Two-substrate Reaction Model for the Heparin-catalyzed Bovine Antithrombin/Protease Reaction*

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The kinetics of the heparin-dependent antithrombin/protease reaction were consistent with an ordered sequential two-substrate reaction model under all circumstances tested. In this model, heparin is the catalyst; while antithrombin is the first substrate and the protease is the second substrate. The first step in this reaction, the heparin-antithrombin interaction, has a $K_m$ of 25 nM but a diffusively determined $K_m$ of about 150 nM regardless of protease substrate. The second step of the reaction, protease interaction with the heparin-antithrombin complex, was fast with a rate constant of $6.8 \times 10^8 \text{M}^{-1} \text{s}^{-1}$ for Factor X and $>8 \times 10^7 \text{M}^{-1} \text{s}^{-1}$ for thrombin. Differences between thrombin and Factor X, at low (nanomolar) concentrations of heparin were evident in the rate constant and the relative affinities for the heparin-antithrombin complex ($K_m$ for Factor X, = 100 nM; $K_m$ for thrombin = 2 nM). In agreement with this difference in $K_m$, regardless of protease substrate, active site-blocked thrombin was a potent inhibitor of the antithrombin reaction; while active site-blocked Factor X, was an ineffective inhibitor. At high heparin concentrations (micromolar), the kinetic parameters for Factor X, were unchanged but the $K_m$ for thrombin increased dramatically to 100 nM. Other kinetic parameters were also estimated. Overall, the two-substrate reaction model provides a versatile approach for studying heparin function.

Antithrombin is one of several protease inhibitors in blood which will inactivate many of the proteases of the coagulation/fibrinolytic system (1, 2). The rate of the antithrombin/protease reaction is greatly enhanced in the presence of the complex polysaccharide heparin (1). Heparin effects this rate enhancement in catalytic amounts (3); and the product of the reaction, an antithrombin-protease stoichiometric 1:1 complex, is identical in both the heparin-dependent and heparin-independent reaction (1). The reaction binding sequence for the antithrombin/thrombin reaction is heparin binding to antithrombin followed by binding of thrombin (4).

When the concentration of heparin is low (nanomolar), a general scheme which is consistent with the catalytic function of heparin as well as the binding sequence of the three components can be written

\[ \begin{align*}
A + P & \rightarrow A \cdot P \\
A \cdot P + H & \rightarrow A \cdot P \cdot H \\
A \cdot P \cdot H & \rightarrow A + P \cdot H \\
A + P \cdot H & \rightarrow A + P
\end{align*} \]

where $H$ represents heparin, $A$, antithrombin; $P$, serine protease; $A \cdot P$, the nondissociable antithrombin-protease complex. This formulation is analogous to a two-substrate enzyme reaction with heparin as the catalyst and with the protease plus antithrombin as the substrates. The reaction can be analyzed by well documented methods (5, 6).

Almost all of the previous kinetic data on the heparin-catalyzed antithrombin/protease reaction have been analyzed and interpreted from the viewpoint of a bimolecular reaction (7-11). This method of analysis may have led to some confusion in understanding the function of heparin. For example, under the most commonly used experimental conditions, the rate-determining step of the antithrombin/thrombin reaction is independent of thrombin and follows normal saturation kinetics with respect to antithrombin (4). The kinetic rate expression for a bimolecular reaction is therefore inappropriate for this reaction under these conditions. However, analysis using the two-substrate reaction in the limit where the concentration of thrombin is saturating would still be valid (4). As the concentration of heparin is increased, the initial velocity of the antithrombin/protease reaction increases and reaches a maximum. For some proteases of which Factor X, is an example, the velocity remains constant with additional increases in the concentration of heparin; for other proteases, such as thrombin, the initial velocity decreases at higher concentrations of heparin (7, 8, 11). Although considerable kinetic data for the antithrombin/protease reaction at high concentrations of heparin have already been published, it is not known if the rate-determining step is the same at high concentrations of heparin as at low concentrations of heparin.

