Four Loops of the Catalytic Domain of Factor VIIa Mediate the Effect of the First EGF-like Domain Substitution on Factor VIIa Catalytic Activity

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The presence of tissue factor is essential for factor VIIa (FVIIa) to reach its full catalytic potential. The previous work in this laboratory demonstrated that substitution of the EGF1 domain of factor VIIa with that of factor IX (FVII(IXegf1)a) results in a substantial decrease in TF-binding affinity and catalytic activity. Supporting simulations of the solution structures of Ca²⁺-bound factor VIIa and FVII(IXegf1)a with tissue factor are provided. Mutants are generated, based on the simulation model, to study the effect of EGF1 substitution on catalytic activity. The simulations show larger Gla-EGF1 and EGF1-EGF2 inter-domain motions for FVII(IXegf1)a than for factor VIIa. The catalytic domain of the chimeric factor VIIa has been disturbed and several surface loops in the catalytic domain of FVII(IXegf1)a (Loop 170s (170-182), Loop 1 (185-188) and Loop 2 (221A-225)) manifest larger position fluctuations than wild-type. The position of Loop 140s (142-152) of FVII(IXegf1)a, near the N terminus insertion site of the catalytic domain, shifts relative to factor VIIa, resulting in a slight alteration of the active site. The results suggest that these four loops mediate the effect of the EGF1 domain substitution on the S1 site and catalytic residues. To test the model, we prepared mutations of these surface loops, including four FVII mutants, D186A, K188A, L144A and R147A, a FVII mutant with multiple mutations (MM3: L144A + R147A + D186A) and a FVII mutant with Loop 170s partially deleted, Loop 170sΔdel. The catalytic activities towards a small peptidyl substrate decreased 2.4, 4.5 and 9-fold for Loop 170sΔdel (a, activated), L144Aa and D186Aa, respectively, while MM3a lost almost all catalytic activity. The combined results of the simulations and mutants provide insight into the mechanism by which tissue factor enhances factor VIIa catalytic activity.

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Keywords: blood coagulation factor; macromolecular simulation; tissue factor; structure-function study; domain substitution

Introduction

Blood coagulation factor VIIa (FVIIa) is a trypsin-like plasma serine protease. FVIIa in its cataly-
tic domains has strong primary sequence identity and tertiary structure similarity with trypsin and chymotrypsin. These enzymes (FVIIa is an example, Figure 1) share common catalytic triad residues, H57, D102 and S195 (the catalytic domain residues being represented with chymotrypsin numbering system, except where indicated), and are largely characterized by their S1-binding sites. FVIIa and trypsin bear an aspartic acid (D189) at the bottom of their S1 pockets, which accounts for the substrate specificity for the positively charged residues, arginine or lysine. The residue at the bottom of S1 site, however, is not sufficient for determination of substrate specificity. Two loops on the surface of trypsin, Loop 1 (185-188) and Loop 2 (221A-225), in concert with Loop 140s (142-152) and the N terminus insertion (16-19) are collectively called “the activation domain”. The active domain is stabilized upon conversion of the zymogen to trypsin and probably also rearranges upon TF binding to FVIIa. These loops and Y172, which is located on Loop 170s (170-182) and is near Loop 2 and the catalytic cleft, have been shown to play important roles in the determination of substrate specificity.

FVIIa is a multi-domain molecule, including a proteolytic domain at the C terminus (residues 16 to 257), and a γ-carboxyglutamic-acid-rich domain (Gla) at the amino terminus. Two epidermal growth-factor-like domains, EGF1 and EGF2, connect the Gla and catalytic domains. The N terminus three domains define the light chain. Once activated, the zymogen FVII is cleaved at the R152L-I16 (L, the light chain) peptide bond into a light chain and a heavy chain (catalytic domain), which remain connected by a disulfide bond. The amino terminus residue I16 of the heavy chain inserts into a groove to form two H-bonds with residue D194 (Figure 1). D194 is adjacent to the catalytic residue S195 and to G193. The amide nitrogen atoms of the latter two residues bind the negatively charged oxygen of the carbonyl cleavage position to stabilize the tetrahedral intermediate.

