Substitution of Valine for Leucine 305 in Factor VIIa Increases the Intrinsic Enzymatic Activity*

Egon Persson‡§, Helle Bak‡, and Ole H. Olsen¶

From ‡Vascular Biochemistry and §Medicinal Chemistry Research IV, Novo Nordisk A/S, Novo Nordisk Park, DK-2760 Måløv, Denmark

Factor VII requires the cleavage of an internal peptide bond and the association with tissue factor (TF) to attain its fully active factor VIIa (FVIIa) conformation. The former event alone leaves FVIIa in a zymogen-like state of relatively low specific activity. We have designed a number of FVIIa mutants with the aim of mimicking the effect of TF, that is, creating molecules with increased intrinsic (TF-independent) enzymatic activity. Based on a possible structural difference between free and TF-bound FVIIa (Pike, A. C. W., Brzozowski, A. M., Roberts, S. M., Olsen, O. H., and Persson, E. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 8925–8930), we focused on the helical region encompassing residues 307–312 and residues in its spatial vicinity. For instance, FVIIa contains Phe-374 and Leu-305, whereas a Phe/Tyr residue in the position corresponding to 374 in homologous coagulation serine proteases is accompanied by Val in the position corresponding to 305. This conceivably results in a unique orientation of this helix in FVIIa. Substitution of Val for Leu-305 in FVIIa resulted in a 3–4-fold increase in the intrinsic amidolytic and proteolytic activity as compared with wild-type FVIIa, whereas the activity in complex with soluble TF remained the same. In accordance with this, L305V-FVIIa exhibited an increased rate of inhibition as compared with wild-type FVIIa, both by D-Phe-Phe-Arg-chloromethyl ketone and antithrombin III in the presence of heparin. The increased FVIIa activity upon replacement of Leu-305 by Val may be mediated by a movement of the 307–312 helix into an orientation resembling that found in factors IXa and Xa and thrombin. The corresponding shortening of the side chain of residue 374 (Phe → Pro) had a smaller effect (about 1.5-fold increase) on the intrinsic activity of FVIIa. Attempts to increase FVIIa activity by introducing single or multiple mutations at positions 306, 309, and 312 to stabilize the 307–312 helix failed.

The association of factor VIIa (FVIIa) with tissue factor (TF), exposed upon vascular injury, triggers blood coagulation (1). Without this complex formation, FVIIa has very low reactivity toward physiologic substrates (2) and is a relatively poor activator (1). Without this complex formation, FVIIa has very low reactivity toward physiologic substrates (2) and is a relatively poor activator (1).

The most logical approach to engineering FVIIa to render it a more efficient catalyst appears to be to impose the conformational changes of TF-bound FVIIa on the free enzyme. Available structural data indicates that the difference between the two states of FVIIa is subtle (4–8), but the presence of an active site inhibitor in all the structures may result in an underestimate of the TF-induced conformational changes. The inhibitor, much like TF, locks FVIIa in an active conformation where the internal salt bridge between Ile-153 and Asp-343 is established. Nevertheless, we observed a difference between free and TF-bound FVIIa in the area of residues 307–312, which are arranged in an a-helix in FVIIa-TP that appears to be distorted in free FVIIa (4). In this report, we give an account of several mutations in FVIIa aimed at increasing the inherent enzymatic activity through stabilization or reorientation of the above-mentioned a-helix.

EXPERIMENTAL PROCEDURES

Reagents and Standard Methods—The preparation of recombinant wild-type FVIIa and recombinant soluble TF (sTF) were carried out as described previously (15, 16). Factor VII (FVII) and FVIIa concentrations were determined using a double monoclonal (both recognizing epitopes in the light chain of FVIIa) enzyme-linked immunosorbent assay and sTF concentrations by absorbance measurements at 280 nm using an absorption coefficient of 1.5 for a 1-mg/ml solution. Factor X (FX) and FXa were from Enzyme Research Laboratories (South Bend, IN), antithrombin III (ATIII) from Hematologic Technologies, Inc. (Essex Junction, VT), D-Phe-Phe-Arg-chloromethyl ketone (FFR-ck) from Calbiochem (La Jolla, CA), and the chromogenic substrates S-2288 and S-2289.

