Tissue Factor Alters the pK_a Values of Catalytically Important Factor VIIa Residues†

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ABSTRACT: Blood coagulation is triggered when the serine protease factor VIIa (fVIIa) binds to cell surface tissue factor (TF) to form the active enzyme−cofactor complex. TF binding to fVIIa allosterically augments the enzymatic activity of fVIIa toward macromolecular substrates and small peptidyl substrates. The mechanism of this enhancement remains unclear. Our previous studies have indicated that soluble TF (sTF; residues 1−219) alters the pH dependence of fVIIa amidolytic activity (Neuenschwander et al. (1993) *Thromb. Haemostasis 70,* 970), indicating an effect of TF on critical ionizations within the fVIIa active center. The p*K*a values and identities of these ionizable groups are unknown. To gain additional insight into this effect, we have performed a detailed study of the pH dependence of fVIIa amidolytic activity. Kinetic constants of Chromozym t-PA ($MeSO_2$-D-Phe-Gly-Arg-*p*NA) hydrolysis at various pH values were determined for fVIIa alone and in complex with sTF. The pH dependence of both enzymes was adequately represented using a diprotic model. For fVIIa alone, two ionizations were observed in the free enzyme (pK_{E1} = 7.46 and pK_{E2} = 8.67), with at least a single ionization apparent in the Michaelis complex (pK_{ES1} ∼ 7.62). For the fVIIa−sTF complex, the pK_a of one of the two important ionizations in the free enzyme was shifted to a more basic value (pK_{E1} = 7.57 and pK_{E2} = 9.27), and the ionization in the Michaelis complex was possibly shifted to a more acidic pH (pK_{ES1} = 6.93). When these results are compared to those obtained for other well-studied serine proteases, K_{E1} and K_{ES1} are presumed to represent the ionization of the overall catalytic triad in the absence and presence of substrate, respectively, while K_{E2} is presumed to represent ionization of the α-amino group of Ile_{153}. Taken together, these results would suggest that sTF binding to fVIIa alters the chemical environment of the fVIIa active site by protecting Ile_{153} from deprotonation in the free enzyme while deprotecting the catalytic triad as a whole when in the Michaelis complex.

The formation of an enzyme−cofactor complex between the serine protease factor VIIa (fVIIa)[1] and the nonenzymatic integral membrane protein tissue factor (TF) serves to trigger the complex series of proteolytic reactions that lead to blood coagulation. In this complex, fVIIa is the trypsin-like serine protease subunit, while TF serves as the regulatory subunit or cofactor (reviewed in ref *1*). The binding of fVIIa to TF greatly enhances its enzymatic activity. Existing studies of the cofactor effect of TF on fVIIa activity have largely focused on examining TF's function with respect to enhancing fVIIa proteolytic activity (i.e., examination of macromolecular substrate hydrolysis and characterization of interactions of these proteins with phospholipid membranes). However, previous studies have demonstrated that TF also enhances fVIIa activity toward small peptidyl substrates (*2*−*4*), suggesting that TF binding to fVIIa also results in important structural changes in the local active center of fVIIa. The mechanism for the enhancement of fVIIa activity and the identities of affected residues remain unclear.

We have previously demonstrated that TF alters the pH dependence of fVIIa activity (*3*). These studies, however, were of a qualitative nature and did not provide information on either the number or identity of the residues responsible. Studies of the pH dependence of other serine proteases such as trypsin, chymotrypsin, and thrombin have demonstrated that these serine proteases exhibit two ionizations important for expression of enzymatic activity: one at a neutral or slightly acidic pH (pH 6−7.5) and a second at a more basic pH (pH 8−9) (*5*). The acidic ionization has generally been attributed to the overall catalytic triad of the protease, specifically the active site His (c57),[2] and is required to be in an unprotonated state for activity (*6*−*8*). The basic

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[1] Abbreviations: TF, tissue factor; sTF, soluble tissue factor (residues 1−219); fVIIa, factor VIIa; fVIIa−sTF, the enzyme−cofactor complex of factor VIIa and sTF; BSA, bovine serum albumin; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Bis-Tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; Veronal, 5,5-diethylbarbituric acid.

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ionization has generally been attributed to the α-amino group of the amino-terminal isoleucine (c16) involved in forming the oxyanion-binding pocket and is active only in the protonated form. The purpose of this study was to perform a detailed kinetic study of the pH dependence of fVIIa activity, both alone and in complex with TF. The data are examined in relation to the pH dependence of other related serine proteases to gain insight into the mechanism of TF enhancement of fVIIa activity and to shed light onto the potential identity of the affected residues in fVIIa.

EXPERIMENTAL PROCEDURES

Materials. Bovine serum albumin (BSA; fraction V and fatty acid free) and 2-(*N*-morpholino)ethanesulfonic acid (Mes; free acid, ULTROL Grade) were from Calbiochem (La Jolla, CA). The amidolytic substrate Chromozym t-PA ($MeSO_2$-D-Phe-Gly-Arg-*p*NA) was from Roche Molecular Biochemicals (Indianapolis, IN). Tris (Trizma base), Hepes, and ethanolamine were from Fisher Scientific (Pittsburgh, PA). Bis-Tris propane and Veronal were from Sigma Chemical Co. (St. Louis, MO). Recombinant human fVIIa and sTF (TF_{1-219}, the extracellular domain of TF lacking the membrane anchor and the carboxyl-terminal cytoplasmic tail) were prepared and purified as previously described (*9*).

