The mechanism of the heparin-promoted reaction of thrombin with antithrombin III was investigated by using covalent complexes of antithrombin III with either high-affinity heparin ($M_c = 15,000$) or heparin fragments having an average of 16 and 12 monosaccharide units ($M_r = 4,300$ and 3,200). The complexes inhibit thrombin in the manner of active site-directed, irreversible inhibitors:

$$E + I \xrightleftharpoons{k_1} E \cdot I \xrightarrow{k_2} E - I$$

That is, the inhibition rate of the enzyme is saturable with respect to concentration of complexes.

The values determined for $k_i = (k_{1} + k_{2})k_{2}$ are 7 nM, 100 nM, and 6 μM when the $M_c$ of the heparin moieties are 15,000, 4,300, 3,200, respectively, whereas $k_2$ (2 s$^{-1}$) is independent of the heparin chain length. The bimolecular rate constant $k_2/k_i$ for intact heparin is $3 \times 10^5$ M$^{-1}$ s$^{-1}$ and the corresponding second order rate constant $k_1$ is $6.7 \times 10^8$ M$^{-1}$ s$^{-1}$, a value greater than that expected for a diffusion-controlled bimolecular reaction. The bimolecular rate constants for the complexes with heparin of $M_c = 4,300$ and 3,200 are, respectively, 2 $\times 10^7$ M$^{-1}$ s$^{-1}$ and $3 \times 10^6$ M$^{-1}$ s$^{-1}$.

Active site-blocked thrombin is an antagonist of covalent antithrombin III-heparin complexes: the effect is monophasic and half-maximum at 4 nM of antagonist against the complex with intact heparin, whereas the effect is weaker against complexes with heparin fragments and not monophasic.

We conclude that virtually all of the activity of high affinity, high molecular weight heparin depends on binding both thrombin and antithrombin III to heparin, and that the exceptionally high activity of heparin results in part from the capacity of thrombin bound nonspecifically to heparin to diffuse in the dimension of the heparin chain towards bound antithrombin III. Increasing the chain length of heparin results in an increased reaction rate because of a higher probability of interaction between thrombin and heparin in solution.

Antithrombin III (heparin cofactor) is a plasma protein that can inhibit several serine proteinases, including thrombin, factors IXa, Xa, XIa, XIIa, and plasmin (1). Antithrombin III is distinguished from other protease inhibitors by the capacity of catalytic amounts of heparin to enhance the rate of enzyme inhibition; the 1:1 stoichiometry of the enzyme-inhibitor reaction is, however, unaffected (2, 3). Three mechanisms have been proposed to account for the activity of heparin. One involves allosteric activation of antithrombin III (3–6) upon binding to heparin (7–10). A second mechanism (11–14) operates via enhancement of the reactivity of thrombin with antithrombin III by binding of heparin to the enzyme. A third hypothesis proposes that a ternary complex, stabilized at least in part by interaction of both proteins with heparin, is required for enhanced inhibition (15–17).

The procoagulant enzymes may be divided into two groups on the basis of their kinetics of inhibition by antithrombin III in the presence of heparin. In one group, e.g. factors Xa and XIIa, the reaction rate is proportional to the concentration of heparin-antithrombin III complex. In the second group, i.e. factor IXa, and thrombin, the inhibition rate increases with increasing heparin concentration but reaches a pseudo-plateau and decreases again at higher heparin concentrations. Furthermore, with thrombin the activity of heparin increases with molecular weight, whereas inhibition of factors Xa and XIIa is enhanced equally by heparin and by heparin fragments with molecular weights as small as 3400 (18, 19). These and related kinetic properties have been used as arguments for a mechanism in which the activated reactant is heparin-antithrombin III complex, with minor additional rate enhancement contributed by interaction of thrombin (but not factor Xa) with the heparin bound to antithrombin III (18, 20, 21).

A major problem in the interpretation of thrombin-antithrombin III kinetics is that, in spite of high binary affinities, measurements are carried out in ternary mixtures of enzyme, antithrombin III, and heparin, where stoichiometries and concentrations of binary complexes must be inferred.

