Residue Met\textsuperscript{156} Contributes to the Labile Enzyme Conformation of Coagulation Factor VIIa*

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Serine protease activation is typically controlled by proteolytic cleavage of the scissile bond, resulting in spontaneous formation of the activating Ile\textsuperscript{16}-Asp\textsuperscript{194} salt bridge. The initiating coagulation protease factor VIIa (VIIa) differs by remaining in a zymogen-like conformation that confers the control of catalytic activity to the obligatory cofactor and receptor tissue factor (TF). This study demonstrates that the unusual hydrophobic Met\textsuperscript{156} residue contributes to the propensity of the VIIa protease domain to remain in a zymogen-like conformation. Mutation of Met\textsuperscript{156} to Gln, which is found in the same position of the highly homologous factor IX, had no influence on the amidolytic and proteolytic activity of TF-bound VIIa. Furthermore, the mutation did not appreciably stabilize the labile Ile\textsuperscript{16}-Asp\textsuperscript{194} salt bridge in the absence of cofactor. VIIa\textsuperscript{Gln156} had increased affinity for TF, consistent with a long range conformational effect that stabilized the cofactor binding site in the VIIa protease domain. Notably, in the absence of cofactor, amidolytic and proteolytic function of VIIa\textsuperscript{Gln156} were enhanced 3- and 9-fold, respectively, compared with wild-type VIIa. The mutation thus selectively influenced the catalytic activity of free VIIa, identifying the Met\textsuperscript{156} residue position as a determinant for the zymogen-like properties of free VIIa.

Allostery regulation of catalytic activity of the serine protease factor VIIa (VIIa)\textsuperscript{1} is utilized as a mechanism to control the initiation of the coagulation pathways (1). VIIa circulates in the blood plasma as zymogen as well as the cleaved two-chain enzyme (2), but proteolytic function only ensues upon binding to its cell surface receptor and catalytic cofactor tissue factor (TF). TF has two distinct effects that regulate proteolysis by VIIa. First, TF provides affinity for macromolecular substrate recognition regions in addition to the active site of VIIa. Second, TF enhances catalytic activity by allosteric effects on the VIIa protease domain. In the absence of cofactor, VIIa has only very low catalytic activity toward small peptidyl substrate mimetics and TF stimulates the amidolytic of VIIa up to 100-fold (1). However, macromolecular substrate factor X scissile bond catalysis is enhanced \( >1000\)-fold (6), indicating that cofactor-induced conformational changes may influence extended macromolecular substrate recognition regions in addition to the S1-S3 subsite that is probed by the small substrates.

The low catalytic activity of free VIIa results from a zymogen-like conformation of the enzyme. Upon zymogen cleavage, serine proteases typically undergo a conformational ordering of loop segments, termed the activation domain (7), resulting in the formation of an activating canonical salt bridge of Asp\textsuperscript{194} with the newly generated amino-terminal Ile\textsuperscript{16} in the absence of cofactor. VIIa shows an increased susceptibility of the amino terminus to chemical modification (8), indicating exposure of Ile\textsuperscript{16} that can result from an alternative conformation or increased flexibility and disorder in the activation pocket of free VIIa. The structural determinants for the propensity of VIIa to stay in a zymogen-like conformation have not been investigated. Available structures of free and TF-bound VIIa (9–13) did not provide mechanistic insight, because in each case the active site of VIIa was occupied with inhibitors that are known to stabilize the Ile\textsuperscript{16} Asp\textsuperscript{194} salt bridge (14) and restrict conformational flexibility in the VIIa protease domain (15). Mutational studies also failed to elucidate the basis for the labile enzyme conformation, because the approaches taken mainly probed the active enzyme in the TF-VIIa complex (16).