Surface Plasmon Resonance. All surface plasmon resonance data were obtained on a Biacore 1000 (Biacore Inc., Piscataway, NJ) at the Biacore Core Facility of the Oklahoma Medical Research Foundation. Soluble TF (100 μg/mL and 25 μg/mL in a 100 mM sodium acetate buffer (pH 4.5) and 100 mM NaCl) was coupled to two CM5 chip sensor surfaces to levels of 641 and 150 resonance units, respectively, using standard amine coupling methods (Biacore Inc). Unreacted sites were blocked with ethanolamine. Binding of fVIIa (0−250 nM) to each sTF surface was examined in triplicate at four pH values using 100 mM Bis-Tris propane (pH 6.5−9.5), 100 mM NaCl, 2 mM $CaCl_2$, and 0.005% polysorbate 20 as the running buffer. Binding was monitored in real time using a flow rate of 10 μL/min (control experiments performed at 30 μL/min showed no change in binding kinetics). Nonspecific binding of the fVIIa to the carboxymethyldextran matrix was minimized by (i) diluting fVIIa into running buffer containing 0.1% BSA and (ii) injecting all samples over the test surface and a control (mock-coupled) surface. The signal from the control surface was subtracted from that of the test surface to correct for any remaining nonspecific binding as well as the refractive index changes due to the BSA. Flow cells were regenerated between fVIIa injections using 10 mM Hepes-NaOH (pH 7.4), 150 mM NaCl, 3 mM EDTA, and 0.005% polysorbate 20. Data obtained at both levels of sTF (not shown) were well-described by a standard 1:1 Langmuir binding model to give rate constants of association (k_{ass}) and dissociation (k_{diss}). These values were used to calculate the equilibrium dissociation constant K_D for each pH ($K_D = k_{diss}/k_{ass}$).

Amidolytic Assays. Kinetic parameters for fVIIa and the fVIIa−sTF complex were determined by amidolytic assay of enzyme activity using the substrate Chromozym t-PA (*3*). Although lacking the transmembrane region of TF, sTF has been shown to behave identically to full-length relipidated TF with respect to its affect on fVIIa amidolytic activity (*4*). Thus, sTF in solution was used in this pH study to circumvent potential complications that would arise from TF incorporated into phospholipid vesicles. Unless indicated otherwise, amidolytic assays were performed in a tribuffer system consisting of 0.1 M Mes, 0.05 M Tris, 0.05 M ethanolamine, 0.2 M NaCl, 0.005 M $CaCl_2$, and 0.1% BSA. This buffer system was used because of its buffering capacity under a wide range of pH values (pH 6.0−10.0). The buffer was titrated to the desired pH at 25 °C (the experimental temperature), and the pH was remeasured following the assay to confirm the appropriateness of the buffer and actual pH value using an Orion model 710 pH meter with a Hamilton Biotrode micro pH electrode containing Proteolyte electrolyte (Hamilton Bonaduz AG, Switzerland).

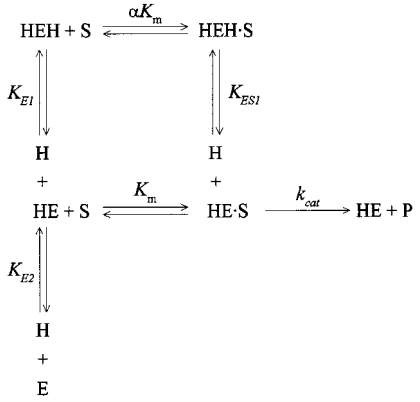
The final volume of all reaction mixtures was 100 μL, and assays were done in 96-well untreated nonsterile polystyrene microplates (Corning, no. 25880-96). Initial rates of substrate hydrolysis were measured by monitoring release of *p*-nitroaniline at 405 nm on a Thermomax kinetic microplate reader or a SpectraMax Plus384 reader (Molecular Devices Inc., Menlo Park, CA) set at 25 °C. The enzyme concentrations used (100 nM fVIIa alone or 10 nM fVIIa plus 100−200 nM sTF) ensured that less than 10% of the substrate was depleted during the assay (5−20 min). Under these conditions, spontaneous hydrolysis of the substrate was negligible (*4*). Initial rates of absorbance (mOD/min) were converted into rates of product generation (μM/min) by comparison to a standard line obtained with various known concentrations of *p*-nitroaniline (Sigma) in the appropriate buffer. The extinction coefficient of *p*-nitroaniline was constant throughout the pH range examined.

Determination of Kinetic Constants. Previous studies examining the kinetic parameters for hydrolysis of Chromozym t-PA (*4*) revealed a very high value of K_m for fVIIa alone (>4 mM). Because of limitations in the solubility of this substrate during experiments using levels greater than 5 mM, the precise determination of kinetic parameters for fVIIa alone was difficult. In this study, all of the kinetic parameters have been determined using both the Michaelis−Menton hyperbolic equation and Hanes−Woolf linear transformation. Although all linear transformations of the Michaelis−Menton hyperbola yield deviations in error, the Hanes−Woolf transformation affects errors the least (*10*). Thus, fits of kinetic data with this equation do not require any form of weighting. Values obtained for k_{cat}, K_m, and k_{cat}/K_m for each data set were the same using either procedure. The linear transformation was used largely to examine the data for non-Michaelis−Menton behavior, for which no evidence was found in any case. Kinetic constants were independently obtained in this way for fVIIa and the fVIIa−sTF complex using a minimum of five substrate concentrations spanning a 10-fold range. For fVIIa alone, this substrate range corresponded to $0.2K_m-2K_m$ at low pH and $0.05K_m-0.5K_m$ at high pH. For the fVIIa−sTF complex, the substrate range corresponded to $0.09K_m-0.9K_m$ at extremes of pH and $0.5K_m-5K_m$ at optimal pH. Hanes−Woolf plots were done in triplicate for determination of the kinetic constants at every pH, and pH titrations were each done in triplicate.

