Hirudin, a potent 65-residue polypeptide inhibitor of α-thrombin found in the saliva of the leech Hirudo medicinalis, and fragments thereof are potentially useful as antithrombotic agents. Hirugen, the synthetic N-acetylated COOH-terminal dodecapeptide (Ac-Asn-Gly-Asp-Phe-Glu-Ile-Pro-Glu-Glu-Tyr(SO_3)_2-Leu) of hirudin was shown in the present study to behave as a pure competitive inhibitor (K_I = 0.54 μM) of human α-thrombin-catalyzed release of fibrinopeptide A from human fibrinogen. In contrast to this inhibitory activity, hirugen slightly enhanced (increased k_cat/K_m 1.8-fold) α-thrombin-catalyzed hydrolysis of the fluorogenic tripeptide substrate N_p-Tosyl-Gly-Pro-Arg-7-amido-4-methylcoumarin. These observations indicate that hirugen binds to α-thrombin at an exosite distinct from the active site, and that interaction with this exosite is a major determinant of the competence of α-thrombin to bind fibrinogen. Consistent with this view, hirugen blocked binding of fibrinogen to α-thrombin.

Studies of the effect of hirugen on the rate of inactivation of α-thrombin by antithrombin III (AT), the major plasma inhibitor of α-thrombin, indicated that binding of hirugen to α-thrombin results in less than a 2.5-fold decrease in the rate of inactivation of α-thrombin by AT, both in the absence and presence of heparin. This behavior is distinct from that of active site-directed competitive inhibitors of α-thrombin which bind to α-thrombin at an exosite, and which block both the rate of inactivation of fibrinogen to fibrin and inactivation of α-thrombin by AT. Hirugen, an exosite-directed competitive inhibitor, blocks the interaction of α-thrombin with fibrinogen while leaving α-thrombin competent to react with AT. Thus, unlike active site-directed competitive inhibitors, hirugen should act in concert with AT and heparin to reduce the amount of fibrinogen that is processed during the lifetime of α-thrombin in plasma.

The serine protease α-thrombin initiates conversion of fibrinogen to fibrin during blood coagulation by catalyzing limited proteolysis of fibrinogen to release a 16-residue peptide (fibrinopeptide A, FPA) from the amino terminus of the Aα chain of fibrinogen. Interestingly, interaction at exosite(s) distinct from the active site of α-thrombin appears to determine the affinity and catalytic specificity of α-thrombin toward fibrinogen (1-6). This exosite region of α-thrombin appears to be lost when α-thrombin is proteolytically modified, as evidenced by the observation that certain proteolytic nicks in α-thrombin result in thrombin forms which have dramatically reduced competence (relative to that of α-thrombin) to process fibrinogen, but essentially undiminished catalytic activity toward small peptide nitroanilide substrates (3, 7-9). Moreover, an antibody directed against residues 62 to 73 of the B chain of human α-thrombin can block α-thrombin-catalyzed hydrolysis of fibrinogen without altering small substrate hydrolysis (5).

Hirudin, a 65-residue polypeptide inhibitor of α-thrombin obtained from the leech Hirudo medicinalis, appears to interact with α-thrombin at both the active site and an exosite of α-thrombin, as evidenced by kinetic studies of the reaction of hirudin with α-thrombin and chemically or proteolytically modified thrombins (10-12). Synthetic peptides corresponding in primary structure to the COOH-terminal domain of hirudin inhibit the ability of α-thrombin to clot fibrinogen with little effect on the activity of α-thrombin toward small substrates (13, 14). This observation suggests that the synthetic peptides corresponding to the COOH terminus of hirudin selectively bind to an exosite of α-thrombin. Substances that reversibly block the fibrinogen-binding exosite of α-thrombin might be expected to have more pronounced effects than reversible active site-directed inhibitors of α-thrombin on the amount of fibrin formed in response to generation of a bolus of α-thrombin in blood plasma. Reversible active site-directed inhibitors would be expected to inhibit equally the rate of conversion of fibrinogen to fibrin and the rate of irreversible inactivation of α-thrombin by antithrombin III (AT), since both processes require an accessible active site. It follows from this realization that the amount of fibrin clot ultimately formed during the plasma lifetime of α-thrombin should be unaffected by the presence of a reversible active site-directed inhibitor under conditions where reaction of α-thrombin with thrombomodulin is insignificant. (Algebraic proof of this conclusion appears later in this paper.) On the other hand, the presence of a reversible exosite-directed inhibitor of α-thrombin might (under the same conditions) lower the amount of fibrin clot generated during the plasma lifetime of α-thrombin, if complexation with the exosite...
directed inhibitor had a greater inhibitory effect on the rate of fibrinogen processing than it had on the rate of the irreversible reaction of α-thrombin with AT.

