Evidence for Allosteric Linkage between Exosites 1 and 2 of Thrombin*

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Investigations to date have demonstrated that ligand binding to exosites 1 or 2 on thrombin produces conformational changes at the active site. In this study, we directly compared the effect of ligand binding to exosites 1 and 2 on the structure and function of the active site of thrombin and investigated functional linkage between the two exosites. Binding studies were performed in solution with fluorescein-Phe-Pro-Arg-CH₂Cl (FPR)-thrombin. Hirudin-(54–65) and sF2, a synthetic peptide corresponding to residues 63–116 of prothrombin fragment 2, were used as ligands for exosites 1 and 2 of thrombin, respectively. The two ligands produce diatomic changes in the fluorescence of fluorescein-FPR-thrombin and also have opposing effects on the rate of thrombin hydrolysis of a number of chromogenic substrates. These results indicate that sF2 and hirudin-(54–65) differentially affect the conformation of the active site. Experiments then were performed to investigate whether both ligands can bind to thrombin simultaneously. When thrombin-bound fluorescein-sF2 is titrated with hirudin-(54–65), complete displacement of fluorescein-sF2 is observed. Likewise, when thrombin-bound fluorescein-hirudin-(54–65) is titrated with sF2, complete displacement occurs. Additional support for reciprocal binding was obtained in fluorescence experiments where both probes were labeled and in experiments monitoring ligand binding to agarose-immobilized thrombin. This mutually exclusive binding of either ligand can be explained by reciprocal, allosteric modulation of ligand affinity between the two exosites. Thus, not only do the two exosites differentially influence the active site, they also affect the binding properties of the opposing exosite.

Thrombin is a trypsin-like enzyme that plays a major role in hemostasis by regulating the procoagulant, anticoagulant, and fibrinolytic pathways (1, 2). A distinguishing feature of thrombin is the presence of two positively charged patches found on opposite sides of the thrombin molecule (3). These regions, termed anion binding exosites 1 and 2, contribute to the specificity of thrombin by serving as binding sites for substrates, cofactors, and other ligands that modulate thrombin activity.

Exosite 1 was initially recognized as the fibrinogen binding site, but more recent studies indicate that it also binds hirudin, heparin cofactor II, and the thrombin receptor (5–9). The docking interaction at exosite 1, used by both substrates and inhibitors, precedes the reaction at the active site. The importance of exosite 1 is demonstrated by the reduced reactivity of γ-thrombin, a proteolytic derivative of thrombin lacking exosite 1, with fibrinogen, hirudin, and heparin cofactor II (9, 10). A second role of exosite 1, revealed by crystallographic and fluorescence studies with hirudin or thrombomodulin, is to confer structural changes in the active site environment that facilitate subsequent binding interactions (11–15). In the case of hirudin, this serves to optimize alignment of the amino-terminal inhibitory domain with the active site of thrombin (11, 15, 16). With thrombomodulin, the allosteric changes serve to alter the substrate specificity of thrombin. This converts thrombin from a procoagulant to a potent anticoagulant enzyme by virtue of its ability to activate protein C (17). Thus, exosite 1 serves both as a substrate/inhibitor binding site and an allosteric regulatory site.

Exosite 2, which has a stronger positive electrostatic field than exosite 1, is known mainly as a glycosaminoglycan binding site (3, 16, 18). Heparin binds to this site to catalyze antithrombin-mediated inhibition of thrombin (19). The chondroitin sulfate moiety of thrombomodulin also binds exosite 2, thereby increasing the affinity of the interaction (13, 14, 20). In addition to binding glycosaminoglycans, exosite 2, together with exosite 1, appears to be important in the interaction of thrombin with factors V and VIII, key cofactors in coagulation (21, 22). Prothrombin fragment 2 (F2),1 a kringle-containing activation fragment of prothrombin, binds to exosite 2 on thrombin (18). This interaction induces conformational changes at the active site, influences the regulation of thrombin activity by calcium, and reduces the rate of thrombin inhibition by antithrombin (20, 23–25). Like exosite 1, therefore, ligand binding to exosite 2 also can induce allosteric changes in the active site that modulate thrombin activity.

