Kinetic Analysis of the Heparin-enhanced Antithrombin III/Thrombin Reaction

REACTION RATE ENHANCEMENT BY HEPARIN-THROMBIN ASSOCIATION

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The distribution of heparin between thrombin and antithrombin III in solutions containing both proteins has been calculated using a heparin-thrombin dissociation constant value, $K_{TH}$, of $1.5 \times 10^{-7}$ m and a heparin-antithrombin III dissociation constant value, $K_{AH}$, of $2.0 \times 10^{-8}$ m. The enhancing effect of heparin on the antithrombin III/thrombin reaction rate appeared to correlate with the binding of heparin to thrombin, suggesting that this is the first step in the mechanism of action of heparin. A decreased effectiveness of heparin at concentrations which were high, relative to the thrombin concentration, was observed. This decrease appeared to correlate with the binding of a second heparin molecule to thrombin with a dissociation constant value of approximately $1.0 \times 10^{-7}$ m. Changes in the antithrombin III/thrombin reaction system which would alter the distribution of heparin between the two proteins, e.g., adding active site-blocked thrombin or increasing the concentration of either protein, had an effect on the ability of heparin to enhance the reaction rate in accord with the binding of heparin to thrombin being the first step of the mechanism of reaction rate enhancement. Increasing the antithrombin III concentration while maintaining fixed thrombin and heparin concentrations inhibited the effectiveness of heparin in enhancing thrombin inactivation. This was consistent with the binding of heparin to the antithrombin III high affinity site, i.e., $K_{AH} = 2.0 \times 10^{-8}$ m, not affecting the rate of the antithrombin III/thrombin reaction other than by displacing heparin from thrombin. The results suggest that the binding of heparin to the high affinity binding site on antithrombin III does not directly enhance or inhibit the antithrombin III/thrombin reaction rate.

The anticoagulant mechanism of action of heparin has been investigated in vitro by studying the effect of heparin on the inactivation of thrombin by antithrombin III. The results of several studies have suggested that enhanced antithrombin III/thrombin reaction rates are due to an interaction of heparin with antithrombin III. Of the data available, three general observations provide evidence for this conclusion. First, chemically modified-antithrombin III, i.e., antithrombin III in which 85% of the lysyl residues have been amidinated, did not bind to heparin-agarose with the same avidity as the unmodified protein (1). Further, when modified-antithrombin III was used to inactivate thrombin, the effectiveness of heparin in enhancing the inactivation reaction rate was decreased. Second, heparin having the property of a high anticoagulant activity also has a high affinity for antithrombin III (2, 3). Third, the binding of heparin to antithrombin III alters the conformation of the protein, which could account for the increased reaction rate between antithrombin III and thrombin (4-7). Results from our laboratory, as well as several others, have indicated, however, that heparin also interacts with thrombin and that this interaction may be significant to the anticoagulant mechanism of action of heparin (8-16). This, of course, raises the possibility that the interaction of heparin with both proteins may be required for the mechanism of action of heparin (17-21).

Most studies have not considered rigorously the fact that regardless of the mechanism of action of heparin, heparin will bind to both thrombin and antithrombin III in a solution containing both proteins. In an attempt to demonstrate the significance of the interaction of heparin with thrombin in a solution containing antithrombin III we recently reported kinetic data showing the effect of adding active site-blocked thrombin to reaction solutions (9). Active site-blocked thrombin does not compete with thrombin for antithrombin III. The effectiveness of heparin in enhancing the reaction rate was markedly diminished, however, presumably due to the binding of heparin to the active site-blocked thrombin. In the present investigation we have expanded our study of the kinetics of the heparin-enhanced antithrombin III/thrombin reaction. We have compared the theoretical binding of heparin to thrombin and antithrombin III in a solution containing both proteins with the observed effect of heparin on the inactivation reaction rate. The results of this study are important in considering steps in the mechanism of action of heparin subsequent to the binding of heparin to thrombin.