We have synthesized covalent 1:1 complexes of antithrombin III and heparin (22, 23). These complexes inhibit factor Xa with second order rate constants very similar to those obtained with the corresponding reversible complexes. Because the system is now binary, kinetic analysis is greatly simplified. Therefore, we have investigated the inhibition of thrombin by antithrombin III coupled to native heparin (average $M_c = 15,000$) or to heparin fragments with $M_r = 3,200$ (h$_{12}$) or $M_r = 4,300$ (h$_{12}$). The results suggest a mechanism

\[ \text{h$_{12}$ heparin fragment obtained by nitrous acid degradation of intact heparin and chromatography on antithrombin III-Sepharose, with average } M_r = 3,200, \text{ and consisting of } 10-14 \text{ monosaccharide units; H, porcine heparin of average } M_r = 15,000 \text{ with high affinity for antithrombin III; h$_{12}$ heparin fragment obtained by nitrous acid degradation of intact heparin and chroma-} \]
involving specific binding of antithrombin III to a unique site on the heparin molecule, and one-dimensional diffusion along the heparin of cationic thrombin bound by electrostatic association to polyanionic heparin. This interaction of thrombin with heparin makes a major contribution to the rate of thrombin inhibition, increasing the inactivation rate by three orders of magnitude. The mechanism of inhibition of thrombin by antithrombin III in the presence of heparin bears some similarities to that of diffusive protein translocation on nucleic acids (24-26).

**EXPERIMENTAL PROCEDURES**

**Materials**

Polyethylene glycol 6000 was obtained from E. Merck (Darmstadt, Federal Republic of Germany), L- amino-α-hexamic acid from BDH (Poole, United Kingdom), and p-aminobenzamide dihydrochloride and Polybrene were from Aldrich (Beemse, Belgium). Benzamidine-Sepharose was prepared as previously described (27). All other reagents were as described elsewhere (22, 23). The synthetic thrombin-inhibitor D-Phe-Pro-Arg-Chl was a kind gift of Dr. E. J. Shaw, Brookhaven National Laboratory, Brookhaven, NY (28).

**Human α-thrombin** was purified from bovine blood (Roussel, Brussels, Belgium) by chromatography on sulfo-propyl (SP)-Sephadex and elution with a linear NaCl gradient (0.05-0.25 M NaCl) in 0.05 M imidazole-HCl buffer, pH 6.3, containing 0.1% polyethylene glycol 6000 and 1 mM benzamidine. The preparation showed one band at 0.1% polyethylene glycol 6000 and 1 mM benzamidine. The preparation showed one band at 280 nm. Factor Xa, high affinity heparin, and the heparin fragments with average chain length of 3000 USP units/mg. Antithrombin III, factor Xa, high affinity heparin, and the heparin fragments with M, = 3200 (h22) and with M, = 4500 (h6) were obtained as described previously (22, 23).

**Human α-thrombin** was the kind gift of Dr. J. W. Fenton, Division of Laboratories and Research, New York State Institute of Health, Albany, NY.

**Extinction coefficients A1% (280 nm) used were 61 for antithrombin III, and 18 for thrombin (29).**

**Methods**

**Cova lent Heparin-Antithrombin III Complexes (AT-H, AT-hh, and AT-hh6)**—The coupling of H and of the heparin fragments (hh and hh6) to antithrombin III was performed as previously described (22, 23). Primary amino groups were introduced in the mucopolysaccharides by reactivity with hexamethylenediamine and the free amino groups of the substituted heparin having a high affinity for antithrombin III was linked to antithrombin III with the bifunctional reagent tolylene-2,4-diisothiocyanate. The covalent complexes were purified by chromatography on DEAE-Sephaloc (23).

