Interaction of the blood clotting proteinase, thrombin, with fibrin monomer and heparin to form a thrombin-fibrin monomer-heparin ternary complex is accompanied by a change in thrombin catalytic specificity. Equilibrium binding interactions in the assembly of the ternary complex were characterized quantitatively using thrombin labeled at the active site with a fluorescent probe and related to changes in thrombin specificity toward exosite I-dependent binding of hirudin and cleavage of fibrinogen. Changes in the active site environment accompanying binding of heparin or fibrin to thrombin in binary complexes were reported by fluorescence enhancements which contributed additively to the perturbation accompanying formation of the ternary complex. Quantitative analysis of the interactions supports a preferentially ordered path of ternary complex assembly, in which initial binding of heparin to thrombin facilitates binding of fibrin monomer with an ~40-fold increased affinity. Binding of fibrin monomer in the ternary complex decreased the affinity of native thrombin for hirudin by >100-fold and inhibited cleavage of fibrinogen, but this inhibition was overcome when fibrinogen-fibrin interactions occurred. These results support a ternary complex model in which heparin binding through exosite II of thrombin facilitates fibrin monomer binding via exosite I, with accompanying changes in thrombin catalytic specificity resulting from perturbations in the active site and reduced accessibility of exosite I to hirudin and fibrinogen.

Proteolytic cleavage of fibrinogen by thrombin to produce fibrin is the culminating step in the blood coagulation cascade (1). In addition, thrombin acts on a large number of other physiological substrates and inhibitors which have essential procoagulant and anticoagulant functions (2). The substrate specificity of thrombin is regulated by interactions involving two regulatory sites on the enzyme, which have been identified as distinct electropositive regions, separate from the catalytic site (1, 3, 4). Exosite I mediates binding of certain protein substrates, regulatory proteins, and inhibitors (1, 5). This site functions in binding of fibrinogen and facilitating its productive interaction with the catalytic site as a specific protein substrate (6). Maintenance of the exosite I binding interaction is primarily responsible for thrombin binding to the reaction products, fibrin I and fibrin II (7–11). This interaction with fibrin I, the initial product of fibrinopeptide A cleavage from fibrinogen, alters the kinetic properties of thrombin (10), and fibrin I and II act as effectors of thrombin in enhancing the activation of factor XIII (9, 12). Similarly to fibrinogen, simultaneous interactions with exosite I and the catalytic site of thrombin are involved in the high affinity and specificity of the inhibitor from leeches, hirudin. The amino-terminal globular domain of hirudin occupies the catalytic site while the extended carboxy-terminal sequence binds to the fibrinogen-recognition exosite (I) (13, 14). The second thrombin regulatory site (exosite II) binds the potent anticoagulant glycosaminoglycan, heparin (4, 15–17), which promotes inactivation of thrombin by its physiological inhibitor, antithrombin, through joint binding of the proteinase and inhibitor (18, 19).

In addition to the individual effects of fibrin and heparin interactions with thrombin, the substrate specificity of the enzyme is also affected by formation of a ternary complex with heparin and fibrin monomer or polymer (20, 21). Thrombin in a ternary complex with heparin and fibrin monomer has decreased activity toward tripeptide chromogenic substrates and prothrombin (21) and markedly reduced reactivity with antithrombin (22). Cleavage of fibrinogen by thrombin in the presence of heparin, however, was not affected under conditions where ternary complexes were predicted to assemble (21). Recent observations indicate that ternary complexes also form in plasma and markedly compromise heparin anticoagulant activity (23).

The influence of thrombin-fibrin-heparin complex formation on the substrate and inhibitor specificity of thrombin and the activity of heparin prompted the present studies to characterize further the assembly of the complex and the properties of the bound thrombin. These studies show that binding of fibrin monomer and heparin result in perturbations of the fluorescence of thrombin labeled at the catalytic site with a tripeptide chloromethyl ketone inhibitor and the probe, 2-anilinophthalene-6-sulfonic acid, providing evidence that changes in the active site environment contribute to changes in the catalytic properties of thrombin bound in the ternary complex. Quantitative studies showed that binding of fibrin monomer and heparin result in perturbations of the fluorescence of thrombin labeled at the catalytic site with a tripeptide chloromethyl ketone inhibitor and the probe, 2-anilinophthalene-6-sulfonic acid, providing...
tative analysis of the ternary complex interactions supports a preferentially ordered route of assembly within the previously described random addition model (20), in which fibrin monomer interacts predominantly with thrombin-heparin binary complex with an enhanced affinity for thrombin. Binding of fibrin monomer to thrombin in the ternary complex reduces the activity of thrombin for exosite I-dependent binding of hirudin and cleavage of fibrinogen, consistent with competition between these interactions for exosite I. The observation that fibrinogen cleavage is inhibited is reconciled with the previous observation that fibrinogen cleavage was unaffected (21) by the finding that inhibition by ternary complex formation with fibrin monomer is overcome when fibrin(ogen)-fibrin interactions occur. These studies indicate that changes in the environment of the thrombin catalytic site and reduced accessibility of thrombin regulatory sites accompany ternary complex formation with fibrin monomer and heparin and contribute to the altered specificity of thrombin for peptide and macromolecular substrates and inhibitors.

