Ca\textsuperscript{2+} Binding to the First Epidermal Growth Factor Module of Coagulation Factor VIIa Is Important for Cofactor Interaction and Proteolytic Function*  

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Epidermal growth factor-like (EGF) domain Ca\textsuperscript{2+} binding sites in the homologous coagulation factors VII, IX, and X stabilize the structural orientation of the \(\gamma\)-carboxyglutamic acid-rich (Gla) domain relative to EGF-1. Site-directed mutagenesis was employed here to analyze the functional importance of Ca\textsuperscript{2+} binding to EGF-1 in factor VIIa (VIIa), which initiates coagulation in complex with its cofactor, tissue factor (TF). Ala replacements for Asp\textsuperscript{63} or Gln\textsuperscript{49} resulted in reduced TF affinity concordant with the number of eliminated Ca\textsuperscript{2+}-coordinating oxygen atoms in the respective side chains. Ca\textsuperscript{2+} binding to EGF-1 had no major direct effect on contacts with TF residue Gln\textsuperscript{110} or on interactions of VIIa residues Arg\textsuperscript{79} and Phe\textsuperscript{40}, suggesting the importance of the high affinity Ca\textsuperscript{2+} coordination in EGF-1 orientation affects overall docking. Gly, Ala, and Glu replacements at Asp\textsuperscript{66}, which is a Ca\textsuperscript{2+}-coordinating residue at the Gla aromatic stack carboxyl terminus, are consistent with the notion that an increased flexibility of the Gla domain relative to EGF-1 contributes significantly to loss of function. Certain mutants in the EGF-1 Ca\textsuperscript{2+} site had reduced proteolytic function, suggesting the importance of the high affinity Ca\textsuperscript{2+} binding site for macromolecular substrate interaction.  

EGF\textsuperscript{1} modules function in Ca\textsuperscript{2+}-dependent extracellular protein-protein interactions mediating cell adhesion and activation of protease cascades. The homologous coagulation factors VII (VII), IX, and X are characterized by one Ca\textsuperscript{2+}-binding epidermal growth factor module (EGF-1) which follows the amino-terminal \(\gamma\)-carboxyglutamic acid (Gla)-rich domain. Functional importance of the EGF-1 Ca\textsuperscript{2+} binding site in factor IX was demonstrated by specific mutations that cause hemophilia B (1) and by mutagenesis studies (2). Structure determinations of EGF modules of factors X (3), IX (4), and VIIa (5) reveal Ca\textsuperscript{2+} binding through two backbone and four side chain oxygen ligands in an octahedral coordination with one free valence. In VIIa, Gly\textsuperscript{17} and Gln\textsuperscript{49} provide backbone coordination, whereas Gln\textsuperscript{49} and Asp\textsuperscript{66} contribute one and Asp\textsuperscript{63} two side chain oxygens to the coordination of Ca\textsuperscript{2+} (5). The Asp\textsuperscript{63} position of EGF-1 is frequently modified by \(\beta\)-hydroxylation, which does not affect Ca\textsuperscript{2+} affinity (6); however, plasma-derived and recombinant VIIa do not contain erythro-\(\beta\)-aspartic acid at this position (7). Ca\textsuperscript{2+} affinity for the EGF-1 modules range from 30 to 250 \(\mu\)M (1, 8), but high affinity binding of the factor X EGF-1 appears to be partially dependent on the carboxyl terminus of the preceding Gla module (8). Structural analysis further demonstrated that the orientation of the Gla versus the EGF-1 module is dependent on Ca\textsuperscript{2+} binding to EGF-1 (9).  