§ To whom correspondence should be addressed: Vascular Biochemistry, Novo Nordisk A/S, Novo Nordisk Park, DK-2760 Måløv, Denmark. Tel.: 45-44-43-43-51; Fax: 45-44-44-44-17; E-mail: egp@novonordisk.com.

‡ The abbreviations used are: FVIIa, activated coagulation factor VII; ATIII, antithrombin III; FFR-ck, D-Phe-Phe-Arg-chloromethyl ketone; FVII, coagulation factor VII; FX, coagulation factor X; FXa, activated coagulation factor X; sTF, soluble tissue factor (residues 1–219); TF, tissue factor; Pipes, 1,4-piperazinediethanesulfonic acid.

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S-2765 were from Chromogenix (Mölndal, Sweden). FVII-deficient human plasma was from Helena Laboratories (Beaumont, TX), Innoven (replidated recombinant human TF) was from Dade Behring (Marburg, Germany), and phosphatidylserine/phosphatidylcholine was from Sigma. SDS-polyacrylamide gel electrophoresis was run on 8––25% gradient gel using the PhastSystem (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Mutagenesis and Protein Expression—The wild-type FVII expression plasmid pLN174 (17) was used as the template for site-directed mutagenesis, except for L305VM306D/D309S-FVII, where a plasmid encoding M306ID309S-FVII was used as the template (12). The Leu-305→ValPhage display (18) Pro mutations were introduced using the QuikChange kit (Stratagene, La Jolla, CA) using the following primers (only sense primers given) with base substitutions in italic and the affected codons underlined: L305V, CGT GCC CCG GTG GAT GAC CCA GGA C; F374P, CCG TGG GCC ACC CTG GGG TGT ACA CC. Plasmids were prepared using the QIAprep spin miniprep and QIAfilter plasmid midi kits (Qiagen, Valencia, CA). The entire cDNA encoding the mutants was verified by sequencing to exclude the presence of additional mutations. Baby hamster kidney cell transfection, selection, and protein expression were carried out as described previously (12).

Mutant Isolation and Activation—Purification, concentration and autophosphorylation of the FVIIa mutants were performed as described (12). The final FVIIa concentrations was determined by enzyme-linked immunosorbent assay.

FVIIa Activity Assays—All proteins were diluted in 50 mM Hepes, pH 7.4, containing 0.1 mM NaCl, 5 mM CaCl2, and 0.1% (w/v) bovine serum albumin, prior to analysis. All assays were run in a final volume of 200 μl. The amidolytic activity was monitored continuously at 405 nm in a SpectraMax 340 microplate spectrophotometer equipped with the software SoftMax PRO, version 2.2 (Molecular Devices Corp., Sunnyvale, CA). To measure the amidolytic activity in the absence of sTF, 100 nM wild-type or mutant FVIIa was mixed with 1 mM S-2288. This analysis was performed at both ambient temperature and at 37 °C and in the absence (CaCl2 replaced by EDTA) and presence of Ca++. The stimulatory effect of sTF on the amidolytic activity was determined by mixing 10 nM FVIIa, L305V-FVIIa, or F374P-FVIIa with 50 nM sTF and by mixing 10 nM L305VM306ID309S-FVIIa with 100 nM sTF, followed by the addition of 1 mM S-2288.

To measure the proteolytic activity in the absence of sTF, a concentration of 10 nM wild-type or mutant FVIIa was used. The effect of sTF on FVIIa-catalyzed FX activation was studied by mixing 10 nM FVIIa or L305V-FVIIa with 50 nM sTF and by mixing 10 nM L305VM306ID309S-FVIIa with 100 nM sTF, followed by the addition of 1 mM S-2288.