In addition to the necessary conformational changes that occur in the regions near the active site, FVIIa must bind its cofactor, tissue factor (TF), in the presence of membrane and calcium ions to exhibit full catalytic activity. TF consists of three sections, an extracellular fragment (1-219), a transmembrane region (220-242) and a small cytoplasmic domain (243-263). X-ray crystallography shows that the FVIIa-TF complex interaction involves all FVIIa domains. The Gla domain, which is pre-

![Figure 1. Simplified ribbon diagram of the catalytic domain of FVIIa.](image)
assumed to contact a membrane surface, is in contact with the extracellular C terminus of TF. The EGF1 domain forms an interface with the central section of TF and the third interface involves the catalytic domain, EGF2 and the N terminus of TF. The catalytic domain-TF interface consists mainly of two small \( \alpha \)-helices, \( \alpha \)-129B-129G and \( \alpha \)-164-167 of FVIIa and the N terminus domain of TF. FVIIa Residues F129F, R134, Q166 and D167 form H-bonds with TF residues. The M164 side-chain inserts into a hydrophobic pocket on TF.\(^5\) Substitution of R134 or M164 with alanine causes a defect in FX activation\(^12\) and in chromogenic substrate hydrolysis and diminishes co-factor enhancement of small-substrate hydrolysis.\(^13\) M164 has been reported\(^14\) to transmit the allosteric changes to the active site caused by TF-binding and conversely to mediate increased TF-binding affinity resulted from small peptide inhibitor binding to the active site. The \( \alpha \)-helix 164-169 is found distorted,\(^15\) and Loop 170s, which follows the \( \alpha \)-helix 164-167 and is located between the catalytic domain-TF interface and the active site, shows high flexibility in the inhibitor-bound TF-free state of FVIIa.\(^{15,16}\)

The EGF1 domain of FVIIa provides most of the binding interaction with TF. The high-affinity calcium-binding site located at the N terminus of the EGF1 domain has been implicated in cofactor activity of TF.\(^17\) Mutation of TF residues contacting the EGF1 domain affects catalytic activity.\(^{18,19,20}\) Previously we\(^21,22\) demonstrated that substitution of the EGF1 domain of TF with that of FIX (FVII\((\text{IXegf1})\)a) greatly reduced TF-binding affinity. Catalytic efficiencies towards a synthetic substrate and FX were also affected\(^21\) by the chimeric substitution in the presence of TF. We subsequently suggested that the allosteric effect of substitution of the EGF1 domain on the enzyme’s catalytic activity is transmitted through the misalignment of the catalytic domain-TF interface to the active site and/or a macromolecular substrate-binding site.\(^21\) It is interesting that an allosteric linkage between the EGF1 domain and the active site has also been observed in free FVIIa using a conformation-sensitive FVII-EGF1-specific antibody.\(^23\)

To study the effect of EGF1 substitution on the catalytic activity of FVIIa, we have performed molecular dynamics (MD) simulations of both wild-type FVIIa and the chimeric FVIIa in the presence of TF using the Amber force field.\(^{24}\) The simulations were performed in solution using the FVIIa-TF complex coordinates\(^5\) to provide the initial system. The simulation results were then tested experimentally using in \textit{vitro} mutagenesis. The experimental evidence supports our hypothesis, derived from the simulation, that four loops, Loop 170s, Loop 1, Loop 2 and Loop 140s, on the surface of the catalytic domain mediate the effect of the EGF1 domain substitution on catalytic activity of FVIIa, suggesting the manner by which TF enhances FVIIa’s enzymatic activity.

### Results

#### RMSD analysis of the simulation

It is important to establish that the simulation system is equilibrated before the simulation structure is analyzed. Evaluating RMSDs has been demonstrated to be an appropriate procedure for monitoring equilibration of the simulation structure.\(^25\) A RMSD analysis is also valuable for observing individual domain stability and inter-domain motion in a multiple-domain macromolecule. The RMSD profile of the wild-type FVIIa-TF simulation for 510 ps is depicted in Figure 2. We used the entire complex FVIIa-TF, FVIIa, or TF, and individual domains (Gla, EGF1, EGF2, the catalytic domain) of the initial energy minimized configuration of FVIIa-TF complex as reference coordinates, respectively, to calculate RMSD values of backbone carbon atoms at half picosecond intervals. In panel a, the RMSD baselines fluctuated around 1.25 Å for TF, 1.4 Å for FVIIa and 1.5 Å for FVIIa-TF, indicating equilibration of the simulation system in solution. The fluctuation of the Gla and the EGF1 RMSDs with respect to their initial structures are approximately 0.6 Å (0.59(± 0.07) Å for EGF1; (b)). The catalytic and the EGF2 domains show equilibration as well (Figure 2(b)). FVIIa alone, or in complex with TF, shows somewhat larger RMSDs relative to each individual domain, suggesting that limited inter-domain motion occurred during simulation. We employed Gla-EGF1, EGF1-EGF2, and EGF2-catalytic domain coordinates of the starting structure as references to examine domain-domain motions in FVIIa. Figure 2(c) demonstrates that limited domain-domain motions occurred between EGF1 and EGF2 and between Gla and EGF1. For the chimeric FVII\((\text{IXegf1})\)a-TF, the simulation RMSDs of more than 800 ps are presented in Figure 3. All sub-domains, except EGF1, shows RMSD fluctuations (Figure 3(b)) similar to the wild-type. The EGF1 domain of FVII\((\text{IXegf1})\)a has an increased RMSD (0.88(±0.07) Å), compared to the EGF1 domain of FVIIa (0.59(±0.07) Å). The p-value is less than 0.001. Likewise FVII\((\text{IXegf1})\)a and FVII\((\text{IXegf1})\)a-TF display larger RMSD profiles (panel a) than those of wild-type FVIIa-TF. TF also shows similar, although smaller, fluctuations. Thus increased domain motions are presented due to the EGF 1 domain substitution. Most of the domain-domain motion occurs between EGF1 and EGF2, and between the Gla and EGF1 domains (Figure 3(c)).