Reversible exosite-directed competitive inhibitors of α-thrombin such as those corresponding to the COOH-terminus of hirudin might exert their inhibitory effect so as to: (i) block the binding of fibrinogen to α-thrombin, (ii) cause fibrinogen to bind nonproductively to α-thrombin, or (iii) incrementally reduce the affinity of α-thrombin for fibrinogen. A study of the effect of the peptides on the initial rate of α-thrombin-catalyzed release of FPA from fibrinogen could distinguish between the possible modes of action of peptides corresponding to the COOH-terminal domain of hirudin. Such a study is presented for the first time in the present paper, where we analyze the effects of hirugen, an N-acetylated synthetic peptide (Ac-Asn-Gly-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr(SO2)-Leu) corresponding in primary structure to that of the COOH-terminal domain of hirudin, on kinetic parameters for the interaction of α-thrombin with fibrinogen, AT, and the small fluorescent α-thrombin substrate N-p-tosyl-Gly-Pro-Arg-7-amido-4-methylcoumarin (tos-GPR-amc). Our observation that hirugen competitively inhibits the interaction of α-thrombin with fibrinogen while only modestly affecting the action of α-thrombin on tos-GPR-amc and the reaction of α-thrombin with AT in the presence or absence of heparin, indicates that: (i) hirugen is an exosite-directed competitive inhibitor of the action of α-thrombin on fibrinogen; (ii) the exosite of α-thrombin that binds hirugen is a critical determinant of the interaction between α-thrombin and fibrinogen; and (iii) hirugen should act in concert with AT and heparin to reduce the amount of fibrin formed during the plasma lifetime of α-thrombin.

**Experimental Procedures and Results**

**Discussion**

The analysis presented under "Results" (see Miniprint) of the effect of hirugen on the initial velocity for the α-thrombin-catalyzed release of FPA from fibrinogen indicated that binding of hirugen and fibrinogen to α-thrombin are mutually exclusive. Analysis of the effect of hirugen as a competitive inhibitor of α-thrombin-catalyzed release of FPA from fibrinogen yielded a value of 0.54 (± 0.02) μM for the dissociation constant of the α-thrombin-hirugen complex. This value is in reasonable agreement with the value (0.64 ± 0.09 μM) that we obtained from an analysis of the hirugen-induced increase in the specificity constant for α-thrombin catalysis of tos-GPR-amc hydrolysis and is similar to the IC50 observed in an earlier study of the hirugen-induced increase in the activated partial thromboplastin time (14). It is unlikely that the observed competitive inhibition by hirugen of the activity of α-thrombin toward fibrinogen is due to the binding of hirugen at the active site, since hirugen does not inhibit the activity of α-thrombin toward tos-GPR-amc and other small peptide substrates (14). The effects of hirugen documented in the present study are consistent with the proposal that hirugen binds to α-thrombin at a site distinct from the active site. The effect of hirugen as a pure competitive inhibitor of the interaction of α-thrombin with fibrinogen suggests that the same exosite that binds hirugen is also a critical determinant of the affinity of α-thrombin for fibrinogen. The observation that fibrin II binds to active site blocked derivatives of α-thrombin (1,34-38) suggested the possibility that the fibrinogen-binding exosite of α-thrombin is also a major determinant of the ability of α-thrombin to bind to fibrin. This hypothesis is supported by our observation that hirugen blocked the binding of α-thrombin to fibrin II.

