Regulation of the Catalytic Function of Coagulation Factor VIIa by a Conformational Linkage of Surface Residue Glu 154 to the Active Site†

Justin Shobe, Craig D. Dickinson, and Wolfram Ruf*

Departments of Immunology and Vascular Biology, The Scripps Research Institute, La Jolla, California 92037

Received August 12, 1998; Revised Manuscript Received December 16, 1998

ABSTRACT: Coagulation factor VIIa is an allosterically regulated trypsin-like serine protease that initiates the coagulation pathways upon complex formation with its cellular receptor and cofactor tissue factor (TF). The analysis of a conformation-sensitive monoclonal antibody directed to the macromolecular substrate exosite in the VIIa protease domain demonstrated a conformational link from this exosite to the catalytic cleft that is independent of cofactor-induced allosteric changes. In this study, we identify Glu 154 as a critical surface-exposed exosite residue side chain that undergoes conformational changes upon active site inhibitor binding. The Glu 154 side chain is important for hydrolysis of scissile bond mimicking peptidyl *p*-nitroanilide substrates, and for inhibition of VIIa's amidolytic function upon antibody binding. This exosite residue is not linked to the catalytic cleft residue Lys 192 which plays an important role in thrombin's allosteric coupling to exosite I. Allosteric linkages between VIIa's active site and the cofactor binding site or between the cofactor binding site and the macromolecular substrate exosite were not influenced by mutation of Glu 154. Glu 154 thus only influences the linkage of the macromolecular substrate binding exosite to the catalytic center. These data provide novel evidence that allosteric regulation of VIIa's catalytic function involves discrete and independent conformational linkages and that allosteric transitions in the VIIa protease domain are not globally coupled.

The serine protease family is essential for a variety of physiological and pathological processes in higher organisms. These enzymes are typically synthesized as zymogen precursors that are activated by proteolytic cleavage of a specific scissile bond, resulting in conformational changes that lead to catalytic activation. The zymogen structures are characterized by several segments, designated the activation domain, that display increased flexibility compared to their enzyme structure counterparts (*1*). The restriction of conformational flexibility, in particular, the ion pair formation of the α-amino group of Ile 16 with Asp 194 (residue numbering is based on chymotrypsin positions), is essential for full catalytic activity of a serine protease domain. The coagulation serine proteases are a subclass of trypsin-like serine proteases with highly restricted substrate specificity. In addition, the proteolytic function of these enzymes frequently depends on protein cofactors that support macromolecular substrate binding and allosterically influence substrate specificity.

Factor VIIa (VIIa)[1] serves as the initiating protease of the coagulation cascade by binding to the cell surface receptor and catalytic cofactor tissue factor (TF), a member of the cytokine receptor family. The structure of the TF·VIIa complex (*2*) and extensive site-directed mutagenesis of TF and VIIa (*3*, *4*) have defined the precise molecular interactions between cofactor and enzyme. The integration of these structure−function studies suggests that macromolecular substrate factor X must assemble through multiple contacts with the complex (*5*). The creation of an extended macromolecular substrate docking site by regions of cofactor and enzyme, in part, explains the low proteolytic activity of VIIa in the absence of cofactor. Furthermore, VIIa's catalytic activity toward small peptide substrates is low, but dramatically enhanced by binding of TF (*6*−*8*), indicating a direct allosteric regulation of the active site by cofactor interactions. Because the amino-terminal Ile 16 is susceptible to chemical modification in free VIIa, but not in the TF-bound enzyme (*9*), a zymogen-like conformation of VIIa in the absence of cofactor is the likely reason for the low amidolytic activity. The cofactor-dependent allosteric activation of the VIIa protease domain is mediated by specific contacts in the interface with TF, in particular Met 164 (*2*, *4*). The localization of this interface adjacent to regions of the activation domain (the segment of residues 182−189) has led to the proposal that cofactor interactions enhance the catalytic function of VIIa by supporting the labile zymogen transition to the active enzyme (*4*, *10*).

Inhibitor binding to the catalytic cleft of VIIa also decreases the susceptibility of Ile 16 to chemical modifications (*10*), indicating that cofactor interactions and active site occupancy induce a similar conformational transition in the protease domain of VIIa. Conformational changes associated with formation of a stabilized amino-terminal Ile 16 ion pair with Asp 194 likely affect the local environment around the insertion site. The amino-terminal insertion site is part of the epitope of a conformation-sensitive monoclonal

† Supported by NIH Grants R01 HL48752 and P01 HL16411. Performed during the tenure of an Established Investigator Award from the American Heart Association (to W.R.).

* Corresponding author: Departments of Immunology and Vascular Biology, IMM-17, 10550 N. Torrey Pines Rd., La Jolla, CA 92037. Telephone: (619)784-2748. Fax: (619)784-8480. E-mail: ruf@scripps.edu.

[1] Abbreviations: TF, tissue factor; VII and VIIa, coagulation factor VII and VIIa, respectively; FFR-VIIa, VIIa modified with the active site inhibitor Phe-Phe-Arg chloromethyl ketone; PC, phosphatidylcholine vesicles; PCPS, phosphatidylcholine/phosphatidylserine vesicles.