Data Analysis. The pH profiles obtained for each of the three kinetic parameters k_{cat}/K_m, K_m, and k_{cat} were fitted to equations based on Scheme 1. This model describes a simple

[2] The letter "c" preceding any residue number designates the chymotrypsinogen numbering system.

Scheme 1: Diprotic Model System Used to Describe the pH Dependence of fVIIa and fVIIa−sTF Enzyme Activity



diprotic system with one productive form of the enzyme (HE) and two nonproductive forms of the enzyme (E, which cannot bind substrate, and HEH, which binds substrate but cannot undergo catalysis to form product). The derivation of the Michaelis−Menton equation for this model has been described by several investigators (*8*, *11*). The pH profile of k_{cat}/K_m for this system is described by the equation

$$k_{cat}/K_m = \frac{k_{cat}/K_{m(ind)}}{1 + [H]/K_{E1} + K_{E2}/[H]} \quad (1)$$

where $k_{cat}/K_{m(ind)}$ is the pH-independent value of k_{cat}/K_m, and K_{E1} and K_{E2} are the two acid equilibrium dissociation constants for the free enzyme. The pH profile of k_{cat} for this system is described by the equation

$$k_{cat} = \frac{k_{cat(ind)}}{1 + [H]/K_{ES1}} \quad (2)$$

where $k_{cat(ind)}$ is the pH-independent value of k_{cat}, and K_{ES1} is the acid equilibrium dissociation constant for the Michaelis complex. The pH profile of K_m for this system is the most complex and involves all three ionizations. This is described by the equation

$$K_m = \frac{K_{m(ind)}(1 + [H]/K_{E1} + K_{E2}/[H])}{1 + [H]/K_{ES1}} \quad (3)$$

where $K_{m(ind)}$ is the pH-independent value of K_m; and K_{E1}, K_{E2}, and K_{ES1} are as described previously. In cases where $K_{E1} = K_{ES1}$, these ionizations cancel each other out and are not visible on K_m pH profiles. In this situation (possibly observed with fVIIa alone), the pH profile of K_m can simply be described by the equation

$$K_m = K_{m(ind)}(1 + K_{E2}/[H]) \quad (4)$$

All nonlinear regression procedures were done using the appropriate equations and the software Slide Write Plus version 5.0 (Advanced Graphic Software, Inc., Encinitas, CA), which uses the Levenberg−Marquardt algorithm for least-squares fitting of experimental data.

RESULTS AND DISCUSSION

pH Dependence of the fVIIa−sTF Interaction. Variation of pH is expected not only to affect the activity of the enzyme, but also, in the case of the fVIIa−sTF complex, to affect the formation of the enzyme−cofactor complex. Thus, initial experiments were aimed at defining the effect of pH on this interaction. Using surface plasmon resonance, we were able to examine the effect of various pH values within our experimental range on the association and dissociation rate constants and the equilibrium binding constant describing the formation of the fVIIa−sTF complex (Figure 1). Although not a major part of the study, this cursory examination of the pH dependence of the binding interaction between fVIIa and sTF shows that there are at least two important ionizable groups involved in this interaction and likely more. This is not surprising considering the potential number of charged residues in both fVIIa and TF that have been proposed to be involved in this interaction (*12*−*17*). In general, the rate constants for association and dissociation of the fVIIa−sTF complex obtained at pH 7.5 agree very well with those of Kelley et al. (*16*); $k_{ass(7.5)} = (5.2 \pm 0.3) \times 10^5\ M^{-1}\ s^{-1}$ and $k_{diss(7.5)} = (1.7 \pm 0.3) \times 10^{-3}\ s^{-1}$. This results in a K_D value of 3.3 ± 0.4 nM at pH 7.5 and agrees well with our previously published value of 4.8 nM obtained from activity studies (*4*) and the value of 6.3 ± 1.2 nM obtained from Biacore studies (*16*). Although extreme pH values of 6.5 and 9.5 were found to greatly reduce the affinity between fVIIa and sTF, the K_D for binding never went above 25 nM. For the purpose of this study, knowledge of the effect of pH on k_{ass}, k_{diss}, and K_D allowed us to ensure that fVIIa would remain fully in complex with sTF throughout the pH range of our studies. On the basis of these data, the experimental conditions used for pH studies on enzymatic activity of the fVIIa−sTF complex ensured that 91−98% of the fVIIa remained in complex with sTF. By using these levels of sTF at all pH values, ionizations involved in the formation of

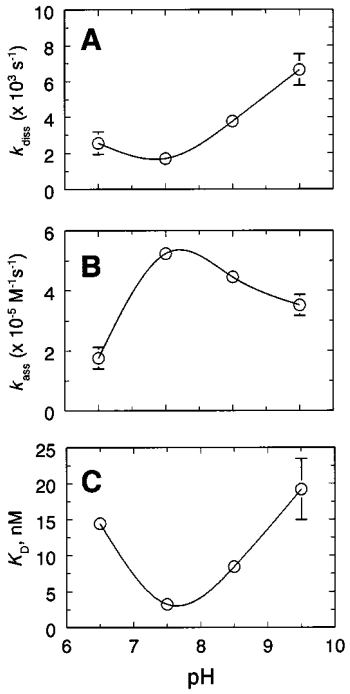


FIGURE 1: Effect of pH on the interaction of fVIIa and sTF. Surface plasmon resonance was used to examine the pH dependence of the fVIIa−sTF interaction, as described under Experimental Procedures: (Panel A) effect of pH on the first-order dissociation rate constant (k_{diss}); (panel B) effect of pH on the second-order association rate constant (k_{ass}); (panel C) effect of pH on the dissociation equilibrium constant (K_D).