**Antithrombin III-heparin complex was purified by gel filtration on Ultrogel ACA 41 in 0.1 M Tris-HCl buffer, pH 7.6, to remove complexes between one heparin molecule and two antithrombin III molecules (22) and most of the unreacted free heparin.**

To remove residual free heparin (about 10%), the preparations, dissolved in 0.05 M NaCl, 0.1 M Tris-HCl, pH 7.6, were applied to columns of concanavalin A-Sepharose equilibrated in the same buffer. About 10% of the heparin but no antithrombin III appeared in the breakthrough. The columns were washed with 1 bed volume of 0.1 M NaCl, 0.1 M Tris-HCl, pH 7.6, and then were eluted with 0.5 M α-methylglucoside, 0.5 M NaCl, 0.1 M Tris-HCl, pH 7.6. The products contained no free heparin detectable by sodium dodecyl sulfate-gel radioelectrophoresis and no free antithrombin III as determined by crossed immunoelectrophoresis (23, 30).

**For all preparations, antithrombin III concentration determined by titration, with thrombin was greater than 85% of that estimated by the method of Lowry et al. (31).**

**Cova lent Heparin-Thrombin Complex—**Heparin was substituted with hexamethylenediamine in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and the high affinity fraction was treated as follows: per ml of substituted heparin (6 mg) diluted to 7 ml with NaCl solution, N,N-dimethyl-N-succinimido buffer, pH 9.2, were added 100 mg of bovine thrombin, activated with FPR. After an interval of 45 °C, the reaction mixture was extracted four times with 7 ml of benzene and then three times with 7 ml of a mixture of heptane/ethyl acetate (2:1). The aqueous phase was then immediately added to 13 mg of bovine thrombin (equimolar amount) in 0.1 M NaHCO3, pH 8.6, containing 0.1% polyethylene glycol 6,000. Sodium dodecyl sulfate-gel electrophoresis of the reaction mixture showed formation of a complex with M, = 45,000 and small concentrations of higher M, complexes. Measurement of radioactivity in the slices revealed that 30-35% of the heparin was complexed with thrombin.

The thrombin-heparin complex was purified by chromatography on a column (1.0 x 20 cm) of heparin-Ultrigel equilibrated with the same buffer. The unbound mixture (containing heparin and thrombin-heparin complex) was then applied to a column (1 x 10 cm) of benzamidine-Sepharose, and eluted with a linear NaCl gradient (0.1-2 M) in 0.05 M imidazole buffer, pH 7.35. Sodium dodecyl sulfate-gel electrophoresis revealed that the complex was of high purity, the final recovery of the complex was 75%. Determination of the specific radioactivity of the heparin, amino acid analysis, and assay of the enzymatic activity with S-2238 revealed that the purified complex had a 1:1 stoichiometry. The final product was stored in a 0.1 M phosphate buffer, pH 6.3, at 80°C.

**Kinetic Analysis of the Inactivation of Thrombin by Antithrombin III—**The inhibition of thrombin was measured by using an excess of antithrombin III (pseudo first order conditions). Antithrombin III (final concentration 10-150 nM) was preincubated with increasing concentrations of H, hh, or hh6 (0.4-5.080 nM) in the presence of the chromogenic substrate S-2238 (40-200 μM) in 0.05 M NaHCO3, pH 7.6. Sodium dodecyl sulfate-gel electrophoresis showed formation of a complex with M, = 1 nM and the residual enzyme activity was measured by recording the absorbance at 405 nm on a Unicam SP 1800 spectrophotometer for 1-15 min. The slope of the curve was measured at different time intervals and plotted versus reaction time on a semilogarithmic scale. Apparent first rate constants (kapp) were calculated from the time to reach 50% inhibition (t50) using the formula

\[ k_{\text{app}} = \frac{\ln 2}{t_{50}} (1 + [S]/K_a) \]