By equilibrium dialysis, two high affinity (\(< 150 \mu\)M) Ca\textsuperscript{2+} sites were found in VIIa (10). These binding sites are also found in VIIa deleted of the amino-terminal Gla domain (des-1–38 VIIa), consistent with binding of Ca\textsuperscript{2+} to EGF-1 and to the protease domain, which has a Ca\textsuperscript{2+} binding motif (11) analogous to the trypsin catalytic domain Ca\textsuperscript{2+} site (12). Whereas fluorescence quenching indicated a \(< 30 \mu\)M affinity for the EGF-1 site, terbium phosphorescence measurements suggested a Ca\textsuperscript{2+} affinity of \(< 2 \mu\)M for the catalytic domain site (11, 13). This higher estimate for the catalytic domain site likely results from the experimental conditions and is also inconsistent with the Ca\textsuperscript{2+}-dependent changes in the amidolytic function of VIIa which are attributable to saturation of the protease domain Ca\textsuperscript{2+} site with a midpoint of \(< 50–250 \mu\)M Ca\textsuperscript{2+} (11, 14, 15).  

The interaction of VIIa with TF is Ca\textsuperscript{2+}-dependent. In the absence of divalent metals, the \(K_D\) of VIIa for TF is \(< 1.5–3 \mu\)M (10, 16). Ca\textsuperscript{2+} titration displays two transitions in the affinity of VIIa for TF: affinities are \(< 50–100 \text{nM}\) at \(50–200 \mu\text{M} \text{Ca}^{2+}\) and \(< 5 \text{nM}\) at \(1–5 \text{mM} \text{Ca}^{2+}\) (17). Ca\textsuperscript{2+} saturation of the Gla domain is likely responsible for the latter increase in affinity, since deletion of Gla in des-1–38 VIIa results in a similar loss of affinity for TF (16, 17). In part, Ca\textsuperscript{2+} saturation of the Gla domain may stabilize energetically important (14) hydrophobic contacts of Gla residues with TF (5). However, the increase in affinity for TF binding at \(\mu\text{M} \text{Ca}^{2+}\) cannot readily be explained from the structure of the TF-VIIa complex (5). The catalytic domain Ca\textsuperscript{2+} binding site is distant from the interface of the protease domain with TF. Ca\textsuperscript{2+} binding to this site may affect TF interaction only indirectly through long range conformational changes. In contrast, the EGF-1 Ca\textsuperscript{2+} site is near the VIIa light chain interface with the carboxyl-terminal module of TF, but neither the Ca\textsuperscript{2+} ion nor side chains involved in Ca\textsuperscript{2+} coordination are in contact with TF. In this study we used site-directed mutagenesis to analyze the contributions of the EGF-1 Ca\textsuperscript{2+} binding site to the interactions of VIIa with TF. We demonstrate that Ca\textsuperscript{2+} binding to this site is responsible for an increased affinity of VIIa for TF and provide evidence in support of the hypothesis that Ca\textsuperscript{2+} coordination in EGF-1 may play a functional role through the stabilization of the orientation of the Gla domain relative to EGF-1.
**VIla EGF-1 Ca Binding Site**

**Characterization of TF Binding by Surface Plasmon Resonance Measurements**

Determination of Kinetic Parameters $K_{d,app}$ and $k_{cat} - K_{d,app}$ and $k_{cat}$ for protein substrate factor X hydrolysis by TF. VIla were determined using relipidated full-length TF (50 pm) which was incubated with excess wild-type or mutant VIIa (5 nm) in Tris-buffered saline, 5 mM CaCl$_2$, 0.1% bovine serum albumin, pH 7.4, for 10 min to allow for complex assembly. Increasing concentrations of factor X (0.015–3.5 μM) were added for 1–5 min at 37 °C, and the reaction was stopped by the addition of 100 mM EDTA. Factor Xa generation was assessed by hydrolysis of chromogenic substrate, and data were fitted to the Michaelis-Menten equation using the program Enzfit (Elsevier Biosoft). For determination of proteolytic function in the absence of TF, 2 μM factor Xa was incubated with 250 nm wild-type or mutant VIIa at 5 mM Ca$^{2+}$ and 37 °C. Rates of factor Xa generation were linear for 1 h, the maximum incubation time used for the calculation of rates of product formation determined in three independent experiments.

