Exosite I of the blood clotting protease, thrombin, mediates interactions of the enzyme with certain inhibitors, physiological substrates and regulatory proteins. Specific binding of a fluorescein-labeled derivative of the COOH-terminal decapeptide of hirudin \((5F)\) Hir\(_{54–65}\) to exosite I was used to probe changes in the function of the regulatory site accompanying inactivation of thrombin by its physiological serpin inhibitor, antithrombin. Fluorescence-monitored equilibrium binding studies showed that \(5F\)Hir\(_{54–65}\) and Hir\(_{54–65}\) bound to human \(\alpha\)-thrombin with dissociation constants of \(26 \pm 2 \text{ nM}\) and \(38 \pm 5 \text{ nM}\), respectively, while the affinity of the peptides for the stable thrombin-antithrombin complex was undetectable \((200\text{-fold weaker})\). Kinetic studies showed that the loss of binding sites for \(5F\)Hir\(_{54–65}\) occurred with the same time-course as the loss of thrombin catalytic activity. Binding of \(5F\)Hir\(_{54–65}\) and Hir\(_{54–65}\) to thrombin was correlated quantitatively with partial inhibition of the rate of the thrombin-antithrombin reaction, maximally decreasing the bimolecular rate constants 1.7- and 2.1-fold, respectively. These results support a mechanism in which thrombin and the thrombin-Hir\(_{54–65}\) complex can associate with antithrombin and undergo formation of the covalent thrombin-antithrombin complex at modestly different rates, with inactivation of exosite I leading to dissociation of the peptide occurring subsequent to the rate-limiting inactivation of thrombin. This mechanism may function physiologically in localizing the activity of thrombin by allowing inactivation of thrombin that is bound in exosite I-mediated complexes with regulatory proteins, such as thrombomodulin and fibrin, without prior dissociation of these complexes. Concomitant with inactivation of thrombin, the thrombin-antithrombin complex may be irreversibly released due to exosite I inactivation.

The activity of the blood clotting enzyme, thrombin, is determined by a balance between proteolytic action on physiological substrates, and irreversible inactivation by the serpin, antithrombin. The substrate specificity of thrombin is regulated by macromolecular effectors which interact with the enzyme at either of two electropositive sites, exosites I and II \((1, 2)\). Exosite I has been defined in crystallographic studies as the site occupied by the extended COOH-terminal sequence of the specific thrombin inhibitor, hirudin \((3–5)\). Exosite II is a distinct site which binds heparin and other ligands \((1)\). Specific binding of COOH-terminal hirudin peptides, particularly \(N\)-acetyl-hirudin\(_{53–64}\) \((\text{Hirugen})\); Ref. \(6)\), to thrombin has been used to establish an important functional role for exosite I in mediating thrombin interactions. The peptide inhibits thrombin hydrolysis of the substrates, fibrinogen \((7)\), factors V and VIII \((8)\), and activation of the thrombin receptor \((9, 10)\); it dissociates thrombin from regulatory interactions with fibrin I and II \((7, 11)\) and thrombomodulin \((12, 13)\); and it inhibits thrombin inactivation by the serpin, heparin cofactor II \((14, 15)\). By contrast to these interactions, the rate of thrombin inactivation by antithrombin is decreased only 1.9-fold by Hirugen \((7)\), contributing to the conclusion that recognition of antithrombin by thrombin does not involve exosite I significantly \((7, 15–18)\). Binding of regulatory macromolecules to exosite I results in similarly modest effects on the rate of thrombin inactivation by antithrombin. Thrombin binding to fibrin I increases the rate 2.8-fold \((19, 20)\), while fibrin II reduces the rate 1.6-fold \((21)\). The rate of inactivation of thrombin bound to thrombomodulin is unaffected or decreased \(-2\)-fold for thrombomodulin lacking covalently attached chondroitin sulfate \((22–25)\), and increased \(2\)-fold for thrombomodulin containing the attached glycosaminoglycan \((23–27)\).

The information available indicates that the catalytic site of thrombin is accessible to antithrombin when the proteinase is bound in exosite I complexes with hirudin peptide, fibrin I and II, or thrombomodulin, and the reaction rate is not greatly affected \((7, 19, 20, 24, 26, 28)\). On the basis of the mechanism of the thrombin-antithrombin reaction \((\text{see Refs. 29 and 30 for reviews})\), this implies that thrombin can associate with antithrombin and undergo irreversible formation of the covalent thrombin-antithrombin complex with exosite I occupied. This suggests that exosite I may remain accessible in the proteinase-inhibitor complex, which could inhibit further exosite I-dependent reactions by accumulation of this bound product. Results of other studies, however, suggest that this is not the case.

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1 The abbreviations used are: Hirugen, \(N\)-acetyl-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr(SO\(_3\))-Leu; T, human \(\alpha\)-thrombin; AT, human antithrombin; T-AT\(^*\), thrombin-antithrombin final complex; Hir\(_{54–65}\), hirudin\(_{54–65}\); Glu-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr(SO\(_3\))\(_{54–65}\) Leu-Glu; \(5F\)Hir\(_{54–65}\), hirudin\(_{54–65}\) labeled at the amino terminus with 5-carboxyfluorescein; FPR-CH\(_2\)Cl, D-Phe-Pro-Arg-CH\(_2\)Cl; ATA-FPR-CH\(_2\)Cl, \(N\)-\(\alpha\)acetylthio)acyl)-(\(\alpha\)-Phe-Pro-Arg-CH\(_2\)Cl; ATA-FFR-CH\(_2\)Cl, \(N\)-\(\alpha\)acetylacyl)-\(\alpha\)-Phe-Phe-Arg-CH\(_2\)Cl; FPR-thrombin, thrombin inactivated with FPR-CH\(_2\)Cl; 4'-APFPR-thrombin, thrombin labeled at the active site with 4'-[(iodoacetamido)methyl]-fluorescein and ATA-FPR-CH\(_2\)Cl; HPLC, high performance liquid chromatography.
The thrombin-antithrombin complex formed in the presence of thrombomodulin does not inhibit the rate of subsequent thrombomodulin-enhanced inactivation of thrombin by antithrombin or activation of protein C, and it has been proposed that loss of affinity of the thrombin-antithrombin complex for thrombomodulin allows the cofactor protein to recycle following thrombin inactivation (22, 27).