The allosteric changes evoked by ligand binding to exosite 1 or 2 may affect different regions of the active site. Binding to exosite 1 is thought to influence the autolysis loop near the south rim of the active site, whereas residues within exosite 2 form part of the S2 subsite of the substrate binding cleft (3, 4). This raises the possibility that ligand binding to one exosite may elicit different effects on the structure and function of the

1 The abbreviations used are: F2, prothrombin fragment 2; sF2, synthetic peptide corresponding to residues 63–116 of F2; FPR, D-Phe-Pro-Arg-CH₂Cl; FPRCK, D-Phe-Pro-Arg-chloromethyl ketone; hirudin-(53–64), synthetic peptide corresponding to hirudin residues 53–64 (Hirugen); hirudin-(54–65), synthetic peptide corresponding to hirudin residues 54–65; I, fluorescence intensity; Chz, Chromozyme; FITC, fluorescein 5-isothiocyanate; GFR-pNA, tosyl-Gly-Pro-Arg-p-nitroanilide; S-2222, benzoylxyacarbonyl-Ile-Glu-(OBz)-Gly-Arg-p-nitroanilide; S-2238, t-NAME-Pip-Arg-p-nitroanilide; S-2251, t-NAME-Val-Leu-Lys-p-nitroanilide; S-2288, t-NAME-Ile-Pro-Arg-p-nitroanilide; S-2444, pyro-Glu-Gly-Gly-Arg-p-nitroanilide; mananyl, 6-N-methylamino-naphthalene-2-sulfonyl chloride.
active site than ligand binding to the other exosite. In addition to their effects on the active site, ligand binding to one exosite may also alter the affinity of the opposing exosite for its ligands. To explore these possibilities we used a synthetic peptide encompassing the carboxyl-terminal half of human F2 as a ligand for exosite 2 (sF2) and a synthetic peptide analogue of the carboxyl terminus of hirudin (hirundin–(54–65)) as a ligand for exosite 1. Herein we report that ligand binding to one exosite directly influences the binding properties of the other, revealing an allosteric linkage between the two regulatory sites on thrombin. We also demonstrate that the two ligands have very different effects on the structure and function of the active site.

