Activation and Active Site Occupation Alter Conformation in the
Region of the First Epidermal Growth Factor-like Domain of Human
Factor VII*

The first epidermal growth factor-like domain (EGF-1) of factor VII (FVII) provides the region of greatest contact
during the interaction of FVIIa with tissue factor. To understand this interaction better, the conformation-
sensitive FVII EGF-1-specific monoclonal antibody (mAb) 231-7 was used to investigate the conformational
effects occurring in this region upon both FVII activation and active site occupation. The binding affinity of
mAb 231-7 was approximately 3-fold greater for thezymogen state than for the active state; a result affected
by the presence of both calcium and the adjacent Gla domain. Once activated, active site inhibition of FVIIa
with a variety of chloromethyl ketone inhibitors resulted in a 10-fold range of affinities of FVIIai molecules
to mAb 231-7. Gla domain removal eliminated this variation in affinity, suggesting the involvement of a Gla/
EGF-1 interaction in this conformational effect. In addition, the binding of mAb 231-7 to FVIIai EGF-1 stimulated
the amidolytic activity of free FVIIai. Taken together, these results imply an allosteric interaction between the
FVIIai active site and the EGF-1 domain that is sensitive to variation in active site occupant structure. Thus,
these present studies indicate that the conformational change associated with FVII activation and active site
occupation involves the EGF-1 domain and suggest potential functional consequences of these changes.

Blood coagulation is initiated when circulating FVII in plasma binds to its essential cofactor, the trans-membrane
lipoprotein receptor TF. Upon binding to cell surface TF, zymogen FVII can be activated to FVIIai by factor IXa (FIXa) (1),
factor Xa (FXa) (2), or in an autocatalytic manner by endogenous FVIIai (3), thus allowing propagation of the coagulation
cascade. Activation of FVII occurs upon proteolytic cleavage of the Arg152–Ile153 bond, giving rise to a 152-amino acid light
chain (−20 kDa) linked by a disulfide bridge to a 254-amino acid heavy chain (−30 kDa) (4). It is the light chain of FVII that
contains the first epidermal growth factor-like domain (EGF-1), a stretch of 37 amino acids with characteristic structure
that has been implicated as the principal site of FVII interaction with TF (5–8). The FVII molecule also requires calcium for
the expression of optimum activity, one molecule of which is bound in each of the protease domains (9, 10) and the EGF-1
domain at a high affinity site (11), with seven more Ca2+ molecules bound with variable affinity by the Gla domain (12).

It has been shown recently that the activation of FVII to FVIIai, as well as occupation of the active site by pseudosubstrate inhibitors, results in conformational changes within the heavy chain with implications on cofactor and substrate interaction (13–15). To date, the conformational effects of these events on the TF-binding EGF-1 domain of the native FVII molecule have not been reported. Relevantly, experiments by Ambrosini et al. (16) have described conformational changes in the region of the EGF-1 domain of the highly homologous FX molecule upon activation which appears to be needed for binding to effector cell protease receptor-1. Chang and co-workers (17) have also observed conformational change in the Gla/EGF-1 region of a FVII/FIX chimera upon proteolytic activation. As well, Persson and others (12, 19, 20) have indicated the presence of an interaction between the Gla/EGF-1 region and the active site of FVII, indicating that active site occupation may also affect conformation within this region.

Conformation-specific monoclonal antibodies (mAbs) with well characterized binding epitopes have been used as effective probes to study the conformational structure of proteins (for a review see Ref. 21), as well as to identify functional consequences of binding to epitopes (22, 23). Recently our laboratory has used a conformation-sensitive mAb, 231-7, to help characterize the nature of the naturally occurring FVII EGF-1 structural variant N57D (24). The epitope specificity of mAb 231-7, within amino acid residues 51–88 of FVII (5), makes it a unique tool to investigate conformational changes specifically involving the FVII EGF-1 domain. An understanding of the mechanisms by which the EGF-1 domain of FVII interacts with TF is of particular relevance, as the interaction of FVII with TF appears to play a critical role not only in coagulation, but in other biological processes such as tumor metastasis and angiogenesis (25, 26). The recent evidence that the interaction of FVIIai with TF elicits signal transduction indicates that these processes likely involve intracellular signaling events (27).