EXPERIMENTAL PROCEDURES

Proteins and Heparin—Human α-thrombin was generously supplied by Dr. John Fenton of the New York State Department of Health, Albany, NY, and its concentration was determined by active site titration (24). ANS-thrombin was prepared by inactivation of human α-thrombin with acetylthiocholine chloride (Sigma), labeling of the NH2OH-generated inhibitor thiol with 2-(4-iodoacetamido)anilino-naphthalene-6-sulfonic acid, following procedures described previously (25, 26). The labeled thrombin contained 1.05 mol of ANS/mol of active thrombin sites and migrated as a single labeled species on SDS-polyacrylamide gels. Fibrinogen and fibrin II monomer were prepared as described previously (22, 27). Recombinant, nonfatted hirudin was supplied by Dr. Paul Johnson of the Stanford Research Institute, Menlo Park, CA. Heparin with high affinity for antithrombin and specific activity of 211 USP units/mg (fraction D, Ref. 28) was provided by Dr. Edgar Sache of the Institute Choay, Paris. Its concentration was determined by weight, using a molecular weight of 20,500.

Fluorescence Studies—Fluorescence was measured on samples in 50 mM HEPES, 0.125 M NaCl, 1 mg/ml PEG, pH 7.4, and at 25 °C with an SLM 8000 spectrofluorimeter, using acrylic fluorescence cuvettes. The pH change due to addition of acetic acid was compensated by titration of the solution containing fibrin with the matched solution lacking fibrin. The fluorescence change occurred rapidly, within the mixing time. Additional measurements at lower fibrin concentrations were made by prior addition of an equal volume of 20 mM acetic acid to a reference cuvette, and again immediately after addition of 0.3 mM ANS-thrombin to both cuvettes. The pH change due to addition of acetic acid was compensated for by prior addition of an equal volume of twice-concentrated buffer, pH 7.7. The fluorescence of ANS-thrombin in the presence and absence of fibrin was corrected for background and scattering from the signals obtained before and after addition of the labeled enzyme. One or two additional measurements at lower fibrin concentrations were made with the concentrations of the other components held constant by diluting the solution containing fibrin with the matched solution lacking it. The fluorescence changes occurred rapidly, within the mixing time. Because GPRP slows but does not prevent fibrin polymerization, measurements were limited to 2 or 3 determinations that could be made in <5 min after addition of fibrin, within the lag phase of polymerization. The relatively low fluorescence yield of ANS-thrombin, trace-level fluorescence impurities present in GPRP, and the high concentrations of fibrin in these experiments contributed to significant corrections for background and scattering which represented a maximum of 30–40% of the initial signal. The validity of the corrections was confirmed by repeating some of the measurements at 0.95 μM ANS-thrombin, a three times higher concentration than that routinely used. The data were expressed as the fractional increase in the initial fluorescence (Fobs - Frest)/Frest and fit by the quadratic equilibrium binding equation to determine the maximum fluorescence change (Fmax - Frest)/Frest and dissociation constant (Kdis), assuming binding to a single site (26)

Binding of Heparin to ANS-thrombin—Titration of ANS-thrombin with heparin were done by measuring the difference in fluorescence of solutions of ANS-thrombin (70 nM) and a mixture of ANS-thrombin (70 nM) and a 4.25-fold molar excess of heparin (298 nM) as a function of their dilution through the concentration range of the dissociation constant. The data were corrected for light scattering of heparin (<3%) from parallel measurements on blanks lacking thrombin. The binding equation for the interaction of heparin with ANS-thrombin under conditions where heparin is present in a fixed molar excess is,

where β = [ANS-T]0 + [H]0, γ = [H]/[ANS-T]0, and Kdis is the dissociation constant. Equation 1 was fit to the data expressed as (Fobs - Frest)/Frest (versus β, with Kdis and Fmax - Frest) the unknown parameters.