10.1021/bi981951g CCC: $18.00
Published on Web 02/10/1999

antibody (F3-3.2A) to the VIIa protease domain (*5*). The affinity of VIIa for F3-3.2A is influenced by active site modification and by cofactor binding. TF binding to VIIa predominantly accelerates the dissociation of VIIa from the antibody by 2−3-fold, whereas active site modification mainly decreases ~10-fold the rate of association of VIIa with the antibody (*5*). Unlike previous studies in which the solvent accessibility of the amino terminus was analyzed (*9*, *10*), the characterization of the conformation-sensitive antibody F3-3.2A thus provided evidence that cofactor binding and active site occupancy induce distinct conformational changes in the VIIa protease domain.

F3-3.2A is a competitive inhibitor for factor X activation (*5*). This property can be explained by the fact that the antibody epitope also overlaps with an electronegative cluster in a position corresponding to thrombin's basic exosite I, and mutagenesis demonstrates that this region in VIIa is involved in macromolecular substrate recognition (*4*). Independent of this effect on macromolecular substrate activation, F3-3.2A inhibits the cleavage of small chromogenic substrates by affecting the catalytic cleft from a distance, indicating an allosteric linkage that can modulate catalytic function of the serine protease domain of VIIa (*5*). We hypothesize that the conformational linkage that inhibits VIIa's amidolytic function upon F3-3.2A binding is related to the effects of active occupancy on the affinity of VIIa for F3-3.2A. To provide support for this hypothesis, we attempted to identify specific residues in the VIIa protease domain that are required for the conformational linkages from the active site to the antibody epitope. We identify one surface-exposed residue side chain that is required for both the inhibition of the TF·VIIa amidolytic function by F3-3.2A and the change in antibody affinity following active site modification. We show that the same residue is not involved in cofactor-induced conformational changes, and thus provide clear evidence for multiple and independent conformational transitions in the VIIa serine protease domain that are important for catalytic function.

EXPERIMENTAL PROCEDURES

Proteins. Full-length recombinant human TF was produced from insect cells and reconstituted into 30% phosphatidylserine/70% phosphatidylcholine (TF/PCPS), or 100% phosphatidylcholine (TF/PC), as described previously (*11*). The soluble extracellular domain of TF, TF_{1-218}, was expressed in *Escherichia coli* and purified and refolded from inclusion bodies, as described previously (*12*). Factor X was purified from plasma, followed by immunoaffinity chromatography on immobilized monoclonal antibody F21-4.2 to reduce the amount of contamination by VII (*4*). Monoclonal antibodies 12C7 and F3-3.2A to VII and VIIa have been characterized previously (*5*). Recombinant wild-type and mutant VII were expressed in Chinese hamster ovary (CHO) cells and purified by sequential monoclonal antibody and ion exchange chromatography, as described previously (*11*). Because of slow conversion to the active enzyme with the VII_{Ala154} and VII_{Ala144} mutants, activation by factor IXa was employed (*13*). Briefly, the mutants were incubated with factor IXa at a 1:10 enzyme:substrate ratio in 20 mM Tris, 50 mM NaCl, and 0.25 mM $CaCl_2$ (pH 8.5) at 37 °C overnight, followed by separation of the activated mutant and factor IXa on MonoQ using a linear NaCl gradient from 50 to 400 mM in 20 mM Tris (pH 7.4). Mutant and wild-type VIIa were active site blocked with D-Phe-L-Phe-Arg chloromethyl ketone (FFR-VIIa) (Calbiochem) to yield a modification level of >98%, as determined by amidolytic assay (*14*). The Gla content of the mutants, determined by amino acid analysis of alkaline hydrolysates performed at Commonwealth Biotechnologies (Richmond, VA), showed a full complement of Gla residues that was indistinguishable from wild-type recombinant VIIa.

Functional Assays. K_M and k_{cat} for factor X hydrolysis by TF·VIIa were determined with a fixed concentration of TF/PCPS or TF/PC (200 pM) and excess wild-type or mutant VIIa (1 nM) in Hepes-buffered saline [HBS, 10 mM Hepes and 150 mM NaCl (pH 7.4)], 5 mM $CaCl_2$, and 0.2% bovine serum albumin. After a brief preincubation (5 min) of TF with VIIa to allow for complex formation, factor X (0.2 nM to 10 μM) was added, and activation was assessed in samples quenched with 100 mM EDTA, using the chromogenic substrate Spectrozyme FXa. Initial rate data were fitted to the Michaelis−Menten equation.

The amidolytic function of wild-type or mutant VIIa (5 nM) was measured in the presence of increasing concentrations of soluble TF_{1-218} (0.5−100 nM) with 0.5 mM chromogenic substrate Chromozyme tPA (Boehringer Mannheim) in Tris-buffered saline, 5 mM $CaCl_2$, and 0.2% bovine serum albumin (pH 8.0). Kinetic parameters for chromogenic substrate hydrolysis were determined at fixed enzyme (30 nM) and cofactor TF_{1-218} (120 nM) concentrations with varying concentrations of substrate (10 μM to 1.5 mM) under the same buffer conditions in a total volume of 150 μL. To determine the extent of inhibition of F3-3.2A on the amidolytic activity of wild-type or mutant VIIa (30 nM) complexed with TF_{1-218} (120 nM), TF·VIIa was incubated at ambient temperature for 15 min with varying concentrations of antibody prior to testing for residual amidolytic function with substrate as described above.

