Effect of Zymogen Domains and Active Site Occupation on Activation of Prothrombin by von Willebrand Factor-binding Protein*

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Prothrombin is conformationally activated by von Willebrand factor-binding protein (vWbp) from Staphylococcus aureus through insertion of the NH₂-terminal residues of vWbp into the prothrombin catalytic domain. The rate of prothrombin activation by vWbp(1–263) is controlled by a hysteretic kinetic mechanism initiated by substrate binding. The present study evaluates activation of prothrombin by full-length vWbp(1–474) through activity progress curve analysis. Additional interactions from the COOH-terminal half of vWbp(1–474) strengthened the initial binding of vWbp to prothrombin, resulting in higher activity and an ~100-fold enhancement in affinity. The affinities of vWbp(1–263) or vWbp(1–474) were compared by equilibrium binding to the prothrombin derivatives prethrombin 1, prethrombin 2, thrombin, meizothrombin, and meizothrombin(des-fragment 1) and their corresponding active site-blocked analogs. Loss of fragment 1 in prethrombin 1 enhanced affinity for both vWbp(1–263) and vWbp(1–474), with a 30–45% increase in Gibbs free energy, implicating a regulatory role for fragment 1 in the activation mechanism. Active site labeling of all prothrombin derivatives with D-Phe-Pro-Arg-\(\text{H}_2\)N-dansyl chloride, analogous to irreversible binding of a substrate, decreased their \(K_D\) values for vWbp into the subnanomolar range, reflecting the dependence of the activating conformational change on substrate binding. The results suggest a role for prothrombin domains in the pathophysiological activation of prothrombin by vWbp, and may reveal a function for autocalysis of the vWbp/prothrombin complexes during initiation of blood coagulation.

As the inactive precursor of the serine proteinase thrombin, the zymogen prothrombin (ProT),³ occupies a central role in the regulation of blood coagulation. Activation of upstream coagulation factors provides a web of feedback reactions that function in the temporal and spatial control of thrombin production, but additional mechanisms exist that directly modify the function of thrombin. Allostery, the regulation of protein function through binding of a ligand or another protein to a site distal from the active site (1), is a principal modulator of proteinase activity, and a large number of allosteric ligands have been identified for thrombin (2). A high degree of allosteric linkage occurs between the catalytic site of thrombin and the two electropositive exosites I and II that mediate substrate, inhibitor, and regulatory cofactor interactions with thrombin. The function of exosite I in particular is linked with proper formation of the active site following proteolysis of ProT at Arg\(^\text{320}\)-Thr\(^{\text{321}}\) (3, 4), from a lower affinity conformation of this exosite (proexosite I) in the zymogen (5, 6).

ProT exhibits a continuum of conformational changes in response to direct proteolytic events that can alter the specificity of exosite I for its ligands, such as the lower activity and affinity for fibrinogen seen with meizothrombin (MzT) (7). Loss of the two kringle domains of ProT (fragments 1 and 2; F1 and F2) during activation exposes exosite II and may produce conformational shifts that affect association of other ligands, although this long distance effect has been debated (8, 9). Conversely, the action of ligand binding to the exosites can change the recognition of substrates by the active site, as illustrated by the capacity of thrombomodulin to switch thrombin from procoagulant to anticoagulant by targeting cleavage of protein C (10). Thrombin ligands can interact with only one of the exosites to affect thrombin activity or with both exosites through different regions of the ligand itself. Such a two-pronged but linked association with the exosites may generate responses unique from those seen with binding of a single exosite. Furthermore, allostery not only influences the interactions of thrombin with substrates, inhibitors, and regulatory macrodomains; MzT, meizothrombin; F1, fragment 1; F2, fragment 2; F1.2, fragment 1.2; FPR-CH\(_2\)Cl, o-Phe-Pro-Arg-CH\(_2\)Cl; H-o-Phe-Pip-Arg-pNA, H-o-phenylalanine-l-pipecolyl-l-arginine-pNA; S-TMRIA, tetramethylrhodamine-5-iodoacetamide dihydroiodide; Hir, hirudin.
molecules, but it also impacts binding of exogenous ligands such as the inhibitor hirudin (2).