Binding of Fibrin to ANS-thrombin—Formation of the ANS-thrombin-fibrin-heparin complex was measured from the enhancement in fluorescence accompanying addition of fibrin to a pre-equilibrated mixture of ANS-thrombin (70 nM) and heparin (298 nM) in the presence of GPRP. The data were corrected for light scattering of fibrin (<5%) and heparin (<3%). In an equilibrium mixture of ANS-T, F, and H, three binary complexes (ANS-T-F, ANS-T-H, and F-H) and the ternary complex (ANS-T-F-H) form according to the random addition model (see Fig. 3). Only those complexes which involve ANS-T contribute to the observed fluorescence (Fobs), which can therefore be expressed as

The equation used for analysis of titrations of ANS-T with fibrin in the presence of heparin was obtained by subtracting Equation 2b from 2a, simplifying by use of the mass conservation expression for [ANS-T]0, and dividing the resulting equation by Equation 2b, which yielded
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rates of hydrolysis of peptide-p-nitroanilide substrates. Chromogenic substrate assays were initiated by addition of thrombin to 200-μl reactions in 50 mM HEPES, 0.125 M NaCl, 1 mg/ml PEG, pH 7.4, at 22 ± 2°C and monitored by the increase in absorbance at 405 nm in a microplate reader. Kinetic parameters for chromogenic substrate hydrolysis were determined by least-squares analyses of full progress curves (31), with the best fits obtained by allowing for competitive product inhibition. The \( K_\text{m} \), \( K_\text{E} \), and \( k_i \) values for IPR-pNA, FPPp-pNA, and GPR-pNA were 1.37 ± 0.39 μM, 5.08 ± 1.51 μM, and 49.5 ± 0.3 s⁻¹; 1.29 ± 0.13 μM, 3.79 ± 0.36 μM, and 76.5 ± 2.2 s⁻¹; and 6.98 ± 0.49 μM, 56.8 ± 6.4 μM, and 117 ± 2 s⁻¹, respectively.

Binding of hirudin to thrombin was determined from its effect on the hydrolysis of a competing chromogenic substrate using the slow, tight binding inhibitor model (32, 33). The inhibition constant for hirudin binding to thrombin (\( K_\text{I} \)) and active inhibitor concentration (\( [I]_0 \)) were determined by analysis of equilibrium titrations of thrombin (0.4 μM) with hirudin in the presence of a fixed concentration of substrate (\( [S]_0 \)), IPR-pNA, 97 μM from measurements of the steady-state rates (34). The apparent inhibition constant (\( K_{\text{app}} \)) for thrombin-hirudin binding in the presence of fibrin and/or heparin was determined by measuring the steady-state velocity of chromogenic substrate hydrolysis in the absence of fibrin (\( v_0 \)) and in the presence of a known concentration of hirudin (\( v_f \)). The apparent inhibitor dissociation constant was obtained from the ratio of the velocities using Equation 3.

\[
K_{\text{app}} = \frac{[I]_0 - [T]_b (1 - \frac{v_f}{v_0})}{v_0 - \frac{v_f}{v_0}} \quad \text{(Eq. 3)}
\]

To determine the inhibition constants for hirudin in the presence of fibrin and heparin, it was necessary to take into account the effects of interactions of these components with thrombin on the chromogenic substrate kinetics (21). The kinetic parameters and equilibrium constants determined previously (20, 21) and in the present work were used to determine the appropriate \( K_\text{m} \) for thrombin hydrolysis of IPR-pNA at any fibrin and heparin concentration and thereby to correct the measured \( K_{\text{app}} \) for the thrombin-hirudin interaction for the competitive effect of the chromogenic substrate using Equation 3.

The effects of heparin and fibrin on the binding of hirudin to thrombin were also analyzed according to the rapid equilibrium inhibition model, in which hirudin can interact with the enzyme species, T, T F, and T F H, where \( K_{\text{app}} \) is described by,

\[
K_{\text{app}} = \frac{k_f [F] + k_h [H]}{k_f [H] + k_h [F]} \quad \text{(Eq. 4)}
\]

The dissociation constants are described as above for ANS-T and \( \phi \) is the factor by which the \( K_\text{app} \) for hirudin is affected when \( T \) is in the ternary complex. This model assumes, as it is demonstrated experimentally, that F and H alone have no discernible effect on inhibition of T by hirudin, under the conditions studied. Although the fibrin and heparin concentrations in Equation 4 are free concentrations, the total concentrations were taken as good approximations of these because the experiments were performed under conditions where \( [H]_0 \gg [F]_0 \), \( [F]_0 \gg [T]_0 \), and binding of F to H is relatively low affinity (\( K_{\text{fH}} = 5.7 \mu M \)) (22), such that F-H complexes were \( \approx 6\% \) of the total F or H concentrations. Equation 4 was fit by nonlinear regression to \( K_{\text{app}} \) determined as a function of fibrin or heparin concentration, with \( K_f \) fixed at 38 μM and \( K_{\text{app}} \) at 3 μM.

The slow kinetics of hirudin binding to thrombin (0.41 nM) in the presence of 391 nM fibrin monomer and 64 nM heparin were measured by the progress of IPR-pNA (144 μM) hydrolysis. Progress curves obtained at five fibrin concentrations (0.25–2.9 nM) were analyzed by nonlinear least-squares fitting to obtain the apparent association and dissociation rate constants (34).