in which [S] represents the initial concentration of S-2238 and K, the Michaelis constant of thrombin for S-2238, which is 9 μM (32). Kinetic Analysis of the Inactivation of Thrombin with Heparin-Antithrombin III Complexes (AT-H, AT-hh, and AT-hh6)—All reactions were carried out at 37 °C in 0.05 M NaCl, 0.1 M bovine albumin, 0.1 M Tris-HCl buffer, pH 7.6. To a flat bottomed polystyrene tube (63 mm inner diameter) was added buffer diluent and antithrombin III reagent (AT-H, AT-hh, or AT-hh6) to a final volume of 450 μl. The mixture was stirred with a 1 x 6-mm stir at about 1000 rpm, and thrombin (5 μl) was added with a positive displacement capillary pipette. After an interval of 2-20 s, 50 μl of 3.6 mM S-2238 and 5 μl (250 μl) of Polybrene were added, and then hydrolysis of substrate was determined spectrophotometrically as the rate of change of A405. With [thrombin] = 1 nM, a rate of 80 μA/min was obtained. The zero point for each experiment was obtained by adding substrate, Polybrene, and maximum AT-H, AT-hh, or AT-hh6 to the mixture before thrombin was added.

**Kinetic Model for Inhibition of Thrombin by Complexes of Antithrombin III and Heparin.** After a short time both T and AT can associate independently with H, catalysis of the reaction

\[ T + AT \rightarrow T \cdot AT \rightarrow T \cdot A \cdot (1) \]

by H can be considered in terms of the following random, bimolecular events (in which H and h6 are interchangeable) that stabilize the intermediate enzyme-inhibitor complex.
Heparin-catalyzed Inhibition of Thrombin by Antithrombin III

This model defines the final transition state as a cyclic ternary complex of T, AT, and H, the formation of which in steady state is described in terms of affinities of T (K_{TH}, (k_{TH} + k_{z})/k_{fTH}) and of AT (K_{ATH}, (k_{Ty} + k_{z})/k_{fTH}) for the same H molecule. Irrespective of heparin chain length, k_{f} is identical in all cases.

Two circumstances arise where the cyclic ternary complex does not occur: one when the heparin chain length is insufficient to accommodate both T and AT (e.g., h_{12}) or, when both T and AT are saturated with H ([H] >> K_{TH} and K_{ATH}). The reactions then reduce to:

\[ T \cdot H + AT \cdot H \rightleftharpoons H \cdot T \cdot AT \]

and

\[ T \cdot H \rightleftharpoons H \cdot T \cdot H \]

The following equilibria may be defined:

\[ K_{TH} = \frac{[T][H]}{[T \cdot H]} \]
\[ K_{ATH} = \frac{[AT][H]}{[AT \cdot H]} \]

with K_{TH}^{C} = K_{ATH}^{C} = K_{TH}^{C} = K_{ATH}^{C}.

At catalytic [14], reaction pathways Equations 1 and 4 do not contribute significantly to the reaction rate. By conservation:

\[ [T]_0 = [T] + [T \cdot H] + [T \cdot AT] + [H \cdot T \cdot AT] \]

Substituting for the different equilibria by using the steady state assumption and factoring [T],

\[ [T]_0 = [T] \left( 1 + \frac{[H]}{K_{TH}^{C}} + \frac{[H][AT]}{K_{ATH}^{C} \cdot K_{TH}^{C}} + [H \cdot AT] \right) \]

Differentiating and rearranging yield

\[ \frac{dT}{dt} = \frac{K_{TH}^{C} \cdot K_{ATH}^{C} \cdot [H][AT]}{K_{TH}^{C} + [H]} \frac{d[H \cdot T \cdot AT]}{dt} \]

Because in steady state

\[ \frac{d[H \cdot T \cdot AT]}{dt} = k_{2} \left[ T \cdot AT \right] \]