#### B-factor analyses

The B-factor is an experimental measure of the thermal harmonic motion of the atoms. The larger the value, the less localized the atom. The (pseudo) B-factors for simulation can be computed from the fluctuations of atoms about their mean positions. We employed the last 100 ps trajectories to calculate B-factors. Figure 4 represents the differ-
ences between FVIIa-TF and FVII_{(IXegf1)}a-TF B-factor profiles. The initial-time structure of each domain was used as the reference in the B-factor calculation so as to eliminate domain-domain motion. Several peaks in the B-factor versus time plots are observed. The FVII residues in the regions of the peaks are located in loops and are not in contact with TF. In general, the B-factors of the residues in these loops are less than 40. The B-factors for residues in the catalytic domain of FVIIa are between 20 and 40. TF residues 78t-80t (t:TF) and 163t-165t have significant thermal fluctuation due to their fragment-terminal location. In addition, the B-factor profile of the FVIIa-TF simulation is similar in pattern to those of several X-ray crystallography studies (data not shown).

The B-factor profile of FVII_{(IXegf1)}a-TF (Figure 4) is similar to that of FVIIa-TF. In the catalytic domain of the chimera (Figure 4(a)), however, B-factors greater than the wild-type FVIIa occur for residues

![Figure 2. Root-mean-square deviations (RMSDs) of the backbone carbon atoms of FVIIa-TF. RMSDs were calculated every 0.5 ps by superimposing the simulation structures. The structure at 0 ps of the plot has undergone a slow heat-up and a cool-down procedure followed by solution simulation for 50 ps of the starting structure. RMSDs are calculated using (a) the entire molecule (FVIIa-TF, FVIIa or TF) as reference; (b) individual domains as reference, and (c) adjacent domains as reference so as to uncover inter-domain motions.](image)

![Figure 3. Root-mean-square deviations (RMSD) of the backbone carbon atoms of FVII_{(IXegf1)}a-TF. The RMSDs were calculated and depicted as in the legend for Figure 2.](image)
170C-175 (see Table 1 for the full-length numbering as used in Figure 4), 185-188B, and 221-224. The B-factor values of these three loops of FVII(IXegf1)a are approximately twice those of FVIIa. In the light chain, two segments of FVII(IXegf1)a with larger B-factors are observed (panel b): residues 86 to 89 and residue 121 to 124. Taken together, we find that the EGF1 domain substitution causes enhanced motion of FVII(IXegf1)a at some surface residues of EGF2 and in the catalytic domain. On the other hand, B-factors of TF in complex with either FVIIa or FVII(IXegf1)a are uniformly similar (Figure 4(c)).

**Local conformational changes**

In general, FVIIa-TF and FVII(IXegf1)a-TF show very similar SAA (solvent accessible areas) profiles (not shown). Only a few small changes of SAA are observed for residues 170 to 170G of the catalytic domain. The EGF1 substitution caused SAA changes at positions 64, 69, 79 and 88 of the light chain. Residue 64, which coordinates the calcium ion located at the interface between the Gla and EGF1 domains and is also involved in TF binding, is more exposed in FVII(IXegf1)a than in FVIIa (50.8 Å² versus 5.6 Å², respectively). The side-chain of I69 was almost completely buried in FVIIa, but E69 (40 Å²) is partially exposed in the chimera. Residue Q88 is located at the junction between EGF2 and TF. The aliphatic part of the Q88 side-chain, in concert with N93, V92, F50t and Y51t, also defines a hydrophobic interaction between EGF2 and TF. The changes in residues 64, 69, 79 and 88 could result in inter-domain motions and/or reduction of TF-binding affinity. Significant

![Figure 4. B-factors of the Cα atoms of FVIIa-TF and FVII(IXegf1)a-TF. The Cα (pseudo) B-factors were calculated with the final 100 ps of simulation. The individual (t=0 ps) domains, Gla, EGF1, EGF2, the catalytic domain, TF-frag 1, TF-frag 2 and TF-frag 3 were used as reference in B-factor calculation of the corresponding regions. The final profiles were obtained by combining individual domains. (a) The catalytic domain of FVIIa, (b) the light chain and (c) TF. The thick lines represent the wild-type FVIIa-TF and the circles represent FVIIa(IXegf1)a-TF.](image-url)
SAA changes for TF are found at residues 50 and 91. F50t is involved in a hydrophobic interaction with the side-chain of Q88. E91t is the N terminus residue of the second fragment of TF and is involved in H-bonding to E178 and R230 of FVIIa. Altering the E91t side-chain interactions (SAA = 9.8 Å² for the chimera versus 41 Å² for the wild-type) could result in important changes for the interaction between the catalytic domain of FVIIa and TF.

