Role of Regulatory Exosite I in Binding of Thrombin to Human Factor V, Factor Va, Factor Va Subunits, and Activation Fragments*

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The blood coagulation proteinase, thrombin, converts factor V into factor Va through a multistep activation pathway that is regulated by interactions with thrombin exosites. Thrombin exosite interactions with human factor V and its activation products were quantitatively characterized in equilibrium binding studies based on fluorescence changes of thrombin covalently labeled with 2-anilinonaphthalene-6-sulfonic acid (ANS) linked to the catalytic site histidine residue by N-acetyl-[acetylthio]acetyl]-D-Phe-Pro-Arg-CH₂Cl (ANS]FPR-thrombin). Exosite I was shown to play a predominant role in the binding of factor V and factor Va from the effect of the exosite I-specific ligand, hirudin54–65, on the interactions. Factor V and factor Va bound to exosite I of [ANS]FPR-thrombin with similar dissociation constants of 3.4 ± 1.3 and 1.1 ± 0.4 μM and fluorescence enhancements of 182 ± 41 and 127 ± 17%, respectively. Native thrombin and labeled thrombin bound with similar affinity to factor Va. Among factor V activation products, the factor Va heavy chain was shown to contain the site of exosite I binding, whereas exosite I-independent, lower affinity interactions were observed for activation fragments E and C₁, and no detectable binding was observed for the factor Va light chain. The results support the conclusion that the factor V activation pathway is initiated by exosite I-mediated binding of thrombin to a site in the heavy chain region of factor V that facilitates the initial cleavage at Arg⁷⁰⁹ to generate the heavy chain of factor Va. The results further suggest that binding of thrombin through exosite I to factor V activation intermediates may regulate their conversion to factor Va and that similar binding of thrombin to the factor Va produced may reflect a mode of interaction involved in the regulation of prothrombin activation.

Blood coagulation factor V is proteolytically processed by thrombin into factor Va through a multistep pathway that is essential for accelerating blood coagulation to the rate required for normal hemostasis (1–5). The single-chain, 330,000 molecular weight human factor V molecule has a homology domain structure of A₁-A₂-B-A₃-C₁-C₂ and is activated by thrombin cleavage at Arg residues 709, 1018, and 1545 within the B domain (4, 6–9). This generates the factor Va heavy chain (A₁-A₂) and light chain (A₃-C₁-C₂) subunits and releases the B domain as two activation fragments: fragment E (residues 710–1018) and fragment C₁ (residues 1019–1545) (4, 6–9). Factor Va is a dimer of the heavy and light chain subunits, which are associated noncovalently in a calcium-dependent interaction that is required for activity (4, 10). Activation of factor V results in an increase in its affinity for factor Xa and prothrombin necessary for efficient assembly on phospholipid membranes of a factor Xa-factor Va-prothrombin catalytic complex that generates thrombin at a ~300,000-fold faster rate than factor Xa alone (4, 11–13). Both associated subunits of factor Va are required for factor Xa binding, whereas prothrombin binds to the factor Va dimer and to the isolated heavy chain subunit (14–17).

The pathway of factor V activation by thrombin follows a kinetically preferred order of bond cleavage, in which products of cleavage at Arg⁷⁰⁹ appear first, followed closely by cleavage at Arg¹⁰¹⁸, and generation of the factor Va light chain by cleavage at Arg¹⁵⁴⁵ as the slowest reaction (4, 8, 9, 18, 19). The interactions responsible for specific binding of factor V to thrombin as a substrate and the mechanisms that regulate the specificity of the reactions on the activation pathway are not completely understood. Recent studies indicate that exosites I and II on thrombin participate in the mechanism of factor V activation (20–22). Exosites I and II are distinct electropositive sites on thrombin that contribute to the specificity of the enzyme by mediating the binding of certain protein substrates and inhibitors, as well as the binding of macromolecular effectors (23, 24). Exosite I has been implicated in factor V activation from the inhibitory effect of the specific peptide ligands, hirudin54–65 (Hir54–65) (Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Tyr-Leu-Gln) or N-acetyl-hirudin53–64 (hirugen) (N-acetyl-Asn-Gly-Asp-Phe-Glu-Ile-Pro-Glu-Tyr(SO₃⁻)-Leu) (20–22). Proteolytic derivatives of thrombin that have lost exosite I function, β- and γ-thrombin, also showed decreased activity in factor V activation (22). The role of exosite II in factor V activation is less clear. Although a decreased rate of bovine factor V activation was observed with an exosite II-disabled thrombin mutant (20), the expected inhibitory effects of the exosite II ligands, heparin and prothrombin fragment 2, have not been observed (20, 22).

Affinity chromatography of factor V activation products on a matrix of thrombin immobilized through its active site was used in previous studies to demonstrate that thrombin binds to factor Va in a previously undescribed, exosite I-dependent interaction with the heavy chain subunit (21). This observation may have significance with respect to the function of factor Va in prothrombin activation as well as the mechanism of factor V activation, as suggested by kinetic studies of prethrombin 2 as a substrate analog of prothrombin (25, 26). Thrombin acts as a

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1 The abbreviations used are: T, human α-thrombin; FPR-CH₂Cl, D-Phe-Pro-Arg-CH₂Cl; ANS, 2-anilinonaphthalene-6-sulfonic acid; factor Va-h, factor Va heavy chain; factor Va-l, factor Va light chain; factor Va-h, isolated two-subunit form of factor Va; Hir, hirudin.
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competitive inhibitor of bovine prethrombin 2 activation through exosite interactions with the factor Xa-factor Va-membrane complex (25, 26), suggesting that thrombin binding to factor Va could be involved.

