Heparin Promotes the Binding of Thrombin to Fibrin Polymer

**QUANTITATIVE CHARACTERIZATION OF A THROMBIN-FIBRIN POLYMER-HEPARIN TERNARY COMPLEX**

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The binding of human α-thrombin (IIa) to fibrin polymer (FnIIp) was studied in the presence and absence of a high affinity 20,300 M\(_{\text{r}}\) heparin (H) at pH 7.4, I 0.15, and 23 °C. In the absence of heparin, thrombin interacts with a high affinity class of binding sites on fibrin polymer with a dissociation constant of 301 ± 36 nM in a manner which is independent of the enzyme active site. Studies of thrombin binding as a function of heparin and fibrin polymer concentrations imply that a ternary thrombin-fibrin polymer-heparin complex (IIa-FnIIp-H) is formed. Assembly of the ternary complex occurs randomly through the interactions of all three possible intermediate binary complexes; IIa-H, IIa-FnIIp, and FnIIp-H. Using an independently determined value of 280 ± 35 nM for the FnIIp-H dissociation constant, global fits of the binding data yield a dissociation constant of 15 ± 6 nM for the IIa-H interaction and 47 ± 9 nM for the IIa-H intermediate binary complex interaction with FnIIp. These studies indicate that heparin enhances the binding of thrombin to fibrin polymer 6.4-fold with an overall dissociation constant for ternary complex formation of 705 nM. The effect of heparin molecular weight on ternary complex formation has also been investigated. Heparins of molecular weights 11,200–20,300 behave similarly with respect to their influence on ternary complex formation, whereas heparins of lower molecular weight are less effective in promoting thrombin binding to fibrin polymer. This effect of heparin is also independent of whether it has high or low affinity for antithrombin III.

The demonstration of the formation of a ternary IIa-FnIIp-H complex complements kinetic evidence indicating the formation of an analogous ternary complex with fibrin II monomer (Hogg, P. J., and Jackson, C. M. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 3619–3623). The possible implications of these findings for the in vivo distribution and actions of thrombin and the clinical efficacy of heparin are also discussed.

Thrombin has central regulatory functions in the maintenance of hemostasis. This serine proteinase interacts with several substrates (fibrinogen, factors V, VIII, XIII, and protein C), inhibitors (antithrombin III, α2-macroglobulin, and heparin cofactor II), and cell surfaces (platelets and endothelial cells) which together contribute to the delicate balance characteristic of the hemostatic process (1).

The ultimate action of thrombin in blood-clotting is the conversion of circulating fibrinogen (Aα2Bβγδ) to the insoluble fibrin matrix of blood clots by the cleavage of Arg-Gly bonds at positions 16–17 of the Aα chain and 14–15 of the Bγ chain to release fibrinopeptides A and B (2, 3). In addition to this enzymatic function, thrombin also interacts with fibrinogen in a manner distinct from the Michaelis complex necessary for fibrinopeptide release. This was demonstrated some time ago by Seegers and co-workers (4) and Liu et al. (5, 6) and more recently by Kaminski and McDonagh (7, 8) who found that thrombin action on small substrates and its inactivation by low-molecular-weight inhibitors is not detectably perturbed by fibrin binding. Recent investigations have further defined the specificity of the interaction, which appears to involve a binding region (9, 10) on the central E domain of fibrinogen (11).

Altered binding of thrombin to fibrin may be responsible for physiological disorders. About 20 cases of dysfibrinogenemia associated with arterial or venous thrombosis have been reported (12, 13) and, in some of these cases, the structural defect in the fibrinogen molecule has been identified. In the cases of fibrinogen New York I (14) and fibrinogen Milano II (15), the underlying cause of thrombosis was attributed to defective binding of thrombin to the fibrin formed from the abnormal fibrinogen.