Table 1. Nomenclature of chymotrypsin and full-length numbering for the partial residues of the catalytic domain of FVIIa

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Chy stands for the chymotrypsin numbering and FL for the Full_length numbering.

The analysis of the simulation shows that the overall structure of FVII(IXegf1)a-TF.810ps remains similar to that of FVIIa-TF.510ps. FVII(IXegf1)a-TF.810ps retains all eight EGF2-catalytic domain H-bonds, and all three catalytic domain-TF-f1 H-bonds of the wild-type FVIIa-TF. However, FVII(IXegf1)a-TF.810ps lacks several important contacts with TF: positions 64 and 79 of the EGF1 domain (Q64-Q110t, two E56t-R79 H-bonds and E24t-R79), that are known to be important for FVIIa function.\textsuperscript{17,21} In a similar way,
only two of six catalytic domain-TF-f2 H-bonds of FVIIa-TF are preserved in FVII(IXEgf1)a-TF.810ps. The H-bonds between the catalytic domain and the TF-f2 fragment in FVIIa-TF.510ps include T165-E91t, two R230-E91t bonds, E91t-E178, T94t-D167 and T96t-D167 (Figure 6(a)). In FVII(IXEgf1)a-TF.810ps only one R230-E91t and T94t-D167 remain (Figure 6(b)), while T165:N-OE1:E91t, T165:OG1-OE1:E91t and Y94t:N-E1:Q166 are formed. These results confirm that the EGF1-TF and catalytic domain-TF interfaces undergo substantial H-bond changes as a result of the chimera simulation.

Intra-catalytic domain H-bonds

FVII(IXEgf1)a-TF.810ps loses a number of intra-catalytic domain H-bonds when compared to FVIIa-TF.510ps. These lost H-bonds may affect:

(i) the S1-binding site and the amino terminus insertion site of the catalytic domain. For instance, D189 is located at the bottom of S1 site. This residue is involved in 3 H-bonds with A221A and V17 (Figure 6(c)). The loss of these H-bonds leads to a deformed S1 site (Figure 6(d)). Also, disruption of the local H-bond network of D170 may be attributed to the increased mobility of the loop 2 (Figure 6(d)); (ii) the conformation of Loop 170s, including D170G-V170E and S170H-V170E H-bonds; (iii) the interactions of Loop 170s with Loop 1 and Loop 2. Disruption of these H-bonds S185-C170A, S170B-Q217 (Figure 6(c) and (d)), and R230-M180 (Figure 6(a)) is likely the cause of increased fluctuation of these loops; finally; (iv) the integrity of Loop 140s and the interaction of Loop 140s with the active site (Figure 7). In chimeric FVIIa, D146 loses two H-bonds to K192. The side-chain of K192 shifts towards the active site. There-
fore, the main chain amide nitrogen atoms (particularly of G193), that determine the catalytic oxyanion hole, shift (Figure 7). This active site change, combined with the position shifting of D189 caused by Si-site deformation, may reasonably affect the catalytic activity of FVIIa.

**Experimental Data by in vitro mutation**

From the simulation analysis, it appears that the EGF1 domain substitution has an effect that is transmitted to the catalytic domain. Alteration of the catalytic domain/TF interface appears
responsible. A major change appears to occur for Loop 170s. The effect resulting from the EGF1 substitution is initiated by perturbation of Loop 170s and is then passed on to the S1-substrate binding site and to Loop 140s, and finally to the residues critical for catalytic activity. To test this hypothesis, we generated four FVIIas with a single alanine substitution for each (L144A, R147A, D186A and K188A) that are located on the presumed TF-binding signal transmitting pathway; a multiple-mutation, MM3, a FVIIa that has mutations L144A, R147A and D186A; and a FVIIa with four residues (Q170, Q170A, S170B and R170C) deleted from Loop 170s (Loop 170sdel). The deletion removes two H-bond interactions of Loop 170s to Loop 1 and Loop 2. The results (Table 2) from the amidolytic activity assay for hydrolysis of the small substrate, S2288, show that the maximum reaction rate for the activated form of Loop 170sdel (Loop 170sdel, a: activated) decreases by more than twofold, for L144Aa by 4.5-fold, for D186Aa by nearly ninefold. R147Aa and K188Aa have slightly higher amidolytic activities than wild-type FVIIa. The multiple-mutation FVIIa, MM3, lost almost all amidolytic activity. The $K_d$ values (representing binding affinities to TF) of all mutants show little difference relative to wild-type FVIIa. The more detailed kinetics analysis data for Loop170sdel, a and the MM3 individual component mutations demonstrate consistent

