The Heparin-enhanced Antithrombin III/Thrombin Reaction Is Saturable with Respect to Both Thrombin and Antithrombin III*

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The heparin-enhanced antithrombin III/thrombin reaction was studied under experimental conditions where the dependence of the reaction velocity on the concentrations of thrombin and antithrombin III could be determined. The results have shown that the reaction is saturable with respect to both thrombin ($K_T = 3.6 \times 10^{-3} \text{ M}$) and antithrombin III ($K_{AT} = 1.0 \times 10^{-2} \text{ M}$) when the heparin concentration is low relative to the initial protein concentrations. The apparent first order rate constant for the rate-limiting step in the reaction was approximately 800 min$^{-1}$. The reaction was subject to inhibition by antithrombin III/thrombin, the product of the reaction. Inhibition appeared to be noncompetitive with respect to antithrombin III with $K_p$, the apparent heparin product dissociation constant, approximately equal to $K_T$. When the heparin-enhanced antithrombin III/thrombin reaction was studied under conditions where the heparin concentration was high relative to the initial protein concentrations the overall reaction was second order. The initial reaction velocity, under any set of experimental conditions, could be described by the general rate equation for a random order bireactant, enzyme-catalyzed reaction, which is mathematically identical with the "template" model for the mechanism of action of heparin (Griffith, M. J. (1982) J. Biol. Chem. 257, 7360-7365).

The mechanism of action of heparin in enhancing the rate of thrombin inhibition by antithrombin III has been the subject of several kinetic studies (1-12). Three general models for the mechanism of action of heparin have been proposed. Two models consider heparin as a positive effector (1-3, 8). In these models, the antithrombin III/thrombin reaction velocity is increased when heparin binds to one of the two reactants, i.e. thrombin (3, 8) or antithrombin III (1, 2). The two models differ primarily in terms of which heparin-protein interaction determines the reaction rate. The third model considers the heparin more as an enzyme in a bireactant system (10, 11). In this model, thrombin and antithrombin III are substrates which react more rapidly when simultaneously bound to the same heparin molecule. Detailed kinetic studies of the heparin-enhanced antithrombin III/thrombin reaction should differentiate among these models and provide a working model for the mechanism of action of heparin.

In a recent report, we examined the heparin-enhanced antithrombin III/thrombin reaction under experimental conditions which were best suited for testing the validity of the models which consider heparin as an effector (11). Our results were not compatible with any of the effector models, but were consistent with the "template" model (13-16). The template model (11) is mathematically identical with a random order bireactant enzyme-catalyzed reaction (17). The present investigation was therefore undertaken to specifically test general enzyme theory against kinetic data obtained for the heparin-catalyzed antithrombin III/thrombin reaction. The results of the present study demonstrate that the reaction is saturable with respect to both thrombin and antithrombin III and subject to product inhibition.

**EXPERIMENTAL PROCEDURES**

*Materials—P-p-tosyl-L-glycyl-L-arginine-p-nitroanilide (TosGlyProArgNaN$^-$) was purchased from Boehringer Mannheim. 1,5-Dimethyl-1,5-diazaoctaneemethylene polymethobromide (Polybrene) was purchased from Aldrich. Polyethylene glycol (M, $= 6,000-7,500$) was purchased from Fisher. Porcine mucosal heparin (165 USP units/mg), which was essentially devoid of protein, was generously provided by Dr. G. van Dodein and E. Coyne. Diosynth B. V., Holland. Heparin was fractionated by gel filtration and antithrombin III-agarose affinity chromatography, as described previously (11) to obtain material with approximately 300 USP units/mg and an apparent $M, = 16,000$. Human a-thrombin (3,600 NIH units/mg) and human antithrombin III were prepared as described previously (11).