The roles of thrombin exosite interactions in factor V activation and factor Va activity have been investigated further in the present studies, where the interactions with human factor V, factor Va, the factor Va subunits, and the activation fragments were characterized in quantitative equilibrium binding studies for the first time. The unique properties of a fluorescent thrombin derivative labeled at the catalytic site with 2-anilinonaphthalene-6-sulfonic acid as a reporter of exosite I interactions have been used to resolve the role of this regulatory site in binding of factor V/Va species. Exosite I is shown to be principally responsible for binding of factor V to active site-labeled thrombin, with a small contribution from exosite I-independent, lower affinity interactions. Examination of thrombin binding to each of the purified factor V activation products identified only one exosite I-mediated interaction of thrombin with a site on the heavy chain subunit of factor Va as the predominant mode of binding, again with small contributions from lower affinity interactions. These studies support a mechanism of factor V activation in which factor V is recognized as a specific thrombin substrate by exosite I-mediated binding at a site on the heavy chain region of factor V, which enhances specific cleavage at Arg709 to initiate the activation pathway. The observation that factor Va retains the affinity for the exosite I interaction indicates that similar thrombin binding to factor V activation intermediates may regulate other steps in the activation pathway. The finding that thrombin binding to factor Va has similar characteristics to those described previously for prothrombin binding to bovine factor Va (15, 16, 27) suggests that these interactions may be related, reflecting a common mode of binding of the substrate and product of prothrombin activation.

EXPERIMENTAL PROCEDURES

Protein Purification and Characterization—Human α-thrombin was purified as described previously (28) or obtained from Dr. John Fenton (New York State Department of Health, Albany, NY). Preparations of thrombin were >95% active by active site titration with p-nitrophenyl p-guanidinobenzoate under the conditions used previously (28) and with the protein concentrations determined from the 280 nm absorbance with a coefficient of 1.74 (mg/ml)·cm⁻¹ and molecular weight of 36,600 (29). Active site-labeled thrombin (2-anilinonaphthalene-6-sulfonic acid-FPR-thrombin ([ANS]FPR-thrombin)) was prepared by inactivation of 46 μM α-thrombin with a 2.5-fold excess of N^6-(acetylimidazole)acetyl]-FPR-CH_{2}Cl and labeling of the NH\_2OH-generated thiol group on the incorporated inhibitor with a 5–6-fold excess of 2-((4-iodacetamido)anilino)naphthalene-6-sulfonic acid at 38 μM enzyme following procedures described previously (28, 30, 31). Preparations of [ANS]FPR-thrombin contained 1.05–1.12 mM of ANS/mol of thrombin.

Factor V was purified from normal human plasma and activated by incubation at 2–6 mg/ml with 50 μM thrombin in 50 mM Hepes, 0.11 mM NaCl, 5 mM CaCl\_2, 1 mg/ml polyethylene glycol 8000, pH 7.4, and at 25 °C. FPR-CH\_2\_Cl (1 μM) was added to the buffer for all titrations except those containing native thrombin. Fluorescence measurements were made with an SLM 8100 fluorometer in the ratio mode. Acrylic cuvettes were coated with polyethylene glycol 20,000 to minimize protein adsorption (34) for measurements of ANS fluorescence, and uncoated cuvettes were used for tryptophan fluorescence. Binding of Hir\_24–65 to thrombin was measured from the changes in tryptophan fluorescence with 295 nm excitation (4 or 8 nm band pass) and 360 nm emission (8 nm band pass). Titrations were done by measuring the fluorescence after successive additions of small volumes of titrant. The observed fluorescence was corrected for dilution (<13%) and for background by subtraction of measurements on solutions lacking the fluorescent species. Fluorescence data were expressed as the fractional change in the initial fluorescence (∆F/ F_0 = (F_{fin} - F_{init})/ F_{init}) Titrations of thrombin with Hir\_24–65 were fit by the quadratic equilibrium binding equation to obtain the maximum fluorescence change (∆F_{max}/ F_0) and dissociation constant (K_{D}), assuming one binding site for Hir\_24–65 on thrombin.

Binding of factor V/Va species to [ANS]FPR-thrombin was measured from changes in ANS fluorescence emission at 450 nm with excitation at 325 nm and using 8 nm band pass. Fixed concentrations of [ANS] FPR-thrombin were titrated with factor V/Va species in the absence of Hir\_24–65 and in the presence of a fixed, near-saturating Hir\_24–65 concentration. Mixtures of [ANS]FPR-thrombin and a fixed level of factor V/Va species were also titrated with Hir\_24–65 under the same conditions. The relatively high protein concentrations required for the titrations necessitated correction for dilution of ∼35%. Dilution of [ANS] FPR-thrombin was included in analysis of the results. However, such corrections had little effect because the concentration of labeled thrombin was below the dissociation constants for the interactions. Corrections of the ANS fluorescence data for background were typically <13%, with the exception of the lowest fluorescence enhancement in the fragment C1 titrations (<20%) and in the native thrombin competition experiments at the highest protein concentrations (∼27%).