Collectively, the observations available present a paradox concerning the mechanism that can accommodate the relatively small, nonessential effects of exosite I interactions on the rate of thrombin inactivation by antithrombin, and also result in loss of affinity of the thrombin-antithrombin complex for exosite I ligands. The present studies were undertaken to investigate this question by determining the fate of exosite I in the thrombin-antithrombin reaction. A fluorescent analog of hirudin (Hi54–65) (Hi54–65), similar to the derivative of hirudin 53–64 described previously by Liu et al. (31), was prepared and used as a specific probe of the function of the exosite. The affinities of fluorescein-labeled Hi54–65 and unlabeled Hi54–65 for exosite I were decreased >200-fold in the thrombin-antithrombin complex. Results of kinetic studies indicate that thrombin with Hi54–65 bound to exosite I can associate with antithrombin, and that effectively irreversible inactivation of exosite I and dissociation of the peptide occur subsequent to the rate-limiting formation of the covalent thrombin-antithrombin complex. These observations support a mechanism in which thrombin bound to regulatory macromolecules through exosite I may be inactivated by antithrombin without the requirement for dissociation of these complexes. Formation of the thrombin-antithrombin stable complex may drive the release of the inactive complex, thereby providing a mechanism for regulatory macromolecules to act as catalytic effectors of thrombin inactivation. This mechanism may function physiologically in clearing thrombin from regulatory macromolecules and participate in localizing thrombin activity.

**EXPERIMENTAL PROCEDURES**

**Protein Purification and Characterization.—** Human α-thrombin was purified as described previously (32) or obtained from Dr. J. Fontenot (New York State Department of Health, Albany, NY). Thrombin concentration was determined by active site titration and absorbance at 280 nm with an absorption coefficient of 1.83 (mg/ml)−1 cm−1 and a molecular weight of 36,600 (33). Preparations of thrombin used in these studies were >95% active. Thrombin inactivated with PFR-CH2Cl (PFR-thrombin) was prepared by incubation of 50 μM enzyme with a 2-fold excess of PFR-CH2Cl for 30 min. at 25 °C, and the residual activity (<0.02%) was determined by chromogenic substrate assay, as described below. Thrombin was labeled at the active site with 4-[([iodoacetyl]amino)methyl]fluorescein and Nα-[acetylthioacyl]lysyl-threo-Pro-Arg-CH2-Cl (4'-AF)PFR (T) and characterized as described previously (34, 35). The preparation used in these studies contained 0.87 μM of fluorescein/mmol of thrombin. Antithrombin was purified from human plasma as described previously (36), and its concentration was determined by absorbance at 280 nm with an absorption coefficient of 0.65 (mg/ml)−1 cm−1 and a molecular weight of 58,000. Preparations of antithrombin were fully active, as measured by thrombin reaction stoichiometries of 1.08 ± 0.08 mol/mol and a second-order rate constant for thrombin inhibition of 0.89 × 10−3 m−1 s−1 under the conditions used previously (37). Thrombin-antithrombin complex (TAT) was prepared by reaction of 28–39 μM thrombin with a 1.7–2.2-fold excess of antithrombin for 10–120 min at 25 °C in 50 mM Hepes, 0.125 M NaCl, 1 mM EDTA, 1 mg/ml polyethylene glycol 8000, pH 7.4. The concentration of TAT was taken as the initial thrombin concentration, consistent with the essentially complete disappearance of thrombin observed by SDS-gel electrophorosis. The residual thrombin concentration (<0.1%) was calculated from the residual chromogenic substrate assay and titrated with antithrombin in the same assay.

**Synthesis and Characterization of 5F-Hir54–65.—** Solutions of the Tyr53-sulfated hirudin dodecapeptide (Sigma or Bachem) were prepared in water and their concentrations calculated from the purity specified by the manufacturers. The fluorescent derivative, [5F]Hir54–65, was prepared by modifications of the procedure described previously for a similar derivative (31). 5-Carboxyfluorescein succinimidyl ester (Molecular Probes) in MeSO was added to 0.4–0.5 mM peptide to give a 5–9-fold molar excess in 50 mM sodium phosphate buffer, pH 8.0, and incubated for 2 h in the dark at 22 °C. The reaction was stopped by addition of 1 M NH4Cl, pH 7.4, to 0.1 M and the labeled peptide was separated from excess dye by chromatography on Sephadex G10 (1 × 118 cm) eluted with water. The labeled peptide was purified by reverse-phase HPLC on a Beckman Ultrasphere 5-μm C18-silica column (4.6 × 150 mm) equilibrated with 0.1% trifluoroacetic acid in water and developed at 0.5 ml/min with 0.1% trifluoroacetic acid in CH3CN. The column was washed with trifluoroacetic acid/H2O for ~20 min after application of the sample, increased to 20% CH3CN over 10 min and washed for ~50 min, and eluted finally with a linear gradient from 20% to 50% CH3CN over 60 min. Fractions containing the major fluorescent peak eluting at ~36% CH3CN were neutralized by addition of 1 M NH4HCO3 to 50 mM and lyophilized. The purified peptide was dissolved in water, and its concentration was determined from the fluorescein absorbance at 490 nm in 10 mM NaOH with an absorption coefficient of 89,125 M−1 cm−1 (38). Purified [5F]Hir54–65 eluted from the above column with a linear gradient as a single peak at 39% CH3CN. Solutions of [5F]Hir54–65 were stored frozen in water. The labeled peptide was stable in water for at least 2 days at room temperature in the dark, as shown by the unchanged HPLC elution profile.