Table 1: Effect of Buffer on the Kinetic Parameters of Chromozym t-PA Hydrolysis by fVIIa and the fVIIa−sTF Complex[a]

buffer, pH	kinetic parameter	fVIIa	fVIIa−sTF
MTE, 6.5	k_{cat} (min^{-1})	$(2.5 \pm 0.6) \times 10^1$	$(1.6 \pm 0.03) \times 10^3$
	K_m (mM)	2.2 ± 0.9	3.6 ± 0.4
	k_{cat}/K_m (mM^{-1} min^{-1})	$(1.2 \pm 0.3) \times 10^1$	$(4.6 \pm 0.6) \times 10^2$
Mes, 6.5	k_{cat} (min^{-1})	$(2.6 \pm 0.1) \times 10^1$	$(1.6 \pm 0.3) \times 10^3$
	K_m (mM)	2.3 ± 0.1	3.4 ± 0.5
	k_{cat}/K_m (mM^{-1} min^{-1})	$(1.1 \pm 0.1) \times 10^1$	$(4.8 \pm 0.2) \times 10^2$
MTE, 7.5	k_{cat} (min^{-1})	$(6.4 \pm 0.4) \times 10^2$	$(3.6 \pm 0.2) \times 10^3$
	K_m (mM)	10 ± 0.6	1.5 ± 0.3
	k_{cat}/K_m (mM^{-1} min^{-1})	$(6.4 \pm 0.1) \times 10^1$	$(2.5 \pm 0.3) \times 10^3$
Tris, 7.5	k_{cat} (min^{-1})	$(5.3 \pm 1.1) \times 10^2$	$(4.1 \pm 1.1) \times 10^3$
	K_m (mM)	6.3 ± 0.05	1.3 ± 0.2
	k_{cat}/K_m (mM^{-1} min^{-1})	$(8.3 \pm 1.8) \times 10^1$	$(3.1 \pm 0.4) \times 10^3$
Hepes, 7.5	k_{cat} (min^{-1})	$(5.6 \pm 1.0) \times 10^2$	$(3.3 \pm 0.5) \times 10^3$
	K_m (mM)	8.0 ± 1.4	1.3 ± 0.3
	k_{cat}/K_m (mM^{-1} min^{-1})	$(7.0 \pm 0.05) \times 10^1$	$(2.5 \pm 0.1) \times 10^3$
MTE, 9.5	k_{cat} (min^{-1})	$(1.6 \pm 0.2) \times 10^2$	$(6.0 \pm 0.1) \times 10^3$
	K_m (mM)	13 ± 1	3.2 ± 0.4
	k_{cat}/K_m (mM^{-1} min^{-1})	$(1.3 \pm 0.01) \times 10^1$	$(1.9 \pm 0.2) \times 10^3$
$EtNH_2$, 9.5	k_{cat} (min^{-1})	$(1.2 \pm 0.2) \times 10^2$	$(6.6 \pm 0.8) \times 10^3$
	K_m (mM)	6.8 ± 1.4	3.0 ± 0.9
	k_{cat}/K_m (mM^{-1} min^{-1})	$(1.7 \pm 0.1) \times 10^1$	$(2.3 \pm 0.4) \times 10^3$
BTP, 9.5	k_{cat} (min^{-1})	$(9.9 \pm 0.7) \times 10^1$	$(6.1 \pm 0.9) \times 10^3$
	K_m (mM)	6.4 ± 1.2	2.9 ± 0.3
	k_{cat}/K_m (mM^{-1} min^{-1})	$(1.8 \pm 0.4) \times 10^1$	$(2.1 \pm 0.6) \times 10^3$
Veronal, 9.5	k_{cat} (min^{-1})	$(1.4 \pm 1.1) \times 10^3$	$(5.4 \pm 0.8) \times 10^3$
	K_m (mM)	50 ± 40	1.7 ± 0.5
	k_{cat}/K_m (mM^{-1} min^{-1})	$(3.0 \pm 0.3) \times 10^1$	$(3.3 \pm 0.6) \times 10^3$

[a] All values are means ±SD from duplicate determinations: MTE, Mes-Tris-ethanolamine (tribuffer) system; $EtNH_2$, ethanolamine; BTP, Bis-Tris propane.

the fVIIa−sTF complex were negligible and not visible in pH profiles.

pH Dependence of the Kinetic Parameters for Hydrolysis of Chromozym t-PA. Previous studies from our group have shown that the pH dependence of fVIIa amidolytic activity is altered upon binding to sTF (*3*). Factor VIIa was found to exhibit optimal activity in the pH range 7.5−8.0, with diminishing activity on either side of this range. The effect of sTF was a general translation of the activity curve to more basic pH values, with a resulting broadening of the pH profile. The qualitative types of curves observed in that study (obtained at only a single substrate concentration) can be explained by the presence of two ionizable groups in fVIIa that are required for its enzymatic activity. However, to quantitatively determine the differences between fVIIa and the fVIIa−sTF complex and to gain insight into the ionizable groups effected by sTF binding, it was necessary to determine the pK_a for each ionizable group involved in catalysis for both enzymes (fVIIa and the fVIIa−sTF complex).

In a simple Michaelis−Menton system, the following three relations hold true. (i) The pH dependence of k_{cat}/K_m will be dependent only on ionizations in the free enzyme and substrate. In this case, it is important to note that the only ionizable group in the substrate used in this study is the guanidino group of arginine (pK_a = 12.5). Thus, within the pH range examined, this substrate behaves as a nonionizing substrate, and only ionizable groups in the free enzyme are observed (K_{E1} and K_{E2}). (ii) The pH dependence of k_{cat} will be dependent only on ionizations in the Michaelis complex (K_{ES1}). (iii) The pH dependence of K_m will be dependent on all ionizations involved in the formation of the Michaelis complex (K_{E1}, K_{E2}, and K_{ES1}) (*6*). By determining the pH dependence of each of these kinetic parameters, information can be obtained concerning the role of various ionizations in each step in the catalytic mechanism.