Surface Plasmon Resonance Analysis. Binding constants were determined on BIAcore 2000 instruments. Monoclonal antibodies to VII (12C7 and F3-3.2A) and to TF (TF9-10H10) were directly immobilized by coupling through free amino groups to a carboxylated dextran matrix, activated with a mixture of *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N*′-[3-(diethylamino)propyl]carbodiimide (EDC). Antibodies were injected at a concentration of 25 μg/mL for coupling, and immobilization densities of 1000−2500 RU were obtained. Typically, samples were injected onto three serial flow cells that contained immobilized antibody at different densities and one flow cell that had no antibody coupled as a control for nonspecific binding. The data presented were derived from the recordings of the first flow cell, but the subsequent cells with lower antibody densities yielded similar results. Association kinetics for binding to F3-3.2A or 12C7 were measured by injecting various concentrations (13 nM to 4 μM) of mutant or wild-type VIIa with or without active site modification onto the antibody-coated sensorchip. To analyze the effect of TF_{1-218} on binding of VIIa to the antibody, the TF concentration was kept at twice the highest VIIa concentration used for association kinetics. TF was also present during the dissociation phases to ensure that dissociation of TF from VIIa did not influence the k_{off} measurements, as described previously (*5*). Binding kinetics of wild-type and mutant VIIa for binding to TF were

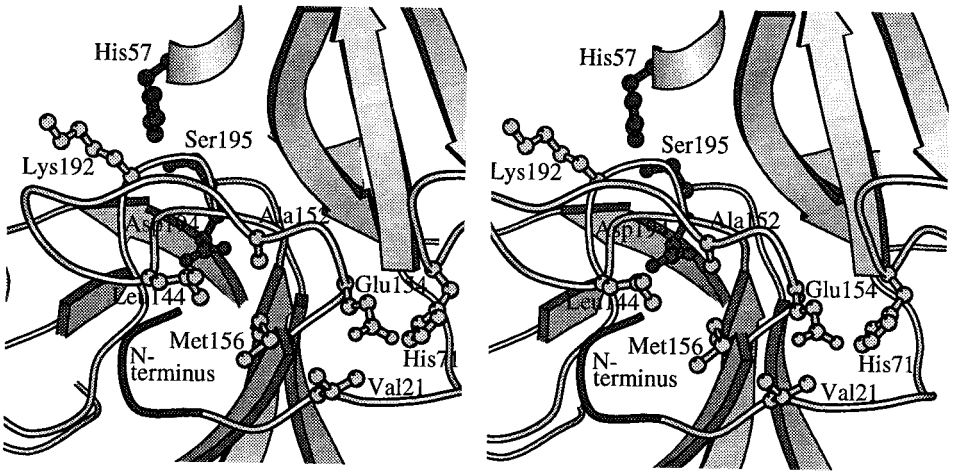

FIGURE 1: Stereoview of the structural environment of residues Leu 144 and Glu 154 in VIIa. The MOLSCRIPT (*30*) representation of the VIIa protease domain amino-terminal insertion site shows important residue side chains as balls and sticks.

determined by capturing full-length TF with the noninhibitory antibody TF9-10H10, as previously described (*14*). Experiments were performed in HBS, 5 mM $CaCl_2$, 0.005% surfactant P20, and 3 mM CHAPS at a flow rate of 30 μL/min. Association rate constants (k_s) were calculated from at least five concentrations of VIIa. The k_{on} was determined from the concentration dependence of k_s and the k_{off} from analysis of the response curve upon return to buffer flow. The association and dissociation kinetics determined in this study for TF are generally in good agreement with our previous publications (*4*, *5*). The association kinetics for the anti-VII antibodies, however, were significantly slower than in the previous study (*5*) which we attribute to subtle differences between batches of monoclonal antibodies and reagents used for the immobilization.

RESULTS

Functional Characterization of Mutants. In this study, we analyze three site specific mutants of VIIa that are potentially relevant to the conformational transitions of the VIIa protease domain. Lys 192 is a position that is known to undergo rearrangements during zymogen to enzyme transition (*1*, *15*) and to play an important role in regulating the substrate specificity of thrombin upon thrombomodulin binding (*16*). In proximity to the amino-terminal insertion site, we had identified by scanning Ala mutagenesis two positions, Leu 144 and Glu 154, in the flexible loop of residues 141−154 (*1*, *15*) that are important for the proteolytic function of VIIa (*4*). Leu 144 is localized in a partially buried hydrophobic environment close to the inserted amino terminus and to the side chains of Ala 152 and Met 156 in the crystal structure of the TF·VIIa complex (*2*) (Figure 1). Since hydrophobic interactions are important in stabilizing the amino-terminal insertion (*17*), we favor the hypothesis that the Leu 144 mutation influences the proper packing of the amino terminus to allow optimal salt bridge formation between Ile 16 and Asp 194. The requirement for proper packing of this region is further illustrated by the hereditary Ala 152 → Val mutation that is a frequent cause of severe clinical VII deficiency (*18*). At the carboxyl terminus of the loop of residues 141−154, the Glu 154 side chain is oriented toward the solvent in the crystal structure of the TF·VIIa complex. Glu 154 is located between residues that flank the amino-terminal insertion (Met 156 and Val 21) and residues (His 71 and Asp 72) that belong to an electronegative region analogous to thrombin's exosite I. The Glu 154 side chain is thus not in direct contact with the amino terminus of the VIIa protease domain, and the loss of function upon mutation is possibly unrelated to a direct influence on the amino-terminal insertion.