Binding of factor V/Va species to [ANS]FPR-thrombin and the effect of Hir\_24–65 were analyzed as a special case of the general situation in which two ligands bind competitively to a fluorescent protein probe and the interactions are accompanied by unequal fluorescence changes. In this model, ligands L and C bind mutually exclusively to n equivalent and independent sites on the probe (P) with dissociation constants K_L and K_C and maximum fluorescence changes relative to the fluorescent of the probe of ∆F_{max,L}/ F_0 and ∆F_{max,C}/ F_0 (Scheme I).

\[
P + nL \quad \overset{K_L}{\underset{[\Delta F_{max,L}/F_0]}{\rightleftharpoons}} \quad nPL\quad \overset{K_C}{\underset{[\Delta F_{max,C}/F_0]}{\rightleftharpoons}} \quad nPC\quad (\text{Scheme I}).
\]

The observed fluorescence change is given by the sum of the contributions from the two probe complexes, weighted by the maximum fluorescence changes associated with their formation (35).

\[
\Delta F = \frac{[PL]}{[P]} \Delta F_{max,L} F_0 + \frac{[PC]}{[P]} \Delta F_{max,C} F_0
\]

(Eq. 1)
The fraction of sites on P occupied by L or C can be expressed in terms of the free ligand concentrations as follows.

$$\left( \frac{[PL]}{n[P]} \right) = \frac{[L]}{K_p + [C]/K_c} + [L]$$  \hspace{1cm} (Eq. 2)

$$\left( \frac{[PC]}{n[P]} \right) = \frac{[C]}{K_p + [L]/K_c} + [C]$$  \hspace{1cm} (Eq. 3)

Equations 1–3 would be adequate for analysis of the fluorescence changes as a function of [L] and [C] if the approximation could be made that the free concentrations of the ligands were equivalent to their total concentrations. However, this assumption is not always justified, and typically only the total concentrations are known in spectroscopic studies. The free concentrations of L and C can be expressed in terms of the total concentrations and the concentration of PL as shown in Equations 4 and 5.

$$[L] = [L]_o - [PL]$$  \hspace{1cm} (Eq. 4)

$$[C] = [C]_o - [PL] + \left( 1 + \frac{K_L}{[L]_o - [PL]} \right)$$  \hspace{1cm} (Eq. 5)

Substitution of these expressions into Equation 2 and rearranging gives the cubic Equations 6–10 for [PL]/n[P], in terms of the total ligand and probe concentrations, the dissociation constants, and the stoichiometric factor.

$$D_1([PL]/n[P])^3 + D_2([PL]/n[P])^2 + D_3([PL]/n[P]) + D_4 = 0$$  \hspace{1cm} (Eq. 6)

$$D_1 = n[P]\left( \frac{K_c - K_L}{K_p} \right)$$  \hspace{1cm} (Eq. 7)

$$D_2 = \left( \frac{K_L - 2K_c}{K_p} \right) + n[P]\left( \frac{K_c - K_L}{K_p} \right) + K_L - K_c - [C]$$  \hspace{1cm} (Eq. 8)

$$D_3 = \left( \frac{[L]_o}{n[P]} \right) \left( \frac{K_L}{K_c} \right)[L]_o + K_c + [C]_o + [L]_o \left( \frac{2K_c - K_L}{K_p} \right)$$  \hspace{1cm} (Eq. 9)

$$D_4 = \left( \frac{[L]_o^2}{n[P]} \frac{K_c}{K_p} \right)$$  \hspace{1cm} (Eq. 10)

Similarly, substitution of the corresponding equations for the free ligand concentrations in terms of [PC]/n[P], (not shown). The observed fluorescence change as a function of the total concentrations of the ligands and the probe is given by Equation 1, with [PL]/n[P] and [PC]/n[P] obtained from the solutions of the two cubic equations. Alternatively, solution of Equations 6–10 for [PL]/n[P], allows [PC]/n[P] to be more easily calculated from Equation 11, which is obtained from the mass conservation equation for P and the definition of the equilibrium constant.

$$\left( \frac{[PC]}{n[P]} \right) = 1 - \left( \frac{[PL]}{n[P]} \right) - \frac{K_p}{[L]_o - n[P]\left( \frac{[PL]}{n[P]} \right)}$$  \hspace{1cm} (Eq. 11)

For the present case, P was [ANS]FPR-thrombin, L was factor V, Va or Vа subunit, and C was Hir54–65. The fluorescence change accompanying Hir54–65 binding to [ANS]FPR-thrombin did not contribute directly to the observed fluorescence change because the maximum fluorescence increase was ≈3% of the change accompanying factor V/Va binding. This allowed the second term of Equation 1 to be set to zero. In the factor V/Va experiments, there was also an exosite I-independent increase in fluorescence that was a linear function of factor V/Va concentration. This was included in the binding model as a term linear in factor V/Va concentration, representing weak interactions of factor V/Va species with labeled thrombin. Simplification of Equation 1 for the present studies and incorporating the exosite I-independent fluorescence change gave Equation 12, where ANS-T represents [ANS]FPR-thrombin and V represents the factor V/Va species.