Although hirugen appears to interact with α-thrombin at a site distinct from the active site, the modest increase in the specificity constant (due to a decrease in the Michaelis constant) for α-thrombin-catalyzed hydrolysis of tos-GPR-amc suggests that binding of hirugen at the exosite induces a conformational change in α-thrombin that alters interactions at the active site so as to modestly decrease the Michaelis constant for α-thrombin-catalyzed hydrolysis of tos-GPR-amc. In accord with this conclusion is the finding that the circular dichroism of α-thrombin is altered upon binding of a nonsulfated peptide from the COOH-terminal domain of hirudin (13).

It is interesting to note that competitive inhibition of the action of α-thrombin on fibrinogen without inhibiting small substrate hydrolysis has been reported previously for thrombomodulin (TM), a macromolecular modifier of α-thrombin which does not bind to the active site (39), and an antibody directed against an α-thrombin fragment thought to comprise an exosite domain (5). It is difficult to exclude the possibility, however, that the effects of these macromolecules reflect steric interactions that prevent fibrinogen from approaching an unoccupied exosite in the complex of α-thrombin with either the antibody or TM, rather than direct competition between the macromolecule and fibrinogen for the fibrinogen-binding exosite of α-thrombin. Our observation that a 12-residue peptide (hirugen) competitively inhibits α-thrombin-catalyzed release of FPA from fibrinogen without inhibiting tos-GPR-amc hydrolysis provides strong evidence that the α-thrombin:fibrinogen interaction is largely determined by interactions at a small contiguous exosite in α-thrombin.

The observation that hirugen binding to α-thrombin effected only a 1.9-fold decrease in the rate of reaction of α-thrombin with AT provides additional evidence that hirugen doesn’t bind to the active site of α-thrombin, since active site-directed competitive inhibitors of α-thrombin block the reaction of α-thrombin with AT (39). In accord with our conclusion that hirugen and fibrin II bind to the same exosite of α-thrombin, fibrin II monomer has been reported to effect a reduction similar to that of hirugen for the rate of reaction of α-thrombin with AT (40).

In contrast to the modest inhibitory effects of hirugen and fibrin II monomer, TM accelerates inactivation of α-thrombin by AT in the absence of heparin (41,42). Heparin-catalyzed inactivation of α-thrombin by AT, however, is inhibited by TM (39). Fibrin II monomer also inhibits heparin-catalyzed inactivation of α-thrombin by AT (40). These observations in conjunction with the observations that hirugen inhibits the clotting of fibrinogen by α-thrombin (43) and decreases the affinity of α-thrombin for hirudin (44) suggested the possibility that heparin binds at the same exosite of α-thrombin as does fibrinogen, TM, and hirugren. Our observation that hirugen causes a similar reduction (1.9- to 2.4-fold) in the reaction between α-thrombin and AT for both the heparin-catalyzed and uncatalyzed reaction suggests that: (i) hirugen alters the intrinsic reactivity of α-thrombin with AT without preventing heparin from bridging AT to α-thrombin in the noncovalent termolecular complex AT:heparin:α-thrombin; and (ii) hirugen does not bind significantly (under our experimental conditions) to the heparin-binding exosite of α-
thrombin that is important for heparin catalysis of the inactivation of α-thrombin by AT. In light of these conclusions, how then can we account for the inhibitory effect of heparin both on the α-thrombin-catalyzed conversion of fibrinogen to fibrin and on the interaction of α-thrombin with hirudin, as well as the ability of TM and fibrin to inhibit heparin-catalyzed inactivation of α-thrombin by AT? Perhaps, in addition to binding to the exosite involved in bridging α-thrombin and AT in a ternolecular complex, heparin also binds to the fibrinogen-binding exosite of α-thrombin. The existence of more than one heparin-binding site in α-thrombin is consistent with the observation that α-thrombin-heparin complexes precipitate in a concentration-dependent manner resembling a cross-linking interaction (45). The effect of TM and fibrin II on heparin-catalyzed inactivation of α-thrombin by AT could of course reflect steric and electronic interactions between α-thrombin-bound heparin and α-thrombin-bound TM or α-thrombin-bound fibrin II. Clearly, further studies will be required to validate these notions.

