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Binding of Exosite Ligands to Human Thrombin

RE-EVALUATION OF ALLOSTERIC LINKAGE BETWEEN THROMBIN EXOSITES I AND II*

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The substrate specificity of thrombin is regulated by binding of macromolecular substrates and effectors to exosites I and II. Exosites I and II have been reported to be extremely linked allosterically, such that binding of a ligand to one exosite results in near-total loss of affinity for ligands at the alternative exosite, whereas other studies support the independence of the interactions. An array of fluorescent thrombin derivatives and fluorescein-labeled hirudin$^{54-65}$ ([5F]Hir$^{54-65}$(SO_3^-)) were used as probes in quantitative equilibrium binding studies to resolve whether the affinities of the exosite I-specific ligands, Hir$^{54-65}$(SO_3^-) and fibrinogen, and of the exosite II-specific ligands, prothrombin fragment 2 and a monoclonal antibody, were affected by alternate exosite occupation. Hir$^{54-65}$(SO_3^-) and fibrinogen bound to exosite I with dissociation constants of 16–28 nM and 5–7 μM, respectively, which were changed ≤2-fold by fragment 2 binding. Native thrombin and four thrombin derivatives labeled with different probes bound fragment 2 and the antibody with dissociation constants of 3–12 μM and 1.8 nM, respectively, unaffected by Hir$^{54-65}$(SO_3^-). The results support a ternary complex binding model in which exosites I and II can be occupied simultaneously. The thrombin catalytic site senses individual and simultaneous binding of exosite I and II ligands differently, resulting in unique active site environments for each thrombin complex. The results indicate significant, ligand-specific allosteric coupling between thrombin exosites I and II and catalytic site perturbations but insignificant inter-exosite thermodynamic linkage.

The specificity of thrombin toward its procoagulant and anticoagulant physiological substrates is allosterically regulated by interactions of macromolecular substrates, inhibitors, and effectors with either of two electropositive sites, exosites I and II, in near-opposition on the enzyme surface (1, 2). Exosite I binds fibrinogen (Fbg)[1] (3), fibrin I and II (3, 4), the 12-residue carboxyl-terminal hirudin$^{54-65}$ sequence (5, 6), thrombomodulin (7), the thrombin receptor (8, 9), and an acidic sequence on the serpin, heparin cofactor II (10, 11). Exosite II binds heparin and other glycosaminoglycans (2, 12, 13), prothrombin activation fragment 2 (F2) (14), the chondroitin sulfate moiety of thrombomodulin (15, 16), the leech peptide hemadin (17), and an exosite II-specific human monoclonal antibody (18). Factors V (19–22), Va (21, 22), and VIII (19), platelet glycoprotein Ibα (23–25), and the snake venom protein bothrojaracin (26) have been reported to interact with both exosites I and II.

Binding of exosite ligands to thrombin is correlated with significant changes in the kinetics of hydrolysis of peptide ester and peptide *p*-nitroanilide substrates (3, 7, 9, 18, 27–31) in addition to profound effects on specificity and reactivity toward its natural macromolecular substrates and inhibitors (10, 11, 15, 32–36). These studies indicate that exosite binding of allosteric effectors is coupled to conformational changes affecting the S1–S3 substrate specificity subsites in the thrombin catalytic site (37–39). Binding studies of F2, thrombomodulin, fibrin, and heparin with various active site-labeled thrombin derivatives in which the S1–S4 subsites were occupied (16, 34, 40), and studies of the effect of exosite ligand binding on the hydrolysis of tripeptide *p*-nitroanilide substrates suggest that structurally different ligands produce ligand-specific changes in the catalytic site. Extreme allosteric linkage between exosites I and II (30, 31, 41) has been reported to prevent simultaneous occupation of exosites I and II (30, 41), whereas other studies provide contrasting evidence for binary and ternary complex formation with similar affinities among thrombin and exosite I and II ligands (18). In favor of inter-exosite linkage, the dissociation constant for fluorescently labeled, Tyr^{63}-sulfated hirudin$^{53-64}$ and bovine thrombin was weakened 10-fold by F2 binding, although ternary complex formation was demonstrated (31). Extremely negative inter-exosite interactions were reported for Tyr^{63}-sulfated hirudin$^{54-65}$ (Hir$^{54-65}$(SO_3^-)) and human F2 or a synthetic peptide, F2$^{63-116}$ (30, 41). The latter studies were concluded to reflect mutually exclusive binding of F2 and Hir$^{54-65}$(SO_3^-) by reciprocal, allosteric modulation of ligand affinity between the two exosites (41). By contrast, binding of an exosite II-specific monoclonal antibody (mAb) did not affect detectably the conformation of thrombin exosite I or its affinity for [5F]Hir$^{54-65}$(SO_3^-) (18).

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[1] The abbreviations used are: Fbg, fibrinogen; ATA-FPR-CH_2Cl, N^{α}-[(acetylthio)acetyl]-D-Phe-Pro-Arg-CH_2Cl; ATA-FFR-CH_2Cl, N^{α}-[(acetylthio)acetyl]-D-Phe-Phe-Arg-CH_2Cl; T, human α-thrombin, T*, human α-thrombin fluorescently labeled at the active site; [5F]FPR-T, [5-(acetamido)fluorescein]-D-Phe-Pro-Arg-thrombin; [6F]FPR-T, [6-(acetamido)fluorescein]-D-Phe-Pro-Arg-thrombin; [4′F]FPR-T, [4′-{[(acetyl)amino]methyl}fluorescein]-D-Phe-Pro-Arg-thrombin; [ANS]FPR-T, [2-[(4′-acetamido)anilino]naphthalene-6-sulfonic acid]-D-Phe-Pro-Arg-thrombin; [ANS]FFR-T, [2-[(4′-acetamido)anilino]naphthalene-6-sulfonic acid]-D-Phe-Phe-Arg-thrombin; F2, human prothrombin fragment 2; mAb, human anti-exosite II monoclonal antibody; Hir$^{54-65}$, Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln; Hir$^{54-65}$(SO_3^-), Tyr^{63}-sulfated hirudin$^{54-65}$; [5F]Hir$^{54-65}$(SO_3^-), Hir$^{54-65}$(SO_3^-) labeled at the amino terminus with 5-carboxy(fluorescein); H*, [5F]Hir$^{54-65}$(SO_3^-); IAANS, 2-[(4′-iodoacetamido)-anilino]naphthalene-6-sulfonic acid; AT, antithrombin.

To understand the mechanism of exosite regulation of thrombin further, the present work was undertaken to resolve whether the affinities of the exosite I-specific ligands, $Hir^{54-65}(SO_3^-)$ and fibrinogen, and of the exosite II-specific ligands, prothrombin fragment 2 and a monoclonal antibody, were affected by alternate exosite occupation. This was an important goal because studies employing hirudin peptides or F2 as probes of exosite involvement in other thrombin interactions could not be interpreted unambiguously, and it was uncertain whether the effects were due to competitive binding of alternate exosite I or II ligands or to extremely negative exosite linkage. Significant differences between human and bovine thrombin have been reported for the affinities of hirudin peptides (6). Also, the broad disagreement among reported affinities of F2 for bovine (42, 43) and human thrombin (30, 40) and its putative linkage to exosite I prompted a detailed quantitative analysis of F2 binding to human thrombin. Binding of Fbg to exosite I and the monoclonal antibody to exosite II were similarly characterized and quantitated for the first time in this context.

The results support the conclusion that binding of the exosite I and II ligands studied here conforms to a model with independent exosite interactions enabling formation of binary and ternary complexes with experimentally indistinguishable affinity. Structurally different exosite ligands produced different effects on the catalytic site, evidenced by different fluorescence changes of active site-labeled thrombins. Non-additive perturbations of the catalytic site accompany the exosite interactions, such that free thrombin and thrombin in binary and ternary complexes have unique properties. Analysis of experimental error in the dissociation constants for an array of ligands and a number of alternate experimental designs provided no evidence to substantiate extremely negative allosteric linkage between the exosites. It is concluded that the affinity of non-interacting, model exosite I and II ligands for thrombin remains unchanged whether the alternative exosite is occupied or not. Changes in binding affinity of factors V and Va, thrombomodulin, fibrin, and high molecular weight heparin affected by model exosite-specific ligands are likely to be due to competitive overlapping binding sites or additional interactions between the ligands themselves, but not to extreme inter-exosite allosteric linkage.

EXPERIMENTAL PROCEDURES

Purification and Characterization of Proteins and Peptides—Human α-thrombin, prepared by activation of prothrombin purified from human plasma (44), was ≥90% active as determined by active site titration (45). Prothrombin fragment 2 (F2) generated by cleavage of prethrombin 1 by factor Xa (40, 46), human fibrinogen (4, 33), antithrombin (47), and the monoclonal antibody (mAb) against thrombin exosite II (51) was purified by the published methods. Protein concentrations were determined at 280 nm with the absorption coefficients and molecular weights of 1.83 $(mg/ml)^{-1}$ cm^{-1} in 0.1 M NaOH or 1.74 in buffer, and 36,700, thrombin (48); 1.25 and 12,900, F2 (49); 1.51 and 170,000, Fbg monomer (4); 0.65 and 58,000, antithrombin (50); 1.35 and 150,000, mAb against thrombin exosite II (51).

Fluorescent thrombin derivatives were prepared by stoichiometric incorporation of ATA-FFR-CH_2Cl or ATA-FPR-CH_2Cl into the active site, and labeling of the NH_2OH-generated free thiol with the fluorescence probes 5-(iodoacetamido)fluorescein (5-IAF), 6-(iodoacetamido)-fluorescein (6-IAF), 4′-{[(iodoacetyl)amino]methyl}fluorescein (4′-IAF), and 2-[(4′-iodoacetamido)-anilino]naphthalene-6-sulfonic acid (IAANS) (Molecular Probes) following published methods (40, 44, 52, 53). Fluorescent meizothrombin-des-F1 was prepared by incorporation of ATA-FPR-CH_2Cl during activation of prethrombin 1 by ecarin and subsequent labeling with IAANS (40). The concentration of nonsulfated Hir^{54-65} and $Hir^{54-65}(SO_3^-)$ (Sigma or Bachem) in water or reaction buffer was determined from the purity and peptide content specified by the manufacturer. [5F]$Hir^{54-65}(SO_3^-)$ was prepared by labeling $Hir^{54-65}(SO_3^-)$ with 5-carboxy(fluorescein) as described previously (35).