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‡ The abbreviations used are: FVII, coagulation factor VII; FVIIai, activated coagulation factor VII; FVIIai, active site-inhibited coagulation factor VII; dGla-FVII, factor VII with the Gla domain proteolytically removed; TF, tissue factor; BFP-Rck, biotinylated n-Phe-Pro-Arg chloromethyl ketone; FP-Rck, n-Phe-Pro-Arg chloromethyl ketone; D-EG-Rck FVII, 1,5-dansyl-Glu-Gly-Arg chloromethyl ketone; FF-Rck, n-Phe-Pro-Arg chloromethyl ketone; EGF-1, first epidermal growth factor-like domain; Gla, the γ-carboxylated domain; FXa, activated coagulation factor X; IXa, activated coagulation factor IX; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; AP, alkaline phosphatase; DTT, dithiothreitol.
In this report, we utilize the mAb 231-7 to investigate the conformational ramifications to the FVII EGF-1 domain upon the activation and active site occupation of the FVII molecule. We provide evidence demonstrating that both modifications are associated with conformational changes involving the FVII EGF-1 region, as well as providing evidence of functional consequences for these changes on cofactor binding and the catastrophic activity of factor VII.

**EXPERIMENTAL PROCEDURES**

**Reagents—**Recombinant human FVIIa was purified from 293 cell culture supernatant using a protocol adapted from Ref. 28. Recombinant human FVII (R152Q) was purified from 293 cell culture supernatant using a protocol slightly modified from that described previously (29). The FVII(R152Q) mutant was chosen because of its inability to become activated during the course of analysis, thus ensuring thezymogen conformation is maintained, as indicated by others (30). Plasma-derived zymogen FVII was obtained from Enzyme Research Labs (South Bend, IN). Isolated EGF-1 peptide, expressed and purified from E. coli and comprising amino acids 45–87 of human factor VII, was the generous gift of Dr. Geoffrey Lee (Genentech, San Francisco, CA). mAb 231-7 was prepared as described previously (31). Human monocytic-derived cathepsin G was obtained from Calbiochem. FPRick, BFPRick, DEGRick, and FFRick were obtained from Calbiochem. Spectrozyme® FVIIa (CH2SO3-6-Ch-Ara-Arg-pNA.AcOH) was obtained from American Diagnostica (American Diagnostica Inc., Montreal, Quebec, Canada). Recombinant human TF (Ortho RecombiPlasTin®) was purchased from Bio-Tek Instruments, Winooski, VT and plotted versus antibody concentration.

**Direct Binding of FVII(R152Q), FVIIa, FPRickFVIIa, and DEGRickFVIIa to Immobilized mAb 231-7—**Briefly, mAb 231-7 (100 ng/well) was immobilized in microtiter wells on a 96-well microtiter plate overnight at 4 °C in carbonate antigen-coating buffer. Nonspecific binding sites were blocked with 2 mg/ml BSA in either 100 mM Tris, pH 8.0, 150 mM NaCl, 10 mM CaCl2 buffer containing 2 mg/ml BSA for ≥3 h at room temperature. After washing 5 times with TBS-spaCa2 buffer, serial dilutions of FVII(R152Q), FVIIa, FPRickFVIIa, and DEGRickFVIIa molecules in TBS-spaCa2 buffer containing 2 mg/ml BSA were incubated in duplicate in the microtiter wells for 2 h at room temperature. Samples to be analyzed in the absence of Ca2+ were incubated in TBS-T buffer, replacing CaCl2 with 10 mM EDTA. Samples were then washed 5 times with TBS-spaCa2 buffer and incubated with biotinylated rabbit anti-human FVII polyclonal antibody (100 μg/ml antibody concentration) in TBS-spaCa2 buffer containing 2 mg/ml BSA for 1 h at room temperature. Sample wells were washed again 5 times with TBS-spaCa2 buffer, followed by incubation with streptavidin-conjugated AP in TBS-spaCa2 buffer containing 2 mg/ml BSA for 1 h at room temperature. After washing 5 times with TBS-spaCa2 buffer, AP substrate was added (100 μl, 1 mg/ml in diethanolamine buffer), and the color reaction was allowed to develop for 30 min. Absorbance was measured at 405 nm on an automated microplate reader, and the results were plotted as absorbance versus FVII concentration.