The human pathogen *Staphylococcus aureus* secretes two proteins that can each interact with proexosite I on ProT, staphylocoagulase and von Willebrand factor-binding protein (vWbp) (11–13). Both are potent procoagulant cofactors capable of triggering rapid clotting of human plasma through direct cleavage of fibrinogen into fibrin by the activator-ProT complexes, but this activity is not reliant on proteolysis of the activation loop of ProT. Instead, conformational activation of thezymogen occurs through direct binding interactions with the activator and, ultimately, insertion of the NH$_2$-terminus of either staphylocoagulase or vWbp into the NH$_2$-terminal binding cleft in the catalytic domain of ProT (13, 14), forming the salt bridge with Asp$^{194}$ (chymotrypsinogen numbering) that is characteristic of proteolytic activation (3). In contrast to the usually minor effects seen with other ligands, binding of staphylocoagulase or vWbp to ProT generates what could be described as a definitive allosteric transition, where an inactive zymogen precursor is completely converted to an active species through a nonproteolytic alternative to its common activation pathway.

We previously described the molecular mechanism of vWbp procoagulant activity, and in addition, identified the role for a substrate-induced hysteretic kinetic mechanism for activation of ProT by vWbp, distinct from staphylocoagulase (13). Hysteresis is classically defined for a number of regulatory metabolic enzymes as a slow transition or conformational change initiated by various processes, including polymerization and ligand displacement (15, 16). The key initiator of hysteretic behavior in vWbp-mediated ProT activation is a tight-binding substrate that alters the slow conformational equilibrium between inactive and active forms of the vWbp-ProT complex (13), which represents the first example of such hysteretic control in serine proteinases.

In addition to the established effect of substrate, preliminary kinetic and binding data indicated that the presence of the fragment domains of ProT, in particular fragment 1, imparts either a steric or an allosteric impediment to fully productive binding of vWbp. The present study defines the contribution to affinity of F1 and F2 and the catalytic domain of ProT and the predominant structural and active forms of the vWbp-ProT complex (13), which represents the first example of such hysteretic control in serine proteinases.

**EXPERIMENTAL PROCEDURES**

**Materials**—d-Phe-Pro-Arg-CH$_2$Cl (FPR-CH$_2$Cl) was purchased from Bachem. H$_2$-D-Phenylalanine-L-pipeolicyl-L-arginine-pNA (H$_2$-D-Phe-Pip-Arg-pNA) was obtained from Diapharma (West Chesterfield, OH). Purified ecarin, the ProT activator from the venom of *Echis carinatus*, was purchased from Pentapharm (Basel, Switzerland), and tetramethylrhodamine-5-iodoacetamide dihydroiodide (5-TMRIA) was from Invitrogen.

**Proteins**—vWbp(1–263) and vWbp(1–474) were expressed and purified as described previously (13). Tyr$^{63}$-sulfated, fluorescein-labeled hirudin (54–65) ([5F]Hir(54–65)) was prepared as described (17). Native human ProT, prethrombin 1 (Pre 1), and thrombin (T) were purified according to established procedures (4, 18). Native human prethrombin 2 (Pre 2) was prepared by a modification of published methods (4, 19), adding a final chromatography step on a HiPrep 16/10 SP XL cation-exchange column (GE Healthcare) in 50 mM MES, 50 mM NaCl, pH 6.0 buffer. Pre 2 was separated from residual inhibited thrombin with a 0–700 mM NaCl gradient, concentrated, and dialyzed into 50 mM HEPES, 125 mM NaCl, pH 7.4 buffer for storage. The active site-blocked (FPR-•) analogs and ProT labeled with 5-TMRIA ([TMR]FPR-ProT) were produced and characterized by established methods (11, 18). Absorption coefficients ($E_{1%}$, 280 nm) and molecular weights of the ProT derivatives used were as follows: Pre T, 1.47 and 71,600; Pre 1, 1.78 and 49,900; Pre 2, 1.73 and 37,000; T, 1.74 and 36,600; MzT, 1.47 and 71,600; meizothrombin (des-fragment 1) (MzT(-F1)), 1.78 and 49,900.

