Ca\(^{2+}\) Binding to the First Epidermal Growth Factor-like Domain of Factor VIIa Increases Amidolytic Activity and Tissue Factor Affinity*

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Coagulation factor VIIa belongs to a family of homologous enzymes, including factors IXa and Xa and activated protein C, composed of two epidermal growth factor-like domains located between an N-terminal domain rich in \(\gamma\)-carboxyglutamic acid residues and a C-terminal serine protease domain. The first epidermal growth factor-like domain in factor VIIa contains a Ca\(^{2+}\) binding site, the function of which is largely unknown. Site-directed mutagenesis of two Ca\(^{2+}\)-liganding Asp residues in this domain abolished Ca\(^{2+}\) binding and resulted in a 2–3-fold decrease in amidolytic activity at optimal Ca\(^{2+}\) concentrations. The lower amidolytic activity persisted in complex with soluble tissue factor, apparently due to a lower \(k_{cat}\) of the mutant factor VIIa. Mutant and wild-type factor VIIa bound to lipitated tissue factor were equally efficient activators of factor X. The dissociation constants, derived from amidolytic activity and surface plasmon resonance measurements, were 2–5 nM and 50–60 nM for the interactions with wild-type and mutant factor VIIa, respectively, and soluble tissue factor. Binding to lipitated tissue factor was characterized by dissociation constants of 7.5 pM for factor VIIa and 160 pM for the factor VIIa mutant. Hence, a functional Ca\(^{2+}\) binding site in the first epidermal growth factor-like domain added 7–8 kJ/mol to the total binding energy of the interaction with both lipitated and soluble tissue factor.

The epidermal growth factor (EGF)-like domain is a widespread building block in extracellular proteins, containing 40–50 amino acid residues with a characteristic disulfide bond pattern. This type of domain is well represented among proteins involved in blood coagulation, and the homologous factors VII (fVII), IX, and X and protein C contain two of them located in the membrane-proximal \(\gamma\)-carboxyglutylgamic acid (Gla)-containing domain and the serine protease domain (1, 2). The first EGF-like domain in these proteins harbors one Ca\(^{2+}\) binding site (3–7). The side chains of two Asp, one of which may be \(\beta\)-hydroxylated, and one Gln residue and two backbone carbonyl oxygens have been identified as Ca\(^{2+}\) ligands in the first EGF-like domain of factors IX and X (8, 9) and the ligands are conserved in fVII and protein C. The affinity of this site in factor X is represented by a dissociation constant (\(K_d\)) of about 0.1 mM in the intact protein (10), in a fragment containing the Gla and first EGF-like domains (11), and in a Gla-EGF fragment from which the N-terminal 28 amino acid residues have been deleted (12). Hence, under physiological conditions, the bound Ca\(^{2+}\) ion and the resulting Ca\(^{2+}\)-dependent structure can be considered as a structural determinant of the protein. In contrast, Ca\(^{2+}\) binding to the isolated EGF-like domain has a 20-fold higher \(K_d\) (4).

The interaction between factor VIIa (fVIIa) and its cell surface receptor tissue factor (TF) is Ca\(^{2+}\)-dependent and involves all four domains in fVIIa (13). The importance of the Ca\(^{2+}\) site in the first EGF-like domain is presently unclear. This is in part due to the complex nature of Ca\(^{2+}\) binding to a total of nine sites in fVIIa (13): seven in the Gla domain (14), one in the first EGF-like domain (7), and one in the protease domain (15). To address this question, we have mutated Asp-46 and Asp-63 in fVIIa to Asn, replacements that, based on studies of Ca\(^{2+}\) binding to synthetic variants of the first EGF-like domain from factor IX, should completely abolish Ca\(^{2+}\) binding to this site (16, 17). We present the results of the characterization of the double mutant (D46N,D63N-fVIIa) and its TF binding properties, as well as the functional status of its complex with TF. Possible functions of Ca\(^{2+}\) binding to the first EGF-like domain, such as structural stabilization of the hinge region between the Gla and first EGF-like domains and proper presentation of residues interacting with TF, are discussed.