Substitution of Equation 9 in Equation 8 yields

\[ \frac{dT}{dt} = -d \ln [T] = k_{app} dt \]

with

\[ k_{app} = \frac{k_{2} \cdot K_{TH}^{C} \cdot K_{ATH}^{C}}{1 + \frac{K_{TH}^{C}}{[AT]} + \frac{K_{ATH}^{C}}{[H][AT]}} \]
between 1 and 4 nM h₁₆, then an increase to reach a pseudo-plateau at 20 nM, and finally a reduction of $k_{\text{app}}$ at higher concentrations. With h₁₂, $k_{\text{app}}$ increases in a monophasic manner to a plateau at $[\text{H}] > 0.2 \mu\text{M}$. According to Equation 12, the decrease in $k_{\text{app}}$ in Fig. 1A and B corresponds to the disappearance of free antithrombin III (thrombin is saturated with H). Equation 18 reveals that plotting $1/k_{\text{app}}$ versus $[\text{H}]$ yields an abcissa intercept equal to $-K_{A\text{TH}}$ and an ordinate intercept of $K_{A\text{H}}/k_{\text{d}}[\text{AT}]_{0}$ (Fig. 2).

With $k_{2} = 2 \text{s}^{-1}$ (see below) and $[\text{AT}]_{0}$ of 10 nM, the ordinate intercepts yield $K_{A\text{TH}} = 20 \text{nM}$ and $K_{A\text{H}} = 160 \text{nM}$. The abcissa intercepts then yield $K_{A\text{TH}} = 70 \text{nM}$. At extreme concentrations of [H] (up to 100 pM), no further decrease occurs in $k_{\text{app}}$ (Fig. 1A, inset), which implies a shift to pathway (4), with an apparent bimolecular rate constant $k_{2}/K_{A\text{TH}} = k_{\text{app}}/[\text{AT}]_{0}$ of $2 \times 10^{8} \text{M}^{-1} \text{s}^{-1}$.

Inhibition of Thrombin by Covalent Complexes of Heparin with Antithrombin III (AT-H, AT-h₁₆, AT-h₁₂)—With AT-H (or AT-h₁₆, AT-h₁₂) concentrations large relative to [T], inhibition of thrombin followed pseudo-first order kinetics until thrombin became undetectable. Fig. 3A shows thrombin inhibition by 1-15 nM AT-H, from which pseudo-first order rate constants (ln $2/\ell_{1/2}$) were obtained and then plotted according to Equation 20 (Fig. 3B). The abscissa intercept indicates that $K_{i} = 7 \text{nM}$, the ordinate intercept indicates that $k_{2} = 2 \text{s}^{-1}$. The bimolecular rate constant calculated as the ratio $k_{2}/K_{i} = k_{1}k_{2}/(k_{1} + k_{2})$ equals $3 \times 10^{6} \text{M}^{-1} \text{s}^{-1}$.

Likewise, pseudo-first order rate constants obtained with AT-h₁₂ (Fig. 4A), yield a linear Kitz-Wilson plot (Fig. 4B) and estimates of $K_{i} = 100 \text{nM}$, $k_{2} = 2 \text{s}^{-1}$ and $k_{2}/K_{i} = 2 \times 10^{9} \text{M}^{-1} \text{s}^{-1}$. Similar analysis of AT-h₁₂ (Fig. 5) yields $K_{i} = 6 \mu\text{M}$, $k_{2} = 1.7 \text{s}^{-1}$, and $k_{2}/K_{i} = 3 \times 10^{9} \text{M}^{-1} \text{s}^{-1}$.

The contribution to the bimolecular reaction rate of the interaction of thrombin with the heparin moiety of the cova-
Heparin-catalyzed Inhibition of Thrombin by Antithrombin III

Inhibition of Covalent Thrombin-Heparin Complex (T-H) by Antithrombin III—Incubation of T-H with antithrombin III results in a monophasic disappearance of enzymatic activity with a bimolecular rate constant $k_{app}/[AT]_0$ of $6.0 \times 10^3$ M$^{-1}$ s$^{-1}$, similar to the value obtained for thrombin inhibition by antithrombin III. Inactivation of T-H by AT-H occurs like-

![Fig. 4. Reaction of thrombin with AT-h16. A, first order plots of thrombin inhibition (see "Methods") at initial [T] = 1 nM and the indicated AT-h16 concentrations; B, the data in A plotted according to Kitz and Wilson (33), with $k_{app} = \ln(2/t_{1/2}).$