EXPERIMENTAL PROCEDURES

Materials

'TosGlyProArgNan' was purchased from Boehringer-Mannheim. 1,5-Dimethyl-1,5-diazaundecamethylene polymethobromide (Polybrene) was purchased from Akrich. Polyethyleneglycol (Mr = 6000 to 7500) was purchased from J. T. Baker. Prothrombin complex concentrates and antithrombin III were obtained from the American Red Cross National Fractionation Center. The antithrombin III was

1 The abbreviations used are: TosGlyProArgNan, N"-p-tosyl-L-glycyl-L-prolyl-L-arginine-p-nitroanilide; PEG 6000, polyethylene glycol (M, = 6000 to 7500); TEA, triethanolamine; VallleProArgCH2Cl, L-valyl-L-isoleucyl-L-prolyl-L-arginine chloromethyl ketone.

2 Antithrombin III and prothrombin complex concentrates, the latter used for the preparation of thrombin, were provided by the American Red Cross National Fractionation Center with the partial support of NIH Grant HL-13881.
judged to be 29% homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein concentrations were determined spectrophotometrically using an extinction coefficient value of 0.61 M⁻¹ cm⁻¹ at 280 nm for human antithrombin III (M, 63,000) (22). In the presence and absence of heparin, 1 mol of antithrombin III was required to inactivate 1 mol of human α-thrombin, indicating 100% activity of the antithrombin III protein. Bovine lung heparin (183 USP units/mg) was obtained from DRS. M. B. Mathews and J. A. Cifonelli, University of Chicago. This material had a molecular weight range of 16,000 as determined by intrinsic viscosity measurements. L-Valyl-l-isoleucyl-l-prolyl-l-arginine chloromethyl ketone was a gift from Drs. C. Kettner and E. Shaw, Brookhaven National Laboratory.

Human α-thrombin was isolated from prothrombin complex concentrates as described previously (23). Fibrinogen clotting activity was determined by adding 0.1 ml of enzyme solution to 0.2 ml of 0.15 M NaCl containing 5.0 mg/ml of fibrinogen at 37°C. Clotting times were converted to NIH units from a standard curve prepared against NIH thrombin standard Lot 3B. Specific activities of 3000 to 3500 NIH units/mg were obtained for the purified enzyme. Protein concentrations were determined spectrophotometrically using an extinction coefficient value of 1.75 M⁻¹ cm⁻¹ at 280 nm for human α-thrombin (M, 39,600) (24). Enzyme solutions contained 0.1% polyethylene glycol to prevent adsorption to surfaces (25).

Heparin-Protein Dissociation Constant Values—For the present investigation a value of 2.0 × 10⁻8 M was determined by fluorescence essentially as described previously (26). Heparin was added to a 0.1 M TEA (pH 8.0) solution containing 0.1% PEG 6000, determined in our laboratory has been reported previously (26). Briefly, heparin enhances the apparent binding affinity of thrombin for the synthetic substrate TosGlyProArgNaN. This effect can be used to titrate the binding of heparin to thrombin and the K₆₇ value determined by Scatchard plot analysis. The heparin-protein dissociation constant value, k³, for the Heparin-enhanced Antithrombin III/Thrombin Reactions—Inactivation of thrombin by a 25-fold molar excess of antithrombin III in the presence of heparin follows pseudo-first order reaction kinetics (9). Antithrombin III was added to reaction solutions containing heparin and thrombin. The reaction solution also contained 0.1 M TEA (pH 8.0) and 0.1% PEG 6000. At timed intervals, 0.1 ml of solution containing 0.1 M NaCl, 0.1% PEG 6000 and 20 mg/ml of Polybrene was added to 0.1 ml of the reaction solution and the remaining thrombin activity was determined. Glacial acetic acid (10%), 3.0 ml, was added to terminate amoidolysis and the amount of p-nitroaniline formed was determined spectrophotometrically using a 5-cm path length cuvette in a Zeiss PM QII spectrophotometer (ε₉₉ = 1.14). The binding of proflavin by heparin (27) may alter the interaction of heparin with thrombin which might explain the difference in binding affinity.

Hypothetical Relationships between Heparin Protein Binding and the Mechanism of Action of Heparin—Several models have been proposed to describe the mechanism of action of heparin. To discuss these models it is important to consider the mechanism of thrombin activation of thrombin by a 25-fold molar excess of antithrombin III in the presence of heparin follows pseudo-first order reaction kinetics. The resulting binding curves are shown in Fig. 1A. Enhancement of the antithrombin III/thrombin reaction rate will increase according to the following equation.

\[
E + H \rightarrow E·H \quad (2)
\]

where k⁺ is the apparent second order rate constant for the reaction. Equation 2 is supported by experimental data demonstrating second order reaction kinetics (11, 28). Second order reaction kinetics are also observed for the inactivation reaction in the presence of heparin (9). The following diagram can be used to describe, in simple terms, the interaction of heparin with the antithrombin III/thrombin reaction system.