This study investigates the role of residue Met\textsuperscript{156} in maintaining the zymogen-like conformation of VIIa. This residue is located within the activation pocket, covering Ile\textsuperscript{16} upon amino-terminal insertion (9). The conformation of the 156 side chain can influence the catalytic activity of serine protease domains. In the case of tissue plasminogen activator (tPA) (17, 18) and vampire bat plasminogen activator (19), Lys\textsuperscript{156} can substitute for Ile\textsuperscript{16} to form an activating salt bridge with Asp\textsuperscript{194}, resulting in efficient catalysis in the absence of zymogen cleavage. However, Lys at this position is found in a large number of serine proteases without conferring catalytic activity in the zymogen precursors, indicating that multiple interactions within the activation pocket determine the activation state of serine protease domains. Although Lys or other hydrophilic side chains are predominant in serine proteases that undergo spontaneous ordering of the activation pocket upon zymogen cleavage, VIIa has a Met residue in the 156 position. We hypothesized that the side-chain property of Met\textsuperscript{156} is one of the determinants that interfere with the acquisition of full catalytic activity of VIIa upon zymogen cleavage. This study demonstrates that replacement of Met\textsuperscript{156} with Gln, the side chain found in factor IX, had little effect on the activity of TF-bound VIIa. However, free

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‡ The abbreviations used are: VII/VIIa, coagulation factor VII/VIIa; TF, tissue factor; Gla domain, \( \gamma \)-carboxyglutamic acid-rich domain; PCPS, phosphatidylcholine/phosphatidylserine; tPA, tissue plasminogen activator; HBS, Hepes-buffered saline; TBS, Tris-buffered saline, CHAPS, 3-\( (3\)-cholamidopropyl)dimethylammonio-1-propanesulfonate.

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VIIaGln156 had enhanced catalytic function toward macromolecular and small peptidyl substrates. These experiments thus identify the first residue side chain that is one of the determinants for thezymogen conformation of the VIIa protease domain.

MATERIALS AND METHODS

Proteins—Wild-type and mutant VIIa were expressed in Chinese hamster ovary cells grown in suspension culture in serum-free medium supplemented with vitamin K. Mutant and wild-type recombinant VII was immunoaffinity purified with a calcium-dependent monoclonal anti-
tibody, affinity purified to VIIa at 4 °C, followed by ion exchange chromatography on MonoQ, as described previously (20). Factor X was purified from plasma with a final monoclonal antibody affinity step to reduce contamination by plasma VII (16). Full-length recombinant human TF, produced from insect cells, was reconstituted into 30% phosphatidyl-
serine/70% phosphatidylcholine (PCPS), as described previously (21). The soluble extracellular domain of TF (TF-(1–218)) was expressed in Escherichia coli and refolded from inclusion bodies (22). The Kunz-
type inhibitor 5L15 selected for VIIa specificity by phage display (23) was kindly provided by Dr. George Vlasuk (Corvas International, San Diego, CA).

Functional Assays—Kinetic parameters for factor X activation were determined by fixed concentration (200 nM) of TF reconstituted in PCPS [TF(PCPS)] and excess wild-type or mutant VIIa (1 nM) in Heps buffer saline (HBS, 10 mM Hepes, 150 mM NaCl, pH 7.4), 5 mM CaCl2, 0.2% bovine serum albumin. After a brief incubation at 37 °C to allow TF-VIIa complex formation, factor X (8 nM to 1 μM) was added, and factor Xa was quantified with the chromogenic substrate Spectrozyme FXa (American Diagnostica, Greenwich, CT) in samples quenched with 100 mM EDTA. Initial rates of factor Xa generation, based on calibration curves made with purified Xa, were fitted to the Michaelis-Menten equation using least squares regression analysis. For the determination of the proteolytic activity of free VIIa, 250 nM VIIa was incubated with 1 μM factor X at 37 °C in the presence or absence of 100 μM PCPS, followed by determination of factor Xa generation by chromogenic assay. Kinetic parameters for chromogenic substrate hydrolysis (Chromo-
zym tPA, Roche Molecular Biochemicals) were determined at fixed enzyme concentration (60 nM in the absence of TF or 30 nM in the presence of 120 nM TF (1–218)) with varying concentrations of substrate (0.02–2 mM) in Triis-buffered saline (TBS, 20 mM Tris, 150 mM NaCl, pH 8.0), 5 mM CaCl2, and 0.2% bovine serum albumin at ambient temperature. Initial rate data were fitted to the Michaelis-Menten equation using least squares regression analysis.