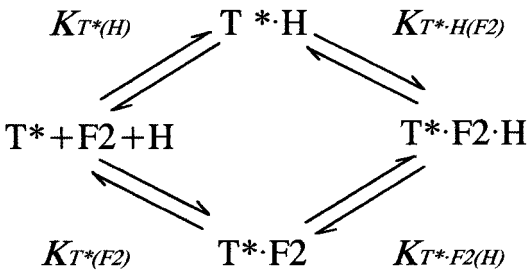
Fluorescence Studies—Fluorescence measurements were made with an SLM 8100 spectrofluorometer, using acrylic cuvettes coated with polyethylene glycol 20,000 except in tryptophan fluorescence experiments. Excitation and emission wavelengths are as follows: [5F]$Hir^{54-65}(SO_3^-)$, 491 and 520 nm, 4–8 nm bandpass; [5F]-, [6F]-, and [4′F]FPR-thrombin, 495 and 520 nm, 4–8 or 8–16 nm bandpass; [ANS]FFR-thrombin and [ANS]FPR-thrombin, 325 and 450 nm, 8–16 nm bandpass; and [5F]- and [6F]FPR-thrombin tryptophan fluorescence, 295 nm excitation and 360 nm emission, 4–8 nm bandpass. Titrations were performed by successive addition of small titrant volumes with ≤13% dilution and corrected for dilution and background. Individual fluorescence measurements were recorded after 5 min of equilibration and were averaged over 10–20 readings. Multiple titrations using overlapping titrant concentrations were combined to eliminate error propagation typical for multiple (>6) additions. Results were expressed as the fractional changes in the initial fluorescence $((F_{obs} - F_o)/F_o = \Delta F/F_o)$ as a function of total titrant concentration and were fit by the appropriate binding equation. Direct binding of F2 to [5F]FPR-thrombin, of competitive F2 binding to unlabeled thrombins, and thrombin inactivation by antithrombin was performed in two buffer systems as follows: 50 mM Hepes, 0.11 M NaCl, 5 mM $CaCl_2$, 1 mg/ml polyethylene glycol, pH 7.4; and the same buffer with 0.125 M NaCl and 1 mM EDTA. FPR-CH_2Cl (1 μM) was added to all titrations except those containing native thrombin.

Binding of F2 to Active Thrombin and Active Site-blocked, Unlabeled and Labeled Thrombin—Fluorescence titrations of direct binding of F2 to [5F]- and [6F]FPR-thrombin were analyzed by the quadratic equation for binding of a single ligand (40), to obtain the maximum fluorescence intensity change $(\Delta F_{max}/F_o)$ and the dissociation constant (K_D), with one binding site assumed on thrombin ($n = 1$). Binding of F2 to native thrombin, and the active site-blocked species ATA-FPR-thrombin and ATA-FFR-thrombin (0, 9, or 25 μM unlabeled thrombin), was measured in competitive binding experiments using [5F]FPR-thrombin (0.26 μM) as a probe. The dependence of $\Delta F/F_o$ on the F2 concentration in the absence and presence of competing unlabeled thrombin were analyzed by least squares fitting of the cubic competitive binding equation defining the fractions $[T^*{\cdot}F2]/(n[T^*]_o)$ and $[T{\cdot}F2]/(n[T]_o)$ for competitive binding of F2 to labeled (T*) and unlabeled thrombin (T), as described previously (54, 55). The observed fluorescence change is given by the contribution of the T*·F2 complex, weighted by the maximum fluorescence change associated with its formation (Equation 1),

$$\frac{\Delta F}{F_o} = \left(\frac{[T^*{\cdot}F2]}{n[T^*]_o}\right)\frac{\Delta F_{max}}{F_o} \qquad \text{(Eq. 1)}$$

in which $[T^*]_o$ is the total concentration of T*, and n is the number of equivalent and independent binding sites for F2. With an assumed 1:1 stoichiometry for the thrombin·F2 complex, the fitted parameters were $\Delta F_{max}/F_o$, and the dissociation constants were $K_{T^*(F2)}$ and $K_{T(F2)}$.

Effect of $Hir^{54-65}(SO_3^-)$ on Binding of F2 to Thrombin—The properties of the thrombin derivatives [5F]FPR-T and [6F]FPR-T in reporting the interactions with F2 and $Hir^{54-65}(SO_3^-)$ with unequal and opposite fluorescence changes were used for monitoring the joint interactions. In separate experiments, 50–100 nM [5F]FPR-thrombin or [6F]FPR-thrombin were titrated with F2, in the absence and presence of 20 μM $Hir^{54-65}(SO_3^-)$. In complementary experiments, the labeled thrombins were also titrated with $Hir^{54-65}(SO_3^-)$ in the absence and presence of 32.5 μM F2. In all of the experiments, the observed fluorescence change was given by Equation 2 for the ternary complex model (Scheme I).



SCHEME I

$$\frac{\Delta F}{F_o} = \left(\frac{[T^*{\cdot}H]}{n[T^*]_o}\right)\frac{\Delta F_{max\ T^*{\cdot}H}}{F_o} + \left(\frac{[T^*{\cdot}F2]}{n[T^*]_o}\right)\frac{\Delta F_{max\ T^*{\cdot}F2}}{F_o} + \left(\frac{[T^*{\cdot}F2{\cdot}H]}{n[T^*]_o}\right)\frac{\Delta F_{max\ T^*{\cdot}F2{\cdot}H}}{F_o} \qquad \text{(Eq. 2)}$$

$[T^*{\cdot}H]$ is the T*·$Hir^{54-65}(SO_3^-)$ complex; $[T^*{\cdot}F2]$ is the T*·F2 complex, and $[T^*{\cdot}F2{\cdot}H]$ is the ternary complex, T*·F2·$Hir^{54-65}(SO_3^-)$. The com-

bined data sets for each labeled thrombin were fit by Equation 2, with the concentrations of the binary and the ternary complexes calculated by simultaneous solution of the expressions for the equilibrium constants defined by the model, and the mass conservation equations. The fitted parameters were the individual $\Delta F_{max}/F_o$ values for the binary and ternary complexes, the dissociation constants for the binary complexes, $K_{T^*(F2)}$ and $K_{T^*(H)}$, and the dissociation constants, $K_{T^*\cdot F2(H)}$ and $K_{T^*\cdot H(F2)}$, for formation of the ternary complex. Because of the small fluorescence changes resulting from the interaction of $Hir^{54-65}(SO_3^-)$ with [5F]FPR-thrombin and [6F]FPR-thrombin, binding was quantitated independently from the changes in tryptophan fluorescence of 100 nM labeled thrombin titrated with $Hir^{54-65}(SO_3^-)$, and $K_{T^*(H)}$ was fixed at the determined value.

To determine the affinity of F2 for unlabeled thrombin species, 100 nM [5F]FPR-thrombin was titrated with F2 in the presence of 10 or 25 μM unlabeled thrombin. This was repeated in titrations with F2 at saturating $Hir^{54-65}(SO_3^-)$ (50 μM). The competition binding data were fit by the cubic equation as described above (54, 55) to obtain $\Delta F_{max}/F_o$ values for the labeled binary and ternary complexes and the dissociation constants for F2 binding to labeled and unlabeled thrombin, respectively, in their free and $Hir^{54-65}(SO_3^-)$-saturated forms.

Effect of F2 on Binding of $Hir^{54-65}(SO_3^-)$ to Labeled and Native Thrombin—[ANS]FFR-T and [4′F]FPR-T exhibited large fluorescence changes upon binding of $Hir^{54-65}(SO_3^-)$, whereas F2 binding caused a significantly smaller effect which allowed the simultaneous interactions to be observed. [ANS]FFR-T (0.19 μM) was titrated with $Hir^{54-65}(SO_3^-)$, in the absence and presence of 32.5 μM F2, and in separate experiments was titrated with F2, in the presence of fixed concentrations of $Hir^{54-65}(SO_3^-)$. [4′F]FPR-T (10 nM) was titrated similarly with $Hir^{54-65}(SO_3^-)$, in the absence and presence of 36 μM F2, and with F2 in the absence and presence of 20 μM $Hir^{54-65}(SO_3^-)$. The combined data sets for each labeled thrombin were fit by Equation 2 and the ternary complex model for binding of F2 and $Hir^{54-65}(SO_3^-)$ to labeled thrombin (Scheme I). In competitive titrations with native thrombin, 10 nM [4′F]FPR-T was titrated with $Hir^{54-65}(SO_3^-)$ in the absence and presence of 143 nM native thrombin. These titrations were repeated in the presence of 36 μM F2.