Table 1: Catalytic Function of VIIa Mutants

(A) Kinetic Parameters for Factor X Activation

	K_M (nM)	k_{cat} (s^{-1})
TF/PC[a]		
wild-type VIIa	12400 ± 1300	1.55 ± 0.15
Lys 192 Ala VIIa	15300 ± 1500	0.15 ± 0.05
Glu 154 Ala VIIa	17600 ± 6500	0.15 ± 0.05
Leu 144 Ala VIIa	20700 ± 2600	0.05 ± 0.05
TF/PCPS		
wild-type VIIa	69 ± 25	7.40 ± 0.45
Lys 192 Ala VIIa	51 ± 12	0.80 ± 0.15
Glu 154 Ala VIIa	59 ± 21	1.25 ± 0.35
Leu 144 Ala VIIa	43 ± 14	0.15 ± 0.05

(B) Kinetic Parameters for Chromogenic Substrate Hydrolysis

	K_M (μM)	v_{max} (relative absorbance units/min)
TF_{1-218}[b]		
wild-type VIIa	560 ± 100	158 ± 9
Lys 192 Ala VIIa	660 ± 160	62 ± 9
Glu 154 Ala VIIa	620 ± 180	56 ± 11
Leu 144 Ala VIIa	1280 ± 90	63 ± 29

[a] TF (200 pM) reconstituted into phosphatidylcholine (PC) or phosphatidylcholine/phosphatidylserine (PCPS) with 1 nM VIIa. [b] VIIa (30 nM) with 120 nM TF_{1-218}.

Factor X activation by the mutants in complex with phospholipid-reconstituted TF occurred with a significantly decreased k_{cat} for each of the mutants, as compared to that of wild-type VIIa (Table 1). On a negatively charged phospholipid surface (PCPS), no difference in macromolecular substrate binding was apparent. To eliminate mass transport effects that result from TF·VIIa independent substrate factor X binding to the negatively charged vesicles, kinetics were also determined on neutral phospholipid (PC). Subtle changes in the K_M for the activation of factor X by the mutants were detected. The K_M slightly increased for the Lys 192 exchange in the catalytic cleft. Somewhat more pronounced changes were detected for the Glu 154 and Leu 144 mutants, with a

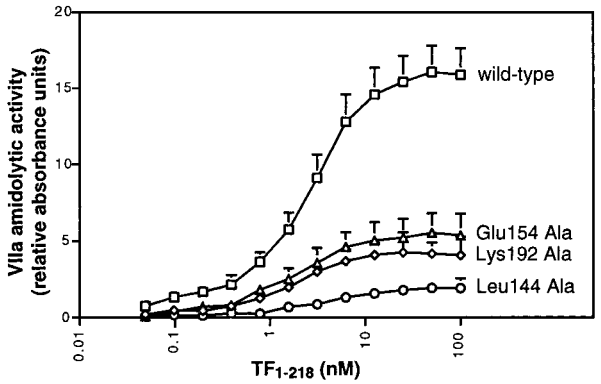

FIGURE 2: Amidolytic function of site-directed mutants in the VIIa protease domain. The level of amidolytic function of mutant or wild-type VIIa (5 nM) in the presence of increasing concentrations of soluble TF_{1-218} was determined at ambient temperature with the *p*-nitroanilide substrate Chromozyme tPA (0.5 mM).

close to 2-fold increase for the latter. This may indicate that mutation of these residues affects the local environment that contributes to macromolecular substrate binding.

The predominant effect of these mutations was on the k_{cat} for factor X activation, indicating allosteric effects on the catalytic site. This notion that scissile bond cleavage is affected by the mutations is further supported by the analysis of the hydrolysis of small peptide substrates that interact with the S1−S3 subsites of the catalytic cleft (Figure 2). All mutants exhibited a decreased level of activation of chromogenic substrates, and the effect was predominantly on the k_{cat}. Only the Leu 144 mutant also displayed a 2-fold decreased affinity for the chromogenic substrate (Table 1B). Since cofactor binding decreases the K_M for hydrolysis of this particular substrate by wild-type VIIa (*19*) and since the cofactor influences the amino-terminal insertion site where Leu 144 is located (Figure 1), the change in the K_M upon mutation of Leu 144 is not an unexpected finding. Rather, this finding is consistent with the hypothesis that the Leu 144 side chain is important for optimal insertion of the amino terminus. The differences in the kinetic parameters of small substrate hydrolysis by $VIIa_{Ala144}$ versus $VIIa_{Ala154}$ further indicate that Glu 154 replacement does not perturb function by the same mechanism as the Leu 144 mutant. It is noteworthy that mutation of Arg 148 in the same loop did not influence the amidolytic function of VIIa (*11*), demonstrating that perturbations of active site catalysis are specific for Glu 154 and Leu 144.