![Fig. 5. Reaction of thrombin with AT-h12. A, first order plots of thrombin inhibition (see "Methods") at initial [T] = 1 nM and the indicated AT-h12 concentrations; B, the data in A plotted according to Kitz and Wilson (33), with $k_{app} = \ln(2/t_{1/2}).$

![Fig. 6. Inhibition by FPR-thrombin of the reaction of thrombin with AT-H, AT-h16, and AT-h12. Reactions were analyzed as described under "Methods" for thrombin inhibition, with $k_{app} = \ln(2/t_{1/2})$ in the absence of FPR-thrombin. Reagent concentrations were: 0.1 nM T, 2 nM AT-H ($k_0 = 0.41$ s$^{-1}$) (●); 1 nM T, 20 nM AT-h16 ($k_0 = 0.38$ s$^{-1}$) (■); 1 nM T, 0.5 μM AT-h12 ($k_0 = 0.10$ s$^{-1}$) (▲) and the indicated concentrations of FPR-thrombin.]}
Heparin-catalyzed Inhibition of Thrombin by Antithrombin III

TABLE I

Kinetic constants of the heparin-catalyzed inhibition of thrombin by antithrombin III

| Reaction | Rate constant | Dissociation constant |
|----------|---------------|-----------------------|
| T + AT | $k_1^{AT}$ | $7.0 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ | >$10^{-5}$ |
| T - H + AT | $k_2^{TH}$ | $6.0 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ | >$10^{-5}$ |
| T + H | $k_1^{THT}$ | $k_2^{THT}$ | $k_3^{THT}$ | $k_4^{THT}$ |
| AT + H | $k_1^{ATH}$ | $k_2^{ATH}$ | $k_3^{ATH}$ | $k_4^{ATH}$ |
| T + h18 | $k_1^{ATH}$ | $k_2^{ATH}$ | $k_3^{ATH}$ | $k_4^{ATH}$ |
| AT + h18 | $k_1^{ATH}$ | $k_2^{ATH}$ |
| T - H | $k_1^{ATH}$ | $k_2^{ATH}$ | $k_3^{ATH}$ |
| T + AT - H | $k_1^{ATH}$ | $k_2^{ATH}$ | $k_3^{ATH}$ |
| T + AT - H | $k_1^{ATH}$ | $k_2^{ATH}$ | $k_3^{ATH}$ |

**Kinetic Constants**

The kinetic constants obtained both with reversible and covalent complexes are summarized in Table I.

**Discussion**

Use of covalent complexes of antithrombin III with heparin and heparin derivatives have enabled an analysis of the kinetics of thrombin inhibition in a binary reaction, where the many competing interactions of the ternary (thrombin-antithrombin-heparin) system have been eliminated. Irrespective of the chain length of the heparin moiety, the complexes inhibit thrombin in the manner characteristic of active site directed, irreversible inhibitors in steady state:

$$E + I \xrightarrow{k_1} E:I \xrightarrow{k_2} E - I$$

shown recently (35) to be the pathway of the thrombin-antithrombin III reaction. In keeping with recent findings of Olson and Shore (35), heparin has little or no effect on $k_3$ and $k_4$. Wise with a bimolecular rate constant of $1.4 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$, similar to that for the inactivation of thrombin by AT-h12 and to that obtained for the inactivation of thrombin by antithrombin III in the presence of saturating concentrations of heparin.

**Kinetic Constants of the Heparin-catalyzed Inhibition of Thrombin by Antithrombin III**—The kinetic constants obtained both with reversible and covalent complexes are summarized in Table I.
Thus, may be regarded as a catalyst that acts by stabilizing the transition state, E-I. However, $K_\text{r} = (k_{-1} + k_0)/k_1$ is strikingly sensitive to the chain length of the heparin moiety, decreasing about 60-fold when the chain length increases from 12 to 16 monosaccharide units, and another 15-fold from 16 to about 40-60 monosaccharide units. Because, as shown by Figs. 1C and 2, the affinity of antithrombin III for heparin is relatively insensitive to chain length, the decreasing $K_r$ must reflect an increasing contribution of the thrombin-heparin interaction.