VII mutants were expressed in transient transfection experiments, and expression levels were determined by immunoassay. Protolytic activities of mutants were analyzed in a functional assay at 37 °C in HBS, 5 mM CaCl2, and 0.2% bovine serum albumin. A fixed concentration of TF/PCPS (5 μM) was saturated with increasing concentrations of mutant or wild-type VIIa, followed by addition of 50 nM factor X and determination of factor Xa generation by amidoanalytic assay. The maxi-
mum rate of Xa generation as a measure of the proteolytic function of the mutant VIIas in complex with TF was calculated based on calibration curves made with purified Xa.

Carbamylation of Ile16 in VIIa—Chemical modification of the amino-terminal Ile16 of wild-type or mutant VIIa was performed at ambient temperature according to Higashi et al. (8). Free VIIa (4 μM) or VIIa (1 μM) in complex with TF-(1–218) (4 μM) was reacted with 0.2 M KCNO in HBS, 5 mM CaCl2 for various times. Samples were withdrawn and diluted 25- or 60-fold for free or TF-bound VIIa, respectively, and the residual amidoanalytic activity was determined with 0.7 mM Chromozym tPA. Rates of inactivation were calculated from a plot of the residual activity (in percent of the initial activity) versus incubation time. Inhibition of VIIa by Antithrombin III/Heparin—To analyze the time dependence of inhibition of free VIIa (100 nM) or VIIa (50 nM) in complex with TF-(1–218) (250 nM) by antithrombin III/heparin, wild-type and mutant VIIa were reacted with 0.5 μM antithrombin III (Hematologic Technologies) in the presence of 5 units/ml unfractionated heparin (Ellkins-Sinn), at 37 °C in TBS, 5 mM CaCl2, and 0.2% bovine serum albumin. After defined times (2–60 min) samples were diluted into the chromogenic substrate Chromozym tPA (1.5 nM), and the residual amidoanalytic activity was immediately determined in a kinetic microplate reader.

Surface Plasmon Resonance Analysis—Binding constants for wild-type and mutant VIIa were analyzed using a BiACore 2000 instrument (Amersham Pharmacia Biotech Biosensor). A noninhibitory anti-TF antibody (TP9–10H10) was directly immobilized by amino-coupling to an activated dextran matrix for capture of full-length recombinant TF, as described previously (24). TF was injected to saturate the antibody, and association data were collected from injections of five concentrations (25 nM to 1 μM) of VIIa in HBS, 5 mM CaCl2, 0.005% surfactant P20, and 3 mM CHAPS. Binding kinetics in the presence of the Kunz-
type inhibitor 5L15 was determined by premixing VIIa with 10 μM 5L15. Dissociation data were collected for 250 s after return to buffer flow, and the chip surface was regenerated with pulses of 0.1 M EDTA and 4 M MgCl2. Dissociation of TF from the antibody could not be detected over a 6-h period under the standard buffer conditions, and the measured dissociation upon injection of VIIa thus reflects dissociation of the TF/VIIa complex, rather than the release of TF from the immo-
obilized antibody. Association and dissociation constants (kₐ and kₐ₋.species) were determined using the software provided by the manufacturer.

RESULTS

Mutational Analysis of Position 156 in VIIa—As a first step to identify the role of the 156 residue position in catalytic activity of VIIa, we characterized the proteolytic function of various side chain replacements in transient transfection experiments. Maximum rates of factor Xa generation were determined by saturating a fixed concentration of phospholipid-reconstituted TF with the goal of defining the permissive mutations that do not interfere with amidoanalytic and proteolytic function of TF-
bound VIIa. Changing the 156 position to Lys, as found in the highly homologous factor X, resulted in diminished function. In contrast, a Gln side chain, as found in factor IX of all species and in the majority of chymotrypsin-like serine proteases, was allowed for normal or slightly enhanced proteolytic function, as compared with wild-type VIIa (Table I). Negatively charged residues, such as Glu and Asp, were consistently less well tolerated than their respective amide counterparts. In the case of Asn, 60% of wild-type activity was retained. Only Gln at 156 produced a fully functional VII molecule, whereas a number of smaller side chain replacements, such as Ala, Val, or Ser, showed significantly reduced proteolytic function. Thus, re-
placement of the hydrophobic Met156 side chain by the more hydrophilic side chain Gln is the only permissive mutation that allows for normal proteolytic function of the TF-VIIa complex.