**Determination of Equilibrium Dissociation Constants (Kd)** for the Binding of the FVIIa, FPRickFVIIa, DEGRickFVIIa, and FFRickFVIIa Molecules to the EGF-1 Domain—**The Kd of mAb 231-7 for FVIIa was determined as described previously (32). Only the Kd of mAb 231-7 for FVII(R152Q), FVIIa, and FPRickFVIIa was determined.

**Chloromethyl Ketone Inhibition of FVIIa—**The chloromethyl ketone inhibitors were reconstituted according to the manufacturers instructions and diluted in reaction buffer immediately prior to reaction. FVIIa samples were diluted to 1 ml in reaction buffer (50 mM Tris, pH 8.6, 150 mM NaCl, 10 mM CaCl2, 100 μg/ml BSA) to a final FVIIa concentration of 1 μM and reacted with a 25 μM excess of chloromethyl ketone inhibitor (1 μl addition) for 30 min. At this time another 25 μM inhibitor was added (50 μM total), and the reaction mix incubated at room temperature for 2 h total. The reaction mixture underwent purification using a protein spin column (Millipore Ultrafine®, molecular mass cut-off at 5 kDa) with 3× buffer exchange into TBS, pH 8.0. The completeness of FVIIa inhibition was verified to be ~99% by using the prothrombin time assay, essentially as described previously (24). Chloromethyl ketone labeling was specific for the protease domain of factor VIIa, as judged by evidence demonstrating that both modifications are associated with conformational changes involving the FVII EGF-1 region, as well as providing evidence of functional consequences for these changes on cofactor binding and the catastrophic activity of factor VII.

**Sensitivity of mAb 231-7 Binding to FVII Conformation—**Relative bindings of native, heat-denatured, and DTT-reduced FVII zymogen to both mAb 231-7 and rabbit anti-human FVII polyclonal antibody were performed using an ELISA technique. Briefly, to prepare reduced FVII, plasma-derived zymogen was boiled for 5 min in 1% SDS followed by snap cooling. Native, DTT-reduced and heat-denatured FVII samples were then incubated overnight at 4 °C in carbonate antigen coating buffer (15 mM Na2CO3, 35 mM NaHCO3, 0.02% NaN3, pH 9.6) in microtiter wells on an Immulon II 96 well microtiter plate. Microtiter wells were washed 3 times with TBS-T buffer, followed by blocking of non-specific binding sites with blocking buffer (5 mg/ml BSA in TBS-spaCa2) for 3 h at room temperature. Serial dilutions of mAb 231-7 or rabbit anti-human FVII polyclonal sera in TBS-spaCa2 buffer, containing 5 mg/ml BSA, were incubated in triplicate in the sample wells for 3 h at room temperature. Sample wells were washed (3 times) with TBS-spaCa2 buffer followed by blocking of non-specific binding sites with blocking buffer. mAb 231-7 or goat anti-rabbit IgG-conjugated AP for mAb 231-7 or goat anti-rabbit IgG-conjugated AP for the rabbit anti-FVII polyclonal sera, for 1 h at room temperature. Sample wells were washed with TBS-spaCa2 buffer (3 times) followed by addition of AP substrate (100 μl of p-nitrophenyl phosphate, disodium, and hexahydrate) at 1 mg/ml in diethanolamine buffer. The absorbance of each sample was measured at 405 nm on an Automated Microplate Reader (model EL 312, Bio-Tek Instruments, Winooski, VT) and plotted versus antibody concentration.
Conformational Changes Involving the FVII EGF-1 Domain

Conformational Sensitivity of mAb 231-7 Binding to FVII—To verify the sensitivity of mAb 231-7 for perturbations in factor VII structure, the relative bindings of mAb 231-7 and polyclonal rabbit anti-human FVII antibody to native, heat-denatured, and DTT-reduced FVII zymogen were compared by ELISA. mAb 231-7 exhibited significantly reduced binding affinity to heat-denatured FVII zymogen compared with that for native FVII zymogen. In addition, the binding of mAb 231-7 to antigen was rendered negligible upon reduction of the FVII zymogen with 10 mM DTT. In contrast, the binding of native, heat-denatured, and DTT-reduced FVII zymogen to polyclonal rabbit anti-human FVII antibody was unaffected by either heat denaturation or DTT reduction treatment of the antigen, indicating that the observed reduction was specific for mAb 231-7 (data not shown).