EXPERIMENTAL PROCEDURES

Materials

Tyr-εsulfated and non-acylated hirundin–(54–65), non-sulfated and non-acylated hirundin–(54–65), streptavidin-agarose, benzamidine-agarose, phosphatidylcholine, phosphatidylserine, and tosyl-Gly-Pro-Arg-β-nitroanilide (tGPR-pNA) were from Sigma. Human α- and β-thrombin, factor Xa, and prothrombin were obtained from Enzyme Research Laboratories (South Bend, IN). n-PhGlu-Arg-chloromethyl ketone (FPRCK) was from Calbiochem. Biotinylated-FPRCK, 5-fluorescein-FPRCK, and factor Va were from Hematologic Technologies Inc. (Essex Junction, VT). Tetramethylrhodamine-5-(and 6)-isothiocyanate, fluorescein-5-isothiocyanate (FITC), 6-(N-methylanilino)naphthalene-2-sulfonic acid (manyl), and 5-iodoacetamidofluorescein were from Molecular Probes Inc. (Eugene, OR). Methoxycarbonyl-n-norleucyl-Gly-Arg-ρ-nitroanilide (Chz-lo fibrinogen (CHO)-Xa) and N'-methyloxysulfonyl-n-Ph-Gly-Arg-ρ-nitroanilide (Chz-TPA) were from Boehringer Mannheim Canada (Laval, Quebec). Benzoxycarbonyl-Ile-Glu(OL)-Arg-Gly-ρ-nitroanilide (S-2251), the β-Ph-Pip-Arg-ρ-nitroanilide (S-2238), and S-val-Val-aspartate-p-nitroanilide (S-2288) were from Chromogenix. Human α- and β-thrombin, factor Xa, and factor Va were from Hematologic Technologies Inc. (Essex Junction, VT). Tetramethylrhodamine-5-(and 6)-isothiocyanate, fluorescein-5-isothiocyanate (FITC), 6-(N-methylanilino)naphthalene-2-sulfonic acid (manyl), and 5-iodoacetamidofluorescein were from Molecular Probes Inc. (Eugene, OR). Methoxycarbonyl-n-norleucyl-Gly-Arg-ρ-nitroanilide (Chz-lo fibrinogen (CHO)-Xa) and N'-methyloxysulfonyl-n-Ph-Gly-Arg-ρ-nitroanilide (Chz-TPA) were from Boehringer Mannheim Canada (Laval, Quebec). Benzoxycarbonyl-Ile-Glu(OL)-Arg-Gly-ρ-nitroanilide (S-2251), the β-Ph-Pip-Arg-ρ-nitroanilide (S-2238), and S-val-Val-aspartate-p-nitroanilide (S-2288) were from Chromogenix. Human α- and β-thrombin, factor Xa, and factor Va were from Hematologic Technologies Inc. (Essex Junction, VT). Tetramethylrhodamine-5-(and 6)-isothiocyanate, fluorescein-5-isothiocyanate (FITC), 6-(N-methylanilino)naphthalene-2-sulfonic acid (manyl), and 5-iodoacetamidofluorescein were from Molecular Probes Inc. (Eugene, OR). Methoxycarbonyl-n-norleucyl-Gly-Arg-ρ-nitroanilide (Chz-lo fibrinogen (CHO)-Xa) and N'-methyloxysulfonyl-n-Ph-Gly-Arg-ρ-nitroanilide (Chz-TPA) were from Boehringer Mannheim Canada (Laval, Quebec). Benzoxycarbonyl-Ile-Glu(OL)-Arg-Gly-ρ-nitroanilide (S-2251), the β-Ph-Pip-Arg-ρ-nitroanilide (S-2238), and S-val-Val-aspartate-p-nitroanilide (S-2288) were from Chromogenix. Human α- and β-thrombin, factor Xa, and factor Va were from Hematologic Technologies Inc. (Essex Junction, VT). Tetramethylrhodamine-5-(and 6)-isothiocyanate, fluorescein-5-isothiocyanate (FITC), 6-(N-methylanilino)naphthalene-2-sulfonic acid (manyl), and 5-iodoacetamidofluorescein were from Molecular Probes Inc. (Eugene, OR). Methoxycarbonyl-n-norleucyl-Gly-Arg-ρ-nitroanilide (Chz-lo fibrinogen (CHO)-Xa) and N'-methyloxysulfonyl-n-Ph-Gly-Arg-ρ-nitroanilide (Chz-TPA) were from Boehringer Mannheim Canada (Laval, Quebec). Benzoxycarbonyl-Ile-Glu(OL)-Arg-Gly-ρ-nitroanilide (S-2251), the β-Ph-Pip-Arg-ρ-nitroanilide (S-2238), and S-val-Val-aspartate-p-nitroanilide (S-2288) were from Chromogenix.
FPR derivatives of the enzymes were used. For sF2 binding studies, 2 ml of 150 nm fluorescein-FPR-α- or γ-thrombin was added to a 1 × 1-cm cuvette, and fluorescence was monitored as described above. The samples were then titrated with aliquots of a solution of 5 mM sF2 (containing 150 nm appropriate fluorescein-FPR-thrombin to prevent probe dilution). After the titration, the time drive profile was analyzed, and the binding parameters were determined as above. For hirudin-(54–65) binding, the fluorescein-FPR-thrombin concentration was 250 nm and the stock hirudin-(54–65) was 100 μM.

Reciprocal Exosite Binding—Reciprocal exosite binding was investigated by observing the influence of increasing ligand occupation at one site on the amount of ligand bound at the other exosite. In the first case, the fluorescein concentration of 10 nm fluorescein-hirudin-(54–65) was monitored before and after the addition of FPR-thrombin to 25 nm, and the sample was then titrated with 3.8 nm sF2 (containing 10 nm fluorescein-hirudin-(54–65)) and changes in fluorescence were monitored in time drive. The intensity values and the $K_a$ were determined as described above. In the second case, the fluorescence of 100 nm fluorescein-sF2 was monitored before and after the addition of FPR-thrombin to 280 nm, and the sample was then titrated with 100 μM hirudin-(54–65) (containing 100 nm fluorescein-sF2).