Effects of Fibrin and Heparin on Cleavage of Fibrinogen by Thrombin—Fibrinogen cleavage by thrombin was quantified by analysis of fibrin polymerization curves according to De Cristofaro and Di Cera (35). Fibrin polymerization was measured by the turbidity increase at 405 nm with time using a Molecular Devices Corp. microplate reader with flat bottom plates (Linbro/Titertek) at 22 ± 2°C. The buffer was 50 mM HEPES, 0.125 M NaCl, 1 mg/ml PEG, pH 7.4. In experiments lacking GPRP, thrombin (0.6–6 nM) was incubated alone or with heparin (1 μM) for 5 min at 22°C before initiation of the fibrinogen reaction by 10-fold dilution into 0.6 μM fibrinogen or 0.6 μM fibrinogen containing 0.1 μM fibrin. In experiments containing GPRP, thrombin (6–47 nM) was incubated with 5 μM or 10 μM GPRP alone or with heparin (1 μM) and/or fibrin (1 μM) for 5 min before 10-fold dilution into 0.6 μM fibrinogen. The clotting time (\( t_c \)) was determined by extrapolating the slope of the maximum change in optical density to the baseline. Under conditions where the fibrinogen concentration is \( \approx K_{1} \) for fibrinopeptide A release (3–6 μM; Refs. 36 and 37), \( t_c \) is related to the ratio, \( K_{[F]/[H]} \) according to Equation 5 (35).

\[
t_c = t_0 + \alpha (K_{[F]/[H]} - 1) [I]_b \quad \text{(Eq. 5)}
\]

\( t_c \) is the asymptotic clotting time for \( [I]_b \rightarrow \infty \), and \( \alpha \) is a constant. Therefore, a plot of \( t_c \) versus \( [I]_b \) has a slope of \( \alpha (K_{[F]/[H]} - 1) \) and an abscissa intercept of \( t_0 \). The effects of heparin and fibrin on the apparent \( k_f / K_f \) were determined from the ratios of the slopes of plots of \( t_c \) versus \( [I]_b \) for reactions containing thrombin alone versus reactions containing fibrin, heparin, or fibrin and heparin, which represent the effects on \( k_f / K_f \). The effects of these components were determined separately for each GPRP concentration because GPRP itself had a small effect on the clotting times. In agreement with the results of previous studies (35), 1 μM GPRP decreased \( k_f / K_f \) 1.9-fold. GPRP (2 μM) had no effect on the initial rate of thrombin hydrolysis of 190 μM VLR-pNA (0.5K_m) (38), indicating that this effect was not due to inhibition of thrombin catalytic activity.

**RESULTS**

**Binding of Fibrin and Heparin to ANS-thrombin**—Changes in the environment of the thrombin active site accompanying binding of fibrin monomer and heparin were characterized by measuring changes in fluorescence of a thrombin derivative that was labeled at the catalytic site with the fluorescence probe, 2-anilinonaphthalene-6-sulfonic acid (ANS-thrombin) (see “Experimental Procedures”; Refs. 25 and 26). Addition of a near-saturating level of a 20,300 molecular weight heparin to ANS-thrombin resulted in a blue shift of 7 ± 1 nm in the fluorescence emission maximum from the initial maximum at 458 nm and an enhancement of \( [F]_o / F_o \) in the probe fluorescence of 2.3 at 448 nm (Fig. 1). Addition of 309 nM fibrin monomer to the ANS-thrombin and heparin mixture in the presence of GPRP, to prevent fibrin polymerization, resulted in a further blue shift to 10 ± 1 nm overall and a fluorescence enhancement of 3.7, relative to ANS-thrombin alone, at the emission maximum of 448 nm. The same concentration of fibrin monomer produced <0.1 relative change in fluorescence (Fig. 1). These results demonstrated ternary complex interactions among ANS-thrombin, heparin, and fibrin monomer and provided the basis for their quantitation.
Spectral studies of fibrin binding to ANS-thrombin at higher concentrations of fibrin monomer were not possible because the accessible concentrations of GPRP did not slow the rate of fibrin polymerization enough to allow collection of spectra. Results of a titration of ANS-thrombin with fibrin monomer, for which the fluorescence measurements could be made before significant polymerization occurred, showed an increase compatible with binding with a dissociation constant ($K_{\text{ANS-T-F}}$) of $3 \pm 3 \mu M$ and maximum fluorescence enhancement of $1.1 \pm 0.4$ (Fig. 2A). Although there was significant experimental error because of the difficulty of the measurements, the satisfactory agreement between results at $0.3 \mu M$ and $0.95 \mu M$ ANS-thrombin supported their validity (Fig. 2A).