**Preparation of Recombinant MzT and FPR-MzT**—A HEK-293 cell line stably transfected with a pcDNA 3.1 expression vector containing the recombinant human ProT(R155Q/R271Q/R284Q) mutant (ProT$^{QQQ}$) construct (20) was kindly provided by Dr. Sriman Krishnaswamy (Children’s Hospital of Philadelphia, PA). The ProT$^{QQQ}$ mutant was expressed and purified as described previously (21, 22) and stored in 5 mM MES, 150 mM NaCl, pH 6.0. To generate stable active MzT, ProT$^{QQQ}$ (20 $\mu$M) was incubated with purified ecarin (6 enzyme units/ml) and 200 $\mu$M dansylarginine N-(3-ethyl-1,5-pentanediyl)amide in 0.1 M HEPES, 0.1 M NaCl, 1 mg/ml PEG 8000, pH 7.4, supplemented with 0.10 volume of 1 M HEPES, pH 7.6, for 2 h at 25 °C. The reaction was quenched by the addition of 50 mM EDTA and immediately diluted 5-fold with 20 mM MES, 20 mM NaCl, pH 6.0 buffer. MzT was separated from residual unreacted ProT$^{QQQ}$ and ecarin by chromatography on a 1-ml Resource Q column (GE Healthcare) in 20 mM MES, pH 6.0 buffer, eluted with a 0–0.5 M NaCl gradient (23). To produce FPR-MzT, a portion of the active enzyme was incubated with a 10-fold molar excess of FPR-CH$_2$Cl for 1 h at 22 °C. Active MzT and FPR-MzT were dialyzed against 5 mM MES, 150 mM NaCl, pH 6.0 buffer, and stored at −80 °C.

**Preparation of FPR-MzT(-F1)**—Native Pre 1 (20 $\mu$M) was activated by ecarin (6 enzyme units/ml) in the presence of FPR-CH$_2$Cl (200 $\mu$M) for 2 h at 25 °C, and activation was quenched by the addition of 50 mM EDTA. The quenched reaction was dialyzed overnight against 25 mM NaH$_2$PO$_4$, pH 6.5, and loaded onto an SP-Sephadex C-50 column equilibrated with the same buffer. Unreacted Pre 1 was eluted with 50 mM NaH$_2$PO$_4$, pH 6.5, followed by elution of FPR-MzT(-F1) with a 50–300 mM NaH$_2$PO$_4$ gradient (23). The peak was pooled, concentrated,
and dialyzed against 5 mM MES, 150 mM NaCl, pH 6.0, for storage.

**Kinetic Titrations of ProT Activation**—Full time-course progress curves for activation of ProT by vWbp(1–474) were collected, monitoring the rate of hydrolysis of H-D-Phe-Pip-Arg-pNA at 405 nm and 25 °C. Reactions contained either 1 nM ProT, varying concentrations of vWbp(1–474), and 200 μM H-D-Phe-Pip-Arg-pNA or 1 nM ProT, 10 mM vWbp(1–474), and varying concentrations of H-D-Phe-Pip-Arg-pNA. Data were collected for 1 h or until A$_{405}$nm = 1.0. The progress curves of p-nitroaniline formation were analyzed by simultaneous non-linear least-squares fitting in KinTek Global Kinetic Explorer using the hysteretic kinetic model described previously (13, 24, 25), assuming diffusion-controlled rapid equilibrium binding steps.

