Antibody-induced Enhancement of Factor VIIa Activity through Distinct Allosteric Pathways

Received for publication, October 10, 2011, and in revised form, January 3, 2012. Published, JBC Papers in Press, January 24, 2012, DOI 10.1074/jbc.M111.312330

Lisbeth M. Andersen‡, Peter A. Andreasen, Ivan Svendsen, Janneke Keemink, Henrik Østergaard, and Egon Persson

From the Novo Nordisk A/S, Novo Nordisk Park, DK-2760 Målev, Denmark and the Department of Molecular Biology and Genetics, Aarhus University, DK-8000 Aarhus C, Denmark

Background: The enzymatic activity of coagulation factor VIIa (FVIIa) is allosterically controlled by tissue factor (TF).

Results: Monoclonal antibodies (mAbs) can allosterically augment the intrinsic activity of FVIIa through mechanisms distinct from that of TF.

Conclusion: Different modes of allosteric activation of FVIIa appear to exist.

Significance: Artificial activators of FVIIa can be tools to study the zymogen-to-protease transition.

In the absence of its cofactor tissue factor (TF), coagulation factor VIIa (FVIIa) predominantly exists in a zymogen-like, catalytically incompetent state. Here we demonstrate that conformation-specific monoclonal antibodies (mAbs) can be used to characterize structural features determining the activity of FVIIa. We isolated two classes of mAbs, which both increased the catalytic efficiency of FVIIa more than 150-fold. The effects of the antibodies were retained with a FVIIa variant, which has been shown to be inert to allosteric activation by the natural activator TF, suggesting that the antibodies and TF employ distinct mechanisms of activation. The antibodies could be classified into two groups based on their patterns of affinities for different conformations of FVIIa. Whereas one class of antibodies affected both the $K_m$ and $k_{cat}$, the other class mainly affected the $K_m$. The antibody-induced activity enhancement could be traced to maturation of the S1 substrate binding pocket and the oxyanion hole, evident by an increased affinity for p-aminobenzamidine, an increased rate of antithrombin inhibition, an increased rate of incorporation of diisopropylfluorophosphate, and an enhanced fraction of molecules with a buried N terminus of the catalytic domain in the presence of antibodies. As demonstrated by site-directed mutagenesis, the two groups of antibodies appear to have overlapping, although clearly different, epitopes in the 170-loop. Our findings suggest that binding of ligands to specific residues in the 170-loop or its spatial vicinity may stabilize the S1 pocket and the oxyanion hole, and they may have general implications for the molecular understanding of FVIIa regulatory mechanisms.

Blood coagulation factor VII (FVII) is a serine protease that plays a predominant role in blood clotting as the initiator of the protein cascade eventually leading to formation of a fibrin clot (1). FVII is synthesized in the liver as an inactive single-chain zymogen that is proteolytically processed to a two-chain form denoted FVIIa. FVIIa possesses very low intrinsic activity both toward its physiological substrate coagulation factor X (FX) (FX-activating activity) and toward small synthetic peptide substrates (amidolytic activity) (2, 3). Upon vascular injury, circulating FVIIa forms a complex with tissue factor (TF), which is a membrane-bound cofactor constitutively expressed by fibroblasts and smooth muscle cells, and achieves its full catalytic potential (4). The transition from the inactive to the active state of FVIIa, which is allosterically mediated by TF, results in conformational changes primarily localized to the activation domain of the enzyme (5). These changes have been addressed by x-ray crystal structural analysis of FVII (6), FVIIa (7), and FVIIa in complex with soluble TF (sTF) (8), and molecular determinants for the catalytically relatively inert nature of FVIIa have been suggested. A prerequisite for enzyme activity is stable insertion of the N terminus into a hydrophobic binding cleft referred to as the activation pocket (9). As opposed to other related serine proteases, FVIIa does not spontaneously acquire the active enzyme conformation, and the N terminus remains exposed to the solvent. Upon binding of TF, the N terminus becomes buried in the interior of the protein where Ile-16(153) forms a salt bridge to Asp-194(343) at the bottom of the pocket (5). This results in ordering of surface-exposed loop regions, including the 170-loop (residues 170a(313)-170i(321)), and the activation domain, consisting of the activation loop (residues 16(153)-21(158)), the autolysis loop (residues 142(285)-152(294)), the oxyanion stabilizing loop (residues 184(332)-194(343)), and the S1 entrance frame (residues 216(365)-223(372)) (8, 10). These changes are associated with maturation of the active site including the substrate binding pockets and the oxyanion hole defined by the main chain amides of Gly-193(342) and Ser-195(344) (11). Essentially the
same structural changes are believed to occur when an active site inhibitor is incorporated into FVIIa (12–14).

TF makes direct contact to Met-164(306) in FVIIa, and this residue has been shown to be a key mediator of the TF-induced allosteric changes (15, 16). The extensive structure-function analyses that have been applied to address the role of TF and to identify allosteric hotspots in FVIIa have contributed to the recent development of FVIIa variants with enhanced intrinsic enzymatic activity (17–20). In this study we explore the possibility of using mAbs as an alternative approach to obtain information about structural features determining the activity of FVIIa. The literature covering antibodies that induce catalytic activity of serine proteases is limited (21–24). We describe the isolation and characterization of two groups of mAbs capable of enhancing the intrinsic activity of FVIIa. The antibodies were characterized with respect to their binding properties and their effects on the enzyme kinetics of FVIIa. Furthermore, the binding epitopes of the mAbs were partly revealed by mutagenesis analysis. This allows us to propose a model for the activation of FVIIa by the mAbs, which clearly differs from the TF-induced activation, although they might represent distinct parts of the same mechanism. These results contribute to general understanding of the molecular mechanisms that govern the zymogen-to-protease transition of FVIIa.