The effects of hirugen on the interactions of α-thrombin with fibrinogen and AT discussed in the present study suggest that hirugen and other exosite-directed competitive inhibitors of α-thrombin may be useful antagonists of fibrin formation in vivo, since they should act in concert with AT. The binding of hirugen to α-thrombin prevents interaction between α-thrombin and fibrinogen without blocking inactivation of α-thrombin by AT. Equation 8 (see "Results" in Miniprint) can be simplified to account for the effect of hirugen on the fraction of fibrinogen (rather than too-GPR-ama) that is converted to product (fibrin I) in the presence of AT. Hirugen and fibrinogen competitively bind to α-thrombin, thus $1/K^m_{H} = 0$ and Equation 8 reduces to Equation 10.

$$f_c = \frac{k_p}{k_K} \frac{[E_c]}{[AT_o]}$$

In this equation, $f_c$ represents the fraction of fibrinogen converted to fibrin concomitant with inactivation of α-thrombin by AT. It can be seen that when the ratio of α-thrombin to antithrombin III ([Eo]/[ATo]) is such that only a small fraction of fibrinogen is converted to fibrin I (conditions where Equation 8 is applicable), the fraction of fibrinogen converted to fibrin I will be reduced by a factor equivalent to $1 + (k'[H]/kK_o)$. That is, the fraction of fibrinogen converted to fibrin I is reduced with increasing concentrations of hirugen ([H]). Similarly, it follows from Equation 9 (see "Results" in Miniprint, setting $1/K^m_{H} = 0$) that when heparin is present hirugen reduces the fraction of fibrinogen converted to fibrin I by a factor equivalent to

$$1 + \frac{k'[H][Hep]}{kK_o} \frac{(k''[Hep][AT_o])}{kK_o(k''[Hep] + [AT_o])}.$$  

It is important to note that although active site-directed competitive inhibitors of α-thrombin decrease the rate of conversion of fibrinogen to fibrin I, they should not alter the amount of fibrinogen that is ultimately converted to fibrin I in solutions containing AT and fibrinogen. This conclusion follows from the fact that blocking the active site of α-thrombin with an active site-directed competitive inhibitor decreases to the same extent the rate of inactivation of α-thrombin by AT and the rate of α-thrombin-catalyzed conversion of fibrinogen to fibrin I. The conclusion that active site-directed competitive inhibitors should not alter the amount of fibrinogen that is ultimately converted to fibrin I in the presence of AT can be proven algebraically by consideration of Equation 9 for the case where H is not hirugen, but rather an active site-directed competitive inhibitor. For such an inhibitor, $k', k''$, and $1/K^m_{H}$ are zero, and Equation 9 reduces to Equation 11, which stipulates that the fraction of fibrinogen that is ultimately converted to fibrin I is a constant, independent of the inhibitor concentration ([H]).

$$f_c = \frac{k_p}{k_K} \frac{[E_c]}{[AT_o]} \frac{1}{1 + \frac{k'[H]}{kK_o} \frac{(k''[Hep][AT_o])}{kK_o(k''[Hep] + [AT_o])}}$$

Thus, in regions of the circulatory system where the major mode of inactivation of α-thrombin is via reaction with AT and similarly reacting serine proteinase inhibitors, exosite-directed competitive inhibitors of α-thrombin may be more effective antagonists of fibrin clot formation than active site-directed competitive inhibitors.

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* This conclusion has been derived previously for irreversible inactivation of an enzyme where substrate and inactivator act as competitive inhibitors (30, 31).

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Supplementary Material

The C-Terminal Domain of Heparin as a Structural Determinant of Competitive Inhibition of the Action of α-Thrombin on Fibrinogen.

