Role of Residue Phe^{225} in the Cofactor-Mediated, Allosteric Regulation of the Serine Protease Coagulation Factor VIIa†

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ABSTRACT: Functional regulation by cofactors is fundamentally important for the highly ordered, consecutive activation of the coagulation cascade. The initiating protease of the coagulation system, factor VIIa (VIIa), retains zymogen-like features after proteolytic cleavage of the activating Arg^{15}−Ile^{16} peptide bond and requires the binding of the cofactor tissue factor (TF) to stabilize the protease domain in an active enzyme conformation. Structural comparison of TF-bound and free VIIa failed to provide a conclusive mechanism for this catalytic activation. This study provides novel insight into the cofactor-dependent regulation of VIIa by demonstrating that the side chain of Phe^{225}, an aromatic residue that is common to allosterically regulated serine proteases, is necessary for optimal TF-mediated activation of VIIa's catalytic function. However, mutation of Phe^{225} did not abolish the cofactor-induced stabilization of the Ile^{16}−Asp^{194} salt bridge, previously considered the primary switch mechanism for activating VIIa. Moreover, mutation of other residue side chains in the VIIa protease domain resulted in a reduced level of or no stabilization of the amino-terminal insertion site upon TF binding, with little or no effect on the TF-mediated enhancement of catalysis. This study thus establishes a crucial role for the aromatic Phe^{225} residue position in the allosteric network that transmits the activating switch from the cofactor interface to the catalytic cleft, providing insight into the highly specific conformational linkages that regulate serine protease function.

Coagulation factors are highly homologous trypsin-like serine proteases that are regulated in their function by protein cofactors. The initiating serine protease of the coagulation cascade, factor VIIa (VIIa),[1] binds to the cell surface receptor and cofactor tissue factor (TF) through multiple interactions involving the amino-terminal γ-carboxyglutamic acid-rich (Gla) domain, two epidermal growth factor-like domains, and the protease domain (*2*, *3*). These interactions with TF orient the VIIa protease domain for scissile bond cleavage of macromolecular substrates that assemble in the plane of a phospholipid membrane. Macromolecular substrate affinity and specificity are primarily determined by recognition through exosites, such as an extended surface, that is created by docking of the VIIa Gla domain with the carboxyl-terminal module of TF (*4*−*8*). The substrate factor X Gla domain interacts with this membrane proximal region of the TF·VIIa complex, allowing for a cooperation of Gla domain-mediated phospholipid membrane mass transport and protein−protein interactions during macromolecular substrate turnover.

Another important aspect of cofactor function can be assessed as the TF-mediated enhancement of VIIa's hydrolysis of small *p*-nitroanilide pseudosubstrates (*9*). This allosteric activation of VIIa is achieved by interactions of the amino-terminal cytokine receptor β-strand module of TF with the VIIa protease domain (*10*). Typically, serine protease zymogens are activated by proteolytic cleavage of the Arg^{15}−Ile^{16} scissile bond[2] that allows for the formation of an internal salt bridge between Ile^{16} and Asp^{194} and resulting spontaneous conformational ordering of a number of loop segments, termed the activation domain, to aquire full catalytic activity (*11*). This transition is only partial upon activation of VII to VIIa, and cofactor interactions are thought to complete the transition of VIIa to an active enzyme conformation (*1*, *12*, *13*). The precise mechanism by which TF allosterically activates the serine protease domain of VIIa remains incompletely defined, despite the availability of TF-bound (*2*, *14*) and free VIIa structures (*15*−*17*). The relatively subtle differences between these structures may result from the crystallization in the presence of either covalent or tight binding inhibitors. Inhibitor binding is known to order the activation domain of serine protease zymogens (*18*) and to change the kinetics of VIIa binding to TF and conformational exosite probes (*10*, *19*, *20*). These changes can be interpreted as restricted conformational flexibility in the protease domain of VIIa.

An evolutionary tree in the serine protease family is suggested from the codon usage for the active site Ser^{195} and the residue side chain properties in position 225 which

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[1] Abbreviations: VII and VIIa, coagulation factors VII and VIIa, respectively; TF, tissue factor; Gla domain, γ-carboxyglutamic acid-rich domain; PCPS, phosphatidylcholine/phosphatidylserine; CHO, Chinese hamster ovary; HBS, Hepes buffer saline; TBS, Tris buffer saline; CHAPS, 3-[(3-cholamidopropyl)dimethylamonio]-1-propanesulfonate.

[2] Residue positions are numbered according to the chymotrypsin scheme described in ref *1*. This numbering differs from the numbering in the TF·VIIa structure (*2*) for certain residues. To avoid ambiguities, residue positions in the mature VII polypeptide are also given in brackets, where necessary.