**Equilibrium Binding Studies.—** All experiments were performed in 50 mM Hepes, 0.125 M NaCl, 1 mM EDTA, 1 mg/ml polyethylene glycol 8000, pH 7.4, and at 25 °C. Fluorescence measurements were made with an SLM 8000 fluorometer in the ratio mode, using acrylic cuvettes (Sarstedt) coated with polyethylene glycol 20,000 to minimize protein adsorption (39). Measurements of [5F]Hir54–65 binding to thrombin were performed with 491 nm excitation (4 nm band pass) and 515 nm emission (8 nm band pass), corresponding to the difference maxima from spectra of 0.2 μM peptide in the absence and presence of saturating (1 μM) thrombin. Fluorescence changes of 4'-AF/PFR-thrombin were measured with 500 nm excitation (4–8 nm band pass) and 519 nm emission (8–16 nm band pass), the excitation and emission difference maxima from spectra of 175 nM labeled thrombin in the absence and presence of 5 μM Hir54–65. Titrations were done by successive addition of small volumes of titrant with <10% dilution, and, when necessary, corrected for background and by subtraction of measurements on solutions lacking the labeled species. Fluorescence data were expressed as the fractional change in the initial fluorescence (ΔF/F0 = (Ffinal−F0)/F0) as a function of the total concentration of thrombin (L) as follows (Equation 1) for obtaining the maximum fluorescence change (ΔFmax/F0), dissociation constant (KL), and stoichiometric factor (n).

\[
\frac{\Delta F}{F_0} = \frac{\Delta F_{\text{max}}}{F_0} - \left[ \frac{(n[L] + K_L)}{n[L] + K_L + n_k^{1/n_o - 4 \Delta F/F_0}} \right] \times \frac{2n_o}{n_o - 1}
\]

For analysis of less extensive data obtained with the same materials, \(\Delta F_{\text{max}}/F_0\) and KL were fit with n fixed at the determined value. Fluorescence titrations of [5F]Hir54–65 (P) with PFR-thrombin (L), and of 4'-AF/PFR-thrombin (P) with Hir54–65 (L) were analyzed similarly. In experiments testing the effect of TAT on PFR-thrombin binding to [5F]Hir54–65, residual active thrombin (<0.1%) present in mixtures of thrombin and antithrombin incubated under the conditions given above for 30 min to form TAT was quantitated by chromogenic substrate assay and rapidly inactivated by addition of 5 μM PFR-CH2-Cl. The concentration of this residual PFR-thrombin was included in the total PFR-thrombin concentration in the titrations. The estimated magnitude of the affinity for the possible competitive binding of TAT (C) and PFR-T (L) to [5F]Hir54–65 (P) was evaluated from the binding curves simulated with Equation 1, with KC increased by a factor of (1 + [TAT]/[KC]), where KC represents the dissociation constant for TAT binding to [5F]Hir54–65.

Fluorescence studies of native thrombin (L) binding to [5F]Hir54–65 (P) with unlabeled Hir54–65 (C) as a competitor were performed by measuring the fluorescence changes after addition of fixed concentrations of thrombin to [5F]Hir54–65, and titrated with Hir54–65. Data collected at a fixed concentration of [5F]Hir54–65 (P) as a function of the total Hir54–65 concentration (ICL) and several fixed total thrombin concentrations (IL), along with data for titration of the same concentration of [5F]Hir54–65 with thrombin alone, were fit simultaneously with Equation 2 to determine the dissociation constant (KL) and stoichiometric factor (n) for competitive binding of Hir54–65 to thrombin, as...
well as $K_p$ and $\Delta F_{max}/F_o$ (40, 41).

$$D_1(\Delta F/\Delta F_{max})^2 + D_2(\Delta F/\Delta F_{max})^3 + D_3(\Delta F/\Delta F_{max}) + D_4 = 0 \quad \text{(Eq. 2)}$$

$$D_1 = n[P_i] \left( \frac{K_r - K_p}{K_p} \right) \quad \text{(Eq. 2a)}$$

$$D_2 = [L_i] \left( \frac{K_r - K_p}{K_p} + n[P_i] \left( \frac{K_r - 2K_p}{K_p} + K_p - K_r - m[C] \right) \right) \quad \text{(Eq. 2b)}$$

$$D_3 = [L_i] \left( \frac{K_p}{K_p} \right) + n[P_i] \left( \frac{K_r}{K_p} + K_r + m[C] \right) \quad \text{(Eq. 2c)}$$

$$D_4 = -[L_i] \left( \frac{K_r}{K_p} \right) \quad \text{(Eq. 2d)}$$

Conditions of these experiments were chosen with $[\text{5F}\text{Hir}^{54-65}]$ to reduce the dependence of the fit on $n$, which was fixed at its separately determined value. The same methods were used to analyze binding of Hirudin analogs to thrombin (C) with native thrombin (B) as a competitor. The magnitudes of the affinities of T-AT* and AT as competitors were evaluated from the binding curves simulated with Equation 2 for given values of $K_p$.

