The kinetics of high affinity heparin binding to human antithrombin III has been studied by stopped flow fluorimetry, using the 40% antithrombin fluorescence enhancement resulting from this interaction. At p 0.15, pH 7.4, and 25 °C, the observed pseudo-first order rate constant varies hyperbolically with heparin concentration with a limiting rate constant of 440 ± 90 s⁻¹, demonstrating that heparin binding is a two-step process involving a conformational change in antithrombin III. An identical dependence is produced when antithrombin is varied, consistent with a symmetrical mechanism in which heparin binding induces a conformational change in antithrombin rather than perturbing an equilibrium between two conformational states of the protein. The rate constant for dissociation of the antithrombin-heparin complex is 1.1-1.5 s⁻¹ at p 0.15, as determined from the ordinate intercept at low heparin concentrations or by dissociation of the antithrombin-heparin complex with iodide. Observation of single pseudo-first order binding rates over a 400-fold heparin concentration range with no detectable lags is compatible with the initial binding step being in rapid equilibrium with a Kd of 4.3 ± 1.3 × 10⁻³ M at p 0.15. Variation in ionic strength primarily affects the Kd for the initial binding step with little effect on the conformational change rate constants, implying that binding involves ionic interactions. Calculation of the overall dissociation equilibrium constant from these rate parameters agrees with the directly determined value of 7.2 ± 1.9 × 10⁻⁸ M at p 0.15. A major function of the conformational change is, thus, to increase the affinity of heparin for antithrombin III greater than 300-fold. The implications of these findings for the mechanism of the heparin-catalyzed inhibition of coagulation proteinases by antithrombin III are discussed.

Heparin accelerates the inactivation of coagulation proteinases by the naturally occurring plasma inhibitor antithrombin III (1, 2), acting catalytically (3, 4) with reported rate enhancements up to 10,000-fold (5). It has been proposed that this large rate enhancement results from the activation of antithrombin III by heparin through an induced conformational change in the protein (1, 2, 5–9). Most of the anticoagulant activity in commercially available heparin resides in a fraction which binds tightly to antithrombin III (10–14). The interaction of this high affinity heparin with antithrombin III has been studied by several spectroscopic techniques. Fluorescence, absorption, and circular dichroism changes have been shown to accompany binding of high affinity heparin to antithrombin III and have been cited as evidence compatible with a conformational change of the protein (5–8, 13–16). This hypothesis has been supported by our finding in the previous paper (17) that the fluorescence changes originate from buried tryptophan residues in antithrombin.

We have examined the kinetics of heparin binding and dissociation by rapid reaction techniques using the 40% enhancement of antithrombin III fluorescence to monitor the binding interaction. These studies were initiated for the following reasons: 1) to ascertain whether there is a conformational change observable in the stopped flow time frame; 2) to determine whether this conformational change is triggered by heparin binding or results from perturbation of a pre-existing equilibrium between different forms of antithrombin III in solution; 3) to evaluate the rate and equilibrium constants for the binding interaction; 4) to resolve the ionic strength effects on the binding step and the conformational change step; and 5) to relate this information to the anticoagulant action of heparin.

MATERIALS AND METHODS

Human antithrombin III was a gift of Dr. Milan Wickerhauser of the American Red Cross. It was homogeneous as judged by sodium dodecyl sulfate gel electrophoresis under reducing conditions and completely active by activity titration with thrombin as described in the preceding paper (17). Protein concentrations were determined using an E₂₅₀ = 6.5 and a molecular weight of 58,000 (18). A purified high affinity heparin fraction (sodium salt) (7), with a reduced polydispersity and molecular weight of 14,300 determined by sedimentation equilibrium, was used for all studies. This material possessed a single binding site for antithrombin as judged from the 1:1 binding stoichiometry when titrated into antithrombin III using a heparin concentration determined by dry weight (6, 7, 19). Heparin solutions were prepared by dilution from a concentrated stock solution (≥1 mM) which was standardized by titration into antithrombin at a concentration greater than 100 times the overall dissociation constant in 0.05 M Tris-Cl, pH 7.4. All other chemicals were reagent grade.