Binding of heparin to ANS-thrombin was characterized further from measurements of the fluorescence change that accompanied this interaction. Titration of ANS-thrombin by addition of increasing concentrations of heparin in the conventional way could not be done because of aggregation of thrombin-heparin complexes. When the molar ratios of heparin to ANS-thrombin were $<2$, stable changes in fluorescence were achieved only very slowly ($>1$ h). In contrast, when the molar ratio of heparin to ANS-thrombin was $>2$ and the ANS-thrombin concentration was $<100$ nM, the fluorescence change was rapidly attained ($<1$ min) and stable for at least 2 h. This behavior was taken to reflect heparin-dependent aggregation of thrombin (39). To quantitate the interaction, titrations were performed by measuring the difference in fluorescence of ANS-thrombin alone and a mixture of ANS-thrombin and a fixed, 4.25-fold molar excess of heparin, as a function of the total concentration, varied by dilution through the concentration range of the dissociation constant. Analysis of the results of such a titration with the binding equation for this experimental design given under “Experimental Procedures” gave a dissociation constant ($K_{\text{ANS-T-H}}$) of $59 \pm 14 \mu M$ and maximum fluorescence enhancement of $3.0 \pm 0.2$ (Fig. 2B). This result represented a molecular dissociation constant, which did not take into account the existence of multiple, nonspecific binding sites for thrombin on heparin (39). The experimental conditions of heparin excess, however, approximated the low thrombin binding density condition ($<0.2$ molecule of thrombin bound/heparin), where the affinity can be ascribed to binding to a single site, and the site binding constant can be obtained by taking into account the total number of binding sites (39). The results obtained with the 20,300 molecular weight heparin containing a calculated 31 overlapping sites gave a value of $1.8 \mu M$ for the ANS-thrombin-heparin intrinsic binding constant. This was identical to the value determined by Olson et al. (39) for a 21,200 molecular weight heparin under similar conditions.

The interaction of fibrin monomer with thrombin in the presence of heparin was studied by titration of a mixture of ANS-thrombin and a near-saturating level of heparin with fibrin monomer, such that the low thrombin-heparin binding density conditions were approximated and the increase in fluorescence represented primarily binding of fibrin monomer to ANS-thrombin-heparin complex. The results (Fig. 2C) indicated a higher affinity of fibrin monomer for ANS-thrombin in the presence of heparin than in its absence (Fig. 2A). These results were analyzed to determine the dissociation constant for fibrin monomer binding to ANS-thrombin-heparin complex and the fluorescence enhancement maximum by fitting of the data with the equations for the ternary complex binding model, with the affinities and fluorescence changes associated with the other complexes fixed at their determined values. This gave $74 \pm 40$ nM for $K_{\text{ANS-TH-F}}$ and an enhancement, relative to ANS-thrombin, of $4.4 \pm 0.4$. This represented an $-40$-fold higher affinity of fibrin monomer for ANS-thrombin when heparin was bound, and the fluorescence enhancement accompanying formation of the ternary complex (4.4 ± 0.4) was accounted for by the sum of the changes for the binary, thrombin-heparin (3.0 ± 0.2), and thrombin-fibrin (1.1 ± 0.4) complexes.

The above results supported the random addition model for ternary complex formation among ANS-thrombin, fibrin mon-
monitor, and heparin from each of the three possible binary complexes, as shown in Fig. 3 (20, 21). Three of the six equilibrium constants were determined in the above experiments, with the additional dissociation constant for the fibrin monomer-heparin interaction determined previously (22), and the remaining two were calculated from detailed balance.

Effect of Fibrin and Heparin on Thrombin Inhibition by Hirudin—The effects of fibrin and heparin binding on the affinity of thrombin for hirudin were investigated in the context of the ternary complex binding model (Fig. 3). Binding of recombinant, nonsulfated hirudin was studied using the tight binding inhibitor kinetic approach (33, 34). The interaction of thrombin with hirudin in the absence of fibrin and heparin was studied first. Analysis of hirudin titrations of thrombin measured from the steady-state rates of chromogenic substrate (IPR-pNA) hydrolysis gave 54 ± 34 fM for $K_i$ (not shown). At fixed hirudin and thrombin concentrations, the apparent inhibition constant showed the predicted linear dependence on substrate concentration up to −250 μM for three substrates, yielding estimates of $K_i$ of 15 ± 3 fM (IPR-pNA), 14 ± 3 fM (GPR-pNA), and 26 ± 4 fM (FPipR-pNA). The best determined value of 54 ± 34 fM was four-fold lower than the 230 fm dissociation constant reported previously for recombinant, nonsulfated hirudin (40). This difference is likely accounted for by the differences in temperature and pH between the two experimental conditions.

The effects of fibrin and heparin on the $K_i$ for hirudin were evaluated from the levels of free thrombin at equilibrium, as measured by the steady-state rates of chromogenic substrate hydrolysis. Neither fibrin alone at concentrations up to 830 nM nor heparin up to 998 nM had a detectable effect on $K_i$ for hirudin binding to thrombin when IPR-pNA was used as the monitoring substrate (Fig. 4). The mean $K_i$ in the presence of heparin or fibrin alone was 38 ± 17 fM, indistinguishable from the value determined in the absence of heparin or fibrin. When similar experiments were done with FPipR-pNA, a 2.8-fold increase in $K_i$ was observed at 1.3 μM heparin, similar to the 3–4 fold increase reported previously in studies with this substrate (41). Because this effect was not observed with IPR-pNA and it was relatively small, it was not studied further.