$$\Delta F = \left( \frac{\Delta F_{\text{max}}}{F_o} \right) \left( \frac{[T]}{n[\text{ANS-T}]} \right) + \Delta F_{\text{exo-ind}} \left( \frac{[V]}{F_o} \right).$$  \hspace{1cm} (Eq. 12)

Fluorescence changes were measured in titrations of [ANS]FPR-thrombin as a function of the total concentration of factor V/Va species in the absence of Hir54–65 and at near-saturating concentrations of Hir54–65, and as a function of Hir54–65 concentration at fixed concentrations of factor V/Va species. The results were fit simultaneously by Equation 12 with [ANS-T/V]/[ANS-T], calculated by solution of the cubic Equations 6–10 using the Newton-Raphson algorithm incorporated into the BAEIC programs Dnrp53 or Dnrpeasy (36, 37). The fitted parameters were the dissociation constant for factor V/Va binding to [ANS]FPR-thrombin (K(V/Va)), the maximum fluorescence change for this interaction, the dissociation constant for Hir54–65 binding, and the slope of the exosite I-independent fluorescence increase (ΔF(exo-ind)/Fo, where “exo-ind” indicates “exosite I-independent”), with a value of 1 assumed for the stoichiometric factor.

The effect of native thrombin on binding of factor Va to [ANS]FPR-thrombin was measured in titrations of [ANS]FPR-thrombin with factor Va in the absence and presence of fixed concentrations of native thrombin and in the absence and presence of near-saturating concentrations of Hir54–65. The results were analyzed by nonlinear least squares fitting of all of the titration data with Equation 12. For this case, which differs from the situation described above, the cubic equation described previously (38, 39) for competitive binding of a ligand (factor Va) to a labeled protein probe ([ANS]FPR-thrombin) and an alternate unlabeled acceptor (native thrombin) was used to calculate [ANS-Trap]/[ANS-T], in Equation 12. The dissociation constants for factor Va binding to [ANS]FPR-thrombin and to native thrombin, the maximum fluorescence change, and the slope of the exosite I-independent fluorescence change were the fitted parameters.

Least squares fitting and simulation were performed with Dnrp53, Dnrpeasy (36, 37), or Scientist software (MicroMath). All reported estimates of error represent ± 2 S.E.

RESULTS

Characterization of 2-Anilinonaphthalene-6-sulfonic Acid-labeled Thrombin as a Probe of Exosite I-dependent Interactions with Factor V/Va Species—Binding of thrombin to factor V and its activation products was characterized quantitatively from fluorescence changes of thrombin that was specifically labeled with ANS covalently linked to the catalytic site histidine residue through N6-[(acetyltihio)acetyl]-n-Phe-Pro-Arg-CH2Cl (28, 30, 31). To determine the role of exosite I in the factor V/Va interactions, specific binding of the nonsulfated hirudin peptide, Hir54–65, to exosite I of [ANS]FPR-thrombin was first characterized. Analysis of intrinsic tryptophan fluorescence changes accompanying Hir54–65 binding to [ANS]FPR-thrombin and native thrombin gave indistinguishable dissociation constants of 0.94 ± 0.14 and 1.1 ± 0.2 μM and maximum fluorescence enhancements of 12.7 ± 0.4 and 13.4 ± 0.6%, respectively (Fig. 1). Under the same conditions, binding of the peptide to [ANS]FPR-thrombin increased the ANS fluorescence by only 2.5 ± 0.2% (Fig. 1). These results indicated that Hir54–65 bound to exosite I of ANS-labeled thrombin and native thrombin with the same affinity but that the ANS probe did not report this interaction with a significant change in fluorescence. The large differential response of [ANS]FPR-thrombin to binding of factor V and its activation products compared with binding of Hir54–65 was used to establish the role of exosite I in the factor V/Va interactions.

Binding of Factor V to [ANS]FPR-thrombin—Addition of single-chain factor V to [ANS]FPR-thrombin resulted in a large, saturable enhancement in the probe fluorescence, signaling factor V binding (Fig. 2). Titration of a mixture of labeled thrombin and factor V with Hir54–65 resulted in a partial return of the enhanced fluorescence toward the initial value, consistent with displacement of thrombin from factor V by the peptide. Titration of [ANS]FPR-thrombin with factor V/Va in the presence of a concentration of Hir54–65 sufficient to essentially saturate exosite I (100 X K(Hir)) produced a linear increase in ANS fluorescence, which represented 21% of the enhancement seen with factor V alone at the highest concentration measured (Fig. 2). These results indicated that the fluorescence enhancement associated with factor V binding was due primarily to an
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Fig. 1. Titrations of [ANS]FPR-thrombin and native thrombin with Hir54–65. The fractional increases in fluorescence (ΔF/F₀) of 0.41 µM [ANS]FPR-thrombin as a function of the total concentration of Hir54–65 ([Hir54–65]₀) monitored by tryptophan fluorescence (○) and ANS fluorescence (▲) are shown in comparison with results for 0.45 µM native thrombin monitored by tryptophan fluorescence (●). The lines for the tryptophan fluorescence titrations represent the least squares fits with the parameters given in the text. The line for the ANS fluorescence titration represents the fit of the maximum fluorescence change with the dissociation constant fixed at the value determined from the tryptophan fluorescence data. Titrations were performed and analyzed as described under “Experimental Procedures.”