Direct Binding to Immobilized mAb 231-7 of FVII(R152Q), FVIIa, FPRckFVIIa, and D-EGRckFVIIa—Immobilized mAb 231-7 specifically bound FVII(R152Q), FVIIa, FPRckFVIIa and D-EGRckFVIIa in both the presence and absence of Ca\(^{2+}\). In the presence of 10 mM Ca\(^{2+}\), mAb 231-7 bound FVII(R152Q), FVIIa, FPRckFVIIa, and D-EGRckFVIIa with distinctly different binding characteristics, indicative of different conformations in the region of the EGF-1 domain for each molecule. FVII(R152Q) bound immobilized mAb 231-7 with greatest affinity, followed by FPRckFVIIa, FVIIa, and D-EGRckFVIIa in descending order (Fig. 1A). In the absence of Ca\(^{2+}\), the binding differential between FVII(R152Q) and FVIIa could not be demonstrated, indicating the activation-dependent conformational change to be calcium-dependent. In the absence of Ca\(^{2+}\), FVIIa bound with greater affinity to immobilized mAb 231-7 than did any of the active site-inhibited FVIIa molecules analyzed (Fig. 1B).

Determination of Equilibrium Dissociation Constants (K\(_D\)) to mAb 231-7 for the Binding of FVIIa, FVII(R152Q), FPRckFVIIa, and D-EGRckFVIIa—Fig. 2 and Table I show the equilibrium binding data of various full-length FVII molecules to mAb 231-7. In the presence of Ca\(^{2+}\), mAb 231-7 bound FVII(R152Q) with a K\(_D\) of 3.8 nM, a 2.5-fold greater affinity than that determined for FVIIa (K\(_D\) = 9.7 nM). In the absence of calcium the affinity of FVIIa increased approximately 2.5-fold, a value similar to the increase in affinity for uninhibited FVIIa.

Determination of Equilibrium Dissociation Constants (K\(_D\)) to mAb 231-7 for the Binding of Gla-FVIIa, Gla-FVII(R152Q), Gla-FPRckFVIIa, and the Isolated EGF-1 Peptide—Table II shows the equilibrium binding data for various FVII molecules with the Gla domain removed, as well for the isolated EGF-1 peptide. Removal of the Gla domain resulted in a 2-fold reduction in binding affinity for Gla-FVIIa for mAb 231-7 compared with that seen for FVIIa in the presence of calcium (19 versus 9.7 nM) (Table III). This contrasts with the apparent increase in mAb 231-7 binding affinity for Gla-FVII(R152Q) compared with that seen for FVII(R152Q) (1.7 versus 3.8 nM). The removal of the Gla domain thus increased the affinity differential between the zymogen and active form of FVII for mAb 231-7 from 2.5- to 10-fold in the presence of calcium and no difference to 10-fold difference in the absence of calcium. Gla removal increased the mAb 231-7 affinity of D-EGRckFVIIa almost 4-fold in the presence of calcium. Gla removal also increased the affinity of D-EGRckFVIIa in the absence of calcium to 12.3 nM, eliminating the calcium-dependent difference. Gla removal decreased the mAb 231-7 affinity of FPRckFVIIa in both the presence (7.2 nM) and absence (10 nM) of calcium, showing no calcium-dependent difference in affinity for mAb 231-7. Gla removal had no significant effect on the mAb 231-7 affinity for FPRckFVIIa in either the presence or absence of calcium (data not shown). As well, the presence or absence of calcium had no significant effect on the mAb 231-7 binding affinity of the isolated EGF-1 peptide.
Effect of Active Site Occupation on Tissue Factor Binding—
Table III shows the effects of active site inhibition on the ability of FVIIa molecules to compete with biotin-labeled FVIIa for TF-binding sites. Active site occupation of FVIIa with D\textsubscript{z}EGRck, FFRck, or FPRck resulted in an increase in ability to inhibit the binding of FVIIa to full-length recombinant relipidated tissue factor in a dose-dependent fashion. The magnitude of the IC\textsubscript{50} was dependent upon the active site inhibitor used. FFRckFVIIa and FPRckFVIIa showed approximately equivalent inhibitory abilities, with IC\textsubscript{50} values of 0.21 and 0.22 nM.