Effect of the Met156 to Gln Mutation on TF Binding—VIIaGln156 was stably expressed, purified, and autoactivated at 4 °C. Autoactivation of the mutant appeared to proceed somewhat faster than wild-type VII, but the purified protein showed electrophoretic mobility and ~95% conversion to the two-chain enzyme indistinguishable from wild-type VIIa. Binding of mu-
tant and wild-type VIIa to antibody-captured full-length TF was analyzed by surface plasmon resonance measurements. Although the mutation had only a minor ~2-fold effect on the association rate, VIIaGln156 dissociated from TF with a signifi-
cantly lower rate (Table II), indicating that the cofactor binding site is in a conformation that allows for tighter binding to TF. Active site occupancy of wild-type VIIa by the Kunz-type inhibitor 5L15 is known to tighten the binding with TF by slowing the dissociation rate (24). Although 5L15 increased affinity for wild-type VIIa 4-fold, only marginal changes were

| n | Wild-type | Met156 to Lys | Met156 to Gln | Met156 to Glu | Met156 to Asn | Met156 to Asp | Met156 to Ala | Met156 to Val | Met156 to Ser |
|---|---|---|---|---|---|---|---|---|---|
| n | 3.0 | 7.9 ± 0.5 | 1.7 ± 0.4 | 10.1 ± 1.2 | 1.2 ± 0.3 | 4.5 ± 0.8 | 0.1 ± 0.02 | 2.6 ± 0.5 | 2.8 ± 0.3 | 2.6 ± 0.6 |

Table I

Factor X activation by VIIa Met156 replacement mutants

*Note.* All values are expressed as the maximal rate of Xa generation (Vmax) as a measure of the proteolytic function of the TF/VIIa complex, rather than the release of TF from the immo-
obilized antibody. Association and dissociation constants (kₐ and kₐ₋) were determined using the software provided by the manufacturer.
observed for VIIa\(^{\text{Gln156}}\). Amidolytic function of mutant or wild-type VIIa were blocked >99% by the inhibitor under the experimental conditions, excluding a loss of inhibitor binding to the mutant as the reason for a lack in the change of binding kinetics. Thus, the conformation of the mutant’s protease domain, independent of active site occupancy, appeared to be in a higher affinity state for TF.

**Normal Cofactor-mediated Stabilization of the Amino-terminal Insertion**—Although free VIIa displays a labile enzyme conformation with the \(\alpha\)-amino group of Ile\(^{16}\) susceptible to chemical modification, cofactor binding induces structural rearrangements resulting in a protected amino terminus. Because carbamylation of Ile\(^{16}\) in VIIa by reaction with KNO\(_2\) inactivates VIIa (8, 25), the decrease in the amidolytic activity can be used to determine the rate of modification of the amino terminus as measure for the stability of the Ile\(^{16}\)-Asp\(^{194}\) salt bridge. Carbamylation of free enzyme showed a similar rate of inactivation of VIIa\(^{\text{Gln156}}\) versus wild-type VIIa (6.3 ± 0.4 versus 7.5 ± 0.3% loss of initial activity/10 min), demonstrating that the residue replacement was not sufficient to completely order the activation pocket and to stabilize the Ile\(^{16}\)-Asp\(^{194}\) salt bridge. In addition, the rate of inactivation of the TF-bound mutant was also indistinguishable from wild-type VIIa (1.1 ± 0.1 versus 1.4 ± 0.1% loss of initial activity/10 min). Thus, the Gln replacement for Met\(^{156}\) does not appreciable influence the stability of the Ile\(^{16}\)-Asp\(^{194}\) salt bridge in free or TF-bound enzyme.