Effect of F2 on Binding of [5F]$Hir^{54-65}(SO_3^-)$ to Thrombin—[5F]$Hir^{54-65}(SO_3^-)$ (50 nM) was titrated with native thrombin in the absence and presence of 25 μM F2, and the combined results from titrations with three separate F2 preparations were pooled. The observed fluorescence change for this situation was given by Equation 3,

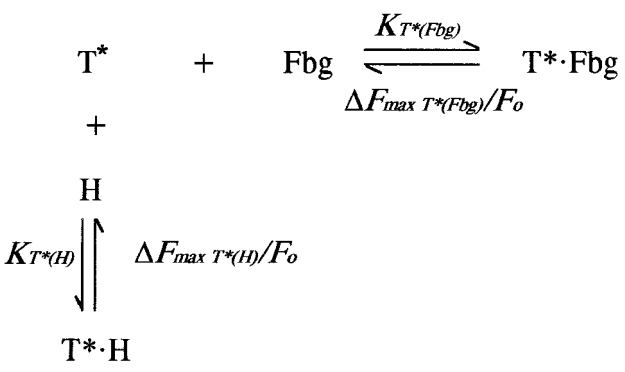
$$\frac{\Delta F}{F_o} = \left(\frac{[T\cdot H^*]}{[H^*]_o}\right)\frac{\Delta F_{max\ T(H^*)}}{F_o} + \left(\frac{[T\cdot F2\cdot H^*]}{[H^*]_o}\right)\frac{\Delta F_{maxT\cdot F2(H^*)}}{F_o} \qquad \text{(Eq. 3)}$$

where H* represents [5F]$Hir^{54-65}(SO_3^-)$; T·H* is the binary thrombin·[5F]$Hir^{54-65}(SO_3^-)$ complex; T·F2·H* is the ternary thrombin·F2·[5F]$Hir^{54-65}(SO_3^-)$ complex; $[H^*]_o$ is the total [5F]$Hir^{54-65}(SO_3^-)$ concentration; and $\Delta F_{maxT(H^*)}$ and $\Delta F_{maxT\cdot F2(H^*)}$ are the maximal relative fluorescence changes for [5F]$Hir^{54-65}(SO_3^-)$ binding to thrombin and the T·F2 complex. The data were fit by Equation 3.

Binding of Fbg to Thrombin and Meizothrombin-des-F1; Effect of F2, $Hir^{54-65}(SO_3^-)$, and Hir^{54-65}—[ANS]FPR-T and [ANS]FPR-meizothrombin-des-F1 reported Fbg binding by a large fluorescence enhancement, whereas these probes were insensitive to binding of Hir^{54-65}, $Hir^{54-65}(SO_3^-)$, and F2. [ANS]FPR-T (0.2 μM) was titrated with Fbg in the absence and presence of fixed concentrations of $Hir^{54-65}(SO_3^-)$ or Hir^{54-65}. These titrations were repeated at 32.5 μM F2.

Similarly, [ANS]FPR-meizothrombin-des-F1 (0.2 μM) was titrated with Fbg in the absence and presence of fixed concentrations of $Hir^{54-65}(SO_3^-)$. Background corrections were 15–30% at the highest protein concentrations. Under conditions of saturation of [ANS]FPR-T with Hir^{54-65} or $Hir^{54-65}(SO_3^-)$, and [ANS]FPR-meizothrombin-des-F1 with $Hir^{54-65}(SO_3^-)$, an exosite I-independent increase in fluorescence was observed as a linear increase in fluorescence with Fbg concentration. In separate experiments, [ANS]FPR-T at near-saturation with 53 μM Fbg was titrated with the exosite I ligands $Hir^{54-65}(SO_3^-)$ or Hir^{54-65}.

Binding of Fbg to [ANS]FPR-T or [ANS]FPR-meizothrombin-des-F1 and the effect of $Hir^{54-65}(SO_3^-)$ and Hir^{54-65} were described by a model (Scheme II) in which two ligands bind competitively to a fluorescent probe, and the interactions were accompanied by unequal fluorescence changes (21), in this case zero for $Hir^{54-65}(SO_3^-)$ and Hir^{54-65} binding. This model was used previously for analysis of competitive binding of $Hir^{54-65}(SO_3^-)$ and factor V/Va to [ANS]FPR-T (21). A linear term in Fbg (protomer) concentration was included to account for the exosite I-independent fluorescence increase. The fluorescence change was described by Equation 4,



SCHEME II

$$\frac{\Delta F}{F_o} = \left(\frac{[T^*\cdot Fbg]}{n[T^*]_o}\right)\frac{\Delta F_{max\ T^*(Fbg)}}{F_o} + \frac{\Delta F_{exo\text{-}ind}}{F_o}[Fbg]_o \qquad \text{(Eq. 4)}$$

Simultaneous least squares fitting of Equation 4 to the data with the cubic equations defining the fractional concentrations of the T*·Fbg complex, $[T^*\cdot Fbg]/(n[T^*]_o)$, and of the T*·hirudin peptide complex, $[T^*\cdot H]/(n[T^*]_o)$, gave the dissociation constants $K_{T^*(Fbg)}$ and $K_{T^*(H)}$ for the binary complexes, the maximum fluorescence change, and the slope of the exosite I-independent fluorescence increase ($\Delta F_{exo\text{-}ind}/F_o$) (Scheme II). The [ANS]FPR-meizothrombin-des-F1 titrations were analyzed using the same equations, in which T* was labeled meizothrombin-des-F1. The titration data at ~87% saturation of [ANS]FPR-T with F2 were analyzed similarly to obtain the dissociation constant for Fbg and $Hir^{54-65}(SO_3^-)$ binding to the T*·F2 complex.

Effect of $Hir^{54-65}(SO_3^-)$ on Binding of an Anti-exosite II Antibody (mAb) to Thrombin—[6F]FPR-T exhibited a large fluorescence quench upon binding of the exosite II-specific mAb, whereas it exhibited a modest enhancement upon binding of $Hir^{54-65}(SO_3^-)$. Binding of the mAb to [6F]FPR-T was studied at probe concentrations of 1.5, 25, and 47 nM, and the dissociation constant and the number of independent thrombin binding sites ($1/n$) on the mAb were determined by simultaneous analysis using the quadratic binding equation (40). [6F]FPR-T was also titrated with antibody in the absence and the presence of 5 μM $Hir^{54-65}(SO_3^-)$. The combined data were fit by Equation 2 and the ternary complex model for binding of mAb and $Hir^{54-65}(SO_3^-)$ to thrombin (Scheme I).

Effect of F2 on the Kinetics of Thrombin Inactivation by Antithrombin—The effect of F2 on thrombin inactivation by antithrombin was measured from the loss of thrombin chromogenic substrate activity, under pseudo first-order conditions ($[AT]_o \gg [T]_o$) in the absence and presence of 5, 10, 15, and 25 μM F2. Residual thrombin ($[T]_t$) was expressed as the fraction of the initial activity ($[T]_o$). In a control reaction, F2 had no effect on the chromogenic assay rate. The progress curves of $[T]_t/[T]_o$ with time were fit by a single exponential decay to obtain the observed pseudo first-order rate constants (k_{obs}) and simultaneously by Equation 5 and the quadratic binding equation, defining $[T\cdot F2]_o/(n[T]_o)$, to obtain the apparent second-order rate constants, k and k', respectively for free thrombin and the T·F2 complex reacting with antithrombin, and the K_D for the T·F2 complex,

$$k_{obs} = k[AT]_o + (k' - k)[AT]_o\left(\frac{[T\cdot F2]_o}{n[T]_o}\right) \qquad \text{(Eq. 5)}$$

Least squares fitting was performed with SCIENTIST Software (MicroMath). All reported estimates of error represent ±2 S.D.

RESULTS

Binding of F2 to Native, Active Site-blocked, Unlabeled, and Labeled Thrombin—The affinity of F2 for an array of fluorescence probe-labeled thrombins was determined previously (40), but the interaction of F2 with native human thrombin has not been characterized quantitatively. To assess the influence of active site labeling on the thrombin-F2 interaction, binding of F2 to human native thrombin, and active site-blocked nonfluorescent ATA-FPR-T and ATA-FFR-T, was characterized in competitive experiments using [5F]FPR-T as a probe. These

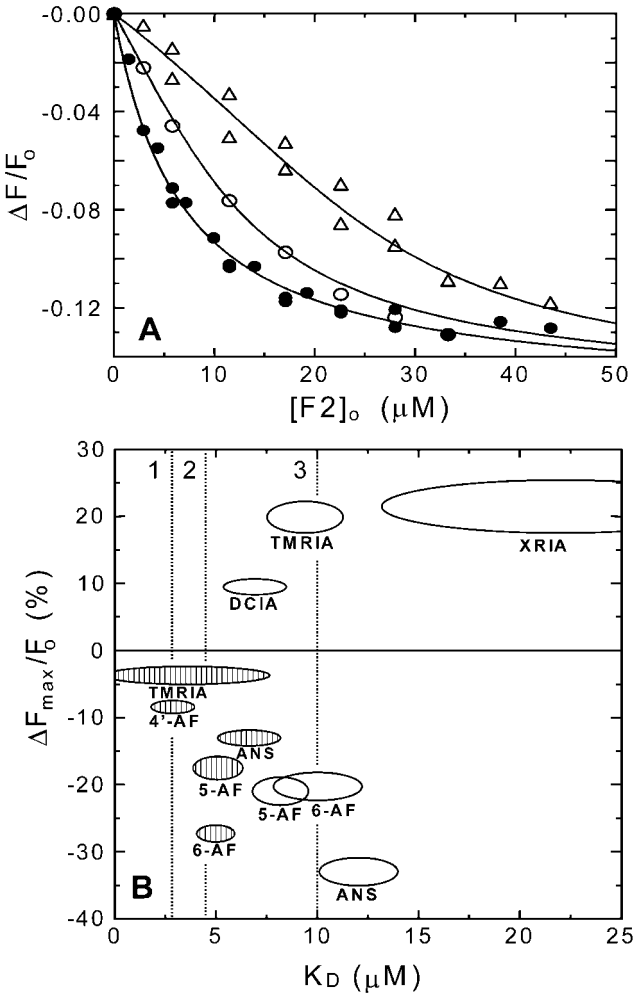


FIG. 1. **Competitive titration of F2 binding to [5F]FPR-T and unlabeled thrombin.** *A*, the fractional change in fluorescence of 0.26 μM [5F]FPR-T ($\Delta F/F_o$) is shown as a function of the total concentration of F2 ($[F2]_o$) in the absence (●) and presence of 9 (○) and 25 μM (△) unlabeled thrombin. Experiments were performed in buffer with EDTA. The *solid lines* represent the least squares fits with the parameters listed in Tables I–III. *B*, comparison of K_D and $\Delta F_{max}/F_o$ values for labeled and unlabeled thrombins. Values of $\Delta F_{max}/F_o$ are shown for F2 binding to fluorescently labeled FPR-thrombins (*shaded ellipses*) and FFR-thrombins (*open ellipses*) plotted against the K_D (40). The fluorescence probes were 4′-{[(iodoacetyl)amino]methyl}fluorescein (*4′-IAF*), 5-(iodoacetamido)fluorescein (*5-IAF*), 6-(iodoacetamido)fluorescein (*6-IAF*), 2-[(4′-iodoacetamido)anilino]naphthalene-6-sulfonic acid (*IAA-NS*), tetramethylrhodamine-5-(and-6)-iodoacetamide (*TMRIA*), rhodamine X iodoacetamide (*XRIA*), and 7-diethylamino-3-[(4′-iodoacetylamino)phenyl]-4-methylcoumarin (*DCIA*). The experimental error in the parameters (±2 S.D.) defines the radii of the ellipses. The *vertical dotted lines* indicate the values for K_D of fragment 2 binding to ATA-FPR-T (*1*), native thrombin (*2*), and ATA-FFR-T (*3*). Titrations were performed and analyzed as described under "Experimental Procedures."