Fig. 2. Determination of equilibrium dissociation constants (K\textsubscript{D}) for the binding to mAb 231-7 of FVII(R152Q), FVIIa, FPRckFVIIa, and D\textsubscript{z}EGRckFVIIa. Scatchard plots of the binding data for FVII(R152Q) (⊗), FVIIa (●), FPRckFVIIa (△), and D\textsubscript{z}EGRckFVIIa (●) in the presence (closed symbols) or absence (open symbols) of Ca\textsuperscript{2+}. Each graph represents the results of a typical experiment. The K\textsubscript{D} values were determined from the inverse of the slope of the linear regression curve of the Scatchard plot, with the mean K\textsubscript{D} values reported in Table I. γ represents the fraction of bound antibody and a the concentration of free antigen, at equilibrium.
TABLE I

| Sample | Presence of Ca2+ | Absence of Ca2+ |
|--------|-----------------|-----------------|
|        | $K_D$ ($nM$)    | $K_D$ ($nM$)    |
| FVIIa  | 9.7 ± 1.0       | 4.2 ± 1.0       |
| FVII(R152Q) | 3.6 ± 0.7     | 3.5 ± 0.6       |
| FPFRckFVIIa | 4.8 ± 1.0   | ND              |
| FPFRckFVIIa | 4.0 ± 1.0     | 5.5 ± 2.0       |
| FFRckFVIIa | 13.0 ± 3.0    | 11.0 ± 1.0     |
| D · EGRckFVIIa | 42.0 ± 9.0 | 19.0 ± 3.0     |

*p < 0.05 when compared with FVIIa, as determined using the Student’s two-sample t test.

TABLE II

| Sample | Presence of Ca2+ | Absence of Ca2+ |
|--------|-----------------|-----------------|
|        | $K_D$ ($nM$)    | $K_D$ ($nM$)    |
| dGlaFVIIa | 19.0 ± 4.0    | 24.0 ± 6.0     |
| dGlaFVII(R152Q) | 1.7 ± 0.2    | 2.5 ± 0.4     |
| dGlaFPFRckFVIIa | 7.2 ± 2.0  | 10.0 ± 7.0     |
| dGlAD · EGRckFVIIa | 11.0 ± 2.0 | 12.0 ± 5.0     |
| FVII EGF-1 | 9.4 ± 3.0   | 14.0 ± 4.0     |

*p < 0.05 when compared with dGlaFVIIa, as determined using the Student’s t test.

TABLE III

| Sample | $IC_{50}$ values (nM) |
|--------|------------------------|
|        | n                     |
| FVIIa  | 2.32 ± 0.38            |
| D · EGRckFVIIa | 0.53 ± 0.24   |
| FFRckFVIIa | 0.21 ± 0.07    |
| FPFRckFVIIa | 0.22 ± 0.03  |

*p < 0.05 when compared with FVIIa, as determined using the Student’s t test.

respectively, representing a 10-fold increase in inhibition over unmodified FVIIa. D · EGRckFVIIa had an $IC_{50}$ value of 0.53 nM, a 4-fold increase in inhibition over unmodified FVIIa. Uninhibited FVIIa had an $IC_{50}$ value of 2.32 nM.