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is an aromatic residue in allosterically regulated enzymes, as compared to Pro in digestive serine proteases (*21*). Mutation of Tyr^{225} to Pro in thrombin abolishes allosteric changes induced by Na^+ binding (*21*), suggesting a more general significance of the position 225 side chain properties in the allosteric regulation of serine protease domains. In VIIa, the aromatic side chain of Phe^{225} is located in proximity to the cofactor binding site (*2*), and we hypothesized that this residue position may be directly involved in the crucial cofactor-mediated allosteric activation of VIIa. This study characterizes the site specific Pro^{225} mutant of VIIa and provides evidence for a role of this residue in transmitting the cofactor effect to the primary specificity site of VIIa. This linkage is independent of effects that stabilize the insertion of the amino terminus in VIIa, providing novel insight into the mechanism of cofactor-mediated regulation of serine protease domains.

EXPERIMENTAL PROCEDURES

Proteins. Factor X was purified from plasma with a final affinity purification step on anti-factor X monoclonal antibody F21-4.2 to reduce the level of contamination by VII (*1*). The soluble extracellular domain of TF (TF_{1-218}) was expressed in *Escherichia coli* and refolded from inclusion bodies (*22*). Full-length recombinant human TF, produced from insect cells, was reconstituted into a 30% phosphatidylserine/70% phosphatidylcholine mixture (TF/PCPS) as described previously (*23*). Protein concentrations were determined by the bicinchoninic acid assay (Pierce Chemical Co) and converted to molar concentrations on the basis of the calculated molecular mass. The Kunitz-type inhibitor 5L15 selected for VIIa specificity by phage display (*24*) was kindly provided by G. Vlasuk (Corvas International, San Diego, CA). Recombinant wild-type or mutant VII was produced in stably transfected Chinese hamster ovary (CHO) cells that were grown in suspension culture in serum-free medium supplemented with vitamin K_3 (*23*). Recombinant VII was immunoaffinity purified with a calcium-dependent monoclonal antibody, activated to VIIa by autoactivation at 4 °C or by incubation with factor IXa at 37 °C, and purified by a final ion exchange chromatography step on MonoQ. Both activation protocols yielded >95% two chain VIIa with no apparent degradation in the light or heavy chains, and activated factor IXa or autoactivated wild-type VIIa behaved identically in functional assays (*25*). VIIa was active site modified with 0.5 mM D-Phe-L-Phe-Arg chloromethyl ketone (Calbiochem) in Hepes buffer saline (HBS) [150 mM NaCl and 10 mM HEPES (pH 7.4)] and 5 mM $CaCl_2$ for 6 h, followed by extensive dialysis against HBS.

Surface Plasmon Resonance Analysis. Binding kinetics were determined on BIAcore 2000 instruments (Pharmacia Biosensor). To characterize the binding of wild-type or mutant VIIa with TF, a noninhibitory anti-TF antibody (TF9-10H10) was directly immobilized by amino coupling to an activated dextran matrix (*10*). Full-length recombinant TF was injected to saturate the antibody, and association data were collected from injections of five concentrations (25 nM to 1 μM) of unmodified or covalently active site-modified VIIa in HBS, 5 mM $CaCl_2$, 0.005% surfactant P20, and 3 mM 3-[(3-cholamidopropyl)dimethylamonnio]-1-propanesulfonate (CHAPS). Binding kinetics in the presence of the Kunitz-type inhibitor 5L15 were determined by premixing VIIa with 10 μM 5L15. Dissociation was monitored for 250 s after the return to buffer flow, and the chip surface was regenerated by alternating injections of 0.1 M EDTA and 4 M $MgCl_2$. To characterize the conformational changes in the epitope of monoclonal antibody F3-3.2A that binds the VIIa protease domain, the antibody was biotinylated and indirectly immobilized to a sensor chip derivatized with streptavidin. Association kinetics for binding of VIIa to the antibody were measured by injecting 0.1−2 μM VIIa or active site-modified VIIa in HBS, 5 mM $CaCl_2$, and 0.005% surfactant P20. To analyze the effect of TF binding on antibody affinity for VIIa, 4 μM TF_{1-218} was included in the injections of VIIa and during the dissociation phase (*20*). Association and dissociation constants (k_{on} and k_{off}, respectively) were calculated using the evaluation software.