Kinetic Studies—The time course of thrombin inactivation by antithrombin was measured under pseudo-first-order reaction conditions ([AT]o $\geq 10$[T]o), from the loss of thrombin chromogenic substrate activity. Chromogenic substrate activity was determined as the initial rate of hydrolysis of 100 M H-N-Phe-Pip-Arg-p-nitroanilide at pH 7.4 and 25°C from the linear increase in absorbance at 405 nm with time. Residual thrombin concentration ([T]o) was expressed as the fraction of the initial activity measured for an identical control reaction mixture lacking AT, to account for small effects of transferred hirudin peptide on the assay rate. Reaction progress curves were fit by Equation 3, for a single exponential decay, to obtain the observed pseudo-first-order rate constant ($k_{obs}$), the reaction amplitude ([T]o), and end point (B).

$$[T]_o = [T]_o e^{-kt} + B \quad \text{(Eq. 3)}$$

For analysis of reactions predicted to deviate from initially exponential behavior, model-independent estimates of the initial $k_{obs}$ ($k_{obs}'$) were obtained by fitting Equation 4 to the data, where the time dependence of the deviation from exponential behavior was approximated by a second degree polynomial.

$$[T]_o = [T]_o e^{-(at^2 + bt)} \quad \text{(Eq. 4)}$$

The data were truncated at 90% of completion for this analysis, and $k_{obs}$ was taken as the least-squares fit value of the coefficient, $b$. The apparent bimolecular rate constants were calculated as $k_{obs}/[AT]_o$.

Kinetic studies of the dissociation of $[\text{5F}\text{Hir}^{54-65}]$ from T upon its reaction with AT were done by first measuring the decrease in fluorescence on addition of thrombin to the fluorescent peptide, and then the return of the quenched fluorescence toward the initial value $F_0$. The fluorescence changes measured with time were transformed into the total concentrations of thrombin binding sites for the peptide (P) at the corresponding times ([T]i) with Equation 5, which is a rearrangement of the binding equation (Equation 1), using the parameters determined in the binding studies.

$$[T]_i = \left( \frac{\Delta F_r}{\Delta F_{max}} \right) \left( \frac{n[P_i]}{1 - \frac{\Delta F_r}{\Delta F_{max}}} \right) + K_p \quad \text{(Eq. 5)}$$

The data were expressed as the fraction of the initial total thrombin concentration remaining ([T]i/[T]o), and the progress curves were analyzed as described for the activity experiments.

Least-squares fitting, numerical integration, and simulation were performed with SCIENTIST software (MicroMath Software). All reported estimates of error represent $\pm 2$ S.E.

**RESULTS**

**Binding of Hirudin Peptides to Thrombin**—A fluorescent analog of Hirudin that was modified at the amino terminus with 5-carboxyfluorescein ([5F]Hir54–65) was synthesized, and its binding to thrombin was characterized from changes in fluorescence intensity. As shown in Fig. 1A, thrombin titrations of fixed concentrations of [5F]Hir54–65 from 10 nM to 500 nM were well described by binding of the peptide to 0.91 ± 0.04 sites on thrombin with a dissociation constant of 26 ± 2 nM, and a maximum decrease in fluorescence of 26 ± 0.3% (Table I). Binding of unlabeled Hirudin to thrombin was examined from its effect on the [5F]Hir54–65 fluorescence, which is a measure of thrombin activity in the presence of unlabeled Hirudin. Binding of Hirudin to mixtures of thrombin and the labeled peptide resulted in return of the quenched fluorescence toward the initial value (Fig. 1B). Simultaneous nonlinear least-squares analysis of titrations with Hirudin at fixed thrombin concentrations ranging from 20 to 600 nM indicated a good fit with the equation for high affinity competitive binding of the labeled and unlabeled peptides, with Hirudin binding to 0.89 ± 0.07 sites on thrombin with a dissociation constant of 38 ± 5 nM (Table I). These results indicated that Hirudin and the fluorescein-labeled analog bound competitively and with similar, high affinity to exosite I of thrombin.

**Binding of Hirudin Peptides to the Thrombin-Antithrombin Complex**—In contrast to the results for thrombin, the thrombin-antithrombin stable complex (T-AT*) or antithrombin (AT) decreased the fluorescence of [5F]Hir54–65 by <3% at concentrations up to 3 μM T-AT* or 10 μM AT (Fig. 2). This indicated either an undetectably low affinity of the peptide for T-AT*, or that the peptide bound but there was no significant change in fluorescence resulting from the interaction. To investigate this further, the effect of the T-AT* complex on binding of [5F]Hir54–65 to thrombin that had been active site-blocked with

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**Fig. 1.** Fluorescence titrations of α-thrombin binding to [5F]Hir54–65 and competitive binding of Hirudin. A, the fractional changes in fluorescence (ΔF/F0) of [5F]Hir54–65 at concentrations of 10 nM (●), 50 nM (○), 250 nM (△), and 500 nM (▲) are plotted as a function of the total thrombin concentration ([T]o). The lines represent the non-linear least squares fit of the equation for a single binding equilibrium (Equation 1) to the data with the parameters given in Table I. B, fluorescence changes of mixtures of 10 nM [5F]Hir54–65 and thrombin at concentrations of 20 nM (●), 100 nM (○), 240 nM (▲), and 460 nM (△) are plotted as a function of the total concentration of unlabeled hirudin peptide ([Hir54–65]). The lines represent the least-squares fit of the equation for competitive binding (Equation 2) with the parameters given in Table I. Titrations were performed and the data were analyzed as described under “Experimental Procedures.”
**Inactivation of Thrombin Exosite I**