Fig. 2. Titrations of [ANS]FPR-thrombin with factor V. The fractional change in fluorescence of 0.40 µM [ANS]FPR-thrombin (ΔF/F₀) is shown as a function of the total factor V concentration ([V]₀) in the absence (○) and presence (▲) of 92 µM Hir54–65. The change in fluorescence of a mixture of 0.33 µM [ANS]FPR-thrombin and 3.1 µM factor V (■) is plotted as a function of the total concentration of Hir54–65 ([Hir54–65]₀). The lines represent the least squares fit of all of the data with the competitive binding model described in the text and the parameters listed in Table I. Titrations were performed and analyzed as described under “Experimental Procedures.”

exosite I-dependent interaction, with a smaller contribution from a lower affinity, exosite I-independent process. The possibility that the exosite I-independent process was due to free fluorescence probe in the labeled thrombin preparations was ruled out by the observations that (a) free probe was not detected in the dialysis buffer from [ANS]FPR-thrombin, and (b) more exhaustive gel filtration or dialysis in the preparation of [ANS]FPR-thrombin did not affect the fluorescence change. The effect of unrelated proteins on the fluorescence of [ANS] FPR-thrombin was examined to evaluate the possibility that the linear fluorescence change was due to nonspecific interactions of factor V with the labeled protein. Ovalbumin and bovine serum albumin also increased the fluorescence of [ANS] FPR-thrombin linearly up to 30–50 µM, with slopes of 0.5 and 0.2% µM⁻¹, respectively (results not shown). These results suggested that nonspecific interactions could be responsible, although the magnitude of the fluorescence change of 7.1% µM⁻¹ seen with factor V was significantly larger. Results of further studies with factor Va described below showed that native thrombin had no detectable effect on the exosite I-independent fluorescence change, indicating that it was due either to nonspecific interactions only exhibited by the probe-labeled protein or to an interaction of factor V/Va at a second site on thrombin with an affinity too low to be determined.

On the basis of these observations, the binding data were analyzed by computer fitting of a model in which factor V and Hir54–65 bound competitively to [ANS]FPR-thrombin, with a large fluorescence change associated with factor V binding and no change for Hir54–65 binding. The model also incorporated weak binding of factor V to thrombin in exosite I-independent interactions characterized by a linear increase in fluorescence. An equation was derived for analysis of the general situation in which two ligands bind competitively to a probe with unequal fluorescence changes accompanying the interactions (see under “Experimental Procedures”). This allowed all of the fluorescence titration results obtained as a function of the total concentrations of both ligands to be fit by nonlinear least squares analysis without the need for the assumption of equivalence of the free and total concentrations. As shown in Fig. 2, the model fit the data well with dissociation constants for factor V binding to [ANS]FPR-thrombin of 3.4 ± 1.3 µM and for competitive binding of Hir54–65 of 0.72 ± 0.19 µM and with a maximum fluorescence enhancement of 182 ± 41% for the factor V interaction (Table I). The good agreement between the dissociation constant for Hir54–65 binding to [ANS]FPR-thrombin obtained from its displacement of factor V and the value of 0.94 ± 0.14 µM measured directly for binding of the peptide to exosite I supported the validity of the binding model.

In this analysis and that described below for other factor V/Va species, the exosite I-independent interactions were included in the model as a linear binding process. As expected, a more detailed model in which these additional interactions were included as individual factor V/Va binding steps did not fit the data with unique parameters for these interactions because of the linearity of the fluorescence increase and the large number of unknowns. However, if equivalent affinity and fluorescence enhancements were assumed for the exosite I-independent interactions, acceptable fits of the results could be obtained when the exosite I-independent interactions had an affinity >5-fold lower than exosite I binding, as judged by the agreement between the fitted values of Kᵥᵥ and the upper error limit of the experimentally determined dissociation constant. These findings confirmed that analysis of the data with more complex models was not justified and supported further the validity of representation of the exosite I-independent interactions in the model as a single, low affinity process.

Binding of Factor Va to [ANS]FPR-thrombin—Titrations of [ANS]FPR-thrombin with thrombin-activated factor V, consisting of a mixture of the two-subunit form of factor Va (Va-hl), fragments E and C1, also produced a fluorescence enhancement (Fig. 3). The enhancement was partially reversed by Hir54–65, and a residual exosite I-independent fluorescence increase was again observed in the presence of a near-saturat-
Parameters were obtained by least squares fitting of the model described in the text. The dissociation constants for factor V/Va binding to [ANS]FPR-thrombin (K_{V/Va}) and for competitive binding of Hir54–65 (K_{Hir}) and the maximum fluorescence change (ΔF_{max}/F_0) are listed for the exosite I-dependent binding mode. The slopes of the linear fluorescence increases with increasing protein concentration (ΔF_{max}/F_0) are listed for the exosite I-independent processes. Experiments were performed and analyzed as described under “Experimental Procedures.”