In contrast to the absence of substantial effects of fibrin or heparin alone, the $K_i$ for hirudin increased with increasing fibrin concentration in the presence of a constant heparin concentration and with increasing heparin at constant fibrin (Fig. 4). To measure the inhibition constants in these experiments from the rates of competing chromogenic substrate hydrolysis, it was necessary to take into account the increase in the $K_m$ for the substrate which accompanies ternary complex formation (21). The results shown in Fig. 4 were obtained by correcting for

![Figure 3](image1.png)

**FIG. 3.** Random addition, ternary complex model for interaction of fibrin and heparin with ANS-thrombin. Formation of the ANS-thrombin-fibrin-heparin ternary complex (ANST.H) through the three possible binary complexes, ANS-thrombin-heparin (ANS-T.H), ANS-thrombin-fibrin (ANS-T.F), and fibrin-heparin (F.H). The dissociation constants $K_{ANS-T.H}$, $K_{ANS-T.F}$, $K_{F.H}$, and $K_{ANS-T.H,F}$ were determined experimentally, while $K_{ANS-T.F,H}$ and $K_{F,H,ANS-T}$ were calculated from detailed balance as described in the text.

![Figure 4](image2.png)

**FIG. 4.** Effect of fibrin and heparin on thrombin inhibition by hirudin. A, effect of fibrin (H) on $K_{app}$ for inhibition of 0.4 nM thrombin by 0.4 nM hirudin in the absence (○) or presence of 325 nM fibrin (●). $K_{app}$ was obtained as described under “Experimental Procedures.” The curves represent the fit of Equation 4 to the data with $K_{H} = 82 ± 39$ nM and $K_{F,app} = 11 ± 3$ nM, and with the other parameters fixed at the values given in the text. The parameter, $\phi$, in Equation 4, which represents the decrease in affinity of hirudin for thrombin in the ternary complex was 100 in the bottom curve (---), and 10$^2$ in the top curve (——). B, effect of fibrin on $K_{app}$ for inhibition of 0.4 nM thrombin by 0.4 nM hirudin in the absence (○) or presence of 64 nM heparin (●) is shown with the fitted curves as described in A. The error bar represents ±2 S.D. for the results of hirudin binding kinetic measurements at 391 nM fibrin and 64 nM heparin, as described in the text.
was increased. As shown by the solid lines in Fig. 4, the results were best fit with 82 ± 39 nM for $K_{TH}$, 11 ± 3 nM for $K_{TH,F}$, and infinitely high values of $\phi$, representing no demonstrable affinity of hirudin for thrombin in the ternary complex. The value of $K_{TH}$ was indistinguishable from that obtained in the binding studies with ANS-thrombin, while that for $K_{TH,F}$ differed from the value of 74 ± 40 nM obtained for ANS-thrombin, and the value determined from analysis of the effects of fibrin monomer and heparin on hydrolysis of tripeptide chromogenic substrate by thrombin, 38 ± 4 nM (21). In view of the complexity of this experimental system, this difference was not considered significant. Examination of the dependence of the fit on the magnitude of the change in hirudin affinity showed that it was relatively insensitive to changes greater than 100-fold, as indicated by comparison of the dotted and solid lines in Fig. 4. With very large values of $\phi$ and under experimental conditions where $[F]_0 \ll K_{TH}$, the dependence of $K_{app}$ on fibrin and heparin given by Equation 4 (see “Experimental Procedures”) is approximated by

$$K_{app} = K \left(1 + \frac{[F][H_b]}{K_{TH,F}[K_{TH} + [H_b]_0]} \right) \quad \text{(Eq. 6)}$$

This predicts a linear dependence of the relative increase in $K_{app}$ on fibrin concentration and hyperbolic dependence on heparin concentration, which is similar to the behavior observed (Fig. 4).

**FIG. 6. Effect of GPRP on fibrin and heparin effects on $(k_c/K_m)_{app}$ for thrombin cleavage of fibrinogen.** Relative change in $(k_c/K_m)_{app}$ for fibrinogen cleavage as a function of GPRP concentration is shown for reactions of thrombin alone (○), and in the presence of 100 nM fibrin monomer (●), 100 nM heparin (△), or 100 nM each of fibrin and heparin together (▲). Experiments were performed and analyzed as described under “Experimental Procedures.”