To rule out specific buffer effects as the pH value is systematically changed, the kinetic parameters were measured at three pH values (6.5, 7.5, 9.5) using either the tribuffer system or the individual "active" buffer component of this system (Mes, pH 6.5; Tris, pH 7.5; or ethanolamine, pH 9.5). The data (Table 1) demonstrate that the tribuffer system used is adequate and that no effect of buffer composition was observed. The potential effect of amines on the kinetic constants was also examined for the amine buffers Tris (pH 7.5) and ethanolamine (pH 9.5) by using the non-amine buffers Hepes (pH 7.5) and Veronal (pH 9.5). Although the Veronal buffer yielded values with very high errors for fVIIa alone, this was found to be due to inconsistent precipitation of the buffer under the conditions used. Thus, the potential effect of ethanolamine was thus further examined using Bis-Tris propane (BTP), pH 9.5. The values obtained for BTP were consistent with other values obtained at pH 9.5, confirming that the ethanolamine buffer in the tribuffer system was not effecting the determination of kinetic constants.

pH Dependence of k_{cat}/K_m: Effect of sTF on Ionizations in the Free Enzyme. Examination of the pH dependence of k_{cat}/K_m yielded the typical bell-shaped pH profile for both fVIIa and fVIIa−sTF. log−log plots of these data (Figure 2) yielded curves with a rising slope of +1 and a falling slope of −1. The unit values indicate that a single proton is defined by each segment. Fits of the data for fVIIa alone with eq 1 yielded mean pK_a values of pK_{E1} = 7.46 ± 0.57 and pK_{E2} = 8.67 ± 0.36. The pH independent value of k_{cat}/

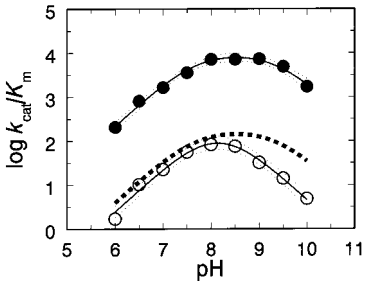


FIGURE 2: pH dependence of k_{cat}/K_m. Values of k_{cat}/K_m (mM^{-1} min^{-1}) were determined in triplicate at each indicated pH value for fVIIa alone (○) and for the fVIIa−sTF complex (●). Solid curves show the fit of the data with eq 1 (Scheme 1). Dotted curves show the 95% confidence limits. The data shown are representative. For fVIIa alone, $pK_{E1} = 7.9$ (95% confidence limits; 7.4−8.3) and $pK_{E2} = 8.4$ (95% confidence limits; 7.9−8.9). For the fVIIa−sTF complex, $pK_{E1} = 7.7$ (95% confidence limits; 7.5−7.8) and $pK_{E2} = 9.4$ (95% confidence limits; 9.1−9.6). Mean values obtained from three independent determinations are given in Table 1. The thick-dashed curve represents the translated fVIIa−sTF curve for comparison purposes.

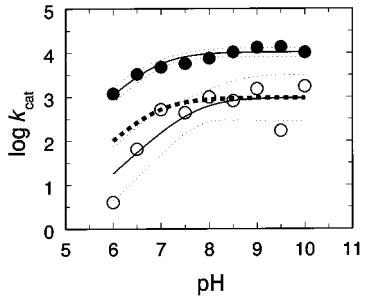


FIGURE 3: pH dependence of k_{cat}. Values of k_{cat} (min^{-1}) were determined in triplicate at each indicated pH value for fVIIa alone (○) and for the fVIIa−sTF complex (●). Solid curves show the fit of the data with eq 1 (Scheme 1). Dotted curves show the 95% confidence limits. The data shown are representative. For fVIIa alone, $pK_{ES1} = 7.7$ (95% confidence limits; 6.8−8.6). For the fVIIa−sTF complex, $pK_{ES1} = 6.9$ (95% confidence limits; 6.7−7.2). Mean values obtained from three independent determinations are given in Table 1. The thick-dashed curve represents the translated fVIIa−sTF curve for comparison purposes.

K_m was determined to be 5.7×10^2 mM^{-1} min^{-1}. In contrast, fits of the data obtained for the fVIIa−sTF complex with eq 1 yielded pK_a values of $pK_{E1} = 7.57 \pm 0.12$ and $pK_{E2} = 9.27 \pm 0.16$, with a pH independent value for k_{cat}/K_m of 2.5×10^4 mM^{-1} min^{-1}. These results are consistent with our previous study (*3*), where sTF was found to alter the pH dependence of fVIIa. The translation of the basic ionization in the free enzyme (described by pK_{E2}) to a slightly more basic pH value can be interpreted as changing the microenvironment of this group to protect it from deprotonation.

Although we have not performed experiments to positively identify the groups and amino acid residues involved, on the basis of work done by others with similar serine proteases (i.e., chymotrypsin and thrombin), it seems reasonable to tentatively assign pK_{E2} to the α-amino group of Ile_{153} (c16). Thus, sTF would seem to place the ionized α-amino group of Ile_{153} into a more stable environment, probably through formation of the salt bridge with Asp_{343} (*17*). This supports the conclusions of Higashi et al. from chemical modification studies done with bovine proteins (*18*, *19*). Although the salt bridge between Ile_{153} and Asp_{343} was also observed in the structure of the inhibited gla-domainless fVIIa in the absence of sTF (*20*), the presence of an inhibitor molecule in the active site is likely to have forced fVIIa into this conformation (*21*). This is consistent with Scheme 1, where substrate binding is predicted to prevent deprotonation of this group and "lock" this residue in the protonated form (deprotonation of HE·S cannot occur).