### Table I

| Exosite I-dependent | Exosite I-independent |
|---------------------|-----------------------|
| K_{V/Va} (μM)       | 3.4 ± 1.3             |
| ΔF_{max}/F_0 (%)    | 152 ± 41              |
| K_{Hir} (μM)        | 0.72 ± 0.19           |
| ΔF_{max}/F_0 (%)    | 7.1 ± 0.9             |
| Hir54–65 (% μM)     | 5.7 ± 1.2             |
| Va-l (μM)           | 1.7 ± 0.2             |
| ΔF_{max}/F_0 (%)    | 110 ± 1.7             |
| Va-h (μM)           | 0.8 ± 0.1             |
| ΔF_{max}/F_0 (%)    | 10.2 ± 0.8            |
| Fragment E (μM)     | 3.1 ± 0.1             |
| ΔF_{max}/F_0 (%)    | 7.5 ± 0.7             |

**Fig. 3. Titrations of [ANS]FPR-thrombin with factor Va.** The fractional change in fluorescence of 0.40 μM [ANS]FPR-thrombin (ΔF/F_0) is shown as a function of the total concentration of factor Va (V_0) in the absence (●) and presence (▲) of Hir54–65. The change in fluorescence of a mixture of 0.33 μM [ANS]FPR-thrombin and 3.2 μM factor Va (C) is shown as a function of the total concentration of Hir54–65 (HiV54–65). The lines represent the least squares fit with the parameters listed in Table I. Titrations were performed and analyzed as described under “Experimental Procedures.”

**Fig. 4. Preparations of factor V and factor V activation products.** SDS gels of reduced samples of preparations of the proteins used in these studies are shown: factor V (–7 μg), factor Va (–9 μg), factor Va-hl (–4 μg), Va-h (–11 μg), Va-l (–7 μg), fragment E (–14 μg), and fragment C1 (–40 μg). The migration positions of molecular weight markers are indicated with the molecular weights in thousands. Gels of fragment C1 were stained with periodic acid-Schiff reagent. Proteins were purified and electrophoresis was performed as described under “Experimental Procedures.”

**Fig. 5. Exosite I-dependent interaction with factor V/Va.** Analysis of the binding of factor Va to [ANS]FPR-thrombin was performed by measuring the fractional change in fluorescence of a mixture of 0.33 μM [ANS]FPR-thrombin and 3.2 μM factor Va (C) in the presence of Hir54–65. The lines represent the least squares fit with the parameters listed in Table I. The dissociation constant for factor V/Va binding to [ANS]FPR-thrombin (K_{V/Va}) and for competitive binding of Hir54–65 (K_{Hir}) and the maximum fluorescence change (ΔF_{max}/F_0) are listed for the exosite I-dependent binding mode. The slopes of the linear fluorescence increases with increasing protein concentration (ΔF_{max}/F_0) are listed for the exosite I-independent processes. Experiments were performed and analyzed as described under “Experimental Procedures.”

**Fig. 6. Binding of Thrombin to Factor V/Va.** The fractional change in fluorescence of a mixture of 0.33 μM [ANS]FPR-thrombin and 3.2 μM factor Va (C) is shown as a function of the total concentration of factor Va (V_0). Experiments were performed and analyzed as described under “Experimental Procedures.”

**Fig. 7. Binding of Exosite I-independent processes.** Experiments were performed and analyzed as described under “Experimental Procedures.”

An exosite I-dependent interaction similar to that seen with unfractionated factor Va, with a dissociation constant of 0.6 ± 0.2 μM and maximum fluorescence enhancement of 113 ± 15% (Fig. 5). These parameters were indistinguishable from those obtained for unfractionated factor Va (Table I), indicating that the exosite I-dependent interaction was accounted for by binding of the factor Va heterodimer to [ANS]FPR-thrombin.

**Interactions of the Isolated Factor Va Subunits and Activation Fragments with [ANS]FPR-thrombin—Comparison of titrations of [ANS]FPR-thrombin with the separated factor Va subunits showed a large fluorescence enhancement for the heavy chain and no significant change (≤3% up to 4 μM) for the light chain (Fig. 6).** Fragments E and C1 produced small increases in fluorescence that were linear with concentration, reaching levels of 12% (fragment E) and 30% (fragment C1) at 4 μM (Fig. 6). Addition of 34–50 μM Hir54–65 to mixtures of [ANS]FPR-thrombin and Va-l, fragment E, or fragment C1 decreased the fluorescence by ≤8%, indicating that these fluorescence changes were exosite I-independent (Fig. 6). Extinction of the titrations with fragment C1 to higher concentrations showed some evidence of curvature, suggestive of a weak interaction with a ~35 μM dissociation constant (results not shown). Analysis of the binding of factor Va-h to [ANS]FPR-thrombin as a function of heavy chain and Hir54–65 concentration (Fig. 7) gave a dissociation constant of 2.1 ± 0.9 μM for exosite I-dependent binding to [ANS]FPR-thrombin and a maximum fluorescence increase of 103 ± 20%. These results indicated that binding of the heavy chain subunit to [ANS]FPR-
Changes in fluorescence units and factor V activation fragments.

The fractional increase in fluorescence ($\Delta F/F_0$) is shown for titrations of 0.38 μM [ANS]FPR-thrombin with factor Va-h (Va-hl) in the absence (○) and presence (▲) of 87 μM Hir54–65. Results are also shown for titration of a mixture of 0.28 μM [ANS]FPR-thrombin and 1.8 μM factor Va-hl (C) as a function of Hir54–65 concentration (Hir54–65). The lines represent the least squares fit of all of the data with the parameters listed in Table I. Titrations were performed and analyzed as described under “Experimental Procedures.”