**EXPERIMENTAL PROCEDURES**

**Enzymes and Reagents**—Recombinant wild-type FVIIa, d-Phe-Phe-Arg chloromethyl ketone (CMK)-inhibited FVIIa (FFR-FVIIa), and sTF were prepared as previously described (14), and antithrombin (AT) was purified from Antithrombin III Baxter® by heparin-Sepharose chromatography. FX and FXa were from Enzyme Research Laboratories (South Bend, IN), and chromogenic substrates S-2288 and S-2765 were from Chromogenix (Mölndal, Sweden). p-Aminobenzamidine (PAB) and hexamethrine bromide (Polybrene) were purchased from Sigma, and diisopropylfluorophosphate ([1,3-3H]DFP) was from PerkinElmer Life Sciences. d-Phe-Phe-Arg chloromethyl ketone (FFR-CMK) and low molecular weight heparin from porcine intestinal mucosa were purchased from Calbiochem.

**Expression and Purification of FVIIa Mutants**—Mutations were introduced into the human FXII expression plasmid pLN174 using QuikChange (Stratagene, La Jolla, CA) (25). Plasmid preparation, baby hamster kidney cell transfection and selection, and protein expression, purification, and activation (autoactivation or catalyzed by factor IXaβ) were performed as described previously (16, 18, 26).

**Antibody Production and Purification**—Mice (RBF; Taconic, Denmark) were immunized with Gla-domainless FVIIa (27) covalently active-site inhibited with FFR-CMK to lock the protease domain in a conformation corresponding to the catalytically active state. The mice were given an initial subcutaneous injection of 20 μg in Freund’s complete adjuvant followed by booster intraperitoneal and subcutaneous tail-base injections of 20 μg of antigen in Freund’s incomplete adjuvant. The spleen was removed aseptically and dispersed to a single cell suspension. Splenocytes were fused to FoxNY myeloma cells using PEG 1500 with a 4:1 fusion ratio of splenocytes to myeloma cells (28). Cells were recloned twice by limiting dilution. Clones were selected and grown in 1-liter shaker flasks for production of mAbs, which were purified by protein A affinity chromatography (GE Healthcare).

**FVIIa Activity Assays**—Activity assays were conducted in 96-well microtiter plates in assay buffer (50 mM Heps, pH 7.4, with 0.1 mM NaCl, 5 mM CaCl2, 0.1% (w/v) bovine serum albumin, and 0.01% Tween 80) in a total volume of 200 μl at 37 °C. The initial reaction rates of substrate hydrolysis were monitored for 20 min as the absorbance increase at 405 nm in a microplate reader. The amidolytic activity was determined by incubating FVIIa (100 nM) or FVIIa (50 nM) with mAb or sTF (0.5–500 nM) and mixing the samples with S-2288 (1 mM). Kinetic parameters were determined by measuring substrate hydrolysis over a range of S-2288 concentrations (0.005–5 mM) in the presence of either FVIIa (100 nM), FVIIa (10 nM), and mAb (500 nM), or FVIIa (10 nM) and sTF (200 nM). Units of absorbance were converted to molarity using a p-nitroaniline standard curve.

The FX-activating activity was determined by incubating FX (200 nM) with FVIIa (100 nM), FVIIa (10 nM) and mAb (0.5–500 nM), or FVIIa (10 nM) and sTF (200 nM) in 100 μl of assay buffer for 1 h at 25 °C. FX activation was stopped by adding 50 μl assay buffer in which CaCl2 had been replaced by 60 mM EDTA. The relative amount of generated FXa was evaluated by measuring initial rates of S-2765 (0.5 mM) hydrolysis. Likewise, kinetic parameters were determined by measuring FX activation over a range of FX concentrations (0.002–2 μM). The experimental procedure was otherwise as described above. The molar generation of FXa was determined from a FXa standard curve in which the initial velocities of S-2765 hydrolysis were plotted against the concentration of purified FXa (0.2–3.0 nM).

**Inhibition of FVIIa by PAB**—The Ki for inhibition of FVIIa by PAB was determined at 37 °C in the absence or presence of sTF or mAbs. FVIIa (10 nM) was incubated with sTF (100 nM) or mAb (100 nM) for 15 min before the addition of buffer or a 2-fold serial concentration range of PAB (9.375–1200 μM) in assay buffer. PAB was allowed to react for 5 min, S-2288 (1 mM) was added, and residual FVIIa activity was determined from the initial reaction velocities monitored as absorbance development at 405 nm. The relative velocities Vr/Vo were plotted against the inhibitor concentration, and Ki values were determined from nonlinear regression using the following equation assuming that PAB is a competitive inhibitor.

\[
\frac{V_r}{V_o} = K_i [S]_o + K_m ([S]_o + K_m (K_i + [I]_o) )
\]  

\( V_r \) and \( V_o \) are the reaction velocities in the presence and absence of inhibitor, \( [S]_o \) and \( [I]_o \) are the total substrate and inhibitor concentrations, \( K_i \) is the inhibition constant, and \( K_m \) is the Michaelis constant for S-2288.

**Inhibition of FVIIa by AT**—FVIIa (50 nM) alone or FVIIa (20 nM) in the presence of mAb (500 nM) was incubated with low molecular weight heparin (10 μM) and AT (500 nM) in assay buffer for different time periods (2–45 min). The reactions were stopped with Polybrene (final concentration 0.6 mg/ml), and residual FVIIa activity was determined by the addition of S-2288 (1 mM). Second-order rate constants were calculated.
from the fits to a single-exponential decay by dividing with the AT concentration.

Carbamoylation Assays—FVIIα (1.2 μM) alone or FVIIα (500 nM) in the presence of mAb (2.5 μM) or sTF (2.5 μM) was incubated in assay buffer without bovine serum albumin supplemented with 0.2 mM KCNO. Samples (20 μl) were withdrawn at different time points and diluted 10-fold in assay buffer containing bovine serum albumin, and residual activity was determined in the presence of S-2288 (1 mM).