**Antithrombin III/Thrombin Reaction Velocity Determination**—Antithrombin III was added to a solution containing 0.1 M triethanolamine (pH 8.0), 0.1 M NaCl, 0.1% polyethylene glycol, thrombin, and heparin. The final protein and heparin concentrations were varied. At timed intervals after the addition of antithrombin III, samples were removed and added to a solution containing 0.1 M triethanolamine (pH 8.0), 0.1 M NaCl, 0.1% polyethylene glycol, 1.5 $\times 10^{-4}$ M TosGlyProArgNaN$^-$, 0.5 mg/ml Polybrene. The hydrolysis of TosGlyProArgNaN$^-$ by thrombin was terminated by adding acetic acid. The amount of substrate hydrolyzed, determined by absorbance at 400 nm, was proportional to the thrombin concentration.

**General Antithrombin III/Thrombin Reaction Velocity Equations**—The present study considers the heparin-enhanced antithrombin III/thrombin reaction to be analogous to a random order, bireactant, enzyme-catalyzed reaction (17). The system can be described by the equilibria below.

$$
H + T \xrightleftharpoons[K_T]{k} H \cdot T + +
\]

$$\begin{array}{c}
AT \\
H \cdot AT + T \xrightleftharpoons[K_{AT}]{k} H \cdot AT \cdot T \\
H + A(T - T) \xrightleftharpoons[K_{AT}]{k} H + A + T - T
\end{array}
$$

In this system, thrombin ($T$) and antithrombin III ($AT$) bind randomly to heparin ($H$). $K_T$ and $K_{AT}$ are the heparin/thrombin and heparin/antithrombin III dissociation constants, respectively, and $k$ is the apparent first order rate constant for the rate-limiting step in product formation. $K_0$ is the dissociation constant for heparin and $k$ the product of the reaction, the stable antithrombin III/thrombin complex ($AT - T$). Assuming rapid equilibrium, the reaction velocity, $v$, is described by the following rate equation (17).

$$
v = \frac{k[H][A(T - T)]}{aK_{AT} + aK_{AT}[T] + aK[H][A(T) + [AT] - [T]}
$$

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$^1$ The abbreviation used is: TosGlyProArgNaN$^-$, N$^\alpha$-p-tosyl-L-glycyl-L-prolyl-L-arginine-p-nitroanilide.
The term $\alpha$ is included to consider the factor by which the binding of one protein to heparin changes the binding affinity of heparin for the second protein. The present study assumes that the value of $\alpha$ is 1.0. Under any set of experimental conditions, $\alpha$ is dependent on the total heparin concentration $[H]$ and the free or unbound antithrombin III $[AT]$ and thrombin $[T]$ concentrations. When the initial antithrombin III concentration $[AT]$, and thrombin concentration $[T]$ are considered much lower than $[H]$, Equation 1, $[AT]/[AT] + [T]/[T]$ = $[T]/[T]$. When the initial antithrombin III and thrombin concentrations are much lower than $[H]$, Equation 1 reduces to

$$v = k \cdot [AT] \cdot [T]/[H]$$

(2)

RESULTS AND DISCUSSION

Kinetics of the Antithrombin III/Thrombin Reaction in the Presence of Low Heparin Concentration—The antithrombin III/thrombin reaction velocity was studied as a function of thrombin concentration in the presence of low heparin concentration. The initial reaction velocities were determined from the data shown in Fig. 1 and plotted as a function of thrombin concentration as shown in the inset (Fig. 1). The data indicate that the heparin-enhanced reaction is saturable with respect to thrombin with $K_T = 3.6 \times 10^{-9}$ M. The apparent maximum reaction velocity, $V_{ax}$, was $1.93 \times 10^{-4}$ M-$\text{min}^{-1}$. Although it is not readily apparent from the data shown in Fig. 1, the decrease in reaction velocity with time was greater than expected for simple substrate(s) consumption to account for. One explanation for the aberrant decrease in reaction velocity is product, i.e. antithrombin III/thrombin, inhibition. Product inhibition is not totally unexpected, based on previous results (8).

The antithrombin III/thrombin reaction velocity was also studied as a function of antithrombin III concentration, in the presence of low heparin concentration. As shown in Fig. 2, the reaction followed apparent first order kinetics. The initial reaction velocities were plotted as a function of antithrombin III concentration as shown in the inset (Fig. 2). The data indicate that the heparin-enhanced reaction is saturable with respect to antithrombin III with $K_{AT} = 1.0 \times 10^{-2}$ M. The apparent maximum reaction velocity, $V_{ax}$, was $3.8 \times 10^{-7}$ M-$\text{min}^{-1}$.