![Figure 7. Alteration of the active site of FVII(IXegf1), TF. The active site is depicted by superimposing the catalytic domains of the final simulation structures of FVIIa (in blue) and FVII(IXegf1), a (in red). The H-bond interactions between residues Q143 and D146 on Loop 140s (right) and between D146 and K192 were interrupted in FVII(IXegf1), TF, which results in the position of the main-chain amide nitrogen atoms shifted in the active site.](image)

Table 2. Amidolytic activities and binding affinities of wt and mutant FVIIAs

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>$R_{max}$ (mOD/min) $^a$</th>
<th>$K_d$ (nM) $^b$</th>
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<tr>
<td>FVIIa</td>
<td>15.08(±0.74)</td>
<td>0.41(±0.10)</td>
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<tr>
<td>Loop170sdel,a</td>
<td>6.39(±0.39)</td>
<td>0.46(±0.02)</td>
</tr>
<tr>
<td>L144Aa</td>
<td>3.32(±0.11)</td>
<td>0.23(±0.02)</td>
</tr>
<tr>
<td>R147Aa</td>
<td>18.58(±0.88)</td>
<td>0.85(±0.20)</td>
</tr>
<tr>
<td>D186Aa</td>
<td>1.74(±0.01)</td>
<td>0.18(±0.02)</td>
</tr>
<tr>
<td>K188Aa</td>
<td>23.78(±1.89)</td>
<td>0.52(±0.33)</td>
</tr>
<tr>
<td>MM3a</td>
<td>0.18(±0.01)</td>
<td>0.49(±0.10)</td>
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$^a$ The maximum reaction rates towards S2288 in the presence of saturation concentration of TF. OD: optical density.

$^b$ $K_d$ represents the tissue factor binding affinity for FVIIa.
results (Table 3). L144Aa and D186Aa rates ($k_{cat}/K_m$, catalytic efficiency) decreased by six- to sevenfold relative to wild-type FVIIa. The differences in catalytic efficiencies resulted largely from the decreases in $k_{cat}$ of the mutants. It is also interesting to examine the effects that the mutants have on the enzymatic activity in the absence of TF. It allows us to examine if these residues are involved in the intrinsic catalytic activity, TF-enhanced catalytic activity or both. The results (Table 3) show that catalytic efficiencies for L144Aa and D186Aa were decreased by 0.4 to fourfold respectively without TF, indicating that L144 and especially D186 are involved in the catalytic activity of free FVIIa. FVIIa increases the catalytic activity of wild-type FVIIa toward S2288 by 36-fold, the mutant D186Aa by 7.93-fold, compared with the catalytic activities with the corresponding domains (superimposed with the individual FVIIa domains and TF for the simulation structures).

The EGF1 domain itself was not as stable in the FVIIa-IXegf1-α-TF simulation as for the wild-type simulation. Larger domain-domain motions relative to FVIIa between Gla and EGF1 and even between EGF1 and EGF2 occurred in FVIIa-IXegf1-α-TF. The RMSD of TF was also affected. The evidence of the domain-domain motions is clearly shown in the Cα distance plot (Figure 5), in which the Gla and EGF1 domains (superimposed with whole molecule as the reference) demonstrate much larger differences in Cα distance compared with the corresponding domains (superimposed with the individual domain as reference). These results indicate that substitution of the EGF1 domain has a significant effect on the local conformation of FVIIa. This effect is transmitted to TF and the catalytic domain, causing several regions to have increased position fluctuations and larger Cα distance deviations between wild-type and chimeric FVIIa. These motions in Gla and EGF1 domains are consistent with the experimental TF binding affinity, which decreases more than a hundredfold21 for the chimeric FVIIa. Furthermore, since two C terminus residues (K165, K166)29,30 and several other residues of Loop 159-16531 of TF are both near the Gla domain and have been shown to be involved in FX binding, the displacement of TF of the chimera (Figure 5) may be reasonably considered to contribute to the decreased interaction rate of FVIIa-IXegf1-α-TF with FX.21