**Competitive Fluorescence Titrations with Active ProT Derivatives**—Continuous fluorescence intensity measurements were taken in 50 mM HEPES, 110 mM NaCl, 5 mM CaCl$_2$, 1 mg/ml PEG 8000, pH 7.4, at 25 °C. Fluorescence was monitored for a buffer blank, [5F]Hir(54–65) alone, after the addition of vWbp(1–263) or vWbp(1–474), and immediately after the addition of competitors of fluorescent peptide binding (Pre 1, Pre 2, T, or MzT). Data were collected with either an SLM 8100 or a PTI QuantaMaster spectrofluorometer at λ$_{ex}$ = 491 nm and λ$_{em}$ = 520 nm. The fractional change in fluorescence was calculated as (F$_{obs}$ - F$_0$)/F$_0$ = ΔF/F$_0$, and the data for each experiment were fit globally with the cubic equation for tight binding to obtain the dissociation constants and the stoichiometric factor for each competitor (17). The Gibbs free energy of binding ($\Delta$G$_{binding}$) was calculated, using the equation $\Delta$G$_{binding}$ = RT lnK$_D$, where R = 1.98 cal mol$^{-1}$ K$^{-1}$ and T = 298.15 K (25 °C).

**In Situ Production of MzT(-F1)**—Because autocatalysis of thrombin-sensitive cleavage sites within ProT or Pre 1 occurs rapidly during conversion to MzT or MzT(-F1), active MzT(-F1) was produced in situ during the fluorescence experiments through activation of native Pre 1 by ecarin. Control time-course gel experiments mimicking the conditions used for the binding assays showed complete conversion of Pre 1 to MzT(-F1) within 20 min, with no detectable degradation of the proteins over this time period. Fluorescence measurements were collected in the same manner as for the active ProT derivatives above, but with inclusion of ecarin (2 enzyme units/ml) in the cuvette before the addition of Pre 1 and measurement of the final fluorescence values at 20 min after initiation of activation.

**Fluorescence Titrations with Active Site-blocked ProT Derivatives**—Titrations were performed in 50 mM HEPES, 110 mM NaCl, 5 mM CaCl$_2$, 1 mg/ml PEG 8000, pH 7.4, 1 mg/ml bovine serum albumin, 10 μM FPR-CH$_2$Cl at 25 °C. Increasing concentrations of either vWbp(1–263) or vWbp(1–474) were titrated in the presence of fixed concentrations of [TM]FPR-ProT and FPR-ProT, FPR-Pre 1, FPR-Pre 2, FPR-T, FPR-MzT, or FPR-MzT(-F1) as the competitor. Data were collected at

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**RESULTS**

**Kinetic Analysis of ProT Activation by vWbp(1–474)**—To confirm the role of hysteresis in the functional activity of full-length vWbp, vWbp(1–474) was employed in ProT activation assays. Although the degree of curvature in the rate of substrate cleavage seen with vWbp(1–474) is less than that of vWbp(1–263), the data were fit very well by the established hysteretic kinetic mechanism (Scheme 1) (Fig. 1). The calculated K$_D$ for the initial formation of the vWbp-ProT complex was 25.3 ± 0.1 nM, ~100-fold tighter binding than vWbp(1–263) (13), verifying that the COOH-terminal half of vWbp contributes greatly to the affinity of vWbp for ProT. The overall equilibrium constant for the conformational change between active vWbp-ProT* and inactive forms of the vWbp-ProT complex was also over 3-fold more favorable for the active form (K$_{con}$ 3.1 ± 1.8), primarily due to ~3-fold slower reverse rate constant (K$_{c2}$ 0.0185 ± 0.0005 s$^{-1}$) with a similar forward rate constant (K$_{c1}$ 0.00592 ± 0.0004 s$^{-1}$) for the slow conformational change (13). The remaining parameters for chromogenic substrate hydrolysis (K$_m$ 3.61 ± 0.01 μM; k$_{cat}$ 63 ± 1 s$^{-1}$) showed an ~6-fold increase in K$_m$ and a 34% decrease in k$_{cat}$ when compared with those determined for vWbp(1–263), giving only a slightly lower (6-fold) specificity constant (k$_{cat}$/K$_m$) (13).