studies were done in buffer containing 1 mM EDTA to allow comparison with previous results and subsequently in buffer containing 5 mM calcium. The [5F]FPR-T data were fit well by the cubic equation (54, 55) for competitive ligand (F2) binding to labeled and unlabeled thrombin. F2 bound to [5F]FPR-T with a K_D of 6 ± 1 μM, and to native human thrombin with a K_D of 5 ± 1 μM, a 1:1 binding stoichiometry, and a maximum fluorescence change of −15.4 ± 0.3%, as shown in Fig. 1*A*. The affinities for ATA-FPR-T and ATA-FFR-T were 3 ± 1 and 10 ± 2 μM, respectively. Experiments in buffer with 5 mM $CaCl_2$ reported indistinguishable F2 binding to labeled and native thrombin. Dissociation constants for ligand binding to active site-labeled thrombins with free and occupied alternate exosites are summarized in Table I; dissociation constants for ligand binding to native and active site-blocked, unlabeled thrombins are listed in Table II; maximal fluorescence changes for active site-labeled thrombins in binary and ternary complexes and for [5F]Hir$^{54-65}$(SO_3^-) binding to free thrombin and T·F2 complex are listed in Table III. Fig. 1*B* compares fluorescence changes and dissociation constants for F2 binding to the unlabeled thrombins with the parameters for the panel of fluorescent thrombins (40). The affinities of F2 for the peptide-inhibited, unlabeled thrombins fell within the range defined by F2 binding to the fluorescent derivatives. The dissociation constants for labeled and unlabeled FFR-thrombins were slightly but consistently higher (~2-fold) than those for FPR-thrombins, although within the joint experimental error. These results indicated a small but reproducible allosteric linkage effect between the affinity for F2 and the structure of the tripeptide occupying the active site.

Effect of Hir$^{54-65}$(SO_3^-) on the Binding of F2 to Thrombin—The effect of Hir$^{54-65}$(SO_3^-) on F2 binding to thrombin was studied with [5F]FPR-T and [6F]FPR-T. These probes reported Hir$^{54-65}$(SO_3^-) binding with small fluorescence increases of 5 and 9% and F2 binding with quenches of −9 and −22%, as shown in Fig. 2 for [6F]FPR-T. No reliable estimate of $K_{T^*(H)}$ for the [5F]FPR-T·Hir$^{54-65}$(SO_3^-) complex could be obtained from the fluorescein fluorescence data alone, due to the large experimental error in the small fluorescence change. However, binding of Hir$^{54-65}$(SO_3^-) to [5F]FPR-T and [6F]FPR-T was determined accurately by independent tryptophan fluorescence titrations with Hir$^{54-65}$(SO_3^-) (Fig. 2, *inset*, and Table I) which gave dissociation constants of 16 ± 12 and 19 ± 8 nM, respectively. These were fixed parameters in the global analysis of the fluorescein fluorescence data. Both thrombin probes were titrated with F2, in the absence and presence of saturating Hir$^{54-65}$(SO_3^-), and in separate experiments titrated with Hir$^{54-65}$(SO_3^-), in the absence and presence of ~85% saturating F2. The combined data for each labeled thrombin were fit simultaneously by Equation 2 for the ternary complex model (see Scheme I). In the absence of Hir$^{54-65}$(SO_3^-), the F2 affinities for [5F]FPR-T and [6F]FPR-T were indistinguishable at 6 ± 1 and 5 ± 1 μM, respectively. In the presence of saturating Hir$^{54-65}$(SO_3^-), the affinities were 3 ± 1 and 5 ± 1 μM for F2 binding to the [5F]FPR-T·Hir$^{54-65}$(SO_3^-) and [6F]FPR-T·Hir$^{54-65}$(SO_3^-) complexes, respectively. In the absence of F2, $K_{T^*(H)}$ for the [6F]FPR-T·Hir$^{54-65}$(SO_3^-) complex was 24 ± 5 nM, and at near-saturating F2, $K_{T^*\cdot F2(H)}$ for Hir$^{54-65}$(SO_3^-) binding to the [6F]FPR-T·F2 complex was an indistinguishable 16 ± 4 nM. Binding of F2 to the labeled thrombins was affected no more than ~2-fold by simultaneous occupation of the alternate exosite with Hir$^{54-65}$(SO_3^-), and binding of Hir$^{54-65}$(SO_3^-) to free and F2-bound thrombin was indistinguishable. The fluorescence amplitudes for individual and simultaneous binding of Hir$^{54-65}$(SO_3^-) and F2 were approximately additive for these two probes (Table III).

Binding of F2 to native thrombin and its complex with Hir$^{54-65}$ (SO_3^-) was quantitated in competition experiments with [5F]FPR-T as a probe. Binding of F2 to native thrombin was characterized by a K_D of 5 ± 1 μM, and to the thrombin·Hir$^{54-65}$(SO_3^-) complex by a K_D of 2 ± 1 μM. In these competition experiments, F2 bound to the [5F]FPR-T·Hir$^{54-65}$(SO_3^-) complex with a K_D of 3 ± 1 μM. These affinities were indistinguishable from the $K_{T^*\cdot H(F2)}$ of 3 μM, obtained by direct titration of the [5F]FPR-T·Hir$^{54-65}$(SO_3^-) complex with F2. These results strongly indicated that at saturating Hir$^{54-65}$(SO_3^-), F2 bound to native thrombin and [5F]FPR-T in a similar fashion.

Effect of F2 on the Binding of Hir$^{54-65}$(SO_3^-) to Labeled and Native Thrombin—[ANS]FFR-T exhibited a 66% quench upon

TABLE I

Dissociation constants for exosite I and II ligand binding to active-site-labeled thrombins with free and occupied alternate exosites

Dissociation constants are listed from global analysis of titrations of the indicated active-site-labeled thrombins with exosite I ($K_{D(\text{exo I})}$) and II ($K_{D(\text{exo II})}$) ligands, in the absence and the presence of saturating alternate exosite ligand, determined as described under "Experimental Procedures." $K_{T(H)}$ for binding of $Hir^{54-65}(SC_3^-)$ to [5F]FPR-T was obtained by tryptophan fluorescence and to [6F]FPR-T by tryptophan (19 ± 8 nM) and fluorescein fluorescence (24 ± 5 nM) (see Fig. 2). $K_{T(F2)}$ for binding of F2 to [5F]FPR-T was obtained by competitive titration with unlabeled and labeled thrombin in EDTA buffer (6 ± 1 μM) and was indistinguishable from the value in buffer containing calcium (6 ± 3 μM) (see Fig. 1*A*). $K_{T(Fbg)}$ for [ANS]FPR-T was determined by competition with $Hir^{54-65}(SO_3^-)$ (7 ± 2 μM) (see Fig. 5*B*) and with Hir^{54-65} (5 ± 2 μM) (see Fig. 5*A*).

Thrombin derivative	Exo I ligand	Exo II ligand	$K_{D(\text{exo I})}$		$K_{D(\text{exo II})}$	
				nM		*μM*
[5F]FPR-T	$Hir^{54-65}(SO_3^-)$		$K_{T(H)}$	16 ± 12		
	$Hir^{54-65}(SO_3^-)$	F2	$K_{T\cdot F2(H)}$	7 ± 3		
		F2			$K_{T(F2)}$	6 ± 1
		F2			$K_{T(F2)}$	6 ± 3
	$Hir^{54-65}(SO_3^-)$	F2			$K_{T\cdot H(F2)}$	3 ± 1
[6F]FPR-T	$Hir^{54-65}(SO_3^-)$		$K_{T(H)}$	19 ± 8		
	$Hir^{54-65}(SO_3^-)$		$K_{T(H)}$	24 ± 5		
	$Hir^{54-65}(SO_3^-)$	F2	$K_{T\cdot F2(H)}$	16 ± 4		
	$Hir^{54-65}(SO_3^-)$	mAb	$K_{T\cdot mAb(H)}$	24 ± 9		
		F2			$K_{T(F2)}$	5 ± 1
	$Hir^{54-65}(SO_3^-)$	F2			$K_{T\cdot H(F2)}$	5 ± 1
		mAb			$K_{T(mAb)}$	0.0018 ± 0.0003
	$Hir^{54-65}(SO_3^-)$	mAb			$K_{T\cdot H(mAb)}$	0.0022 ± 0.0009
[4′F]FPR-T	$Hir^{54-65}(SO_3^-)$		$K_{T(H)}$	150 ± 16		
	$Hir^{54-65}(SO_3^-)$	F2	$K_{T\cdot F2(H)}$	114 ± 28		
		F2			$K_{T(F2)}$	5 ± 2
	$Hir^{54-65}(SO_3^-)$	F2			$K_{T\cdot H(F2)}$	6 ± 4
[ANS]FFR-T	$Hir^{54-65}(SO_3^-)$		$K_{T(H)}$	27 ± 4		
	$Hir^{54-65}(SO_3^-)$	F2	$K_{T\cdot F2(H)}$	54 ± 28		
		F2			$K_{T(F2)}$	12 ± 4
	$Hir^{54-65}(SO_3^-)$	F2			$K_{T\cdot H(F2)}$	25 ± 15
[ANS]FPR-T	Fbg		$K_{T(Fbg)}$	7000 ± 2000		
	Fbg		$K_{T(Fbg)}$	5000 ± 2000		
	Fbg	F2	$K_{T\cdot F2(Fbg)}$	13,000 ± 4000		
	$Hir^{54-65}(SO_3^-)$	F2	$K_{T(H)}$	17 ± 7		
	$Hir^{54-65}(SO_3^-)$		$K_{T(H)}$	19 ± 7		
	Hir^{54-65}		$K_{T(H)}$	550 ± 340		
[ANS]FPR-MzT(-F1)	Fbg		$K_{MzT(-F1)(Fbg)}$	25,000 ± 3000		
	$Hir^{54-65}(SO_3^-)$		$K_{MzT(-F1)(H)}$	27 ± 4		