Surface Plasmon Resonance Analyses—All analyses were conducted on a Biacore T100 instrument (Biacore AB, Uppsala, Sweden) at 25°C. An anti-mouse IgG CM5 sensor chip was prepared using a mouse antibody capture kit (Biacore AB) according to the manufacturer’s instruction. The levels of immobilization were between 10,000 and 14,000 response units (RU). mAbs (1.5 μg/ml) were injected in running buffer (10 mM Hepes, pH 7.4, containing 150 mM NaCl, 5 mM CaCl₂, and 0.005% Tween 20) at a flow-rate of 10 μl/min and a contact time of 60 s. After a stable base line had been achieved, FVIIα, FFR-FVIIα, zymogen FVII, or FVIIα-sTF was injected in a 2-fold serial concentration range (3.125–200 nM) at a flow rate of 30 μl/min and a contact time of 120 s. The dissociation was followed for 600 s. Between each run the chip was regenerated with regeneration buffer (10 mM glycine-HCl, pH 1.7) at a flow rate of 10 μl/min and a contact time of 180 s. The kinetic parameters $k_{on}$ and $k_{off}$ were determined using the Biacore T100 evaluation software and a 1:1 binding model. Binding measurements of mAbs F36 and F37 to FVIIα mutants were conducted essentially as described above, except that the mAbs were injected at a concentration of 5 μg/ml and FVIIα mutants were injected at a single concentration of 100 nM. The binding level for each mutant was normalized to that obtained for wild-type FVIIα. FFR-FVIIα variants were prepared by incubating FVIIα variant (100 nM) with FFR-CMK (1 μM) overnight at 5°C in running buffer. The samples were subsequently injected over the immobilized antibodies.

Detection and Quantification of [1,3-3H]DFP-labeled FVIIα—Samples containing FVIIα (0.6 μM) alone or with sTF (3 μM) or mAb (3 μM) were incubated with [1,3-3H]DFP (50 μM) for 0, 2, 4, or 24 h at room temperature. Unbound [1,3-3H]DFP was eliminated by precipitating the proteins with trichloroacetic acid. The precipitated proteins were dissolved in SDS sample buffer and fractionated by nondenaturing polyacrylamide gel electrophoresis. Each gel lane was cut into seven equally sized pieces, and according to a molecular marker FVIIα was contained in gel piece 4. Individual gel pieces were treated with trypsin to facilitate release of protein-bound [1,3-3H]DFP. Scintillation solution (3 ml) was added, and the samples analyzed in a scintillation counter.

RESULTS

Isolation of Antibodies That Stimulate the Activity of FVIIα—Mice were immunized with Gla-domainless FFR-CMK inhibited FVIIα (FFR-FVIIα). This antigen was chosen because it presents the active FVIIα conformation to the immune system and thereby increases the likelihood of generating antibodies that recognize and stabilize this particular state. We also avoid generating antibodies against the Gla domain, which are unlikely to influence the enzymatic activity. Of the antibody-expressing hybridoma cell lines tested, as much as eight percent were found to express antibodies that stimulated the activity of FVIIα measured as increased hydrolysis of a small peptidyl substrate (S-2288). Furthermore, ~25% of the stimulatory clones also positively affected the TF-independent FX-activating activity of FVIIα (supplemental Fig. S1). The three clones that stimulated the FX-activating activity of FVIIα most markedly and the five clones with the most pronounced effect on the amidolytic activity of FVIIα were chosen for further characterization. Based on the subsequent initial biochemical characterization, which included determination of binding kinetics to different ligands as well as effects on enzyme kinetics, it was clearly established that these eight antibodies belonged to two groups according to their different functional effects on FVIIα and antigen binding kinetics (data are provided in supplemental Tables S1 and S2). F37 and F36, respectively, were chosen as representative examples of these two groups, and the results of the characterization of these two antibodies are presented in the following sections.

Effect of Antibodies on Amidolytic Activity of FVIIα—Initially, the effect of antibody on the intrinsic activity of FVIIα was assessed by measuring hydrolysis of the small peptidyl substrate S-2288. The relative catalytic efficiency was increased 170- and 150-fold in the presence of saturating amounts of F36 and F37, respectively (Table 1). The enhancement of catalytic potency was caused by both a decrease in the $K_m$ value and an increase in $k_{cat}$. F36 decreased the $K_m$ almost 24-fold, and the $k_{cat}$ was increased 7-fold, whereas F37 decreased the $K_m$ almost 100-fold but had only a small impact on the $k_{cat}$ (less than 2-fold). By comparison, sTF only increased the overall catalytic efficiency by 48-fold.

It has previously been reported that Met-164(306) in FVIIα is pivotal for the allosteric regulation of FVIIα by TF. Replacing this residue with Asp almost abolishes the TF-induced stimulation of the catalytic potency without a significant effect on the binding of FVIIα to TF (16). When bound to F36 and F37, the relative catalytic efficiency of M164(306)D-FVIIα was increased 270- and 200-fold, respectively. As expected from previous results (16), sTF was found to induce only a modest 2.2-fold increase in catalytic efficiency, and this was mainly caused by lowering of the $K_m$ (Table 1). Based on these results, the antibodies seem to stimulate FVIIα by a mechanism different from that of TF.

| Enzyme          | $K_m$ (nM) | $k_{cat}$ (s⁻¹) | Relative catalytic efficiency |
|-----------------|------------|----------------|-----------------------------|
| FVIIα           | 11.8 ± 0.45| 13 ± 0.7       | 1                           |
| FVIIα-sTF       | 2.1 ± 0.01 | 110 ± 8.8      | 48                          |
| FVIIα-F36       | 0.5 ± 0.03 | 95 ± 9.5       | 170                         |
| FVIIα-F37       | 0.12 ± 0.001| 20 ± 0.1       | 150                         |
| M164(306)-D-FVIIα | 10.2 ± 1.9| 3.7 ± 0.7     | 7                           |
| M164(306)-D-FVIIα-sTF | 6.3 ± 3.4| 5.1 ± 2.2     | 2.2                         |
| M164(306)-D-FVIIα-F36 | 0.53 ± 0.14| 51 ± 0.7   | 270                         |
| M164(306)-D-FVIIα-F37 | 0.14 ± 0.01| 10 ± 0.7     | 200                         |