Using an iterative process, these equations were simultaneously satisfied. Using an iterative process, these equations were simultaneously satisfied. Using an iterative process, these equations were simultaneously satisfied. Using an iterative process, these equations were simultaneously satisfied.

RESULTS

Heparin-Protein Binding and the Enhancement of the Antithrombin III/Thrombin Reaction Rate—The binding of heparin to thrombin and antithrombin III in systems containing both proteins was calculated over 1,000-fold concentration range of heparin. The resulting binding curves are shown in Fig. 1A. Enhancement of the antithrombin III/thrombin reaction rate was investigated by a factor of a if the binding of heparin to thrombin is involved in the mechanism of action of heparin or by a factor of 2 if the binding of antithrombin III to thrombin is involved. More to the point, the antithrombin III/thrombin reaction rate will increase according to the following equation.

\[
E + I \rightarrow E·I \quad (1)
\]

This suggests that the enzyme, E, and the inhibitor, I, form a dissociable complex, EI, which is subsequently stabilized, possibly by bond formation between K and I, by a step or series of steps to yield EI*. In the case of thrombin and antithrombin III it would appear that k₁ ≫ k₂ ≫ k₃ which was inferred from the observation that the affinity of thrombin for antithrombin III is low, K₆₇ = 10⁻³ M (see "Results"). This reduces Equation 1 to a simple second order equation.

\[
E + I \rightarrow E·I \quad (2)
\]

where k⁺ is the apparent second order rate constant for the reaction. Equation 2 is supported by experimental data demonstrating second order reaction kinetics (11, 28). Second order reaction kinetics are also observed for the inactivation reaction in the presence of heparin (9). The following diagram can be used to describe, in simple terms, the interaction of heparin with the antithrombin III/thrombin reaction system.
Heparin-Thrombin Interaction

**FIG. 1.** Heparin-protein binding and the enhancement of the antithrombin III/thrombin reaction rate. Panel A, theoretical heparin-protein binding. The binding of heparin to thrombin and antithrombin III in a solution containing both proteins was calculated as described under "Experimental Procedures." The thrombin concentration was set at $4.0 \times 10^{-9}$ M and the $K_{AT}^{th}$ value was $1.5 \times 10^{-9}$ M. The antithrombin III concentration was set at $1.0 \times 10^{-1}$ M and the $K_{AT}^{th}$ value was $2.0 \times 10^{-8}$ M. The amount of thrombin bound by heparin was determined relative to the total thrombin concentration. The amount of antithrombin III bound by heparin was determined relative to the total antithrombin III concentration. Rapid equilibrium was assumed. It was also assumed that heparin bound to thrombin does not require binding to the high affinity site on antithrombin III. If the latter assumption was not valid, -- -- would predict the inhibition of the antithrombin III/thrombin reaction due to heparin saturation of both proteins. --- - -- predicts complete inhibition of the heparin enhancing effect due to the binding of a second molecule of heparin to thrombin with a $K_{AT}^{th}$ value of $1.0 \times 10^{-7}$ M. Panel B, heparin enhancement of the antithrombin III/thrombin reaction rate. Inactivation of thrombin by antithrombin III in the presence of heparin was determined as described under "Experimental Procedures." The final concentration of thrombin was $4.0 \times 10^{-9}$ M. The final concentrations of antithrombin III were: 0, $1.0 \times 10^{-6}$ M; and 1, $1.0 \times 10^{-7}$ M.

Theoretical curves were applied to the data using the binding parameters above. An 80% inhibition due to the apparent binding of a second molecule of heparin to thrombin was used to calculate the theoretical line in Fig. 1B. The data points fall very near the theoretical line. These results indicate that heparin binds to antithrombin III in the presence of thrombin with an affinity described by a $K_{AT}^{th}$ value of $2.0 \times 10^{-8}$ M, but that this binding does not participate directly in the mechanism of action of heparin in accelerating the antithrombin III/thrombin reaction.