Analysis of Binding at Both Exosites—Rhodamine-hirudin-(54–65) and fluorescein-sF2 were used to monitor simultaneous binding to both exosites. A 2-ml sample containing 150 nm FPR-thrombin and 100 nm fluorescein-sF2 was titrated with 18.3 μM rhodamine-hirudin-(54–65). The rhodamine and rhodamine fluorescence of the sample was monitored with $\lambda_m$ of 532 nm and $\lambda_m$ of 575 nm for rhodamine and for fluorescein-hirudin-(54–65) at $\lambda_m$ of 650 nm and $\lambda_m$ of 575 nm for rhodamine at each step in the titration. A blank titration lacking FPR-thrombin was used to correct the data for spectral overlap of the two probes. The displacement of fluorescein-sF2 was quantified by analysis of the $I/I_0$ values as described above. Binding of rhodamine-hirudin-(54–65) to thrombin was determined by calculating the difference in intensity of rhodamine-hirudin-(54–65) in the absence and presence of FPR-thrombin, which was taken to represent the thrombin-bound fraction of rhodamine-hirudin-(54–65). Binding of both sF2 and hirudin-(54–65) was analyzed by nonlinear regression as described above. To graphically display displacement of fluorescein-sF2 by rhodamine-hirudin-(54–65) titration, the amount of fluorescein-sF2 bound was converted to a percent of that initially bound to thrombin, prior to rhodamine-hirudin-(54–65) addition. To display rhodamine-hirudin-(54–65) binding, the amount bound was calculated as the percent of maximal rhodamine-hirudin-(54–65) binding at saturation.

Thrombin-Agarose Binding Studies—Ligand binding to immobilized thrombin was used to supplement the fluorescence studies. FITC-labeled peptides could not be used in these experiments because high salt concentrations needed in the final wash step influenced the fluorescence intensity. Instead, the effect of hirudin-(54–65) on sF2 binding to thrombin-agarose was assessed by first binding mansyl-sF2 to immobilized thrombin and then monitoring mansyl-sF2 fluorescence in the absence and presence of FPR-thrombin, which was taken to represent the thrombin-bound fraction of rhodamine-hirudin-(54–65). Binding of both sF2 and hirudin-(54–65) was analyzed by nonlinear regression as described above. To graphically display displacement of fluorescein-sF2 by rhodamine-hirudin-(54–65) titration, the amount of fluorescein-sF2 bound was converted to a percent of that initially bound to thrombin, prior to rhodamine-hirudin-(54–65) addition. To display rhodamine-hirudin-(54–65) binding, the amount bound was calculated as the percent of maximal rhodamine-hirudin-(54–65) binding at saturation.

Chromogenic Activity—The chromogenic activity of 20 nM thrombin was assessed by first binding mansyl-sF2 to immobilized thrombin-agarose and then monitoring mansyl-sF2 fluorescence in the absence and presence of FPR-thrombin, which was taken to represent the thrombin-bound fraction of rhodamine-hirudin-(54–65). Binding of both sF2 and hirudin-(54–65) was analyzed by nonlinear regression as described above. Binding of rhodamine-hirudin-(54–65) to thrombin was determined by calculating the difference in intensity of rhodamine-hirudin-(54–65) in the absence and presence of FPR-thrombin, which was taken to represent the thrombin-bound fraction of rhodamine-hirudin-(54–65). Binding of both sF2 and hirudin-(54–65) was analyzed by nonlinear regression as described above. To graphically display displacement of fluorescein-sF2 by rhodamine-hirudin-(54–65) titration, the amount of fluorescein-sF2 bound was converted to a percent of that initially bound to thrombin, prior to rhodamine-hirudin-(54–65) addition. To display rhodamine-hirudin-(54–65) binding, the amount bound was calculated as the percent of maximal rhodamine-hirudin-(54–65) binding at saturation.

