Kinetics of the Heparin-enhanced Antithrombin III/Thrombin Reaction

EVIDENCE FOR A TEMPLATE MODEL FOR THE MECHANISM OF ACTION OF HEPARIN

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The heparin-enhanced antithrombin III/thrombin reaction was studied under a variety of conditions designed to determine the validity of kinetic models for the mechanism of action of heparin. The observed second order rate constant $k_2^*$ values for thrombin inhibition by antithrombin III were determined as a function of heparin concentration in the presence and absence of varying amounts of active site-blocked thrombin. In the absence of active site-blocked thrombin, the $k_2^*$ value increased as the concentration of heparin was increased to approximately 10$^{-7}$ M. With higher heparin concentration, the $k_2^*$ value progressively decreased. In the presence of active site-blocked thrombin, the heparin concentration dependence curve was shifted to the right at low heparin concentration (less than 10$^{-7}$ M). With higher heparin concentration (greater than 5.0 X 10$^{-7}$ M) there was no measurable effect of active site-blocked thrombin on the rate of thrombin inhibition. Experiments were also conducted in the presence and absence of active site-blocked thrombin to determine the antithrombin III concentration dependence for the heparin-enhanced antithrombin III/thrombin reaction. The results suggested that the enhanced rate of thrombin inhibition by antithrombin III in the presence of heparin is due to the simultaneous binding of both proteins to the same heparin molecule. Of the previously reported models for the mechanism of action of heparin, only the template (ternary complex) model appeared to describe the experimental results. The template model was, therefore, derived in mathematical terms and theoretical data calculated to test the possible validity of the model. Using heparin-thrombin and heparin-antithrombin III dissociation constant values of 3.5 X 10$^{-8}$ M and 1.0 X 10$^{-7}$ M, respectively, a very reasonable correlation between theoretical data and experimental data was obtained. With this model, the reaction is first order with respect to the concentration of the ternary (antithrombin III-heparin-thrombin) complex, with an apparent first order rate constant $k'$ value of 800 min$^{-1}$. It was concluded that, at the present time, the template model provides the simplest working model for the mechanism of action of heparin in accelerating thrombin inhibition by antithrombin III.

In 1939, Brinkhous et al. (1) reported that heparin requires a plasma component to effect its anticoagulant activity. The plasma component was termed heparin cofactor and later shown to be the same protein responsible for antithrombin III activity (2, 3). While it has been shown that two distinct heparin cofactor proteins are normal components of plasma (4-6), the term heparin cofactor has been used primarily in reference to antithrombin III. Perhaps because of the terminology, it has been presumed that heparin and antithrombin III interact in such a way that the inhibitory activity of antithrombin III toward the blood coagulation proteases increases. Suggestive evidence supporting this concept was reported by Rosenberg and Damus in 1973 (3). These workers found that chemically modifying the lysyl residues (~53%) of antithrombin III reduced both the affinity of antithrombin III for heparin-agarose and the heparin cofactor activity of the inhibitor. Recent studies from several laboratories have shown, however, that heparin does not accelerate the rate of inhibition of chemically modified thrombin (7-9) or chemically modified factor Xa (10) by antithrombin III. Since chemically modified antithrombin III reacts normally with thrombin in the absence of heparin and chemically modified thrombin reacts normally with antithrombin III in the absence of heparin, it would appear that the interaction of heparin with both proteins is involved in the mechanism of action of heparin (11-15).

Heparin, unfortunately, is not a discrete chemical entity, but rather a heterogeneous mixture of molecules ranging in molecular weight from around 4,000 to greater than 30,000. All molecules of heparin do not appear to have anticoagulant activity. Those molecules which have the greatest activity bind tightly to antithrombin III (16, 17), which has proved useful in fractionating heparin into high and low activity populations (16). Apparent size classes of heparin have been prepared by gel filtration (18) and, when combined with antithrombin III affinity fractionation, better preparations of heparin have been obtained to investigate the kinetics of the antithrombin III/thrombin reaction in the presence of heparin (19, 20).