Fluorescence measurements were made in the ratio mode using an SLM 8000 photon-counting spectrophotometer with a thermostatted cell compartment. Titrations of antithrombin with heparin were performed at 25 °C using λe = 280 nm and λm = 340 nm with excitation and emission bandpasses at 4 nm each. Typically, 8–10 10-s integrations were averaged for each fluorescence measurement. All data were corrected for dilution and analyzed by a nonlinear least squares binding program as previously described (17). The largest error in these fits arises from the experimental uncertainty in the antithrombin and heparin concentrations, estimated at ±5% and ±10%, respectively. The error in the equilibrium binding constant was, thus, estimated by repeating the fitting procedure using the protein and ligand concentration limits which gave the highest and lowest binding constants. Protein concentrations employed were 0.1–0.2 μM.
Stopped flow fluorescence measurements were made on an instrument with a dead time of 1-2 ms constructed by Dr. David Ballou of the University of Michigan. Excitation was at 280-285 nm with an emission filter which cut on at 320 nm. Reaction traces were transferred to a Data General Nova 2/10 computer using an interface provided by On-Line-Instrument-Systems, Athens, GA. Up to 16 traces could be averaged to improve signal/noise at low protein concentrations. This permitted us to measure 10% changes in fluorescence at antithrombin concentrations as low as 10⁻⁶ M. To avoid protein adsorption to glass at these low concentrations, the glass surface of the stopped flow syringes was siliconized. All measurements were made in degassed 0.02 M NaP buffers at 25 °C, pH 7.4. The ionic strength was varied by addition of NaCl.

The kinetics of heparin binding to antithrombin III was studied at varying concentrations of the two components, always maintaining a considerable excess of one component to provide pseudo-first order conditions. When the heparin concentration was in excess, it ranged between 0.1 and 40 μM, with the antithrombin concentration varying between 0.02 and 2 μM. In these experiments, the heparin to antithrombin ratio was 5:1 at the lowest heparin concentrations (no more than the first three points of each plot in Figs. 2 and 4) and otherwise 10:1 or higher. Observed rate constants were indistinguishable within the ±10% measurement error of the rate constant when the heparin to antithrombin ratio was varied from 5:1 to 40:1 at a constant heparin concentration. This indicates that even at a 5-fold excess, the pseudo-first order approximation is valid within the accuracy of our rate constant determinations. Thus, the observed rate constants depend solely on the concentration of the component in excess. In other experiments, antithrombin was in excess over heparin and was varied from 0.5-6 μM. Due to the large background fluorescence in these experiments, the heparin concentration was maintained at one-fifth the antithrombin concentration.

Reaction traces were transferred from computer memory to graph paper with an X-Y recorder and analyzed as first order plots. Values of kobs as a function of heparin concentration (Ho) were fit to the hyperbolic function kobs = klim Ho/(K0.5 + Ho) (See "Discussion") using a nonlinear least squares computer program which varied the parameters klim and K0.5 to produce a minimum in the sum of the residuals (20). To allow for the increase in absolute error as the rate constants increased, triplicate data points at each heparin concentration were individually entered in the least squares program. klim represents the limiting rate approached at high heparin concentration and K0.5 represents the heparin concentration required to reach one-half of klim. The use of this equation assumes that the amount of heparin bound in the experiment is negligible compared to the total heparin concentration used; i.e. this is equivalent to satisfying the pseudo-first order condition. In the few instances where only a 5-fold excess was employed, which was necessary to improve the signal/noise level, a 20% change in heparin concentration is expected at the end of the reaction. However, since kobs was determined in these cases from the first two half-lives, any deviations from linearity in the first order plot over this time are averaged, resulting in an underestimate in kobs of less than 10%. This was verified by plotting the data from a few good reaction traces where a 5-fold excess was employed as a second order plot (i.e. log [AT]/[H] versus time). Comparison of the second order rate constant obtained from this plot with that calculated from the pseudo-first order plot indicated an underestimate no greater than 6-8% in kobs.