**TABLE I**

| Interaction                  | Method                                | Binding constants | Kinetic constants |
|------------------------------|---------------------------------------|-------------------|------------------|
|                              |                                       | $K_D$ (nm) | $\Delta F_{max}/F_0$ (%) | $K_P$ (nM) | $k$ (s$^{-1}$) | $k'$ (s$^{-1}$) |
| Fluorescent hirudin peptide  |                                       |               |                  |            |               |               |
| [5F]Hir$^{54-65}$            | T                                     | 26 ± 2       | 0.91 ± 0.04      | 26 ± 0.3  |            |               |
|                             | FPR-T                                 | 26 ± 2       | 0.75 ± 0.03      | 30 ± 0.2  |            |               |
|                             | T-AT$^*$                              | >5200         |                   |            |            |               |
|                             | T+AT$^*$                              |                 |                   |            |            |               |
|                             | T+AT$^*$                              |             |                   |            |            |               |
| Unlabeled hirudin peptide    |                                       |              |                  |            |               |               |
| [4'-AF]FPR-T                | T                                     | 38 ± 5       | 0.89 ± 0.07      | 52 ± 0.6  |            |               |
|                             | T                                      | 136 ± 7      | 1.20 ± 0.05      | 52 ± 0.6  |            |               |
|                             | AT                                    | 35 ± 9       | 0.94 ± 0.08      |            |            |               |
|                             | T-AT$^*$                              | >7000         |                   |            |            |               |
|                             | T+AT$^*$                              |                 |                   |            |            |               |
|                             | T+AT$^*$                              |             |                   |            |            |               |
|                             | T+AT$^*$                              |             |                   |            |            |               |

**FIG. 2. Fluorescence titrations of [5F]Hir$^{54-65}$ with thrombin, antithrombin, and the thrombin-antithrombin complex.** The changes in fluorescence ($\Delta F/F_0$) of 110 nm [5F]Hir$^{54-65}$ are shown as a function of the log of the total concentrations of thrombin (T), antithrombin (AT), or thrombin-antithrombin complex (T-AT$^*$). The solid line represents the fit of the thrombin titration; with a $-27 \pm 1\%$ maximum decrease in fluorescence accompanying binding of the peptide to 0.92 ± 0.16 sites with a 23 ± 7 nm dissociation constant. Titrations were performed and the data analyzed as described under “Experimental Procedures.”

$\alpha$-Phe-Pro-Arg-CH$_2$Cl (FPR-thrombin) was examined. Active site-blocked thrombin was used in these experiments to prevent reaction with excess antithrombin present in the mixture of thrombin and antithrombin used to form the T-AT$^*$ complex. As shown by the titrations of 11 nm and 110 nm [5F]Hir$^{54-65}$ with FPR-thrombin in Fig. 3, binding of the peptide to active site-blocked thrombin was accompanied by a slightly larger, 30 ± 0.2% change in fluorescence when compared with native thrombin, and the same dissociation constant (Table I). The presence of 4.2 µM T-AT$^*$ complex had no significant effect on titrations of the labeled peptide with FPR-thrombin (Fig. 3). Simulation of the effect of competitive binding of [5F]Hir$^{54-65}$ to T-AT$^*$ and FPR-thrombin indicated that an affinity of the labeled peptide for T-AT$^*$ complex up to 200-fold lower than the affinity for thrombin would have been detected (Fig. 3).

To determine if the low affinity of the T-AT$^*$ complex for [5F]Hir$^{54-65}$ was due to the presence of the fluorophore on the peptide, binding of unlabeled Hir$^{54-65}$ was studied using an active site-labeled fluorescent thrombin derivative as a binding probe. To identify a fluorescent derivative with suitable properties, six derivatives were prepared by active site-specific inactivation of thrombin with either of the two thioester tripeptide chloromethyl ketones, ATA-FPR-CH$_2$Cl and ATA-FPR-CHCl$_2$ (32, 34). The thiol generated from the thioester on the incorporated peptides was labeled with each of three structural isomers of fluorescein-iodoacetamide in which the iodoacetamide group was linked through the 5-, 6-, or 4’-positions of the fluorescein ring system (34). Results of screening these thrombin derivatives for those that signaled binding of Hir$^{54-65}$ showed, remarkably, that five of the six derivatives exhibited small, $-1 \to +8\%$ changes in fluorescence (data not shown), while only [4'-AF]FPR-thrombin reported the interaction with a large fluorescence change. Titrations of [4'-AF]FPR-thrombin with Hir$^{54-65}$ showed an enhancement of 52 ± 0.6% accompanying binding of the peptide with a dissociation constant of 136 ± 7 nm (Fig. 4). Analysis of the effect of native thrombin on binding of Hir$^{54-65}$ to [4'-AF]FPR-thrombin (Fig. 4) was consistent with competitive binding of the peptide to native thrombin with a dissociation constant of 35 ± 9 nm, in agreement with the value of 38 ± 5 nm determined from the previous experiments (Table I). The presence of 4.0 µM T-AT$^*$ or 10.1 µM AT had no significant effect on binding of Hir$^{54-65}$ (Fig. 4). Simulation of the effect of competitive binding indicated that, similar to the previous results with [5F]Hir$^{54-65}$ the affinity of Hir$^{54-65}$ for T-AT$^*$ was at least 200-fold lower than the affinity for native thrombin (Fig. 4). Titrations of the active site-labeled thrombin in the absence of Hir$^{54-65}$ with up to 2 µM native thrombin, 4 µM T-AT$^*$, or 10 µM AT produced $\pm 1\%$ changes in fluorescence (data not shown), indicating no significant nonspecific interactions.