EXPERIMENTAL PROCEDURES

Materials: The α-CGR-αm and N-pTos-α-Thr-Pro-Arg-αm were purchased from Sigma. The concentration of the reaction mixture contained 150 mM NaCl, 0.05% (v/v) Triton X-100, 0.5 M N-methylimidazole, 0.5 M NaCl, 0.1 M NaOH, pH 7.8 using 0.02% Tween 80 (v/v) (16). Fibrinogen was prepared from human plasma using repeated precipitation with 95% ethanol. The final precipitate was dissolved in and dialyzed against 0.3 M NaCl, 0.1 M sodium phosphate, pH 7.5, then chromatographed on hydroxyapatite and gelatin-agarose to remove platelets (18) and fibrinogen (19). Fibrinogen concentrations were determined using an anion exchange column (18, 19) in 0.3 M NaCl and at pH 6.0 of 0.02% NaCl (20). Procedures described previously were used to prepare AP (21), high affinity heparin (22), and monomer (23). The ATP concentration was determined using an α-Endo G (24) at 0.1 M NaCl and at pH 6.0 of 0.02% NaCl (25). The concentration of α-thrombin was calculated by comparison to a standard curve of initial rates measured at known α-thrombin concentrations. To prevent α-thrombin autoactivation due to freeze (26) cycles, α-thrombin was stored at -80°C as small aliquots. Before each experiment an aliquot was thawed and immediately diluted into buffer containing PEG.

Section Kinetics measurements were carried out under the following standard conditions: 37°C, 700 mM sodium phosphate, 6.8 mM NaCl, pH 7.4, 0.1% TSE. The activation of α-thrombin on the fluorescein-α-CGR-αm was monitored with an SLM-9000 spectrophotometer, 380 nm excitation, 400 nm emission. All initial rates were measured after 10% of the initial concentration of α-thrombin substrate (α-CGR-αm, N-pTos-α-Thr-Pro-Arg-αm, or fibrinogen) was used.

Concentration of α-thrombin from heparin. In these experiments the initial rate of FPA release was measured in samples containing constant concentrations of fibrinogen and hirugen that ranged from 2.5 μM (based on the amidolytic activity) and 0.5 μM (based on the amidolytic activity). Fibrinogen concentrations were determined using anion exchange column and at 0.1 M NaCl, 0.02% NaCl (24). The samples were then centrifuged and filtered through a 0.45 μm filter. FPA was then assayed by high performance liquid chromatography (24). Initial rates were measured from slope linear plots of FPA concentration versus time. FPA concentration was determined from the chromogenic peak height. A linear relationship between peak height and chromogenic peak height is established using a standard curve (25). FPA that was released from a known concentration of fibrinogen with excess α-thrombin, versus the FPA concentration. Conversions of α-CGR-αm into hirugen in the Presence of Heparin. Initial rates of α-CGR-αm hydrolysis were measured under standard conditions. Reactions were initiated by addition of a aliquot of α-TH to a reaction mixture containing 100 μM α-TH, 0.5 M NaCl, 0.1 M NaOH, 0.02% Tween 80 (v/v) and 0.005% (v/v) hirugen. Initial rates were determined from the slope linear plots of fluorescence versus the time of incubation. When λmax/λ780 was determined experimentally, the λmax was determined as the half-maximal effective concentration. This was confirmed by control experiments which indicated a direct proportionality of the initial rate to the stoichiometry of hirugen.

Kinetics of α-thrombin Inhibition of AT in the Presence of Heparin with and without Heparin. Reactions were initiated by addition of α-thrombin to a reaction mixture containing 100 μM AT, 0.5 M NaCl, 0.1 M NaOH, and 0.5 M NaCl. The concentration of α-thrombin was varied from 0.05 μM to 0.1 μM. The concentration of hirugen was varied from 0.05 μM to 0.1 μM. The concentration of hirugen was varied from 0.05 μM to 0.1 μM. The concentration of hirugen was varied from 0.05 μM to 0.1 μM. The concentration of hirugen was varied from 0.05 μM to 0.1 μM.

RESULTS

Equations 1 and 2 provide a basis for analysis of the effects of hirugen on α-thrombin-mediated platelet aggregation.

