Active Site Modification of Factor VIIa Affects Interactions of the Protease Domain with Tissue Factor*

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In the initiation of coagulation, tissue factor (TF) allosterically activates the serine protease factor VIIa (VIIa) through specific interactions with protease domain residues. These interactions, and consequently affinity for TF, may be influenced by conformational changes in the protease domain that result from zymogen-enzyme transition or occupancy of the active site by tight binding inhibitors. In functional competition and direct binding analysis, we determined affinities for zymogen and enzyme species of wild-type VII and of mutants at protease domain residues that contact TF. We demonstrate that TF binding is not influenced by zymogen activation, indicating that the protease domain of zymogen and enzyme dock similarly with TF. In contrast, active site occupancy enhanced the affinity for TF by predominantly decreasing the dissociation rate of the TF-VIIa complex. Of the three interface residues studied, only Met306 played a major role in the inhibitor-induced increase in affinity. Met306 is also important for transmitting the allosteric changes from TF to the active site, resulting in enhanced catalysis. This study thus provides evidence for a bidirectional conformational interdependence of the interface residue Met306 and the active site of VIIa.

Factor VII (VII)† binding to tissue factor (TF) at the cell surface is the major initiating step in the blood coagulation cascade (for a review, see Ref. 1). Besides its function in cell surface localization and acceleration of the activation of VII, TF is the cofactor for VIIa, enhancing proteolytic and amidolytic activity of the bound protease (2). Recently, the crystal structure of a complex of soluble TF and VIIa inhibited with Phe-Phe-Arg chloromethylketone (FFR-VIIa) demonstrated that each domain of VIIa makes contact with TF (3). The γ-carboxyglutamic acid-rich domain of VIIa docks with the carboxy-terminal fibronectin type III domain of TF, the first epidermal growth factor-like domain of VIIa contacts both domains of TF, and the second epidermal growth factor-like domain and protease domain form a continuous surface that sits upon the amino-terminal domain of TF. TF contacts with the protease domain are distant from the active site and also from the amino-terminal insertion by which residue Ile153(c16)2 forms a salt-bridge with Asp243(c194) that is critical for enzymatic activity of trypsin-like serine proteases. Because of the solvent accessibility of the insertion site, proteolytic activation of zymogen VII may proceed even if VII is docked to TF in a manner identical to the enzyme VIIa.

However, some studies reported a difference in the affinity for TF when zymogen VII was compared with the active protease (4–6). Higashi et al. (7) furthermore proposed that binding epitopes in bovine zymogen VII are not accessible for binding to TF, unless VII is activated. We hypothesized that conformational consequences of zymogen activation likely affect interactions of the protease domain of VIIa with TF. By alanine scanning mutagenesis, we had previously identified residues in the VIIa protease domain that are involved in binding TF (8). By comparing the binding characteristics of zymogen and enzyme forms of mutants at residue positions that contact TF, we here address the question of whether specific residue side chains in the interface with TF play different roles in the interaction of zymogen as compared with enzyme.

The binding residues in the interface with TF also transmit the allosteric changes that result in the catalytic activation of VIIa (8). Moreover, a reverse effect of active site occupancy and TF binding exists, as demonstrated by the increased TF affinity upon modification of the active site of VIIa with tight binding inhibitors (7) or upon binding of substrates (9, 10). We hypothesized that these conformational effects transmit to specific binding residues in the interface with TF. By characterizing the effect of active site modification of replacement mutants in the interface with TF, we identify one residue side chain that is influenced by inhibitor occupancy of the active site of VIIa. This analysis thus provides insight into the molecular details of the conformational changes in the protease domain of VIIa that affect TF interactions.

MATERIALS AND METHODS

Proteins—Full-length recombinant human TF from insect cells was reconstituted in 30% phosphatidylserine, 70% phosphatidylcholine as described (11). Plasma factor X was purified according to Fair et al. (12) followed by an immunoaffinity step to remove residual VII (8). The Kunitz-type inhibitor 5L15, which was selected from bovine pancreatic trypsin inhibitor by random mutagenesis and phage display for affinity for VIIa (13), was kindly provided by Dr. George Vlasuk (Corvas International, San Diego, CA).