**RESULTS**

**Expression of Site-directed Mutants in EGF-1**—To reduce the affinity for Ca$^{2+}$ binding by EGF-1, we generated individual Ala replacement mutants for Gln$^{49}$ and Asp$^{63}$ which provide one and two side chain oxygen atoms, respectively, for the coordination of Ca$^{2+}$ (Fig. 1). The mutants were transiently expressed in mammalian cells and tested for TF binding and proteolytic activity in a linked functional assay (23). Elimination of a single coordination in VIIaAla$^{49}$ had little impact on proteolytic function (Table I). In contrast, the expression levels typically achieved in transient transfection experiments were below concentrations needed to achieve full saturation of TF by the Asp$^{63}$ → Ala mutant, indicating a severe reduction in TF binding and possibly diminished proteolytic function (Table I).

For further study of these mutants, recombinant proteins were purified from serum-free culture supernatant of stably transfected cells using a two-step procedure involving monoclonal affinity and ion exchange chromatography employing Ca$^{2+}$ gradient elution to select for fully γ-carboxylated proteins. The final preparations had the expected percentage of Glu residues modified for carboxylation. VIIaAla$^{49}$ had 97%, VIIaAla$^{63}$ had 100%, and wild-type recombinant VIIa had 9.9 Gla residues. Plasma-derived VIIa purified by the same protocol was used as a control and showed the expected 10.0 Gla residues. VIIaAla$^{49}$ yielded activated VIIa during the purification, as typically observed for wild-type VIIa, whereas VIIaAla$^{63}$ only partially converted to VIIa, despite elution from the ion exchange resin by Ca$^{2+}$ at a concentration identical to that of wild-type VIIa. The normal chromatographic profile suggested
that the Gla domain of VIIAla63 adopted a conformational transition upon Ca\textsuperscript{2+} binding similar to wild-type VII. Ca\textsuperscript{2+}-dependent changes in the intrinsic protein fluorescence did not reveal differences between mutant and wild-type proteins, and we also found identical Ca\textsuperscript{2+} dependence for binding of both mutants and wild-type VIIa to a monoclonal antibody directed to a Ca\textsuperscript{2+}-sensitive epitope in the Gla domain (data not shown). We thus were unable to detect a defect in the conformation of the Ca\textsuperscript{2+}-saturated Gla domain of this mutant. Activation of the TF-bound mutant zymogen by factor Xa occurred with rates indistinguishable from wild-type VIIa (data not shown), indicating that the mutation in the EGF-1 Ca\textsuperscript{2+} site selectively affected the “autoactivation” of free VIIAla63 (26) which occurs during the purification.

Activation of VIIAla63 was achieved by cleavage with factor IXa in fluid phase. Purified proteins were analyzed in the functional assay at 5 mM Ca\textsuperscript{2+} (Table I). Consistent with data from transient transfection experiments, elimination of the single Ca\textsuperscript{2+} coordination provided by Gln 49 did not impair factor X activation and caused no detectable change in the K_D,app. The affinity of TF binding was reduced >100-fold as a consequence of the Asp63 mutation, and at saturation of cofactor a 4-fold reduction in the rate of factor X activation was apparent (Table I). The loss of function was similar with the zymogen form and the activated enzyme, excluding the possibility that a defect in the conversion of VII to VIIia additionally contributes to the functional defect of VIIAla63. We conclude from these results that the EGF-1 Ca\textsuperscript{2+} site is important for optimal interaction of VIIa with macromolecular substrate. It is noteworthy that elimination of a Ca\textsuperscript{2+}-coordinating residue side chain (Glu\textsuperscript{220}) in the protease domain Ca\textsuperscript{2+} site reduced catalytic function without diminishing the affinity for TF (Table I). Thus, Ca\textsuperscript{2+} binding to the catalytic domain has little impact on the TF interaction, despite a structural role in maintaining a catalytically active conformation of the VIIa protease domain.