The effect of sTF on the pK_a of Ile_{153} would establish that the microenvironment of this group is altered upon TF binding to fVIIa. On the basis of our data and Scheme 1, this must occur before substrate binding (in the free enzyme). Thus, it is probable that one of the functions of TF is to place fVIIa in this favorable conformation before substrate arrival to help facilitate substrate docking. This is qualitatively similar to the proposal of Higashi et al. that fVIIa exists in two forms (inactive and active) and that TF binds preferentially to the active form of fVIIa (*18*, *19*). Clarification of whether TF binding energy is used to alter the conformation of fVIIa into the active form or if TF binds only to the active form must await further study.

pH Dependence of k_{cat} and K_m: Effect of sTF on Ionizations in the Michaelis Complex. Examination of the pH dependence of k_{cat} yielded a crude half-bell-shaped pH profile for both fVIIa and the fVIIa−sTF complex. log−log plots of these data (Figure 3) supported a single ionization event for the pH range examined. Fits of the data for fVIIa alone with eq 2 yielded mean values of $pK_{ES1} = 7.62 \pm 0.13$ and $k_{cat(ind)} = 3.6 \times 10^3$ min^{-1}. For the fVIIa−sTF complex, the values obtained were $pK_{ES1} = 6.93 \pm 0.01$ and $k_{cat(ind)} = 1.8 \times 10^4$ min^{-1}. The difference in the measured pK_{ES1} value for fVIIa versus the fVIIa−sTF complex suggests that sTF may affect this ionization by facilitating its deprotonation. However, the wide 95% confidence limits observed for fVIIa preclude a definitive conclusion based on these data alone.

In an attempt to gain further insight into the potential effect of sTF on pK_{ES1}, we closely examined the pH dependence of K_m, which shows ionizations in all species in the reaction scheme. It is important to note that, in plots of pK_m versus pH, upward-bending curves indicate ionizations in the enzyme−substrate (Michaelis) complex, while downward-bending curves indicate ionizations in the free enzyme (*8*). Thus, for a simple diprotic model with a k_{cat}/K_m pH profile as observed in Figure 2 and in which substrate binding does not affect the pK values, four potential pK_m profiles are possible (Figure 4, parts A−D). (i) If both ionizations affect k_{cat}/K_m (free enzyme) and k_{cat} (enzyme−substrate complex) equally, the resultant profile will be a horizontal line (Figure 4A). (ii) If neither ionization is important for k_{cat}, the profile will look similar to that obtained for k_{cat}/K_m (Figure 4B). (iii) If only one of the ionizations is important for k_{cat}, the profile for pK_m will only show the other ionization (Figure 4, parts C and D). This behavior becomes more complex in cases where one of the ionizations is affected by substrate binding. For instance, if substrate binding affects pK_{ES1} in such a way as to translate it to a more acidic pH, the pK_m profile will reveal three bends (Figure 4, parts E and F): an upward bend corresponding to the pK for the ES complex (k_{cat}) and two downward bends corresponding to the pK values observed in the free enzyme.

Careful examination of the pH dependence of pK_m for the fVIIa−sTF complex suggests that substrate binding indeed affects the ionization of K_{ES1} (Figure 5, closed circles), the

