Transient Kinetics of Heparin-catalyzed Protease Inactivation by Antithrombin III

CHARACTERIZATION OF ASSEMBLY, PRODUCT FORMATION, AND HEPARIN DISSOCIATION STEPS IN THE FACTOR X, REACTION

(Received for publication, September 27, 1988)

Paul A. Craig, Steven T. Olson‡, and Joseph D. Shore
From the Division of Biochemical Research, Henry Ford Hospital, Detroit, Michigan 48202

The kinetics of α-factor X, inhibition by antithrombin III (AT) were studied in the absence and presence of heparin (H) with high affinity for antithrombin by stopped-flow fluorometry at I 0.3, pH 7.4 and 25 °C, using the fluorescence probe p-aminobenzamidine (P) and intrinsic protein fluorescence to monitor the reactions. Active site binding of p-aminobenzamidine to factor X, was characterized by a 200-fold enhancement and 4-nm blue shift of the probe fluorescence emission spectrum (λmax 372 nm), 29-nm red shift of the excitation spectrum (λmax 323 nm), and dissociation constant (K_D) of about 80 μM. Under pseudo-first order conditions ([AT]_0/[H]_0/[P]_0 ≫ [X,]_0), the observed factor X, inactivation rate constant (kObs) measured by p-aminobenzamidine displacement or residual enzymatic activity increased linearly with the "effective" antithrombin concentration (i.e. corrected for probe concentration) up to 300 μM in the absence of heparin, indicating a simple bimolecular process with a rate constant of 2.1 × 10^5 M^-1 s^-1. In the presence of heparin, a similar linear dependence of kObs on effective AT-H complex concentration was found up to 25 μM whether the reaction was followed by probe displacement or the quenching of AT-H complex protein fluorescence due to heparin dissociation, consistent with a bimolecular reaction between AT-H complex and free factor X, with a 300-fold enhanced rate constant of 7 × 10^8 M^-1 s^-1. Above 25 μM AT-H complex, an increasing dead time displacement of p-aminobenzamidine and a downward deviation of kObs from the linear dependence on AT-H complex concentration were found, reflecting the saturation of an intermediate X,-AT*H complex with a K_D of 200 μM and a limiting rate of X,-AT product complex formation of 140 s^-1. Kinetic studies at catalytic heparin concentrations yielded a kObs/K_m for factor X, at saturating antithrombin of 7 × 10^8 M^-1 s^-1 in agreement with the bimolecular rate constant obtained in single heparin turnover experiments. These results demonstrate that 1) the accelerating effect of heparin on the AT/X, reaction is at least partly due to heparin promoting the ordered assembly of antithrombin and factor X, in an intermediate ternary complex and that 2) heparin catalytic turnover is limited by the rate of conversion of the ternary complex intermediate to the product X,-AT complex with heparin dissociation occurring either concomitant with this step or in a subsequent faster step.

Heparin, a highly sulfated glycosaminoglycan, possesses a potent anticoagulant activity which derives from its ability to catalyze the inactivation of blood coagulation proteases by their primary inhibitor, antithrombin III (AT)1 (1). Studies of the mechanism by which heparin accelerates the inhibition of these proteases by antithrombin have suggested that the mode of heparin action may not be the same for all proteases (1, 2). Thus, in the case of thrombin, substantial evidence has accumulated in favor of a surface catalytic or "approximation" mechanism in which binding of both antithrombin and protease to the heparin polysaccharide surface promotes an initial interaction between the two proteins prior to their reaction to form a stable inhibitor-protease complex. This evidence includes: 1) the requirement for catalytic activity of a heparin chain length corresponding to the minimum size which can accommodate both proteins simultaneously (i.e. ≥ 18 saccharide residues) (3–5); 2) the ability to markedly reduce heparin's accelerating effect at high heparin concentrations where binding of antithrombin and protease to separate heparin molecules is favored (6–9); and 3) the selective loss of heparin accelerating activity by natural mutation or chemical modification of amino acid residues in either antithrombin or protease which are presumably essential for heparin binding (10–14). Rapid kinetic studies have provided direct evidence for the assembly of thrombin and antithrombin on the heparin surface prior to their reaction to form the product thrombin-AT complex. Such studies have demonstrated that heparin acceleration is due to heparin promoting the initial encounter of thrombin and antithrombin in the initial assembly step rather than enhancing the rate of the subsequent product formation step (15, 16). Later studies showed that heparin is released concomitant with product complex formation and does not limit the rate of heparin catalytic turnover (17).