The magnitude of the rate enhancement that depends on interactions of thrombin with heparin may be addressed in two ways. First, the capacity of FPR-thrombin to inhibit the reaction of the covalent heparin-antithrombin III with thrombin must arise from competition for the heparin moiety, as thrombin with an occupied catalytic center does not inhibit the reaction of thrombin with free antithrombin III (18, 36), and competition of FPR-thrombin for the antithrombin III binding site is precluded by the covalent coupling of the inhibitor to heparin. Compared with that of free antithrombin III, the maximum bimolecular rate constant of $3 \times 10^8$ M$^{-1}$ s$^{-1}$ obtained with AT-H or with high-affinity heparin saturated with antithrombin III represents an increase in reaction rate by about 50,000-fold, the greatest so far observed. Because even AT-h$_2$ is partially inhibited by FPR-thrombin, we may conclude that no less than 1,000-fold, or 99.9% of the rate enhancement catalyzed by high molecular weight, high-affinity heparin depends on noncovalent bonds between heparin and thrombin.

From a comparison between the values of $K^{\text{ATH}}_T$ (80 nM) and $K^{\text{ATH}}_{T_2}$ (160 nM) on the one hand and $K^{\text{ATH}}_T = K^{\text{ATH}}_0$ (70 nM) on the other hand, it can be assumed that the stability of the final transition state is equal but not higher than predicted by the electrostatic interactions between proteins and heparin. Therefore the reaction can be interpreted in terms of electrostatic interactions of thrombin with a site on the heparin chain adjacent to antithrombin III. Thus, in $K^{\text{ATH}}_T = (k^{\text{ATH}}_0 + k_0)/(k^{\text{ATH}}_T)$, $k^{\text{ATH}}_0$ equals that fraction of the association rate constant between thrombin and heparin that is directed to the site adjacent to antithrombin III. Similarly, $k^{\text{ATH}}_T$ equals the same fraction of the corresponding dissociation rate constant. In addition, the interaction between FPR-thrombin and AT-H (Fig. 6) is suggestive of a single association between both molecules. Therefore, the IC$_{50}$ of 4 nM represents the dissociation constant of the equilibrium as follows.

$$T + \text{AT-H} \rightleftharpoons \text{T-H-AT} \quad \text{with} \quad \frac{k_{\text{off}}}{k_{\text{on}}} = 4 \text{nM}$$

Combining $(k^{\text{ATH}}_0 + k_0)/(k^{\text{ATH}}_T) = (1/k_{\text{off}} + k_0)/(f \cdot k_{\text{on}}) = 7 \text{nM}$ and $f$, $k_{\text{off}}/k_{\text{on}} = 4 \text{nM}$, yields a value of $k^{\text{ATH}}_0 = 6.7 \times 10^8$ M$^{-1}$ s$^{-1}$ and of $k^{\text{ATH}}_T = 2.7 \times 10^8$ s$^{-1}$.

From Fig. 1B it appears that h$_2$ consists of two slightly different heparin populations. Yet, assuming that the IC$_{50}$ (60 nM) (Fig. 6) represents the average dissociation constant of the equilibrium

$$T + \text{AT-h}_2 \rightleftharpoons \text{T-h}_2 \text{-AT} \quad \text{with} \quad \frac{k_{\text{off}}}{k_{\text{on}}} = 4 \text{nM}$$

it can similarly be calculated that $k^{\text{ATH}}_{T_2} = 5.0 \times 10^7$ M$^{-1}$ s$^{-1}$ and that $k^{\text{ATH}}_{T_0} = 3 \times 10^7$ s$^{-1}$.