**RESULTS**

The enhancement of antithrombin III protein fluorescence produced by heparin binding was used to monitor the kinetics of the binding interaction by stopped flow fluorimetry. Initially, heparin was varied under pseudo-first order conditions at approximately physiological ionic strength (0.15). Fig. 1 shows a typical stopped flow trace with a first order plot of the data. Single exponentials were observed from 0.1-40 μM heparin with no detectable lags. Furthermore, amplitudes were proportional to the expected equilibrium concentration of antithrombin-heparin complex. Observed rate constants obtained from the slopes of first order plots are presented in Fig. 2 as a function of heparin concentration (open squares). It is apparent from this plot that the observed rates signifi-

![Fig. 1. Stopped flow trace of the rate of heparin binding to antithrombin III monitored by the antithrombin III fluorescence enhancement. Heparin was mixed with antithrombin III in the stopped flow to give final concentrations of 5 μM and 1 μM, respectively, in 0.02 M NaP, and 0.1 M NaCl, pH 7.4, 25 °C. The inset shows a first order plot of these data on the same time scale.](image-url)
cantly depart from linearity above 8 \( \mu \text{M} \) heparin, consistent with approach to a limiting rate. Fig. 3 shows a double reciprocal plot of these data after subtracting the small ordinate intercept of 1.3 s\(^{-1}\), an average value obtained from the ordinate intercepts of Figs. 4 and 5. The plot is linear with a finite intercept, indicating that the heparin dependence follows a hyperbolic function. A limiting rate constant of 440 s\(^{-1}\) is obtained from the intercept of this plot, while the concentration of heparin producing one-half this velocity, \( K_{0.5} \), obtained from the slope/intercept is 4.3 \( \times 10^{-5} \) M. To evaluate the error in these parameters, the data were fit to a hyperbolic function using a nonlinear least squares computer program. The solid lines in Figs. 2 and 3 correspond to the computer fits and Table I provides a range of error equal to 2 S.D. (95% confidence interval).

Binding of heparin to antithrombin is strongly dependent on ionic strength (6, 13). Fig. 2 shows the results of experiments conducted at different ionic strengths ranging from 0.05–0.36. At ionic strengths lower than 0.15, the hyperbolic dependence of the rate constants is clearly evident up to the limits at which we could accurately measure the rate constant (about 200 s\(^{-1}\)). and the initial linear portion of the curves shows increased slopes as ionic strength is lowered. The linear double reciprocal plots with finite intercepts shown in Fig. 3 confirm the hyperbolic dependence. As shown in Table I, the limiting rates obtained at these lower ionic strength values were similar to that found at physiological ionic strength. The \( K_{0.5} \) value, however, decreased markedly as the ionic strength was lowered. This trend in \( K_{0.5} \) is also evident from the corresponding increase in the initial slope of the hyperbolic plot (Table I). At the lowest ionic strength, we interchanged the roles of heparin and antithrombin by varying antithrombin in excess over heparin. Although these rate constants were more difficult to measure due to the small relative change in fluorescence, they are indistinguishable within experimental error from the curve produced by nonlinear least squares fitting of the data obtained by varying heparin.