Effect of Binding of mAb 231-7 on FVIIa Amidolytic Activity—mAb 231-7 increased the amidolytic activity of human FVIIa toward the tri-peptide chromogenic substrate Spectrozyme FVIIa reproducibly and in a dose-dependent fashion (Fig. 3). FVIIa/mAb 231-7 activity reached a maximal increase of 41 ± 11% (n = 5) over FVIIa alone in the presence of approximately a 3-fold molar excess of IgG-binding sites to FVIIa molecules. An increase in FVIIa activity was detectable with as little as a 1:5 molar ratio of monoclonal binding sites to FVIIa. The stimulatory ability of mAb 231-7 also appeared to increase with increasing pH (9.9, data not shown), an effect also known to occur for TF (35). Incubation of FVIIa with a nonspecific monoclonal antibody of the same IgG subclass did not result in any increase in the amidolytic activity of FVIIa toward Spectrozyme FVIIa (Fig. 3) nor did mAb 231-7 alone elicit any amidolytic activity (data not shown).

DISCUSSION

Previous studies from our laboratory have shown that mAb 231-7 is specific for the EGF-1 domain of factor VII (5) and is sensitive to structural alterations involving this domain (24). The conformation specificity of mAb 231-7 was thus used to characterize the conformational behavior of the FVII EGF-1 domain upon zymogen activation, as well as the occupation of the active site of FVIIa with various active site-specific chloromethyl ketones.

The decrease in affinity for mAb 231-7 to FVIIa compared with that observed for the zymogen FVII(R152Q) in the presence of calcium was observable in both the direct binding assay and the homogenous solution phase assay. This effect was reproducibly quantified to give ~3-fold change in magnitude of binding affinity. Thus, these data indicate that the zymogen conformation of the EGF-1 domain differs from that of the catalytically active form, FVIIa. As the binding epitopes of mAb 231-7 and TF are known to overlap within EGF-1 (5), it is likely that this activation-dependent conformational change affects the manner in which FVII interacts with TF. This hypothesis is consistent with the findings of Chang and co-workers (17), who showed an activation-dependent conformational change in a protein chimera containing the Gla/EGF-1 domains of FVII that affected TF binding ability ~4-fold. Such domain-specific conformational changes may go undetected when one measures the overall affinity of factor VII for TF, due to the complexity of the interaction (7), an effect we overcome through the use of the EGF-1-specific mAb 231-7. Our observations thus extend the findings of Chang et al. (17) to the native FVII molecule. We have further localized the activation-dependent conformational change occurring in the light chain of FVII to involve amino acids 51–88 of the EGF-1 domain.

The observation that the activation-dependent conformational change occurring between FVII and FVIIa is dependent on the presence of calcium (Table I) indicates that there is clearly involvement of a calcium-binding region. The fact that the isolated EGF-1 domain did not show any calcium dependence in its interaction with mAb 231-7 indicates that the pres-
ence of another domain is needed to facilitate this interaction. Given the proximity of the calcium-binding Gla region and its effect on calcium binding to the EGF-1 domain in FVII (36), we investigated the possible role of the Gla domain in the observed activation-dependent conformational change. Proteolytic removal of the Gla domain eliminated any change in the FVIIa affinity to mAb 231-7 due to calcium, indicating that the calcium-associated binding was facilitated in the presence of the Gla domain and that the protease domain was not responsible for the observed calcium dependence. We feel that the Gla/Ca\(^{2+}\)-dependent EGF-1 conformational changes occurring upon FVII activation may be important for the development of optimum activity of the factor VIIa molecule, as is hypothesized for the calcium-mediated Gla/EGF-1 conformational change seen in factor Xa (37).

Analysis of the impact of occupation of the active site of FVIIa on the binding of mAb 231-7 allowed us to investigate conformational changes in the EGF-1 region once the catalytic activity of the enzyme had been established. Incorporation of FPRck into the active site of FVIIa in the presence of Ca\(^{2+}\) is associated with an apparent 3-fold increase in mAb 231-7 affinity compared with that observed upon FFRck inhibition. The fact that the two inhibitors bind in an identical manner to the active site (7) would suggest that the conformational changes propagated to the EGF-1 domain from the active site likely involved at least the S2 sub-site. (S2, P2, etc. nomenclature used in this discussion is that of Schecter and Berger (44).) In comparison, D-EGRck inhibition of FVIIa resulted in a 10-fold decrease in mAb 231-7 affinity compared with that observed upon FPRck inhibition. This effect may also be mediated by the different P2 residues present in the two inhibitors, although concomitant differences in P3 and the amino-terminal cap leave open the possibility of propagation through other interactions, such as at the aryl binding site. Importantly, there is precedence for allosteric linkages at the S2 and S3 sub-sites in the serine proteases, as these sites appear to be involved in the modulation of the thrombin active site by thrombomodulin (38, 39).

