of Tyr^{374} (225) and between the side

chains of Tyr^{374} (225) and Gln^{313} (170A) may also have been introduced. Furthermore, the L305V mutation may mediate a shift of the 307–312 (165–170) helix into the orientation observed in constitutively active proteases (e.g. FXa) as discussed in a previous report [18]. Together, the mutations may increase the loop stability and optimize the helix orientation, and the increased amidolytic activity of L305V/S314E/F374Y-FVIIa indicates that the mutations partly mimic the effect of TF binding. Interestingly, an increased intrinsic activity has also been achieved in FVIIa variants containing a shorter loop, grafted from trypsin, replacing residues 310–329 (168–182) [22,23]. The plausible reason again appears to be improved structural rigidity. In contrast to $FVIIa_{VEAY}$, which has a loop composed of the native residues, substantial alteration of substrate specificity accompanied the activity enhancement of these graft mutants. The explanation for this is most likely that other residues than those in FVIIa (and in $FVIIa_{VEAY}$) occupy positions participating in upstream substrate recognition sites.

The rate of carbamylation of $FVIIa_{VEAY}$ (and of K337A-FVIIa [19]) is similar to that of wild-type FVIIa, but the K337A mutation still significantly enhances the amidolytic activity, both as a single mutation and in the tetramutant. The longer lysine side chain may act as a gatekeeper at the entrance to the activation pocket, sterically interfering with N-terminal insertion, whereas an alanine side chain permits easier access to the pocket. This suggests that the effect of the K337A mutation on the N-terminus, if any, is mediated by a decrease of the energy barrier for its insertion rather than a lowering of the free energy of the inserted state. This would result in a faster in-and-out transition without affecting the propensity to form a salt bridge with Asp^{343} (194) and the protection from chemical modification. We have found that the K337A mutation induces some maturation of the S1 pocket, manifested as a decreased K_i for PABA (results not shown), which might be the reason for the activity increase induced by this mutation. In contrast, in $FVIIa_{IIa}$, the N-terminus is believed to be tethered to the body of the protease domain by the introduced Asp and Gln residues at positions 158 (21) and 298 (156), respectively, and the terminal amino group is more protected from carbamylation [19]. This suggests that a gain in free energy accompanies the insertion of the N-terminus in $FVIIa_{IIa}$. We propose that the substitution of Ala for Lys^{337} (188) results in an effect on the kinetics (lower energy barrier) of insertion, while the changes in $FVIIa_{IIa}$ result in a thermodynamic effect (lower free energy).

In conclusion, an increasing body of experimental data supports the notion that FVIIa can be coaxed into becoming a more efficient enzyme in several ways. We have only begun to explore the determinants of the zymogen-like behaviour of free FVIIa and ways to improve the enzymic activity, but we believe that the helix-loop segment discussed in this study holds the promise of being one of the crucial regulatory elements, possibly involved in the natural activation mechanism. The structural explanations for the higher intrinsic activity of the FVIIa variants described here and elsewhere [17–23] await the crystal structures of free, uninhibited wild-type and mutant FVIIa.

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