TABLE II

Dissociation constants for binding of F2, $Hir^{54-65}(SO_3^-)$, and [5F]$Hir^{54-65}(SO_3^-)$ to native and unlabeled, active-site-blocked thrombins

Dissociation constants ($K_{D(\text{exo I})}$) for [5F]$Hir^{54-65}(SO_3^-)$ binding to native thrombin were from global analysis of fluorescence titrations in the absence and the presence of saturating F2, as described under "Experimental Procedures." Binding constants of $Hir^{54-65}(SO_3^-)$ and F2 to native thrombin, and of F2 to unlabeled active-site-blocked thrombins ($K_{D(\text{exo II})}$) were determined by competitive titrations with fluorescent thrombins in the presence of EDTA or calcium. Binding of F2 to native thrombin was identical (5 ± 1 μM) in the absence and presence of EDTA. $K_{T(F2)}$ values for ATA-FPR-T and ATA-FFR-T were determined in buffer with EDTA.

Thrombin derivative	Exo I ligand	Exo II ligand	$K_{D(\text{exo I})}$		$K_{D(\text{exo II})}$	
				nM		*μM*
Native, unlabeled	[5F]$Hir^{54-65}(SO_3^-)$		$K_{T(H)}$	18 ± 3		
	[5F]$Hir^{54-65}(SO_3^-)$	F2	$K_{T\cdot F2(H)}$	20 ± 7		
	$Hir^{54-65}(SO_3^-)$		$K_{T(H)}$	28 ± 14		
	$Hir^{54-65}(SO_3^-)$	F2	$K_{T\cdot F2(H)}$	23 ± 18		
		F2			$K_{T(F2)}$	5 ± 1
	$Hir^{54-65}(SO_3^-)$	F2			$K_{T\cdot H(F2)}$	2 ± 1
ATA-FPR-T		F2			$K_{T(F2)}$	3 ± 1
ATA-FFR-T		F2			$K_{T(F2)}$	10 ± 2

binding of $Hir^{54-65}(SO_3^-)$, and reported F2 binding by a 31% quench, dissimilar signals that were used to examine the effect of F2 on binding of $Hir^{54-65}(SO_3^-)$. Fig. 3*A* shows the combined results for $Hir^{54-65}(SO_3^-)$ and F2 binding to [ANS]FFR-T and the fit by the ternary complex model (Equation 2). Binding of $Hir^{54-65}(SO_3^-)$ to free [ANS]FFR-T and the [ANS]FFR-T·F2 complex was characterized by indistinguishable dissociation constants of 27 ± 4 and 54 ± 28 nM, respectively, whereas F2 bound to free [ANS]FFR-T and the [ANS]FFR-T·$Hir^{54-65}(SO_3^-)$ complex with dissociation constants of 12 ± 4 and 25 ± 15 μM, respectively. Maximum fluorescence changes for separate and simultaneous binding of $Hir^{54-65}(SO_3^-)$ and F2 were non-additive (Table III), indicating that the thrombin active site sensed separate and simultaneous exosite binding differently, but this was not linked to a significant change in affinity.

Similar experiments with [4′F]FPR-T reported $Hir^{54-65}(SO_3^-)$ binding with a 50% enhancement (Fig. 3, *B* and *C*). Titrations of [4′F]FPR-T and competing native thrombin with Hir^{54-65} (SO_3^-) were done in the absence and presence of ~88% saturating F2. The *inset* (Fig. 3*C*) shows titrations of [4′F]FPR-T with F2, in the absence and presence of saturating $Hir^{54-65}(SO_3^-)$. The global fit demonstrated binding of $Hir^{54-65}(SO_3^-)$ to

TABLE III

Maximal fluorescence changes of binary and ternary complexes of active-site-labeled thrombins with exosite I and II ligands

Maximal fluorescence changes ($\Delta F_{max}/F_o$) were from global analysis of fluorescence titrations of active-site-labeled thrombins with exosite I (exo I) and II (exo II) ligands, in the absence (binary complexes) and the presence (ternary complex) of saturating alternate exosite ligand, as described under "Experimental Procedures." Fluorescence changes for F2 binding to [5F]FPR-T in buffer with and without EDTA were −15.4 and −9%, respectively. Fluorescence changes for Fbg binding to [ANS]FPR-T were 102 and 91% from competition experiments with $Hir^{54-65}(SO_3^-)$ and Hir^{54-65}, respectively. Exosite I-independent fluorescence changes caused by Fbg binding to [ANS]FPR-T in the presence of saturating $Hir^{54-65}(SO_3^-)$ were 0.012 ± 0.001 μM^{-1} at 0 μM F2 and 0.007 ± 0.001 μM^{-1} at 32 μM F2, and in the presence of saturating Hir^{54-65}, 0.013 ± 0.002 μM^{-1} at 0 μM F2. The exosite I-independent fluorescence change for [ANS]FPR-meizothrombin-des-F1 was 0.007 ± 0.001 μM^{-1}.

Thrombin derivative	Exo I ligand	Exo II ligand	$(\Delta F_{max}/F_o)_{exo\ I}$ binary complex	$(\Delta F_{max}/F_o)_{exo\ II}$ binary complex	$\Delta F_{max}/F_o$ ternary complex
			%	%	%
[5F]FPR-T	$Hir^{54-65}(SO_3^-)$		5.5 ± 0.4		
		F2		−15.4 ± 0.3	
		F2		−9 ± 1	
	$Hir^{54-65}(SO_3^-)$	F2			−8 ± 1
[6F]FPR-T	$Hir^{54-65}(SO_3^-)$		9.2 ± 0.3		
		F2		−22 ± 1	
		mAb		−22 ± 1	
	$Hir^{54-65}(SO_3^-)$	F2			−17 ± 1
	$Hir^{54-65}(SO_3^-)$	mAb			−14 ± 1
[4′F]FPR-T	$Hir^{54-65}(SO_3^-)$		50 ± 2		
		F2		−7 ± 1	
	$Hir^{54-65}(SO_3^-)$	F2			34 ± 2
[ANS]FFR-T	$Hir^{54-65}(SO_3^-)$		−66 ± 1		
		F2		−31 ± 3	
	$Hir^{54-65}(SO_3^-)$	F2			−71 ± 4
[ANS]FPR-T	Fbg		102 ± 10		
	Fbg		91 ± 13		
		F2		0	
	Fbg	F2			103 ± 13
	$Hir^{54-65}(SO_3^-)$		0		
	Hir^{54-65}		0		
[ANS]FPR-MzT(-F1)	Fbg		173 ± 12		
	$Hir^{54-65}(SO_3^-)$		0		
Native thrombin	[5F]$Hir^{54-65}(SO_3^-)$		−29 ± 1		
		F2			
	[5F]$Hir^{54-65}(SO_3^-)$	F2		0	−28 ± 1

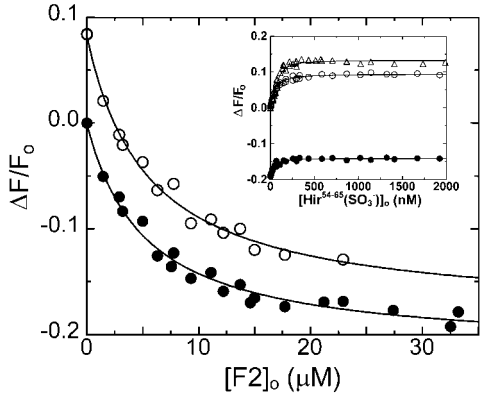


FIG. 2. **Binding of F2 and $Hir^{54-65}(SO_3^-)$ to [6F]FPR-T.** The fractional change in fluorescence of 50 nM [6F]FPR-T ($\Delta F/F_o$) is shown as a function of the total concentration of F2 ($[F2]_o$) in the absence (●) and presence (○) of 20 μM $Hir^{54-65}(SO_3^-)$. The *inset* shows titration of 50 nM [6F]FPR-T measured by fluorescein fluorescence as a function of the total concentration of $Hir^{54-65}(SO_3^-)$ ($[Hir^{54-65}(SO_3^-)]_o$) in the absence (○) and the presence (●) of 32.5 μM F2, and titrations of 100 nM [6F]FPR-T tryptophan fluorescence (△) in the absence of F2. Experiments were performed in buffer containing 5 mM $CaCl_2$. The *solid lines* represent the least squares fits with the parameters listed in Tables I and III. Titrations were performed and analyzed as described under "Experimental Procedures."

free [4′F]FPR-T and the [4′F]FPR-T·F2 complex with indistinguishable dissociation constants of 150 ± 16 nM and 114 ± 28 nM, respectively. F2 bound to free [4′F]FPR-T and the [4′F]FPR-T·$Hir^{54-65}(SO_3^-)$ complex with affinities of 5 ± 2 and 6 ± 4 μM. Fluorescence amplitudes for individual and simultaneous binding of $Hir^{54-65}(SO_3^-)$ and F2 were also non-additive (Table III). Competitive binding of $Hir^{54-65}(SO_3^-)$ to native thrombin and [4′F]FPR-T was characterized by dissociation constants of 28 ± 14 and 150 ± 16 nM. $Hir^{54-65}(SO_3^-)$ binding to the respective thrombin·F2 complexes was indistinguishable, with affinities of 23 ± 18 nM for the native thrombin·F2 complex and 117 ± 22 nM for the [4′F]FPR-T·F2 complex. The affinity of $Hir^{54-65}(SO_3^-)$ for [4′F]FPR-T was reduced ~5-fold compared with [5F]-, [6F]-, and [ANS]-labeled thrombins, which were very similar to the affinity for native thrombin. This was the largest linkage effect observed between the catalytic site probe and the affinity of exosite I for $Hir^{54-65}(SO_3^-)$. However, binding of the complementary exosite ligand affected minimally the affinity of $Hir^{54-65}(SO_3^-)$ and F2 for [ANS]FFR-T, [4′F]FPR-T, and native thrombin.