The investigations of the calcium dependence of the conformational changes observed in FVIIa EGF-1 upon active site inhibition indicate variations in the calcium effect, depending on the inhibitor used. The affinities to mAb 231-7 upon FPRck and FFRck inhibition were not significantly affected by calcium removal, whereas the mAb 231-7 affinity measured upon D-EGRck inhibition showed a similar 2-fold affinity increase as that observed for uninhibited FVIIa. This variation between these inhibitors may relate to differences in the mechanism of allosteric propagation, as the P2–P4 positions of D-EGRck likely interact differently in the active site of FVIIa compared with FPRck and FFRck (40). Regardless of calcium involvement, we chose to investigate the effect of the presence of the adjacent calcium-binding Gla domain, as data from Persson and others (19, 20) have indicated interactions involving both the Gla domain and the EGF-1 calcium-binding site with the FVII active site. Removal of the Gla domain abolished the differences in affinity among the inhibited molecules to mAb 231-7, indicating that the Gla domain was involved in the observed allosteric linkage of the active site to the EGF-1 domain. The fact that the inhibited dGla-FVIIai molecules still maintained a slight but significantly different affinity compared with uninhibited dGla-FVIIa indicates that the allosteric linkage may not be totally Gla-dependent.

Once the presence of an allosteric linkage extending from the active site of FVIIa to the EGF-1 domain was established, we then sought to investigate the functional consequences of such an interaction on the FVII molecule. Active site inhibition increased the ability of FVIIa to inhibit the FVIIa/TF interaction, consistent with the observation of increased affinity of FVIIai molecules for TF when compared with uninhibited FVIIa reported by others (14, 30, 41). Thus conformational changes occurring in FVIIai upon active site occupation that affect the interaction with TF are consistent with the conformational changes we observed in the EGF-1 domain. Relevantly, recent evidence has indicated that at least some of the changes in TF binding affinity can be attributed to changes in TF-binding residues in the protease domain (14, 15), although changes to TF-binding regions in the light chain were not precluded by these studies.

As the mAb 231-7 binding epitope overlaps with that of TF in the EGF-1 domain (5) and the binding of TF elicits a large increase in FVIIa catalytic activity (42), we sought to investigate whether mAb binding at EGF-1 would have any affect on FVIIa activity. We observed that the binding of mAb 231-7 to EGF-1 stimulated the catalytic activity of FVIIa toward a tripeptide substrate in a dose-dependent manner. This result verified the reciprocal nature of the allosteric linkage between EGF-1 and the active site of FVIIa. The relevance of this effect is not obvious; however, the variation in mAb 231-7 binding affinities toward the various active site inhibitors suggests a role for this linkage in substrate/inhibitor specificity. The involvement of the S2 sub-site of FVIIa is consistent with this hypothesis, as the S2 sub-site has also been shown previously to be involved in substrate/inhibitor specificity for the highly homologous activated protein C and FXa serine proteases (43) as well as for thrombin (39).

The results of our study support the hypothesis that activation of FVII causes calcium-dependent conformational changes that involve the region of the EGF-1 domain. Once FVII is activated, an allosteric linkage can be observed between EGF-1 and the active site that involves the adjacent Gla domain, affecting the catalytic properties of the active enzyme and possibly cofactor binding. These observations shed light on conformational effects during the development of FVII catalytic activity and provide further evidence of the existence of independent conformational linkages that regulate the catalytic function of FVIIa, such as those shown by others (15, 18, 22), extending this model specifically to include the region of the EGF-1 domain of FVII.

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