In the present report, the kinetics of the heparin-enhanced antithrombin III/thrombin reaction have been investigated using a fraction of crude heparin obtained by gel filtration and antithrombin III affinity chromatography. Data were obtained under a variety of conditions designed to determine the significance of the binding of heparin to thrombin and antithrombin III in the mechanism of action of heparin. Of particular importance to this study was the use of active site-blocked thrombin in varying the concentration of thrombin binding sites for heparin. Previous work has shown that active site-blocked thrombin does not interact with antithrombin III, but can decrease the effectiveness of heparin in enhancing the antithrombin III/thrombin reaction rate (21). The two models for the mechanism of action of heparin, i.e. the heparin-antithrombin III model (19, 20) and the heparin-thrombin
model (22-27), which have been rigorously derived, were tested by comparing the experimental to the theoretical data predicted by the models. In addition, the model suggesting that heparin simultaneously binds both proteins, i.e. the template (ternary complex) model (11-15), to accelerate thrombin inhibition has been rigorously derived and considered in view of the experimental data. The results indicate that the heparin-thrombin and heparin-antithrombin III models cannot completely describe the data under all conditions. The template model, however, appears to fit all the data in the present report and also to be compatible with most, if not all, data in the literature previously used to support the other models.

EXPERIMENTAL PROCEDURES

Materials—TosGlyProArgNaN³ was purchased from Boehringer-Mannheim. 1,5-Dimethyl-1,5-diazaundecamethylene polyethylene imine (Polybrenen) was purchased from Aldrich. Polyethylene glycol (M, = 6000-7500) was from J. T. Baker. Sulfopropyl (SP)-Sephadex was purchased from Pharmacia; DEAE-cellulose was from Whatman, and Echis carinatus venom was from Sigma. Frozen human plasma was kindly provided by Dr. M. M. Mozen, Cutter Laboratories. Porcine mucosal heparin (165 USP units/mg) was generously provided by Mr. W. van Deem, and E. Coyne, with purified E. carinatus venom (30). Thrombin (3,600 NIH units/mg) was isolated by SP-Sephadex column chromatography as described previously (29). Protein concentrations were determined spectrophotometrically using an extinction coefficient value of 1.75 ml-mg⁻¹-cm⁻¹ at 280 nm for human antithrombin (M, = 36,600) (30). Thrombin solutions contained 0.1% polyethylene glycol to prevent adsorption to surfaces (31).

Hunan antithrombin III was isolated from barium citrate-adsorbed plasma by heparin-agarose affinity chromatography essentially as described by the literature previously used to support the other models. The model states that (1) heparin binds to thrombin (T) with an affinity described by the dissociation constant, KfH; (2) heparin binds to antithrombin III (AT) with an affinity described by the dissociation constant, Kf; (3) thrombin and antithrombin III can simultaneously bind to the same heparin molecule to form a ternary (antithrombin III-heparin-thrombin) complex (AT-H-T); 4) the rate of thrombin inhibition is dependent on the AT-H-T concentration; 5) the reaction is first order with respect to AT-H-T, described by the apparent first order constant k; 6) the product of the reaction is a stable, essentially irreversible (35), antithrombin III-thrombin complex (AT-T). Also shown in Scheme 1 is the interaction of active site-blocked thrombin (T') with heparin. It is assumed that the heparin-active site-blocked thrombin dissociation constant is equivalent to KfH, but T' does not react with antithrombin III (21). To test the model, the model must be derived in mathematical terms and theoretical data calculated for comparison with experimental data. The rate of thrombin inhibition can be described by the following:

\[
\frac{-d[T]}{dt} = k \cdot [AT \cdot H \cdot T]
\]

(1)

Under any set of conditions, the concentration of AT-H-T is described by

\[
[AT \cdot H \cdot T] = \frac{[T \cdot H^+] \cdot [AT \cdot H^*]}{[H^*]}
\]

(2)

where [H'] is the total heparin concentration in the reaction solution. The heparin-thrombin-(T') concentration is related to the total (unreacted) thrombin (T) as described by the following:

\[
[T \cdot H^+] = \frac{[T \cdot [H^*] \cdot K_{f_{H}}]}{[K_{f_{H}} + [H^*]]}
\]