FIG. 5. Titrations of [ANS]FPR-thrombin with the two-subunit form of factor Va. The fractional increase in fluorescence ($\Delta F/F_0$) is shown for titrations of 0.38 μM [ANS]FPR-thrombin with factor Va-h (Va-hl) in the absence (○) and presence (▲) of Hir54–65. Results are also shown for titration of a mixture of 0.28 μM [ANS]FPR-thrombin and 1.8 μM factor Va-hl (C) as a function of Hir54–65 concentration (Hir54–65). The lines represent the least squares fit of all of the data with the parameters listed in Table I. Titrations were performed and analyzed as described under “Experimental Procedures.”

Thrombin accounted substantially for the exosite I-dependent interaction seen with the factor Va heterodimer (Table I).

Effect of Native Thrombin on Factor Va Binding to [ANS]FPR-thrombin—The effect of native thrombin on the binding of factor Va to [ANS]FPR-thrombin was examined to assess the effect of the presence of the label in the thrombin active site on the interaction. Binding of factor Va was resolved into exosite I-dependent and -independent components by titrations in the presence and absence of a near-saturating concentration of Hir54–65, as in the previous experiments. The results of these titrations in the absence and presence of 4.6 μM and 9.1 μM native thrombin are shown in Fig. 8, in which the fitted linear, exosite I-independent fluorescence change has been subtracted from all of the results to illustrate the effects more clearly. Native thrombin decreased the affinity of exosite I-dependent binding of factor Va, whereas it had no detectable effect on the exosite I-independent fluorescence change (Fig. 8). In the absence of factor Va, native thrombin increased the fluorescence of [ANS]FPR-thrombin by ≤ 3.5% at concentrations up to 10 μM, indicating no significant nonspecific interaction. The effect of native thrombin on the exosite I-dependent interaction was well described by competitive binding of native thrombin and [ANS]FPR-thrombin to factor Va, with a ~2.5-fold higher dissociation constant of 2.9 ± 0.7 μM for native thrombin (Fig. 8 and Table I). Simulation of the effect of competitive binding would have on the slope of the exosite I-independent fluorescence change indicated that a ≤15 μM dissociation constant would have been detected. Thus, these results indicated that the linear fluorescence increase either did not represent an interaction of native thrombin at all or represented a low affinity interaction with a dissociation constant of >15 μM.

DISCUSSION

The results of quantitative characterization of the binding of active site-labeled thrombin to human factor V/Va species support the conclusion that binding of thrombin to a site on the factor V/Va heavy chain through exosite I is the predominant mode of nonenzymatic interaction. The unique properties of thrombin labeled at the catalytic site with ANS linked by N"-(acetylthio)acetyl-FPR-CH2Cl in signaling binding of factor V/Va but not Hir54–65 allowed the contribution of exosite I to the fluorescence changes to be resolved from exosite I-independent changes. The difference in the fluorescence changes for factor V/Va and Hir54–65 reflects the dependence of the pertur-
The results for the exosite I-independent component are plotted along with FPR-thrombin. Results of titrations of 0.41 μM thrombin and native thrombin to factor Va with dissociation constants of squares fit of the exosite I-dependent competitive binding of [ANS]FPR-thrombin fluorescence observed in titrations with 12%.

The possibility that these changes represent nonspecific interactions with the covalently attached probe was demonstrated by the similar effect of high concentrations of unrelated proteins. The magnitudes of the fluorescence changes for factor V/Va species were larger, however, and the possibility that they represent significant interactions through other sites on thrombin cannot be excluded. The absence of an observable effect of native thrombin on the exosite I-independent fluorescence changes indicates that if such interactions occur with the native enzyme, they have an affinity at least 5-fold lower than exosite I-dependent binding.

Comparison of the interactions of each of the isolated factor V activation products with [ANS]FPR-thrombin demonstrated one exosite I-dependent interaction with the factor Va heavy chain, and exosite I-independent interactions of lower affinity for fragments E and C1. The light chain showed no detectable affinity for [ANS]FPR-thrombin, in agreement with previous affinity chromatography results (21). Binding of the isolated factor Va heterodimer to [ANS]FPR-thrombin accounted for the results obtained with the complete mixture of factor V activation products, and the heavy chain subunit bound with an indistinguishable fluorescence enhancement and only 2–4-fold lower affinity than factor Va. It is concluded from these results that the binding site for thrombin on factor Va is contained in the heavy chain alone, with little or no significant interaction with the light chain or effect of association of the subunits in the factor Va dimer.

The factor Va heavy chain contains two acidic sequences within residues 659–698 that are homologous to hirudin54–65 and include one or more sulfated tyrosine residues (7, 46, 47). Tyrosine sulfate occurs frequently in exosite I-binding sequences, and sulfation of Tyr63 in hirudin54–65 results in a 5–10-fold increase in affinity of the peptide for thrombin (45, 46). One of the hirudin54–65-like sequences in the factor Va heavy chain likely represents the site that interacts directly with exosite I. However, the reported linkage between binding of certain ligands to exosites I and II (43) raises the alternative possibility that factor V/Va binds to a different site on thrombin and that binding of Hir54–65 to exosite I results in a conformational change that greatly reduces the affinity of factor V/Va binding at the linked site. As is generally the case, if the changes in affinity are large, this possibility cannot be easily distinguished from simple competitive binding. A second sequence homologous to hirudin54–65 precedes the Arg1545 cleavage site in fragment C1 and has also been proposed as a possible thrombin binding site, whereas no similar sequence neighbors the Arg1018 site in fragment E (7, 46–48). The fluorescence results for fragment E agreed with the observation of no detectable affinity of this fragment for thrombin by affinity chromatography (21). Although no evidence for an exosite I interaction with fragment C1 was obtained, this fragment produced the largest exosite I-independent increase in fluorescence, which could represent a lower affinity interaction with a different site on thrombin.