The specific clotting activity of the FVIIa variants was measured in one-stage assays and the clotting times were recorded on an ACL 300 Research coagulometer (Instrumentation Laboratory, Milan, Italy). In the TF-dependent clotting assay, 40 μl of FVIIa (wild-type or mutant FVIIa in 50 mM Pipes, pH 7.2, containing 0.1 mM NaCl, 2 mM EDTA, and 1% (w/v) bovine serum albumin) was mixed with 40 μl of FVII-deficient plasma, and clotting was initiated by adding 80 μl of 12.5 mM CaCl2 containing Innoven (dissolved according to the manufacturer’s instructions and subsequently diluted 100-fold in Pipes buffer and mixed with an equal volume of 25 mM CaCl2). Dilutions of pooled normal human plasma were used to construct a standard curve (clotting times: ~40–60 s), and the specific clotting activity of wild-type FVIIa was set to 100%. In the TF-free clotting assay, 55 μl of test sample, containing wild-type or mutant FVIIa in Pipes buffer, was mixed with an equal volume of 12.5 mM CaCl2 containing phosphatidylcholine/phosphatidylserine vesicles (total phospholipid concentration 50 μM; 80% phosphatidylcholine/20% phosphatidylserine), and clotting was started by adding 55 μl of FVII-deficient plasma. Dilutions of wild-type and mutant FVIIa (0.1–100 μg/ml) were used to construct a standard curve, giving clotting times between 60 and 170 s, from which the specific activities of the mutants were derived.

FVIIa Inhibition Assays—These assays were carried out in the same buffer as the activity assays. The inhibition by FFR-crk was measured by incubating wild-type or mutant FVIIa (100 nM) with the inhibitor (5 μM) for various time periods followed by the addition of S-2288 (1 mM) to measure the residual activity at 405 nm as described above. The inhibition of 100 nM FVIIa variant alone or of 10 nM FVIIa plus 100 nM sTF by ATIII (100 μg/ml) in the absence or presence of heparin (unfractionated, 1 unit/ml; Heparin Leo, Leo Pharmaceutical, Ballerup, Denmark) was measured in an analogous manner.

Surface Plasmon Resonance Measurements—The conditions for sTF immobilization (~1000 resonance units) in the Biacore 1000 instrument (Biacore AB, Uppsala, Sweden), regeneration of the sTF-coated surface, and evaluation of binding data were as described previously (18, 19). Wild-type, L305V-, and L305VM306ID309S-FVIIa were injected for 7 min at a concentration of 30–150 nM in 50 mM Hepes, pH 7.5, containing 0.15 mM NaCl, 5 mM CaCl2, and 0.02% Tween 80, followed by a 10-min dissociation phase. The flow rate was 5 μl/min and the temperature 25 °C.

Structural Analysis—Analyses (and figure preparations) using x-ray crystallographic data were performed in Quanta (Molecular Simulations, San Diego, CA). Superpositioning of the protease domains was based on homology, and the main chain atoms of identical amino acid residues in the sequences were superimposed. The protein structures were coagulation FXa (PDB code 1heg), coagulation factor IXa (PDB code 1px), coagulation FVIIa (PDB code 1dai), thrombin (PDB code 1tom), and trypsin (PDB code 3ptn).