**EXPERIMENTAL PROCEDURES**

*Proteins and Standard Methods—*The isolation of recombinant fVIIa (18) and D46N,D63N-fVIIa (19) was carried out as described, and the Gla-domainless forms were prepared by cleavage with \(\alpha\)-chymotrypsin (20) followed by purification on a column of Q Sepharose (Pharmacia Biotech Inc.). The protein concentrations were determined using a fVIIa (20) followed by purification on a column of Q Sepharose (Pharmacia Biotech Inc.). The protein concentrations were determined using a fVIIa enzyme-linked immunosorbent assay and by absorbance measurements using an absorption coefficient (\(\varepsilon_1\)) of 13.2. Amino acid analyses were performed as described (4). The resistance of fVIIa and D46N,D63N-fVIIa against proteolytic cleavage was tested by incubation with cathepsin G (ICN Biomedicals, Inc.) and \(\alpha\)-chymotrypsin (Sigma), using 0.1% (w/v) enzyme, in 50 mM Tris, 0.1 mM NaCl, pH 8.0, containing 2 mM EDTA, 0.3 mM CaCl\(_2\), or 1.5 mM CaCl\(_2\). The production and isolation of soluble TF (sTF) has been described (21, 22), and the concentration was estimated using an absorption coefficient of 15.0. Full-length TF was purchased from American Diagnostica Inc. and repurified as described (23) using 75% phosphatidylcholine, 25% phosphatidylethanolamine (Sigma). Factors X and Xa were from Enzyme Research Laboratories. SDS-PAGE was run in 12% gels using ingredients from Serva and Bio-Rad (24). Agarose gel electrophoresis (0.8% gels) was run in 75 mM Tris, 25 mM 5,5-diethylbarbiturate, pH 8.6, containing either

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10 mM CaCl$_2$ or 2 mM EDTA, using agarose purchased from Litex.

**FVII cDNA Construction, Transfection, and Expression**—The wild-type FVII expression plasmid, pLN174, has been described previously (19). For the construction of the mutant cDNA (D46N,D63N), we used FVII cDNA inserted in the cloning vector pBlueScript II KS$^+$ (Stratagene, La Jolla, CA) as described by PCR (28) with the following two primers: 5′-GCC ATT CTG GCA TGG ACT TGA GGC ACA CTG GTC CCC ATT ACT GTA AGA-3′ and 5′-GCC TCA AGT CCA TGC CAG AAT GGG GCC TTC TGG AAC AAC CAC CTC CAG-3′. In brief, the template plasmid was denatured by treatment with NaOH followed by PCR with Pwo polymerase (Boehringer Mannheim). Escherichia coli were transformed with the resulting PCR product, and clones were screened for the presence of the mutations. The sequence was verified between the BamHI site before the initiation codon and the XhoI site (base number 534 of the FVII coding sequence) for one clone containing the desired mutation. The BamHI-XhoI fragment was removed from the plasmid pLN174 and replaced with the corresponding fragment from the mutant plasmid. The presence of the mutations in the final expression construct was verified by sequencing. The baby hamster kidney cell line BHK570 (ATCC CRL 1632) was used for transfection and expression of mutant FVII protein as described earlier (19). Cell culture materials were from Life Technologies, Inc.

**Amidolytic Assays**—The amidolytic activity of FVIIa and D46N,D63N-FVIIa and their Gla-domainless counterparts (final concentration 150 nM) was measured in the presence of 1 mM S-2288 (Chromogenix) in 20 mM Hepes, 0.1 mM NaCl, pH 7.4, containing 1 mg/ml BSA and either 2 mM EDTA or 5 mM CaCl$_2$ (26). The absorbance at 405 nm was monitored for 45 min.

The sTF-induced amidolytic activity enhancement was determined by mixing 10 nM sTF or 10 nM D46N,D63N-FVIIa with various concentrations of sTF (0–2 μM) at 5 mM CaCl$_2$ and the conditions described above. Alternatively, FVIIa or D46N,D63N-FVIIa (0–500 nM) was added to 10 nM sTF. The absorbance at 405 nm was monitored for 30 min. The activity of FVIIa or D46N,D63N-FVIIa alone was subtracted.