**Kinetics of Thrombin Inactivation by Antithrombin and the Loss of Affinity for the Hirudin Peptide—Addition of antithrombin to a mixture of thrombin and the fluorescent hirudin peptide resulted in a time-dependent return of the quenched fluorescence of [5F]Hir$^{54-65}$ to the initial value, consistent with dissociation of the peptide from thrombin upon its reaction with antithrombin. On the basis of the previous results, the changes in fluorescence were taken as measures of the concentrations of thrombin containing the peptide binding site, which were calculated using the binding parameters that had been determined (see “Experimental Procedures”). As shown in Fig. 5, the loss of thrombin binding sites for [5F]Hir$^{54-65}$ closely paralleled the loss of thrombin activity following addition of antithrombin, over the full course of the reaction. Similar comparisons were done at other concentrations of [5F]Hir$^{54-65}$ (see below). The measured fluorescence changes of 5–17% for these reactions corresponded to calculated changes in the concentration of thrombin able to bind the peptide that were indistinguishable from the initial thrombin concentrations (89 ± 30%),
Inactivation of Thrombin Exosite I

The large error in this calculation reflected the diminishing reaction amplitude at higher concentrations of the fluorescent peptide, and a deviation of the fluorescence amplitudes of $\leq 2\%$ from the predicted maxima. For all of the reactions, the final fluorescence was within $1\%$ of the initial fluorescence of the peptide alone, indicating complete dissociation of the peptide. 

**Effect of Hirudin Peptides on the Kinetics of Thrombin Inactivation by Antithrombin**—Kinetic studies were undertaken to characterize the mechanism of the effect of the hirudin peptides on the reaction of antithrombin with thrombin, from the time courses of enzyme inactivation and peptide dissociation. The initial results, together with previous observations (7), suggested that the reactions under bimolecular conditions would follow the mechanism shown in Scheme I.

\[
\begin{align*}
AT + T & \xrightarrow{k} T-AT^* \\
\text{P} + \text{P} & \xrightarrow{K_P} \text{P} \\
AT + T & \xrightarrow{k'} T-AT^* \\
\text{P} & \xrightarrow{K_P} \text{P}
\end{align*}
\]

**Scheme I**

In this mechanism, thrombin (T) and the thrombin-peptide (P) complex in rapid equilibrium can react with AT with different second-order rate constants, $k$ and $k'$, to form the stable T-AT* complex. The reaction of the thrombin-peptide complex with AT is accompanied by a large decrease in affinity for the peptide. The differential rate equation for the disappearance of active thrombin ([T]$_o$) by this mechanism, under conditions of large excess of antithrombin over thrombin, such that [AT] $\approx$ [AT]$_{eq}$, is shown in Equation 6.

\[
-d[\text{T}]_o/dt = k[\text{AT}][\text{T}]_o + (k' - k)[\text{AT}]_o[\text{TP}]
\]  

(Eq. 6)

The high affinity of the peptide for thrombin and its release over the course of the reaction implied that the disappearance of thrombin would not follow a strictly exponential time course under all of the experimentally accessible conditions. This was due to the disproportionate changes in [TP] and [T]$_o$, accompanying the decrease in total thrombin concentration over the course of the reaction. In the initial phase of the reaction where the approximation can be made that [TP]/[T]$_o$ $\approx$ [TP]/[AT]$_{eq}$, the differential Equation 6 becomes Equation 7.

\[
-d[\text{T}]_o/dt = k[\text{AT}][\text{T}]_o + (k' - k)[\text{AT}][\text{TP}]/([\text{TP}]_o/[\text{AT}]_o)
\]  

(Eq. 7)

This integrates to an exponential function characterized by an initial, pseudo-first-order rate constant ($k_{obs}$) (shown in Equation 8).

\[
k_{obs} = k[\text{AT}]+(k'-k)[\text{AT}]/([\text{TP}]_o/[\text{AT}]_o)
\]  

(Eq. 8)

The fraction of thrombin sites occupied by the peptide is given by the binding equation (Equation 9).

\[
[\text{TP}]_o = (K_r + n[\text{T}]_o + [\text{P}]) - \sqrt{K_r + n[\text{T}]_o + [\text{P}]}x - 4n[\text{T}]_o[\text{P}]/2n[\text{T}]_o
\]  

(Eq. 9)

To allow for the predicted deviation from exponential behavior following the initial reaction phase, the reaction progress curves were fit by the exponential function with a second degree polynomial in time as the exponent. This approximation was valid because the deviations from exponential behavior were small (Fig. 5), although significant enough to affect the analysis. This method allowed for deviation of the reactions from first-order behavior and permitted estimation of the pseudo-first-order rate constant for the initial portion of the reaction, $k_{obs}$, from the fitted coefficient of the first-order term of the polynomial. As shown in Fig. 6A, the apparent bimolecular rate constant, $k_{obs}/[\text{AT}]_o$, decreased with increasing concentration of [5F]Hir$^{54-65}$, approaching a limiting value at high concentrations. The rate constant for the fluorescence-monitored peptide dissociation was indistinguishable from the rate constant for activity loss, for all of the reactions. The pseudo-first-order rate constants increased linearly with the antithrombin concentration from 500 to 2000 nM in the presence of 45 nM and 150 nM [5F]Hir$^{54-65}$, or 400 nM Hir$^{54-65}$ (data not shown), indicating that the loss of activity and dissociation of the peptide were both second-order reactions. For the reactions in the absence of the peptide, which were known to be pseudo-first-order processes, there was good agreement ($\leq 6\%$) between $k_{obs}$ and the rate constants obtained by fitting of single exponentials, which supported the validity of the analysis. In the pres-
concentration (limiting bimolecular rate constant at saturating peptide concentration) was indistinguishable from the 26 nM dissociation constant. Activity and fluorescence were measured as described under “Experimental Procedures.” Fluorescence data were transformed into thrombin binding site concentrations with Equation 5 described under “Experimental Procedures.” Fluorescence data were transformed into thrombin binding site concentrations with Equation 5 (see “Experimental Procedures”) and normalized to the initial value. The dashed line represents the fit of the combined data by an exponential with $k_\text{obs,AT}/[\text{AT}]_o$ of 0.57 $\times 10^4$ M$^{-1}$ s$^{-1}$. The solid line represents the fit of the data up to 90% of completion with Equation 4 under “Experimental Procedures” and $k_\text{obs,AT}/[\text{AT}]_o$ of 0.62 $\times 10^4$ M$^{-1}$ s$^{-1}$.