**Enhanced Amidolytic Activity of VIIa\(^{\text{Gln156}}\) in the Absence of Cofactor**—The higher affinity binding of VIIa\(^{\text{Gln156}}\) to TF may reflect conformational changes in the cofactor binding site that are associated with increased catalytic function of the mutant in the absence of cofactor. To address this issue, the catalytic activities of wild-type or mutant VIIa were analyzed with small peptidyl substrates. The catalytic efficiency of hydrolysis of amido group of Ile \(^{16}\) susceptible to chemical modification, cofactor binding induces structural rearrangements resulting in a protected amino terminus. Because carbamylation of Ile\(^{16}\) in VIIa by reaction with KNO\(_2\) inactivates VIIa (8, 25), the decrease in the amidolytic activity can be used to determine the rate of modification of the amino terminus as measure for the stability of the Ile\(^{16}\)-Asp\(^{194}\) salt bridge. Carbamylation of free enzyme showed a similar rate of inactivation of VIIa\(^{\text{Gln156}}\) versus wild-type VIIa (6.3 ± 0.4 versus 7.5 ± 0.3% loss of initial activity/10 min), demonstrating that the residue replacement was not sufficient to completely order the activation pocket and to stabilize the Ile\(^{16}\)-Asp\(^{194}\) salt bridge. In addition, the rate of inactivation of the TF-bound mutant was also indistinguishable from wild-type VIIa (1.1 ± 0.1 versus 1.4 ± 0.1% loss of initial activity/10 min). Thus, the Gln replacement for Met\(^{156}\) does not appreciable influence the stability of the Ile\(^{16}\)-Asp\(^{194}\) salt bridge in free or TF-bound enzyme.

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**Increased Proteolytic Function of Free VIIa upon Replacement of Met\(^{156}\) with Gln**—Factor X activation by free VIIa was analyzed in the presence and absence of a negatively charged phospholipid surface (PCPS). In both cases, activation of factor X by VIIa\(^{\text{Gln156}}\) was enhanced 9-fold compared with wild-type VIIa (Table IV). However, only subtle changes in the activation of factor X by VIIa\(^{\text{Gln156}}\) in complex with phospholipid-reconstituted TF were detected (Table IV), both in regard to \(K_m\) and \(k_{cat}\). These data demonstrate that macromolecular substrate binding as well as scissile bond cleavage are not affected by the mutation after complex formation with the catalytic cofactor and after acquisition of full catalytic activity. In free VIIa, however, the catalytic function toward the macromolecular substrate is enhanced, consistent with the data for small substrate hydrolysis that also demonstrated selectively increased amidolytic activity of free VIIa\(^{\text{Gln156}}\).
Gln replacement is permissive for function of the TF-bound respective proteases, providing a structural rationale why the same space is occupied by the Met and Gln side chains in the activation pocket. Second, VIIa Gln156 had higher amidolytic change of the residue replacement of Met156 by Gln in the appearance to be altered, indicating a long range conformational angles with 0.5 μM antithrombin III/heparin. Residual amidolytic activity was determined after the indicated times of incubation of 100 nM free VIIaGln156 was little different from wild-type VIIa. How-

![Figure 1](http://www.jbc.org/Downloaded from)

**FIG. 1.** Inhibition of wild-type VIIa (squares) or VIIaGln156 (triangles) by antithrombin III/heparin. Residual amidolytic activity was determined after the indicated times of incubation of 100 nM free VIIa (filled symbols) or 50 nM VIIa in complex with 250 nM TF-(1–218) (open symbols) with 0.5 μM antithrombin III in the presence of 5 units/ml unfractionated heparin at 37 °C in TBS, 5 mM CaCl₂, and 0.2% bovine serum albumin.