To investigate differences between FVIIa and D46N,D63N-FVIIa in K$_m$ for S-2288, we subjected 0.1–2 mM substrate to hydrolysis by 100 nM FVIIa, 100 nM D46N,D63N-FVIIa, 10 nM FVIIa/10 nM sTF, or 10 nM D46N,D63N-FVIIa/100 nM sTF. The hydrolysis rate at different substrate concentrations was divided by the rate at 2 mM S-2288. The Ca$^{2+}$ dependence of FVIIa and D46N,D63N-FVIIa binding to sTF was studied at the same protein concentrations in the Hepes buffer containing gelatin instead of BSA.

In the factor X activation assay, FVIIa and D46N,D63N-FVIIa (final concentration 2–5 nM) were mixed with a fixed effective concentration of sTF (2.5 μM assuming quantitative reconstitution and that half the molecules are oriented outward from the vesicles) in 50 μl of 20 mM Hepes, pH 7.4, containing 0.1 mM NaCl, 5 mM CaCl$_2$, and 1 mg/ml BSA. The reaction was started by adding factor X (final concentration 175 nM), giving a final volume of 100 μl. After 5 min, the reaction was terminated by adding 50 μl buffer containing 20 mM EDTA instead of CaCl$_2$. The generated factor Xa was quantified by subsequent incubation of 50 μl of 2 mM S-2765 (Chromogenix), and the absorbance at 405 nm was measured after 10 min. The factor Xa concentration was derived from a standard curve.

**Surface Plasmon Resonance Measurements**—The conditions of sTF immobilization and regeneration of the sTF-coated surface in the BIAcore instrument (BIAcore AB, Uppsala, Sweden) have been described (27). All experiments were performed at a flow rate of 5 μl/min, and the interaction between FVII and sTF at 5 mM CaCl$_2$ was also monitored at 10 μl/min without any sign of mass transport limitation. For other experimental details, see the legend to Fig. 4. Association and dissociation phases lasted for 7 and 5 min, respectively. The presence of 2 μM sTF in solution during the dissociation phase did not affect the dissociation rate, showing that there was no rebinding of released FVIIa. Binding data was fitted by non-linear regression to a one-site model using BIAevaluation 2.1 supplied with the instrument. In the Ca$^{2+}$ dependence study, 50 nM FVIIa or 200 nM D46N,D63N-FVIIa was injected at different Ca$^{2+}$ concentrations over 1760 resonance units of sTF and the amount of bound protein after 15 min of association was measured.

**Modeling**—The structures of FVIIa residues 1–85 were built using Modeler implemented in the modeling package Quanta (Molecular Simulations, Inc., San Diego, CA). The models of the apo and 1 Ca$^{2+}$ forms were based on the apo and 1 Ca$^{2+}$ structures of the corresponding part of bovine factor X (entry codes 1WHE and 1WHF, respectively) (28). The model of the Ca$^{2+}$-loaded Gla domain was based on the Ca$^{2+}$ structure of the prothrombin Gla domain (entry code 2PF2) (29) and used to produce the Ca$^{2+}$-loaded form of the Gla-EGF frag-

![FIG. 1](image374x625to498x729)

**RESULTS**

Purification and Characterization of D46N,D63N-FVIIa—The single-step affinity-chromatographic purification resulted in a homogeneous preparation of D46N,D63N-FVIIa with a yield of 1.3 mg/liter of medium. The ability of the mutant to bind to the antibody (which recognizes a Ca$^{2+}$-dependent epitope in the Gla domain) employed as affinity ligand indicated that it was properly γ-carboxylated (26), and amino acid analysis showed 9.7 mol of Gla/mol of D46N,D63N-FVIIa (the calculated number according to sequence data is 10). SDS-PAGE showed that the isolated mutant was in the two-chain, activated form with the bands corresponding to the heavy and light chains of FVIIa (data not shown). The conversion from single-chain D46N,D63N-FVII appeared to occur during purification. The starting material contained no amidolytic activity enhancable by the addition of sTF, whereas the activity of the purified protein could be stimulated by sTF (31, 32).