Effect of Active Site Modification on the Conformation of the Mutants. Inhibitor binding to the active site of VIIa influences the cofactor binding site (*14*). The binding of the mutants to TF was analyzed by surface plasmon resonance (Table 2A). The kinetics of binding of unmodified mutants to TF did not significantly differ from those of wild-type VIIa. Mutant and wild-type VIIa were covalently active site modified with D-Phe-L-Phe-Arg chloromethyl ketone, resulting in a similar 2−3-fold decrease in the rates of dissociation from TF. These data demonstrate that the mutants have unaltered binding function for TF and that active site modification of the mutants produces conformational changes in the cofactor binding site, as observed for wild-type VIIa. The mutated residue side chains are therefore not involved in the conformational linkages between the cofactor binding site and the catalytic cleft.

Table 2: Binding of VIIa to TF and Monoclonal Antibodies

	(A) Binding to TF_{1-263} k_{on} ($\times 10^5$ M^{-1} s^{-1})	k_{off} ($\times 10^{-3}$ s^{-1})
VIIa		
wild-type	1.9 ± 0.5	1.6 ± 0.3
Lys 192 Ala	2.7 ± 1.5	1.7 ± 0.7
Leu 144 Ala	4.6 ± 0.9	2.1 ± 0.4
Glu 154 Ala	2.6 ± 0.2	1.9 ± 0.2
FFR-VIIa		
wild-type	3.8 ± 0.8	0.5 ± 0.1
Lys 192 Ala	2.7 ± 1.0	0.9 ± 0.2
Leu 144 Ala	3.6 ± 0.8	0.7 ± 0.0
Glu 154 Ala	2.4 ± 0.3	0.5 ± 0.2
	(B) Binding to F3-3.2A k_{on} ($\times 10^4$ M^{-1} s^{-1})	k_{off} ($\times 10^{-2}$ s^{-1})
VIIa		
wild-type	9.8 ± 0.6	2.5 ± 0.4
Lys 192 Ala	5.7 ± 2.3	3.9 ± 0.4
Leu 144 Ala	9.4 ± 1.5	2.2 ± 0.2
Glu 154 Ala	9.2 ± 0.6	3.3 ± 0.5
Arg 134 Ala	8.4 ± 0.5	2.6 ± 0.0
VIIa and TF_{1-218}		
wild-type	5.6 ± 0.5	7.2 ± 1.0
Lys 192 Ala	3.2 ± 0.7	7.4 ± 0.2
Leu 144 Ala	5.6 ± 0.5	6.1 ± 1.0
Glu 154 Ala	5.4 ± 1.9	8.4 ± 2.1
Arg 134 Ala	5.3 ± 0.6	2.9 ± 0.2
FFR-VIIa		
wild-type	1.0 ± 0.2	1.3 ± 0.1
Lys 192 Ala	1.0 ± 0.7	0.9 ± 0.1
Leu 144 Ala	1.6 ± 0.2	1.0 ± 0.1
Glu 154 Ala	11.1 ± 2.2	2.3 ± 0.8
	(C) Binding to 12C7 k_{on} ($\times 10^5$ M^{-1} s^{-1})	k_{off} ($\times 10^{-4}$ s^{-1})
VIIa		
wild-type	1.2 ± 0.1	5.2 ± 0.8
Glu 154 Ala	0.9 ± 0.1	6.0 ± 0.7
FFR-VIIa		
wild-type	1.0 ± 0.1	7.0 ± 0.5
Glu 154 Ala	0.9 ± 0.2	6.6 ± 1.1

The affinity of VIIa for monoclonal antibody F3-3.2A is dependent on conformational changes in the protease domain. The binding of free, unmodified VIIa with antibody F3-3.2A was not influenced by any of the mutations analyzed in this study (Table 2B), although our mapping of the F3-3.2A epitope indicates that Glu 154 and Leu 144 are within the epitope boundary of this antibody (*5*). In the presence of soluble TF_{1-218}, the dissociation rate increased 2−3-fold in the case of the Leu 144, Glu 154, and Lys 192 mutants, as observed for the wild-type VIIa control. This increase in the dissociation rate upon TF binding is due to specific effects, as demonstrated by Ala replacement of Arg 134 which is an important contact between TF and the VIIa protease domain (*4*). This mutant did not display accelerated dissociation from F3-3.2A following TF binding, although an increased response in the sensorgrams provided evidence for binding of the complex of VIIa and TF to the immobilized antibody (Table 2B). This clearly supports a specific conformational change in the monoclonal antibody epitope that is dependent on interaction of TF with Arg 134. Glu 154 and Leu 144 located within the antibody epitope boundaries are not involved in this allosteric linkage from the exosite to the cofactor binding site.

Our previous study demonstrated that active site modification has effects on the F3-3.2A epitope conformation that

are different from TF-induced allosteric changes (*5*). We tested each of the active site-modified mutants for binding to the immobilized antibody. Modification of the catalytic cleft Lys 192 and the insertion site Leu 144 mutant changed the association and dissociation kinetics, concordant with the effect of active site inhibition of wild-type VIIa (Table 2B). In contrast, there were no significant changes in the binding kinetics of the active site-modified Ala replacement mutant of Glu 154 in comparison to those of the unmodified enzyme. Truncation of the Glu 154 side chain thus abolished the effects of active site modification on the antibody affinity. This finding indicates that inhibitor binding to the active site of VIIa induces a reorientation of the Glu 154 side chain toward a position that is less favorable for antibody binding with VIIa. Therefore, the Glu 154 side chain must be critical for the linkage of active site occupancy to the conformation of the antibody epitope. The kinetics of binding of Glu 154 to a different antibody with a conformation-insensitive epitope in the VIIa protease domain were indistinguishable from those of wild-type VIIa. This excludes global conformational changes in protease domain conformation of the free or modified mutant (Table 2C). More importantly, active site modification of $VIIa_{Ala154}$ had effects on the kinetics of binding with TF that were equivalent to those of inhibitor binding to wild-type VIIa (Table 2A). Inhibitor binding to the active site of $VIIa_{Ala154}$ is thus unaltered and is capable of eliciting conformational changes that perturb the TF binding site in $VIIa_{Ala154}$. Mutation of Glu 154 thus affects only the conformational linkage between the active site and the F3-3.2A epitope.