Increasing the concentration of thrombin resulted in a shift in the heparin concentration-dependent curve to the right and a decrease in the maximum reaction rate enhancement by heparin. These results are shown in Fig. 2A. Similar results were obtained when the total thrombin concentration was increased by adding active site-blocked thrombin. These results are shown in Fig. 2B. Since active site-blocked thrombin affects the reaction kinetics by competing for heparin (not antithrombin III) (9), the decreased maximum reaction rate enhancement appeared to be due to a nonspecific thrombin-thrombin interaction, possibly aggregation. When this decrease in the maximum reaction rate is considered, the shift to the right in the heparin concentration-dependent curve by increasing the thrombin concentration with either native or active site-blocked thrombin is as predicted by the theoretical binding curves described in the legend to Fig. 1.

**Inhibition of the Heparin-enhanced Antithrombin III/Thrombin Reaction by Antithrombin III**—In the preceding section it was suggested that the high affinity binding of heparin to antithrombin III does not participate directly in the mechanism of action of heparin. The binding of heparin to antithrombin III does occur, however, as shown in Fig. 1B and cannot be ignored when studying the mechanism of action of heparin. In the absence of heparin the rate of thrombin inactivation can be described by the following equation derived from Equation 2.

$$\frac{-dE}{dt} = (E)(AT)k^e$$  

(3)
Integrating,

\[
\ln \left( \frac{[E]}{[E_0]} \right) = k''(AT) \cdot t
\]

When the antithrombin III concentration is large relative to thrombin, pseudo-first order reaction conditions will be achieved and the pseudo-first order rate constant, \( k' \), will be described as follows.

\[
k' = k''(AT)
\]

The plot of \( k' \) values versus antithrombin III concentration will be linear with a slope equal to \( k'' \). These results are shown in Fig. 3. The \( k'' \) value was approximately \( 6.7 \times 10^8 \text{ M}^{-1} \cdot \text{min}^{-1} \). The \( k'' \) value is an apparent value describing only the rate-limiting step for thrombin inactivation. The observation that the line in Fig. 3 is linear over the antithrombin concentration range used indicates that the dissociation constant value for a dissociable thrombin-antithrombin III complex, if it exists, must be \( \geq 10^{-5} \text{ M} \). When heparin is present, however, a hyperbolic curve, suggesting a saturation phenomenon, results as the antithrombin III concentration was increased (Fig. 3). Double reciprocal plots of data obtained at several heparin concentrations are linear. These results are shown in Fig. 4. The lines drawn through the points are the theoretical lines calculated using a \( K_{\text{H}} \) value of \( 1.5 \times 10^{-8} \text{ M} \) and a \( K_{\text{H}^2} \) value of \( 2.0 \times 10^{-8} \text{ M} \) where the observed reaction rate is determined by the relative amount of thrombin bound to heparin.

**DISCUSSION**

The mechanism by which heparin enhances the antithrombin III/thrombin reaction is not entirely understood at the present time. The majority of investigations have attempted to correlate the binding of heparin to either thrombin or antithrombin III with the mechanism of action. In this regard, it has been shown that heparin which binds tightly to antithrombin III has a much greater anticoagulant activity than heparin which does not bind to the protein (2, 3). In addition, heparin induces a conformational change in antithrombin III which could result in the protein being more reactive with...
thrombin (4–7). These observations argue for the binding of heparin to antithrombin III as the first step in the mechanism of action of heparin. If this is so, then in solutions containing thrombin and antithrombin III, which both bind heparin very tightly, the enhancing effect of heparin on the inactivation reaction should correlate with the binding of heparin to antithrombin III. The results of the present study suggest, however, that the enhancing effect of heparin correlates, instead, with the binding of heparin to thrombin.

