Localization of the Thrombin-binding Domain on Prothrombin Fragment 2*

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Co-crystallographic studies have shown that the interaction of human prothrombin fragment 2 (F2) with thrombin involves the formation of salt bridges between the kringle inner loop of F2 and anion-binding exosite II of thrombin. When F2 binds to thrombin, it has been shown to evoke conformational changes at the active site and at exosite I of the enzyme. Using plasma, recombinant, and synthetic F2 peptides (F2, rF2, and sF2, respectively) we have further localized the thrombin-binding domain on F2. F2, rF2-(1–116), rF2-(55–116), and sF2-(63–116), all of which contain the kringle inner loop (residues 64–93) and the acidic COOH-terminal connecting peptide (residues 94–116), bind to thrombin-agarose. In contrast, analogues of the kringle inner loop, sF2-(63–90), or the COOH-terminal connecting peptide, sF2-(92–116), do not bind. Thus, contrary to predictions from the crystal structure, the COOH-terminal connecting peptide as well as the kringle inner loop are involved in the interaction of F2 with thrombin. F2 and sF2-(63–116) bind saturably to fluorescently labeled active site-blocked thrombin with $K_d$ values of 4.1 and 51.3 $\mu$m, respectively. The affinity of sF2-(63–116) for thrombin increases about 5-fold ($K_d = 10 \mu$m) when Val at position 78 is substituted with Glu. F2 and sF2-(63–116) bind to exosite II on thrombin because both reduce the heparin-catalyzed rate of thrombin inhibition by antithrombin ~4-fold. In contrast, only F2 slows the uncatalyzed rate of thrombin inactivation by antithrombin. Like F2, sF2-(63–116) induces allosteric changes in the active site and exosite I of thrombin because it alters the rates of thrombin-mediated hydrolysis of chromogenic substrates and displaces fluorescently labeled hirudin$_{4-45}$ from active site-blocked thrombin, respectively. Both peptides also prolong the thrombin clotting time of fibrinogen in a concentration-dependent fashion, reflecting their effects on the active site and/or exosite I. These studies provide further insight into the regions of F2 that evoke functional changes in thrombin.

Prothrombin, a 581-amino acid plasma glycoprotein, is converted to the serine protease thrombin in the final stages of the blood coagulation cascade. The proteolytic conversion of prothrombin to thrombin is catalyzed by prothrombinase, an enzyme complex composed of the serine protease factor Xa, the cofactor Va, phospholipids, and calcium (1, 2). During this reaction, prothrombin is cleaved into three fragments: fragment 1 (F1)$^1$ (consisting of a $\gamma$-carboxyglutamic acid (Gla) domain and the kringle 1 domain), fragment 2 (F2) (consisting of the kringle 2 domain), and the catalytic domain (3). The Gla domain of F1 facilitates calcium-dependent binding of the proenzyme to phospholipid surfaces (4). Although the F2 domain has been shown to interact with factor Va (5), recent studies indicate that F2-factor Va interactions are not required for factor Va to enhance the catalytic efficiency of factor Xa within the prothrombinase complex (6). Rather, the function of F2 in prothrombin may be to alter the conformation of the proenzyme so that its scissile bond(s) is more complementary to the active site of factor Xa (6).

After release from prothrombin, F2 retains its ability to bind thrombin and influences thrombin function. Thus, F2 has been reported to slow the rate of thrombin inactivation by antithrombin (AT) (7), alter the environment of the catalytic site (8), enhance the esterolytic activity of thrombin (9), inhibit the clotting activity of thrombin (10) and modulate the calcium dependence of protein C activation (11). Crystallographic and chemical modification studies have shown that F2 interacts with a highly electropositive region in the COOH terminus of thrombin, also known as anion-binding exosite II or the heparin-binding region of thrombin (12–14). The crystallographic structure of F2 complexed with active site-blocked thrombin reveals numerous ionic interactions between the anionic inner loop of the F2 kringle and the COOH-terminal helix of thrombin, a region abundant in arginine and lysine residues (12–16).

In this study, we set out to further characterize the thrombin-binding domain within F2. Using plasma, recombinant, and synthetic F2 peptides (F2, rF2, and sF2, respectively), we report that, contrary to what would have been predicted from the crystallographic data, residues in the COOH-terminal connecting peptide as well as the inner loop of the F2 kringle are necessary for F2 interaction with thrombin. In addition, to identify the regions in F2 that evoke functional changes in thrombin, we also compared sF2-(63–116), a thrombin-binding domain on F2.