Determination of the Kinetic Parameters for Substrate Hydrolysis. Kinetic parameters for chromogenic substrate (0.02−2 mM) Chromozyme tPA (Roche) hydrolysis by wild-type or mutant VIIa were determined at a fixed enzyme concentration (60 nM in the absence of TF or 30 nM in the presence of 120 nM TF_{1-218}) in Tris-buffered saline (TBS) [150 mM NaCl and 20 mM Tris (pH 8.0)], 5 mM $CaCl_2$, and 0.2% bovine serum albumin at ambient temperature. To exclude effects of Na ions on the measurements of amidolytic activity, stock solutions of wild-type VIIa and TF_{1-218} were extensively dialyzed against a Na^+-free buffer consisting of 100 mM choline chloride, 5 mM $CaCl_2$, and 10 mM Tris (pH 8.0). Proteins were diluted into 10 mM Tris (pH 8.0), 5 mM $CaCl_2$, and either NaCl or choline chloride at 200 mM to evaluate the effect of Na ion concentration independent of ionic strength on the hydrolysis of the chromogenic substrate Chromozyme tPA (0.5 mM).

Kinetic parameters for factor X activation were determined in HBS, 5 mM $CaCl_2$, and 0.2% bovine serum albumin at 200 pM TF/PCPC with excess VIIa (1 nM) at 37 °C. Factor X (8 nM to 1 μM) was added after a brief preincubation to allow for TF·VIIa complex formation, and the reaction was quenched with 100 mM EDTA. Factor Xa was quantified with the chromogenic substrate Spectrozyme FXa (American Diagnostica, Greenwich, CT), based on calibration curves with purified factor Xa, and initial rates of factor Xa generation were calculated. Initial rate data were fitted to the Michaelis−Menten equation using least-squares regression analysis.

Determination of the K_i for Inhibition of VIIa by p-Aminobenzamidine. After a brief preincubation of 30 nM wild-type or mutant VIIa with 120 nM TF_{1-218} in HBS, 5 mM $CaCl_2$, and 0.2% bovine serum albumin, 0.008−1 mM *p*-aminobenzamidine was added along with 0.5 mM chromogenic substrate Chromozyme tPA for the determination of the rates of *p*-nitroanilide release at ambient temparature. Apparent K_i values were calculated by fitting to a model of competitive inhibition at a fixed substrate concentration, taking into account the calculated parameters for chromogenic substrate hydrolysis.

Carbamylation of Ile^{16} by Reaction with KNCO. Wild-type or mutant VIIa was reacted with 0.2 M KNCO in HBS and 5 mM $CaCl_2$ at ambient temperature according to the method of Higashi et al. (*13*). After chemical modification of free VIIa (4 μM) or VIIa (1 μM) in complex with TF_{1-218} (4 μM) for various periods of time, samples were withdrawn and diluted 25- and 60-fold for free and TF-bound VIIa,

respectively, into 0.7 mM Chromozyme tPA for determination of the residual amidolytic activity of the unreacted enzyme. Rates of inactivation were calculated from a plot of the residual activity (percentage of initial) versus the incubation time.

RESULTS

Loss of Proteolytic Function of VIIa$_{Pro}^{225}$. Residue Phe[225] in VIIa was mutated to Pro by site-directed mutagenesis, and the mutant was expressed by stable transfection of Chinese hamster ovary cells. Although the expression levels for this mutant were significantly lower than those obtained for previously generated mutant and wild-type VII expressing cells lines, VII$_{Pro}^{225}$ could be purified and activated under standard conditions, yielding >95% activated two-chain enzyme. Both chains exhibited electorphoretic mobilities that were indistinguishable from those of wild-type VIIa, excluding major conformational alterations or increased protease sensitivity of the mutant protein. VIIa$_{Pro}^{225}$ in complex with phospholipid-reconstituted TF activated the macromolecular substrate factor X with a K_M that was comparable to the K_M obtained for wild-type VIIa (48 ± 4 vs 55 ± 8 nM). These data indicate that macromolecular substrate docking is not influenced by the mutation. However, the mutant exhibited a 3-fold reduction in the k_{cat} (3.2 ± 0.4 vs 9.4 ± 0.9 s^{-1}), indicating that the mutant exhibited a reduced level of scissile bond cleavage due to a direct influence on the catalytic cleft or due to perturbation of the cofactor-induced conformational changes that enhance the catalytic function of VIIa.