Several aspects of the mechanism of action of heparin are evident from the results. It is not likely, for example, that the high affinity binding of heparin to antithrombin III has a significant role in the mechanism of action of heparin in the purified system. This conclusion can be seen with the aid of Fig. 5. Four species of heparin are proposed; free heparin, heparin-thrombin, heparin-antithrombin III, and heparin-thrombin-antithrombin III. At low heparin concentration, because of its higher affinity for thrombin, the majority of bound heparin is associated with thrombin. As the concentration of heparin is increased, thrombin becomes saturated with heparin and the formation of a ternary heparin-thrombin-antithrombin III complex is favored. At higher concentrations of heparin, both heparin-thrombin and heparin-antithrombin III complexes are favored. This scheme assumes that each active heparin molecule has a high affinity site for each protein. If formation of a ternary complex (as described in Fig. 5) were required for heparin to enhance the antithrombin III/thrombin reaction, then enhancement of the reaction would have to correlate with the binding of antithrombin III to the heparin-thrombin complex. In other words, enhancement of the antithrombin III/thrombin reaction rate would correspond to the binding of heparin to antithrombin III even though the heparin-thrombin complex was formed first. This was not experimentally observed. In addition, inhibition of the formation of the ternary complex would occur at high heparin concentration because of the saturation of both proteins by free heparin. This was also not experimentally observed. The data in the present report suggest that heparin does bind with a high affinity to antithrombin III, but the formation of this complex does not affect the interaction with heparin-thrombin complexes and inactivation of the enzyme.

The best evidence that heparin-antithrombin III complexes are formed in the presence of thrombin comes from the observation that the amount of heparin-thrombin complex and, therefore, the reaction rate is, in effect, competitively inhibited by antithrombin III in accordance with the heparin-antithrombin III dissociation constant value of $2.0 \times 10^{-8} \text{M}$ which was determined by fluorescence measurements in the absence of thrombin.

Under certain conditions, the effect of heparin on the antithrombin III/thrombin reaction can appear to correspond with the binding of heparin to antithrombin III. For this to happen, however, the high affinity binding of heparin to thrombin cannot be considered. For example, Jordan and co-workers (26) recently reported the heparin concentration dependence for the enhancement of the antithrombin III/thrombin reaction rate. They compared these data to the binding of heparin to antithrombin III which was determined by the fluorescence technique used in the present report. A very reasonable correlation appeared to exist. The heparin-antithrombin III binding determination was done in the absence of thrombin, however. If the heparin-antithrombin III binding curve were corrected for the amount of heparin which binds to thrombin in the reaction solutions there would not have been a correlation between heparin-antithrombin III binding and heparin enhancement of the antithrombin III/thrombin reaction rate.

At the present time the data are sufficient to conclude very little about steps in the mechanism of action of heparin subsequent to the binding of heparin to thrombin. Two general models are shown in Fig. 6. In one model, heparin bound to thrombin does not bind antithrombin III, whereas in the other

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**Fig. 5.** Binding of heparin to thrombin and antithrombin III.

The scheme diagrams the distribution of heparin between thrombin and antithrombin III at four relative concentrations of heparin. The scheme indicates that each active heparin molecule has a high affinity binding site for thrombin (●) and antithrombin III (▲). It is presumed that the rate of thrombin inactivation is dependent on the initial concentration of the ternary complex shown. Assuming concentrations of thrombin and antithrombin III of $4.0 \times 10^{-9}$ and $1.0 \times 10^{-7}$ M, respectively, the following approximate concentrations of heparin can be assigned: i, $5.0 \times 10^{-9}$ M; ii, $1.0 \times 10^{-8}$ M; iii, $5.0 \times 10^{-8}$ M; and iv, $5.0 \times 10^{-7}$ M.

**Fig. 6.** Models for the mechanism of action of heparin. I, binding of heparin to thrombin induces a conformational change in the enzyme enhancing a subsequent step in the reaction. II, binding of heparin to thrombin forms a part of the binding site for antithrombin III to the heparin-enzyme complex. A, dissociable complex; B, stabilized complex.
model, heparin forms a part of the thrombin binding site for antithrombin III. There are two extremes possible with these models which are consistent with the data. Either there is no change in the thrombin-antithrombin III dissociation constant value of \(10^{-5} M\), thus making the subsequent complex stabilization rate (possibly bond formation) (29) the point of the heparin effect, or the thrombin-antithrombin III dissociation constant value is tremendously lowered by heparin such that bond formation is no longer the rate-limiting step. Thermodynamic studies such as those reported by Machovich and Aranyi (28) may help resolve these models.

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REFERENCES

Kinetic analysis of the heparin-enhanced antithrombin III/thrombin reaction.  
Reaction rate enhancement by heparin-thrombin association.  
M J Griffith  