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1 The abbreviations used are: F1, prothrombin fragment 1; F2, prothrombin fragment 2; sF2, synthetic F2 peptides; rF2, recombinant F2 peptides; AT, antithrombin; ANS, anilino naphtalene-6-sulfonic acid; FPR, d-phenylalanyl-l-propyl-l-arginine chloromethyl ketone; FITC, fluorescein 5-isothiocyanate; ATA-FPR, N-(acetyltihio)acetyld-Phe-Pro-Arg-CH$_2$CI; PAGE, polyacrylamide gel electrophoresis; PFR, polymerase chain reaction.

2 Sequential numbering of amino acids in F2 begins at the first amino acid of F2 (corresponds to amino acid 218 of prothrombin). The chymotrypsinogen numbering system is used for thrombin (15).

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ing analogue encompassing the kringle inner loop and the COOH-terminal connecting peptide, with F2 in terms of their ability to modulate thrombin function.

**EXPERIMENTAL PROCEDURES**

**Materials**—Oligonucleotides were synthesized by the Institute for Molecular Biology and Biotechnology, McMaster University, p-Phenylalanine-1-prolyl-1-arginine chloromethyl ketone (FPR) was from Novabiochem Int'l. (San Diego, CA). Biotin-FPR was from Hematologic Technologies Inc. (Essex Junction, VT). Monospecific polyclonal IgGs against human prothrombin and AT, isolated from human plasma by affinity chromatography, were from Affinity Biologicals Inc. (Hamilton, ON). Heparin, hirudin, anti-sheep IgG alkaline phosphatase, streptavidin-digoxigenin-agarose, and N-p-tosyl-Gly-Pro-Arg p-nitroanilide were from Sigma. Fluorescein-5-isothiocyanate (FITC) was from Molecular Probes Inc. (Eugene, OR). N-Methylsulfonyl-l-Phe-Gly-Arg p-nitroanilide (Chx-tPA) was from Boehringer Mannheim Canada (Laval, PQ). Benzoylxy-carbonyl-He-Glu-(OR)-Gly-Arg p-nitroanilide (S2222), H-p-Phe-Pip-Arg p-nitroanilide (S2238), pyro-Glu-Gly-Arg p-nitroanilide (S2444), l-pyroglutamyl-l-Pro-l-Arg p-nitroanilide hydrochloride (S2366), and H- l-Ile-Pro-Arg p-nitroanilide (S2288) were from Chromogenix (Helena Laboratories, Mississauga, ON). Cellulose ester dialysis membranes were from Spectra/Por (Houston, TX). All other chemicals were of the highest grade commercially available.

**Synthetic Peptides**—Cyclized synthetic peptides, purified by preparative reverse phase high performance liquid chromatography, were prepared by Chiron Mimotopes Peptide Systems (San Diego, CA) as described by Federnburg et al. (17). The cyclized peptides were resuspended in TBS (20 mM Tris-HCl, pH 7.4, 150 mM NaCl), and titrated to prepared by Chiron Mimotopes Peptide Systems (San Diego, CA) as

| Primer | Sequence | Amino acid hybridization site in F2 |
|--------|----------|-----------------------------------|
| A      | 5′-GGATCATGGCGGAGCGTTCAGTGAT-3′ | 116 |
| B      | 5′-GGATCATGGCGGAGCTGGCAGTTGAGGAG-3′ | 55 |
| C      | 5′-GGATCATGGCGGAGCTGGCAGTTGAGGAG-3′ | 55 |
| D      | 5′-GGATCATGGCGGAGCTGGCAGTTGAGGAG-3′ | 55 |
| E      | 5′-GGATCATGGCGGAGCTGGCAGTTGAGGAG-3′ | 55 |
| F      | 5′-GGATCATGGCGGAGCTGGCAGTTGAGGAG-3′ | 55 |

**To ensure proper disulfide bond formation and to prevent the formation of intermolecular complexes, PCR mutagenesis was used to convert Cys76 and Cys93 to serine residues. Briefly, plasmid pB5-hFII was PCR amplified using primer B (Table I), which hybridizes downstream of the mutagenizing site of plasmid pB5-hFII, at a concentration of 1 mg/ml were subjected to electrophoresis in 15% SDS-polyacrylamide gels under reducing conditions (19), transferred to nitrocellulose, and visualized by Ponceau S staining and immunoblotting as described previously (20).**