In the case of factor Xa, several observations suggest that the surface catalytic mechanism elucidated for the heparin-enhanced thrombin/AT reaction cannot explain heparin's accelerating effect on the inactivation of this protease by antithrombin. Thus, heparin oligosaccharides containing the unique pentasaccharide necessary for high affinity binding of

---

1 The abbreviations used are: AT, antithrombin III; Xa, factor Xa; P, p-aminobenzamidine; H, heparin with high affinity for antithrombin III; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
the inhibitor and as small as this pentasaccharide, effectively accelerate antithrombin inactivation of factor X_{r}, despite their lack of activity with thrombin as the protease (3-6, 18). Moreover, high heparin concentrations do not appear to decrease heparin’s accelerating effects on the AT/X_{r} reaction as they do with the AT/thrombin reaction (3, 6). Such observations are consistent with factor X_{r} binding to heparin not being essential for heparin rate enhancement and have suggested that the conformational change induced in antithrombin upon binding to the specific pentasaccharide site on heparin may make antithrombin a better inhibitor of factor X_{r} (1, 2, 6). That this allosteric mechanism is insufficient to completely account for antithrombin’s accelerating effect is suggested, however, from the increased effectiveness of heparins of increasing chain length in accelerating the AT/X_{r} reaction (5, 19). It is thus possible that a surface catalytic mechanism may contribute to the rate enhancement produced by larger heparin chains.

Because of the success of the rapid kinetic approach in resolving the elementary mechanistic steps in the heparin-catalyzed AT/thrombin reaction (e.g. intermediate complex assembly, conformational change, product formation, and heparin dissociation steps), we have examined the heparin-catalyzed AT/X_{r} reaction using a similar approach as a basis for delineating mechanistic differences between these two protease reactions. The present studies were undertaken to determine: 1) whether the initial assembly of an intermediate AT-X_{r} complex and subsequent formation of the product inhibitor-protease complex could be resolved in the presence and absence of heparin; 2) whether heparin accelerated the AT/X_{r} reaction by promoting the assembly step or the product formation step; and 3) whether antithrombin conformational change or heparin dissociation steps limited the rate at which heparin could recycle as a catalyst. The results of these studies indicate that the heparin-catalyzed AT/X_{r} reaction can be described by the same sequence of reaction steps previously found to characterize the heparin-enhanced AT/thrombin reaction although clear quantitative differences in binding and rate parameters as well as heparin effects on individual reaction steps are evident in these two reactions. The implications of our results for the different hypothesized modes of heparin action are discussed.

**MATERIALS AND METHODS**

**RESULTS**

**Kinetics of Factor X_{r} Inhibition by Antithrombin**—The kinetics of antithrombin III (AT) inhibition of factor X_{r} were monitored continuously in the presence of p-aminobenzamidine (P) from the decrease in fluorescence accompanying the displacement of the probe from the factor X_{r} active site by the inhibitor. Assuming that antithrombin inhibits factor X_{r} in a two-step process involving an initial reversible formation of a noncovalent X_{r}-AT complex followed by the irreversible formation of a covalently linked X_{r}-AT complex (Scheme 1), and that equilibration of X_{r}, P and intermediate X_{r}, AT complexes is rapid relative to the rate of covalent X_{r}-AT complex formation, then displacement of p-aminobenzamidine is predicted to occur in two phases: 1) an initial rapid displacement of a fraction of the bound probe due to equilibrium formation of the intermediate X_{r}, AT complex, followed by 2) a slower displacement of the remaining bound probe due to quantitative conversion of factor X_{r} to the covalent X_{r}-AT complex (15).