**Equilibrium Binding of vWbp(1–263) or vWbp(1–474) to Active ProT Derivatives**—The affinity of vWbp(1–263) for native ProT was previously determined with [5F]Hir(54–65) as a competitive ligand for proexosite I, supporting the relatively weak affinity determined from kinetic analysis with the same
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...with the covalent, active site-specific inhibitor FPR-CH2Cl. The occupation on the affinity of ProT and its derivatives for vWbp, molar (T) (Table 1).

vWbp(1–263) revealed a similar relationship, native ProT to vWbp(1–263) decreased with values ranging from 1.6-fold from −7.6 kcal/mol for native ProT to −12.0 kcal/mol for the active enzyme forms (T, MzT, MzT(-F1)). vWbp(1–474) revealed a similar relationship, with values ranging from −9.4 kcal/mol (ProT) to −12.9 kcal/mol (T) (Table 1).

**Binding of vWbp(1–263) or vWbp(1–474) to Active Site-blocked ProT Derivatives**—To isolate the effect of active site occupation on the affinity of ProT and its derivatives for vWbp, both the zymogen and the active enzyme forms were labeled with the covalent, active site-specific inhibitor FPR-CH2Cl. The conformational changes associated with labeling with the inhibitor mimic the catalytic transition state of a bound substrate (26), a process predicted to substantially increase the affinity of vWbp for ProT due to the influence of substrates on the kinetic mechanism of activation. The resulting higher affinities for the active site-blocked analogs precluded the use of the same experimental approach used for the active derivatives. The fluorescent hirudin peptide could not effectively compete with either vWbp construct for binding because of the massively higher affinity of the FPR derivatives. To address this problem, [TMR]FPR-ProT was employed as a competitive ligand with the FPR analogs due to its similar high affinity for vWbp. Both vWbp(1–263) and vWbp(1–474) bound to all active derivative experiments, the calculated parameters support formation of a 1:1 molar complex between the proteins (0.7–1.2 mol of vWbp/mol of ProT derivative). Free energy values were nearly identical for all active site-inhibited forms, for both vWbp(1–263) (−12.9 to −13.3 kcal/mol) and vWbp(1–474) (−13.2 to −15.9 kcal/mol) (Table 1, Fig. 6).

**DISCUSSION**

The present results reveal a substantial role for both steric and allosteric changes in the mechanism of binding and activation of ProT by vWbp. Previous work characterized vWbp as...
interacting with proexosite I on ProT due to its ability to compete for binding with the COOH-terminal peptide of hirudin, Hir(54–65) (13). Similarly to many exosite I-specific ligands, vWbp demonstrates increased affinity for ProT derivatives with a proteinase-like conformation, with the COOH-terminal region of vWbp also contributing to binding affinity and complex activity. The preference of vWbp for active site-occupied binding partners emphasizes the importance of substrate in the hysteretic activation mechanism. Together, these results indicate that vWbp is capable of exploiting the physiological mechanisms that govern thrombin recognition of other ligands.

Although vWbp is capable of binding (pro)exosite I and potentially altering the specificity of ProT, a thorough analysis of the kinetics of ProT activation by vWbp(1–474) indicates that other regions of the zymogen interact with the COOH-terminal half of vWbp. Although the NH2-terminal half of vWbp (vWbp(1–263)) is predicted to have a secondary structure composed of two α-helical bundles (27), multiple prediction analyses on the COOH-terminal half of vWbp assign almost no regular structure, apart from a helical segment (~50 residues) at the distal end (not shown). The higher activity and ~100-fold higher affinity of vWbp(1–474) in forming the initial vWbp-ProT complex revealed in the kinetic analysis likely results from additional binding interactions, which may affect the conformation of proexosite I or facilitate insertion of the NH2 terminus of vWbp into the activation pocket on ProT. The unstructured region of vWbp may sterically alter the fragment domains to increase the overall affinity of vWbp for the zymogen. Whether this process is mediated by the largely disordered region or the smaller COOH-terminal helical portion of vWbp remains unknown.