From Equation 1, $V_{ax}$ and $V_{ax}$ are related to $k$, the apparent first order rate constant for the reaction, according to $V_{ax} = k \cdot [H]/(1 + K_{AT}/[AT])$ and $V_{ax} = k \cdot [H]/(1 + K_{P}/[T])$. Under the experimental conditions used to determine $V_{ax}$, the calculated value of $k$ is 804 M-$\text{min}^{-1}$. Under the experimental conditions used to determine $V_{ax}$, the calculated value of $k$ is 767 M-$\text{min}^{-1}$.

The observation that the antithrombin III/thrombin reaction follows first order kinetics under the experimental conditions described in Fig. 2 is not consistent with the rate equation, Equation 1. As suggested earlier, the reaction may be subject to product inhibition. To examine this further, the experiments described in Fig. 2 were repeated in the presence of antithrombin III/thrombin. When the initial reaction velocities were plotted as a function of antithrombin III concentration (Fig. 2, inset), it appeared that the $K_{AT}$ value was not changed significantly. The $V_{ax}$ value, however, was approximately 2-fold lower $(1.8 \times 10^{-7}$ M-$\text{min}^{-1})$. These results suggest that antithrombin III/thrombin, under these conditions, is a noncompetitive inhibitor of the reaction with respect to antithrombin III. Although not rigorous, the $K_P$ for antithrombin III/thrombin can be approximated from $V_{ax}/V_{ax} = K_P/(K_P + [AT-T])$, if it is assumed that antithrombin III/thrombin is actually a noncompetitive inhibitor of the reaction with respect to antithrombin III, $K_P = 3.2 \times 10^{-10}$ M.

The similarity between $K_T$ and $K_P$ values can provide an explanation for the first order kinetics observed in Fig. 2. When $[AT] \gg [T]$, the antithrombin III concentration will not change significantly during the course of the reaction and the rate equation (Equation 1 with $[AT] = [AT]$, $[T] = [T]$) can be modified to

$$v = k \cdot [T]/(K_T + [T])$$

(3)

where $k' = k \cdot [H] \cdot [AT]/(K_P + [AT-T])$. Equation 3 is further modified to consider product inhibition as indicated by

$$v = k' \cdot [T]/(K_T \cdot (1 + [AT-T]/K_P) + [T])$$

(4)

which can be integrated to

$$-k't = K_T \cdot (1 + [T]/K_P) \cdot \ln [T]/(T) + (1 - K_T/K_P) \cdot [AT-T]$$

(5)
If \( K_T = K_P \), then
\[
-k' \approx (K_T + [T_\text{I}]) \cdot \ln [T]/[T_\text{I}]
\] (6)
which fits the general form of the integrated first order rate law. In other words, the heparin-enhanced antithrombin III/thrombin reaction would appear to be a pseudo-first order reaction when \([AT_\text{I}] \gg [T_\text{I}]\), as a consequence of the interaction of heparin with antithrombin III/thrombin.

**Kinetics of the Antithrombin III/Thrombin Reaction in the Presence of High Heparin Concentration**—In the presence of relatively high heparin concentration, both thrombin and antithrombin III will be essentially completely bound to heparin, and the initial reaction velocity will be described by Equation 2. The reaction should follow first order kinetics when \([AT_\text{I}] \gg [T_\text{I}]\), as shown in Fig. 3, and second order kinetics when \([AT_\text{I}] \approx [T_\text{I}]\), as shown in Fig. 4. The \( k \) values, calculated according to Equation 2, for the data shown in Figs. 3 and 4 are summarized in Table I.