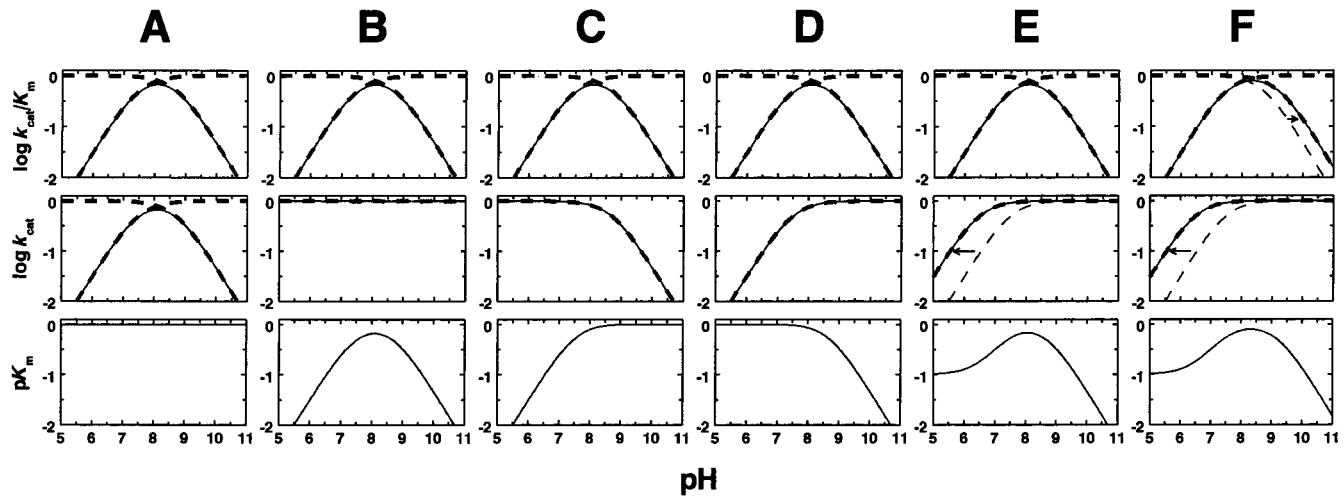


FIGURE 4: Potential effects of ionizations on plots of pK_m versus pH in a diprotic system (Scheme 1). A diprotic model in which the log k_{cat}/K_m profile shows two ionizations in the free enzyme (top panels) can produce different pK_m profiles (bottom panels) on the basis of the concomitant effect of these ionizations on log k_{cat} plots (middle panels). Columns A−D show the four predictions resulting from the ionizations having the same effect on k_{cat} (column A), no effect on k_{cat} (column B), or either ionization alone effecting k_{cat} (columns C and D). Complexity arises when substrate binding can cause a shift in one (or more) of the pK_a values from what is observed in the free enzyme (column E). The effect of sTF is indicated in column F (the fVIIa−sTF complex). The pH dependence of fVIIa alone is postulated to be represented by column D (see text). Dashed lines represent the individual ionizations, and the solid lines represent the resulting activity profile. The arrows in columns E and F represent shifts in the individual ionizations from column D. Curves were drawn using eqs 1−4 with pK_a values of pK_1 = 7.5 and pK_2 = 8.5. For columns E and F, the values used were pK_1 = 7.5 → 6.5 and pK_2 = 8.5 → 9.5.

Table 2: Summary of pK_a Values and pH-Independent Constants for fVIIa and the fVIIa−sTF Complex[a]

enzyme	kinetic constant	pK_{E1}	pK_{E2}	pK_{ES1}	pH-independent value
fVIIa	k_{cat}/K_m	7.46 ± 0.57	8.67 ± 0.36		5.7×10^2 mM^{-1} min^{-1}
	K_m		8.70 ± 0.37		7.4 mM
	k_{cat}			7.62 ± 0.13	3.6×10^3 min^{-1}
fVIIa−sTF	k_{cat}/K_m	7.57 ± 0.12	9.27 ± 0.16		2.5×10^4 mM^{-1} min^{-1}
	K_m	7.19 ± 0.22	9.19 ± 0.17	6.30 ± 0.31	0.9 mM
	k_{cat}			6.93 ± 0.01	1.8×10^4 min^{-1}

[a] Obtained from fitting data sets with eqs 1−4 (Scheme 1). Values of pK_a are shown ± SD (n = 3).

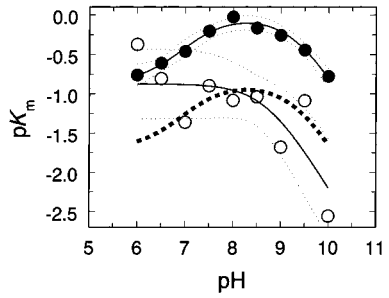


FIGURE 5: pH dependence of K_m. Values of K_m (mM) were determined in triplicate at each indicated pH value for fVIIa alone (○) and for the fVIIa−sTF complex (●). Curves show the fit of the data with eq 3 (●) or eq 4 (○) (Scheme 1). Dotted curves show the 95% confidence limits. The data shown are representative. For fVIIa alone, pK_{E2} = 8.7 (95% confidence limits; 7.8−9.6). For the fVIIa−sTF complex, pK_{E1} = 7.3 (95% confidence limits; 6.7−7.8), pK_{E2} = 9.3 (95% confidence limits; 9.0−9.6), and pK_{ES1} = 6.9 (95% confidence limits; 5.9−7.1). Mean values obtained from three independent determinations are given in Table 1. The thick-dashed curve represents the translated fVIIa−sTF curve for comparison purposes.

pH dependence being a composite of all three of the ionizations observed in the free enzyme and the enzyme−substrate complex. Fits of these data with eq 3 yielded values of pK_{E1} = 7.19 ± 0.22, pK_{E2} = 9.19 ± 0.17, pK_{ES1} = 6.30 ± 0.31, and $K_{m(ind)}$ = 0.9 mM. The pK_a values are completely consistent with those obtained independently from plots of log k_{cat}/K_m (free enzyme) and log k_{cat} (Michaelis complex) versus pH (cf. Figures 2 and 3, closed circles) and lend critical support to the values obtained and their interpretation in the context of Scheme 1.

The analysis of the K_m data for fVIIa alone was more difficult because of the relatively high K_m values obtained. Qualitatively, however, the data does not show the same pattern as observed for the fVIIa−sTF complex and, in fact, strictly support only a single ionization event within the pH range examined. This ionization event is in the free enzyme, based on the downward curvature produced in the plot. While eq 3 could not adequately describe the data, fitting the data with eq 4 yielded values of $K_{m(ind)}$ = 7.4 mM and pK_{E2} = 8.70 ± 0.37. The latter value is consistent with the value of pK_{E2} obtained independently from the plot of log k_{cat}/K_m versus pH (cf. Figure 2, open circles).

It is important to note that interpretation of plots of pK_m versus pH is often very complex, because the pH dependence of K_m involves ionizations in all precatalytic species. As such, because of the noise of the data obtained with fVIIa alone, we cannot definitively rule out other important ionizations. However, despite the inconclusiveness of the individual observations, we feel that an examination of the data as a whole with respect to the model is suggestive and worthy of mention. (i) The values obtained with fVIIa for pK_{E1} and pK_{ES1} from two independent fits agree well (Figures 2 and 3; Table 2). (ii) The shape of the pK_m plot is not indicative

of a downward curvature at acidic pH values but is consistent with the similarity of pK_{E1} and pK_{ES1}. (iii) The pK_m data are better described by eq 4 (cf. Figure 4D) than eq 3 (cf. Figure 4E). (iv) The pK_{E2} value obtained from the fit with eq 4 compares well with that obtained independently in Figure 2. On the basis of these data, we propose that substrate binding does not affect pK_{ES1} in fVIIa alone as it does in the fVIIa−sTF complex (cf. Figure 4F).