Importance of the Glu 154 Side Chain for Inhibition of Catalytic Function by F3-3.2A. F3-3.2A inhibits the amidolytic function of the TF·VIIa complex by ≥80% (*5*). Since the epitope of the antibody is distant from the catalytic cleft, we infer that this functional inhibition is due to allosteric effects on the active site. To provide insight about which residue side chains of VIIa participate in this effect of F3-3.2A on the catalytic cleft, we analyzed each of the mutants for inhibition of amidolytic function by F3-3.2A. F3-3.2A produced a dose-dependent inhibition of the Lys 192 and the Leu 144 mutant, although the dose−response curve for Leu 144 was shifted to lower concentrations of the antibody (Figure 3). Since the Leu 144 mutant had a lower affinity for the chromogenic substrate, we tested whether the degree of substrate occupancy can influence the degree of inhibition. The degree of inhibition by a fixed concentration (2 μM) of F3-3.2A was compared at chromogenic substrate concentrations that were close to the K_M for the mutant Leu 144 VIIa (1.5 mM) and for wild-type VIIa (0.5 mM Chromozyme tPA). The degree of functional inhibition of mutant and wild-type VIIa at the respective substrate concentrations was similar (48 vs 50%). Substrate occupancy thus influences the equilibrium of free and antibody-bound VIIa, consistent with the changes in affinity observed in the direct binding measurements following active site occupancy with covalent inhibitors (Table 2B).

The amidolytic function of the Glu 154 mutant was only slightly inhibited by the antibody. F3-3.2A bound to TF·$VIIa_{Ala154}$ with the same affinity as it did to the wild-type TF·VIIa complex, and the affinity of the antibody for the active site-modified mutant was even higher than that of the wild-type control (Table 2B). Thus, the poor functional inhibition of the mutant cannot be attributed to a lack of binding by F3-3.2A, but rather demonstrates a major role of the Glu 154 side chain in influencing the catalytic activity of VIIa in response to antibody binding. However, the antibody inhibited the amidolytic function of wild-type VIIa to a level that is clearly lower than the level of amidolytic function of the Glu 154 mutant (compare Figures 2 and 3). The Glu 154 side chain thus accounts for the majority of the inhibition that is induced by F3-3.2A, but other residue side chains or subtle influences on the backbone conformation within the antibody epitope may have additional although minor effects.

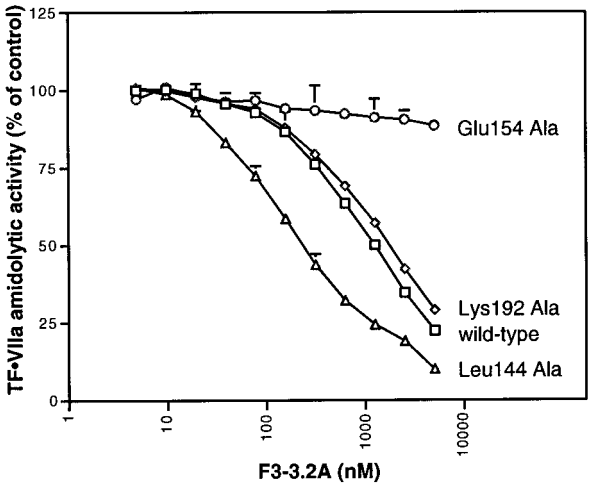

FIGURE 3: Inhibition of TF·VIIa amidolytic function by anti-VIIa monoclonal antibody F3-3.2A. Wild-type VIIa or the indicated mutant of VIIa (30 nM) in complex with soluble TF_{1-218} (120 nM) was incubated with varying concentrations of F3-3.2A for 15 min, and the residual amidolytic activity was determined with the chromogenic substrate Chromozyme tPA (0.5 mM).

DISCUSSION

Flexible regions in the protease domain are critical for the regulation of catalytic activity of serine proteases. The mechanism of zymogen activation provides an example of a global conformational transition in which several loop segments become structurally more ordered. These changes result in optimal positioning of the charge-relay system of the catalytic triad, formation of the oxyanion hole, and maturation of the S1 specificity site. Similar conformational changes are important for the cofactor-dependent enhancement of VIIa's catalytic function, since VIIa appears to retain zymogen-like features in the absence of cofactor (*5*, *9*). Binding of tetrahedral transition state mimicking inhibitors, such as Phe-Phe-Arg chloromethyl ketone, stabilizes the amino-terminal insertion and enhances the cofactor affinity of VIIa. This indicates that binding of cofactor and ordering of the serine protease domain of VIIa for optimal transition state binding of the scissile bond are intimately linked. However, analysis of the conformation-sensitive monoclonal antibody F3-3.2A provided unexpected evidence that cofactor binding and active site occupancy have qualitatively unique effects on the extended region involving the amino-terminal insertion site and the macromolecular substrate binding exosite (*5*). This study defines the molecular basis for the allosteric linkage of this region of VIIa by identifying the solvent-exposed side chain of Glu 154 as a critical residue

that is specifically linked to the catalytic cleft. This allosteric linkage is unrelated to cofactor binding, providing evidence for an independent and novel mechanism for regulating VIIa's catalytic function through a surface residue distant from the catalytic cleft.