In this publication, we report that the heparin-dependent antithrombin/protease reaction can be modeled as an ordered two-substrate heparin-catalyzed reaction (Equation 1) over a wide range of experimental conditions. Differences in the initial velocities under various conditions are explained largely by the $K_m$ for the protease substrates.

**EXPERIMENTAL PROCEDURES**

*Proteins and Heparin*—Bovine $\alpha$-thrombin was generated from prothrombin using a prothrombinase complex and purified as previously described (4). The specific clotting activity of the thrombin was 2600-2800 NIH units/mg when compared to NIH standard thrombin Lot J (12). Factor X was purified from bovine plasma by minor modifications of standard techniques (13). Factor X, was formed by treatment of Factor X with Russell’s viper venom (Sigma) at pH 7.8 for 1 h at 37°C. A ratio of Factor X to lyophilized venom of 200:1 was used. The Factor X, was subsequently purified by ion exchange chromatography on DEAE-Sephadex using 0.1 M phosphate buffer (pH 6.9) and a linear gradient of 0.1 to 0.66 M NaCl. Polyacrylamide gel electrophoresis indicated that the product corresponded to Factor...
Heparin-catalyzed Antithrombin/Protease Reaction

Xₐ (14). Bovine antithrombin was purified as previously described (4).

Active-site blocked proteases were prepared by incubating the purified protease with active-site reagent in 0.1 M Tris-HCl (pH 8.4) containing 0.1% polyethylene glycol 4000 at 25°C until no amidolytic activity could be detected. The solution was then dialyzed to remove the active-site reagent. Thrombin was incubated with 1 mM DFP (Sigma) and 1 mM TLCK (Sigma) and Factor Xa was incubated with 10 mM DFP.

Extinction coefficients (ε₀/con) for the determination of protein concentrations were 19.5 for thrombin (16), 9.4 for Factor Xa (17), and 6.0 for antithrombin (15).

Fractionated porcine mucosal heparin, kindly donated by Hiker Laboratories, 3M Company (Northridge, CA) (specific activity, 101 units/mg) was used in all antithrombin/protease reactions. The molar concentration of heparin was estimated by titrating the heparin-dependent fluorescence change of antithrombin by the method of Nordenmann and Bjork (18).

Antithrombin/Protease Inactivation Studies — Procedures for the antithrombin/thrombin reaction and for determining the concentration of thrombin activity have been detailed previously (4). Procedures for the antithrombin/Factor Xa reaction were identical with those of the antithrombin/thrombin reaction (4). The remaining Factor Xa activity was determined using the chromogenic substrate N-benzoyl-t-isoleucyl-t-glutamylglycyl-t-arginine-p-nitroanilide (S-2222) (Kabi Diagnostics, Greenwich, CT). The Factor Xa specific activity was 140 nmol-nmol⁻¹-s⁻¹. This value is somewhat higher than that reported by the supplier (110 nmol-nmol⁻¹-s⁻¹).

Kinetinc Analysis — The initial velocity of the antithrombin/protease reaction was estimated directly from plots of protease activity remaining versus time (4). Less than 20% of the protease was inactivated during the time required to estimate the initial velocity except in the experiments where the concentrations of antithrombin exceeded 140 nM. In these few cases up to 30% of the protease was consumed. All velocities were corrected for the heparin-independent reaction. Double reciprocal plots of initial velocity versus substrate concentration were employed to determine various kinetic parameters. The kinetics of the antithrombin/protease reaction were analyzed in a manner analogous to a two-substrate enzyme reaction as given below (5, 6).

Fraction reaction lifetimes were used to establish the order of rate-determining steps by published procedures (19, 20). In this method, a logarithmic plot of the fractional lifetime as a function of the initial concentrations of the reactant is made. The slope of the line is related to the order of the reaction by:

Order of the reaction = –slope + 1

The fractional lifetime is the time required for some given fraction of the substrate to be consumed.