(3)

\[
T_f \text{ is the total free (unbound) thrombin and } H^* \text{ is heparin not bound to thrombin.}
\]

Since

\[
[T_f] = \frac{[T]}{[1 + [H^*]/K_{f_{H}}]}
\]

(4)

Equation 3 becomes

\[
[T \cdot H^+] = \frac{[T \cdot [H^*] \cdot K_{f_{H}} + [H^*]]}{[K_{f_{H}} + [H^*]]}
\]

(5)

In a similar manner, the heparin-antithrombin III (AT-H') concentration can be related to the total (unreacted) antithrombin III (AT).

\[
[AT \cdot H^*] = \frac{[AT \cdot [H^*] \cdot K_{f_{H}}]}{[K_{f_{H}} + [H^*]]}
\]

(6)

As indicated in Scheme 1, thrombin binding to heparin is independent of the binding of antithrombin III to heparin and vice versa. The total heparin concentration is described by [H'] = [H'] + [T'] + [AT-H] + [AT-H-T].

\[
\text{With respect to thrombin, the total heparin concentration is described by } [H'] = [H'] + [T'] + [AT-H] + [AT-H-T].
\]

Likewise, with respect to antithrombin III, [H'] = [H'] + [AT-H'] and [AT-H'] = [AT-H] + [AT-T].
HII is heparin not bound to antithrombin III. Substituting Equations 5 and 6 into Equation 2, Equation 1 can be rewritten to describe the rate of thrombin inhibition in terms of T, A'T, and HII.

\[
-\frac{dT}{dt} = k' \left( \frac{[HII]}{K_{D,II} + [HII]} \right) \left( \frac{[HII]}{K_{D,II} + [HI]} \right) \left( \frac{T}{[AT]} \right) \tag{7}
\]

It is apparent from Equation 7 that the rate of thrombin inhibition is dependent on the concentrations of thrombin and antithrombin III which will change as the reaction proceeds. If the concentrations of HI and HII do not change significantly during the reaction, then the reaction will follow second order kinetics with the observed second order rate constant kobs, described by the following:

\[
k_{obs} = k' \left( \frac{([HII]/(K_{D,II} + [HII])) - \left( \frac{1}{[HI]} \right) \right) \tag{8}
\]

Experimental evidence has been reported which indicates that the reaction follows second order kinetics (19, 22). In the present study, experiments were conducted under pseudo-first order reaction conditions, i.e. [AT] >> [T]. Under these conditions, the concentration of HI does not change significantly. The observed pseudo-first order rate constant kobs is described by the following:

\[
k_{obs} = k' \left( \frac{[AT]}{K_{D,II} + [HI]} \right) \left( \frac{[AT]}{[HI]} \right) \tag{9}
\]

The reaction will follow first order kinetics if the concentration of HI does not change significantly during the reaction. The concentration of HI will change if the concentration of thrombin binding sites for heparin does not change during the reaction. We have reported evidence that the antithrombin III-thrombin complex binds heparin as tightly as thrombin (21). We have also shown (21, 36), as have many others, that the reaction follows first order kinetics in the presence of heparin when [AT] >> [T]. It should be appreciated that HI would increase if the concentration of thrombin binding sites decreased during the reaction. This would increase kobs, a phenomena which we have not observed in any of our experiments. It is concluded that the concentration of HI does not change significantly during the course of the reaction, presumably due to the binding of heparin to the antithrombin III-thrombin complex with a dissociation constant value equivalent to K_{D,II}. The binding of heparin to A'T-T is, therefore, indicated in Scheme 1(A'T-T|HII).