**Effect of EGF-1 Mutations on Amidolytic and Proteolytic Function of VIIa—**Mutant and wild-type VIIa was saturated with increasing concentrations of soluble TF\textsubscript{1–218} at 5 mM Ca\textsuperscript{2+}, and the amidolytic function of the complex was determined with chromogenic p-nitroanilide substrate. The calculated apparent dissociation constants for these experiments demonstrated a significant reduction in TF binding of VIIAla63 compared with wild-type VIIa. Since these experiments were conducted with soluble TF in the absence of lipids, the results are consistent with the notion that mutation of Asp63 affected protein-protein interactions with TF. At saturation of the mutants with TF\textsubscript{1–218}, there was no difference in chromogenic substrate hydrolysis between either VIIAla49 or VIIAla63 and wild-type VIIa (Table II). Kinetic parameters for macromolecular substrate factor X activation demonstrated that VIIAla49 had normal proteolytic function at 5 mM Ca\textsuperscript{2+}. In contrast, VIIAla63 was defective in factor X activation resulting predominantly from a 3-fold decrease in Kcat with unchanged K_m,app (Table II). In the absence of TF and phospholipid, VIIAla63 activated factor X with a 4-fold reduced rate (14 ± 4 pmol factor Xa generated/min) compared with wild-type VIIa (52 ± 11 pmol factor Xa generated/min) when tested at the same enzyme concentration (250 nM). These results establish that VIIAla63 is selectively defective in macromolecular substrate activation independent of cofactor interactions.

**Surface Plasmon Resonance Analysis of TF Binding by Mutant and Wild-type VIIa—**The Ca\textsuperscript{2+} dependence of VIIia binding to soluble TF\textsubscript{1–218} was analyzed by surface plasmon resonance detection. Ca\textsuperscript{2+} saturation increased the affinity of wild-type VIIa for TF by 3 orders of magnitude, reflected in a decrease of the K_D from 4.7 ± 2.1 nM in 10 mM EDTA to 5.8 ± 2.0 nM at 5 mM Ca\textsuperscript{2+} (Table III). The association rate constant (k_a) increased only modestly by ~4-fold, whereas a >100-fold decrease in the dissociation rate constant (k_d) was largely responsible for the tighter binding of VIIa to TF. Ca\textsuperscript{2+} binding to VIIa

![Molecular model of the EGF-1 Ca\textsuperscript{2+} site. Homology models (32) of the EGF-1 and Gla domain aromatic stack were built based on the structures of the factor IX EGF-1 (4) and prothrombin Gla domain (33), respectively. The models were connected in an orientation consistent with the docked structure of VIIia in the TF-VIIia complex (5). Ca\textsuperscript{2+}-coordinating side chains Asp\textsuperscript{63}, Gln\textsuperscript{49}, and Asp\textsuperscript{63} are shown as well as the side chains of the noncoordinating Asp\textsuperscript{63} and the Cys\textsuperscript{20}·Cys\textsuperscript{63} disulfide (unshaded). Important residues in the interface of EGF-1 with TF (Arg\textsuperscript{79}, Phe\textsuperscript{71}, Ile\textsuperscript{69}, and Gln\textsuperscript{64}) and aromatic stack residues Arg\textsuperscript{36}, Ser\textsuperscript{43}, and Phe\textsuperscript{65}, which contact the carboxyl-terminal module of TF, are shaded dark. The interaction of Arg\textsuperscript{46} with TF is uncertain because of low resolution in the crystal structure (5).](http://www.jbc.org/)

**Table I**

| Effect of Ala mutations of VII residues Gln\textsuperscript{49} and Asp\textsuperscript{63} |
|-----------------------------------------------|-----------------------------------------------|
| Mutants from transient transfection experiments or as purified zymogen VII (>90% VII), enzyme spontaneously activated during purification (VIIia) or activated with factor IXa (VIIa, IXa activated) were tested for TF (5 ps in phospholipid) binding in a functional assay at 37 °C and in the presence of 5 mM Ca\textsuperscript{2+}. The maximum rate of factor X (100 nm) activation was extrapolated from the curve fitting to saturation of cofactor and provides an estimate for proteolytic function of the VII mutant (mean ± S.D., n = 3). |}