Recombinant VII—The generation of VII mutants in the interface of the protease domain with TF has been described (8). Protein was purified from serum-free (Excel 301 supplemented with vitamin K3)
culture supernatant by antibody affinity on a Ca\(^{2+}\)-dependent antibody to the γ-carboxyglutamic acid-rich domain, as described (11). The protein obtained from this purification was predominantlyzymogen VII, and part of the protein preparation was set aside for characterization of thezymogen forms. The remaining protein was dialyzed against 50 mm NaCl, 25 mm CHAPS, 20 mm Tris/HCl, pH 8.5, for further HPLC fractionation on Mono Q employing a CaCl\(_2\) gradient elution (0.25–100 mm) in the same buffer. This latter step resulted in >80% activation to the active enzyme. The degree of γ-carboxylation in the final preparation was determined by amino acid analysis after alkaline hydrolysis (14). These analyses were performed by Dr. Robert Harris (Commonwealth Biotechnologies, Richmond, VA). M306A VIIa had 10.8 γ-carboxylated Glu residues, R277A VIIa 9.3, D309A VIIa 9.9, and wild-type VIIa 9.9.

Using oligonucleotide-directed mutagenesis with selection against a unique restriction site (15), we further generated a mutant with increased stability as zymogen by replacing the P1 (16) Arg with Gln (R152Q VII) and a mutant that lacked catalytic activity by Ala replacement of the active site Ser (S344A VII). The latter mutant also incorporated two replacements that precluded N-linked glycosylation (N145A and N322D). Elimination of N-linked glycosylation by these mutations in wild-type VIIa affected neither proteolytic function nor TF binding (data not shown). S344A VII was converted to S344A VIIa by incubation with factor IXa at a 1:10 enzyme:substrate ratio in 50 mm NaCl, 0.25 mm CaCl\(_2\), 20 mm Tris/HCl, pH 8.5, overnight at 37 °C followed by purification on Mono Q employing a NaCl (20–500 mm) gradient elution in 20 mm Tris/HCl, pH 8.5.

To test the effect of active site occupancy, mutants and wild-type VIIa were reacted with 0.5 mm dansyl-Glu-Gly-Arg chloromethylketone or N-Phe-1-Arg chloromethylketone (Calbiochem) in HEPES-buffered saline (HBS), 5 mm CaCl\(_2\) for 2–4 h. Subsequently, protein samples were extensively dialyzed against HBS with six buffer exchanges over a 24-h period to remove unreacted peptide inhibitor.

**Competitive Functional Assay to Determine Affinity for TF**—In a linked functional assay (8), we measured the ability of catalytically inactive VIIa derivatives or nonactivatable VII mutant to compete with active VIIa for TF binding. In an initial incubation of 20–60 min at 37 °C, 20 pm wild-type VIIa and varying concentrations of competitor were allowed to bind to a limiting concentration of 10 pm phospholipid-reconstituted TF in HBS (150 mm NaCl, 15 mm HEPES, pH 7.4), 5 mm CaCl\(_2\), 0.2% bovine serum albumin. Factor X was added to 100 nm to measure catalytically active TF/VIIa complexes for 10 min at 37 °C, followed by quenching with 50 mm EDTA and determination of factor Xa formation by chromogenic substrate (Spectrozyme FXa, American Diagnostica, Greenwich, CT) hydrolysis at ambient temperature. Data were fitted by a nonlinear least squares algorithm to the equilibrium binding equation assuming competition of VII and VIIa to the same binding site. The \(K_D\) of active VIIa was 5 pm, as determined from a dose titration in the functional assay (8).

Competition was also analyzed in an amidolytic assay with recombinant soluble TF (1–218) expressed in Escherichia coli (17). Dansyl-Glu-Gly-Arg chloromethylketone-inactivated wild-type or mutant VIIa at varying concentrations was incubated with 10 nm VIIa and 10 nm TF (1–218) in HBS, 5 mm CaCl\(_2\), 0.2% bovine serum albumin for 1 h at 37 °C to allow for complex formation. Chromozyme tPA (Boehringer Mannheim) was added to 1 nm, and initial rates of substrate hydrolysis were recorded in a kinetic microplate reader. Data were fitted to a competitive model of ligand binding, as above, assuming a \(K_D\) (app) of 2.8 nm for active VIIa binding to soluble TF (1–218) (8).

**Surface Plasmoc Resonance Analysis**—Rate and equilibrium binding constants of VII and VIIa binding to TF were obtained by surface plasmon resonance using a BIAcore 2000, as described previously (8). A noninhibitory anti-TF monoclonal antibody (TF9–10H10) was immobilized by amine coupling to the N-hydroxysuccinimide ester surface to give approximately 1000 response units. The standard flow buffer was HBS, 5 mm CaCl\(_2\), 1 mm CHAPS, and all proteins were diluted in this buffer. Full-length recombinant TF (1–218) was injected to saturate the monoclonal antibody on the sensor surface. Association data were collected from 40-µl injections of varying concentrations of VII or VIIa at a flow rate of 10 µl/min. Dissociation data were collected for 250 s after return to buffer flow. Injections of 100 nm EDTA served to regenerate the chip surface by destabilizing the TF/VIIa interaction. Association rate constants were determined from the early portion of the dissociation phase following injection of 1 mm VII or VIIa. Binding kinetics for wild-type and mutant VIIa in the presence of the Kunitz-type inhibitor 5L15 were determined by premixing VIIa with 10 µM 5L15. At this concentration, the inhibitor blocked amidolytic function of free VIIa, indicating complex formation in the absence of TF.