Amino acid substitutions in the isolated first EGF-like domain from factor IX (16, 17) suggest that the two Asp → Asn mutations in D46N,D63N-FVIIa should abolish Ca$^{2+}$ binding to the first EGF-like domain. However, it is known that the Ca$^{2+}$ site in the first EGF-like domain has a higher affinity in the intact protein due to the presence of additional ligands and/or increased structural stability. To confirm that the mutations had eliminated Ca$^{2+}$ binding, the electrophoretic mobility of Gla-domainless mutant and wild-type FVIIa was analyzed on agarose gels in the presence and absence of Ca$^{2+}$. Mutant and wild-type Gla-domainless FVIIa should contain one and two Ca$^{2+}$ binding sites, respectively. The removal of two negative charges in the mutant reduced its anodal migration rate in the absence of Ca$^{2+}$ compared with that of FVIIa (Fig. 1). In the presence of 10 mM Ca$^{2+}$, the mobility of both proteins was further reduced. The Ca$^{2+}$-induced mobility shift was twice as big for FVIIa compared with D46N,D63N-FVIIa, indicating that the mutagenesis had successfully abolished Ca$^{2+}$ binding to the EGF-like domain. The extra Ca$^{2+}$-bound to FVIIa compensated for its two additional negative charges, resulting in identical mobilities of the two FVIIa forms in the presence of Ca$^{2+}$.

In the absence of Ca$^{2+}$, D46N,D63N-FVIIa and FVIIa had similar specific amidolytic activities and the two proteins displayed similar, low affinity binding to sTF as measured by surface plasmon resonance (data not shown). Hence, there are no Ca$^{2+}$-independent differences between them. However, the amidolytic activity of FVIIa was stimulated by Ca$^{2+}$ to a 2–3-
fold higher level than that of D46N,D63N-fVIIa (Fig. 2), suggesting that a functional Ca\textsuperscript{2+} site in the EGF-like domain is required for full activity. Interestingly, the Gla-domainless forms of fVIIa and D46N,D63N-fVIIa were similarly stimulated by Ca\textsuperscript{2+}. The data suggest that the presence of the Gla domain increases the activity in the case of a functional Ca\textsuperscript{2+} site in the EGF-like domain.

The interaction between D46N,D63N-fVIIa and TF and Activity of the Complex—The ability of sTF to enhance the activities of fVIIa and D46N,D63N-fVIIa allows the use of an amidolytic assay for the estimation of their affinities for the cofactor. In our system, the amidolytic activity of fVIIa was maximally enhanced at sTF concentrations above 50 nM and half-maximum was observed between 5 and 10 nM (Fig. 3). The data yielded a $K_d$ of $3.7 \pm 0.7$ nM. D46N,D63N-fVIIa displayed half-maximum around 100 nM sTF and was fully stimulated at sTF concentrations above 1 \mu M. A $K_d$ of $54 \pm 8$ nM was calculated for the mutant, i.e., 25-fold higher than that of fVIIa. Moreover, the mutant displayed lower activity than fVIIa at saturating concentrations of sTF, similar to what we observed in the absence of sTF. Almost identical results were seen when titrating a constant amount of sTF with fVIIa or D46N,D63N-fVIIa (Fig. 3). However, the sTF-independent activity prevented us from going higher than 500 nM in D46N,D63N-fVIIa. To elucidate the reason for the lower amidolytic activity of D46N,D63N-fVIIa-sTF, substrate hydrolysis was measured at varying concentrations of S-2288. Although the $K_m$ for S-2288 appears to be high and could not be accurately determined, the relative rate of hydrolysis obtained with fVIIa-sTF and D46N,D63N-fVIIa-sTF was identical when compared with the respective rate at the highest substrate concentration (which was lower for D46N,D63N-fVIIa-sTF). This indicates that fVIIa-sTF and D46N,D63N-fVIIa-sTF have similar $K_m$ values for S-2288 and that the difference in catalytic efficiency appears to be due to a lower $k_{cat}$ of the mutant in complex with sTF. The Ca\textsuperscript{2+} dependence of fVIIa and D46N,D63N-fVIIa binding to sTF was also investigated. At our protein concentrations, low levels of Ca\textsuperscript{2+} (0.1–0.2 mM) resulted in a larger absolute increase in fVIIa-sTF activity, due to the higher affinity of this complex, than in D46N,D63N-fVIIa-sTF activity. Nevertheless, these Ca\textsuperscript{2+} concentrations were sufficient to stimulate D46N,D63N-fVIIa-sTF, but not fVIIa-sTF, half-maximally (not shown). This suggests that the Ca\textsuperscript{2+} dependence of D46N,D63N-fVIIa was mediated solely by the Ca\textsuperscript{2+} site in the protease domain and that the Glu-EGF region has no influence in the absence of the Ca\textsuperscript{2+} site in the first EGF-like domain.