**FIG. 5. Effect of fibrin and heparin on cleavage of fibrinogen by thrombin in the presence of GPRP.** A, progress curves for thrombin-catalyzed fibrin polymerization measured from the optical density change at 405 nm are shown for reactions containing 23 nM thrombin, 600 nM fibrinogen, and 0.5 mM GPRP in the absence (a) and presence of 100 nM fibrin monomer (b), 100 nM heparin (c), or 100 nM each of fibrin monomer and heparin together (d). The clotting time ($t_c$) was obtained by extrapolation of the maximum slope to the baseline as shown by the dotted line for curve d. B, plots of clotting time versus inverse thrombin concentration ([T]$^{-1}$) from polymerization curves as described in A at final concentrations of 6–47 nM thrombin in the absence (○) and presence of fibrin (●), heparin (△), and fibrin plus heparin (▲). The lines represent the linear regression fits.

**DISCUSSION**

The results of these studies support a model for ternary complex formation of thrombin with fibrin monomer and heparin in which binding of heparin to regulatory exosite II of thrombin promotes assembly by enhancing the affinity of fibrin monomer binding through exosite I, with both interactions maintained within the ternary complex. Competition between heparin-facilitated fibrin monomer binding and exosite I-de-
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P. J. Hogg and C. M. Jackson, unpublished data.

pendent interactions with the catalytic site is sufficient to explain the decrease in thrombin affinity for hirudin and inhibition of fibrinogen cleavage. Conformational changes induced in the thrombin catalytic site as a result of heparin and fibrin binding may also contribute to changes in thrombin catalytic efficiency and inhibitor binding affinity.

Results of the fluorescence studies of the ternary complex interactions showed that binding of fibrin or heparin alone resulted in changes in the environment of the thrombin catalytic site, and the fluorescence enhancement accompanying ternary complex formation was accounted for by the sum of the perturbations associated with the binary interactions. This additivity results in each complex displaying a characteristically different active site environment from that of free thrombin and also implies that the binary complex interactions are maintained within the ternary complex. Thermodynamic analysis of the interactions supports the previously described, random addition, ternary complex model (20), and the fluorescence changes correlate with changes in substrate specificity of thrombin associated with binding of these two effectors (21, 22). This supports the conclusion that the ANS probe reports functionally significant perturbations in the environment of the active site. The results of analysis of the component binding interactions are in good agreement with previous studies. Fibrinogen, fibrin I, and fibrin II share a common mode of interaction with thrombin, mediated by regulatory exosite I (7–11). Consistent with this, the 3 μM dissociation constant determined in the present studies with fibrin II monomer is similar to the K_d for the thrombin-fibrinogen reaction of 3–6 μM (10, 36, 37). Other studies of thrombin-fibrin binding have reported similar dissociation constants of ~2 μM (42–44), with variation in the values due in part to heterogeneity of thrombin binding sites on polymeric fibrin (20, 43). Binding of heparin to thrombin is mediated by the second thrombin regulatory exosite (4, 15–17). The dissociation constant obtained for this interaction with ANS-labeled thrombin (59 nM) agrees with the value of 40 nM determined for native thrombin by affinity chromatography2 and the results of other studies (39) and is marginally greater than the 15 ± 6 nM inferred from a combination of binding and kinetic studies (20, 21). On the basis of the current understanding of the modes of the individual heparin and fibrin interactions with thrombin, the results of the present studies support a model for the ternary complex in which thrombin is bound simultaneously to heparin via exosite I and fibrin monomer through exosite I. Formation of the ternary complex is promoted by linkage between the binary interactions, where preferential binding of heparin to thrombin with high affinity enhances the affinity of thrombin for fibrin monomer by ~40-fold, resulting in a preferred route of assembly. The source of the enhanced affinity is not known, although the existence of fibrin-heparin interactions (22) suggests that this is likely to be responsible.