**Western Blot Analysis of Proteins Recovered from the Sensor Chip**—Because zymogen VII is readily activated to VIIa when bound to TF (18), we wanted to characterize the protein that was bound specifically to the immobilized TF. After allowing binding and return to buffer flow, 50 nm EDTA was injected to recover the specifically bound VII/VIIa in a single fraction. The recovered protein was reduced, separated by SDS-polyacrylamide gel electrophoresis, and transferred to Immobilon-P (Millipore Corp.) for Western blotting. The VIIa light chain was detected by horseradish peroxidase-conjugated secondary antibody and chemiluminescence.

**RESULTS**

**Comparison of VII and VIIa in a Competitive Functional Assay**—Recent data indicated that bovine zymogen VII bound with reduced affinity to TF as compared with active enzyme (7). Since the described difference may be attributable to the different assay formats used for determining TF binding by enzyme VIIa (direct amidolytic assay) versus zymogen VII (competitive assay), we produced a catalytically inactive mutant VIIa by active site Ser\(^{344}\) → Ala replacement. S344A VIIa was generated by activation of the zymogen form by factor IXa followed by repurification using ion exchange chromatography. We also generated a zymogen VII (R152Q VII) with increased stability by replacing the P1 residue Arg\(^{152}\) with Gln with VIIa to essentially abolish activation during purification and in functional assays. These mutants allowed us to compare zymogen and enzyme forms of human VII in a competitive functional assay. Zymogen and enzyme forms showed virtually identical dose titrations in competition with wild-type VIIa (Fig. 1). The calculated affinities for zymogen and enzyme were indistinguishable, providing evidence that the affinity of VII was not appreciably altered following zymogen activation to enzyme. The affinities also matched the functionally determined \(K_D\) (app) (5 pm) (8) for VIIa binding to TF in the same assay format. In contrast, wild-type VIIa inactivated with FFR-VIIa showed a markedly enhanced inhibition and thus an increased affinity for TF, in accordance with previous studies analyzing active site-modified VIIa (7).

**Comparison of Zymogen and Enzyme Forms by Surface Plasmon Resonance Binding Analysis**—A limitation of the competitive assay approach is the presence of substrate that could differentially affect TF binding by the VIIa variants. We therefore used surface plasmon resonance analysis to directly compare TF binding by VII and VIIa in the absence of substrate.
FIG. 2. Kinetics of VII and VIIa binding to immobilized TF measured by surface plasmon resonance. Upper panels, sensorgram obtained from injection of nonactivable R152Q VII (A) or wild-type VIIa (B) at concentrations from 12.5 to 800 nM over a surface containing immobilized TF-(1–263). Middle panels, dependence of concentration on values of $k_d$ determined from the association data for R152Q VII (C) or wild-type VIIa (D). A linear least squares analysis of the data fitted to the equation $k_d = k_{wa} C + k_{wu}$ (where $C$ represents the concentration of VII or VIIa) is shown as a solid line. Lower panels, sensorgrams of dissociations following injections of 800 nM R152Q VII (E) or wild-type VIIa (F) are shown as dashed lines. Nonlinear least squares curve fits of data to single exponential decay are shown as dashed lines.

| $k_a \times 10^7$ | $k_d \times 10^{-1}$ | $K_D$ |
|-----------------|-----------------|------|
| R152Q VII | 2.1 ± 1.0 | 7.8 ± 0.8 | 3.7 |
| Wild-type VIIa | 1.4 ± 0.2 | 5.6 ± 1.2 | 4.1 |
| S344A VII | 1.9 ± 0.7 | 4.6 ± 1.6 | 2.5 |
| S344A VIIa | 2.1 ± 0.9 | 7.4 ± 1.9 | 3.6 |
| R277A VII | 2.0 ± 0.8 | 21.3 ± 4.7 | 10.5 |
| R277A VIIa | 1.8 ± 0.6 | 29.0 ± 7.0 | 16.3 |
| M306A VII | 1.9 ± 0.6 | 17.2 ± 3.7 | 8.8 |
| M306A VIIa | 1.8 ± 0.5 | 14.5 ± 2.6 | 8.1 |
| D309A VII | 1.7 ± 0.4 | 13.5 ± 2.9 | 7.8 |
| D309A VIIa | 2.4 ± 0.2 | 18.8 ± 3.0 | 8.0 |

TABLE I
Comparisons of binding kinetics to TF of zymogen and enzyme species of wild-type and mutant VII

Mean and S.D. ($n \geq 3$) for the association ($k_a$) and dissociation rate ($k_d$) are shown. $K_D = k_d/k_a$.