Heparin-Antithrombin Dissociation Constant — The dissociation constant for the heparin-antithrombin interaction was defined as the concentration of free heparin at half-maximal heparin-induced intrinsic antithrombin protein fluorescence change. This is similar to methods used previously (7, 21). Heparin was added to solutions of antithrombin at 25°C and fluorescence monitored in an SLM model 4800 spectrofluorometer (SLM Instruments, Urbana, IL). Excitation was at 290 nm (slit = 2 nm) and emission was monitored at 340 nm (slit = 8 nm). The added heparin partitions into free heparin and antithrombin-bound heparin. The concentration of bound heparin was estimated by (fraction of maximum fluorescence change) × (concentration of antithrombin). The free heparin is the difference between bound and total heparin added. Four different titrations at antithrombin concentrations of 12.5, 25, 50, and 100 nM all gave similar dissociation constants at 25 ± 5°C heparin. This dissociation constant is similar to that reported by Griffith (7) (20 nM) who used the same source of heparin and approximately the same conditions. Similar dissociation constants at different protein concentrations confirm a 1:1 stoichiometry for heparin-antithrombin interactions.

RESULTS

Heparin-dependent Antithrombin/Factor Xa Reaction — The heparin-dependent antithrombin/Factor Xa reaction was modeled as a two-substrate heparin-catalyzed reaction. Using steady state assumptions, the rate for a two-substrate reaction becomes, in the notation of Dalziel (5):

$$
\frac{[H]}{v} = \frac{\phi_0 + \phi_A/[A] + \phi_P/[P] + \phi_{AP}}{[A][P]}
$$

A summary of the relationships between the rate constants (Equation 1) and the experimentally determined $\phi$ coefficients (Equation 3) is included for the reader’s convenience (Table I).

Double reciprocal plots of initial velocity versus antithrombin concentration at various Factor Xa concentrations fit a family of lines which intersected in the third quadrant (Fig. 1). This family of intersecting lines established the reaction as a sequential two-substrate reaction but did not distinguish between random or ordered addition of the substrates. In the heparin-catalyzed antithrombin/thrombin reaction, the addition of substrates is ordered with thrombin as the second substrate (4). These results suggest that Factor Xa would also be the second substrate. Moreover, the heparin-Factor Xa binding, which is a prerequisite for random addition of substrates, is quite weak ($K_D = 8 \times 10^{-9}$ M) (22). These data suggest that the reaction sequence where Factor Xa is the

![Fig. 1. Double reciprocal plots of the velocity of Factor Xa inactivation as a function of antithrombin concentration. The concentration of heparin was 2.4 nM; the initial concentration of Factor Xa was varied over the 4-fold range indicated. Replots of intercepts and slopes are shown in inset A and B, respectively. Linear least squares analyses were used to determine the slopes and intercepts.](image-url)
second substrate would make the greatest contribution to the overall velocity of the reaction.

The kinetic parameters for the antithrombin/Factor X, heparin-catalyzed reaction were obtained using the standard analysis for a two-substrate reaction (Table II) (5, 6). Inter- section of the double reciprocal plots (Fig. 1) in the third quadrant indicated that the $K_D$ for heparin-antithrombin binding was less than the $K_m$ for antithrombin (6). The $K_D$ for heparin-antithrombin binding, determined independently by changes in the intrinsic fluorescence of antithrombin, was 25 nM (see "Experimental Procedures"), whereas the $K_m$ for antithrombin was 150 nM. The $K_D$ calculated from the $V$ parameters was 40 nM (Table II) which is comparable to the independently determined value. These $K_D$ values were determined by very different techniques and therefore provide strong corroborative evidence that the heparin-dependent fluorescence change of antithrombin is important to the kinetics of the reaction.

For comparative purposes, kinetic parameters for the antithrombin/thrombin reaction which follows the single substrate limit of the two-substrate reactions, are included in Table II. The $K_m$ values for antithrombin for both reactions were similar. In contrast, the $K_m$ for Factor X, was at least 50-fold higher than the $K_m$ for thrombin.