RESULTS

Enzymatic Activity and sTF Binding of FVIIa Mutants—After purification from cell culture medium and autoactivation as described (12), the FVIIa mutants were virtually homogeneous and, after reduction, indistinguishable from wild-type FVIIa as judged by SDS-polyacrylamide gel electrophoresis followed by silver staining of the gel (not shown). The amidolytic activity of L305V-FVIIa and F374P-FVIIa at ambient temperature and in the absence of TF was found to be ~3-fold and 1.4-fold that of wild-type FVIIa, respectively (Fig. 1). The result with F374P-FVIIa is in agreement with a recent report (20). When the Leu-305→Val mutation was introduced into M306ID309S-FVIIa, a mutant previously shown to have an amidolytic activity similar to wild-type FVIIa (12), a 3-fold increase in activity was again obtained. All FVIIa variants had a slightly lower activity at 37 °C as compared with at room temperature, resulting in a similar enhancement of the activity of the mutants as compared with wild-type FVIIa at both temperatures. This indicates that the thermal stability was not affected by the mutations. The omission of calcium ions in the assay buffer resulted in a loss of more than 80% of the activity of all FVIIa mutants, a behavior similar to that of wild-type FVIIa. The ability of the mutants to activate FX in the absence of TF and phospholipid was then assessed. L305V-FVIIa and L305VM306ID309S-FVIIa catalyzed FXa generation at a 3–4-fold increased rate as compared with wild-type FVIIa (Table 1). This resulted solely from an increased substrate turnover, with K values identical to that of wt-FVIIa. In contrast, F374P-FVIIa activated FX at a 2-fold slower rate than the

FIG. 1. Amidolytic activity of FVIIa variants. The activity in the absence of sTF and in the presence of saturating concentrations of the cofactor are shown. All activities are relative to that of wild-type FVIIa in the absence of sTF, which is arbitrarily set to 1 (also denoted by the dotted line).
wild-type enzyme (not shown). The relative rates of FX activation, as compared with wild-type FVIIa, were not altered by increasing the temperature to 37 °C.

The affinity of L305V-FVIIa and L305V/M306D/D309S-FVIIa for sTF was measured by surface plasmon resonance and found to be characterized by dissociation constants of 3.4 and 10.6 nM, respectively, values slightly higher than that obtained for wild-type FVIIa (Table II). Because TF stabilizes the active conformation of FVIIa, a higher affinity of the superactive mutants for sTF might be anticipated. However, the mutations are located at or in the proximity of contact points with sTF, and this is the plausible reason for the slightly negative impact on the FVIIasTF interaction. F374P-FVIIa has been found by others to have a slightly higher affinity than wild-type FVIIa for TF (20). The protein concentrations in the following activity measurements, performed in the presence of sTF, were chosen to virtually saturate the mutants with cofactor. In complex with sTF, L305V-FVIIa had both an amidolytic (92%, Fig. 1) and a proteolytic (80%, data not shown) activity similar to that of the wild-type FVIIasTF complex. This obviously results from a relatively smaller TF-induced enhancement of the activity of L305V-FVIIa as compared with wild-type FVIIa. The amidolytic and proteolytic activity of L305V/M306D/D309S-FVIIa was virtually unaffected by the presence of sTF (1.1-fold increase). This is in agreement with previous data on M306D-FVIIa and M306D/D309S-FVIIa and apparently a consequence of the Met-306 → Asp mutation (12). The stimulation of F374P-FVIIa by sTF was significantly impaired as reported previously (20).

The specific clotting activity of L305V-FVIIa, F374P-FVIIa, and L305V/M306D/D309S-FVIIa in the presence of TF was 93 ± 10%, 50 ± 4%, and about 1% of that of wild-type FVIIa, respectively, reflecting the various degrees of reduction of the proteolytic activity in complex with TF as compared with that of wild-type FVIIa. In the absence of TF, the specific clotting activity of L305V-FVIIa was about 10-fold higher than that of wild-type FVIIa, whereas F374P-FVIIa had a 1.5-fold higher activity. This is in agreement with the observed superactivity of free L305V-FVIIa in the experiment looking at the activation of purified FX, a superactivity that appears to be amplified in the clotting assay.