To monitor binding interactions at exosite 2, we prepared a synthetic peptide corresponding to a portion of F2. The 54-amino acid peptide (sF2) is derived from residues 218 to 271 of human prothrombin (residues 63–116 of F2) in which the Cys$^{218}$–Cys$^{243}$ disulfide bond is maintained, whereas Cys$^{231}$ and Cys$^{248}$ have been replaced by Ser. In addition, Lys$^{236}$ is changed to Gln, the residue found in bovine F2, to facilitate specific FITC labeling at the amino terminus. This peptide corresponds to the inner kringle loop and the contiguous 23-residue acidic carboxyl-terminal connecting peptide of F2. To confirm its similarity to F2, binding studies were performed using a FITC derivative, fluorescein-sF2, and FPR-thrombin (Fig. 1). Addition of FPR-thrombin to fluorescein-sF2 resulted in a 1.6 ± 0.2% increase (mean ± S.D. of 6 determinations) in fluorescence intensity ($I$). When the sample was then titrated with plasma-derived F2, the $I$ returned to slightly beyond the base-line value corresponding to unbound fluorescein-sF2. This indicates that sF2 and plasma-derived F2 bind to the same site on thrombin. Analysis of fluorescein-sF2 displacement by F2 revealed a $K_d$ value of 6.8 μM, comparable to the value of 4 μM obtained in direct binding studies with F2 (not shown; see Ref. 24). To further confirm the specificity of sF2 binding, FPR-thrombin was added to a FITC-labeled scrambled homologue of sF2, containing an intact disulfide bond. There was no change in fluorescence of fluorescein in the presence of FPR-thrombin suggesting that there was no binding of the scrambled homologue to thrombin.

To assess interactions at exosite 1, FITC-hirudin-(54–65) was titrated with FPR-thrombin. The experiment yielded a $K_d$ value of 31 nM and $I/I_0$ value of 0.73 (not shown). When active thrombin was substituted for FPR-thrombin, indistinguishable results were obtained. These results are comparable to those obtained by Liu et al. (12) for the binding of fluorescein-hirudin-(53–64) to active thrombin ($K_d$ of 96 nM, $I/I_0$ of 0.82). Therefore, the FITC derivative of hirudin-(54–65) is a suitable probe for monitoring binding to thrombin.

Interactions between the Active Site and the Exosites—Binding of unlabeled sF2 to fluorescein-FPR derivatives of α- or

**FIG. 1. Synthetic F2 (sF2) competes with F2 for binding to FPR-thrombin.** The fluorescence intensity ($I$) of 100 nm fluorescein-sF2 (∗) was determined ($\lambda_m$ of 492 nm, $\lambda_m$ of 522 nm). Addition of FPR-thrombin to 280 nm increased $I$ by 1.6% (●). Aliquots of prothrombin fragment 2 (F2) were added to the cuvette, and the $I$ was determined after each addition. This led to a saturable and dose-dependent decrease in $I/I_0$, to approximately the base-line value of unbound sF2 (∗). The line shows the fit of the data to the binding isotherm and reveals a $K_d$ value of 6.8 μM for F2 binding to thrombin.
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**Fig. 2. Binding of sF2 and hirudin-(54–65) to α- or γ-fluorescein-FPR-thrombin.** A, the fluorescence intensity of 150 nM fluorescein-FPR-α- or γ-thrombin was monitored at 522 nm. Aliquots of sF2 were added and the intensity (I) determined after each addition. The ratio of the measured intensity (I) to the initial intensity (I₀) is plotted versus the sF2 concentration. Both titrations led to saturable decreases in I. Regressive analyses revealed Kᵢ values for α- and γ-thrombin of 46 and 20 μM, respectively. B, the fluorescence intensity of 150 nM fluorescein-FPR-α- or γ-thrombin was monitored at 522 nm. When fluorescein-FPR-α-thrombin was titrated with hirudin-(54–65) there was a saturable increase in I. In contrast, titration of fluorescein-FPR-γ-thrombin with hirudin-(54–65) resulted in no change in I. Regression analysis revealed a Kᵢ value of 62 nM for hirudin-(54–65) binding to fluorescein-FPR-α-thrombin. *hirudin*(54–65) bound to fluorescein-FPR-γ-thrombin.

**Fig. 3. Influence of sF2 or hirudin-(54–65) on thrombin chromogenic activity.** The chromogenic activity of 20 nM thrombin was determined with the indicated chromogenic substrates (0.5 mM). The rates of hydrolysis were normalized relative to the control thrombin activity in the absence of ligand. sF2 (80 μM) and hirudin-(54–65) (10 μM) produced opposing effects on thrombin activity with the substrates S-2238, Chz-Xa, Chz-μPA, S-2222, and S-2224. In contrast, both sF2 and hirudin-(54–65) increased thrombin activity with tGPR-pNA and S-2222.