<table>
<thead>
<tr>
<th>K_D,app</th>
<th>ΔG</th>
<th>Maximum rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>pm</td>
<td>kcal/mol</td>
<td>s\textsuperscript{-1}</td>
</tr>
<tr>
<td><em>Wild-type</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>4.8 ± 1.1</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>Gln\textsuperscript{49} → Ala</td>
<td>5.1 ± 1.1</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Asp\textsuperscript{63} → Ala</td>
<td>&gt;500</td>
<td>&gt;2.3</td>
</tr>
<tr>
<td>Purified VII</td>
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<tr>
<td><em>Wild-type</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>4.3 ± 1.0</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>Asp\textsuperscript{63} → Ala</td>
<td>230 ± 41</td>
<td>2.5</td>
</tr>
<tr>
<td>VIIa, IXa activated</td>
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<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>5.6 ± 0.9</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>Asp\textsuperscript{63} → Ala</td>
<td>291 ± 12</td>
<td>2.2</td>
</tr>
<tr>
<td>VIIa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>5.1 ± 1.3</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>Gln\textsuperscript{49} → Ala</td>
<td>5.1 ± 0.8</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>Glu\textsuperscript{220} → Ala</td>
<td>4.1 ± 2.0</td>
<td>0.1 ± 0.1</td>
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Table II
Catalytic function of VIIaAla49 and VIIaAla63

<table>
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<th>Function</th>
<th>VIIa</th>
<th>VIIaAla49</th>
<th>VIIaAla63</th>
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<tr>
<td>Amidolytic assay</td>
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<td></td>
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<tr>
<td>$K_{D, app}$</td>
<td>$\Delta G$</td>
<td>$k_{cat}$</td>
<td>$n$</td>
</tr>
<tr>
<td>Wild-type VIIa</td>
<td>3.8 ± 1.8</td>
<td>7.8 ± 1.8</td>
<td>8</td>
</tr>
<tr>
<td>$\pm 1.8$</td>
<td></td>
<td>48 ± 6</td>
<td>5</td>
</tr>
<tr>
<td>VIIa (IXa activated)</td>
<td>2.3 ± 0.9</td>
<td>6.1 ± 0.7</td>
<td>9</td>
</tr>
<tr>
<td>$\pm 0.7$</td>
<td></td>
<td>71 ± 13</td>
<td>4</td>
</tr>
<tr>
<td>VIIaAla49</td>
<td>5.5 ± 1.5</td>
<td>9.6 ± 0.4</td>
<td>6</td>
</tr>
<tr>
<td>$\pm 0.4$</td>
<td></td>
<td>49 ± 10</td>
<td>3</td>
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<tr>
<td>VIIaAla63</td>
<td>309 ± 91</td>
<td>7.1 ± 0.7</td>
<td>6</td>
</tr>
<tr>
<td>$\pm 91$</td>
<td></td>
<td>39 ± 4</td>
<td>5</td>
</tr>
<tr>
<td>Wild-type VIIa</td>
<td>3.8 ± 1.8</td>
<td>7.8 ± 1.8</td>
<td>8</td>
</tr>
<tr>
<td>VIIaAla49</td>
<td>5.5 ± 1.5</td>
<td>9.6 ± 0.4</td>
<td>6</td>
</tr>
<tr>
<td>VIIaAla63</td>
<td>309 ± 91</td>
<td>7.1 ± 0.7</td>
<td>6</td>
</tr>
<tr>
<td>Wild-type VIIa</td>
<td>3.8 ± 1.8</td>
<td>7.8 ± 1.8</td>
<td>8</td>
</tr>
<tr>
<td>VIIaAla49</td>
<td>5.5 ± 1.5</td>
<td>9.6 ± 0.4</td>
<td>6</td>
</tr>
<tr>
<td>VIIaAla63</td>
<td>309 ± 91</td>
<td>7.1 ± 0.7</td>
<td>6</td>
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Table III
Kinetic parameters for binding of mutant and wild-type VIIa to TF