Effect of F2 on Binding of [5F]$Hir^{54-65}(SO_3^-)$ to Native Thrombin—Titration of [5F]$Hir^{54-65}(SO_3^-)$ with native thrombin, in the absence and the presence of near-saturating F2, gave near-identical fluorescence decreases as shown in Fig. 4. The combined data were fit by the ternary complex model (Scheme I) with $K_{T(F2)}$ fixed at 5 μM, the mean value for F2 binding to native thrombin. Binding of [5F]$Hir^{54-65}(SO_3^-)$ to active thrombin and the thrombin·F2 complex were characterized by indistinguishable binding constants of 18 ± 3 and 20 ± 7 nM, respectively. The fluorescence change was unaffected by binding of F2 that was devoid of traces of contaminating pre-

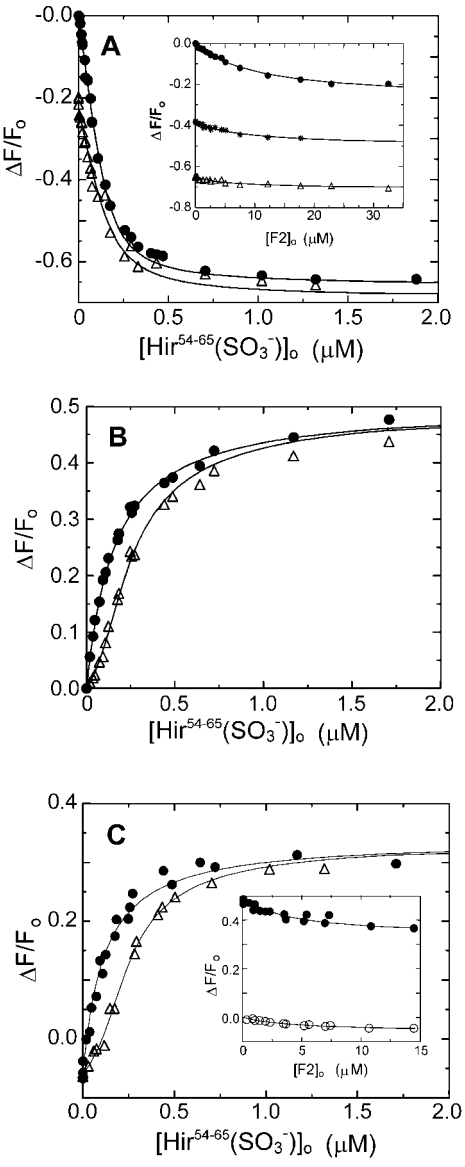


FIG. 3. **Binding of Hir$^{54-65}$(SO$_3^-$) and fragment 2 to [ANS]FFR-T.** *A*, the fractional change in fluorescence of 190 nM [ANS]FFR-T ($\Delta F/F_o$) is shown as a function of the total concentration of Hir$^{54-65}$(SO$_3^-$) ([*Hir$^{54-65}$ (SO$_3^-$)*]$_o$) in the absence (●) and presence (△) of 32.5 μM F2. The *inset* shows $\Delta F/F_o$ as a function of the total concentration of F2 ([*F2*]$_o$), in the absence (●) and presence of 0.15 μM (*) and 20 μM (Δ) Hir$^{54-65}$(SO$_3^-$). Experiments were performed in buffer containing EDTA. The *solid lines* represent the least squares fits with the parameters listed in Tables I and III. *B*, competitive titration of [4′F]FPR-T and native thrombin with Hir$^{54-65}$(SO$_3^-$). $\Delta F/F_o$ of 10 nM [4′F]FPR-T is shown as a function of the total concentration of [Hir$^{54-65}$(SO$_3^-$)]$_o$ in the absence (●) and presence (△) of 0.143 μM native thrombin in buffer with 5 mM $CaCl_2$. The *solid lines* represent the least squares fit with the parameters listed in Tables I–III. *C*, competitive titration of [4′F]FPR-T and native thrombin with Hir$^{54-65}$(SO$_3^-$) and effect of F2. $\Delta F/F_o$ of 10 nM [4′F]FPR-T is shown as a function of [Hir$^{54-65}$(SO$_3^-$)]$_o$ in the absence (●) and presence (△) of 0.143 μM native thrombin and in the presence of 36.6 μM F2. The *inset* shows $\Delta F/F_o$ as a function of [F2]$_o$ in the absence (○) and presence of 20 μM (●) Hir$^{54-65}$(SO$_3^-$). Experiments were performed in buffer with 5 mM $CaCl_2$. The *solid lines* represent the least squares fits with the parameters listed in Tables I–III. All titrations were performed and analyzed as described under "Experimental Procedures."

thrombin 2 by SDS-gel electrophoresis. Equivalent affinities of [5F]Hir$^{54-65}$(SO$_3^-$) and Hir$^{54-65}$(SO$_3^-$) for human thrombin have been demonstrated previously (6). Hence, the almost identical results for [5F]Hir$^{54-65}$(SO$_3^-$) binding to thrombin and the thrombin·F2 complex found here were a reflection of the similar

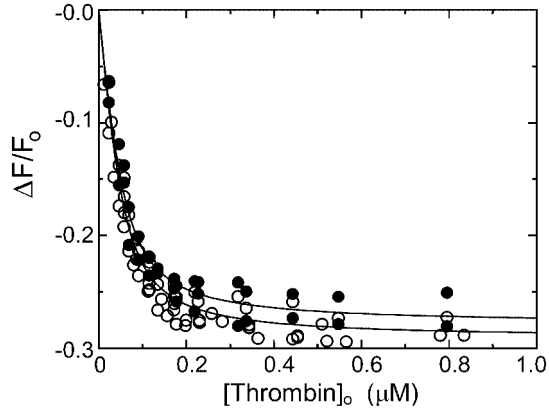


FIG. 4. **Effect of F2 on binding of native thrombin to [5F]Hir$^{54-65}$(SO$_3^-$).** The fractional change in fluorescence of 50 nM [5F]Hir$^{54-65}$(SO$_3^-$) ($\Delta F/F_o$) is shown as a function of the total concentration of native thrombin ([*Thrombin*]$_o$) in the absence (○) and presence (●) of 25 μM F2. Experiments were performed in buffer with $CaCl_2$. The *solid lines* represent the least squares fits with the dissociation constants listed in Table II. Titrations were performed and analyzed as described under "Experimental Procedures."

affinities of Hir$^{54-65}$(SO$_3^-$) binding. Moreover, they were in excellent agreement with the data for Hir$^{54-65}$(SO$_3^-$) binding to unlabeled thrombin and thrombin·F2 complex, determined with [4′F]FPR-T as a probe as described above.

Binding of Fbg to Thrombin and Meizothrombin-des-F1; Effect of F2, Hir$^{54-65}$(SO$_3^-$), and Hir$^{54-65}$—Kinetic studies of the pathway of Fbg cleavage by thrombin have shown that cleavage is inhibited competitively by hirudin peptides (3). This reflects the predominant role of exosite I in mediating productive Fbg binding as a substrate and in determining the K_m of 7.5 μM (4). The ligand-selective reporting properties of [ANS]FPR-T and [ANS]FPR-meizothrombin-des-F1 were used to investigate Fbg binding directly for the first time. Fbg binding to active site-blocked [ANS]FPR-T, in competition with Hir$^{54-65}$(SO$_3^-$) and Hir$^{54-65}$, respectively, was signaled by maximal fluorescence enhancements of 102 ± 10 and 91 ± 13% (Fig. 5*A*), whereas Fbg binding to [ANS]FPR-meizothrombin-des-F1 gave 173 ± 12% (Fig. 5*C*). Binding of F2, Hir$^{54-65}$, and Hir$^{54-65}$(SO$_3^-$) to [ANS]FPR-T and [ANS]FPR-meizothrombin-des-F1 did not result in detectable fluorescence changes. [ANS]FPR-T was titrated with Fbg in the absence and presence of Hir$^{54-65}$(SO$_3^-$) or Hir$^{54-65}$ (Fig. 5*A*) and in complementary titrations in the presence of ~83% saturating F2 (Fig. 5*B*). [ANS]FPR-meizothrombin-des-F1 was titrated with Fbg in the absence and presence of Hir$^{54-65}$(SO$_3^-$) (Fig. 5*C*). The peptides progressively decreased Fbg binding, revealing the exosite I-dependent interaction. At saturating Hir$^{54-65}$(SO$_3^-$) or Hir$^{54-65}$, titration with Fbg of [ANS]FPR-T and [ANS]FPR-meizothrombin-des-F1 demonstrated an additional exosite I-independent fluorescence change, as indicated by a linear fluorescence increase with Fbg concentration. In the competitive binding experiments, [ANS]FPR-T in the presence of ~89% saturating Fbg was titrated with Hir$^{54-65}$(SO$_3^-$) and with Hir$^{54-65}$. Analysis of the data by Equation 4 showed that Fbg bound to [ANS]FPR-T in the exosite I-mediated interaction with a dissociation constant of 7 ± 2 μM, calculated from the data set with competing Hir$^{54-65}$(SO$_3^-$), and the indistinguishable value of 5 ± 2 μM, calculated from the data set with competing Hir$^{54-65}$. Fbg bound to the [ANS]FPR-T·F2 complex with a binding constant of 13 ± 4 μM, indicating a possible ~2-fold effect of F2 on the affinity for Fbg. In the presence of Fbg, the affinities of Hir$^{54-65}$(SO$_3^-$) for [ANS]FPR-T and the [ANS]FPR-T·F2 complex were indistinguishable, 17 ± 7 and 19 ± 7 nM, respectively. These studies demonstrated that when the active site of thrombin