### Table 3. Kinetics study of wild-type and mutant FVIIa with or without TF

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<tr>
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<th>$K_m$ (mM) (±SD)</th>
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<td><strong>With TF</strong></td>
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<tr>
<td>FVIIa</td>
<td>30.45 (±7.93)</td>
<td>0.83 (±0.26)</td>
<td>3.67 × 10$^4$</td>
</tr>
<tr>
<td>L144Aa</td>
<td>8.26 (±2.25)</td>
<td>1.52 (±0.11)</td>
<td>5.43 × 10$^3$</td>
</tr>
<tr>
<td>R147Aa</td>
<td>36.66 (±3.76)</td>
<td>0.65 (±0.22)</td>
<td>5.64 × 10$^4$</td>
</tr>
<tr>
<td>D186Aa</td>
<td>5.64 (±1.23)</td>
<td>0.95 (±0.39)</td>
<td>5.93 × 10$^3$</td>
</tr>
<tr>
<td>Loop 170sdelα</td>
<td>20.13 (±2.53)</td>
<td>1.43 (±0.42)</td>
<td>1.40 × 10$^4$</td>
</tr>
<tr>
<td><strong>No TF</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FVIIa</td>
<td>1.40 (±0.22)</td>
<td>1.36 (±0.18)</td>
<td>1.03 × 10$^5$</td>
</tr>
<tr>
<td>L144Aa</td>
<td>0.30 (±0.04)</td>
<td>0.44 (±0.16)</td>
<td>6.82 × 10$^3$</td>
</tr>
<tr>
<td>R147Aa</td>
<td>1.29 (±0.57)</td>
<td>0.88 (±0.22)</td>
<td>1.46 × 10$^4$</td>
</tr>
<tr>
<td>D186Aa</td>
<td>0.12 (±0.06)</td>
<td>0.47 (±0.39)</td>
<td>2.55 × 10$^3$</td>
</tr>
<tr>
<td>Loop 170sdelα</td>
<td>0.69 (±0.15)</td>
<td>2.15 (±0.63)</td>
<td>3.21 × 10$^3$</td>
</tr>
</tbody>
</table>

* Amidolytic activities towards S2288.
The EGF2 domain of FVIIa has a hydrophobic interaction with TF near the catalytic domain-TF interface. This interaction involves side-chains of Q88L, N93L, E94L, V92L, F50t, Y51t and K41t. The largely hydrophobic area interacts with Loop 121-124L through residues N95L, C98L, E99L and S126L. The latter residue forms two H-bonds with loop residue D123L, which are essential for Loop 121-124L stability. In FVIIα(IXegf1)a-TF, however, F50t and Y51t of TF are displaced in such a manner that the hydrophobic interaction in this region is diminished. Also the two H-bonds of S126L to D123L are lost, resulting in increased fluctuation of Loop 121-124L (Figure 4). Furthermore, because of the loss of the H-bond N93L-L89L for FVIIα(IXegf1)a-TF, the Q88L-K85L bond, which is responsible for stability of the hinge between EGF1 and EGF2, was also lost, resulting in higher B-factors for residues 85L to 89L of the chimera relative to the FVIIa-TF simulation. These changes in the EGF2 domain may contribute to the decreased TF-binding affinity of the chimeric FVIIa. No significant rotation of the EGF2 domain relative to the catalytic domain occurred in the chimeric FVIIα simulation. The observed EGF2 domain rotation of free FVIIa15 relative to the protease domain of TF-bound FVIIa could be caused by the disordered EGF1 domain of the Gla-domainless FVIIa.

The surface loops of FVIIa-TF and its chimera have substantial fluctuations (Figure 4). For the chimera complex the B-factors at residues 170C to 175, 185 to 188 and 221A to 224 are roughly twice those of FVIIa-TF, indicating that these areas are most susceptible to the alterations in the catalytic domain-TF interface. The position shifting of these loops15 relative to the FVIIa-TF complex and disordering of Loop 170s16 are observed in free FVIIa. These three loops are spatially adjacent and connected via several H-bond interactions (Figure 6(c)). It is likely that the greater fluctuations are caused by disruption of the catalytic domain-TF interface,15 resulting in a breakdown of the local H-bond network among these loops (Figure 6(d)). This structural difference may account for the decreased activity of FVIIa in the absence of TF.

D186 is one of critical residues for maintaining the integrity of Loop 1 conformation. In wild-type FVIIa, residue D186 is involved in three H-bonds (Figure 6(c)). In FVIIα(IXegf1)a-TF, only S188A-D186 was formed (Figure 6(d)). Due to the loss of the S185-Q170A and Q217-S170B H-bonds and G223-S185 H-bond formation, Loop 1 refolded. It is reasonable to conclude that impairment of the local H-bond network involving Loop 1 leads to deformation of the S1-binding site. This speculation is supported by in vitro mutagenesis of D186 in this study (Tables 2 and 3) and by the work of Petrovan and Ruf.13 Mutant D186Aa is defective in protection of the N-terminal terminus from the chemical modification in the presence of TF.13 The mutation K188A, however, showed no effect and even a small increase in activity, probably because the mutant retained significant D186 H-bond interactions. The importance of Loop 2 has also been observed.13 F225, a partially buried residue and located in the end of Loop 2, has been suggested as an independent linkage to transmit the activation signal from the protease-TF interface to the active site.13 Consistent with that study, the position of F225 was significantly moved from wild-type (Figure 5), indicating that both presumed mechanisms15 (enhancing FVIIa's catalytic activity with/without affecting the N terminus insertion respectively) could be in response to the substitution of the EGF1 domain on the catalytic activity of FVIIa. E154, which is located in the macromolecule binding exosite of the catalytic domain, may also be involved.26