The ability of heparin, a complex glycosaminoglycan isolated from a variety of natural sources, to enhance the inactivation of coagulation proteinases has been established (16–19) and has proven clinical value (20). Of the proteinases which are modulated by heparin action, experiments in plasma (21–25) indicate that the heparin-catalyzed inactivation of thrombin is of most importance. It has been concluded that this is primarily because of the importance of thrombin for activation of Factors VIII and V. However, the inability of heparin to prevent coronary reocclusion in patients treated with tissue plasminogen activator (26–28) suggests physiological or pathophysiologic circumstances where the efficacy of heparin is compromised. It has been demonstrated that fibrin monomer binding to thrombin decreases the second-order rate constant for the inactivation of thrombin by heparin-antithrombin III more than 300-fold at physiological fibrin concentrations (29). These studies suggested that thrombin forms a ternary complex with fibrin and heparin and that the thrombin in this complex possesses altered reactivity toward its substrates and inhibitors. From measurements of thrombin binding as a function of heparin and fibrin polymer concentrations, we demonstrate and quantify the formation of a ternary thrombin-fibrin-polymer-heparin complex and discuss 1) the implications of this binding for the clinical efficacy of
heparin, 2) the possible influence of fibrin on the interaction of thrombin with cell surface glycosaminoglycans, and 3) how the formation of an analogous ternary complex with fibrin monomer can explain the combined effects of heparin and fibrin monomer on the kinetic properties of thrombin. To facilitate understanding the quantitative studies described below, the three possible pathways for the formation of the ternary thrombin-fibrin polymer-heparin complex are shown in Scheme 1.

EXPERIMENTAL PROCEDURES

RESULTS

Formation of the Binary, Thrombin-Fibrin Polymer, and Fibrin Polymer-Heparin Complexes—The dissociation constants for the binary complexes between thrombin and fibrin polymer, $K_{\text{IIa FnIIp}}$, and between fibrin polymer and heparin, $K_{\text{FnIIp H}}$, have been characterized under two limiting conditions. Fibrin polymer is expressed as fibrin monomer concentration. When experiments are performed under conditions where $[\text{FnIIp}] \gg [\text{IIa}]$, rectangular hyperbolic binding is observed which is characterized by a dissociation constant of $301 \pm 37\, \text{nM}$ (Fig. 1A). This value is independent of whether a fixed thrombin concentration of $3.87$ or $15.63\, \text{nM}$ is employed. These results imply that, under these low binding density conditions ([FnIIp] $\gg$ [IIa] $< 15.63\, \text{nM}$), thrombin is interacting essentially exclusively with a single high affinity class of binding sites on fibrin polymer. This interaction is unaffected by the presence of $1.5\, \text{mM Ca}^{2+}$ ions. The binding data reported in Fig. 1B are from experiments of more conventional design, i.e. varying thrombin concentration at a fixed fibrin polymer concentration. The results are indicative of an interaction involving heterogeneous binding sites (see inset, Fig. 1B) and agree well with the findings of Liu et al. (5), who examined the binding of radiolabeled thrombin to a fibrin clot. The data have been fitted to a model for two classes of independent, nonequivalent binding sites with the $K_d$ for the high affinity site fixed at $301\, \text{nM}$. Inset, Scatchard plot of the data (63); $r$ (mol thrombin/mol fibrin) is expressed as free concentration of fibrin monomer. The solid line was calculated from a least-squares fit of the data to a rectangular hyperbola which is unaffected by the presence of $1.5\, \text{mM Ca}^{2+}$ ions. The binding data reported in Fig. 1B are from experiments of more conventional design, i.e. varying thrombin concentration at a fixed fibrin polymer concentration. The results are indicative of an interaction involving heterogeneous binding sites (see inset, Fig. 1B) and agree well with the findings of Liu et al. (5), who examined the binding of radiolabeled thrombin to a fibrin clot. The data have been fitted to a model for two classes of independent, nonequivalent binding sites with the $K_d$ for the high affinity site fixed at $301\, \text{nM}$. Inset, Scatchard plot of the data (63); $r$ (mol thrombin/mol fibrin) is expressed as free concentration of fibrin monomer. The solid line was calculated from a least-squares fit of the data to a rectangular hyperbola which is unaffected by the presence of $1.5\, \text{mM Ca}^{2+}$ ions.