The interaction between D46N,D63N-fVIIa and sTF was then compared with that between fVIIa and sTF in a BIAcore instrument. The real-time biosensor analysis of the fVIIa-sTF interaction yielded a $K_d$ of 4.6 nM, in agreement with previous studies (19, 27, 33). The mutant D46N,D63N-fVIIa associated about 3-fold slower with sTF ($1.1 \times 10^5$ s\textsuperscript{-1} s\textsuperscript{-1}) and dissociated about 3.5-fold faster from sTF ($5.9 \times 10^{-3}$ s\textsuperscript{-1}) which resulted in a $K_d$ of 54 nM (Fig. 4). The $K_d$ values are in agreement with those derived from the sTF-dependent amidolytic activity assay. In addition, the Ca\textsuperscript{2+} dependence of the binding of fVIIa and D46N,D63N-fVIIa to sTF in the BIAcore closely resembled the Ca\textsuperscript{2+} dependence of fVIIa-sTF and D46N,D63N-fVIIa-sTF amidolytic activity (data not shown).

Wild-type fVIIa and D46N,D63N-fVIIa also had different affinities for phospholipid-embedded TF. The $K_d$ values were estimated to be 7.5 ± 0.4 pM for fVIIa, in good agreement with earlier studies (14, 34), and 158 ± 16 pM for D46N,D63N-fVIIa (Fig. 5). In our system, with a fixed concentration of TF, maximal factor X activation was observed above 200 pM fVIIa and 2 nM D46N,D63N-fVIIa. There was no difference in the maximal rate of factor Xa generation between fVIIa-TF and D46N,D63N-fVIIa-TF. Both complexes catalyzed the generation of approximately 300 mol of factor Xa/mol of TF/min, assuming that all TF molecules were successfully reconstituted
Ca$^{2+}$ in the First EGF Domain of Factor VIIa

FIG. 4. Sensorgrams for the interactions between wild-type fVIIa (top curve) and D46N,D63N-fVIIa (bottom curve) and immobilized sTF. 50 nM of analyte in 20 mM Hepes, pH 7.4, containing 0.1 mM NaCl, 5 mM CaCl$_2$, and 0.02% Tween 80, was injected over 1620 resonance units (RU) of sTF. The signals obtained when injecting the samples over a blank biosensor surface have been subtracted.

FIG. 5. Factor X activation by fVIIa-TF and D46N,D63N-fVIIa-TF. The indicated concentrations of fVIIa (○) or D46N,D63N-fVIIa (●) were mixed with relipidated TF and factor X (see “Experimental Procedures” for details). The amount of factor Xa generated was quantified employing S-2765 amidolyis and given as percent of maximal activation. Each point represents the mean of two experiments. The data were fitted to a one-site model using GraFit 3.0 (Erithacus Software Ltd., Staines, Middlesex, United Kingdom).

into phospholipid vesicles and that 50% of them were functionally available on the outside of the vesicles.