<table>
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<tr>
<th>Wild-type VIIa</th>
<th>VIIaAla49</th>
<th>VIIaAla63</th>
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<tbody>
<tr>
<td>$k_{a} \times 10^{4}$</td>
<td>$k_{d} \times 10^{-3}$</td>
<td>$K_D$</td>
</tr>
<tr>
<td>$s^{-1}$</td>
<td>$s^{-1}$</td>
<td>$nM$</td>
</tr>
<tr>
<td>10 mM EDTA</td>
<td>4.3 ± 0.3</td>
<td>700 ± 38</td>
</tr>
<tr>
<td>50 mM Ca$^{2+}$</td>
<td>8.9 ± 1.0</td>
<td>24 ± 2</td>
</tr>
<tr>
<td>200 mM Ca$^{2+}$</td>
<td>9.7 ± 0.5</td>
<td>35 ± 12</td>
</tr>
<tr>
<td>1 mM Ca$^{2+}$</td>
<td>16 ± 3</td>
<td>3.8 ± 0.5</td>
</tr>
<tr>
<td>5 mM Ca$^{2+}$</td>
<td>19 ± 8</td>
<td>1.0 ± 0.4</td>
</tr>
</tbody>
</table>

resulted in a $-4$ kcal/mol change of the calculated free energy of TF binding, and 75% of the change was observed at $50-200$ mM Ca$^{2+}$ (Fig. 2). This Ca$^{2+}$ dependence is characteristic of high affinity Ca$^{2+}$ binding sites located outside the Gla domain, and thus the energetic contributions to TF binding which result from Ca$^{2+}$ binding are only to a lesser extent attributable to Ca$^{2+}$ saturation of the Gla domain. Indeed the approximately 1 kcal/mol increase upon addition of 1 mM Ca$^{2+}$ is consistent with the modest loss of binding function following truncations that remove the Gla domain from VIIa (14, 17).

There was no difference in TF binding when VIIaAla49 was compared with wild-type VIIa in the presence of the divalent cation chelator EDTA, but a subtle 2-fold increase in the dissociation rate constant for VIIaAla63 was found (Table III). This subtle difference could indicate that the Asp46 side chain, in part, may contribute to TF binding independent of its role in Ca$^{2+}$ coordination. Both mutants were severely defective compared with wild-type VIIa at 50 and 200 mM Ca$^{2+}$, reflected in a shift of the saturation curves displayed in Fig. 2. Whereas a defect in the association rate constant was observed only at $50$ mM Ca$^{2+}$ for VIIaAla49, the association rate for VIIaAla63 did not change with increasing Ca$^{2+}$, resulting in a 4-fold lower rate compared with wild-type TF at 5 mM Ca$^{2+}$. The defect in the dissociation rate constant and consequently in the $K_D$ was most pronounced at 10 mM EDTA (Fig. 2A). The Gibbs free energy of binding was calculated from data given in Table III according to $\Delta G = -RT \ln(K_D)$, and the absolute change in $\Delta G$ relative to the values determined at 10 mM EDTA ($\Delta \Delta G$) is shown in dependence of the Ca$^{2+}$ concentration.

Asp$^{46}$ plays a local structural role in the EGF-1 Ca$^{2+}$ site, but it does not directly coordinate Ca$^{2+}$. Ala exchange of this residue decreased the affinity for TF $10$-fold, a loss of function intermediate between the severe reduction observed with VIIaAla49 and the subtle defect of VIIaAla63. Asp$^{46}$, being localized in the Gla domain at the carboxyl terminus of the helical aromatic stack region, provides the fourth Ca$^{2+}$-coordinating side chain oxygen atom (5). Unlike the subtle defect resulting from the elimination of a side chain with a single coordination in the case of Glu$^{49}$, the Asp$^{46}$ mutation more severely reduced TF binding and VIIaAla63 proteolytic function (Table IV). The more prominent defect of the Asp$^{46}$ mutation points to a function of the EGF-1 Ca$^{2+}$ site in orienting the Gla domain including the aromatic stack region relative to EGF-1. Replacing the Asp$^{46}$ side chain with Glu resulted in mutant protein with a less severe defect in TF binding and proteolytic function. Our interpretation is that providing Ca$^{2+}$ coordination alone is not
TABLE IV
Functional characterization of TFAla110 and mutants in the EGF-1 Ca\(^{2+}\) site of VII