An estimate of the dissociation constant for the formation of the ternary complex between fibrin polymer and heparin, $K_{\text{FnIIp H}}$, was made under conditions where [H] $\ll$ [FnIIp] by equilibrating varying fibrin polymer concentrations (0.06–2.5 $\mu\text{M}$) with a constant concentration of heparin (49.9 $\text{nM}$) and measuring free heparin concentration at equilibrium based on its ability to enhance the inactivation of bovine Xa by antithrombin III (see “Methods and Materials,” Ref. 42). The results are reported in Fig. 2. The data were fitted by least-squares analysis (46) to a rectangular hyperbola which is $60$
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1.0 2.0

FIG. 2. The binding of an M. 20,300 high affinity heparin (49.9 nM) to fibrin polymer, expressed as free concentration of fibrin monomer. The solid line was calculated from a least-squares fit of the data to a rectangular hyperbola with $K_d$ equal to 280 ± 35 nM (46).

0' I

0

20 40 60 80 100

[Heparin], nM

FIG. 3. The binding of thrombin (3.87 nM), expressed as % Thrombin Bound (Equation 4), to four fixed concentrations of fibrin polymer (0, 63.5 nM; 121.7 nM; 190.4 nM; 317.4 nM, expressed as fibrin monomer concentration) as a function of M. 20,300 high affinity heparin concentration (0–99.8 nM). The free concentrations of heparin are calculated using Equation 3. The solid lines are calculated from the global least-squares fit of the binding data to Equation 2 using values of 301 and 280 nM for the $K_{IIa,n}$ and $K_{FnIIp,H}$ dissociation constants, respectively. The data for 121.7 nM fibrin polymer (+) is from experiments in the presence of 1.5 mM Ca$^{2+}$ ions.

characterized by a dissociation constant of 280 ± 35 nM. This dissociation constant is 20-fold lower than the dissociation constant for the interaction of heparin with fibrin II monomer determined from quantitative affinity chromatographic studies of this interaction, 5.7 μM (29). The higher affinity of heparin for fibrin polymer than fibrin monomer perhaps reflects steric or protein conformational differences between monomeric and polymerized fibrin or may be a result of additional interactions of the heparin with neighboring fibrin molecules in the polymerized fibrin.

Formation of the Binary Thrombin-Heparin and the Ternary Thrombin-Heparin-Fibrin Polymer Complexes—Results of experiments where the amount of thrombin bound ([IIa]$_T$ = 3.87 nM) to four fixed concentrations of fibrin polymer (63.5, 121.7, 190.4, and 317.4 nM) was measured as a function of heparin concentration (0–99.8 nM) are reported in Fig. 3. The data for 121.7 nM fibrin polymer (+) is from experiments in the presence of 1.5 mM Ca$^{2+}$ ions. The data for 121.7 nM fibrin polymer (+) is from experiments in the presence of 1.5 mM Ca$^{2+}$ ions.

Experimental Validation of Ternary Complex Formation—A diagnostic feature of ternary complex formation is the ability of one reactant at high concentrations, e.g. heparin, to inhibit competitively the binding of another reactant (47), e.g. thrombin. This arises because the other two components in the system, e.g. thrombin and fibrin polymer, become saturated with this reactant at high concentration resulting in the formation of noninteracting binary complexes, e.g. IIa-H and FnIIp-H. An example of this phenomenon, as it relates to this system (Scheme 1), is seen in Fig. 4. Heparin at high concentrations (>100 nM) competitively inhibits the binding of thrombin (4.14 nM) to fibrin polymer (87.9 nM). The descending limb of this curve represents the formation of FnIIp-H binary complexes. The solid line is the theoretical fit to the data using Equation 2 (Miniprint Supplement) with the values for the binary and ternary dissociation constants that have been calculated from the experiments described in the previous two sections.