These findings indicate that the dissociation rate constant of thrombin from the antithrombin III binding site ($k_0$) on heparin is independent of the chain length of the remaining heparin part, whereas the second order rate constant ($k_0$) of the association between enzyme and inhibitor on the heparin chain is strongly dependent on the total chain length.

The exceptionally high second order rate constant of $6.7 \times 10^8$ M$^{-1}$ s$^{-1}$ suggests that the reaction is proceeding faster than might be expected for a diffusion-limited bimolecular reaction between two macromolecules (37). Because thrombin carries a net charge at pH 7.6 of about +9 (38), the initial encounter of thrombin with heparin may represent geometrically random ion pairing. If thrombin then were much more likely to diffuse in the dimension of the heparin chain than to dissociate, the probability that any interaction of thrombin with heparin may lead to reaction with bound antithrombin III is greatly enhanced. Thus, steric restrictions of the initial encounter are minimized or eliminated, and as the heparin chain elongates, the probability of an interaction increases, and may exceed the usual expectations of second order reactions. An analogous mechanism, termed "sliding" and reviewed recently by Berg et al. (25) accounts for the rapid association of regulatory proteins with their target base sequences on DNA (24). Also consistent with such a process is the relatively narrow maximum in the second order rate constant of thrombin inhibition when ionic strength is varied (Fig. 1A) (24) and the insensitivity to heparin chain length of the rate of reaction of the anionic enzyme, factor Xa (23). In addition, preventing restricted diffusion of thrombin on the heparin chain by cova lent attachment results in abolishment of the heparin catalysis as evidenced by the reduction of the inhibition rate to the value observed in the absence of heparin.

Because the covalent and noncovalent complexes yield virtually identical bimolecular rate constants, the findings with the binary system may now be used to rationalize the ternary system of heparin, antithrombin III, and thrombin. From the values of the various equilibrium and inhibition constants, it is clear that, as concluded recently by Griffith (39), the pathway of the reaction is random but may be restricted by the appropriate concentrations of any of the three components. This is illustrated by Fig. 1. The ascending limb in Fig. 1A can be explained by considering both the 10-fold greater affinity of thrombin than antithrombin III for heparin, and the 10-fold greater reaction rate of heparin-antithrombin III with thrombin than that of heparin-thrombin with antithrombin III. Upon saturation of thrombin with heparin, the formation of ternary complex shifts from a random to a sequential pathway, in which heparin-thrombin is inhibited by antithrombin III. Thus, the descending limbs in Fig. 1, A and B, reflect a decreasing concentration of antithrombin III, which is being saturated with heparin. At complete saturation of both proteins the reaction rate minimizes to a rate comparable to the reaction of AT-H with T-H, which interestingly compares with similar rates of reaction of either covalent or noncovalent h$_2$-antithrombin III with either free thrombin or thrombin saturated with heparin. Furthermore, the reaction of factor Xa with heparin-antithrombin III proceeds at only a slightly faster rate (23). This convergence of rate constants to between $10^7$ and $10^8$ M$^{-1}$ s$^{-1}$ suggests the minimum rate above which additional rate enhancement arises only from additional interactions of the thrombin with the heparin chain.

Acknowledgments—We are grateful to one of the reviewers of this paper for the suggestion to remove residual free heparin from covalent antithrombin III-heparin complexes by chromatography on concanavalin A-Sepharose. This procedure efficiently eliminated residual free heparin and simplified the interpretation of the data.

REFERENCES

1. Rosenberg, R. D. (1976) in Heparin Chemistry and Clinical Usage (Kakkar, V. V., and Thomas, D. P., eds) pp. 101-117, Academic Press, London

2. Bjork, I., and Nordenman, B. (1976) Eur. J. Biochem. 68, 507-511
Involvement of heparin chain length in the heparin-catalyzed inhibition of thrombin by antithrombin III.

M Hoylaerts, W G Owen and D Collen

J. Biol. Chem. 1984, 259:5670-5677.

Access the most updated version of this article at http://www.jbc.org/content/259/9/5670

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/259/9/5670.full.html#ref-list-1