**Expression and Purification of Recombinant F2 Peptides—**Recombinant F2 peptides were expressed in *Escherichia coli* BL21(DE3) by isopropyl-1-thio-D-galactoside induction using the histidine-tag pET system (Novagen, Inc., Madison, WI). The expression vector used, pET22b(+), directs the recombinant proteins to the periplasmic space thereby promoting proper folding and disulfide bond formation. The periplasmic fraction was obtained using the cold osmotic shock method described by Ausubel et al. (18). Histidine-binding Ni²⁺ resin (Novagen, Inc.) was used to purify the recombinant proteins. The isolated proteins at a concentration of 1 mg/ml were subjected to electrophoresis in 10% SDS-polyacrylamide gels under reducing conditions (19), transferred to nitrocellulose, and visualized by Ponceau S staining and immunoblotting as described previously (20).
ting as described above. The protein elution profiles were obtained by laser densitometry scans of immunoblots using the UltraScanTM XL laser densitometer (Pharmacia LKB Biotechnology). The density of F2 in each fraction was expressed as a percentage of the total F2 density in the complete elution profile.

Results—

The dissociation of F2 and rF2-(1–116)—The disulfide bonds in F2 and rF2-(1–116) were reduced and alkylated as described (22). The peptides were resuspended in 50 mM dithiothreitol at a concentration of 1 mg/mL and placed at room temperature for 30 min. Iodoacetamide (made up fresh in sterile distilled water) was then added to 50 mM and the reaction mixture was placed at room temperature for 45 min in the dark. The reaction mixture was then dialyzed in HBSE buffer, incubated with 10-fold molar excess Tris-HCl, pH 7.4, 150 mM NaCl, 0.01% Tween 20 using Spectra/Per cellulose ester dialysis membrane (10-kDa cutoff) and the protein concentration determined as described above for intact F2.

Determination of the Affinity of F2 Peptides for Thrombin—The association between F2 peptides and thrombin was monitored by the F2-dependent fluorescence intensity change of anilinonaphthalene-6-sulfonic acid-thrombin (ANS-FFP-thrombin). ANS-FFP-thrombin was prepared using ATA-FFP-N-(acetylthio)acetyl-b-Pro-Arg-Ch(2,3)Cl) as described by the supplier (Molecular Innovations Inc., Royal Oak, MI). Briefly, 10.6 μM thrombin was incubated for 30 min at room temperature with 2.5-fold molar excess ATA-FFP in HBSE buffer (20 mM HEPES, pH 7.0, 100 mM NaCl, 1 mM EDTA). The reaction mixture was then dialyzed in HBSE buffer, incubated with 10-fold molar excess 2-(4-iodoacetamide)anilino)naphthalene-6-sulfonic acid (Molecular Probes Inc.) in the presence of hydroxylamine for 60 min at room temperature in the dark, and then dialyzed. 500 μL of 100 nM ANS-FFP-thrombin was added to the same buffer in a semi-micro quartz cuvette. Using a Perkin-Elmer LS50B luminescence spectrometer with excitation and emission wavelengths set to 328 and 450 nm, respectively, and excitation and emission slit widths set to 12 nm, and an emission filter of 390 nm, readings were taken of ANS-FFP-thrombin alone (Io). Known quantities of F2 peptides (3.8 μM) were then added to the cuvette and, after mixing, the change in fluorescence was monitored (I).

As a control, a scrambled variant of the 54-amino acid peptide was titrated to the same concentration, and any change in fluorescence due to nonspecific binding was used to correct the binding curve. The Kd values were calculated by plotting Io versus F2 peptide concentration. The parameters Kd and α were calculated by nonlinear regression (TableCurve, Jandel Scientific, San Rafael, CA) using the equation: 

$$\frac{I}{Io} = 1 + \frac{Kd}{[F2]}$$

where α is the maximum fluorescence change and assuming a stoichiometry of 1 (17).

Dissociation of Fluorescein-hirudin54-65 from F2 peptide. Fluorescein-hirudin54-65 was labeled with FITC as described by Liu et al. (23). 2 mL of TBS containing 10 nM FITC-hirudin54-65 was added to a 1 cm × 1 cm quartz cuvette in a Perkin-Elmer LS50B luminescence spectrometer. Excitation and emission wavelengths were set to 492 and 522 nm, respectively, and excitation and emission slit widths were both set to 8 nm. The fluorescence intensity of thrombin (1 μM) was measured before (Io) and after (I) the addition of either 20–40 μL aliquots of 161 μM F2 or 2–5 μL aliquots of 3.8 μM F2 (63–116). Both F2 and sF2-(63–116) solutions contained 10 μM FITC-hirudin54-65 so that the concentration of FITC-hirudin54-65 remained constant. Titration was continued up to 43 μM F2 or 300 μM sF2-(63–116). After the experiment, intensity values were read from time drive profiles and Io values were calculated and plotted versus the peptide concentration. As a control, the fluorescence intensity was measured before and after the addition of up to 100 μM reduced and alkylated sF2-(63–116).