An experiment to further test for ternary complex formation was performed (Fig. 5). In this experiment the amount of thrombin bound ([IIa]$_T$ = 6.25 nM) as a function of fibrin polymer concentration was measured at three fixed concen-

![Fig. 4. The binding of thrombin (4.14 nM), expressed as % Thrombin Bound (Equation 4), to fibrin polymer (87.9 nM fibrin monomer concentration) as a function of M. 20,300 high affinity heparin concentration (0–1.2 μM). The free concentrations of heparin were calculated using Equation 3. The solid line is calculated from Equation 2 using the values reported in Scheme 2.](http://www.jbc.org/)

Values of 2.3 and 2.5 nM for $K_{IIa,FnIIp,H}$ and $K_{FmIIp,H,IIa}$ ternary dissociation constants are, therefore, calculated (Scheme 2).
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Fig. 5. The binding of thrombin (6.25 nM), expressed as % Thrombin Bound (Equation 4), to fibrin polymer, expressed as total concentration of fibrin monomer, at three fixed M, 20,300 high affinity heparin concentrations (○, 99.8 nM; ♦, 499 nM; ■, 2.00 μM). The solid lines are calculated from Equation 2 using the values reported in Scheme 2.

Fig. 6. A, the binding of thrombin (3.87 nM), expressed as % Thrombin Bound (Equation 4), to fibrin polymer (190.4 nM fibrin monomer concentration) as a function of different molecular weight high affinity heparins: ●, pentasaccharide, M, 1,720; ○, M, 6,700; ▼, M, 15,400; □, M, 20,300, and a low affinity heparin, ◼, M, 11,200. The solid lines are calculated from least-squares fits of the data (○ and ▼) to a rectangular hyperbolae (46). B, plot of the maximum % Thrombin Bound at saturating heparin concentration versus heparin molecular weight.

trations of heparin (99.8 nM, 499 nM, and 2.00 μM). Equation 2 (Miniprint Supplement) predicts that the isotherm for binding of thrombin to fibrin polymer at high heparin concentrations ([H] >> K dissociation) is effective in binding heparin polymer 2. This feature is a result of the formation of FnH. H binary complexes at high heparin concentrations which, like the situation described above in relation to Fig. 4, competitively inhibit the binding of thrombin to fibrin polymer. The solid lines in Fig. 5 are the theoretical fits to the data using Equation 2 with the values for the binary and ternary dissociation constants that have been calculated from Figs. 1–3.

Effect of Heparin Molecular Weight and Heparin Affinity for Antithrombin III on Ternary Complex Formation—The effect of heparin molecular weight on ternary complex formation has been investigated on the basis of its ability to enhance thrombin ([IIa]T = 3.87 nM) binding to fibrin polymer (190 nM). Heparin of molecular weight 11,200-20,300 behave similarly with respect to their influence on ternary complex formation (Fig. 6). This is judged on the basis of the extrapolated extent of thrombin binding at saturating heparin (Fig. 6). Whether the heparin has high or low affinity for antithrombin III appears to have no influence on its ability to promote thrombin binding to fibrin polymer. The heparin of molecular weight 11,200 has low affinity for antithrombin III (Fig. 6). Heparin species of molecular weight <11,200 are much less effective in promoting thrombin binding to fibrin polymer.

Discussion

The binding of thrombin to fibrin was first demonstrated by Søegers et al. (4). At that time they associated it with a type of antithrombin mechanism (antithrombin I) whereby the capturing of thrombin by the clot reduces thrombin action on circulating fibrinogen. Indeed, defective binding of thrombin to fibrin has been associated with thrombosis in some cases of dysfibrinogenemia (14, 15). From these studies we find that thrombin interacts with a high affinity class of sites on fibrin polymer (expressed as fibrin monomer concentrations) with a dissociation constant of 301 ± 36 nM (Fig. 1A). At high thrombin concentrations heterogeneous binding is observed (Fig. 1B). This agrees well with results of Liu et al. (5), when their data were refitted by nonlinear regression (46) to take explicitly into account the two classes of sites observed by them. The heterogeneous binding of thrombin to fibrin polymer illustrated in Fig. 1A indicates approximately 0.34 high affinity site on fibrin polymer for thrombin. The dissociation constant determined for this interaction, 301 nM, is a stoichiometric constant (Kd) and relates to the intrinsic or site-binding constant (k0) by the relationship, k0 = nKd (48) where n is the number of binding sites, 0.34. The site binding constant is therefore calculated to be 102 nM. Assuming that thrombin interacts with one, or perhaps two, sites on a fibrin monomer these findings indicate that the polymerization of fibrins results in a loss of thrombin binding sites; or stated alternatively, only one of every three fibrin monomer sites is accessible on polymerized fibrin. This masking of binding sites upon fibrin polymerization may have important implications for the effects of fibrin on the kinetic actions of thrombin because it alters the partition of thrombin between solution and fibrin polymer during the course of fibrin monomer polymerization (see the accompanying article (64)).