Inhibition by Active Site-blocked Proteases—The antithrombin/protease reaction was inhibited by active site-blocked proteases (Fig. 2) (11). The extent of inhibition depended upon the active site-blocked protease but was independent of the substrates. For example, TLCK-thrombin was a potent inhibitor of both the antithrombin/thrombin and the antithrombin/Factor X, reaction; while DFP-Factor X, was an ineffective inhibitor for both reactions. These results suggest that the proteases compete for the same active site even though the $K_m$ values are quite different. The inhibition by DFP-thrombin was indistinguishable from that of TLCK-thrombin.

Order of the Reactions at Low and High Heparin—The overall order of the heparin-dependent antithrombin/Factor X, reaction with respect to total protein was the same at both low and high concentrations of heparin. (Heparin = 2.4 nM, slope = −0.45; heparin = 800 nM, slope = −6.35; Fig. 3). These data suggest that the two-substrate catalyzed reaction mechanism formulated for low concentrations of heparin would also apply at high concentrations of heparin. In addition, the experimentally determined initial velocity for the antithrombin/Factor X, reaction in the presence of 800 nM heparin compared quite closely to the value calculated from the kinetic parameters determined at the low concentration of heparin (Table III). These results are another indication that the reaction mechanism is the same at both low and high concentrations of heparin. Rapid reaction kinetic techniques will be needed to rigorously establish that the reaction mechanisms remain the same at even higher heparin concentrations.

### Table II

Summary of kinetic parameters for the heparin-dependent antithrombin/protease reaction at low heparin

<table>
<thead>
<tr>
<th>Kinetic parameter</th>
<th>Factor X,</th>
<th>Thrombin</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ for antithrombin</td>
<td>150 nM</td>
<td>120 nM&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>$K_m$ for protease</td>
<td>100 nM</td>
<td>&lt;3 nM&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>$k_1$</td>
<td>0.7 s&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>0.16 s&lt;sup&gt;−1&lt;/sup&gt;</td>
</tr>
<tr>
<td>$k_2$</td>
<td>$4.5 \times 10^5$ M&lt;sup&gt;−1&lt;/sup&gt;s&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>1.33 $\times 10^5$ M&lt;sup&gt;−1&lt;/sup&gt;s&lt;sup&gt;−1&lt;/sup&gt;</td>
</tr>
<tr>
<td>$k_3$</td>
<td>0.17 s&lt;sup&gt;−1&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>$k_4$</td>
<td>$6.8 \times 10^5$ M&lt;sup&gt;−1&lt;/sup&gt;s&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>$&gt;8 \times 10^7$ M&lt;sup&gt;−1&lt;/sup&gt;s&lt;sup&gt;−1&lt;/sup&gt;</td>
</tr>
<tr>
<td>$K_D$</td>
<td>40 nM</td>
<td></td>
</tr>
<tr>
<td>$K_D$</td>
<td>20 nM</td>
<td></td>
</tr>
</tbody>
</table>
For the antithrombin/thrombin reaction at low concentrations of heparin the order of the rate-determining step varied from first (slope = 0) to zero (slope = 1) order with respect to total protein (Fig. 4). Details of this reaction have been previously published (4). In summary, these studies showed that the rate-determining step is independent of thrombin and follows normal saturation kinetics with respect to antithrombin (Table II). At intermediate concentrations of heparin the overall order of the reaction increased from first order (slope = 0) at 80 nM heparin to 1.3 (slope = −0.3) at 400 nM heparin.