* Efficiency ($k_{cat}/K_m$) compared with that of free wild-type FVIIα or free M164(306)D-FVIIα (which are given the arbitrary value 1).
**Effect of Antibodies on FX-Activating Activity of FVIIa**—Given the enhancement of amidolytic activity, it was of interest to investigate whether the activation of the macromolecular substrate factor X was correspondingly augmented. However, whereas both antibodies stimulated the amidolytic activity, only F37 was able to stimulate the FX-activating activity of FVIIa. The effect of F37 could be ascribed to a 6-fold lowering of \( K_m \) and a 4-fold increase of \( k_{cat} \), resulting in a 24-fold increase in catalytic efficiency. By comparison, sTF increased \( k_{cat} \) by more than 100-fold but had only a moderate effect (1.6-fold decrease) on \( K_m \) (Table 2).

**Antibody Binding Kinetics to Different Conformational States of FVIIa**—The profound stimulation of FVIIa activity could suggest that the antibodies preferentially bind the active FVIIa conformation. To get an impression of how selective the antibodies are in their recognition of different forms of FVII(a), the binding kinetics of F36 and F37 to FFR-FVIIa, FVIIa, FVIIa-sTF, and FVII were determined by surface plasmon resonance measurements. Although FVIIa is believed to exist in a conformational equilibrium between active and inactive states, strongly favoring inactive conformations, FFR-FVIIa represents the active conformation of FVIIa and is, due to the presence of an active site inhibitor (FFR-CMK), stably locked in this state. The single-chain zymogen form, FVII, represents an inactive conformational state of the enzyme. Both F36 and F37 bound with high and comparable affinities in the low nanomolar range to FFR-FVIIa (Table 3). In contrast, the profiles for binding to FVIIa differed between the two antibodies (Fig. 1). F36 exhibited 150-fold reduced affinity for FVIIa compared with FFR-FVIIa, caused by both a slower \( k_{on}(16\text{-fold}) \) and a faster \( k_{off}(9\text{-fold}) \). Likewise, F37 bound FVIIa with a lower affinity compared with FFR-FVIIa. However, opposite to F36, this was entirely caused by a faster \( k_{off} \) (Table 3). F36 bound the FVIIa-sTF complex with a \( K_d \) of ~5 nM. Only little binding of F37 to FVIIa-sTF was detected, most likely reflecting binding to the small fraction of FVIIa not bound to sTF. F37 bound to FVII and FVIIa with comparable affinities and binding kinetics (Table 3). In contrast, very low binding of F36 to FVII was measured, possibly reflecting the presence of small amounts of FVIIa in the FVII preparation (Fig. 1).

**Effect of Antibodies on Carbamylation of N Terminus of Protease Domain**—The exposure of the N terminus to the solvent was investigated to obtain mechanistic information about the mode by which the antibodies enhance the activity of FVIIa. The susceptibility of the N terminus to carbamylation has been shown to be influenced by the binding of TF. TF promotes and stabilizes the insertion of the N terminus in the activation pocket, which thereby becomes less accessible for chemical modification by KCNO (5, 13). The conformational changes occurring as a consequence of insertion of the N terminus have

---

**TABLE 2**

| Enzyme                | \( K_m \)       | \( k_{cat} \)     | Relative catalytic efficiency* |
|-----------------------|-----------------|-------------------|--------------------------------|
| FVIIa                 | 5.4 ± 3.4       | \( 1 \times 10^{-5} \) s\(^{-1} \) | 1                             |
| FVIIa-sTF             | 3.4 ± 0.9       | 110 ± 23          | 175                           |
| FVIIa-F37             | 0.90 ± 0.07     | 4.0 ± 0.6         | 24                            |

*Efficiency \((k_{cat}/K_m)\) is calculated relatively to wild-type FVIIa, which has been assigned the arbitrary value 1.

**TABLE 3**

| Analyte   | \( k_{on} \) \( \times 10^5 \text{ M}^{-1} \text{ s}^{-1} \) | \( k_{off} \) \( \times 10^{-4} \text{ s}^{-1} \) | \( K_d \) nM | \( k_{on} \) \( \times 10^5 \text{ M}^{-1} \text{ s}^{-1} \) | \( k_{off} \) \( \times 10^{-4} \text{ s}^{-1} \) | \( K_d \) nM |
|-----------|------------------------------------------------------------|-------------------------------------------------|-------------|------------------------------------------------------------|-------------------------------------------------|-------------|
| FFR-FVIIa | 1.6 ± 0.1                                                  | 0.8 ± 0.7                                       | 0.5         | 1.0 ± 0.3                                                  | (3.5 ± 2.2) \( \times 10^{-5} \)              | 0.4         |
| FVIIa     | 0.1 ± 0.03                                                 | 7.5 ± 1.7                                       | 75          | 1.0 ± 0.4                                                  | (1.0 ± 0.2) \( \times 10^{-5} \)              | 100         |
| FVIIa-sTF | 0.7 ± 0.27                                                 | 3.2 ± 0.5                                       | 4.6         | 1.2 ± 0.4                                                  | (1.8 ± 0.5) \( \times 10^{-2} \)              | 150         |
| FVII      | Low binding*                                               |                                                 |             |                                                            |                                                 |             |

*Less than 20 RU detected at the highest concentration of ligand (200 nM) (Fig. 1).

*Less than 20 RU detected at the highest concentration of ligand (200 nM) (Fig. 1).
been directly linked to the activation of the protease. In all cases the activity loss during the inactivation with KCNO followed mono-exponential kinetics (Fig. 2). At the concentrations used, the FVIIa-sTF complex lost its activity more than 5-fold slower than FVIIa alone. Interestingly, the FVIIa-F36 complex lost its activity as slowly as the FVIIa-sTF complex, whereas the FVIIa-F37 complex displayed an intermediate inactivation rate (Table 4). Hence, both antibodies, and especially F36, appear to promote insertion of the N terminus into the activation pocket.