The structure of the TF·VIIa complex inhibited by Phe-Phe-Arg chloromethyl ketone (*2*) has provided detailed molecular insight into the enzyme−cofactor interaction and the sites for interaction of the macromolecular substrate. Macromolecular substrate binding to enzyme−cofactor complexes, such as the prothrombinase complex (*20*), appears to precede scissile bond docking. The Glu 154 side chain is located within the macromolecular substrate exosite binding region, and the linkage of this residue to the catalytic cleft has important implications for understanding the function of cofactor-dependent serine proteases. If the conformational changes induced by active site inhibitors are representative of the changes mediated by scissile bond docking, one would predict a conformational effect on the macromolecular substrate exosite during transition state formation. This conformational rearrangement may facilitate macromolecular product release from the acyl−enzyme intermediate, consistent with the reduced k_{cat} for factor X activation demonstrated for $VIIa_{Ala154}$. The extent of cleavage of small peptidyl substrates was also reduced for $VIIa_{Aa154}$, indicating an intimate link between the conformation of the exosite and the function of the catalytic center. It is noteworthy that the conformational changes in the F3-3.2A epitope in response to active site inhibitors were dominant over the conformational changes induced by cofactor binding (*5*), suggesting that the conformational cross-talk between the active site and the macromolecular substrate exosite is indeed operational in a functional TF·VIIa complex. Analogously, the macromolecular substrate exosite I of thrombin is also involved in conformational linkages to the active site (*15*, *21*). The binding of thrombomodulin to exosite I changes the substrate specificity toward protein C activation by perturbing Glu 192 (*15*, *16*). Yet in VIIa, the Lys 192 side chain is clearly not involved in the conformational link to the F3-3.2A epitope, suggesting that the Glu 154 position influences catalysis by a mechanism that is distinct from thrombin's regulation of substrate specificity by reorientation of the position 192 side chain.

Glu 154 mutation may influence two regions that have the potential to regulate catalytic function. Met 156 is located adjacent to Glu 154, but more toward the amino-terminal insertion site. A Lys residue in the 156 position of tissue plasminogen activator is critical to regulation of the catalytic function of the zymogen by forming a salt bridge with Asp 194 (*22*, *23*). The specific local conformation of this insertion site in VIIa possibly accounts for the labile zymogen to enzyme transition. Since cofactor interactions are necessary to activate VIIa, catalytic function is likely regulated by conformational changes at the amino-terminal insertion site. This notion is supported by the finding that mutation of the insertion site residue Leu 144 did indeed severely reduce the level of amidolytic function of VIIa. However, mutation of Leu 144 and Glu 154 resulted in quite distinct changes in the kinetics of small substrate hydrolysis, suggesting that the effect of Glu 154 is not through an indirect perturbation of the amino terminus. Moreover, TF-induced changes in the F3-3.2A epitope are unaffected by the Glu 154 mutation, consistent with a linkage of Glu 154 that is independent of cofactor-dependent conformational changes. We consider it thus unlikely that Glu 154 functions primarily by influencing the amino-terminal insertion.

On the opposite side, Glu 154 contacts the side chain of His 71, located in the high-affinity Ca^{2+}-binding loop. Reducing the Ca^{2+} affinity of this binding site by mutagenesis of coordinating residue side chains severely impairs F3-3.2A binding (*5*), demonstrating that the surface residue side conformation is dependent on Ca^{2+} coordination. Mutation of the Ca^{2+}-binding loop also diminishes the level of amidolytic function of VIIa (*13*, *24*). We speculate that the effect of Glu 154 mutation on amidolytic function could operate through an effect on structures that are conformationally linked with the Ca^{2+}-binding loop. Factor Xa (*25*) and activated protein C (*26*) are also somewhat modulated in their amidolytic activities by Ca^{2+}. Ala replacement of thrombin residue His 71 in the high-affinity Ca^{2+}-binding loop of residues 70−80 reduces the level of amidolytic function approximately 2-fold (*27*). Since surface-exposed side chains in the loop of residues 70−80 contribute to macromolecular substrate recognition, the importance of the Ca^{2+}-binding site for amidolytic function may be related to a more general conformational link between macromolecular substrate docking and scissile bond catalysis in serine protease domains. Docking to this region may even serve as a direct mechanism for allosteric activation of serine protease zymogens, as suggested by the recent report of the streptokinase−plasmin complex crystal structure (*28*) that demonstrates binding of the zymogen activator to the surface of the Ca^{2+}-binding loop and the adjacent base of the autolysis loop.