The finding that thrombin bound in the ternary complex has significantly lower affinity for hirudin supports the proposed model for the interactions. A decrease in affinity of ~20-fold was observed directly, with the dependence on fibrin monomer and heparin concentrations well described by the equilibrium binding model, and the condition that affinity for hirudin was decreased measurably only in the ternary complex. Analysis of the results indicated that the magnitude of the decrease in affinity was >100-fold and indistinguishable from essentially complete loss of affinity. It should be noted, however, that it was not possible to define further the upper limit of the affinity of hirudin for thrombin bound in the ternary complex, and it remains possible that hirudin binds with a dissociation constant in the picomolar range. The established mode of hirudin binding requires interaction of the carboxyl-terminal sequence with exosite I of thrombin to facilitate high affinity inhibitory interaction of the amino-terminal domain with the catalytic site (13, 14). The separate hirudin domains bind individually with greatly reduced affinity (45). The effects on hirudin affinity observed for the component interactions of the ternary complex indicate that binding of fibrin monomer to thrombin is required for the decrease in affinity. Heparin alone at concentrations sufficient to saturate thrombin had no demonstrable effect of the inhibition constant for hirudin, consistent with the absence of significant overlap between exosites I and II in the thrombin structure (1, 13, 14). This observation, however, differed from the previous finding that saturating levels of heparin decreased the affinity of thrombin for hirudin by 3–4-fold (41), and the suggestion from other studies that large heparins bound primarily through exosite II may interact weakly with exosite I (6, 39). In the present studies, observation of an effect of heparin on hirudin affinity was related to the chromogenic substrate used to monitor the reaction, with no change observed with IPR-pNA and a 2.8-fold increase with FFpIP-pNA. The source of this difference has not been determined, and, because of the relatively small magnitude of the effect, it was not studied further. No effect of the thrombin-fibrin monomer component interaction on the affinity for hirudin was observed, but the 3 μM dissociation constant did not allow sufficient saturation of thrombin to be achieved to determine whether this interaction alone affected hirudin binding. Although the role of fibrin monomer binding by itself could not be directly established, the absence of a substantial effect of heparin in comparison with the effect of fibrin monomer in the presence of heparin suggests that the thrombin-fibrin interaction is largely responsible for the decrease in hirudin affinity. These results suggest that competitive binding of fibrin monomer and the hirudin carboxyl-terminal sequence to exosite I is sufficient to explain the loss of affinity. Although it is considered less likely, it is not possible with the results presently available to distinguish this possibility from the alternative that the lower affinity for hirudin is due to conformational changes in the thrombin catalytic site that reduce affinity for the hirudin amino-terminal domain.

The observation that thrombin bound to fibrin monomer and heparin has significantly reduced activity toward fibrinogen is compatible with the model for the ternary complex interactions. The interpretation of the effects of fibrin and heparin is complicated by the generation of fibrin as a product of the reactions and by the unexpected influence of fibrinogen(ogen)-fibrin interactions on the results. Previous studies showed no effect of heparin on release of fibrinopeptides A and B by thrombin under conditions where thrombin-fibrin polymer-heparin ternary complexes form (21). This was confirmed in the present studies by the lack of significant inhibition of thrombin cleavage of fibrinogen by fibrin and heparin in the absence of GPRP. Examination of the dependence of these effects on GPRP concentration, however, showed that under conditions were fibrinogen(ogen)-fibrin interactions were greatly diminished, the combination of fibrin and heparin had the maximal inhibitory effect on thrombin cleavage of fibrinogen, while fibrin alone had no detectable effect, and heparin reduced the rate only a small amount. These results are compatible with competition between thrombin-fibrin monomer-heparin ternary complex formation and productive interaction of fibrinogen with exosite I as the basis for the inhibition. The smaller inhibitory effect seen with heparin alone is thought to reflect ternary complex formation with fibrin formed during the course of fibrinogen
cleavage. The dependence of thrombin inhibition on GPRP implies that fibrin(o)gen-fibrin interactions act in opposition to the inhibitory effect of ternary complex formation. The mechanism of this effect is unknown. The higher affinity of thrombin and heparin for fibrin polymer compared to fibrin monomer can result in a different distribution of thrombin among the binary and ternary complexes (20). Under the conditions of the present studies, however, it is unlikely that inhibition of fibrin-fibrin interactions by GPRP resulted in significantly more thrombin bound in the ternary complex. Instead, inhibition of fibrinogen-fibrin interactions by GPRP may be involved. It is possible that fibrinogen-fibrin interactions inhibit the formation of the ternary complex, or that thrombin shows a preference for binding of fibrinogen over fibrin when fibrinogen and fibrin interact. Additional studies of these complex interactions will be needed to determine the source of this effect.

The results of further characterization of thrombin interactions with fibrin and heparin provide additional information for evaluating the potential in vivo significance of the interactions. Ternary complex formation and the favorable linkage between the interactions may account for the effectiveness of fibrin monomer in inhibiting the anticoagulant activity of heparin in plasma (23). The observation that fibrinogen cleavage was inhibited by ternary complex formation only when fibrin(o)gen-fibrin complexes were prevented from forming, coupled with the protection of thrombin from heparin-catalyzed inactivation by antithrombin (22), suggest that the net effect of ternary complex formation in vivo will be prothrombotic. These interactions probably contribute to the ineffectiveness of heparin in inhibiting clot-bound thrombin (46). Clinical situations in which clot-bound thrombin is important and in which heparin has limited efficacy include venous thrombosis (47), coronary thrombosis, and angioplasty and unstable angina (48). Hirudin and its derivative hirulog are being investigated as alternative anticoagulants in these clinical situations (47, 48). The results of the present studies imply that co-administration of heparin with hirudin might reduce hirudin efficacy as an anticoagulant by promoting assembly of the ternary complex and thereby reducing affinity of thrombin for hirudin.

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Binding of Fibrin Monomer and Heparin to Thrombin in a Ternary Complex Alters the Environment of the Thrombin Catalytic Site, Reduces Affinity for Hirudin, and Inhibits Cleavage of Fibrinogen

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