Resistance of fVIIa and D46N,D63N-fVIIa against Proteolytic Degradation—Cleavage sites in the hydrophobic cluster in the C-terminal part of the Gla domain have been exploited for more than a decade to produce Gla-domainless clotting factors. Since the Ca$^{2+}$ binding site in the first EGF-like domain is located close to the hydrophobic stack (or C-terminal helix of the Gla domain) and might affect the conformation of this hinge region between the Gla and first EGF-like domains, we examined if the mutations had any effect on the rate of enzymatic cleavage. fVIIa was rapidly cleaved in the hydrophobic stack by chymotrypsin and cathepsin G in the absence of Ca$^{2+}$ as monitored by SDS-PAGE, and considerable protection against degradation. Identical results of the chymotrypsin-cathepsin G in the absence of Ca$^{2+}$ should give more than 70% saturation of the Ca$^{2+}$ binding site in the first EGF-like domain of fVIIa, but no structural effects were observed when the mutations were introduced into phospholipid vesicles. However, 0.3 mM Ca$^{2+}$, which should give more than 70% saturation of the Ca$^{2+}$ site in the first EGF-like domain of fVIIa, did not result in any protection against degradation. Identical results of the chymotrypsin-catalyzed cleavage were obtained with D46N,D63N-fVIIa, whereas cathepsin G was unable to cleave the mutant under any of our conditions. Hence, no evidence for a specific protective effect of an intact Ca$^{2+}$ binding site in the EGF-like domain was obtained.

DISCUSSION

The first EGF-like domain of fVIIa is known from biochemical studies (35–37) as well as from the x-ray crystallographic structure of the fVIIa-sTF complex (13) to interact with TF, but the importance of the Ca$^{2+}$ site in this domain is unclear. Based on Ca$^{2+}$ structures of EGF-like domains (8, 9) and their homology and high degree of sequence identity with the corresponding domain in fVIIa, the Asp residues in positions 46 and 63 in fVIIa were replaced by Asn to abolish and investigate the role of Ca$^{2+}$ binding to the first EGF-like domain. D46N,D63N-fVIIa was shown not to bind Ca$^{2+}$ in the EGF-like domain, and no structural effects were observed when the mutations were put into our model of fVIIa (22). D46N,D63N-fVIIa and fVIIa displayed similar amidolytic activity and similar, poor binding to sTF in the absence of Ca$^{2+}$, indicating that the proteins only differed when in their Ca$^{2+}$ conformations.

A 2–3-fold difference in specific amidolytic activity between fVIIa and D46N,D63N-fVIIa was observed in the presence of Ca$^{2+}$, both in the absence of and in complex with sTF. Thus the stimulatory effect of sTF, which is known primarily to result from an increase in $k_{cat}$ (31, 38), appears to be the same on both forms of fVIIa. However, the reasons for the impaired activity in the absence and presence of sTF might be different. The lower amidolytic activity of D46N,D63N-fVIIa is in agreement with a study of factor IXa mutants in which the residue corresponding to Asp-63 was replaced by Lys, Glu, or Val (39). Ca$^{2+}$ binding to the first EGF-like domain appears to be involved in the Ca$^{2+}$-induced increase in amidolytic activity, perhaps through an interaction between a Ca$^{2+}$-dependent structure in the Gla-EGF region and the protease domain. The similar maximal factor X activation rates by fVIIa and D46N,D63N-fVIIa in complex with TF suggest that factor X and/or the phospholipid membrane is able to stabilize the optimally active conformation of both complexes (40).