The H-bonds D189-V17 and V17-D189 of Loop 1 provide support for the catalytic domain N terminus insertion (Figure 6(c)), based on their positions adjacent to the N terminus insertion residue I16. The H-bond K20-V157 may also be involved. The N terminus insertion is thought to be associated with the mechanism of Sp activation32 and stabilized by TF-binding FVIIa.13,33-36 Active site occupation by inhibitor has also been suggested to provide stabilization for the N terminus insertion.16 The N terminus insertion of FVIIα(IXegf1)a might be expected to have a significant shift in position, due to the loss of the H-bonds K20-V157, V17-D189 and D189-V17. However, the morphology of the insertion region shows little change in the chimera. Therefore, other factors must play key roles in maintaining the integrity of the N terminus insertion. In addition to Loop 1, the insertion region consists of Loop 140s and residues G18 and V17. M156 is also near the insertion in this hydrophobic region (Figure 1). This residue is considered to be a determinant for the zymogen-like properties of FVIIa.36 The residues G142, Q143, L144 and L145 might be important for maintaining the stability of the N terminus insertion, since they are either fully or partially buried and nearby the insertion. In general, the structure surrounding the N terminus insertion remains similar between wild-type and chimeric molecules. The Cα-distances of residues 144 to 150 of Loop 140s between the chimera and the wild-type FVIIa show some deviations (Figure 5), which appear to be caused by loss of the H-bonds K20-V157, V17-D189 and D189-V17. Although the motion of Loop 140s (Figure 5) of the chimera results in only small alteration of the I16-D194 interaction, an intra-loop H-bond Q143-D146 and two H-bonds of D146 to K192 are lost (Figure 7). Disruption of the H-bond interaction of K192 with Loop 140s may affect the formation of the oxyanion hole of the active site, since K192 is adjacent to G193, which provides stabilization of the oxyanion hole. K192 has been shown to play a role in FX activation12,26 and chromogenic-substrate hydrolysis.26

The importance of Loop 140s for FVIIa catalytic activity has been demonstrated in another X-ray crystal study28 of FVIIα in complex with a peptide
Concluding comments

Several X-ray crystallography studies have been performed to study the mechanism by which TF enhances FVIIa's catalytic activity.\textsuperscript{5,15,16,37} Because of the crystal packing and the inhibitor occupation in the active site, it is difficult to observe inhibitor-free FVIIa and inhibitor-free TF-complexed FVIIa. Our simulations employ FVIIa/FVII\textsubscript{(IX\textsubscript{EGF1})}a-TF complexes without inhibitor to investigate the conformational changes that result from the EGF1 domain substitution and thereby modify the TF-mediated enhancement of FVIIa's catalytic activity. We find that, in addition to significant inter-domain displacements that occur in the light chain of FVIIa, the most flexible regions in the catalytic domain of FVII\textsubscript{(IX\textsubscript{EGF1})} are those of Loop 1, Loop 2 and Loop 170s. The N terminus insertion has not been appreciably affected by these more flexible loops, although Loop 140s results in a slight alteration of the active site. This work demonstrates a complementary approach (simulation) for studying the mechanism of EGF1 domain substitution on the catalytic activity of FVIIa. Our combined simulations and experiments suggest that the stability and appropriate positioning of the light chain is important for full cofactor activity. Loop 1, Loop 2, Loop 140s and Loop 170s in the catalytic domain of FVIIa transmit the TF-mediated effect through the catalytic domain-TF interface. Alteration of these regions appears to cause deformation of the S1 substrate binding site and to destabilize the N terminus insertion, with a concomitant effect on the active site. Our model thus provides new insight into the mechanism by which TF enhances FVIIa's catalytic activity.