Effect of Active Site Occupancy on the Affinity for TF—Previous studies (7, 9, 10) show that active site occupancy of VIIa influences cofactor affinity. By surface plasmon resonance, we analyzed TF binding of wild-type and mutant VIIa modified covalently with either dansyl-Glu-Gly-Arg chloromethylketone (EGR-VIIa) or FFR-VIIa. We also analyzed the effect of 5L15, a tight binding noncovalent inhibitor derived by phage maturation from bovine pancreatic trypsin inhibitor (13). When mutant or wild-type VIIa was compared before and after active site modification, the association rates were reduced 2-fold for EGR-VIIa, increased 2–3-fold for FFR-VIIa, and unchanged in
the presence of the 5L15 (Table II). There was no difference in the association rates when comparing mutant with wild-type VIIa either free or with each of the specific modifications. These experiments did not identify a molecular mechanism of how inhibitor binding influences the association rate of VIIa with TF.

The binding defect resulting from mutations of protease domain residues at the TF-binding interface is attributable to an increased dissociation rate of the mutant enzymes relative to wild-type VIIa (Table II). A lower dissociation rate was a common characteristic of active site modification by inhibitors. Inhibitor binding to wild-type VIIa slowed dissociation 6-fold for FFR-VIIa, 4-fold for EGR-VIIa, and 3-fold with 5L15 present. Inhibitor binding to R277A VIIa and D309A VIIa decreased the rate of dissociation to an extent that was similar to wild-type VIIa (Table II). In contrast, the dissociation rates of M306A VIIa were not significantly affected by binding of 5L15 or by active site modification with dansyl-Glu-Gly-Arg chloromethylketone. M306A FFR-VIIa dissociation from TF decreased 3.5-fold as compared with 6-fold for FFR-VIIa, demonstrating a partial response to binding of this inhibitor. Diminished response of M306A VIIa to inhibitor binding is also demonstrated by changes in the calculated free energy of binding relative to free enzyme (Table II). With each inhibitor, modification of M306A VIIa failed to produce a 0.5 kcal/mol decrease in the free energy of binding as compared with wild-type VIIa, R277A VIIa, or D309A VIIa. These data link Met306 to conformational changes in the active site of VIIa.

**Competition of M306A EGR-VIIa with Wild-type VIIa**—The loss of TF binding affinity of active M306A VIIa and R277A VIIa were similar, as measured by amideolytic assay (8), linked proteolytic assay (8), or surface plasmon resonance (Table I). Both mutants exhibited a similar 0.5–0.6 kcal/mol loss in free energy of binding relative to wild-type VIIa (Table I). Following active site modification, the difference in free energy of binding was unchanged for R277A EGR-VIIa (0.6 kcal/mol) as compared with wild-type EGR-VIIa, whereas M306A EGR-VIIa showed a 1.4 kcal/mol difference in free energy of binding relative to wild-type EGR-VIIa. Since M306A did not respond to active site modification with dansyl-Glu-Gly-Arg chloromethylketone as measured by surface plasmon resonance (Table II), we predicted that M306A EGR-VIIa should be a less potent competitor for VIIa binding to TF compared with R277A EGR-VIIa. The inhibition of active VIIa binding to soluble TF (Fig. 4) by M306A EGR-VIIa required significantly higher concentrations than the inhibition by R277A EGR-VIIa (Fig. 4), reflected in a 3-fold higher $K_i$ for the mutant at Met306. The calculated difference in the free energy of binding between mutant and wild-type EGR-VIIa are in good agreement with the data from the surface plasmon resonance experiments, demonstrating a 1.2 kcal/mol difference for M306A EGR-VIIa as compared with 0.5 kcal/mol for R277A EGR-VIIa. The competition experiment thus provides independent evidence for a prominent role of the Met306 side chain in increasing the affinity for TF following active site inhibition.