The above analysis of the kinetics supported the mechanism of Scheme I that was 1.7-fold lower than the value in the absence of the peptide and an apparent peptide dissociation constant ($K_P$) of 16 $\pm$ 13 nM (Fig. 6A, Table I). The latter value was indistinguishable from the 26 $\pm$ 2 nM dissociation constant determined for free thrombin in the binding studies, supporting the mechanism in Scheme I.

The effect of Hir54–65 on the kinetics of the thrombin-antithrombin-catalytic activity. Results of these experiments showed similar behavior (Fig. 6B), with a decrease in the rate constant of 2.1-fold and an apparent dissociation constant of 31 $\pm$ 24 nM, in agreement with the value of 38 $\pm$ 5 nM determined for binding of the unlabeled peptide to thrombin (Table I).

The above analysis of the kinetics supported the mechanism of Scheme I but resulted in rather large uncertainties in the parameters because of the small effect of the peptides on the rate, and error inherent in the measurements. To test the mechanism further, all of the full time course fluorescence and activity data collected as a function of [5F]Hir54–65 concentration, represented by the results in Fig. 6A, were fit without approximation by numerical integration of the differential equation (Equation 6) to solve for the thrombin concentration as a function of time, with changes in the concentration of the thrombin-peptide complex with time calculated with Equation 9. This analysis gave second-order rate constants indistinguishable from those determined by the initial exponential analysis (Table I) and an apparent peptide dissociation constant of 19 $\pm$ 6 nM, in closer agreement with the dissociation constant obtained in the equilibrium binding studies. Application of the same analysis to all of the progress curve data for Hir54–65 gave a dissociation constant of 37 $\pm$ 7 nM and rate constants that were similarly in good agreement with the values obtained in the preceding analysis and binding studies (Table I). These results supported the adequacy of the mechanism in Scheme I as a quantitative description of the effect of the hirudin peptides on the kinetics of thrombin inactivation and peptide dissociation.

The effect of the peptides on the inhibition reaction stoichiometry was examined to determine if binding of the peptides to thrombin decreased the rate by enhancing an alternative substrate reaction of antithrombin with thrombin (42, 43). The stoichiometry of 1.01 $\pm$ 0.03 mol of AT/mol of T obtained in the absence of peptide was unchanged by 5.3 $\mu$M [5F]Hir54–65 (1.00 $\pm$ 0.01 mol/mol) or 4.9 $\mu$M Hir54–65 (0.98 $\pm$ 0.06 mol/mol), indicating no significant effect of the peptides on the reaction stoichiometry.

**DISCUSSION**

These studies were undertaken to investigate possible changes in the function of regulatory exosite I of thrombin accompanying its inactivation by antithrombin. The current understanding of the mechanism of antithrombin inactivation of thrombin provides a foundation for interpretation of the results. Previous studies support a branched-pathway, suicide substrate mechanism (42, 44), as shown in the upper part of

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**FIG. 5.** Time courses of activity loss and [5F]Hir54–65 dissociation accompanying reaction of thrombin with antithrombin. Thrombin activity (●) and the concentration of binding sites for [5F]Hir54–65 (○), expressed as fractions of the initial concentrations ([T]$_o$)/([AT]$_o$), are shown with time after addition of 500 nM AT to 50 nM T and 30 nM [5F]Hir54–65. Activity and fluorescence were measured as described under “Experimental Procedures.” Fluorescence data were transformed into thrombin binding site concentrations with Equation 5 (see “Experimental Procedures”) and normalized to the initial value. The dashed line represents the fit of the combined data by an exponential with $k_\text{obs,AT}/[\text{AT}]_o$ of 0.57 $\times 10^4$ M$^{-1}$ s$^{-1}$. The solid line represents the fit of the data up to 90% of completion with Equation 4 under “Experimental Procedures” and $k_\text{obs,AT}/[\text{AT}]_o$ of 0.62 $\times 10^4$ M$^{-1}$ s$^{-1}$.

**FIG. 6.** Effect of hirudin peptides on rate constants for thrombin-antithrombin reactions. A, apparent bimolecular rate constants ($k_\text{obs,AT}/[\text{AT}]_o$) measured by the loss of activity for reactions of 50 nM thrombin with 500–515 nM antithrombin (●), and measured by the fluorescence changes representing peptide dissociation (○) are shown as a function of the concentration of fluorescent peptide ([5F]Hir54–65). The solid line represents the fit of the data with the equations given in the text and the parameters in Table I. B, the dependence of the apparent bimolecular rate constant for inactivation of thrombin by antithrombin on the concentration of unlabeled hirudin peptide ([Hir54–65]) is shown, along with the least-squares fit of the data (solid line) with the parameters listed in Table I. Kinetics were measured and analyzed as described under “Experimental Procedures.”
A rapidly reversible Michaelis-type of enzyme-inhibitor complex (T-AT*) is formed initially between the protease active site and the reactive-site sequence of the inhibitor, exposed on a flexible loop (45–48). The reactive-site bond is subsequently engaged chemically by the protease as a normal protein substrate, in an irreversible reaction that results in a covalent intermediate complex (T-AT). A conformational change in the T-AT complex results finally in irreversible trapping of thrombin in the stable product complex (T-AT*). In parallel with this reaction, the covalent intermediate can undergo completion of the substrate reaction, ultimately deacylating to yield reactive-site cleaved inhibitor (AT_m) and active protease (42–44, 50, 51). The rate-limiting step of thrombin inactivation is the formation of the covalent T-AT intermediate, which is thought to trigger the subsequent, faster serpin conformational change (52, 53).