Effect of F2 on Thrombin-mediated Clotting of Fibrinogen—Thrombin, at a final concentration of 0.6 μM, was mixed with either F2 (0–50 μM) or sF2-(63–116) (0–300 μM) in TSTW buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.01% Tween 20) in a final volume of 75 μL. 25 μL of human fibrinogen and CaCl2 were then added to final concentrations of 4 μM and 2 mM, respectively, and the times for half-maximal increase in turbidity (T50) were determined at 23 °C by continuous measurement of absorbance at 405 nm using a Molecular Devices plate reader.

Effect of F2 on Thrombin-mediated Hydrolysis of Chromogenic Substrates—The thrombinolytic activity of 20 nM thrombin in TBS was determined with 0.7–14 μM of various chromogenic substrates, Nε-γ-carboxy-Ser-Pro-Arg-GLY-Pro-Arg-p-nitroanilide in 200 μL of TBS containing 10 mg/mL Polybrene. Thrombin inhibitory activity in the presence of 1 unit/mL heparin was determined using thrombin and AT concentrations of 5 and 50 nM, respectively. Residual thrombin activity was then calculated by measuring absorbance for 5 min at 405 nm using a Molecular Devices plate reader. The pseudo first-order rate constants (kₕ) for thrombin inhibition were determined by fitting the data to the equation: 

$$k_{cat} = \ln([Pf]/[P]),$$

where [Pf] is initial thrombin activity and [P] is thrombin activity at time t (24). The second-order rate constant, kₕ, was determined by dividing kₕ by the AT concentration.

RESULTS

Purification of F2 Peptides—F2 is a 116-amino acid prothrombin activation fragment that consists of a 14-residue interkringle peptide (region A), a 79-residue kringle (regions B, C, D, and E), and a 25-residue acidic COOH-terminal kringle-catalytic domain-connecting peptide (region F) (Fig. 1, panel A). For simplicity, the latter is referred to as the COOH-terminal connecting peptide. We mapped the thrombin-binding domain of F2 using deletion and point mutants prepared by recombinant and synthetic techniques. Schematic diagrams of the F2 peptides are shown in Fig. 1 (panel B) where F2, sF2, and rF2 denote plasma, synthetic, and recombinant F2 peptides, respectively. The deletion mutants encompass various portions of F2 including the kringle inner loop (residues 63–90), the kringle outer loop (residues 1–55), the COOH-terminal connecting peptide (residues 92–116), and the COOH-terminal half of F2 (residues 55–116).

The predicted molecular masses of F2, rF2-(1–116), and the three deletion derivatives rF2-(1–55), rF2-(55–116), and rF2-(1–93) are 14, 15, 6, 7, and 11 kDa, respectively. These predicted values are consistent with the apparent molecular masses of the peptides as determined by SDS-PAGE and Western blot analysis (Fig. 2). No immunoreactive material was found in lysates of E. coli transformed with pET22b (+), the parent vector lacking the F2 cDNA (data not shown). The presence of disulfide bonds in the F2 peptides was verified by comparing the electrophoretic mobilities of reduced versus non-reduced peptides. Disulfide bond-containing peptides migrated more slowly under reducing conditions than under non-reducing conditions (data not shown).