Thrombin has a high-affinity binding site for a Na^+ ion that was suggested to be linked to a global conformational transition from the pro- to the anticoagulant form of the enzyme (*29*). A signature aromatic residue at position 225 is essential for this conformational change, and Phe 225 in VIIa could seemingly permit similar mechanisms of allosteric regulation. However, there is no evidence that VIIa's conformational linkages are globally coupled, as suggested for thrombin. Rather, we find that selected surface residues are thermodynamically linked to specific functional regions. For example, Met 164 mediates the increased affinity from the active site to the cofactor binding site and, vice versa, regulates the catalytic activity of VIIa dependent on TF interactions (*14*). In thrombin, specific salt bridges connect the flexible 141−154 loop residue Glu 146 to Arg 221A and the active site cleft residue Glu 217 to Lys 224, resulting in coupling to the Na^+-binding loop of residues 220−225 (*29*). There are no equivalent ionic interactions in VIIa which may account for uncoupling of conformational linkages from the active site to the cofactor binding site and to the macromolecular substrate exosite. By identifying a novel linkage between Glu 154 and the catalytic cleft, we thus substantiate the hypothesis that the catalytic function of VIIa is regulated by highly selective allosteric linkages between discrete functional regions of the protease domain.

ACKNOWLEDGMENT

We thank Jennifer Royce and David Revak for the production of recombinant proteins.

REFERENCES

1. Huber, R., and Bode, W. (1978) *Acc. Chem. Res. 11*, 114−122.
2. Banner, D. W., D'Arcy, A., Chène, C., Winkler, F. K., Guha, A., Konigsberg, W. H., Nemerson, Y., and Kirchhofer, D. (1996) *Nature 380*, 41−46.
3. Martin, D. M. A., Boys, C. W. G., and Ruf, W. (1995) *FASEB J. 9*, 852−859.
4. Dickinson, C. D., Kelly, C. R., and Ruf, W. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 14379−14384.
5. Dickinson, C. D., Shobe, J., and Ruf, W. (1998) *J. Mol. Biol. 277*, 959−971.
6. Ruf, W., Kalnik, M. W., Lund-Hansen, T., and Edgington, T. S. (1991) *J. Biol. Chem. 266*, 15719−15725.
7. Higashi, S., Nishimura, H., Fujii, S., Takada, K., and Iwanaga, S. (1992) *J. Biol. Chem. 267*, 17990−17996.
8. Lawson, J. H., Butenas, S., and Mann, K. G. (1992) *J. Biol. Chem. 267*, 4834−4843.
9. Higashi, S., Nishimura, H., Aita, K., and Iwanaga, S. (1994) *J. Biol. Chem. 269*, 18891−18898.
10. Higashi, S., Matsumoto, N., and Iwanaga, S. (1996) *J. Biol. Chem. 271*, 26569−26574.
11. Ruf, W. (1994) *Biochemistry 33*, 11631−11636.
12. Stone, M. J., Ruf, W., Miles, D. J., Edgington, T. S., and Wright, P. E. (1995) *Biochem. J. 310*, 605−614.
13. Kelly, C. R., Dickinson, C. D., and Ruf, W. (1997) *J. Biol. Chem. 272*, 17467−17472.
14. Dickinson, C. D., and Ruf, W. (1997) *J. Biol. Chem. 272*, 19875−19879.
15. Vijayalakshmi, J., Padmanabhan, K. P., Mann, K. G., and Tulinsky, A. (1994) *Protein Sci. 3*, 2254−2271.
16. Le Bonniec, B. F., and Esmon, C. T. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7371−7375.
17. Hedstrom, L., Lin, T. Y., and Fast, W. (1996) *Biochemistry 35*, 4515−4523.
18. Arbini, A. A., Bodkin, D., Lopaciuk, S., and Bauer, K. A. (1994) *Blood 84*, 2214−2220.
19. Neuenschwander, P. F., and Morrissey, J. H. (1995) *Biochemistry 34*, 8701−8707.
20. Betz, A., and Krishnaswamy, S. (1998) *J. Biol. Chem. 273*, 10709−10718.
21. Ye, J., Esmon, N. L., Esmon, C. T., and Johnson, A. E. (1991) *J. Biol. Chem. 266*, 23016−23021.
22. Tachias, K., and Madison, E. L. (1997) *J. Biol. Chem. 272*, 28−31.
23. Renatus, M., Engh, R. A., Stubbs, M. T., Huber, R., Fischer, S., Kohnert, U., and Bode, W. (1997) *EMBO J. 16*, 4797−4805.
24. Wildgoose, P., Foster, D., Schiodt, J., Wiberg, F. C., Birktoft, J. J., and Petersen, L. C. (1993) *Biochemistry 32*, 114−119.
25. Rezaie, A. R., and Esmon, C. T. (1994) *J. Biol. Chem. 269*, 21495−21499.
26. Rezaie, A. R., Mather, T., Sussman, F., and Esmon, C. T. (1994) *J. Biol. Chem. 269*, 3151−3154.
27. Tsiang, M., Jain, A. K., Dunn, K. E., Rojas, M. E., Leung, L. L. K., and Gibbs, C. S. (1995) *J. Biol. Chem. 270*, 16854−16863.
28. Wang, X. Q., Lin, X. L., Loy, J. A., Tang, J., and Zhang, X. J. C. (1998) *Science 281*, 1662−1665.
29. Di Cera, E., Dang, Q. D., and Ayala, Y. M. (1997) *Cell. Mol. Life Sci. 53*, 701−730.
30. Kraulis, P. J. (1991) *J. Appl. Crystallogr. 24*, 946−950.

BI981951G