Equation 1:

\[ E + S \overset{k_1}{\rightarrow} ES \overset{k_2}{\rightarrow} E + P \]
Exosite-directed Competitive Inhibition of \(\alpha\)-Thrombin

Fig. 1 illustrates plots for the dependence of the reciprocal of the initial velocity on the reciprocal of the thrombin concentration at several concentrations of hirugen. To account for any small difference in the Y intercept that might occur, initial rates were measured at higher thrombin and hirugen concentrations (Fig. 1, inset). These lines also intersect at a common point on the ordinate. The observation that the Y intercept of Eq. (1) and (2) is constant in the presence of hirugen indicates that \(k_{\text{cat}}\) for thrombin (i.e., in the absence of hirugen) is independent of the hirugen concentration, with the two data sets correlating well (Fig. 1, inset). An analysis assuming that the second-order rate constant is dependent on the hirugen concentration yielded a value of 6.4 \(\times\) 10^{-6} M^{-1} s^{-1} for \(k_{\text{cat}}\). The second-order rate constant obtained in the absence of hirugen is 0.6 \(\times\) 10^{-6} M^{-1} s^{-1}.

Fig. 3. Dependence of the initial rate of \(\alpha\)-thrombin-catalyzed hydrolysis of tos-GPR-amc on the concentration of hirugen. Initial rates (\(v\)) were measured at constant concentrations of tos-GPR-amc (7.5 \(\mu\)M) and 0.74 \(\mu\)M tos-GPR-amc. The curves represent a nonlinear least squares fit of the data to Equation 7 where \(K_{\text{D}}\) represents the observed dissociation constant.

Since the hirugen bound \(\alpha\)-thrombin to the inhibitor, it was of interest to determine whether hirugen blocked binding of \(\alpha\)-thrombin to fibrin. When fibrin (100 \(\mu\)M in 50 mM Tris/20 mM NaCl, pH 7.4) was added to a solution of hirugen (3.3 \(\mu\)M) in 50 mM Tris/20 mM NaCl, formation of a fibrin clot was accompanied by a decrease in the concentration of hirugen in the supernatant solution from 3.3 to 1.8 \(\mu\)M due to the binding of hirugen to the fibrin clot. Interestingly, fibrin (100 \(\mu\)M) blocked the binding of \(\alpha\)-thrombin to fibrin as evidenced by the observation that fibrin formation was not accompanied by a detectable decrease in the concentration of free \(\alpha\)-thrombin when hirugen was present (a decrease of 8% would have been detected).

Fig. 4. Double reciprocal plot for the effect of hirugen on the kinetics of \(\alpha\)-thrombin-catalyzed hydrolysis of tos-GPR-amc on the concentration of hirugen. The curves represent a nonlinear least squares fit of the data to Equation 7 where the substrate (S) is now tos-GPR-amc.

Although hirugen behaved as a competitive inhibitor of \(\alpha\)-thrombin-catalyzed hydrolysis of tos-GPR-amc, hirugen was observed to associate with a thrombin/hirugen complex. The potency of the peptide substrate, tos-GPR-amc, on the concentration of hirugen (Fig. 3) shows the dependence of the rate of tos-GPR-amc hydrolysis on the concentration of hirugen under conditions where the concentration of tos-GPR-amc was well below \(K_{\text{D}}\). Under these conditions, Equations 5-6 reduce to Equation 7, where the substrate (S) is now tos-GPR-amc.

The constants \(K_{\text{D}}\), \(K_{\text{D}}\), and \(K_{\text{D}}\) are the specificity constants for substrate (tos-GPR-amc) and byproducts caused by \(\alpha\)-thrombin and the exosite/hirugen complex, respectively. The expression for the total rate of hydrolysis of tos-GPR-amc. The expression for the total rate of hydrolysis of tos-GPR-amc is given by:

\[
\frac{1}{v} = \frac{1}{v_{\text{max}}} + \frac{1}{K_{\text{D}} + [S]} + \frac{1}{K_{\text{D}} + [S]} + \frac{1}{K_{\text{D}} + [S]}
\]

Although both plots display constant Y intercepts, Fig. 5 shows that both curves decrease with increasing hirugen concentration in a linear manner. The curves represent a nonlinear least squares fit of the data to Equation 7, where the substrate (S) is now tos-GPR-amc.