Thrombin binds to a variety of negatively charged polysaccharides. The nonspecific binding of heparin to thrombin, in addition to its specific interaction with antithrombin III, has been demonstrated to be a prerequisite for the catalytic efficiency of this glycosaminoglycan in enhancing the inactiva-

tion of thrombin by antithrombin III (19). The global best-fit estimate for the thrombin-heparin dissociation constant, 15 ± 6 nM (1 S.D.), is in agreement with an independently determined estimate of this constant, 32 ± 10 nM (1 S.D.), from unrelated quantitative affinity chromatographic studies. Thrombin similarly interacts with the cell-surface glycosaminoglycans (GAG) of vascular endothelial cells (49-52). It has been proposed that the binding of thrombin to these structures regulates the actions of this proteinase (53). Several studies of the binding of thrombin to cell surface GAG indicate that the Kd for this interaction is also in the low nanomolar range, 20-30 nM (49-52).

In addition to characterizing the binary complex interactions between fibrin and thrombin and between heparin and thrombin, we have examined the consequences of the combined interactions of these two reactants on the distribution of thrombin in equilibrium mixtures of thrombin, fibrin polymer, and heparin. The findings are summarized in Scheme 2 and are concordant with the predicted behavior for a binding model in which a ternary thrombin-fibrin-heparin complex is formed. In Scheme 2, the assembly of the ternary complex occurs randomly through the interactions of all three
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possible intermediate binary complexes: IIa-H, IIa-FnIlp, and FnIlp-H. Upon comparison of the constants for formation of the three binary complexes, however, the higher affinity of the IIa-H interaction compared to the affinities of the other two binary complexes indicates that the assembly of the ternary complex will occur predominantly through the interaction of intermediate IIa-H binary complexes with fibrin polymer.

A distinguishing feature of ternary complex formation is the ability of one reactant at high concentrations to inhibit competitively the binding of another reactant (47). The results of Figs. 4 and 5 demonstrate that heparin, at high concentrations, will inhibit competitively the binding of thrombin to fibrin polymer. These experiments are important in that they further establish the adequacy of the model proposed here for the interactions of thrombin, fibrin polymer, and heparin.

Heparins of molecular weight 11,200-20,300 behave similarly with respect to their influence on ternary complex formation (Fig. 6). As expected for a nonspecific interaction, the ability of heparin to enhance the binding of thrombin to fibrin polymer is independent of whether the heparin has high or low affinity for antithrombin III (Fig. 6). Because the molecular weight distribution of therapeutic heparin spans the region from ~5,000 to 30,000 (54, 55) the studies reported here will be relevant to the type of heparin that is used clinically. The large size of the GAG which have been isolated from vascular endothelium similarly suggests that these interactions with thrombin and fibrin will also be supported by these structures.

Heparins of lower molecular weight (<11,200) are less effective in promoting thrombin binding to fibrin polymer. Assuming an equivalent model (Scheme 1) for the interactions of lower molecular weight heparins, the data suggest that the effect of reduction in molecular weight is a result of an increase in the $K_d$ of IIa-H and $K_d$ (H,FnIlp) dissociation constants. This is reflected as a decrease in the apparent half-maximal saturation value for heparin and an extrapolated lower maximal extent of thrombin binding, respectively (Fig. 6). The heparin molecular weight dependence demonstrated here resembles the molecular weight dependence of heparin with high affinity for antithrombin III in catalyzing the inactivation of thrombin by antithrombin III (56). This may reflect a correspondence between the heparin molecular weight and affinity of heparin for thrombin, as indicated here, and the ability of the heparin to enhance the inactivation of thrombin by antithrombin III. Correlation between heparin molecular weight and its catalysis of thrombin inactivation by antithrombin III has been suggested by Hoylaerts et al. (18).