Inhibition of FVIIa Mutants—It has been shown that FVIIa-TF is much more rapidly inhibited than free FVIIa by ATIII (21, 22), presumably reflecting the maturation of the active site of FVIIa upon cofactor binding. We compared the rate of binding of ATIII and FFR-ck to the wild-type and mutant FVIIa as an indication of the relative accessibility of a mature active site. ATIII, in the presence of heparin, inhibited L305V- and L305V/M306D/D309S-FVIIa more rapidly than it inhibited wild-type FVIIa (Fig. 2). The time required for inhibition of 50% of the activity was reduced from −50 min for wild-type FVIIa to about 10 min for the mutants. In the absence of heparin, no significant inhibition of either FVIIa variant was observed after 120 min (not shown). When bound to sTF in the presence of heparin, L305V-FVIIa and wild-type FVIIa were much more rapidly and equally efficiently inhibited by ATIII with only about 5% remaining amidolytic activity after 10 min (not shown). In contrast, L305V/M306D/D309S-FVIIa in complex with sTF was inhibited at a rate indistinguishable from that of the free mutant, reflecting its inability to be stimulated by sTF. This clearly shows that sTF does not contribute directly to ATIII binding and inhibition of FVIIasTF and that the rate of inhibition only depends on the state of the active site. Using FFR-ck as the inhibitor, an increased rate of inhibition was also observed for the mutants (Fig. 3). The time required for inhibition of 50% of the activity was about 5 min for wild-type FVIIa and below 1 min for both mutants. Thus the increased intrinsic proteolytic and amidolytic activity of the mutants was mirrored in an increased susceptibility to inhibition by physiological (macromolecular) as well as low molecular weight inhibitors.

**DISCUSSION**

A structural comparison of free (4) and TF-bound FVIIa (7, 8) reveals that the salient effect of cofactor binding appears to be on the helical secondary structure containing residues 307–312 in FVIIa. However, the effect is far from conspicuous, if at all visible, when looking at the two other available structures of free FVIIa (5, 6). The differing observations may be related to crystallization conditions and to the crystal packing. Nevertheless, the region around residues 307–312 clearly gives an im-

![Figure 2](http://www.jbc.org/)

**Figure 2.** Inhibition of FVIIa variants by ATIII/heparin. The residual activity of wild-type FVIIa (circles), L305V-FVIIa (squares), and L305V/M306D/D309S-FVIIa (diamonds) after various incubation times with the inhibitor is shown.

![Figure 3](http://www.jbc.org/)

**Figure 3.** Inhibition of FVIIa variants by FFR-ck. The residual activity of wild-type FVIIa (circles), L305V-FVIIa (squares), and L305V/M306D/D309S-FVIIa (diamonds) after various incubation times with the inhibitor is shown.

| FVIIa variant | $k_{cat}$ | $K_m$ |
|--------------|----------|------|
| Wild-type    | 0.0817 ± 0.0007 | 2.7 ± 0.3 |
| L305V-FVIIa  | 0.029 ± 0.004 | 2.9 ± 0.5 |
| L305V/M306D/D309S-FVIIa | 0.022 ± 0.006 | 3.0 ± 0.4 |

| FVIIa variant | $K_d$ |
|--------------|------|
| Wild-type    | 10.6 nM |
| L305V-FVIIa  | 3.4 ± 0.6 |
| L305V/M306D/D309S-FVIIa | 10.6 ± 3.0 |

**Table I**

**Inhibition of FVIIa variants in the absence of sTF**

Values are means ± S.E. of the mean (n = 3).