Computational Procedure

Model constructions and simulation protocol

The initial structure of FVIIa-TF (residues 1 to 142, 153 to 406 of FVIIa and residues 6 to 80, 91 to 158 and 163 to 210 of TF) for modeling was obtained from entry 1dan in RCSB Protein Data Base (PDB). Because of the subtilisin treatment (residues <6, residues >210, and the cleavage loop 85-89) and disorder of several loops (residues 135 to 138, 158 to 164, and 181 to 183), the coordinates for residues 1 to 5, 81 to 90, 159 to 162 and after 210 were missing, resulting in the extracellular three fragments, fragment 1(TF-f1, residues 6 to 80), fragment 2 (TF-f2, 91-158) and fragment 3 (TF-f3, 163-210). TF-f1 and TF-f3 are located within the carboxyl and N-terminal domains, respectively, of TF for residues 1 to 5, 81 to 90, 159 to 162 and 210. TF-f1 and TF-f3 consist of sections of the C and N domains. The simulation was performed as described by Perera \textit{et al.}\textsuperscript{25,38} All nine Ca\textsuperscript{2+} coordinates assigned in the X-ray crystal form, including 239 crystallographic water molecules, were preserved in the initial structure. Necessary hydrogen atoms and counter ions were added to the X-ray crystallographic structure, followed by energy
minimization of the side-chains (500 steepest descent plus 10,000 conjugate gradient steps). For construction of the FVIIa chimera with FIX’s EGF1 domain (FVII(IXegf1)a), 12 residues in FVIIa, which differ in FVII and FIX EGF1 domains, were sequentially mutated, one at a time, to FIX’s residues, with sequential energy minimization of the side-chains (500 steepest descent and followed by 10,000 conjugated gradient steps) for each mutation. After all mutations were completed, the energy minimization of the side-chains, counterions, calcium and crystal water was carried out for 10,000 steps, followed by energy minimization of the entire system.

The proteins, along with the crystallographic water molecules, the calcium ions and the counter ions, were then solvated in a box of water molecules, for which the boundaries were 12.5 Å away from any protein atom. Water molecules were excluded if any atom was less than 2.0 Å from the proteins. The simulation box contained 587 residues, 25,195 water molecules for FVIIa-TF (25,432 for FVII(IXegf1)a-TF), 9 calcium ions and counter ions Cl⁻ (seven for FVIIa-TF, four for FVII(IXegf1)a-TF). The counter ions ensure electrical neutrality. The added water molecules were then energy minimized at constant volume. All of the water molecules were then subjected to energy minimization. Finally, the entire system was energy minimized. The system was subsequently subjected to a slow heat-up procedure to bring the temperature of the system to 325 K. After 25 ps of a constant volume/constant temperature simulation, the system was brought back to 300 K, followed by a constant T/V simulation for 50 ps. In the final, major step, a constant T/P protocol was adopted to simulate 510 ps dynamics for wild-type FVIIa-TF and 810 ps for FVII(IXegf1)a-TF (the coordinates are available from jjin@unc.edu).

Here, we used the AMBER version 5.0 in conjunction with the particle mesh Ewald method to accommodate long-range interaction (both Lennard-Jones and electrostatic interactions) in all solution simulations. The time step was 1 fs, with the non-bonded interactions updated at every step. The translational and rotational motions of the center of mass were removed at the beginning and regular 2.5-ps intervals. All covalent bonds involving hydrogens were constrained by a modified form of SHAKE.

### Experimental Materials and Methods

#### Materials

The recombinant TF apoprotein (residues 1 to 243) was a generous gift from Dr Gordon Vehar (Genentech, Inc., South San Francisco, CA). Chromogenic substrate S-2288 (H-D-isoleucyl-L-prolyl-L-arginine-p-nitroanilinedihydrochloride: H-D-Ile-Pro-Arg-pNA-2HCl) was purchased from DaiPhama Group, Inc. (West Chester, OH). The E.I.A/RIA plates for amidolytic assays were purchased from Costar Corporation (Cambridge, MA). Oligonucleotides were synthesized by GIBCO BRL Life Technologies (Grand Island, New York). Restriction enzymes, ligase and DNA polymerase were purchased from New England Biolabs (Beverly, MA). Vitamin K was from Abbott Laboratories (North Chicago, IL). Insulin-transferrin-sodium selenite tissue culture supplement was from Boehringer Mannheim (Indianapolis, IN). Dulbecco’s Modified Eagle Medium (D-MEM/F-12) was from GIBCO BRL Life Technologies (Grand Island, New York).

#### Site-directed mutagenesis

A human FVII cDNA construct was used as the starting material to create mutant FVII’s. A megaprimer PCR was performed to generate constructs with mutations. The PCR consisted of two steps which were as follows: the first step was to generate a megaprimer (200 bp < megaprimer < 500 bp) by using a pair of oligonucleotide primers, one of which contained the mutation desired; the PCR product from the first round PCR was isolated from an agarose gel; after purification, the megaprimer was used to perform the second round of PCR with another oligonucleotide primer, that was complementary to the opposite strand DNA relative to the first primer. The expected mutations and full sequence of the constructs were confirmed by sequencing performed by the UNC-CH Sequencing Facilities.

#### Expression, purification, activation and characterization of proteins

Wild-type and mutant FVII’s were expressed, purified and activated essentially as described by Jin et al.

#### Amidolytic activity assay and kinetics analysis

The assays were performed as described by Jin et al.

### Acknowledgments

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### References


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