The fragment domains of ProT present a potential hindrance to binding by a macromolecular ligand such as vWbp, but release of these domains is normally strictly regulated during ProT activation. When it is not bound to a membrane surface through Ca2+-mediated interactions between γ-carboxyglutamic acid residues and phosphatidylserine (28), the orientation of F1 relative to the rest of the zymogen is largely undefined, although crystal structures of F1, F2, Pre 1, and MzT(–F1) have been solved (28–31). Release of F1 through cleavage at Arg155 is greatly inhibited in the presence of Ca2+ (32, 33), and F1 typically remains covalently linked to F2 in fragment 1.2 (F1.2) (34). Loss of F1 or F1.2 not only relieves potential blocking interactions for binding of vWbp, but also produces conformational changes within the activation domain of ProT itself (35, 36) that could favor ligand-exosite association.

A role for both steric and allosteric processes is indicated by the results of the competitive binding studies using ProT analogs with unmodified active sites, which revealed substantial increases in the affinity of vWbp as F1 and F2 are removed from ProT. The greatest enhancement results from loss of F1, with ~30-fold higher affinity for vWbp(1–263) and a 6-fold change with vWbp(1–474). It is worth noting that although vWbp(1–474) is capable of binding to all of the ProT derivatives with higher affinities than vWbp(1–263), there is still an effect from the loss of F1 despite the significant contribution of the COOH-terminal region of vWbp to activity and affinity. A direct comparison of the ΔG values shows a 12–26% increase in binding free energy upon loss of F1 alone (Pre 1) for both vWbp constructs, but only a 4–6% increase upon subsequent loss of F2 (Pre 2).

**TABLE 1**

**Summary of binding parameters**

Dissociation constants ($K_d$), stoichiometries (n), fluorescence change magnitudes ($\Delta F_{max}/F_o$), Gibbs free energy (ΔG), and change in ΔG ($\Delta G_{obs} = \Delta G_{Pre}$) are listed for competitive equilibrium binding of [5F]Hir(54–65) and vWbp(1–263) or vWbp(1–474) to unlabeled ProT derivatives. Also listed are parameters obtained using [TMR]FPR-ProT as the probe with active site-blocked ProT analogs. Errors represent ± 2 S.D. Gder, Gibbs free energy of ProT derivative.

| ProT derivative | Probe | $K_d$ (nM) | mol of vWbp/mol of ProT | $\Delta F_{max}/F_o$ | ΔG (kcal/mol) | $\Delta G_{obs} = \Delta G_{Pre}$ (ΔG) (% of change) |
|----------------|-------|------------|-------------------------|---------------------|--------------|---------------------------------|
| vWbp(1–263)    | [5F]Hir(54–65)   | 2501 ± 336 | 1 (fixed) | -0.19 ± 0.01 | -7.6 | 0.0 |
| Pre 1          | [5F]Hir(54–65)   | 84 ± 23 | 0.78 ± 0.05 | -0.22 ± 0.01 | -9.6 | -2.0 (26%) |
| Pre 2          | [5F]Hir(54–65)   | 55 ± 21 | 0.81 ± 0.05 | -0.24 ± 0.01 | -9.9 | -2.3 (30%) |
| T              | [5F]Hir(54–65)   | 1.5 ± 0.3 | 0.74 ± 0.02 | -0.29 ± 0.01 | -12.0 | -4.4 (58%) |
| MzT            | [5F]Hir(54–65)   | 1.6 ± 0.6 | 0.85 ± 0.03 | -0.37 ± 0.01 | -11.9 | -4.3 (57%) |
| MzT(–F1)       | [5F]Hir(54–65)   | 1.3 ± 0.7 | 0.76 ± 0.04 | -0.30 ± 0.01 | -12.1 | -5.3 (70%) |
| Pre 1 (TMR)    | [TMR]FPR-ProT    | 0.153 ± 0.078 | 1.18 ± 0.07 | 1.00 ± 0.05 | -13.3 | -5.7 (57%) |
| Pre 2 (TMR)    | [TMR]FPR-ProT    | 0.161 ± 0.088 | 1.24 ± 0.09 | 0.98 ± 0.05 | -13.3 | -5.7 (57%) |
| FPR-T          | [TMR]FPR-ProT    | 0.210 ± 0.160 | 1.06 ± 0.12 | 1.00 ± 0.07 | -13.1 | -5.5 (72%) |
| MzT(–F1)       | [TMR]FPR-ProT    | 0.318 ± 0.118 | 0.79 ± 0.07 | 0.98 ± 0.02 | -12.9 | -5.3 (70%) |
| Pre 2 (FPR-MzT) | [TMR]FPR-ProT    | 0.252 ± 0.091 | 1.04 ± 0.07 | 0.95 ± 0.03 | -13.0 | -5.4 (71%) |