Due to limitations in the supply of our purified heparin fraction, we were unable to extend our measurements at ionic strengths higher than 0.15 to concentrations where the hyperbolic curvature could be seen. The slopes of the lower two curves in Fig. 2, however, follow the decreasing trend with increasing ionic strength as noted above. The lines drawn through these data are linear least squares fits and the slopes are shown in Table I. Data were also obtained at 0.56 and 1.05 \( \mu \text{M} \) by mixing 1 \( \text{M} \) or 2 \( \text{M} \) NaCl in 0.02 \( \text{M} \) NaPi, respectively, with antithrombin-heparin complex in 0.02 \( \text{M} \) NaPi, in the stopped flow instrument to give a final pH of 7.4. The final antithrombin concentration was 0.2 \( \mu \text{M} \), while the final heparin concentration varied between 1 and 7 \( \mu \text{M} \) in these experiments. Since the excess of heparin employed provides pseudo-first order conditions (see “Materials and Methods”), even large perturbations of the antithrombin-heparin equilibrium will produce a first order relaxation with the same hyperbolic dependence of \( k_{\text{obs}} \) on heparin concentration. Only the initial linear portion of the hyperbola was seen over the heparin concentration range examined. A slope of 0.27 \( \mu \text{M}^{-1} \text{s}^{-1} \) was obtained at 0.56 \( \mu \text{M} \). At 1.05 \( \mu \text{M} \), however, the rate constant was independent of heparin concentration up to \( 7 \mu \text{M} \), indicating that heparin does not bind significantly at this salt concentration and that the observed rate constant represents dissociation of the heparin-antithrombin complex with no contribution from association.

Ionic strength effects on the binding rates made it necessary to consider the possibility that the observed curvature in Fig. 2 was due to an increase in ionic strength resulting from the negatively charged heparin species. The maximum ionic strength contributed by heparin would result by considering

\[ \begin{array}{c|c|c|c|c|c}
\mu & K_{eq} & \text{Initial slope} & K_{0.5} & k_{obs} & \text{Ordinate intercept} \\
\hline
0.05 & 6.2 \times 10^{-9} & 42.2 & 8.5 \pm 1.2 \times 10^{-4} & 378 \pm 34 & 0-1 \\
0.1 & 1.9 \times 10^{-8} & 23.5 & 20.2 \pm 3.4 \times 10^{-4} & 507 \pm 65 & 0-1 \\
0.15 & 7.2 \times 10^{-7} & 8.3 & 43 \pm 19 \times 10^{-4} & 438 \pm 89 & 1.3 \pm 0.5 \\
0.25 & 2.0 \times 10^{-7} & 3.3 & 17 \pm 6 \times 10^{-4} & 17 \pm 6 & 1.7 \pm 0.6 \\
0.36 & 9.2 \times 10^{-7} & 1.9 & 1.6 \pm 0.6 \\
\end{array} \]
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Since the ordinate intercepts in Fig. 2 are indistinguishable from the origin, we examined the low heparin concentration range in more detail to provide an accurate value of this intercept. Fig. 4 shows the data obtained between 0.1 and 4 \( \mu \text{M} \) heparin at 0.15 \( \mu \). \( k_{\text{obs}} \) exhibits a linear dependence on heparin concentration over this concentration range (\( R^2 = 0.966 \)) and the least squares line intersects the ordinate axis at 1.5 s\(^{-1}\). An alternative method of obtaining the ordinate intercept at this ionic strength is to use the salt relaxation method discussed previously. If antithrombin-heparin complex at low ionic strength is partially dissociated by mixing in the stopped flow instrument with NaCl to give physiological ionic strength, the observed rate constant will be the same as that measured when equivalent concentrations of heparin and antithrombin are mixed from separate syringes, provided pseudo-first order conditions are employed. To increase the degree of dissociation, yet maintain physiological ionic strength, we used NaI which inhibits heparin binding much better than chloride, as shown in the preceding paper (17). Fig. 5 shows a stopped flow trace obtained by partially dissociating the antithrombin-heparin complex with NaI at a final ionic strength of 0.15. To achieve pseudo-first order conditions, the heparin concentration (varying between 0.5 and 2.5 \( \mu \text{M} \) after mixing) was at least 5-fold higher than the antithrombin concentration, which varied between 0.1 and 0.2 \( \mu \text{M} \) after mixing. A first order decrease in fluorescence intensity is noted, reflecting heparin displacement. The observed rate constant should follow a hyperbolic dependence on heparin concentration, but with a much smaller initial slope due to the weaker binding in the presence of iodide. Variation of the heparin concentration under pseudo-first order conditions produced the data shown in Fig. 6. Only the initial linear portion of the presumed hyperbola is seen over this concentration range. The linear least squares line gives a slope of 2.2 \( \mu \text{M}^{-1} \text{s}^{-1} \) which is much smaller than that seen with chloride at the same ionic strength (Table I). The ordinate intercept of 1.1 s\(^{-1}\), however, agrees within its graphically estimated error with that found in Fig. 4, an average of these two values being reported in Table I.