Thrombin-agarose Affinity Chromatography—The affinities of F2 peptides for thrombin were qualitatively assessed by subjecting the peptides to thrombin-agarose affinity chromatography. Biotin-FFP-treated thrombin was coupled to streptavidin-agarose as described under “Experimental Procedures.” This directed coupling procedure ensures that all surface basic residues are unmodified and that all the thrombin molecules are coupled in the same fashion. F2 peptides were applied to the column in 10 mM NaCl, 20 mM Tris-HCl, pH 7.4, and the column was then washed with increasing concentrations of NaCl. Aliquots from the eluates were analyzed by SDS-PAGE and immunoblotting. The protein elution profiles were determined by laser densitometry scans of immunoblots. Intact F2 and rF2-(1–116) were blocked on the thrombin-agarose column at concentrations up to 300 and 250 mM NaCl, respectively (Fig. 3, panel A). Upon reduction and alkylation of the disulfide bonds, both F2 and rF2-(1–116) eluted from the thrombin-agarose column as sharp peaks at 80 mM NaCl (Fig. 3, panel A). Bovine serum albumin, used as a negative control for thrombin-agarose binding, eluted from the column at 60 mM NaCl (data not shown). Like rF2-(1–116), the deletion derivatives rF2-(55–116) and sF2-(63–116) were retained on the thrombin-
agarose column at NaCl concentrations up to 250 and 300 mM NaCl, respectively (Fig. 3, panel B). Both of these peptides contain the kringle inner loop (residues 63–93) as well as the COOH-terminal connecting peptide (residues 94–116). In contrast, deletion derivatives that contain only the kringle inner loop, sF2-(63–90), or the COOH-terminal connecting peptide, sF2-(92–116), did not bind to thrombin-agarose. Furthermore, neither the NH2-terminal interkringle peptide (residues 1–14) nor the outer loop of the kringle (residues 15–55) is required for thrombin-agarose binding since rF2-(1–55) and rF2-(1–93) bound weakly to thrombin-agarose (Fig. 3, panel B).

Determination of the Affinities of F2 Peptides for Thrombin—
The affinities of F2 peptides for ANS-FPR-thrombin were determined quantitatively by monitoring the change in probe fluorescence during peptide titration as described by Bock (8). After titration, the I/Io values were plotted versus F2 peptide concentration and the data analyzed by nonlinear regression. Because of the low yields of rF2 peptides, Keq values were only determined for F2 and sF2 peptides. F2 and sF2-(63–116), both of which contain the kringle inner loop as well as the COOH-terminal connecting peptide, bind saturably to ANS-FPR-thrombin with Keq values of 4.1 and 51.3 μM, respectively (Fig. 4, Table I). The binding of F2 and sF2-(63–116) to ANS-FPR-thrombin is specific because there was no change in fluorescence intensity of ANS-FPR-thrombin in the presence of a scrambled variant of sF2-(63–116) (data not shown). Previous
results, demonstrating (a) competitive binding of F2 and sF2-(63–116) to FPR-thrombin and (b) binding of both F2 and sF2-(63–116) to γ-thrombin, a proteolytic derivative of thrombin lacking exosite I, reveal that F2 and sF2-(63–116) bind to exosite II of thrombin (17).

Further truncation of sF2-(63–116) to peptides which only span the kringle inner loop, sF2-(63–90) and sF2-(63–94), or only encompasses the COOH-terminal connecting peptide, sF2-(92–116), results in derivatives that do not bind to ANS-FPR-thrombin (Table II). A mixture of sF2-(63–90) and sF2-(92–116) also does not affect the fluorescence intensity of ANS-FPR-thrombin, indicating that the COOH-terminal connecting peptide must be contiguous with the kringle inner loop for effective interaction with thrombin (Table II).

In an attempt to modify the number of salt bridges formed with exosite II of thrombin, five separate amino acid substitutions were introduced into sF2-(63–116), and the affinities of the mutant peptides for ANS-FPR-thrombin were measured (Table II). Substitution of Val78 with Glu increased the affinity of sF2-(63–116) for ANS-FPR-thrombin 5-fold (Kd decreased from 51.3 to 10.0 μM), whereas substitution of Tyr 87 with Glu produced only a marginal increase (Kd decreased from 51.3 to 40.7 μM). In contrast, F85E and K81Q mutations decreased the affinity of sF2-(63–116) for ANS-FPR-thrombin 2-fold, whereas substitution of Lys81 with Glu abolished binding. These studies indicate that specific amino acid substitutions within the kringle inner loop can affect the affinity of F2 for thrombin.

**Functional Comparison of F2 and sF2-(63–116)**—To identify the F2 domains that evoke conformational changes in thrombin, we compared the effects of F2 and sF2-(63–116) on

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**FIG. 3. Thrombin-agarose affinity chromatography.** Panel A, elution of native and reduced forms of F2 and rF2-(1–116) from thrombin-agarose. F2 peptides were subjected to thrombin-agarose affinity chromatography and eluted with an NaCl gradient from 10 to 500 mM. Aliquots from the eluates were analyzed by SDS-PAGE and immunoblotting. The protein elution profiles were obtained by laser densitometry where the density of each lane was expressed as a percentage of the combined density of all the lanes in a given elution. Symbols: ●, reduced F2; ○, reduced rF2-(1–116); ■, native F2; and □, native rF2-(1–116). Panel B, elution of F2 derivatives from thrombin-agarose. F2 derivatives were subjected to thrombin-agarose affinity chromatography and eluted with an NaCl gradient as described above. Symbols: ●, sF2-(63–90); ○, sF2-(92–116); ■, rF2-(1–55); □, rF2-(1–93); △, rF2-(55–116); and Δ sF2-(63–116).