γ-thrombin was performed to identify the sF2 binding site on thrombin. SF2 binding to both α- and γ-fluorescein-FPR-thrombin was characterized by a saturable decrease in I (Fig. 2A), and Kᵢ values of 46 and 20 μM were calculated for sF2 interaction with α- and γ-thrombin, respectively. Thus, sF2 binds to a site found on both α- and γ-thrombin. However, the sF2 peptide has about a 10-fold lower affinity for thrombin than plasma-derived F2 (Kᵢ values of 46 versus 4 μM, respectively). Binding studies with a probe for exosite 1 also were performed with fluorescein-FPR derivatives of α- and γ-thrombin. As a ligand for exosite 1, a 12-amino acid peptide corresponding to a carboxyl-terminal segment of hirudin, hirudin-(54–65), was used. This peptide binds to thrombin with an affinity similar to that of hirudin-(53–64), a peptide also known as Hirugen (7, 33). Hirudin-(54–65) binds to FPR-α-thrombin with high affinity (Kᵢ of 62 nM and I₀ of 1.07, Fig. 2B) and shows no binding to FPR-γ-thrombin. These results are consistent with the concept that hirudin-(54–65) binds to exosite 1, whereas sF2 binds to exosite 2, in agreement with the known specificity of exosites 1 and 2. Furthermore, the FITC derivatives of hirudin-(54–65) and sF2 each report binding interactions through changes in I revealing that they are responsive probes that can be used as specific ligands for thrombin exosites 1 and 2, respectively.

The binding studies with the fluorescein-FPR derivatives of thrombin (Fig. 2) suggest that ligand binding to either exosite causes conformational changes at the active site. This is supported by numerous studies demonstrating alterations in chromogenic activity of thrombin in the presence of hirudin-(54–65) or F2 (7, 12, 15, 34). In the current study, the influence of these ligands on thrombin chromogenic activity was compared directly (Fig. 3). Hirudin-(54–65) and sF2 produced opposing effects on thrombin chromogenic activity with S-2238, Chz-Xa, Chz-μPA, S-2222, and S-2224. Increased activity was induced by sF2 on two of these substrates and by hirudin-(54–65) on three substrates. With tGPR-pNA, the two ligands produced similar effects, whereas activity with S-2222 was promoted more by hirudin-(54–65) than by sF2. Although these data confirm previous reports that ligand binding to either exosite 1 or 2 affects thrombin chromogenic activity, they also demonstrate that the active site can be differentially regulated depending on the exosite specificity of the ligand.

**Interactions between Exosites**—To determine whether the two exosites are allosterically linked, the fluorescence of an FITC-labeled ligand bound to FPR-thrombin was monitored throughout a titration with an unlabeled ligand specific for the opposing exosite. In Fig. 4A, the influence of sF2 on thrombin-bound fluorescein-hirudin-(54–65) was determined. Addition of 25 nM FPR-thrombin to 10 nM fluorescein-hirudin-(54–65) resulted in a −10% decrease in I. Subsequent titration of the sample with sF2 returned the I to the original value obtained with unbound fluorescein-hirudin-(54–65). This indicates complete displacement of fluorescein-hirudin-(54–65) from thrombin. The inset shows III values for the sF2-induced displacement of thrombin-bound fluorescein-hirudin-(54–65). The line represents a nonlinear regression analysis of the sF2 binding data revealing a Kᵢ of 202 μM. This corresponds to a 4-fold reduction in the affinity of thrombin for sF2 in the presence of fluorescein-hirudin-(54–65). In the complementary experiment, thrombin-bound fluorescein-sF2 was titrated with hirudin-(54–65) (Fig. 4B). The fluorescence of 100 nM fluorescein-sF2 increased 1.7% upon addition of 250 nM FPR-thrombin, an increase similar to that observed in Fig. 1. Subsequent
ligands could bind simultaneously. To accomplish this, thrombin, it was of interest to determine whether the two hirudin-(54–65) and sF2 report binding interactions with the opposing exosite.