Fig. 5. Dependence of \(K_{\text{D}}\) for the \(\alpha\)-thrombin-catalyzed hydrolysis of tos-GPR-amc on the concentration of hirugen. The curves represent a nonlinear least squares fit of the data to Equation 7 where the substrate (S) is now tos-GPR-amc.

Although both plots display constant Y intercepts, Fig. 5 shows that both curves decrease with increasing hirugen concentration. The curves represent a nonlinear least squares fit of the data to Equation 7, where the substrate (S) is now tos-GPR-amc.

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Exosite-directed Competitive Inhibition of α-Thrombin

\[ \frac{1}{E} \frac{d[E]}{dt} = \frac{1}{K_{D}} \frac{K_{D} + [I]}{K_{D} + [I] + K_{M}} \]

\[ k_{cat} \frac{[S]}{[E][I]} = \frac{k_{cat}}{K_{M}} \]

\[ k_{cat} \frac{[S]}{[E][I]} = \frac{k_{cat}}{K_{M}} \]

[ΔT, A, and [I]] are the initial concentrations of α-thrombin, AT, and heparin, respectively, and kcat and kcat are the second order rate constants for reaction of AT with free α-thrombin and heparin-bound α-thrombin, respectively. Eq. 9 describes the dependence of the observed fraction of heparin-susceptible α-thrombin on the concentration of heparin.

A non-linear least squares fit to the data with a quadratic model yielded a value of 8.1 ± 0.2 M⁻¹·s⁻¹ for kcat when the values of kcat, kcat, Kcat, and Kcat were set at their previously determined values of 3.4 ± 0.2 M⁻¹·s⁻¹, 3.4 ± 0.2 M⁻¹·s⁻¹, and 3.4 ± 0.2 M⁻¹·s⁻¹, respectively, and the value of kcat was set to the value of 15.6 ± 0.2 M⁻¹·s⁻¹ that was obtained from the fit of Equation 9 to the data from substrate conversion experiments performed with heparin in the absence of α-thrombin. Comparison of the values of kcat and kcat revealed that AT reacts 1.9-fold more slowly with the heparin-bound α-thrombin complex than with free heparin (see Table 2).

We also investigated the effect of heparin on heparin-catalyzed reaction of the heparin-thrombin complex with the nucleotides. Heparin was found to be a potent inhibitor of the heparin-thrombin complex with an inhibition constant of 2.6 ± 0.1 M⁻¹·s⁻¹ (see Table 2).

Equation 9 differs from Equation 8 by the two additional terms in the denominator accounting for the heparin-catalyzed inactivation of free and heparin-bound α-thrombin. kcat and kcat are the second order rate constants for reaction of heparin-bound α-thrombin with free heparin and AT, respectively. The effect of heparin on the fraction of free-GPIIb-AT heparin-converted to produce competent with heparin-accelerated inactivation of α-thrombin is shown in Fig. 7. Setting the constants kcat, kcat, Kcat, Kcat, and Kcat to their previously determined values of 1.1 ± 0.2 M⁻¹·s⁻¹, 1.1 ± 0.2 M⁻¹·s⁻¹, 1.1 ± 0.2 M⁻¹·s⁻¹, and 1.1 ± 0.2 M⁻¹·s⁻¹, respectively, and setting kcat = kcat = Kcat = Kcat to the value of 6.0 ± 0.2 M⁻¹·s⁻¹ determined from Equation 9 and the value of the fraction of substrate conversion observed upon heparin-catalyzed inactivation of α-thrombin by AT in the absence of heparin, a non-linear least squares fit of the data to Equation 9 yielded a value of K = 2.6 ± 0.1 M⁻¹·s⁻¹. Comparison of the values of kcat, kcat, Kcat, Kcat, and kcat, kcat, Kcat, Kcat indicated that heparin-bound α-thrombin reacts 2.9-fold more slowly with the heparin-competent AT complex than free α-thrombin (see Table 2).