Since we were unable to measure rate constants below 0.1 \( \mu \text{M} \) heparin, we could not determine an accurate ordinate intercept at ionic strength values lower than 0.15. At higher ionic strength, however, a finite intercept could be measured, with values obtained for the two lowest curves in Fig. 2 being given in Table I. At 0.56 and 1.05 \( \mu \), intercept values of 2.5 s\(^{-1}\) and 5.2 s\(^{-1}\), respectively, were found. Thus, in the ionic strength range where significant heparin binding occurs and for which values of this intercept could be obtained (0.15-0.56), the intercept varied from 1.3 s\(^{-1}\) (average) to 2.5 s\(^{-1}\) compared with greater than a 30-fold decrease in the initial slope. This further emphasizes the weak ionic strength dependence of both the ordinate intercept and the limiting rate constant as compared with \( K_{\text{obs}} \). The ionic strength dependence of the overall dissociation equilibrium constant was also determined and the results are given in Table I.

**DISCUSSION**

Before our kinetic data can be evaluated, we need to consider the expected kinetic behavior that distinguishes a simple one-step binding of high affinity heparin to antithrombin III from a binding mechanism involving a protein conformational change. The simple binding equilibrium mechanism

\[
AT + H \stackrel{k_1}{\rightleftharpoons} AT - H
\]

predicts a linear dependence of the observed rate constant when heparin is varied under pseudo-first order conditions with a slope equal to \( k_1 \) and an intercept equal to \( k_{-1} \) (27, 39).
If heparin binding induces a conformational change in antithrombin III, binding becomes a two-step process. This could occur in either of two ways:

\[
AT + H \xrightleftharpoons[k_1]{k_{-1}} AT - H \xrightleftharpoons[k_2]{k_{-2}} AT^* - H
\]

(2)

\[
AT^* + H \xrightarrow[k_3]{k_{-3}} AT^* - H
\]

(3)

In mechanism 2, binding of heparin to the native antithrombin conformation induces a conformational change in the protein. Alternatively, as depicted in mechanism 3, heparin may shift a pre-existing conformational equilibrium by binding exclusively to one form.

The differential equations describing the general two-step mechanism

\[
A^2 \xrightarrow{k_{1-2}} B \xrightarrow{k_{2-3}} C
\]

(4)

can be solved (33), the time dependence for appearance of species C being given by:

\[
[C] = [A_0] \left\{ \frac{k_1k_2}{\lambda_1\lambda_2} + \frac{k_1k_2}{\lambda_1(\lambda_1 - \lambda_2)} e^{-\lambda_2 t} - \frac{k_1k_2}{\lambda_2(\lambda_1 - \lambda_2)} e^{-\lambda_1 t} \right\}
\]

(5)

The observed rate constants for the two exponential terms, \( \lambda_1 \) and \( \lambda_2 \), are functions of the intrinsic rate constants, their sum and products being the simple expressions:

\[
\lambda_1 + \lambda_2 = k_1 + k_{-1} + k_2 + k_{-2}
\]

(6)

\[
\lambda_2\lambda_3 = k_1k_2 + k_{-1}k_{-2} + k_2k_{-2}
\]