At high concentrations of heparin (800 nM) the rate-determining step of antithrombin/thrombin reaction was essentially second order (slope = −0.9) with respect to protein (Fig. 4). The rate-determining step was first order with respect to both thrombin (slope = 0) and antithrombin (slope = 0) (Fig. 5). At high concentrations of heparin, the antithrombin is saturated with heparin; consequently, the reaction should conform to a single substrate reaction with the heparin-antithrombin complex as a single turn-over catalyst and thrombin as the substrate. In the limit where the concentrations of the heparin-antithrombin complex and the thrombin are well below the dissociation constant for the heparin-antithrombin-thrombin complex, the rate-determining step should approach first order with respect to each protein. These results were observed (Fig. 5).

Under these conditions of high heparin, a \( k_{cat} \) and a \( K_m \) for thrombin can be obtained from double reciprocal plots. The results (Fig. 6) indicated a \( K_m \) for thrombin of about 100 nM and a \( k_{cat} \) of 0.1 s\(^{-1}\). These values were obtained by consider-able extrapolation and are therefore subject to some error; however, due to high reaction velocities, reliable initial rates could not be estimated in experiments with higher concentrations of protease. Nevertheless, the results indicate that the \( k_{cat} \) for the antithrombin/thrombin reaction at high concentrations of heparin is similar to the \( k_{cat} \) at low heparin; but the \( K_m \) has dramatically increased. In addition, 40 nM DFP-thrombin had no detectable effect on the initial reaction at high concentrations of heparin (Fig. 6) (11). These results show that the affinity of DFP-thrombin for the active complex is also decreased at high concentrations of heparin (compare with the results in Fig. 2). This low affinity correlates with the low affinity of the thrombin substrate under the same conditions.

**Discussion**

The experimental kinetic results for both the antithrombin/
Factor X, and antithrombin/thrombin reactions, determined over a broad range of heparin concentrations, were consistent with an ordered sequential two-substrate heparin-catalyzed reaction. The reaction scheme for this two-substrate model is given by:

\[
A \overset{k_1}{\rightleftharpoons} P \\
H \overset{k_2}{\rightleftharpoons} H \cdot A \\
H \cdot A \cdot P \overset{k_3}{\rightleftharpoons} H \cdot A \cdot P \overset{k_4}{\rightleftharpoons} H \cdot A + P \\
H + A \overset{k_5}{\rightleftharpoons} H + A \overset{k_6}{\rightleftharpoons} H + A
\]

where \( P \) represents protease; \( A \), antithrombin; \( H \), heparin, and \( A \sim P \), the nondissociable complex.

At low concentrations of heparin, as predicted by the two-substrate model, similar ternary complexes are formed for either protease; i.e. the proteases bind to the same active site formed by heparin plus antithrombin. For example, DFP-
thrombin inhibited both the inactivation of thrombin and of Factor X, to the same extent. In contrast, active site-blocked Factor X, was a poor inhibitor of both reactions (Fig. 2). This difference in inhibition properties closely correlated with the

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**Table III**

Initial velocity of the heparin-dependent antithrombin/factor X, reaction

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration (nM)</th>
<th>Initial Velocity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Experimental</td>
</tr>
<tr>
<td>X,</td>
<td>3</td>
<td>1.6 x 10^-10 M-sec^-1</td>
</tr>
<tr>
<td>A</td>
<td>6</td>
<td>7.8 x 10^-10 M-sec^-1</td>
</tr>
<tr>
<td>X,</td>
<td>9</td>
<td>18</td>
</tr>
<tr>
<td>A</td>
<td>2</td>
<td>40</td>
</tr>
<tr>
<td>X,</td>
<td>1</td>
<td>80</td>
</tr>
</tbody>
</table>

*Heparin concentration is 800 nM. Values were estimated using the kinetic parameters from Table I.
Relative affinities of thrombin and Factor Xa for the heparin-antithrombin complex.

The two-substrate model was also applicable under conditions where the concentration of heparin was high. Under these conditions, the reaction is in the limit of saturating catalyst; therefore, the heparin-antithrombin complex behaves as a single turnover catalyst and the protease as the single substrate. The kinetics of the antithrombin/Factor Xa reaction were consistent with this mechanism, and the $K_m$ and $k_{cat}$ for Factor Xa inactivation appeared independent of the heparin concentration over the range tested.