From the discussion above, kobs is related to the initial concentrations of HI and HII. By assuming rapid equilibrium binding, [HI] and [HII] were calculated for various reaction conditions from K_{D,II} and K_{D,III}, respectively. For [HI], Equation 3 was expanded and put in the form of the quadratic equation.

\[
[T-H] = \sqrt{K_{D,II} + [T] + [HI]} \pm \sqrt{[K_{D,II} + [T] + [HI]^2 - 4[H][HI]} \tag{10}
\]

Equation 10 was solved and [HI] calculated by subtracting [T-H] from [HI]. A similar process was followed to calculate [HII]. To calculate theoretical data, k', K_{D,II}, and K_{D,III} values must be known. The K_{D,II} value (1.0 × 10^{-5} M) was determined as described earlier. K_{D,III} and k' values were determined by fitting Equation 9 to experimental data obtained under one set of experimental conditions (see Fig. 1). Briefly, K_{D,III} values were inserted into Equation 9 and k' values calculated assuming a k' value of 1.0. The ratio of k' to k_{D,III} was determined for heparin concentrations of 5.0 × 10^{-9} to 5.0 × 10^{-5} M. With a K_{D,II} value of 3.5 × 10^{-4} M, an essentially constant ratio of 1.900 was obtained. The effect of increasing or decreasing K_{D,II} by 5.0 × 10^{-9} M was sufficient to distinguish the range of K_{D,III} values possible to fit Equation 9 to the data (see Fig. 1). From this, it was estimated that the K_{D,II} value is 3.5 × 10^{-4} M and the k' value is 800 min^-1. These values were then used to calculate theoretical data for the remaining experimental data. Theoretical data are indicated as dashed lines in all figures. In experiments where active site-blocked thrombin was included, [HI] was calculated on the basis of the total concentration of thrombin binding sites for heparin, i.e. [T] + [T].

**Antithrombin III/Thrombin Reaction Rate Determination** — The rate of thrombin inhibition by antithrombin III is very fast in the presence of heparin, making it difficult to accurately determine rate constants for the reaction. Synthetic substrates effectively slow the rate of thrombin inhibition by antithrombin III (37). In the present study, rate constants were determined by measuring the rate of thrombin inhibition in the presence of TosGlyProArgNaN as described previously (36). Briefly, thrombin was added to solutions containing antithrombin III, 0.1 M TEA (pH 8.0), 0.1 M NaCl, 0.1% PEG 6000, 1.5 × 10^{-4} M TosGlyProArgNaN. The final thrombin concentration was always at least 10-fold lower than the antithrombin III concentration. Polybrene was added to the solution at a set time after the addition of thrombin and the solution was placed in a cuvette. The change in absorbance at 400 nm was monitored using a Beckman Acta CIII recording spectrophotometer. The observed first order rate constant kobs value was derived from the following:

\[
\ln \frac{v'}{v} = \frac{-k_{obs}}{(1 + [S]/K_a)} t \tag{11}
\]

where v is the rate of substrate hydrolysis when Polybrene is added to the solution before thrombin and v' is the rate of hydrolysis when Polybrene is added to the solution at time t after thrombin. The K_a for thrombin with TosGlyProArgNaN, determined as described previously (38), was 5.0 × 10^{-6} M in this buffer system.

**RESULTS AND DISCUSSION**

**Effect of Heparin Concentration on the Rate of Thrombin Inhibition by Antithrombin III in the Presence and Absence of Active Site-blocked Thrombin** — The rate of thrombin inhibition by antithrombin III was determined as a function of heparin concentration. The results are shown in Fig. 1. Increasing the heparin concentration from 5.0 × 10^{-5} to 1.0 × 10^{-3} M increased the rate of thrombin inhibition. Increasing the heparin concentration above 10^{-3} M, however, appeared to decrease the effectiveness of heparin in accelerating the antithrombin III/thrombin reaction. Previous reports have suggested that the decrease in reaction rate at high heparin concentration is due to the binding of heparin to thrombin (19, 20, 22). If true, then adding active site-blocked thrombin to the reaction solution should shift the heparin concentration...
The curves obtained with three concentrations of active site-blocked thrombin appear to converge as the heparin concentration is increased above \(10^{-7}\) M. In addition, the maximum rate of the reaction decreased with increasing active site-blocked thrombin concentration. Increasing the concentration of antithrombin III had a similar effect on the heparin concentration dependence curve. These results are shown in Fig. 2. The curves again appear to converge as the heparin concentration is increased above \(10^{-7}\) M. Since the thrombin concentration was not changed in the experiments described in Fig. 2, the complete heparin concentration curve should have been shifted to the right with the same maximum second order rate constant value being attained, independent of the antithrombin III concentration. The results argue against a simple heparin-thrombin interaction being responsible for the decreased effectiveness of heparin at concentrations greater than \(10^{-7}\) M.