(7)

Furthermore, the ratio of the amplitudes of the first to the second exponential term is equal to \( \lambda_2/\lambda_1 \). Thus, in general, biphasic reactions are expected for such a mechanism. When the two exponentials are widely separated in rate, however, (i.e. \( \lambda_1 \gg \lambda_2 \)), the faster exponential exhibits a negligible amplitude relative to the slower exponential, so that one observes only the slower exponential term with an observed rate constant \( \lambda_2 \). The observed rate constant can be solved in terms of the intrinsic rate constants of the mechanism as follows: since \( \lambda_1 \gg \lambda_2 \), equation 6 reduces to:

\[
\lambda_2 = k_1 + k_{-1} + k_2 + k_{-2}
\]

(8)

Equation 7 can then be solved for \( \lambda_2 \):

\[
\lambda_2 = \frac{k_1k_2k_{-1}k_{-2} + k_1k_{-2} + k_2k_{-1}}{k_1} = \frac{k_1k_2k_{-1}k_{-2} + k_2k_{-2}}{k_1 + k_{-1} + k_2 + k_{-2}} = k_{obs}
\]

(9)

If the bimolecular step in the binding mechanisms 2 and 3 is reduced to a pseudo-first order reaction by employing a considerable excess of heparin over antithrombin, the expression for the observed rate constant in the case where \( \lambda_1 \gg \lambda_2 \) can be obtained from equation 9 by substituting, for mechanism 2, \( k_1H_0 \) and for mechanism 3, \( k_2H_0 \). The resulting expressions for \( k_{obs} \) become for mechanism 2:

\[
k_{obs} = k_1H_0 (k_2 + k_{-2} + k_{-1}k_{-2})
\]

(10)

and for mechanism 3:

\[
k_{obs} = \frac{k_1k_2H_0 + (k_1 + k_{-1})k_{-2}}{k_1 + k_{-1} + k_2H_0 + k_{-2}}
\]

(11)

Both mechanisms predict a nonlinear dependence of the observed rate constants when the heparin concentration is varied, which distinguishes them from the simple one-step equilibrium. Furthermore, in both mechanisms, as the heparin concentration approaches infinity, the observed rate constant approaches a limiting value characteristic of the unimolecular step. For mechanism 2, this limiting rate is \( k_2 + k_{-2} \), while for mechanism 3, the limiting rate is \( k_1 \). Finally, both equations reduce to hyperbolic form if \( k_{-2} \) is small relative to the other rate constant terms. The observed nonlinear dependence of \( k_{obs} \) on heparin concentration rules out the simple bimolecular equilibrium of mechanism 1. The data, thus, demonstrate that heparin binding to antithrombin III is a two-step process and is consistent with the involvement of a conformational change in the binding mechanism. Since single exponentials were observed, the dependence of \( k_{obs} \) on heparin concentration should follow either equation 10 or 11 depending on which mechanism applies.

A distinguishing feature of these two mechanisms is the symmetry of antithrombin and heparin in mechanism 2, not present in mechanism 3. Thus, mechanism 2 predicts the same nonlinear plot should be generated when antithrombin is varied under pseudo-first order conditions. On the contrary, mechanism 3 predicts a linear dependence with varied antithrombin concentration assuming the concentration of \( AT^* \) is constant (26). The generation of an identical nonlinear dependence when antithrombin was varied is, thus, consistent with mechanism 2 (Figs. 2 and 3).