**FIG. 4. Binding of F2 and sF2-(63–116) to ANS-thrombin.** Increasing amounts of F2 (panel A) or sF2-(63–116) (panel B) were mixed with 100 nM ANS-thrombin and changes in fluorescent intensity were monitored. I/Io is plotted versus peptide concentration, where I is the fluorescent intensity at a given peptide concentration and Io is the initial fluorescence intensity. The Kd values were determined by nonlinear regression analyses of the data (line).

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| Peptide          | Kd (μM) |
|------------------|---------|
| F2               | 4.1     |
| sF2-(63–116)     | 51.3    |
| sF2-(63–90)      | No binding |
| sF2-(63–94)      | No binding |
| sF2-(92–116)     | No binding |
| sF2-(63–90) and sF2-(92–116) | No binding |
| V78E             | 10.0    |
| K81E             | No binding |
| Y87E             | 40.7    |
| F85E             | 99.8    |
| K81Q             | 94.0    |

**TABLE II**

Dissociation constants for the interaction of F2 peptides with ANS-FPR-thrombin

The affinities of F2 peptides for ANS-FPR-thrombin were determined by monitoring the F2 peptide-dependent fluorescent intensity changes of ANS-FPR-thrombin as described under “Experimental Procedures.”

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rates of thrombin inhibition by AT, (b) the rates of thrombin-mediated hydrolysis of chromogenic substrates, (c) thrombin clotting times, and (d) displacement of fluorescein-labeled hirudin$_{54-65}$ from FPR-thrombin. Like F2, saturating amounts of sF2-(63–116) reduce the heparin-catalyzed rate of thrombin inhibition by AT approximately 4-fold, indicating that both peptides compete with heparin for binding to exosite II on thrombin (Fig. 5). In the absence of heparin, however, only F2 slows the rate of thrombin inhibition by AT (Fig. 5). Upon reduction and alkylation, neither F2 nor sF2-(63–116) has any inhibitory effect on the rate of thrombin inhibition by AT either in the absence or presence of heparin (data not shown). As shown in Fig. 6, both F2 and sF2-(63–116) increase the chromogenic activity of thrombin with substrates S2238, ChtPA, and S2244. With chromogenic substrates S2366 and S2288, opposing effects were induced by the two peptides.

Both F2 and sF2-(63–116) also cause a concentration-dependent increase in the thrombin clotting time of fibrinogen (Fig. 7). Upon reduction and alkylation, sF2-(63–116) not only loses its ability to increase the thrombin clotting times, but appears to promote clotting to a small extent (Fig. 7). These data confirm previous reports that ligand binding to exosite II alters the conformation and function of the active site. However, the effect of the peptides on thrombin clotting times may also reflect ligand-induced changes at exosite I since both F2 and sF2-(63–116) displace fluorescein-labeled hirudin$_{54-65}$ from FPR-thrombin. As shown in Table III, titration of FPR-thrombin with up to 300 μM sF2-(63–116) causes complete displacement of fluorescein-labeled hirudin$_{54-65}$ from FPR-thrombin. The effect of F2 and sF2-(63–116) on hirudin$_{54-65}$ binding to thrombin was examined by binding fluorescein-hirudin$_{54-65}$ to FPR-thrombin and monitoring changes in the fluorescence intensity when the FPR-thrombin was titrated with F2, sF2-(63–116), and reduced and alkylated sF2-(63–116).

![Fig. 5. Effects of F2 and sF2-(63–116) on the rates of thrombin inhibition by AT.](image1)

![Fig. 6. Influence of F2 and sF2-(63–116) on the chromogenic activity of thrombin.](image2)

![Fig. 7. Effect of F2 and sF2-(63–116) on thrombin clotting times.](image3)

**Table III**

| Peptide       | Concentration | % Displacement of fluorescein-hirudin$_{54-65}$ from FPR-thrombin | $K_d$ μM |
|---------------|---------------|--------------------------------------------------------------------|---------|
| sF2-(63–116)  | 300 μM        | 100                                                                | 200 μM  |
| F2            | 41            | 45                                                                 | ND      |
| Reduced sF2   | 100           | 0                                                                  | No binding |