**Effect of Antithrombin III Concentration on the Rate of Thrombin Inhibition in the Presence of Heparin**—Although the apparent second order rate constant for the heparin-enhanced antithrombin III/thrombin reaction decreased as the antithrombin III concentration was increased (Fig. 2), the observed first order rate constant \(k_{\text{obs}}\) increased in a manner suggesting a saturation phenomenon. These results, shown in Fig. 3, suggest that the reaction rate is dependent on the heparin-antithrombin III concentration, which approaches a maximum when the antithrombin III concentration is increased above the dissociation constant value for the complex. Since the heparin concentration \((1.25 \times 10^{-8}\) M) was well below the antithrombin III concentration, a rough approximation of the apparent dissociation constant for the heparin-antithrombin III complex can be derived from the data shown in Fig. 3. \(K_{\text{DIII}}^{\text{N}}\) was 1.0 \(\times 10^{-7}\) M. This value is the same as the \(K_{\text{DIII}}^{\text{N}}\) value determined by measuring the fluorescence intensity of antithrombin III as a function of heparin concentration (see "Experimental Procedures"). Increasing the concentration of thrombin from \(5.0 \times 10^{-8}\) to \(2.0 \times 10^{-8}\) M did not appear to significantly alter the concentration of antithrombin III required to attain the maximum rate (Fig. 3). The maximum rate, however, was significantly decreased. Similar results were obtained when active site-blocked thrombin was present at a concentration of \(1.5 \times 10^{-8}\) M (Fig. 3). These results suggest that the reaction rate is dependent on the concentration of thrombin binding sites for heparin.

**Models for the Mechanism of Action of Heparin in Accelerating the Inhibition of Thrombin by Antithrombin III**—Models for the mechanism of action of heparin in purified antithrombin III protease systems have been proposed previously (11, 19, 20, 22). The heparin-thrombin model, which postulates that heparin binds to thrombin to accelerate the antithrombin III/thrombin reaction rate, and the heparin-antithrombin model, which postulates that heparin binds to antithrombin III to accelerate the antithrombin III/thrombin reaction, have been described in detail (19, 20, 22), while the template (ternary complex) model has been described in general terms (11). The validity of each of these models can be evaluated by comparing the experimental data in the present report with the predictions each model makes for changing experimental conditions. Both the heparin-thrombin model and the heparin-antithrombin model postulate that the decrease in reaction rate at high heparin concentration is due to the binding of heparin to thrombin. The results (Figs. 1 and 2) argue against this possibility. In addition, both models predict that active site-blocked thrombin should be a competitive inhibitor with respect to heparin binding. The results (Figs. 1 and 3) argue against this possibility. Both models have been rigorously derived and theoretical data calculated for each model for the experimental conditions described in Figs. 1–3. As expected from the general predictions, neither model could generate theoretical data which fit the experimental data under all conditions (not shown).\(^1\) It is therefore concluded that the heparin-thrombin and heparin-antithrombin III models do not describe the mechanism of action of heparin.

The predictions made by the template model for the mechanism of action of heparin appeared to be qualitatively compatible with the experimental data. Specifically, the rate of thrombin inhibition could be related to the relative concentration of the ternary, antithrombin III-heparin-thrombin complex. To evaluate the template model more rigorously,