**TABLE IV**

| Factor X activation by VIIa bound to phospholipid-reconstituted TF (n = 7). |
|-------------------------------------------------|
| **Kinetic parameters**                         | **VIIa** | **VIIa + PCPS** |
| **K_{cat} nmol of Xa/min/μmol of VIIa**         | 1.3 ± 0.1 | 23.9 ± 1.1 |
| **K_{m} nmol of VIIa**                         | 0.15 ± 0.02 | 2.6 ± 0.1 |
| **k_{cat}/K_{m} × 10^{3} M^{-1} s^{-1}**       | 1.9 ± 1.5 | 1.3 ± 1.0 |

![Figure 2](http://www.jbc.org/Downloaded from)

**FIG. 2.** A, view of the activation pocket of VIIa in complex with TF (9). The position of the amino terminus of the protease domain is marked by NH₂. Key residues (positions 21, 154, and 156) that differ between VIIa and other serine proteases, such as factor IXa, are shown in a surface representation (at 80% of the actual size). The backbone is shown in a ribbon representation, colored to highlight residues 16–20 (white) and 142–153 (yellow), and the atoms of residues 21, 154, and 156 are color-coded: red, oxygen; blue, nitrogen; green, carbon; yellow, sulfur. B, replacement of Met156 by one possible rotamer position of Gln in the structure of VIIa is shown in relation to residues Glu154 and Val153.

in the TF-VIIa complex (9) demonstrate that essentially the same space is occupied by the Met and Gln side chains in the respective proteases, providing a structural rationale why the Gln replacement is permissive for function of the TF-bound VIIa. However, free VIIaGln156 displayed increased function compared with wild-type VIIa. First, the mutant had higher affinity for TF and affinity was not appreciably influenced by active site inhibitor binding that typically slows dissociation for wild-type VIIa and for most of the previously characterized mutants (15). The cofactor binding site of VIIaGln156 thus appears to be altered, indicating a long range conformational change of the residue replacement of Met156 by Gln in the activation pocket. Second, VIIaGln156 had higher amidolytic and proteolytic activity in the absence of cofactor. Amidolytic activity was enhanced 3-fold, although activation of factor X was increased 9-fold in the presence or absence of phospholipid. The difference in enhancement may result from an additional ordering of regions that influence macromolecular substrate scissile bond cleavage, but not the hydrolysis of p-nitroanilide chromogenic substrates, which bind primarily to the S1-S3 subsites of the catalytic cleft.

Because stabilization of the amino-terminal Ile²¹⁶-Asp¹⁹⁴ salt bridge is generally considered to be the major determinant for the activation state of the VIIa protease domain, it was surprising that chemical modification of the amino-terminal Ile¹⁵⁶ in free VIIaGln¹⁵⁶ was little different from wild-type VIIa. However, we have found poor correlation of cofactor-mediated catalytic enhancement and susceptibility of the amino terminus to chemical modification in a number of other VIIa mutants (29).

The local environment at the 156 position in VIIa differs from other homologous coagulation factors such as factor IXa. Although porcine factor IXa has a hydrophobic Ile residue in the 154 position along with a pair of charged residues at position 21 (Asn) and 156 (Gln), VIIa has a charged Glu¹⁵⁴ and a pair of hydrophobic residues, i.e. Val¹⁵³ and Met¹⁵⁶ (Fig. 2A). One or two water molecules (not shown) are consistently resolved between the Glu¹⁵⁴ and Met¹⁵⁶ side chain in structures of the TF-VIIa complex (9, 10). Fig. 2B shows Met¹⁵⁶ replaced by Gln in one possible rotamer position that orients the amide of the Gln side chain toward Glu¹⁵⁴. The low catalytic function of acidic side chain replacement mutants and the high functional activity of amide counterparts (Table I) indirectly supports the notion that such an orientation of the introduced Gln¹⁵⁶ is most compatible with optimal activity. The charge complementarity of the Gln¹⁵⁶ and Glu¹⁵⁴ side chains may stabilize the local conformation of this region in free VIIaGln¹⁵⁶, possibly involving the coordination of a water...
molecule between the two side chains. The activating effect of the Gln\textsubscript{156} mutation may thus be mediated through an effect on the Glu\textsubscript{154} position rather than the adjacent amino-terminal insertion.

In wild-type VIIa, Ala mutation of Glu\textsubscript{154} reduces amidolytic function 3-fold and proteolytic function 6- to 10-fold, displaying some divergence of the mutational effect on amidolytic function 3-fold and proteolytic function 6- to 10-fold, displaying

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