**Effect of Antibodies on Inhibition of FVIIa by PAB**—PAB is a nonspecific competitive inhibitor of serine proteases that binds in the S1 pocket, thereby blocking the access of the substrate to the active site (29). The interaction with PAB is dependent on a structurally well defined S1 pocket. The $K_i$ value for inhibition of FVIIa is an indicator of S1 maturation, and any changes are interpreted as structural alterations affecting the S1 pocket (e.g. maturation of the S1 pocket is seen as a decrease in $K_i$). As previously shown, FVIIa was inefficiently inhibited by PAB with a $K_i$ of 3.2 mM. In the presence of sTF, the affinity for PAB was greater, and the $K_i$ value decreased to 150 $\mu$M. The binding of F36 or F37 to FVIIa appeared to have an effect on the S1 site at least as dramatic as that induced by sTF with decreases of $K_i$ down to 38 and 29 $\mu$M, respectively (Table 4).

**Effect of Antibodies on Inhibition of FVIIa by AT**—AT belongs to the serpin family and is a physiological inhibitor of FVIIa. The interaction between the enzyme and the serpin requires a well defined active site, and especially interactions in the substrate binding pockets S1-S4 seem to be crucial (30). We found that FVIIa alone is relatively slowly inhibited by AT with a second-order rate constant of 310 $\text{m}^{-1}\text{s}^{-1}$. In the presence of F36, this is increased to 1900 $\text{m}^{-1}\text{s}^{-1}$, whereas the effect of F37 is even more pronounced with a second-order rate constant of 7200 $\text{m}^{-1}\text{s}^{-1}$.

**Effect of Antibodies on Incorporation of [1,3-$^3$H]DFP into FVIIa**—DFP is a potent irreversible inhibitor of serine proteases. It reacts with the hydroxyl group of the serine in the catalytic triad and occupies the oxyanion hole formed by main chain amides from Gly-193(342) and the mentioned Ser-195(344) (31). The reactivity of serine proteases toward DFP depends on the integrity of the oxyanion hole, and we used DFP to assess the antibody-induced effects on this region. We found consistent, clear effects of sTF, F36, and F37 on the incorporation of [1,3-$^3$H]DFP into FVIIa, which was markedly increased in the presence of these ligands as compared with FVIIa alone. After 24 h of incubation with [1,3-$^3$H]DFP in the presence of F36 and sTF, the amount of incorporated [1,3-$^3$H]DFP was more than 3-fold higher compared with FVIIa alone, whereas only a 2-fold higher incorporation was observed in the presence of F37 (Fig. 3).

**Mapping Binding Sites for F36 and F37 by Mutagenesis**—Given the different functional consequences of sTF, F36, and F37 binding, it was of interest to map the binding regions on FVIIa. To this end, the binding of F36 and F37 to two superactive FVIIa variants was analyzed by surface plasmon resonance. FVIIa$_\text{DVQ}$ (V21(158)D/E154(296)V/M156(298)Q) and FVIIa$_\text{VEAY}$ (L163(305)V/Y164(306)V/M307(347)D/Q) have three mutations located near the activation pocket. These mutations cause a stabilization of the N terminus inserted into the activation pocket, resulting in increased catalytic activity (18). F36 or F37 were immobilized on an anti-mouse IgG antibody CM5 chip. The binding levels, i.e. maximum binding measured as RU after a single injection of FVIIa$_\text{DVQ}$ (100 nm), to F36 and F37 was increased 3- and 1.5-fold, respectively, compared with wild-type FVIIa (100 nm) (data not shown). FVIIa$_\text{VEAY}$ (L163(305)V/S170b(314)E/K188(337)A/F225(374)Y) has mutations situated in and in spatial vicinity of the 170-loop, leading to stabilization of this relatively long and flexible region. In contrast to the situation in FVIIa$_\text{DVQ}$, these mutations have no effect on the insertion and stabilization of the N terminus in the activation pocket.

**TABLE 4** Effects of antibodies on the inhibition of FVIIa by PAB, the rate of AT inhibition, and carbamylation of the protease domain N-terminus

| Enzyme       | $K_i$ for PAB inhibition | Second-order rate constant for AT inhibition $\text{m}^{-1}\text{s}^{-1}$ | Rate of inactivation by KCNO $\times 10^{-3} \text{min}^{-1}$ |
|--------------|-------------------------|-------------------------------------------------|-------------------------------------------------|
| FVIIa        | 3200 ± 370              | 310 ± 25                                        | 17 ± 0.4                                        |
| FVIIa-sTF    | 150 ± 15                | ND*                                             | 30 ± 0.3                                        |
| FVIIa-F36    | 38 ± 3.6                | 1900 ± 100                                      | 2.9 ± 0.7                                       |
| FVIIa-F37    | 29 ± 0.7                | 7200 ± 430                                      | 9.5 ± 0.3                                       |

*Not determined.

this is increased to 1900 $\text{m}^{-1}\text{s}^{-1}$, whereas the effect of F37 is even more pronounced with a second-order rate constant of 7200 $\text{m}^{-1}\text{s}^{-1}$.

**FIGURE 2.** The rate of inactivation of FVIIa by carbamylation in the presence of sTF, F36, and F37. The relative activities were plotted against the incubation times in a semi-logarithmic plot. The slopes were derived from linear regression to obtain the rate constants for the decay of enzyme activity. The curves shown are representative examples of three independent experiments.