Binding of thrombin to the heavy chain region of factor V through exosite I is concluded to play an important role in factor V activation by mediating factor V binding as a specific thrombin substrate. This interaction is thought to enhance the specificity of cleavage at Arg979 that initiates the factor V activation mechanism by approximating thrombin to the cleavage site and possibly by inducing changes in the thrombin catalytic site that affect substrate specificity. The characteristics of factor V and factor Va binding to [ANS]FPR-thrombin were slightly different, with factor V producing a 1.4-fold larger fluorescence enhancement and binding with 3-fold lower affinity. These relatively small differences reflect the effects of the activation bond cleavages and dissociation of the activation fragments on the environment of the thrombin active site and its binding site in the complexes with the intact protein substrate and the final reaction product. The Km of 0.07 μM reported for factor V activation (9) can be taken as an approximation of the overall dissociation constant for factor V binding as a substrate. The 49-fold and 16-fold higher dissociation
constants for factor V and factor Va binding, respectively, represent estimates of only the affinity of the exosite I-mediated interactions because the catalytic site and S1-S3 substrate binding subsites are occupied in the [ANS]FPR-thrombin derivative. With the assumption that the overall affinity includes contributions from both exosite I and catalytic site interactions, exosite I-mediated binding would account for 76% of the free energy of factor V binding as a substrate.

The small changes in affinity for the exosite I interaction accompanying conversion of factor V to factor Va suggest that thrombin binds similarly to factor V activation intermediates and that these interactions may regulate other steps in the activation pathway. The activation cleavage reactions are not strictly sequential but follow the preferred order of Arg709 first to generate the heavy chain, followed by Arg1018 and Arg1545 last to produce the light chain (4, 8, 9, 18, 19). Cleavage at Arg709 is important for rapid initiation of factor V activation, whereas development of full factor Va activity requires cleavage at Arg1545 (8, 18, 19, 48). Studies of recombinant factor Va mutants indicate that the cleavages at Arg709 and Arg1018 generate partially active intermediates and that these steps enhance the rate of cleavage at Arg1545 (8, 18, 19, 48). The effect of inhibiting exosite I interactions with Hir54–65 or hirugan indicated that the Arg709 and Arg1545 reactions were exosite-I-dependent, whereas the results for Arg1018 were less clear (20, 21). Cleavage at Arg1545 has also been shown to depend on the presence of the region of fragment C1 containing the sequence that is a putative binding site for thrombin (48). The absence of evidence for an exosite I-dependent interaction of [ANS]FPR-thrombin with isolated fragment C1 suggests that an interaction of thrombin with this site may be expressed differently in factor V activation intermediates and that the affinity may be lost in the released fragment. An alternative possibility is that thrombin binding to the heavy chain could regulate cleavage at Arg1545 in addition to Arg709. A precedent for this possibility is the mechanism of conversion of fibrinogen to fibrin, in which thrombin binding through exosite I facilitates fibrinogen binding and cleavage of two bonds to release fibrinopeptides A and B (50–52). The exosite I interaction is maintained with essentially the same affinity for the substrate, fibrinogen; the intermediate, fibrin I; and the final product, fibrin II (50–52).

The properties of thrombin binding to factor Va suggest that the binding site on factor Va may be related to the site of prothrombin binding, which functions in the cofactor-assisted interaction of prothrombin as a substrate of the factor Xa-factor Va complex. In parallel with the results for thrombin, bovine prothrombin has been shown to bind to a site on the factor Va heavy chain in a calcium-independent interaction, and it binds with essentially the same affinity to the isolated subunit and the factor Va dimer (15, 16, 27). Kinetic studies of prothrombin 2 activation by the bovine factor Xa-factor Va-membrane complex have shown that substrate recognition is mediated by exosites present on the enzyme complex (25, 26). Active site-blocked thrombin inhibits prothrombin 2 activation through competitive exosite binding, and the inhibitory interaction is greatly diminished in affinity by hirugan (26). The present results with the human proteins suggest that the site of thrombin binding through exosite I on factor Va may overlap one of the exosites in the enzyme complex that contributes to productive substrate interactions. This explanation is apparently in conflict with the results of additional studies with proteolytic derivatives of thrombin, which indicated that exosites I and II were not required for inhibition of the factor Xa-factor Va-membrane complex (26). It has been proposed that thrombin binding as an inhibitor occurs through a site that is distinct from exosite I but is conformationally linked to hirugan binding, such that the affinity of the inhibitory interaction is greatly reduced (26). Another possibility is that the substrate and product interactions with exosites expressed in the factor Xa-factor Va-membrane complex include thrombin binding to factor Va in addition to other modes of binding.

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