In a recent report, Pletcher and Nelsestuen (12) concluded that the heparin-enhanced antithrombin III/thrombin reaction is independent of thrombin. A rather classical kinetic approach was used by these investigators to analyze data and obtain the reaction order with respect to thrombin. In the approach, the logarithmic plot of the fractional lifetime of the reaction against the initial thrombin concentration is used to determine the reaction order with respect to thrombin. As shown in the *inset* (Fig. 4), the logarithmic plot of the reaction half-time, \( t_{1/2} \), against the antithrombin III (plus thrombin) concentration is linear, with a slope of \(-1.0\) for the data from both Figs. 3 and 4. As predicted by equation 2, this indicates that the overall reaction is second order when the initial heparin concentration is high relative to \( K_T, K_{AT}, \) and the initial thrombin and antithrombin III concentrations.

Previous results from our laboratory have shown that the antithrombin III/thrombin reaction velocity can be predicted for any heparin concentration if \( K_T, K_{AT}, \) and \( k \) values of 3.5 \( \times 10^{-9} \) M, 1.0 \( \times 10^{-7} \) M, and 800 min\(^{-1}\), respectively, are substituted into a rate equation which is functionally analogous to Equation 1 (11). In our previous study, however, \( K_T \) and \( k \) values were empirically derived. The good agreement between the \( K_T \) and \( k \) values determined in the two studies provides reasonable support for the template model for the mechanism of action of heparin (13-16). It should be emphasized that both of our studies were conducted using the same preparation of heparin. Heparin, as obtained commercially, is heterogeneous with respect to size, antithrombin III affinity (18, 19), and charge density (20, 21) and the catalytic properties of heparin can be expected to vary depending on how heparin is fractionated prior to kinetic study.

Finally, two assumptions have been made in evaluating the kinetic data in the present study. First, it was assumed that the binding of thrombin to heparin does not change the binding affinity of heparin for antithrombin III (and vice versa), *i.e.* \( \alpha \) in Equation 1 is equal to 1.0. Second, it was assumed that product inhibition was noncompetitive with respect to antithrombin III and, therefore, competitive with respect to thrombin. While the data are consistent with Equation 1 when these assumptions are made, neither assumption is intuitively obvious. When it is considered that the solution structure of heparin is not likely to be as stable as that of an enzyme, it would seem reasonable that the binding of one protein to heparin would affect, in some way, the binding of the second protein. It would also seem reasonable that the

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**Table I**

| \([AT_\text{I}]\) | \([T_\text{I}]\) | \( t_{1/2} \) | \( k \) |
|---|---|---|---|
| \( \text{nm} \) | \( \text{nm} \) | \( \text{M/min} \) | \( \text{min}^{-1} \) |
| **I.** | | | |
| 40 | 2.0 | 7.2 | 813 |
| 32 | 1.6 | 4.6 | 832 |
| 24 | 1.2 | 2.5 | 768 |
| 16 | 0.8 | 1.1 | 784 |
| 8 | 0.4 | 0.3 | 877 |
| **II.** | | | |
| 40 | 20 | 62.4 | 702 |
| 30 | 15 | 35.1 | 702 |
| 20 | 10 | 15.6 | 702 |
| 10 | 5 | 3.9 | 702 |

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**Fig. 3.** Heparin-enhanced antithrombin III/thrombin reaction: first order kinetics. Thrombin inhibition by antithrombin III in the presence of heparin (9.0 \( \times 10^{-5} \) M) was determined as described under "Experimental Procedures." The \([AT_\text{I}]/[T_\text{I}]\) was 20:1 in each experiment.

**Fig. 4.** Heparin-enhanced antithrombin III/thrombin reaction: second order kinetics. Thrombin inhibition by antithrombin III in the presence of heparin (9.0 \( \times 10^{-5} \) M) was determined as described under "Experimental Procedures." The \([AT_\text{I}]/[T_\text{I}]\) was 20:1 (II) (data from Fig. 3), or 2:1 (O) (present data). The plot can be used to determine the overall reaction order according to reaction order = -slope + 1.0 (22).
binding of product (antithrombin III/thrombin) to heparin would affect, in some way, the binding of antithrombin III, as well as thrombin. The assumptions, therefore, should be studied more rigorously. Given the working model for the mechanism of action of heparin described in the present study, these assumptions can be tested.

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