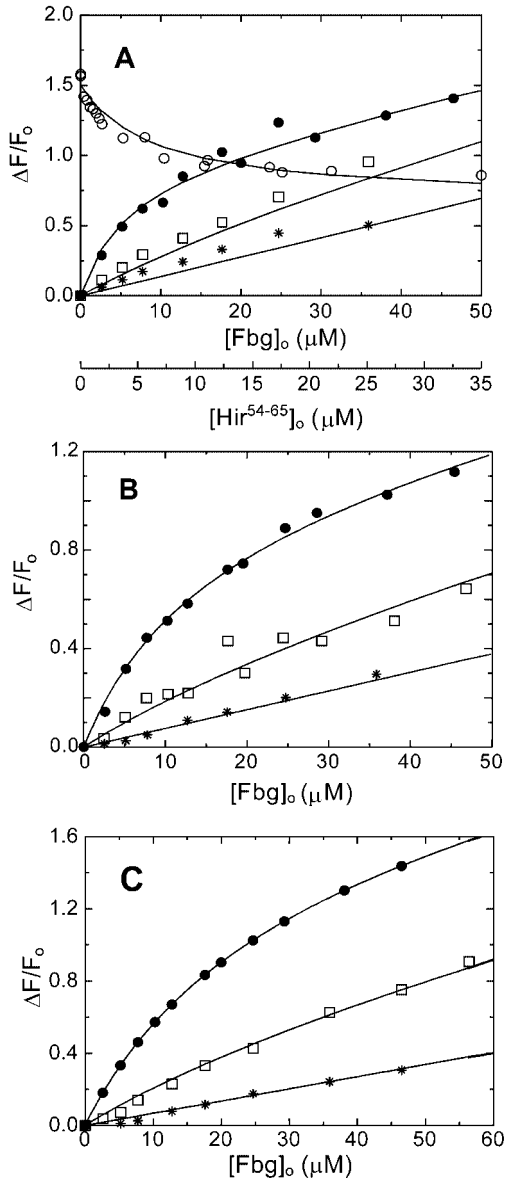


FIG. 5. **Titrations of [ANS]FPR-T with Fbg and Hir$^{54-65}$.** *A*, the fractional change in fluorescence of 200 nM [ANS]FPR-T ($\Delta F/F_o$) is shown as a function of the total concentration of Fbg ($[Fbg]_o$) in the absence (●) and presence of 0.25 μM (□) and 100 μM (*) Hir$^{54-65}$. The change in fluorescence of a mixture of 200 nM [ANS]FPR-T and 53 μM Fbg (○) is shown as a function of the total concentration of Hir$^{54-65}$ ($[Hir^{54-65}]_o$). The *solid lines* represent the least squares fits with the parameters listed in Tables I and III. *B*, effect of F2 on the binding of Fbg and Hir$^{54-65}$(SO_3^-) to [ANS]FPR-T. $\Delta F/F_o$ of 200 nM [ANS]FPR-T, in the presence of 32.5 μM F2, is shown as a function of the total concentration of Fbg ($[Fbg]_o$) in the absence (●) and presence of 0.25 (□) and 100 μM (*) Hir$^{54-65}$(SO_3^-). The *solid lines* represent the least squares fits with the parameters listed in Tables I and III. *C*, titrations of [ANS]FPR-meizothrombin-des-F1 with Fbg and Hir$^{54-65}$(SO_3^-). $\Delta F/F_o$ of 212 nM [ANS]FPR-meizothrombin-des-F1 is shown as a function of $[Fbg]_o$ in the absence (●) and presence of 0.25 μM (□) and 100 μM (*) Hir$^{54-65}$(SO_3^-). The *solid lines* represent the least squares fits with the parameters listed under "Results." All experiments were performed in buffer with $CaCl_2$ and analyzed as described under "Experimental Procedures."

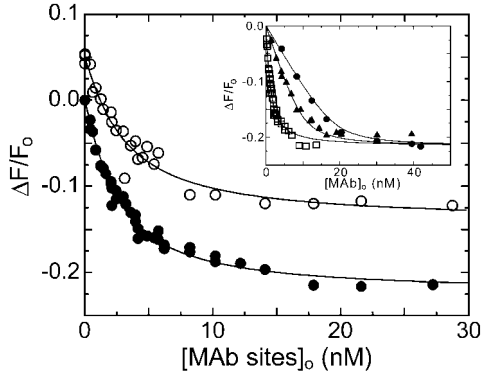


FIG. 6. **Titration of [6F]FPR-T with monoclonal antibody and effect of Hir$^{54-65}$(SO_3^-).** The fractional change in fluorescence ($\Delta F/F_o$) of 1.5 nM [6F]FPR-T is shown a function of the total concentration of monoclonal antibody sites ($[Mab\ sites]_o$) in the absence (●) and presence (○) of 20 μM Hir$^{54-65}$(SO_3^-). The *inset* shows $\Delta F/F_o$ of [6F]FPR-T at 1.5 (□), 25 (▲), and 47 nM (●) as a function of the total concentration of mAb ($[Mab]_o$). Experiments were performed in buffer with $CaCl_2$. The *solid lines* represent the least squares fits with the parameters listed in Tables I and III. Titrations were performed and analyzed as described under "Experimental Procedures."

was blocked by the tripeptide label, Fbg bound exosite I with an affinity equivalent to the K_m of Fbg for native thrombin (7.5 μM) (4). [ANS]FPR-meizothrombin-des-F1 bound Fbg with a dissociation constant of 25 ± 3 μM and Hir$^{54-65}$(SO_3^-) with a dissociation constant of 27 ± 4 nM. These results indicated that meizothrombin-des-F1 was capable of binding Fbg through exosite I, in agreement with crystallographic studies showing that the Fbg recognition site is accessible on meizothrombin-des-F1 (56).

Effect of an Anti-Exosite II Antibody on the Binding of Hir$^{54-65}$(SO_3^-) to Thrombin Exosite I—mAb bound to [6F]FPR-T (Fig. 6, *inset*) with a dissociation constant of 1.7 ± 0.2 nM, an amplitude of −22 ± 1%, and n = 0.40 ± 0.02 mol of mAb/mol of [6F]FPR-T, consistent with two independent, non-interacting binding sites for thrombin on each antibody molecule. [6F]FPR-T titrations with mAb, in the absence and the presence of 5 μM Hir$^{54-65}$(SO_3^-) shown in Fig. 6, were analyzed by the ternary complex model (Scheme I). $K_{T^*(H)}$ for Hir$^{54-65}$(SO_3^-) binding was fixed at 19 nM, as determined independently by tryptophan fluorescence titration. Hir$^{54-65}$(SO_3^-) bound to the binary [6F]FPR-T·mAb complex with a dissociation constant of 24 ± 9 nM, indistinguishable from the affinity in the absence of mAb. The affinities of mAb for the free probe and the binary [6F]FPR-T·Hir$^{54-65}$(SO_3^-) complex were also indistinguishable at 1.8 ± 0.3 and 2.2 ± 0.9 nM. Amplitudes of fluorescence changes for the binary and the ternary complexes were additive (Table III). These results were in excellent agreement with a previous study reporting the absence of any effect of mAb binding on the affinity of [5F]Hir$^{54-65}$(SO_3^-) for native thrombin (18), and with the affinity of mAb for native thrombin determined in functional assays (18).

Effect of F2 on the Kinetics of Thrombin Inactivation by Antithrombin—Previous kinetic studies of thrombin inactivation by antithrombin have shown that F2 decreases the reactivity by ~3-fold (36). To determine whether the measured F2 interactions directly reflected this functional change, the kinetics of the fractional loss of thrombin activity ($[T]_t/[T]_o$) were characterized under pseudo first-order conditions ($[AT]_o \gg [T]_o$), in the absence and the presence of fixed F2 concentrations (Fig. 7). The dependence of the observed rate constant on F2 concentration was fit by Equation 5 and the quadratic equation defining the fraction of thrombin sites occupied by F2. In buffer containing EDTA, the dissociation constant for T·F2 interaction was 4 ± 2 μM, and the apparent bimolecular rate constants, k and k', were 6.3 ± 0.3 and 1.7 ± 0.5 $\mu M^{-1}\ s^{-1}$, respectively (Fig. 7, *inset*). In buffer with $CaCl_2$ (results not shown), the dissociation constant for F2 was 5 ± 1 μM, and k and k were 7.3 ± 0.1 and 2.5 ± 0.3 $\mu M^{-1}\ s^{-1}$, respectively. These results agreed with the previously reported effects of F2 on the thrombin-antithrombin reaction and verified that the

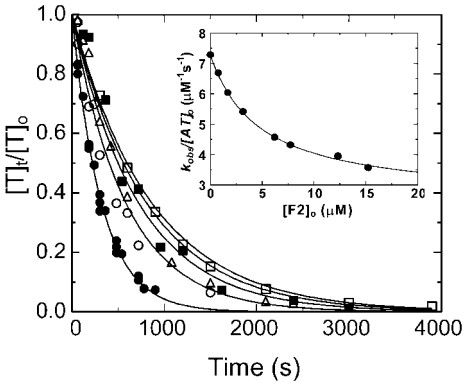


FIG. 7. **Effect of F2 on rate constants for inactivation of thrombin by antithrombin.** Thrombin activity, expressed as a fraction of the initial concentration ($[T]_t/[T]_o$), is shown with time, for reactions of 500 nM AT and 64 nM T, in the absence (●) and the presence of 5 (○), 10 (△), 15(■), and 25 μM (□) F2. *Solid lines* represent the fit of the data with Equation 5 under "Experimental Procedures." The *inset* shows the dependence of the apparent bimolecular rate constant ($k_{obs}/[AT]_o$) on the total concentration of F2 ($[F2]_o$) and the least-squares fit of the data (*solid line*) with the parameters listed under "Results." These data were obtained in buffer with EDTA as described under "Experimental Procedures."

interactions we observed were responsible for the same interaction of F2 with thrombin.