**TABLE II**

**Binding of FVIIa variants to sTF**

| FVIIa variant | $k_{on} \times 10^3 M^{-1} \text{s}^{-1}$ | $k_{off} \times 10^{-3} M^{-1}$ | $K_d$ |
|--------------|---------------------------------|-----------------|------|
| Wild-type    | 2.2 ± 0.2 | 3.3 ± 0.6 |
| L305V-FVIIa  | 2.7 ± 0.1 | 10.6 ± 3.0 |
| L305V/M306D/D309S-FVIIa | 2.7 ± 0.1 | 10.6 ± 3.0 |
expression of a relatively high flexibility (as indicated by high crystallographic B factors). The helix containing residues 307–312 in FVIIa definitely has a unique orientation relative to the body of the protease domain, whereas in related enzymes the corresponding helices fill a virtually identical space different from that in FVIIa (Fig. 4A). The explanation possibly rests in the residues occupying positions 305 and 374 in FVIIa, namely Leu and Phe, respectively. To our knowledge, no related enzyme has the same combination. The corresponding positions are rather occupied by Leu and Pro or by Val and Phe/Tyr (Fig. 4, B and C). Thus the combined length of these two side chains is the longest in FVIIa, and it is about one carbon atom shorter in the other enzymes. This might in turn influence the chirality of the Cys-310–Cys-329 disulfide bond and the relative orientation of the 307–312 helix. With the focus on this region, structural comparisons suggest that the creation of superactive FVIIa variants requires either stabilization (as in TF-bound FVIIa) or reorientation (as in FXa and thrombin) of the 307–312 helix.

Attempts to generate FVIIa variants with enhanced intrinsic activity by stabilizing the helix through the introduction of new N-capping residues in position 306 (12, 23), or by forming new intrahelical hydrogen bonds by combining Met-306→Ser with Asp-309→Glu (24), or by combining new N-capping side chains in position 306 (Ser, Thr, Asp, or Asn) with a Gln-312→Asn mutation (25), uniformly failed. Assuming that at least one of the above modifications to FVIIa suffices to stabilize the α-helix encompassing residues 307–312 in a conformation similar to that when bound to TF, the lack of a positive effect on the intrinsic activity, in combination with the increased activity obtained when substituting Val for Leu-305, indicates that the chirality of the Cys-310–Cys-329 disulfide bond and/or the orientation of the helix is more important than its stability, at least in the free form of FVIIa. In other words, the observed increase in activity for L305V-FVIIa arises from changing the local structure into one resembling for instance FXa and thrombin rather than mimicking TF-bound FVIIa. If the hypothesis is correct that a reorientation of the 307–312 helix is involved in the enhanced intrinsic activity of L305V-FVIIa, the data obtained with L305V-FVIIa and F374P-FVIIa indicates that the sequentially adjacent Leu-305 governs the helix orientation to a larger extent than the spatially proximal Phe-374. Even though the activity of L305V-FVIIa is similar to that of FVIIa, this mutant and, in particular, F374P-FVIIa suffer from sub-optimal sTF-induced activity enhancement. This indicates that the two residues are on the same allosteric pathway. The reason why F374P-FVIIa displays a lower specific activity in the free form and is less stimulated by sTF as compared with L305V-FVIIa may be because of backbone restrictions imposed by the Pro residue.

The effect of the Leu→Val mutation in position 305 of FVIIa has been described on two different backgrounds (wild-type and M306D/D309S-FVIIa), generating mutants displaying unique profiles. L305V-FVIIa has enhanced activity in the free form and normal activity in complex with TF, whereas L305VM306D/D309S-FVIIa has a similarly increased intrinsic activity which is virtually unaffected by the presence of TF (i.e. far below that of wt-FVIIa-TF). The reason why the latter mutant is unable to be stimulated by TF has been described previously (12). The free form of both mutants is more efficiently targeted than wt-FVIIa by inhibitors. The allosteric effect of the Leu→Val mutation on FVIIa’s active site is as long ranging as that induced by TF, which is assumed to originate from Met-306 (10, 12). The truncation of residue 305 may induce an increased activity either by using the 307–312 helix as a spring to influence the 313–321 loop, whose C-terminal part helps defining the S4 subsite, or via Phe-374 which has been shown to be important for the allosteric stimulation of FVIIa by TF (20), or by yet another mechanism. Although we have shown that the region containing Leu-305 influences the intrinsic activity of FVIIa, we are convinced that mutations in this region are not the sole key to an improved FVIIa activity and that distant residues are also involved in the entrapment of the free enzyme in a state of low activity.

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