<table>
<thead>
<tr>
<th>Mutant</th>
<th>KD,(\text{app}) ((\mu)M)</th>
<th>(\Delta G) (kcal/mol)</th>
<th>Maximum rate (s(^{-1}))</th>
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</thead>
<tbody>
<tr>
<td>TF Wild-type</td>
<td>3.2 ± 1.2</td>
<td>1.1 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>VIIa Wild-type</td>
<td>459 ± 37</td>
<td>3.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Glu110 → Ala</td>
<td>11.3 ± 5.4</td>
<td>0.8</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>Glu110 → Ala</td>
<td>1,600 ± 273</td>
<td>3.9</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>VIIa Wild-type</td>
<td>3.9 ± 2.6</td>
<td>1.9 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Asp46 → Ala</td>
<td>28 ± 10</td>
<td>1.3</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>Asp46 → Ala</td>
<td>12 ± 1</td>
<td>0.7</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Asp46 → Gly</td>
<td>63 ± 18</td>
<td>1.7</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Arg79 → Ala</td>
<td>35 ± 13</td>
<td>1.3</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>Phe40 → Ala</td>
<td>28 ± 3</td>
<td>1.2</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>Asp46/Arg79 → Ala</td>
<td>212 ± 57</td>
<td>2.5</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Asp46/Phe40 → Ala</td>
<td>122 ± 19</td>
<td>2.1</td>
<td>0.4 ± 0.1</td>
</tr>
</tbody>
</table>

From the mutational analysis of the VIIa EGF-1 Ca\(^{2+}\) binding site, we arrive at the following conclusions. (i) Approximately 75% of the increase in TF affinity caused by Ca\(^{2+}\) binding to VIIa is observed at 50–200 \(\mu\)M Ca\(^{2+}\); this Ca\(^{2+}\) concentration range is consistent with estimates for the affinity of the EGF-1 site for Ca\(^{2+}\) (8, 10, 13, 28). (ii) Mutations of Ca\(^{2+}\)-coordinating residues in EGF-1 result in significantly reduced affinity for TF, particularly at 50 and 200 \(\mu\)M Ca\(^{2+}\). (iii) Mutations of the aromatic stack residue Asp46 result in mutational defects that are larger than those at the Gln\(^{110}\) position, which in the crystal structure of the complex (5) also provides a single Ca\(^{2+}\)-coordinating oxygen atom. (iv) Ca\(^{2+}\) binding to EGF-1 does not affect the VIIa interaction with TF residue Gln\(^{110}\). (v) Certain mutants had defects in proteolytic function, suggesting that the EGF-1 Ca\(^{2+}\) site functions to support macromolecular substrate interaction with TF-VIIa.

Whereas Ca\(^{2+}\) has little impact on the conformation of the TF extracellular domain (17), Ca\(^{2+}\) binding to the Gla domain is important for high affinity binding to TF. Several studies have provided convergent evidence that the Ca\(^{2+}\)-saturated conformation of the Gla domain is responsible for an approximately 1 kcal/mol increase in the free energy of binding for TF-VIIa protein-protein interactions (14, 16, 17). The contribution of the Gla domain, however, does not fully account for the effect of Ca\(^{2+}\) on VIIa binding to TF. Indirect evidence from competition experiments with synthetic peptides analogous to VIIa protease domain sequences (10) or with a Glu\(^{220}\) → Ala mutant of VII (11) was interpreted to indicate that the protease domain Ca\(^{2+}\) binding site contributes to TF interactions. These studies did not provide a direct binding analysis of the mutants in the catalytic domain binding site. The functional \(K_{D,\text{app}}\) for VIIa\(^{\text{Ala220}}\) determined in our experiments did not reveal defects in TF binding, although the amidolytic and proteolytic function of our mutant preparation was severely impaired, consistent with previous analysis of this mutant (11). The decreased proteolytic function (Table I) indicates incomplete Ca\(^{2+}\) saturation of the site under our experimental conditions. We thus interpret the unchanged \(K_{D,\text{app}}\) as evidence that the protease domain Ca\(^{2+}\) site, consistent with its location distant from the TF interface (5), has little importance for TF binding and that Ca\(^{2+}\) binding to EGF-1 is largely responsible for the demonstrated Ca\(^{2+}\) dependence of the TF-VIIa interaction (10, 15, 17). Notably, even at the Gla domain saturating concentration sufficient to restore function fully. The Gly replacement for Asp46 displayed a binding defect similar to the Ala mutant and a more pronounced defect in proteolytic function, an indication that flexibility at this position is detrimental. Indeed, the Asp at this position may function as a bridge connecting two structures: the EGF-1 through calcium coordination and the Gla domain by virtue of the Asp46 position at the end of the helix which harbors critical residues that constitute the hydrophobic stack. However, parts of the aliphatic side chain of Asp46 may contribute directly to macromolecular substrate interaction, and the mutagenesis results would also be consistent with this interpretation.