DISCUSSION

These studies were undertaken to evaluate the degree of thermodynamic linkage between exosites I and II by quantitative analysis of binding to thrombin of a panel of small and large exosite I and II ligands, and to characterize the effect of binding on the environment of the catalytic site. The studies addressed the previous interpretation (30, 41) that exosite I and II ligand binding to human thrombin was mutually exclusive, based on binding of fluorescent derivatives of $Hir^{54-65}(SO_3^-)$, F2, and a synthetic F2 peptide. These studies concluded that noninteracting exosite ligands affected binding of the alternate exosite ligand through extreme inter-exosite linkage (30, 41). In this mechanism, exosite ligand binding is thought to induce a conformational change in the alternate exosite, resulting in a near-total loss in affinity for the alternate ligand. Thus, $[5F]Hir^{54-65}(SO_3^-)$ was concluded to be released from thrombin by F2 binding to exosite II and vice versa. In the present study a large data set was analyzed so that an assessment of experimental error could be applied to accept or reject the inter-exosite linkage hypothesis. All of the results in the present study were explained by independent exosite binding and no detectable inter-exosite linkage. This outcome indicates that the effects of alternate exosite ligands can be explained by competitive binding. Thus, our results indicate that hirudin peptides and Fbg bind competitively to exosite I, whereas F2 and mAb exosite II ligands bind competitively to exosite II. The basis for the divergent results between studies is not known. Some of the discrepancies may be caused by differences between human and bovine thrombin (6, 31). The previous studies with human thrombin also used primarily a synthetic F2 peptide derivative (30, 41), which may not retain all of the properties of native F2, and involved measurement of small fluorescence changes (30). Moreover, native F2 preparations containing as little as 1% prethrombin 2 produced fluorescence amplitude decreases in the titrations of thrombin with $[5F]Hir^{54-65}(SO_3^-)$, linear with increasing F2 concentration (data not shown). These decreases in amplitude were artifacts caused by ignoring the small but significant decreases in the initial probe fluorescence as the result of $[5F]Hir^{54-65}(SO_3^-)$ binding to the contaminating prethrombin 2.[2]

The main conclusions of this study are supported by results of previously investigated thrombin interactions. $[5F]Hir^{54-65}(SO_3^-)$ was shown to bind to human thrombin with the same affinity in the absence and in the presence of saturating concentrations of a tight binding monoclonal antibody against exosite II, suggesting formation of a ternary complex (18). Simultaneous binding of fibrin monomer or polymer to exosite I and high molecular weight heparin to exosite II in a ternary complex with thrombin was facilitated by binding interactions between fibrin and heparin, resulting in a respective 6–40-fold enhanced affinity of polymer and monomer for the thrombin-heparin complex, compared with free thrombin (34, 57). However, these substantial increases in affinity are not attributed to allosteric inter-exosite linkage but are the consequence of additional heparin-fibrin interactions. In subsequent studies, interactions among heparin cofactor II, fibrin, and heparin have been attributed to ternary complex formation rather than allosteric linkage (58, 59). Similarly, thrombomodulin binds simultaneously to thrombin exosite I through its growth factor domains and to exosite II through a chondroitin sulfate moiety (7, 15). Fluorescein-labeled $hirudin^{53-64}$ forms a complex with bovine thrombin in the presence of F2 and with meizothrombin-des-F1, which has F2 attached (31). In addition, our results show competition between Fbg and $Hir^{54-65}(SO_3^-)$ for binding to exosite I of human meizothrombin-des-F1. Ternary complex formation thus appears to be the rule rather than the exception.

Due to the very broad range of binding constants reported for the interaction of F2 with bovine and human thrombin (30, 40, 42, 43), and the possible physiological role of the interaction in regulation of thrombin activity by antithrombin (36) and prothrombin activation (60), the affinity of this exosite ligand for thrombin was evaluated in direct binding studies with an array of labeled thrombin derivatives and in competitive binding studies with unlabeled and native thrombin. Dissociation constants ranged from 3 to 12 μM for F2 binding to thrombins carrying a fluorescently labeled or unlabeled tripeptide chloromethyl ketone in the active site, whereas native thrombin bound with a dissociation constant of 5 μM. Fluorescent thrombins with a FPR-inhibitor spacer sequence typically displayed slightly lower binding constants than those with an FFR-spacer sequence (40), indicating linkage between exosite II and the catalytic site, in agreement with binding studies with labeled thrombins (16, 18), kinetic studies of tripeptide substrate hydrolysis (18, 30, 42), and thrombin inactivation by antithrombin (36). The effects obtained here were relatively small (~2-fold), however, and close to the estimated experimental uncertainty. No significant changes in the binding constants for F2 were observed when exosite I was occupied with $Hir^{54-65}(SO_3^-)$ or Fbg.

F2 has been shown to inhibit the rate of the thrombin-antithrombin reaction ~3-fold, suggesting a potential role for F2 in the mechanism of regulation of thrombin in plasma (36). On the basis of the 5 μM dissociation constant for this process, however, it is concluded that the F2 interaction by itself is probably not physiologically significant because of the predicted dissociation of the complex at plasma protein levels. In bovine prothrombin activation, in the presence of factor Va, phospholipids, and calcium, accumulation of a membrane-bound thrombin-fragment 1.2 product complex has been demonstrated (60). Although not directly shown here, our results suggest that the F2-human thrombin interaction alone would

[2] P. J. Anderson, A. Nesset, and P. E. Bock, unpublished results.

play a minor role in stabilizing an analogous human product complex. Previous studies (6, 31) used bovine proteins that have different exosite I and II affinities. The results suggest that interactions with other components would be needed to stabilize the postulated complex consisting of the human proteins.

Fbg bound to exosite I of active site-blocked thrombin in the absence and presence of near-saturating F2 with dissociation constants of 7 and 13 μM, respectively, and was displaced competitively by $Hir^{54-65}(SO_3^-)$ and Hir^{54-65}. The results were consistent with the formation of a ternary complex in which exosite II was occupied by F2 and exosite I was occupied by Fbg. The K_D value for exosite I binding was remarkably similar to the kinetically determined K_m value for Fbg cleavage (7.5 μM) (4). This confirmed previous studies of the kinetics of Fbg cleavage concluding that Fbg substrate recognition is dominated by exosite I binding (4) and suggested that exosite II may play a secondary role. In addition, Fbg bound to [ANS]FPR-meizothrombin-des-F1 with a K_D value of 25 μM and was competitively displaced by $Hir^{54-65}(SO_3^-)$, which bound with a K_D value of 27 nM, in agreement with independent binding studies.[2] These results were in keeping with crystallographic studies of the thrombin-F2 complex (14, 61) and meizothrombin-des-F1 (56), which concluded that F2 does not cover exosite I or interfere directly with the active site. Meizothrombin-des-F1, which contains F2 covalently attached, has activity toward tripeptide chromogenic substrates, but little activity toward clotting of Fbg (62). Although the mechanism has not been determined precisely, the reduced activity of meizothrombin-des-F1 toward Fbg may be primarily an effect on catalytic efficiency rather than on binding, on the basis of the results indicating that Fbg binds with only a 2-fold lower affinity to the thrombin·F2 complex and ~4-fold weaker to [ANS]FPR-meizothrombin des-F1 than to free thrombin. This lower affinity implies a 2–4-fold effect of F2 on Fbg binding that may be the result of steric constraints or other unfavorable interactions. A comparison of the crystal structures of human FPR-thrombin and FPR-thrombin·F2 complexes indicates that thrombin accommodates F2 with little change in its folded structure (14, 61). If allosteric inter-exosite linkage were to occur, larger structural changes might have been expected.

Binding of exosite I and II ligands to various active site-labeled fluorescent thrombin derivatives showed non-additive changes in the maximum fluorescence for the individual binary complexes compared with the fluorescence change for the ternary complex, for 2 of the 5 thrombin derivatives. This indicated that the active site environments for the individual binary complexes and the ternary complex were different, such that each binary and ternary thrombin complex could be expected to display unique catalytic properties. This is borne out by previous studies of the effects of exosite occupation on the reactivity of thrombin toward peptide chromogenic substrates (7, 9, 18, 27–31, 42, 43). In the present study, consistent with previous findings (40), F2 bound weaker to fluorescent and non-fluorescent thrombin derivatives containing the FFR tripeptide in the active site, compared with the FPR derivatives, suggesting negative linkage between binding of F2 and the presence of a Phe residue occupying the S2 specificity subsite. $Hir^{54-65}(SO_3^-)$ bound ~5-fold weaker to [4′F]FPR-T than to native thrombin and any other active site-labeled thrombin used in this study, displaying the largest linkage effect between exosite I binding and occupation of the S4 subsite which is thought to be near the probe, again consistent with previous findings (35). These results indicated significant linkage between binding to the individual exosites and the S1–S4 specificity subsites for the pairs of ligands studied, whereas linkage between exosites I and II was not evident.

The previously unresolved relationship between exosite binding and possible inter-exosite linkage confounds the interpretation of multiple thrombin interactions because it is unclear whether apparent competitive binding effects, such as hirudin peptides on thrombin binding of factor V and Va (21), Fbg (3, this study), and thrombomodulin (7), are due to the presence of competitive overlapping sites for alternate exosite ligands or due to extreme conformational linkage. Our results for the ligands studied here demonstrate the predominance of relatively independent exosite interactions and exosite-specific binding of ligands that form binary and ternary complexes with indistinguishable affinity.

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ENZYME CATALYSIS AND REGULATION:

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