The observation that the affinity of thrombin for either hirudin-(54–65) was determined by nonlinear regression analysis. Thus, the affinity of thrombin for hirudin-(54–65) bound to exosite 1 on thrombin there was concomitant displacement, respectively. Therefore, as rhodamine-hirudin-(54–65) bound to exosite 1 on thrombin there was concomitant displacement of fluorescein-sF2 from exosite 2. As noted above, the affinity of thrombin for hirudin-(54–65) was reduced in the presence of sF2. To exclude the possibility that the fluorescence data reflected environmental changes of the labeled ligand rather than true displacement, we used agarose-immobilized thrombin to examine the ability of the ligands for exosites 1 and 2 to displace each other. Binding to thrombin-agarose was assessed by measuring the free labeled ligand remaining in the supernatant after brief centrifugation. These measurements were performed throughout the titration of the sample with the other, unlabeled ligand. Mansyl-sF2 and 125I-hirudin-(54–65) were used in place of the fluorescein-labeled derivatives for this study because the final, high salt elution step altered the fluorescence intensity of the fluorescein. Thrombin was bound to the support via biotin-FPR such that all molecules were coupled through their active site, leaving the exosites free to bind their ligands. With mansyl-sF2, about 46% bound to a 500-μl suspension of thrombin-agarose containing about 2 μM thrombin. Titration of the sample with hirudin-(54–65) resulted in displacement of the bound mansyl-sF2, as evidenced by almost...
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**FIG. 6. Ligand binding to thrombin-agarose.** A, a 500-µl suspension of thrombin-agarose was incubated with 0.5 µm mannsyl-sF2. After the sample was washed twice, the supernatant was monitored for mannsyl fluorescence and returned to the agarose. Aliquots of hirudin-(54–65) were added, and the fluorescence of the supernatant was determined after each addition. The total amount of bound mannsyl-sF2, determined after the addition of NaCl to 2 M, was used to calculate the percent bound at each step in the titration. The line shows the nonlinear regression analysis of the data. Hirudin-(54–65) bound to thrombin-agarose, as monitored by mannsyl-sF2 displacement, with a Ki of 2 µM. B, 125I-hirudin-(54–65), bound to thrombin-agarose as described in A, was titrated with fluorescein-sF2. The radioactivity in the supernatant was determined after each addition. The total amount of 125I-hirudin-(54–65) was determined and used to calculate the percent of hirudin-(54–65) bound. The data were analyzed by nonlinear regression analysis (line), yielding a Ki of 35 µM.

In this study we used specific ligands for exosites 1 and 2 on thrombin to demonstrate an allosteric linkage between the two exosites and to show that ligand binding to one exosite produces changes at the active site different from those induced by ligand binding to the other exosite. Hirudin-(54–65), which has properties similar to hirudin-(53–64) (7, 33), was used as a ligand for exosite 1 since there is crystallographic evidence of its specificity (11) and a fluorescein derivative has been used successfully in previous binding studies (12, 20). As a probe for exosite 2, prothrombin fragment 2 (F2) was utilized because of its defined composition and the crystallographic evidence of its specificity for exosite 2 (18, 24). To facilitate reproducible and unique fluorescent labeling, a synthetic peptide corresponding to the carboxyl-terminal half of human F2 (prothrombin residues 218–271) was synthesized. Both probes displayed binding consistent with their expected specificities since hirudin-(54–65) binds only to α-thrombin, whereas sF2 binds to both α- and γ-thrombin (Fig. 2). The integrity of exosite 2, but not exosite 1, in γ-thrombin is consistent with its ability to bind heparin (10) but not hirudin (35).