The kinetics of the antithrombin/thrombin reaction were also consistent with this model (Fig. 6). The $k_{cat}$ (about 0.1 s$^{-1}$) appeared to be independent of heparin concentration. However, at high concentrations of heparin the $K_m$ (100 nM) for thrombin was at least 50-fold larger than the $K_m$ for thrombin at low concentrations of heparin. Consequently, under most experimental conditions at low concentrations of heparin, the rate-determining step was independent of thrombin but dependent on antithrombin, while at high concentrations of heparin the reaction was dependent upon both antithrombin and thrombin. The velocity of the reaction therefore decreased at high heparin even though the $k_{cat}$ was unchanged.

For the antithrombin/thrombin reaction at low concentrations of heparin, determination of the second order kinetic rate constant and are 20- to 100-fold greater than the estimated value of a diffusionally controlled rate constant. Kinetic results presented here. Filling this site with heparin would eliminate others have concluded from experiments using small heparin fragments that such a binding site contributes to the overall inactivation of thrombin (31, 32). The second order rate constant for heparin-antithrombin interaction slightly exceeded the theoretical value and implies a slightly larger than 1-nm collisional overlap.

For the antithrombin/thrombin reaction at low concentrations of heparin, determination of the second order kinetic rate constant by either the bimolecular model or the two-substrate model is not straightforward. The $K_m$ for thrombin is low (<2 nm). Consequently, under readily accessible experimental conditions, the concentration of thrombin is saturating. This means that the second order rate constants reported in the literature were probably determined under conditions where the bimolecular reaction model is not applicable and therefore represent a lower limit for the true second order rate constants. The second order rate constants, $2.8 \times 10^8$ (8) and $3 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ (7) reported at 37°C are somewhat lower than the minimum value of $8 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ estimated here (Table I). However, the significant point is that these kinetically determined values are lower limits for the second order rate constant and are 20- to 100-fold greater than the estimated theoretical physical value for a diffusion-limited reaction rate constant. These values are still below the collisional rate constant and are physically possible. They imply that the required collisional overlap region between thrombin and the antithrombin-heparin complex must be much greater than a 1-nm circle.

There is no direct evidence for how the protease associates in the ternary complex; however, many models have been proposed (7, 8, 11, 28-31). A model where the heparin binding site on thrombin is utilized in binding thrombin to the heparin-antithrombin complex is consistent with the kinetic results presented here. Filling this site with heparin would eliminate this interaction and decrease the contact region between the reactants which in turn would increase the diffusionally controlled $K_m$. Others have concluded from experiments using small heparin fragments that such a binding site contributes to the overall inactivation of thrombin (31, 32). The second order rate constant for heparin-antithrombin interaction ($k_b$, Table I) also approximated the theoretical value of a diffusionally controlled rate constant. Kinetic results clearly established that this interaction is diffusionally controlled ($K_m$ is larger than $K_b$, Table I). The values of $k_b$ differed somewhat for these two proteases but need not be identical since they are kinetic instead of physical parameters. For example, the difference could be due to thrombin reacting with only a subpopulation of the heparin-antithrombin complex.
plex or to thrombin forming a nonproductive ternary complex with the heparin-antithrombin complex. The explanation for this difference will require further study.

In summary, the two-substrate heparin-catalyzed reaction model (Equation 1) appears to be a general one. It applies over a wide range of heparin concentrations and to two protease substrates. Kinetic differences between the two proteases are readily explained in terms of their respective $K_{m}$ values. It seems probable that the antithrombin reaction with other proteases of the coagulation-fibrinolytic system would fit this reaction mechanism. Furthermore, this model may be most useful in evaluating fractionated preparations of heparin; changes in $k_{cat}$ or $K_{m}$ with heparin preparation may provide insight into the nature of the active site and/or contact sites of heparin.

REFERENCES