**DISCUSSION**

This study analyzes the effect of conformational changes in the VII protease domain on binding to cofactor TF. VIIa is among the few trypsin-like serine proteases that require cofactor binding for full catalytic function. In part, the low catalytic activity of free VIIa may result from a labile activating insertion of the newly formed amino terminus afterzymogen activation (6). Based on the data from the surface plasmon resonance experiments, demonstrating a 1.2 kcal/mol difference for M306A EGR-VIIa as compared with 0.5 kcal/mol for R277A EGR-VIIa. The competition experiment thus provides independent evidence for a prominent role of the Met306 side chain in increasing the affinity for TF following active site inhibition.

**Table II**

|                | $k_1 \times 10^6$ | $k_2 \times 10^{-4}$ | $K_i$ | $\Delta\Delta G$ |
|----------------|-------------------|----------------------|------|-----------------|
| Wild type      | 1.4 ± 0.1         | 0.5 ± 0.2            | 4.1  | 4.1             |
| VIIa 5L15      | 1.4 ± 0.1         | 1.8 ± 0.2            | 1.3  | 0.6             |
| EGR-VIIa       | 0.8 ± 0.1         | 1.3 ± 0.1            | 1.7  | 0.4             |
| FFR-VIIa       | 4.2 ± 0.2         | 0.9 ± 0.1            | 0.2  | 0.1             |
| R277A 5L15     | 2.0 ± 0.8         | 21.3 ± 4.7           | 10.5 | 1.1             |
| VIIa 5L15      | 1.5 ± 0.1         | 6.9 ± 1.1            | 4.7  | 0.4             |
| EGR-VIIa       | 0.8 ± 0.1         | 4.8 ± 0.6            | 5.8  | -0.4            |
| FFR-VIIa       | 4.0 ± 1.0         | 2.4 ± 0.5            | 0.6  | 0.7             |
| M306A VIIa     | 1.9 ± 0.6         | 17.2 ± 2.7           | 8.8  | 0.5             |
| EGR-VIIa       | 1.1 ± 0.4         | 11.8 ± 1.3           | 10.8 | +0.1            |
| FFR-VIIa       | 3.3 ± 0.1         | 4.9 ± 0.7            | 1.5  | 0.1             |
| D309A VIIa     | 1.7 ± 0.4         | 13.5 ± 2.9           | 7.8  | 0.9             |
| EGR-VIIa       | 1.3 ± 0.1         | 5.2 ± 0.6            | 3.9  | -0.4            |
| FFR-VIIa       | 4.2 ± 0.1         | 1.7 ± 0.1            | 0.4  | -1.8            |

This study analyzes the effect of conformational changes in the VII protease domain on binding to cofactor TF. VIIa is among the few trypsin-like serine proteases that require cofactor binding for full catalytic function. In part, the low catalytic activity of free VIIa may result from a labile activating insertion of the newly formed amino terminus afterzymogen activation (6). Based on the data from the surface plasmon resonance experiments, demonstrating a 1.2 kcal/mol difference for M306A EGR-VIIa as compared with 0.5 kcal/mol for R277A EGR-VIIa. The competition experiment thus provides independent evidence for a prominent role of the Met306 side chain in increasing the affinity for TF following active site inhibition.
for the protease domain of VIIa as opposed to VII. Three major energetically important contact residues in the protease domain contribute to TF-VIIa complex formation (8). We determined binding kinetics for zymogen and enzyme form of each of the mutants at position Arg\(^{277}\) (c134), Met\(^{306}\) (c164), and Asp\(^{309}\) (c167). Since binding affinities for each mutant zymogen and enzyme form were indistinguishable, it is reasonable to conclude that the protease domain of VII or VIIa docks similarly with TF. Based on this finding, we speculate that the overall structure of the TF-VII complex is quite similar to the TF-VIIa structure (3) with the exception of the uncleaved amino terminus of the protease domain, which would be surface-exposed. In the suggested position, the scissile bond is easily accessible to activating proteases, such as TF(VIIa) (23, 24) and factor Xa (18, 25), providing a rationale for the preferred activation of TF-bound zymogen VII.

Consistent with earlier studies (7), we found that active site occupancy of VIIa resulted in tighter binding to TF. Each inhibitor had different effects on the association rate of VIIa. Covalent modifications with dansyl-Glu-Gly-Arg chloromethylketone had the opposite effect. The Kunitz-type inhibitor 5L15 influenced the association rate insignificantly. We thank Dr. Curtis Kelly for advice with the BIAcore experiments. We are grateful for help in the recombinant protein expression and analysis by Cindi Biazak, Jennifer Royce, Pablitio Tejada, and David Revak and for preparation of the manuscript by Jenny Robertson. We thank Dr. George Vlasuk for the purified 5L15 inhibitor.

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