**Interactions between the Exosites and the Active Site—**Previous studies have demonstrated that F2 (12, 20, 24, 36) and hirudin-(53–64) (12, 15, 37) affect the structure and function of thrombin. In the present study, direct comparison reveals that these two ligands induce diatomic changes in the active site of thrombin. Whereas sF2 caused a 16% decrease in the intensity of fluorescein-FPR-thrombin, hirudin-(54–65) induced a 7% increase (Fig. 2). Furthermore, the two ligands also produced opposing effects on the activity of thrombin with five chromogenic substrates. The fluorescence results reveal changes in the environment of the fluorophore, which may be located up to 15 Å away from the catalytic serine residue (3, 13). Since it is unlikely that the fluorophore is proximal to both exosites, these results suggest that the intensity changes are mediated by residues in or around the thrombin binding pocket and not by contact of the fluorophore with the ligand. The data with the chromogenic substrates also point to changes in the substrate binding pocket since hirudin-(53–64) has been observed to influence the Km of thrombin for low molecular weight substrates (7). Although the fluorescence and the functional data cannot be directly correlated, taken together they suggest that the active site is differentially modulated depending on which exosite is occupied. This points to the possibility that exosites 1 and 2 play distinct roles in regulating thrombin activity. This concept is supported by studies examining the role of exosite 2 in the activation of protein C by thrombin bound to thrombomodulin. Binding of chondroitin sulfate or F2 to exosite 2 or mutation of basic residues in exosite 2 reduces the rate of protein C activation by thrombin complexed with chondroitin sulfate-deficient thrombomodulin and alter the calcium dependence of the reaction (20, 38). These data suggest that ligands for exosites 1 and 2 have different effects on the active site.

Crystallographic studies of the F2 or hirudin-(53–64) complex with FPR-thrombin show no major changes in thrombin structure (11, 18). However, the allosteric changes detected by solution-phase binding studies may reflect movement of flexible loops of thrombin that are poorly defined in the crystal structure (18, 39). Two of these flexible loops reside in the vicinity of the active site and thus are good candidates for regulatory domains (3, 11, 18).

**DISCUSSION**

In this study we used specific ligands for exosites 1 and 2 on thrombin to demonstrate an allosteric linkage between the two exosites and to show that ligand binding to one exosite produces changes at the active site different from those induced by ligand binding to the other exosite. Hirudin-(54–65), which has properties similar to hirudin-(53–64) (7, 33), was used as a ligand for exosite 1 since there is crystallographic evidence of its specificity (11) and a fluorescein derivative has been used successfully in previous binding studies (12, 20). As a probe for exosite 2, prothrombin fragment 2 (F2) was utilized because of...
Our findings are supported by the work of other investigators. F2 has been reported to reduce the affinity of thrombin for hirudin-(53–64) and thrombomodulin (12, 20), to reduce thrombin clotting activity (36), and to inhibit thrombomodulin-dependent protein C activation (36, 38). Since exosite 1 is involved in all these reactions, these results suggest that F2 has the capacity to modulate the function of exosite 1. Ligands other than F2 can also influence exosite 1 function. For example, heparin reduces the rate at which hirudin inhibits thrombin (40), and thrombin binding to heparin-agarose is abrogated by hirudin (41). These results suggest that the allosteric linkage between the exosites constitutes a general regulatory mechanism that is not solely confined to hirudin-(54–65) and F2.

Although the data presented herein suggest that only one exosite can be occupied at a time, there are examples of simultaneous occupation of both exosites. Thrombomodulin binds to exosite 1 via its growth factor domains and to exosite 2 via the chondroitin sulfate moiety (20). Both exosites contribute to binding because intact thrombomodulin binds thrombin more tightly than chondroitin sulfate-deficient thrombomodulin (12). Another example of concomitant occupation of both exosites is the ternary thrombin-fibrin-heparin complex in which fibrin binds to exosite 1 and heparin to exosite 2 of thrombin (42). In both of these complexes, the glycosaminoglycan serves to augment affinity for thrombin by binding to exosite 2 as well as to the ligand occupying exosite 1. This demonstrates another mechanism by which thrombin utilizes exosites 1 and 2 and reveals that different ligands may play distinct roles in thrombin activity.

Conclusions—The current study provides three major observations regarding the role of exosite 2 in the regulation of thrombin. The first observation is that exosite 2 serves as an allosteric modulator of thrombin structure and function. This highlights its similarity to exosite 1, where considerable support exists for an allosteric linkage between exosite 1 and the active site (7, 8, 12–15, 43, 44). The second observation is that ligands for exosites 1 and 2 have different effects on the active site of thrombin, as judged by both fluorescence and functional studies. This suggests that the two exosites play distinct regulatory roles, possibly by invoking unique conformational changes. The third observation is that there is direct allosteric linkage between the two exosites. Conformational linkage between the two exosites and between the exosites and the active site could be exploited by thrombin to evoke ligand-specific responses. These findings point to a greater complexity in modulation of thrombin function and suggest new avenues of physiological and pharmacological regulation of thrombin activity.

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