Apparent Effect of sTF on the Acidic Ionization. The acid dissociation equilibriums defined by K_{E1} and K_{ES1} in Scheme 1 refer to the same proton but in the absence and presence of substrate, respectively. Assuming rapid equilibrium for amino-terminally blocked peptidyl substrates (*5*), the cyclical nature of Scheme 1 predicts that if $pK_{E1} = pK_{ES1}$, then the K_m for substrate binding will be the same for HE and HEH (i.e., $\alpha = 1$). Conversely, different values for pK_{E1} and pK_{ES1} predict a different K_m for substrate binding for HE and HEH (i.e., $\alpha < 1$ if $pK_{ES1} > pK_{E1}$, and $\alpha > 1$ if $pK_{ES1} < pK_{E1}$). This can be written in mathematical terms as $K_m = K_m' \times (K_{E1}/K_{ES1})$, where $K_m' = (K_{ES1}/K_{E1}) \times K_m = \alpha K_m$ (Scheme 1). For the fVIIa−sTF complex, $pK_{E1} \neq pK_{ES1}$; thus, substrate binding affects this protonic equilibrium, and all three ionizations were apparent in the plot of pK_m versus pH. Once again, on the basis of work done by others with similar serine proteases, it seems reasonable to tentatively assign pK_{E1} and pK_{ES1} to the overall active site of fVIIa (*6*−*8*). For purposes of discussion, we take this as being the imidazole group of His_{193} (c57). The effect of sTF binding to fVIIa would be to lower the pK_a of this group only in the presence of substrate. This would result in an overall increase in the ionization of His_{193} at physiological pH. While it is likely that such a relatively small change in pK_a of His_{193} would have little effect on its reactivity as a Brønsted base in catalysis, the unprotonated His_{193} may be more favorable for substrate binding, as has been previously observed with thrombin (*5*, *22*). Indeed, the $K_{m(ind)}$ value obtained with the fVIIa−sTF complex was roughly 8-fold lower than that obtained with fVIIa alone. In addition, it is noteworthy that this effect on K_m is entirely accounted for by the sTF-dependent change in pK_{ES1}, which resulted in a value for α of 7.9. Thus, for this substrate, $\alpha K_{m(fVIIa-sTF)} = K_{m(fVIIa)}$.

The observation of a larger effect of sTF on K_m than on k_{cat} is consistent with our previous studies (*3*, *4*, *23*). The fact that no effect of sTF was previously observed in the rate of acylation of fVIIa using an ester substrate (4-methylumbelliferyl-*p*′-guanidinobenzoate) (*23*) suggests that the alteration of the environment of the active site by sTF would not greatly affect the catalytic steps following formation of the Michaelis complex. Alternatively, and of greater potential consequence, the observation of an effect of sTF on substrate binding and subsequent catalytic steps may be substrate specific. Because the binding of substrate is known to alter the microenvironment of the enzyme active site, this effect of sTF may vary with substrate. This notion is partially supported by the fact that not all amide substrates examined with fVIIa showed an altered K_m in the presence of sTF (*24*), suggesting that some synthetic amide substrates may not induce this sTF-dependent shift in pK_{ES1}. More importantly, it raises the intriguing possibility that this effect of TF may be used to various extents by the natural substrates of fVIIa. This may allow the fVIIa−TF complex to achieve much greater increases in k_{cat} with macromolecular substrates (*25*, *26*) than is possible with small peptide substrates (*3*, *4*, *23*, *27*).

pH Dependence of fVIIa and fVIIa−sTF Amidolytic Activity. In general, we have found that the pH dependence of both fVIIa and the fVIIa−sTF complex was well-described by a simple diprotic system (Scheme 1, and eqs 1−3) and that it was not necessary to use a more complex model. This model describes an enzyme whose activity is dependent on two critical protonic equilibriums. The active enzyme state is the monoprotonated (HE) form, which can bind substrate as well as undergo catalysis to form product. In contrast, the diprotonated (HEH) form can bind substrate but cannot undergo catalysis, and the unprotonated (E) form is not capable of binding substrate and is thus nonproductive. The pK_a values and the values of the pH-independent constants obtained from fitting to this model for fVIIa and the fVIIa−sTF complex are shown collectively in Table 2. Additional support for the model is provided by the values obtained for the pH-independent kinetic constants, which agree very well with previously published values obtained at pH 7.5 and 8.3 (*3*, *4*, *23*). Soluble TF was found to increase k_{cat} roughly 5-fold and to reduce K_m roughly 8-fold, resulting in a ~40-fold enhancement in k_{cat}/K_m for this substrate.

In conclusion, it is perhaps somewhat surprising that a more complex model was not required to describe the pH dependence of fVIIa activity and the effect of sTF. While trypsin and chymotrypsin each have much simpler substrate binding requirements, fVIIa requires a substrate with a longer peptide sequence. This more extended substrate-binding site in fVIIa might have been expected to contain a greater number of ionizable groups important for binding and catalysis. Indeed, other charged residues have been implicated from our studies as well as the studies of others (*13*, *15*, *27*−*36*). In addition, one might have expected that charged amino acids in fVIIa or sTF involved in allosteric activation would be observed in pH plots. However, this proved not to be the case. This is likely a result of the use of saturating levels of sTF at all pH values, which may have negated the effect of any ionization involved in allosteric activation aside from those involved directly in catalysis. This would imply that once sTF is bound to fVIIa, ionizations that may be important for expression of allosteric effects are rendered unimportant. Alternatively, these ionizations may be important in catalysis only with macromolecular substrates. Identification of other potential ionizations must await future studies.

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