A further distinction between these two mechanisms is that the limiting rate constant, \( k_1 \), of mechanism 3 should be independent of the nature of the heparin ligand, while the limiting rate constant for mechanism 2, \( k_2 + k_{-2} \), could vary with the nature of the heparin species. Weaker binding forms of heparin and heparin-like species such as dextran sulfate are known to bind to the same site as high affinity heparin (29, 30). An attempt to determine the limiting rate with dextran sulfate failed since the reaction occurred in the dead time of the stopped flow instrument, presumably the result of a larger dissociation rate constant. It is known, however, that saturation with these weaker binding heparins produces fluorescence and other spectral changes qualitatively similar, but much smaller in magnitude (6, 7). If mechanism 3 were correct, weaker binding heparin should produce identical spectral changes at saturation, whereas a decrease in the conformational equilibrium constant \( k_3/k_{-3} \) in mechanism 2 could explain the smaller spectral changes with different heparin species. Our results, therefore, support the induced conformational change mechanism 2, with the conformational change giving rise to the observed fluorescence enhancement. This would be in keeping with the conclusions of our preceding paper, which postulates a conformational change based on the finding that the fluorescence yield of buried tryptophan residues increases when heparin is bound (17).

The observed hyperbolic dependence of the heparin binding rate constant can be accounted for if \( k_{-2} \) in mechanism 2 is small relative to the other rate constants. Neglecting \( k_{-2} \) in the expression for \( k_{obs} \) simplifies equation 10 to the hyperbolic function:

\[
k_{obs} = k_1H_0 ((k_{-1} + k_{-2})/k_1 + H_0)
\]

(12)

The above equation indicates that the limiting rate constant is the conformational change rate constant, \( k_2 \). The observed limiting rates ranging between 380 and 510 s\(^{-1}\), thus, imply a

\[ \text{(S. T. Olson and J. D. Shore, unpublished experiments.)} \]

The alternative possibility where \( k_{-2} \) is comparable to or greater than \( k_1 \) would require a relatively small \( k_{-2} \) to generate the hyperbolic dependence. This situation is considered unlikely in that the observed dissociation rate constant would be controlled by \( k_{-1} \) and would, therefore, show a significant dependence on ionic strength (32), which is not observed (Table 1).
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highly favorable conformational equilibrium; i.e., \( k_{-1}k_{2} < 1 \). Since the overall dissociation equilibrium constant, \( K_{eq} \), is equal to \( k_{-1}k_{2}/k_{2} \), this would require that \( k_{-1}/k_{1} \gg K_{eq} \) or the binding constant for the initial binding step must be much weaker than the overall equilibrium constant.

The other kinetic parameter derived from the double reciprocal plots, \( K_{eq} \), is equal to \((k_{-1} + k_{2})/k_{1} \) from equation 12. Two possible limiting cases of mechanism 2 are expected to produce monomeric kinetics (31). If \( k_{2} \gg k_{-1} \), then a steady state condition exists where the intermediate \( AT-H \) reacts much faster than it is formed. The opposite case in which \( k_{-1} \gg k_{2} \) is the rapid equilibrium condition where the initial binding step equilibrates much more rapidly than the subsequent conformational change. Analog computer simulations of mechanism 2 by Strickland et al. (31) have shown that the steady state condition is distinguishable from the rapid equilibrium condition by the presence of significant lags. The failure to observe lags over a 400-fold concentration range of heparin is, thus, compatible with the initial binding step being in a rapid equilibrium relative to the subsequent conformational change. In the case of a rapid equilibrium, \( K_{eq} \) becomes \( k_{-1}/k_{1} \) for the initial binding step. Since \( K_{eq} \) is found to be nearly three orders of magnitude greater than \( K_{eq} \) in all cases (Table I), this condition fulfills the requirement for an initial weak binding step.