Role of TF Residue Gln\(^{110}\)—The defect in TF binding upon mutations of Asp46 could be explained by local conformational effects on an important contact with TF. Gln\(^{110}\) in EGF-1 and Ser\(^{43}\) in the Gla domain (Fig. 1) share Gln\(^{110}\) as a common contact in TF (5), and loss of Ca\(^{2+}\) coordination may position Ser\(^{43}\) or Gln\(^{110}\) in an unfavorable orientation for interaction with TF. Mutation of TF residue Gln\(^{110}\) reduced the affinity for wild-type VIIa ∼3-fold (Table IV), a loss of function consistent with the study by Kelley et al. (25), but less than the 1.4 kcal/mol decrease in the free energy of binding reported by Gibbs et al. (27). Binding of VIIa\(^{\text{Ala63}}\) to TF\(^{\text{Ala110}}\) occurred with a \(K_{D,\text{app}}\) of 1,600 \(\mu\)M, significantly higher than the 1.4 \(K_{D,\text{app}}\) of Table IV, a loss of function consistent with the study by Kelley et al. (25). Binding of VIIa\(^{\text{Ala220}}\) determined in our experiments did not reveal defects in the catalytic domain binding site. The functional \(K_{D,\text{app}}\) for VIIa\(^{\text{Ala220}}\) determined in our experiments did not reveal defects in TF binding, although the amidolytic and proteolytic function of our mutant preparation was severely impaired, consistent with previous analysis of this mutant (11). The decreased proteolytic function (Table I) indicates incomplete Ca\(^{2+}\) saturation of the site under our experimental conditions. We thus interpret the unchanged \(K_{D,\text{app}}\) as evidence that the protease domain Ca\(^{2+}\) site, consistent with its location distant from the TF interface (5), has little importance for TF binding and that Ca\(^{2+}\) binding to EGF-1 is largely responsible for the demonstrated Ca\(^{2+}\) dependence of the TF-VIIa interaction (10, 15, 17). Notably, even at the Gla domain saturating concentration.
of 5 mM Ca\(^{2+}\), a significant binding defect remained for VIIa\(_{\text{Ala63}}\) and VIIa\(_{\text{Ala46}}\), indicating that cooperative effects of Ca\(^{2+}\) binding to the Gla domain do not substitute for the requirement of Ca\(^{2+}\) binding to EGF-1.

Since the crystal structure of the TF-VIIa complex reveals no direct role of the EGF-1 Ca\(^{2+}\) site in TF binding, the binding defects resulting from the mutation of Ca\(^{2+}\)-coordinating residues must be explained by an indirect effect on the VIIa light chain conformation. These indirect effects could be predominately on EGF-1 or on Gla domain interactions with TF. In the first case, Gln\(_{64}\) is the most likely residue to be affected by loss of Ca\(^{2+}\) binding to EGF-1 because the carbonyl oxygen of Gln\(_{64}\) participates in Ca\(^{2+}\)-coordination (5), and loss of Ca\(^{2+}\) binding to EGF-1 may affect the contact of the Gln\(_{64}\) side chain with Lys\(_{165}\) and Lys\(_{166}\) in the carboxyl-terminal domain. It is possible that the proteolytic defect is unrelated to the significant binding defect also displayed a defect in proteolytic function of TF-VIIa (30). This may suggest that the predominant functional role of the EGF-1 Ca\(^{2+}\) site is to stabilize an optimal orientation of the Gla domain relative to EGF-1 even after docking of the two domains with TF.

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