**FIGURE 3.** Time-dependent [1,3-$^3$H]DFP incorporation into FVIIa in the presence of sTF, F36, and F37. FVIIa (0.6 $\mu$m) was incubated alone or together with sTF, F36, or F37 (3 $\mu$m) in the presence of [1,3-$^3$H]DFP (50 $\mu$m). Samples were withdrawn at different time points and fractionated by non-denaturing polyacrylamide gel electrophoresis. The content of [1,3-$^3$H]DFP in the gel pieces containing FVIIa was determined by scintillation measurements. The maximum theoretical incorporation of [1,3-$^3$H]DFP with the FVIIa concentration used is 9400 cpm.
Factor VIIa-stimulating Antibodies

To determine whether the mutated residues are directly involved in the binding of the antibodies or, alternatively, introduce conformational changes that are deleterious for the binding, FFR-CMK inhibitor was irreversibly incorporated into the active site of the mutants. Because the inhibitor presumably constrains all the FVIIa variants in the same active conformation, any effect on binding of the antibodies could be ascribed to the mutated residue and place it in the binding epitope. FVIIaVEAY, L163(305)V-FVIIa, S170b(314)E-FVIIa, and F225(374)Y-FVIIa inhibited with FFR-CMK all maintained binding to F36 and F37. The binding of this mutant to F37 was partly restored when FFR-CMK was incorporated (from no binding to 59% compared with wild-type), but the binding to F36 remained low (5% compared with wild-type) (Fig. 4). The finding that some mutations affect the binding of both F36 and F37 suggests that the antibodies have overlapping, but not identical, epitopes. This was supported by the fact that F36 and F37 were incapable of binding simultaneously to FVIIa. However, even though we have used an active site inhibitor to lock FVIIa in the active conformation, we cannot entirely rule out the possibility that the two epitopes are distant but conformationally linked.

DISCUSSION

FVIIa possesses poor intrinsic catalytic activity and is predominantly in a zymogen-like conformation even after the endoproteolytic cleavage that yields the two-chain form. That is a notable exception from other serine proteases, which spontaneously become maximally active after cleavage of the peptide bond between residues 15 and 16. The catalytic activity of the zymogen versus the activated form of serine proteases is believed to be determined by the equilibrium distribution of active and inactive conformational species (for reviews, see Refs. 31 and 32). From this we inferred that an antibody recognizing and stabilizing the active conformation of FVIIa potentially could enhance the catalytic activity by shifting the conformational equilibrium toward the active state of the protease. To optimize the possibility of obtaining an antibody with these properties, we immunized with FFR-FVIIa, which is constrained in an active conformation. Eight percent of the antibodies from the generated hybridoma cell lines were indeed capable of increasing the intrinsic activity of FVIIa upon binding. Interestingly, the antibodies cluster in two distinct groups, exemplified by F36 and F37. The antibodies display different binding kinetics and differential effects on the enzymatic properties of FVIIa, suggesting that they operate through different mechanisms.

F36 and F37 bind FFR-FVIIa with high affinities in the subnanomolar range and with similar binding kinetics. Although both antibodies bind FVIIa with substantially decreased affinities compared with FFR-FVIIa, this is in the case of F36 mainly caused by a reduced association rate constant ($k_{on}$) and in the case of F37 entirely due to an increased dissociation rate constant ($k_{off}$). The affinity of F36 to FVIIa is increased upon complex formation with sTF, whereas the binding of F37 is almost completely abolished, suggesting that sTF sterically or conformationally hinders the association of this particular antibody. FVII, which represents the zymogen conformation of the enzyme, binds F37 with comparable affinity and binding kinetics as FVIIa, whereas only low binding to F36 was detected (Fig. 1 and Table 3). Together, these binding studies imply that F36 binds to an epitope on FVIIa that is not fully exposed or assembled in the inactive zymogen form. Because

Pocket but rather induce activity by stabilizing the substrate binding pockets S1, S2, and S3 (17). The binding of FVIIaVEAY to F36 was ~30% compared with wild-type FVIIa, whereas no binding to F37 was detected. This indicated that the epitopes had been disrupted either directly by one or more of the individual point mutations or indirectly by a conformational change. Further analysis showed that three of the four mutations in FVIIaVEAY (L163(305)V, S170b(314)E, and F225(374)Y), when introduced individually into FVIIa, affected the binding of F36 and F37. The single mutants displayed 21, 27, and 58%, respectively, binding to F36 and 8, 25, and 5%, respectively, binding to F37 compared with wild-type FVIIa. In addition, the mutation G223(372)A only affected the binding of F36 (40% binding compared with wild-type FVIIa) but not that of F37 (Fig. 4).

FIGURE 4. Epitope analysis using surface plasmon resonance. mAbs F36 or F37 were captured on a CM5 chip coated with anti-mouse IgG antibody and the binding, measured as maximum RUUs obtained after a single injection of wild-type FVIIa and FVIIa variants (100 nM), was monitored. The data were normalized to the binding level obtained for wild-type FVIIa, which was arbitrarily set to 1. The binding of ligands to F36 is depicted by blue bars; dark blue is in the absence of FFR-CMK, and light blue is in the presence of FFR-CMK. Likewise, binding of ligands to F37 is shown by gray bars. Dark and light gray is in the absence and presence of FFR-CMK, respectively.
Factor VIIa-stimulating Antibodies

only a small fraction of FVIIa is believed to exist in an active conformation, we propose that F36 preferentially recognizes the active fraction leading to the observed decrease in $k_m$, of FVIIa compared with FFR-FVIIa. F37 binds to FFR-FVIIa, FVIIa, and FVII and notably with similar association rate constants, indicating that the epitope of F37 is common to all three forms.