Analysis of Cofactor Binding by Surface Plasmon Resonance Analysis. Affinity of VIIa for TF is in part mediated by an extended interface of the VIIa protease domain. To analyze the effects of the Phe[225] mutation on the interaction of the protease domain with TF, binding parameters were determined in the presence and absence of active site inhibitors. Without active site occupancy, VIIa$_{Pro}^{225}$ dissociated from TF with a significantly reduced rate compared to that of wild-type VIIa (Table 1). Active site occupancy decreases the level of dissociation of wild-type VIIa from cofactor, likely by stabilizing the interface where VIIa residue Met[164] contacts TF (*10*). The active sites of mutant or wild-type VIIa were modified either covalently with Phe-Phe-Arg chloromethyl ketone or by co-injection of the reversible Kunitz-type inhibitor 5L15 (*24*). Under the experimental conditions, the amidolytic activity of the mutant and wild-type VIIa was inhibited >99%, demonstrating complete active site occupancy by the inhibitors. Active site modification of wild-type VIIa resulted in the typical 3− 6-fold decrease in the dissociation rate, depending on the inhibitor that was used. In contrast to wild-type VIIa, binding of the Kunitz-type inhibitor 5L15 failed to further slow dissociation of the mutant from TF and covalent modification with Phe-Phe-Arg chloromethyl ketone had only a minor <2-fold effect on the dissociation rate (Table 1). These data indicate that the thermodynamic linkage from the active site to the VIIa protease domain cofactor binding site is perturbed by mutation of residue Phe[225].

The extended VIIa protease domain epitope of monoclonal antibody F3-3.2A is influenced by conformational changes that result from active site modification or cofactor binding (*20*). By Biacore analysis, active site modification of VIIa results in significantly reduced association kinetics through a conformational change that involves Glu[154] in VIIa. Furthermore, in active site-modified VIIa, cofactor-mediated conformational changes in the antibody epitope are no longer detectable, indicating a restriction of conformational flexibility (*26*). Binding kinetics of the active site-modified mutant with the antibody were indistinguishable from those of wild-type VIIa, in both the presence and absence of TF (Table 1). Inhibitor binding thus appeared to induce a similar conformation of the VIIa protease domain surface within the antibody epitope in mutant and wild-type VIIa and to restrict conformational flexibility, as indicated by the identical binding kinetics of active site-modified VIIa's in the presence of soluble TF.

Table 1: Binding of VIIa to TF and Monoclonal Antibody F3-3.2A[a]

	Binding to TF_{1-263}	
	k_{on} ($\times 10^5$ M^{-1} s^{-1})	k_{off} ($\times 10^{-4}$ s^{-1})
wild-type VIIa	2.9 ± 0.1	7.8 ± 0.2
VIIa$_{Pro}^{225}$	1.5 ± 0.5	2.3 ± 0.2
with 5L15		
wild-type VIIa	3.0 ± 0.1	2.7 ± 0.3
VIIa$_{Pro}^{225}$	1.2 ± 0.1	2.4 ± 0.1
Phe-Phe-Arg-modified		
wild-type VIIa	4.4 ± 0.4	2.2 ± 0.2
VIIa$_{Pro}^{225}$	2.4 ± 0.1	1.3 ± 0.1
	Binding to F3-3.2A	
	k_{on} ($\times 10^4$ M^{-1} s^{-1})	k_{off} ($\times 10^{-2}$ s^{-1})
free		
Phe-Phe-Arg-VIIa	1.6 ± 0.3	1.3 ± 0.1
Phe-Phe-Arg-VIIa$_{Pro}^{225}$	1.6 ± 0.1	1.0 ± 0.1
with TF_{1-218}		
Phe-Phe-Arg-VIIa	1.4 ± 0.2	1.8 ± 0.6
Phe-Phe-Arg-VIIa$_{Pro}^{225}$	1.8 ± 0.4	1.8 ± 0.1
free		
wild-type VIIa	13.7 ± 4.4	2.5 ± 0.3
VIIa$_{Pro}^{225}$	4.2 ± 0.1	1.7 ± 0.5
with TF_{1-218}		
wild-type VIIa	3.5 ± 0.9	7.1 ± 1.2
VIIa$_{Pro}^{225}$	15.6 ± 4.2	3.2 ± 0.4

[a] Values are means ± the standard deviation ($n \geq 3$).

Compared to active site occupancy, cofactor binding induces a more subtle reduction of the association rate and also an accelerated dissociation of wild-type VIIa from antibody F3-3.2A (*20*). Mutation of Arg[134] in the interface with the cofactor prevented the accelerated dissociation without influencing the changes in association kinetics (*26*), providing evidence for two different effects of cofactor interaction on the antibody binding kinetic. Cofactor interaction with VIIa$_{Pro}^{225}$ induced a faster dissociation of the mutant from F3-3.2A, as observed with wild-type VIIa (Table 1). However, the association of free VIIa$_{Pro}^{225}$ was significantly slower than that of wild-type VIIa, and TF had an effect on the association kinetics of the mutant that is the opposite of that of wild-type VIIa (Table 1). With regard to the conformational changes that are reflected by the association kinetics, it appears that the free mutant displays a conformational state that is similar to the conformation of TF-bound wild-type VIIa, whereas the TF-bound mutant resembles free wild-type VIIa. Despite this unexplained difference from wild-type VIIa, the accelerated dissociation of the mutant upon cofactor binding nevertheless provides evidence that the cofactor is capable to induce conformational changes within the F3-3.2A epitope. Thus, the unchanged kinetics of dissociation of VIIa$_{Pro}^{225}$ from TF upon active site