*ND, not determined.*

**DISCUSSION**

Previous co-crystallization studies of F2 with active site-blocked thrombin revealed that F2 makes contacts of less than
4 Å with Arg⁹¹, Arg⁹⁷, Arg¹⁰¹, and Arg¹⁷⁵ of the heparin-binding region of thrombin² (12, 13). These positively charged residues on thrombin form salt bridges with the anionic motif DGDEE (residues 68–72) in the kringle inner loop of F2. In this study, we experimentally defined the regions of F2 required for thrombin binding. Our data indicate that, in addition to the kringle inner loop (residues 64–93), the COOH-terminal connecting peptide (residues 94–116) also plays a role in the interaction of F2 with thrombin. The requirement of the COOH-terminal connecting peptide of F2 for F2-thrombin interactions would not have been predicted from the F2-thrombin co-crystallized structure because the F2 interkringle connecting peptides (regions A and F, Fig. 1) were disordered in the crystal (12, 13). However, recent molecular modelling suggests that the COOH-terminal connecting peptide is in contact with basic residues within exosite II (25), a concept supported by our data.

The interaction of F2 with thrombin is conformation-dependent and ionic in nature because the binding of F2 and rF2-(1–116) to thrombin-agarose is abolished when the peptides are reduced and alkylated and binding of F2 deletion mutants to thrombin-agarose is dependent on the NaCl concentration. In support of the ionic nature of the F2-thrombin interaction, the reported $K_d$ values for the binding of F2 to thrombin in low ionic strength buffer (0.05 M Tris-HCl, pH 8.1) and in physiological ionic strength buffer (0.05 M Tris-HCl, pH 7.5, 150 mM NaCl) are 770 μM (9) and 5 μM (8), respectively. In the physiological ionic strength buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl) used in our studies, F2 and sF2-(63–116) bind saturably to ANS-FPR-thrombin with $K_d$ values of 4.1 and 51.3 μM, respectively. The $K_d$ values determined by fluorescence (4.1 μM in this work, 5 μM in Ref. 8), are in agreement with $K_d$ values reported for the influence of F2 on AT inhibition (7), fibrinogen clotting (10), and protein C activation (10). Thus, at physiological ionic strength, F2 binds thrombin with a $K_d$ of about 5 μM. In contrast, F2 has been reported to bind prethrombin 2 with a $K_d$ of 33 nM (6). These differences could reflect structural diversity between prethrombin 2 and thrombin.

The $K_d$ values were determined by monitoring the changes in ANS-FPR-thrombin fluorescence intensity during peptide titration. Although both F2 and sF2-(63–116) elicit changes in the thrombin active site environment upon binding, the changes in fluorescence signal are diametric (Fig. 4). The diametric changes may reflect differences in the conformation of the COOH-terminal connecting peptide of sF2-(63–116) relative to full-length F2. However, three lines of evidence suggest that the structural integrity of the COOH-terminal connecting peptide is maintained in sF2-(63–116). First, the COOH-terminal connecting peptide alone does not bind to thrombin; only when this peptide is contiguous with the kringle inner loop of F2 does binding occur (Table II). Second, like F2, sF2-(63–116) loses its ability to bind thrombin-agarose upon reduction and alklylation of disulfide bonds (data not shown). Third, rF2-(1–93), which contains all but the COOH-terminal connecting peptide of F2, fails to bind to thrombin-agarose (Fig. 3).

The diametric changes in the thrombin active site environment elicited by the binding of F2 and sF2-(63–116) may instead arise from fewer sites of contact with thrombin for the truncated peptide relative to full-length F2. This is supported by the weaker affinity of rF2-(63–116) for thrombin compared with F2. The lower affinity of sF2-(63–116) may reflect the absence of residues in loop C (Leu⁶⁷, His⁴⁰, and Gln⁴¹) that form interdomain contacts of less than 4 Å with thrombin (12, 13). However, because rF2-(1–55) and rF2-(1–93), both of which contain loop C, fail to bind to thrombin-agarose, the NH₂-terminal half of F2 likely plays only an indirect role in thrombin binding, either by contributing to the stability of F2 or by providing secondary contacts with thrombin. The distinct structural changes that occur in thrombin when it associates with F2 and sF2-(63–116) are similar to unique binding interactions of thrombin with different forms of thrombomodulin. Although thrombin binds to the fifth and sixth epidermal growth factor-like domains of thrombomodulin with high affinity (26), the fourth through sixth epidermal growth factor-like domains are needed for optimal protein C activation by thrombin (27, 28) and for induction of structural changes in the active site of thrombin (29).