The amidolytic activity of FVIIa is increased to a similar extent by F36 and F37 (170- and 150-fold, respectively). However, the kinetic parameters are influenced differently by the two antibodies. Whereas F36 causes changes to both $K_m$ and $k_{cat}$, F37 mainly affects $K_m$. Notably, the increase in catalytic efficiency is more pronounced in the presence of antibodies compared with the natural activator sTF due to larger effects on $K_m$ (Table 1). These results have been related to the conformational state of important structural elements in the presence of the different ligands. Carbamylation of the N-terminal Ile-16(153) is dramatically reduced in the presence of F36, in fact to a similar level as seen with sTF (Fig. 2 and Table 4), suggesting that the antibody stabilizes the N terminus inserted into the activation pocket. Furthermore, we found that the rate of AT inhibition of FVIIa was increased 6-fold in the presence of F36 (Table 4). Moreover, incorporation of [1,3-3H]DFP (Fig. 3) and binding of PAB (Table 4) is increased substantially by F36, implying maturation of the oxyanion hole and the S1 pocket, respectively. On the other hand the oxyanion hole was found to be relatively less stabilized by F37 as judged from the intermediate incorporation of [1,3-3H]DFP (Fig. 3), and this antibody has only a modest influence on the N terminus (Fig. 2 and Table 4). Despite the limited effects on the N terminus and the oxyanion hole, F37 decreases the $K_i$ for PAB inhibition dramatically and to a level below that observed in the presence of sTF and F36 (Table 4). In addition, the second-order rate constant for AT inhibition of FVIIa was increased more than 20-fold in the presence of F37 (Table 4). Based on these results we propose a link between the buried N terminus and maturation of the oxyanion hole with a subsequent increase in $k_{cat}$. The maturation of the substrate binding pockets appears, at least in part, to occur independently of the former structural changes leading to increased binding of PAB, increased rate of AT inhibition, and lowering of $K_m$ for S-2288 hydrolysis. This is supported by substantially lower $K_i$ and $K_m$ values in the presence of F36 compared with sTF despite similar protection of the N terminus and incorporation of [1,3-3H]DFP in the presence of the two ligands. In addition, F37 decreases $K_m$ and $K_i$ without any notable increase in $k_{cat}$. The FX-activating activity of FVIIa is only stimulated by F37 and not by F36, and the effect is moderate compared with sTF and mainly caused by a decrease in $K_m$ (Table 2). The fact that F36 does not promote FX activation could suggest that the antibody disturbs important exosite interactions between FVIIa and FX. Even though F36, from a functional perspective, seems to resemble sTF by stabilizing the N terminus in the activation pocket, we found one apparent and conspicuous difference. Met-164(306) has been identified as a key mediator of the allosteric activation of FVIIa by TF (16). However, the activity of M164(306)D-FVIIa, which is inert to stimulation by sTF, is enhanced 270- and 200-fold by F36 and F37, respectively, indicating the existence of alternative allosteric mediators in FVIIa. F36 and F37 appear to have overlapping epitopes in an area encompassing the 170-loop supported by the observation that the two antibodies cannot engage in simultaneous binding to FVIIa. Specifically, residues Ser-170b(314), Phe-225(374), and Leu-163(305) seem to be part of the epitopes (Figs. 4 and 5). Furthermore, mutations at positions 164(306), 165(307), 170c(315), and 223(372) also seem to negatively affect the binding. However, especially for the latter mutants a substantial gain in binding is observed in the presence of FFR-CMK, indicating that global conformational changes induced by the mutations, at least in part, is responsible for the decreased binding (Fig. 4). Among the tested mutants we found one (R170c(315)A) that clearly affected binding of F36 but not F37 (Fig. 4). This residue is located in the S1 entrance frame (residues 216(365)-223(372)) (Fig. 5), which is known to be associated with conformational changes upon activation of FVIIa (10, 26, 33, 34). F36 could potentially associate directly with the activation domain through binding to the S1 entrance frame and in that way influence the N terminus. F37 may not directly contact the activation domain and, therefore, have a less pronounced effect on insertion of the N terminus.

In summary, we hypothesize that F36 acts globally on the enzyme by stabilizing the entire activation domain, presumably through stabilization of the N terminus, whereas F37 acts locally by stabilizing loop regions that form part of the substrate binding pockets. They both have epitopes in and around the 170-loop, but the exact differences in binding modes will require structural characterization.
Factor VIIa-stimulating Antibodies

Acknowledgments—We thank Anette Østergaard for expert technical assistance and Kasper Lambeth for hybridoma screening.

REFERENCES

1. Davie, E. W., Fujikawa, K., and Kisiel, W. (1991) The coagulation cascade. Initiation, maintenance, and regulation. Biochemistry 30, 10363–10370

2. Pedersen, A. H., Nordfang, O., Norris, F., Wiberg, F. C., Christensen, P. M., Moeller, K. B., Meidahl-Pedersen, J., Beck, T. C., Norris, K., Hedner, U., and Kisiel, W. (1990) Recombinant human extrinsic pathway inhibitor. Production, isolation, and characterization of its inhibitory activity on tissue factor-initiated coagulation reactions. J. Biol. Chem. 265, 16786–16793

3. Silverberg, S. A., Nemerson, Y., and Zur, M. (1977) Kinetics of the activation of bovine coagulation factor X by components of the extrinsic pathway. Kinetic behavior of two-chain factor VII in the presence and absence of tissue factor. J. Biol. Chem. 252, 8481–8488

4. Monroe, D. M., and Key, N. S. (2007) The tissue factor-factor VIIa complex. Procoagulant activity, regulation, and multitasking. J. Thromb. Haemost. 5, 1097–1105

5. Higashi, S., Nishimura H., Aita, K., and Iwanaga, S. (1994) Identification of regions of bovine factor VII essential for binding to tissue factor. J. Biol. Chem. 269, 18891–18898

6. Eigenbrot, C., Kirchhofer, D., Dennis, M. S., Santell, L., Lazarus, R. A., Stamos, J., and Ultsch, M. H. (1996) The crystal structure of bovine trypsinogen at 1.8Å resolution. II. Crystallographic refinement, and comparison with bovine trypsin. J. Biol. Chem. 271, 3109–3119

7. Pike, A. C., Brzozowski, A. M., Roberts, S. M., Olsen, O. H., and Persson, E. (1999) Structure of human factor VIIa and its implications for the triggering of blood coagulation. Proc. Natl. Acad. Sci. U.S.A. 96, 8925–8930

8. Banner, D. W., D’Arcy, A., Chêne, C., Winkler, F. K., Guha, A., Königsberg, W. H., Nemerson, Y., and Kirchhofer, D. (1996) The crystal structure of the complex of blood coagulation factor VIIa with soluble tissue factor. Nature 380, 41–46