Table 2: Chromogenic Substrate Hydrolysis[a]

	k_{cat}/K_m ($\times 10^{-3}$ M^{-1} s^{-1})			inhibition of TF·VIIa by *p*-aminobenzamidine K_i (μM)
	VIIa	TF·VIIa	enhancement	
wild-type VIIa	1.5 ± 0.1	49.1 ± 7.2	33-fold	111 ± 22
$VIIa_{Pro}^{225}$	2.5 ± 0.1	10.7 ± 1.9	4-fold	266 ± 29
$VIIa_{Ala}^{134}$	1.0 ± 0.1	12.2 ± 0.7	12-fold	384 ± 73
$VIIa_{Ala}^{164}$	0.7 ± 0.1	7.3 ± 2.8	11-fold	339 ± 60
$VIIa_{Ala}^{185B}$	<0.1	4.0 ± 0.2	>50-fold	779 ± 76
$VIIa_{Ala}^{217}$	0.3 ± 0.1	9.0 ± 0.6	26-fold	333 ± 29

[a] Values are means ± the standard deviation ($n \geq 3$).

modification cannot be attributed to a general restriction of the conformational flexibility of the protease domain upon mutation of Phe^{225}.

Reduced Level of Cofactor-Mediated Enhancement of the Catalytic Function of $VIIa_{Pro}^{225}$. The reduced level of scissile bond cleavage of the macromolecular substrate indicated diminished catalytic function of $VIIa_{Pro}^{225}$. Catalytic activities of free and cofactor-bound wild-type and mutant enzymes were analyzed with small chromogenic pseudosubstrates. Because the Na ion concentration can influence the amidolytic activity of thrombin and because some of the mutants analyzed here may perturb a potential Na^+-binding site analogous to the site in thrombin (*27*), the effect of Na ions on the activity of wild-type VIIa was analyzed. There was no difference in the amidolytic activity when free VIIa was analyzed in the presence of 200 mM NaCl, as compared to the same concentration of choline chloride (rate of hydrolysis at 60 nM VIIa: 1.2 ± 0.1 vs 1.2 ± 0.1 μmol/min, $n = 3$). Na ions had also no effect on the cofactor-mediated enhancement of the amidolytic function of VIIa, resulting in similar rates of hydrolysis in the presence and absence of 200 mM NaCl (rate of hydrolysis at 30 nM TF·VIIa: 13.3 ± 0.5 vs 14.9 ± 1.5 μmol/min, $n = 3$). Thus, under our experimental conditions, Na ions do not appreciably influence VIIa's catalytic activity, and mutational perturbation of Na ion binding is an improbable cause for reductions in amidolytic activity.

The hydrolysis of the chromogenic substrate Chromozyme tPA by free $VIIa_{Pro}^{225}$ was enhanced <2-fold compared to that of wild-type VIIa, which is in contrast to the slightly decreased k_{cat}/K_M for previously characterized mutants in the interface with TF (Table 2). The k_{cat}/K_M for small substrate cleavage of TF-bound $VIIa_{Pro}^{225}$ was reduced 5-fold compared to that of wild-type VIIa, which is in reasonable agreement with the above-described reduction of scissile bond cleavage of the macromolecular substrate. The function of TF-bound mutants of key interface residues Arg^{134} and Met^{164} was reduced 4−7-fold relative to that of wild-type VIIa. Since these mutants had also reduced functions as free enzymes, the calculated TF-mediated enhancement of small substrate hydrolysis was diminished only approximately 3-fold in comparison to the cofactor effect on wild-type VIIa. In contrast, $VIIa_{Pro}^{225}$ as a free enzyme had higher function, similar to that of wild-type VIIa, and the calculated catalytic enhancement was thus only 4-fold, a 8-fold reduction in comparison to the 33-fold cofactor enhancement of wild-type VIIa (Table 2).