Fig. 6. Dependence of the fraction of free-GPIIb-AT heparin-converted to produce competent with heparin-catalyzed inactivation of α-thrombin by AT in the concentration of heparin. The fraction of free-GPIIb-AT heparin-converted to produce competent with heparin-catalyzed inactivation of α-thrombin (1.3 ± 0.2 M⁻¹·s⁻¹) at AT (2.2 M) in the presence of free-GPIIb-AT (2.2 M) and heparin (1.3 ± 0.2 M⁻¹·s⁻¹) at the potted thrombin concentrations. The curve represents a non-linear least squares fit of the data to Equation 9 with kcat, kcat, Kcat, Kcat, and Kcat set to the previously determined values of 2.6 ± 0.1 M⁻¹·s⁻¹, 2.6 ± 0.1 M⁻¹·s⁻¹, 2.6 ± 0.1 M⁻¹·s⁻¹, 2.6 ± 0.1 M⁻¹·s⁻¹, and |ΔH|, M⁻¹, respectively. The best fit yielded a value of K = 6.0 ± 0.1 M⁻¹·s⁻¹.

Fig. 7. Dependence of the fraction of free-GPIIb-AT heparin-converted to produce competent with heparin-catalyzed inactivation of α-thrombin by AT in the concentration of heparin. The fraction of free-GPIIb-AT heparin-converted to produce competent with heparin-catalyzed inactivation of α-thrombin (2.2 M) at AT (2.2 M) in the presence of free-GPIIb-AT (2.2 M) and heparin (0.5 M) at the potted thrombin concentrations. The curve represents a non-linear least squares fit of the data to Equation 9 with kcat, kcat, Kcat, and Kcat set to the previously determined values of 2.6 ± 0.1 M⁻¹·s⁻¹, 2.6 ± 0.1 M⁻¹·s⁻¹, 2.6 ± 0.1 M⁻¹·s⁻¹, and 2.6 ± 0.1 M⁻¹·s⁻¹, respectively. The best fit yielded a value of K = 6.0 ± 0.1 M⁻¹·s⁻¹.

Table 1

| Reaction Conditions | AT + Heparin bound - α-Thrombin with | free-GPIIb-AT + Heparin bound-AT |
|--------------------|-------------------------------------|----------------------------------|
| Fractures          | [α-S2] + [H] + [I] | [α-S2] + [H] + [I] |
|                   | E + free-GPIIb-AT | 2.0 ± 0.1 M⁻¹·s⁻¹ |
|                   | E + Heparin bound-AT | 2.0 ± 0.1 M⁻¹·s⁻¹ |
|                   | E + AT + Heparin bound-AT | 2.0 ± 0.1 M⁻¹·s⁻¹ |

Table 2

| Heparin concentration (I) | E + Heparin bound-AT |
|---------------------------|----------------------|
| 0.5 M                     | 2.0 ± 0.1 M⁻¹·s⁻¹ |
| 1.0 M                     | 2.0 ± 0.1 M⁻¹·s⁻¹ |
| 1.5 M                     | 2.0 ± 0.1 M⁻¹·s⁻¹ |

[ΔH, or the total concentration of α-thrombin (0.5 M) and Heparin (0.5 M) is the initial α-thrombin concentration at any time (0) is the experimentally measured second order rate constant for inactivation of α-thrombin by AT. Substitution of Equations 13 to 18 into the initial rate expression for fluorescamine substrate hydrolysis (Equation 13) followed by integration assuming initial rate conditions (23) is constant over the boundaries t = 0, t = d and dividing by the initial substrate concentration ([Sub]) yields Equation 20.

Multiplying the numerator and denominator of Equation 20 by 1/2 and Equation 9 of the results section. This equation describes the heparin dependent fraction of substrate converted to product during the time course of exosite inactivation by AT.

The absorption of heparin (150 µg) on Sepharose 4B matrix affinity eluted the heparin-dependent and heparin-independent inhibitors by 2/3 and Equation 9 of the results section. This equation is identical to Equation 2b except for the absence of the terms in the denominator containing heparin.