In an attempt to modify ionic interactions between the kringle inner loop of F2 and exosite II of thrombin, five separate amino acid substitutions were introduced into sF2-(63–116) and the affinities of the resultant peptides for thrombin were determined. Substitution of Val⁷⁸ with Glu increased the affinity of sF2-(63–116) for ANS-FPR-thrombin approximately 5-fold ($K_d$ decreased from 51.3 to 10.0 μM) giving it an affinity similar to that of F2 ($K_d$ = 4.1 μM), whereas substitution of Tyr⁶⁷ with Glu produced only a modest increase in affinity ($K_d$ decreased from 51.3 to 40.7 μM). Both of these mutations likely increase the number of ionic interactions formed between F2 and thrombin. In contrast, substitution of Lys⁸¹ with Gln decreased the affinity of sF2-(63–116) for thrombin approximately 2-fold and substitution of Lys⁸¹ with Glu abolished the binding of sF2-(63–116) to ANS-thrombin. Since Lys⁸¹ was not observed to make ionic contact with thrombin (12, 13), one possible explanation for the decreases in thrombin affinity is that mutations in the second inner loop of the kringle distort the hairpin β-turn which has been shown to pivot at Val⁷⁸ and Asp⁸¹ (12, 13). The stability of F2 appears to be enhanced by aromatic stacking components since substitution of Phe⁸⁵ with Glu decreases the affinity of sF2-(63–116) for thrombin approximately 2-fold ($K_d$ increased from 51.3 to 99.8 μM).

Functional comparison of F2 with sF2-(63–116), both of which contain the kringle inner loop (loops D and E) as well as the COOH-terminal connecting peptide (loop F), indicates that both peptides influence the chromogenic activity of thrombin (Fig. 6), producing similar effects on four substrates and opposing effects on two others. Although these results illustrate changes in the active site environment of thrombin due to ligand binding to exosite II, they also suggest that there may be additional or altered contacts of F2 compared with sF2-(63–116). Both F2 and sF2-(63–116) displace fluorescein-labeled hirugen from FPR-thrombin, indicating that binding of either ligand to exosite II affects the binding properties of exosite I, a phenomenon previously demonstrated by Fredenburgh et al. (17). F2 and sF2-(63–116) also prolong the thrombin clotting time of fibrinogen in a dose-dependent manner (Fig. 7). That F2 appears to be a more potent inhibitor of thrombin clotting activity likely reflects its higher affinity for thrombin. The effect of the peptides on thrombin clotting times may reflect conformational changes at the active site and/or allosteric changes at exosite I that result in decreased fibrinogen binding.

Walker and Eason (7) reported that F2 slows the rate of thrombin inactivation by AT because it inhibits AT binding to thrombin. In this study, we demonstrated that, unlike F2, sF2-(63–116) has no inhibitory effect on the rate of thrombin inhibition by AT in the absence of heparin. One possible explanation for these findings is that the NH₂-terminal connecting peptide or possibly the outer loop of F2, both of which are missing from sF2-(63–116), sterically block access of AT to the active site of thrombin. Alternatively, F2, but not sF2-(63–116), may evoke conformational changes in thrombin that limit its reactivity with AT. This is supported by the distinct changes in fluorescence of ANS-FPR-thrombin induced by F2 and sF2-(63–116) (Fig. 3) and by the work of others who proposed that
individual residues within exosite II mediate various functions of the exosite (30). In contrast to their different effects in the absence of heparin, both F2 and sF2-(63–116) reduce the heparin-catalyzed rate of thrombin inactivation by AT about 4-fold likely by competing with heparin for binding to exosite II on thrombin.

In summary, our findings indicate that the functional changes produced by sF2-(63–116) binding to thrombin are quantitatively different from those evoked by F2. Some of these differences may simply reflect the 12-fold higher affinity of F2 for thrombin (Kd values of 4.1 and 51.3 μM, respectively). How-

ever, it is unlikely that thrombin binding affinity alone ac-

counts for (a) the diametric changes in fluorescence signal elicited by the two ligands when they bind to thrombin, or (b) their differential effects on the uncatalyzed rate of thrombin inhibition by AT. Rather, these observations more likely reflect additional contacts of F2 relative to the smaller sF2-(63–116) peptide and suggest that ligand binding to various subsites within exosite II may have different effects on thrombin function.

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