These mutants had reduced affinity for the competitive S1-occupying inhibitor *p*-aminobenzamidine in the presence of cofactor (Table 2), consistent with the notion that TF increases the affinity for the P1 residue in the catalytic cleft (*28*). We had previously found in transient transfection experiments that mutations of the Asp^{185B} (VII334) or Ser^{187} (VII336) side chains reduced proteolytic function (*1*). These side chains form a surface hydrogen bond that likely stabilizes the conformation of the 180−189 loop and thus possibly Asp^{189} in the primary specificity site. The loss of function upon mutation of these residues might indicate importance of the 180−189 loop in transmitting the cofactor effect to the S1 subsite. Free $VIIa_{Ala}^{185B}$ had extremely low amidolytic activity, but TF induced an approximately 50-fold increase in the level of catalysis (Table 2). TF-bound $VIIa_{Ala}^{185B}$ had a severely reduced affinity for *p*-aminobenzamidine (Table 2), arguing that the S1 subsite is indeed destabilized upon residue exchange. However, destabilizing the 180−189 loop, including the primary specificity determinant Asp^{189}, did not prevent cofactor-mediated enhancement of catalysis. $VIIa_{Ala}^{217}$ (*29*) is another example of a mutant that is efficiently activated by cofactor, despite alterations in the catalytic cleft that result in lower affinity for the small substrate and *p*-aminobenzamidine (Table 2). This mutant follows the recurrent theme that reductions in the catalytic activity of the bound enzyme are associated with diminished activity of the free enzyme, resulting in normal or close to normal induction by cofactor. Mutation of Phe^{225} produced a distinct effect which is impaired cofactor-mediated enhancement without effects on the catalytic activity of the free enzyme, demonstrating a specific role of this residue in the cofactor-mediated activation of VIIa's catalytic function.

Normal Cofactor-Mediated Stabilization of the Amino-Terminal Insertion of $VIIa_{Pro}^{225}$. The α-amino group of Ile^{16} is more susceptible to chemical modification in free VIIa than in the cofactor-bound enzyme (*30*). This may indicate that TF-mediated stabilization of the Ile^{16}−Asp^{194} salt bridge is the primary mechanism underlying the cofactor effect. To determine the effect of the mutations on the stability of the amino-terminal insertion, free and TF-bound mutant or wild-type VIIa was reacted with KNCO. Since carbamylation of Ile^{16} is known to inactivate VIIa by preventing the optimal positioning of Asp^{189} for binding of the P1 residue (*30*, *31*), the loss of amidolytic function can be utilized to determine the rate of modification of the amino terminus as a measure for the stability of the Ile^{16}−Asp^{194} salt bridge. The rate of carbamylation of free $VIIa_{Pro}^{225}$ was slightly reduced in comparison to that of wild-type VIIa, but TF interaction resulted in a similar stabilization of the Ile^{16}−Asp^{194} salt bridge, as evidenced by identical rates of inactivation by KNCO (Table 3). Thus, stabilization of the amino-terminal insertion appears to be insufficient for achieving the full cofactor-induced catalytic activity of $VIIa_{Pro}^{225}$. The S2 subsite

Table 3: Effect of Point Mutations on the Inactivation by Carbamylation of Ile[16] [a]

	rate of carbamylation (% inactivation/10 min)	
	VIIa	TF·VIIa
wild-type VIIa	6.7 ± 0.4	0.9 ± 0.1
$VIIa_{Pro^{225}}$	5.1 ± 0.6	1.0 ± 0.1
$VIIa_{Ala^{99}}$	5.8 ± 0.8	0.6 ± 0.3
$VIIa_{Ala^{134}}$	5.5 ± 0.4	5.0 ± 0.2
$VIIa_{Ala^{164}}$	7.2 ± 0.6	5.2 ± 0.2
$VIIa_{Ala^{185B}}$	not feasible	6.0 ± 0.6

[a] Values are means ± the standard deviation ($n \geq 4$).

mutant $VIIa_{Ala^{99}}$ is another example for a mutant that displayed a normal cofactor-induced protection of the amino-terminal Ile[16] from chemical modification (Table 3) along with a significant reduction in the extent of catalysis of scissile bond mimicking *p*-nitroanilide substrates (*8*). Thus, perturbations within the catalytic cleft do not necessarily destabilize the amino-terminal insertion site.

In contrast, mutants of TF interface residues Arg[134] and Met[164] or $VIIa_{Ala^{185B}}$, which have only partially reduced or normal enhancement of catalytic function caused by TF, exhibited marked defects in the protection of the amino terminus from chemical modification in the presence of cofactor. In the case of $VIIa_{Ala^{134}}$, the rate of inactivation was essentially identical in the presence and absence of cofactor. These data argue that the cofactor enhances catalytic function, in part, through conformational changes that do not require a stabilization of the amino-terminal insertion. These three mutants displayed a pronounced increase in the K_i for inhibition by *p*-aminobenzamidine, consistent with an incomplete maturation of the S1 subsite upon cofactor binding. The incomplete stabilization of the Ile[16]−Arg[194] salt bridge upon cofactor binding may thus result indirectly from a destabilized Asp[189] position.