9. Huber, R., and Bode, W. (1978) Structural basis of the activation and action of trypsin. Acc. Chem. Res. 11, 114–122

10. Rand, K. D., Jorgensen T. J., Olsen, O. H., Persson, E., Jensen, O. N., Stennicke, H. R., and Andersen, M. D. (2006) Allosteric activation of coagulation factor VIIa visualized by hydrogen exchange. J. Biol. Chem. 281, 23018–23024

11. Fehlhammer, H., Bode, W., and Huber, R. (1977) Crystal structure of bovine trypsinogen at 1.8Å resolution. II. Crystallographic refinement, refined crystal structure, and comparison with bovine trypsin. J. Mol. Biol. 111, 415–438

12. Dickinson, C. D., and Ruf, W. (1997) Active site modification of factor VIIa affects interactions of the protease domain with tissue factor. J. Biol. Chem. 272, 19875–19879

13. Higashi, S., Matsumoto, N., and Iwanaga, S. (1996) Molecular mechanism of tissue factor-mediated acceleration of factor VIIa activity. J. Biol. Chem. 271, 26569–26574

14. Sørensen, B. B., Persson, E., Freskgård, P. O., Kjalke, M., Ebzan, M., Williams, T., and Rao, L. V. (1997) Incorporation of an active site inhibitor in factor VIIa alters the affinity for tissue factor. J. Biol. Chem. 272, 11863–11868

15. Dickinson, C. D., Kelly, C. R., and Ruf, W. (1996) Identification of surface residues mediating tissue factor binding and catalytic function of the serine protease factor VIIa. Proc. Natl. Acad. Sci. U.S.A. 93, 14379–14384

16. Persson, E., Nielsen, L. S., and Olsen, O. H. (2001) Substitution of aspartic acid for methionine-306 in factor VIIa abolishes the allosteric linkage between the active site and the binding interface with tissue factor. Biochemistry 40, 3251–3256

17. Persson, E., Bak, H., Østergaard, A., and Olsen, O. H. (2004) Augmented intrinsic activity of factor VIIa by replacement of residues 305, 314, 337, and 374. Evidence of two unique mutational mechanisms of activity enhancement. Biochem. J. 379, 497–503

18. Persson, E., Kjalke, M., and Olsen, O. H. (2001) Rational design of coagulation factor VIIa variants with substantially increased intrinsic activity. Proc. Natl. Acad. Sci. U.S.A. 98, 13583–13588

19. Persson, E., Bak, H., and Olsen, O. H. (2001) Substitution of valine for leucine 305 in factor VIIa increases the intrinsic enzymatic activity. J. Biol. Chem. 276, 29195–29199

20. Petrovan, R. J., and Ruf, W. (2002) Role of zymogenicity-determining residues of coagulation factor VII/VIIa in cofactor interaction and macromolecular substrate recognition. Biochemistry 41, 9302–9309

21. Kerschbaumer, R. J., Riedrich, K., Kral, M., Varadi, K., Dorner, F., Rosing, J., and Scheffl fanger, F. (2004) An antibody specific for coagulation factor IX enhances the activity of the intrinsic factor X-activating complex. J. Biol. Chem. 279, 40445–40450

22. Ménez, R., Michel, S., Muller, B. H., Bossus, M., Ducancel, F., Jolivet-Reynaud, C., and Stura, E. A. (2008) Crystal structure of a ternary complex between human prostate-specific antigen, its substrate acyl intermediate, and an activating antibody. J. Mol. Biol. 376, 1021–1033

23. Scheffl fanger, F., Dockel, M., Rosing, J., and Kerschbaumer, R. I. (2008) Enhancement of the enzymatic activity of activated coagulation factor IX by anti-factor IX antibodies. J. Thromb. Haemost. 6, 315–322

24. Yoshihara, E., Gotoh, N., Nishino, T., and Nakae, T. (1996) Protein D2 porin of the Pseudomonas aeruginosa outer membrane bears the protease activity. FEBS Lett. 394, 179–182

25. Persson, E., and Nielsen, L. S. (1996) Site-directed mutagenesis but not γ-carboxylation of Glu-35 in factor VIIa affects the association with tissue factor. FEBS Lett. 385, 241–243

26. Persson, E., and Olsen, O. H. (2009) Activation loop 3 and the 170 loop interact in the active conformation of coagulation factor VIIa. FEBS J. 276, 3099–3109

27. Nicolaisen, E. M., Petersen, L. C., Thim, L., Jacobsen, J. K., Christensen, L., Kragelund, B. B., and Stennicke, H. R. (1992) Generation of Gla-domainless FVIIa by cathepsin G-mediated cleavage. FEBS Lett. 306, 157–160

28. Köhler, G., and Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256, 495–497

29. Markwardt, F., Landmann, H., and Walsmann, P. (1968) Comparative studies on the inhibition of trypsin, plasmin, and thrombin by derivatives of benzylamine and benzamidine. Eur. J. Biochem. 6, 502–506

30. Larsen, K. S., Østergaard, B., Bjelke, J. R., Olsen, O. H., Rasmussen, H. B., Christensen, L., Kragelund, B. B., and Stennicke, H. R. (2007) Engineering the substrate and inhibitor specificities of human coagulation factor VIIa. Biochem. J. 405, 429–438

31. Kraut, J. (1977) Serine proteases. Structure and mechanism of catalysis. Annu. Rev. Biochem. 46, 331–358

32. Hedstrom, L. (2002) Serine protease mechanism and specificity. Chem. Rev. 102, 4501–4524

33. Olsen, O. H., Rand, K. D., Østergaard, H., and Persson, E. (2007) A combined structural dynamics approach identifies a putative switch in factor VIIa employed by tissue factor to initiate blood coagulation. Protein Sci. 16, 671–682

34. Persson, E., and Olsen, O. H. (2010) Current status on tissue factor activation of factor VIIa. Thromb. Res. 125, S11–S12