*Parameters from previous analysis determined in Ref. 13.*
The same pattern is not seen upon examination of the three active enzyme forms, T, MzT, and MzT-F1). vWbp(1–263) binds indistinguishably to all three species ($K_D$, $1.3–1.6$ nM), suggesting that the existence of a proteinase-like activation domain promotes an equally favorable structure for ligand binding. An identical relationship can be seen with vWbp(1–474), where although it is capable of binding the active enzymes with ~5-fold tighter $K_D$ values than vWbp(1–263), the presence of the fragment domains has no obvious effect. These results are consistent with previous studies on the influence of F1 and F2 on the expression of exosite I, where loss of F1 in Pre 1 increases the affinity of the exosite 7-fold for a fluorescently labeled hirudin peptide, but any modulating effect of F1 is canceled out by proteolytic activation of the zymogen (35).

What does this binding behavior indicate within the context of the hysteretic mechanism of activation by vWbp? An optimal ProT conformation that supports initial high affinity binding of vWbp (conformational selection) would seem to be implied from the binding studies alone (37). In contrast, the key step in the model of activation by vWbp occurs upon binding of a substrate into what is presumed an imperfectly formed, inactive active site within a relatively low affinity vWbp-ProT complex. Substrate serves to generate a more catalytically competent, high affinity complex, consistent with substrate- and cofactor-mediated induced fit mechanisms of activation. The activity of the complement protease factor D is regulated by binding of its substrate, C3b-complexed factor B (38, 39). Similarly, factor VIIa exists in a state of incomplete activation with a conformation resembling an intermediate stage between zymogen and protease (40–42). Requirement for binding its cofactor tissue factor (43) shows parallels to the unfavorable equilibrium that exists for the vWbp-ProT complex in the absence of substrate (Scheme 1).

The concept of substrate binding as a fundamental amplifier of ProT activation by vWbp is further strengthened by the equilibrium binding results with FPR-blocked ProT derivatives. Structural studies have shown that inhibition of thrombin by FPR-CH$_2$Cl gives a similar orientation of residues in the substrate-binding cleft as is seen with binding of substrates, but distinctly different from changes seen with binding of hirugen to exosite I (44). Thus, corresponding FPR analogs of all the enzyme and zymogen derivatives of ProT were produced through either direct inhibition with FPR-CH$_2$Cl or formation of a reversible, conformationally activated complex of the zymogen with staphylocoagulase (27), allowing incorporation of the inhibitor into the induced active site on the zymogen-activator complexes. The substrate-bound structural mimicry of the FPR analogs allowed vWbp(1–263) and vWbp(1–474) to bind with 4–6-fold higher affinity, even with the zymogens containing F1/F2. Therefore, the vWbp-ProT complex does not
simply require an “enzyme-like” orientation of the activation domain between the catalytic residues, oxyanion hole, and substrate specificity site, but it also calls for occupation of the S1 site by an appropriate substrate, consistent with the hysteretic mechanism of activation.