DISCUSSION

Coagulation factor VIIa is an unusual serine protease that remains in a conformational state of low catalytic activity after proteolytic zymogen to enzyme conversion. This property is of possible physiological significance for activation of the coagulation cascade by ensuring a prolonged plasma half-life of VIIa (*32*) due to inefficient inactivation by plasma serine protease inhibitors (*33*, *34*). As a mechanism for controlling the activation of the enzyme cascade, the typical proteolytic zymogen to enzyme conversion is thus replaced by functional regulation of VIIa through cofactor interactions. The structural determinants that cause the zymogen-like conformation of VIIa are not defined in detail, but likely include disorder in the loop segments of the activation domain, as evidenced by an increased susceptibility of the amino-terminal Ile[16] to chemical modification. Whereas the formation of the salt bridge between Ile[16] and Asp[194] is required for catalysis by VIIa (*30*), it is unclear whether stabilization of the labile amino-terminal insertion is the sole determinant for cofactor-mediated activation of VIIa.

This study identifies Phe[225] as a residue that is important for the cofactor-mediated allosteric regulation of the VIIa protease domain. Phe[225] sits in a buried pocket that is created by the side chains of Leu[163], Ser[185A] (VII333), Val[223], and the Cys[168]−Cys[182] disulfide bond (Figure 1A). This pocket is in proximity to surface residues that contact TF, most notably, Met[164] and Asp[167], both of which are important for catalytic function (*1*). Removal of the aromatic side chain of Phe[225] likely perturbs this local environment and influences the interaction with TF, as evidenced by the slower dissociation of $VIIa_{Pro^{225}}$ from TF. In wild-type VIIa, active site modification reduces the level of dissociation from TF, and this effect involves the Met[164] side chain (*10*). Active site modification of $VIIa_{Pro^{225}}$ with a Kunitz-type inhibitor failed to further decrease the extent of dissociation from TF, indicating that the local environment of Met[164] in $VIIa_{Pro^{225}}$ is already in a conformational state that allows for tighter binding of TF and that active site inhibition did not produce additional conformational changes that influence the affinity for TF. These data can be interpreted as a severed conformational linkage between the cofactor binding site and the

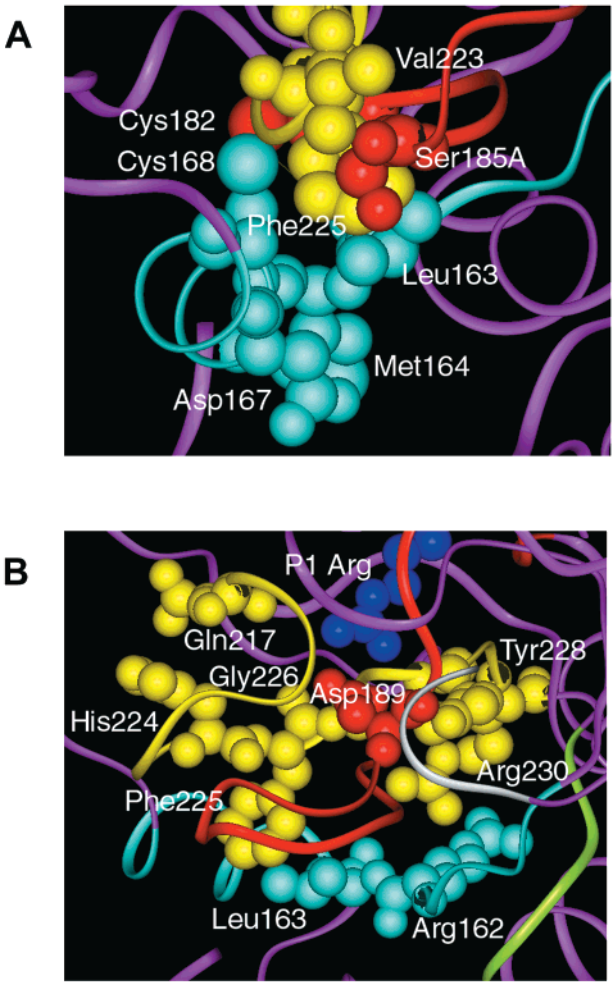

FIGURE 1: Location of Phe[225] in the structure of the TF·VIIa complex (*2*). The following segments, including side chains, are highlighted according to the following color scheme: dark blue, the P1 Arg residue of the active site inhibitor; white, residues 16−19; green, residues 134−138; light blue, residues 158−170; red, residues 182−195; and yellow, residues 217−230 (chymotrypsin numbering based on ref *1*). The backbone is shown as a ribbon; certain residues, including side chains, are shown with a solid surface reduced to 60%. (A) Proximity of Phe[225] highlighting contact residues Leu[163], Ser[185A], and Val[223] and the Cys[168]−Cys[182] disulfide bond. The TF contact residues Met[164] and Asp[167] are shown to the right of the 165−170 helix. (B) Relationship of Phe[225] to the catalytic cleft. Highlighted are a potential link involving His[224] and Gln[217], the packing of Asp[189] with Gly[226] and Tyr[228], the stacking of Arg[230] and Arg[162]. For orientation, the Leu[163] side chain is also shown.

active site, which results in the functional correlate of reduced enhancement of catalytic function of $VIIa_{Pro^{225}}$ by TF.