The involvement of ionic interactions in the binding of antithrombin and heparin is apparent from the sensitivity of \( K_{eq} \) to ionic strength (Table I). The importance of charge interactions between the highly negatively charged heparin polysaccharide and a presumed lysine-rich binding site on antithrombin (1) has been demonstrated (34). This ionic component should be reflected primarily in the \( K_{D} \) for the initial binding interaction. Ionic strength markedly influences \( K_{eq} \) and the initial slopes of the hyperbolars, while relatively minor ionic strength effects are seen in \( k_{2} \) and the ordinate intercepts of the hyperbolars (Table I). These observations are compatible with a rapid equilibrium for, in this case, \( K_{eq} \) is the \( K_{D} \) for the initial binding step, the initial slopes of the hyperbolars are \( k_{2}/K_{D} \), and the ordinate intercepts become \( k_{-1} \). Thus, the ionic strength effects are accounted for in the rapid equilibrium case by a strong ionic strength dependence of \( K_{D} \) and a relatively weak dependence of the conformational change rate constants as would be expected. Our kinetic data are, therefore, in accord with an initial rapid equilibrating binding step preceding the conformational change step. \( K_{eq} \) is, thus, the \( K_{D} \) for the initial binding step and the ordinate intercept represents \( k_{-1} \), the latter rate constant controlling the rate of heparin dissociation.

The kinetic parameters measured for the binding mechanism at physiological ionic strength are summarized in the following scheme:

\[
AT + H \rightleftharpoons K_{D} \xrightarrow{43 \mu M} AT - H \rightleftharpoons 440 \text{s}^{-1} \xrightarrow{1.3 \text{s}^{-1}} AT^{*} - H
\]

In order to satisfy the rapid equilibrium condition, \( K_{eq} \) for the binding step would have to be much greater than the conformational change rate of 440 s\(^{-1}\), thereby requiring \( K_{eq} \) for this step to be much greater than \( 10^{7} \text{M}^{-1} \text{s}^{-1} \). The bimolecular step is, therefore, in the expected range of a diffusion-controlled reaction (27).

Having obtained the most extensive data at an ionic strength of 0.15, the validity of the mechanism and the measured rate parameters can be checked by comparing the overall equilibrium constant which they predict with that measured by direct titration. The calculated equilibrium constant would be:

\[
K_{eq} = \frac{k_{-1} k_{2}}{k_{1} k_{2}} = K_{D} \times k_{-1} / k_{1} = 43 \mu M \times 1.3 \text{s}^{-1} / 440 \text{s}^{-1} = 1.3 \times 10^{-5} \mu M
\]

From the estimated errors of each parameter (Table I), the error bounds of this calculation would range between 0.5 and 2.7 \( \times 10^{-5} \mu M \). This is within the error limits of the directly measured \( K_{eq} \) of 0.72 \( \pm 0.19 \times 10^{-5} \mu M \).

Binding of heparin to antithrombin, thus, involves the initial formation of a relatively weak complex which subsequently undergoes a highly favorable conformational change \( (k_{2}/k_{-1} > 300) \), resulting in tight binding. This may be an important function of the conformational change. A tight binding interaction is known to be essential for antithrombin anticoagulant activity (10-14), probably because it favors the approximation of antithrombin and an activated clotting factor on the heparin surface (5). While an approximation mechanism may account for a faster inactivation of a clotting factor by antithrombin in the presence of heparin, it does not explain the known ability of heparin to act catalytically in this reaction (3,4,13,14). This would require rapid dissociation of heparin after the inactivation reaction which is incompatible with an initial tight binding interaction between heparin and antithrombin. The binding interaction must, therefore, become weaker after the antithrombin-protease complex has formed. This could be accomplished by a further conformational change of antithrombin (in complex with the clotting protease) to a form similar to the native conformation. In support of this idea, the \( K_{D} \) for heparin binding to the antithrombin-thrombin complex has been estimated to be greater than 10 \( \mu M \) at 0.2 \( \mu M \) (13) which is the same order of magnitude \( K_{D} \) we find for heparin binding to the native antithrombin conformation. A dissociation constant of this magnitude would be compatible with rapid dissociation of heparin from the antithrombin-thrombin complex and account for the catalytic effect of heparin. Conformational changes of antithrombin may, therefore, play an important role in modulating heparin binding affinity during the catalytic cycle.

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