The involvement of substrate in the mechanism is clear, but does the presence of F1/F2 have any detectable effect on the binding of vWbp to derivatives containing an active catalytic domain? The design of the binding assays with the active ProT derivatives allowed observation of changes in fluorescence immediately upon the addition of the ProT analog to the [5F]Hir(54–65) and vWbp ligand mixture, and most of the assays showed very rapid equilibrium between the competing proteins. The only discrepancies were witnessed upon either the addition of active MzT or in situ activation of Pre 1 to MzT(-F1), where a noticeably longer time period was required to reach equilibrium and a stable fluorescence change. Fig. 7A shows two representative fluorescence traces for MzT binding, with the assay containing vWbp(1–474) requiring an additional ~50 s to reach equilibrium. The fact that vWbp(1–263) does not show a lag suggests that the COOH-terminal half of vWbp has opposition from F1.2 in MzT and that the kinetics of binding are affected but not the fundamental equilibrium dissociation constant.

A different mechanism may be at work during conversion of Pre 1 to MzT(-F1) (Fig. 7B), where vWbp(1–263) and vWbp(1–474) both alter the kinetics of activation by ecarin to different degrees when compared with Pre 1 in the presence of [5F]Hir(54–65) alone. Whether this is a consequence of partial blockage of areas on the zymogen required for interaction with ecarin is unknown, but it could also be influenced by conformational changes in Pre 1 induced by vWbp that may alter accessibility of the activation bond or orientation of the F2 domain. These findings suggest that F1 and F2 introduce some degree of hindrance to vWbp, even with an active catalytic domain and an exosite I conformation that favors high affinity binding.

Although vWbp can form an active complex with ProT exclusively through NH2-terminal insertion and substrate-dependent changes in conformation, previous studies of both vWbp (13) and staphylocoagulase (11) have identified a pattern of autocatalysis consistent with cleavage at two thrombin-sensitive sites. In the presence of vWbp, production of Pre 1 from ProT has been detected in reaction mixtures at both high and low concentrations of ProT, with formation of a species called Pre 2 after extended incubations at high ProT concentrations (13). The sequence of cleavage in vWbp-ProT mixtures corresponds with what is known about the mechanisms of feedback proteolysis in MzT and MzT(-F1), where both intramolecular and intermolecular events contribute to formation of proteol...
yasis products (45). This indicates that the proteolytic activity of vWbp-ProT* is consistent with thrombin-like behavior.

If vWbp can form a procoagulant complex with ProT that is not only capable of cleaving fibrinogen into fibrin, but also contains the potential to rid itself of its regulatory zymogen domains, what implications would this have for the pathological capacity of vWbp in a blood-borne staphylocoaguloc infection? The most vital function of F1 for ProT is to mediate binding of the zymogen to the activated platelet or endothelial cell membrane (46), permitting association with factor Xa and factor Va in the prothrombinase complex for thrombin production. If vWbp can bind to membrane-bound ProT, as well as free ProT in the blood, premature release of high affinity procoagulant vWbp-ProPre 1* complexes from membranes could occur in a pathological dissemination mechanism. This would have the amplified effect of liberating serpin-insensitive protein complexes to downstream locations where they could freely associate with both von Willebrand factor and fibrinogen, setting up new foci for fibrin deposition and bacterial colonization. Indirect effects related to other regulatory ligands would also occur, including blockage of all exosite I-specific ligands by the presence of vWbp and reduction of exosite II interactions from the presence of either F1.2 or F2. The end result would be vWbp-ProT* or vWbp-ProPre 1* complexes with thrombin-like activity that are nearly impervious to inhibition or down-regulation, but that exhibit high specificity for the single substrate fibrinogen due to the driving force of the substrate-mediated mechanism of vWbp zymogen activation.

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