$VIIa_{Pro^{225}}$ had normal activity as a free enzyme, but had reduced proteolytic and amidolytic function in the presence of TF. The normal activity of free $VIIa_{Pro^{225}}$ argues that the integrated network that enables catalytic function of the VIIa protease domain is not disrupted in a global and nonspecific manner in this mutant. Rather, the functional defect is limited to the TF-mediated enhancement of function, emphasizing a specific role of this buried side chain in the cofactor-induced allosteric changes. The comparison of free and cofactor-bound structures of Phe-Phe-Arg chloromethyl ketone-inactivated VIIa revealed an extension of the 165−170 α-helix upon TF binding (*15*). Associated with these changes were movements of the more buried 184−190 and 221−225 loop segments, both of which are significant for substrate specificity in the chymotrypsin-like serine protease family (*35*). Interactions of TF with Met^{164} and the 165−170 helix may conceivably cooperate in stabilizing these buried loop segments by ordering the hydrophobic environment surrounding Phe^{225} (Figure 1A). Mutation of Val^{223} and Leu^{163} (*1*) as well as reduction of the Cys^{168}−Cys^{182} disulfide bond (*36*) severely impaired function, pointing to a possible role of these residues in linking the TF interface to the Phe^{225} position.

The reduced affinity of $VIIa_{Pro^{225}}$ for the S1-occupying inhibitor *p*-aminobenzamidine suggests a defect in shaping the primary specificity site upon cofactor binding to the mutant. The different mutational effect at the Asp^{185B} (VII334) position is evidence that does not support a linkage from Phe^{225} to the primary specificity site through the 182−189 loop and stabilization of Asp^{189}. Mutation of Phe^{225} or of residues within the 182−189 loop could similarly perturb a water channel and the connected Na^+ ion binding site, based on the homology with thrombin (*27*). However, the different mutational effect of Phe^{225} and Asp^{185B} would also not support perturbation of the water channel as a common mechanism to explain the loss of function. Phe^{225} may influence the S1 pocket by indirect effects involving the 220s loop. The S1 pocket is in part formed by the loop segment preceding Phe^{225} leading to Cys^{220}, which is close to the 142−154 loop of the activation domain. Phe^{225} may have a long-range effect on the 220s loop residue His^{224} that links to Gln^{217} and the bottom of the catalytic cleft (Figure 1B). Mutation of Gln^{217}, however, did not reduce the cofactor effect on catalysis (Table 2), arguing against an effect on these extended recognition determinants in the catalytic cleft. A short-range conformational effect of Phe^{225} on the primary specificity site can be considered. The backbone of Phe^{225} is located in proximity to the primary specificity site, and the adjacent residues Gly^{226} and Tyr^{228} shape the bottom of the pocket within <5 Å of one of the guanidino groups of the P1 Arg residue (Figure 1B). The importance of the stability of the 220s loop segments for catalytic activity of VIIa is further emphasized by the loss of function upon mutation of Arg^{230} (*1*) that stacks against Arg^{162}, another critical residue found mutated in hereditary VIIa deficiencies (*37*, *38*). Through the contact with Leu^{163}, Phe^{225} may stabilize Arg^{162}, resulting in a cooperative effect on Tyr^{228} and Gly^{226} that shape the S1 pocket (Figure 1B).

Measurements of the susceptibility of the amino terminus to chemical modification demonstrate two distinct mechanisms by which cofactor-mediated enhancement of catalytic activity of VIIa is perturbed. Phe^{225} mutation disrupted the enhancement without influencing the stability of the amino-terminal insertion. On the other hand, mutants at residue positions in the interface with TF failed to stabilize Ile^{16}−Asp^{194} salt bridge formation, but did not completely abolish cofactor-mediated enhancement of VIIa's catalytic activity. Thus, stabilization of the Ile^{16}−Asp^{194} salt bridge is only one of the conformational effects by which cofactor binding enhances catalysis. A mechanism for cofactor-mediated enhancement of catalysis of VIIa emerges from these data. TF interface contact residues appear to cooperate in an ordering of the amino-terminal insertion and the Asp^{189} position, while an independent effect is transmitted to the active site through an allosteric network involving an aromatic position 225 residue that characterizes allosterically regulated serine proteases.

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