We find that D46N,D63N-fVIIa binds both sTF and lipidated TF with approximately 20-fold higher $K_a$ than does wild-type fVIIa, showing that Ca$^{2+}$ binding to the first EGF-like domain is essential for optimal cofactor binding. The affinity of D46N,D63N-fVIIa for sTF is similar to that of the most defective Ala mutant (I69A) in the EGF-like domain (41). In addition, the kinetics of the interaction between D46N,D63N-fVIIa and sTF closely resemble those of the interaction between K79Q-fVIIa and sTF (33). Arg-79 is a key TF-interactive residue located in the C-terminal part of the first EGF-like domain (13). However, in contrast to D46N,D63N-fVIIa, this mutant displayed a lower rate of factor X activation than fVIIa but similar amidolytic activity. The replacement of Asp-47 or Asp-64 in the first EGF-like domain from factor IX (corresponding to Asp-46 and Asp-63 in fVIIa) by Asn is known to abolish or severely impair Ca$^{2+}$ binding (16, 17). Mutations at these positions result in very low biological activity of factor IXa causing hemophilia B (39, 42–47). The primary reason for the low activity appears to be a defective interaction with factor VIIIa. The effects of mutating Ca$^{2+}$-coordinating residues on the fVIIa-TF and factor IXa-factor VIIIa interactions suggest that Ca$^{2+}$ binding to the first EGF-like domain in the vitamin K-dependent clotting factors is important for optimal recognition of their cofactors.

Recently it was found, using a Gla-EGF fragment from factor X, that the relative orientation of the Gla and first EGF-like domains was altered upon Ca$^{2+}$ binding to the latter domain.
This strongly suggests that the folding of the Gla domain over the Ca\(^{2+}\)-binding site in the first EGF-like domain is a general feature of these proteins (Fig. 6). In addition, calorimetric and chromatographic data supportive of a Ca\(^{2+}\)–induced interaction between the Gla and first EGF-like domains in factor IX have been presented (50, 51) and a Ca\(^{2+}\)–dependent interaction stabilizing the disulfide bonds in the two domains has been demonstrated in factor X (52). In contrast, Gla-EGF from protein Z, which contains the Trp residue in the hydrophobic stack hinge region, does not exhibit any fluorescence increase (53). We therefore postulate that Ca\(^{2+}\) binding to D46N,D63N-fVIIa, occurring outside the first EGF-like domain, is not responsible for the relative orientation of the Gla and first EGF-like domains, whereas Ca\(^{2+}\) binding to fVIIa may affect the domain orientation.

What is the exact role of the Ca\(^{2+}\) site in the first EGF-like domain of fVIIa? Since the Ca\(^{2+}\)–induced orientation of the Gla and first EGF-like domains in Gla-EGF from factor X (28), likely to be present in fVIIa but not in D46N,D63N-fVIIa, is not found in fVIIa in complex with sTF (Fig. 6) (13). In the complex, the orientation of these domains is closer to that seen in the apo structure of the factor X Gla-EGF fragment. Hence, Ca\(^{2+}\) binding to the first EGF-like domain in fVIIa does not obviously facilitate the interaction with TF, but it cannot be ruled out that the angle between the Gla and EGF-like domains is critical in the docking of fVIIa with TF. Subsequently, the Gla-EGF region may be forced into another conformation by TF, changing the structure of the hydrophobic stack hinge region as inferred from CD experiments (22). In a physiological environment, the membrane surface may also be involved in this reorientation of domains through its interaction with the Gla domain. A recent energy transfer study suggests that the distance between the active site of fVIIa and the membrane surface decreases upon tissue factor factor binding (54). Considering that the Gla-EGF part of fVIIa appears to adopt a more upright conformation when bound to TF, this implies a dramatic reorientation of the protease domain relative to the membrane upon association with TF. However, rotational movement of the protease domain could explain at least part of the energy transfer change observed upon TF binding. Ca\(^{2+}\) binding to the first EGF-like domain in fVIIa definitely decreases the flexibility around the hydrophobic stack and thereby probably facilitates TF binding. It is probably also pivotal for optimal positioning of TF-interactive residues both in the first EGF-like domain and in the Gla domain. We also speculated that Ca\(^{2+}\) binding to the first EGF-like domain might be important for fVIIa activity in vivo by protecting it from proteolytic degradation in the hydrophobic stack (55–57), a rapid process in the absence of Ca\(^{2+}\).
However, our conclusion is that FVIIa and D46N,D63N-FVIIa are equally susceptible to enzymatic removal of the Gla domain at any given Ca$^{2+}$ concentration. This supports the idea that the Ca$^{2+}$-induced protection against proteolysis in the hydrophobic stack is mediated by Gla-dependent sites (53).

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