The thrombin-antithrombin mechanism can be expanded to include all of the exosite I binding equilibria as shown in Scheme 2. The results of the binding studies indicate that the affinity of thrombin in the product, T-AT*, complex, for the hirudin peptides is reduced at least 200-fold compared with free thrombin. Characterization of binding of the fluorescein-labeled hirudin44–65 peptide to human α-thrombin gave results consistent with those reported previously for bovine thrombin and an analogous derivative of hirudin53–64 in which the probe was coupled through slightly different chemistry (31). The ~4-fold higher affinity of the peptide used here and slightly larger fluorescence change are probably accounted for by the structural differences between the peptides and the lower affinity of such peptides for bovine thrombin compared with the human enzyme (6). In the present studies, [5F]Hir54–65 and the unlabeled peptide bound competitively and with similar, high affinity to a single site on thrombin, while [5F]Hir54–65 had no detectable affinity for T-AT* in direct fluorescence titrations or in competitive binding experiments with FPR-thrombin. Evidence of a similar loss of affinity for the unlabeled peptide was obtained in experiments in which active site labeled thrombin was used as a macromolecular fluorescence probe of binding. These results demonstrated that the fluorescence changes of [5F]Hir54–65 reported the peptide binding equilibrium, rather than perturbations due to nonspecific interactions. The absence of detectable binding of the peptides to the T-AT* complex is concluded to be due to loss of specific interactions with exosite I.

Although the thrombin-antithrombin reaction intermediates in Scheme 2 were not resolved in the present studies, the results of the kinetic studies support the conclusion that the loss of affinity of exosite I for the hirudin peptide occurs in the rate-limiting chemical reaction or a subsequent step. This is supported by the correspondence between the time courses of peptide dissociation and loss of thrombin activity, and between the dissociation constants for the peptides determined kinetically and by direct binding. The relatively small inhibition of the reaction, with 1.7- and 2.1-fold lower bimolecular rate constants at saturating peptide, rules out mechanisms of inhibition in which peptide binding competes with antithrombin for formation of the T-AT complex, or prevents the subsequent chemical reaction (Scheme 2). These mechanisms would show complete inhibition at saturating peptide concentration. These findings are in agreement with the previously reported effect of Hirugen (7), and the conclusion that recognition of antithrombin by thrombin does not depend significantly on exosite I (7, 15–17). The significant residual rate at saturating peptide concentrations implies that the thrombin-peptide complex reacts with antithrombin and undergoes conversion to the covalent P-T-AT complex. On this basis, it is concluded that dissociation of the peptide occurs in a step following either the irreversible reaction that produces the covalent complex, or following a subsequent step that occurs at a faster rate (Scheme 2).

Because the kinetic studies were restricted to bimolecular reaction conditions, the 1.7- and 2.1-fold inhibition of the rate by binding of the peptides could be due to an increase in the dissociation constant for antithrombin binding to the thrombin-peptide complex, or to a decrease in the rate constant for covalent complex formation. Previous observations indicate that initial recognition of antithrombin by thrombin involves binding of the reactive-site sequence at the primary substrate specificity site and secondary subsites (45, 48, 53–55). In other studies, binding of hirudin peptides to exosite I has been shown to change thrombin specificity toward tripeptide substrates, which interact at these sites (12, 31, 56). This linkage between interactions at exosite I and the active site is thought to account for the difference observed here in the affinity of the hirudin peptide for [4^F]-FPR-thrombin and native thrombin (Table I). This linkage may be similarly responsible for the decrease in the bimolecular rate constant for thrombin reaction with antithrombin.

The results of the kinetic studies do not resolve whether the loss of affinity of thrombin for hirudin peptides occurs as a result of the formation of the covalent T-AT complex, or the subsequent serpin conformational change leading to the final T-AT* complex (Scheme 2). The apparently small effect of the peptide on steps prior to the chemical reaction implies that the exosite is not greatly affected by the initial proteinase-inhibitor association, and more likely is disrupted or blocked as a result of the major structural changes which accompany the conformational change. The conformational change is thought to involve incorporation of the reactive site loop into β-sheet A of antithrombin, accompanied by a significant movement of the proteinase relative to the inhibitor (30, 47, 48, 57), and loss of specific binding affinity of antithrombin for heparin (52, 58). Other studies indicate that a conformational change also occurs in the thrombin component of the thrombin-antithrombin complex, as evidenced by an increased susceptibility to proteolysis by free thrombin (59, 60). An initial site of cleavage was identified as Arg68 (Arg73 in the chymotrypsin numbering system) (60), which is located in the β-loop that forms an integral part of exosite I involved in binding of hirudin peptides (1, 4, 5). Cleavage of this loop in α-thrombin to produce β-forms of thrombin, or mutation of Arg68 are accompanied by loss of exosite I functions, including cleavage of fibrinogen, and binding of hirudin and thrombomodulin (18, 61). These observations suggest that exosite I is accessible to thrombin cleavage in the stable thrombin-antithrombin complex and that this occurs at an enhanced rate, supporting the idea that conformational changes in this region of the proteinase are likely responsible for the loss of affinity. Significant levels of degradation of the T-AT* complex requires excess thrombin and is relatively slow.
can act catalytically as an effector of thrombin inactivation. This mechanism may play an important role physiologically in localizing the activity of thrombin by coupling the inactivation of the protease at the sites of its exosite I-mediated interactions to the obligatory release of the proteinase-inhibitor complex. Such coupling would maintain the competency of regulatory proteins to bind thrombin and prevent dissemination of the enzyme from sites of vascular injury.

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