\(^1\) The derivations and theoretical data for the heparin-thrombin and heparin-antithrombin III models are available upon request.
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the model was derived in mathematical terms from which theoretical data could be calculated (Equation 9, "Experimental Procedures"). To calculate the theoretical data, $K^A_{H/III,II}^H, K^O_{H/III,II}^H$, and $k'$ values had to be determined. The $K^A_{H/III,II}^H$ value (1.0×10^{-7} m) was determined directly (see "Experimental Procedures") and appeared to correlate well with the $K^O_{H/III,II}^H$ value estimated from experimental data (Fig. 3). The $K^O_{H/III,II}^H$ value had to be estimated from experimental data. It appeared (Fig. 1, inset) that active site-blocked thrombin must bind heparin relatively tightly, $K^O_{H/III,II}^H \leq 10^{-7}$ m. Evaluation of $K^O_{H/III,II}^H$ values lower than $10^{-7}$ m, according to the template model, indicated that a value of 3.5×10^{-7} m provides the best fit to the experimental data (see "Experimental Procedures"). The corresponding $k'$ value was 800 min^{-1}. Theoretical data for the template model are shown in each figure as a dashed line. It can be seen that the template model provides a reasonable description of the results. While this does not provide a definitive proof for the template model, it does provide a good working model for the mechanism of action of heparin.

The template model would appear to be consistent with most of the experimental data in the literature previously used to support other models for the mechanism of action of heparin. Since heparin binds to both proteins, chemical modification of either thrombin (7-9) or antithrombin III (3) could affect heparin binding and decrease the reaction rate in the presence of heparin. Pomerantz and Owen (11) proposed the template model for the mechanism of action of heparin on the basis of chemical modification data. Since only a fraction of commercial heparin appears to bind tightly to antithrombin III (16, 17), only this fraction should be active in terms of enhancing the antithrombin III/thrombin reaction rate, as has been observed (16, 17, 19, 20). Since both proteins must simultaneously bind to the same heparin molecule, there should be a positive correlation between increasing activity and increasing size of the heparin. There is good evidence that this is true (14, 18). Heparin appears to alter the conformation of antithrombin III (39-44). There is no evidence that this conformational change is required for antithrombin III to inhibit thrombin at an accelerated rate in the presence of heparin, i.e., for antithrombin III to become a better inhibitor. Recent evidence suggests, however, that the tight binding of heparin to antithrombin III is a consequence of a heparin-induced conformational change in the protein (43, 44). Since tight binding of heparin is required by the template model, the conformational change in antithrombin III may very well be required. It should be noted, however, if heparin binding to antithrombin III or thrombin were to increase the reactivity of the proteins, this would not be incompatible with the template model.

It is important to note that binding measurement techniques, such as those used to determine $K^A_{H/III,II}^H$, measure only the interaction of heparin with antithrombin III which, in this case, increases the fluorescence intensity of the protein. Since there may be an interaction of heparin with antithrombin III which is kinetically important but does not affect the intrinsic fluorescence, it is significant that the $K^O_{H/III,II}^H$ value estimated from the kinetic data is similar to the $K^A_{H/III,II}^H$ value determined by fluorescence intensity measurements.

An alternative model to the template model has been derived and theoretical data calculated which also provides a reasonable description of the experimental data. This model, however, is considerably more complex than the template model, requiring the formation of a nonproductive heparin-thrombin-antithrombin III complex. Since there is no direct experimental evidence for such a complex, the model has not been presented. Other kinetic models are also likely, which serves to illustrate that the usefulness of deriving models is in testing the possibilities of action of heparin and not in proving the mechanisms. The alternative model and corresponding theoretical data are available upon request.

The anticoagulant mechanism of action of heparin, i.e., how heparin prevents the clotting of blood in vitro, is not known and should not be derived from the results of the present study alone. It is clear that heparin accelerates thrombin inhibition by antithrombin III in the purified system, as well as accelerating the rate of inhibition of other blood coagulation proteases (20). This may be one aspect of the anticoagulant mechanism of action of heparin. Other aspects may also be important, however. For example, factor IX and factor Xa bind relatively tightly to heparin-agarose, suggesting that they may form a complex with heparin in solution. It could be postulated that heparin would directly inhibit blood coagulation by preventing factor IXa from interacting with factor VIIIa or factor X. Ofosu et al. (45) have recently shown that heparin significantly decreases the rate of clotting of antithrombin III-deficient plasma. This would not invalidate the conclusions of Brinkhous et al. (1) when it is considered that the anticoagulant effect of heparin may be 2-fold. First, heparin may bind to the coagulation proteases (and cofactors) such that the interaction with substrates and cofactors is blocked, and second, enhancing the rate of inactivation of the proteases by antithrombin III.

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