In addition to its interaction with cell-surface GAG, thrombin also interacts with thrombomodulin (TM), an endothelial cell intrinsic membrane glycoprotein that binds thrombin with high affinity and modulates its substrate specificity (see Ref. 57 for a recent review). With a knowledge of the affinities and the approximate number of TM and GAG binding sites on the endothelial cell for thrombin it is possible to calculate the partition of thrombin between these two cell-surface receptors. This enables an assessment of the influence of fibrin on the partition of thrombin and therefore some idea of the relative importance of these ternary complex interactions for the distribution of thrombin between these two endothelial receptors. There are approximately 100,000 TM molecules/endothelial cell that bind thrombin with a $K_d$ of approximately 0.7 nM (57, 58). Studies of thrombin binding to endothelial cell GAG indicate approximately 300,000 GAG binding sites/endothelial cell (50). Assuming a $K_d$ for the thrombin-GAG interaction of 15 nM measured here which is in the range of values reported for this interaction (49-52), and the $K_d$ values for the ternary complex interactions indicated in Scheme 2, calculation of the distribution of 0.1 nM thrombin (approximately 0.7 unit) between TM and GAG as a function of fibrin concentration results in the following observations. In the absence of fibrin, assuming microcirculation TM and GAG concentrations of 0.5 nM and 1.5 pM, respectively (50, 57), approximately 88% of the thrombin will be bound to TM and only 12% to GAG. In the presence of 70 nM fibrin, which corresponds to conversion of only 1% of the local fibrinogen concentration (7 μM) to fibrin, 77% of the thrombin will be bound to TM and 23% to GAG. Similarly, at 10% conversion (0.7 μM fibrin) 37% of the thrombin will be associated with TM and 62% with GAG. These results suggest that fibrin, as a result of ternary complex interactions with thrombin and GAG, may alter the distribution of thrombin between these two receptors and thereby influence the binding of thrombin to the endothelial surface. In the accompanying paper (64), we demonstrate that thrombin binding in this ternary complex differentially affects its actions on its many substrates, and thus this partitioning process may have a very important role in regulating thrombin action. The initial observation that more thrombin is bound to fibrin in the presence of heparin than in its absence was made many years ago (59-61). Although the significance of this phenomenon for the clinical efficacy of heparin is still not known, it may have important implications for the regulation of thrombin action on fibrinogen and protection of thrombin from inactivation by antithrombin III (29). There are multiple mechanisms in blood clotting for localizing and compartmentalizing reactions (62). During hemostasis and perhaps arterial thrombosis the endothelial and platelet surfaces, in conjunction with fibrin and von Willebrand factor, interact to constrain spatially the coagulation reactions to the injury site. The enhanced binding of thrombin to fibrin polymer in the presence of heparin, or GAG on endothelial cells adjacent to the injury site, may be a mechanism whereby diffusion of thrombin away from the growing clot is restricted thus preventing dissemination of this proteinase throughout the circulation. The protection of thrombin from inactivation by heparin-antithrombin III by fibrin (29) will also act to prolong the action of thrombin on fibrinogen in the region of a clot, in contrast to the situation in the circulation where thrombin is subject to rapid inactivation. In this way heparin and cell surface GAG may facilitate the formation of a fibrin canopy at the site of injury thereby limiting hemostatic plug and thrombus progression. The protection by fibrin monomer may be of clinical importance as it possibly explains the limited efficacy of heparin in preventing coronary reocclusion in patients treated with fibrinolytic agents (26-28). The combined effects of these two reactants on some other kinetic properties of thrombin are explored in the following paper (64).

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SUPPLEMENTAL MATERIAL TO
HEPARIN PROMOTES THE BINDING OF THROMBIN TO FIBRIN POLYMER: QUANTITATIVE CHARACTERIZATION OF A THROMBIN-FIBRIN POLYMER-HEPARIN TERNARY COMPLEX
Philip J. Hogg and Craig M. Jackson

In solution containing an equilibrium mixture of FII, FIII, and H the concentration of FII in the solution phase, [FII]s, is given by

\[ [\text{FII}]_s = [\text{FII}]_0 + (K_{\text{III}} + K_{\text{H}})[\text{III}]_0 + [\text{H}]_0 \]

and is related to the total concentration of FII, [FII]T, and the free concentrations of FIII, [FIII], and H, [H], according to Equation 1:

\[ [\text{FII}]_0 = \frac{[\text{FII}]_s - [\text{FII}]_0 - (K_{\text{III}} + K_{\text{H}})[\text{III}]_0 + [\text{H}]_0}{1 + K_{\text{III}} + K_{\text{H}}} \]

Equation 2 predicts that, when \([\text{FIII}]_0 > [\text{FII}]_0 + [\text{H}]_0\), the concentration of thrombin bound to a fixed concentration of fibrin polymer will increase hyperbolically with heparin concentration with half-maximal binding occurring at the magnitude of \([\text{FIII}]_0\) and the maximal extent of binding proportional to the magnitude of \([\text{FIII}]_0 + [\text{H}]_0\). Higher heparin concentrations, \([\text{H}]_0 > [\text{FIII}]_0 + [\text{H}]_0\), will competitively displace the binding of thrombin to fibrin polymer because of the formation of non-interacting FIII-H and FIII-H heparin complexes.

The results for thrombin binding to fibrin polymer in the absence or presence of heparin, when \([\text{FIII}]_0 > [\text{FII}]_0\), are expressed in terms of percent thrombin bound to afford a more familiar representation of the binding data. The dependent variable in Equation 2 is simply expressed as follows:

\[ \% \text{Thrombin Bound} = \frac{[\text{FII}]_0}{[\text{FII}]_0 + [\text{H}]_0} \times 100\%
\]

Experimental Procedures

Protein - Human thrombin was supplied by Dr. John Fantus of the New York State Department of Health, Albany, New York. The active enzyme concentration was determined by active-site titration (32). Human Factor Xa was a gift of Dr. Paul Boeck of the American Red Cross, Detroit, Michigan. It was prepared by activation of purified Factor X with the proteinase K from Russell's viper venom (33), buffered by sodium oxalate into Sepharose affinity chromatography, and activated by tritiated (32).

Recombinant Factor Xa was obtained by activation of purified Factor X (31) with the same protease from Russell's viper venom (33), modified by Sephadex G-200 chromatography, and activated in vitro (31,33).

Pro-Pro-Arg-thrombin was prepared by incubating human thrombin (3.25 mg/ml) with approximately three-fold molar excess of Pro-Pro-Arg-thrombin (32

Heparin was prepared from human fresh plasma by the method of Jakobsen and Kastrup (34), and the thrombin clotting times in the presence of heparin were determined by the thrombin-ceftriaxone method in the absence of heparin or by colorimetric assays in the presence of heparin.

Fibrinogen was reconstituted in 0.5 M NaCl, pH 7.4, and the final diazot was adjusted to 0.15 M NaCl with 0.1 M NaOH, pH 7.4. This fibrinogen was rechromatographed on Sephacryl S-300 HR (Pharmacia) in the same buffer at 35°C to remove any aggregated fibrinogen or thrombin-fibrinogen complexes. Standard fibrinogen (0.02 M) was then freeze and stored at -70°C.

Antithrombin III was prepared from human cryoprecipitate by means of a modification of procedures of Miller-Ayers et al. (35) and Thacher and Jacobson (36) as described by Lafore and Jacobson (36). The final product was dialyzed against 0.1 M NaCl, 0.1 M EDTA, pH 7.5 buffer, then freeze and stored at -70°C. Antithrombin III concentration was calculated using an E280 of 0.4 and a molecular weight of 50,000.
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