\[
K_{X_{r},P} = \frac{K_{X_{r},X_{r}}}{K_{X_{r},P}} \quad K_{X_{r},AT} = X_{r}, AT \rightarrow k_{X_{r},AT}
\]

Scheme 1

Under the pseudo-first order conditions, [AT]_{0} >> [X_{r},] ≪ [P]_{0}, the latter phase will be observed as a simple exponential decrease in bound p-aminobenzamidine fluorescence with a hyperbolic dependence of the pseudo-first order rate constant (k_{obs}) as well as the reaction amplitude on the “effective” antithrombin concentration, [AT]_{0}/(1 + [P]_{0}/K_{X_{r},P}), i.e. corrected for the competitive effect of the probe (Equations 1 and 2) (15).

\[
k_{obs} = \frac{k_{X_{r},P}(1 + [P]_{0}/K_{X_{r},P})}{K_{X_{r},AT} + [AT]_{0}/(1 + [P]_{0}/K_{X_{r},P})} \quad \Delta F = \frac{K_{X_{r},AT}(1 + [P]_{0}/K_{X_{r},P})}{K_{X_{r},AT} + [AT]_{0}/(1 + [P]_{0}/K_{X_{r},P})}, \Delta F_{max}
\]

where \(\Delta F_{max}\) represents the maximum fluorescence change achieved when factor X_{r} is saturated with p-aminobenzamidine.

Fig. 2 (left panel) shows factor X_{r} reactions with three different concentrations of antithrombin monitored by p-aminobenzamidine displacement under pseudo-first order conditions. A simple exponential decay of bound probe fluorescence was observed for all reactions. Pseudo-first order rate constants determined from the data of Fig. 2 increased linearly with [AT]_{0}, whereas reaction amplitudes were independent of [AT]_{0} and equal to the initial enhanced fluorescence of the factor X_{r}-bound probe. These results implied that the effective antithrombin concentrations employed were well below the dissociation constant for the putative X_{r}-AT intermediate complex (i.e. [AT]_{0}/(1 + [P]_{0}/K_{X_{r},P}) ≪ K_{X_{r},AT}) (15).

To confirm the simple competitive model of Scheme 1 as well as validate p-aminobenzamidine as a reporter of this reaction, the dependence of k_{obs} on [P]_{0} was determined at fixed concentrations of factor X_{r} and antithrombin under conditions where k_{obs} was a linear function of the effective

---

2 Portions of this paper (including “Materials and Methods,” part of “Results,” and Figs. 1 and 3) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full-size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
antithrombin concentration (Fig. 4). In accordance with the predicted behavior of Equation 1, $k_{on}$ decreased hyperbolically with increasing [P], and approached an asymptote of zero (Fig. 3, in Miniprint). A nonlinear least squares fit of the data to the simplified form of Equation 1 (see legend to Fig. 3) yielded a bimolecular rate constant, $k/K_{X,AT}$, of $2.9 \pm 0.2 \times 10^8$ M$^{-1}$ s$^{-1}$ and $K_{X,P}$ of $85 \pm 10$ $\mu M$. The former value agrees well with the bimolecular rate constant of $2.5 \pm 0.1 \times 10^8$ M$^{-1}$ s$^{-1}$ determined for the AT/X$_e$ reaction either in the presence or absence of Polybrene by discontinuous assay of residual factor X$_e$ enzymatic activity under the same solution conditions (see “Materials and Methods,” in Miniprint), while the latter value is indistinguishable from the $K_c$ determined in Fig. 1B by direct binding measurements. These results thus justified the use of p-aminobenzamidine as a reporter of the AT/X$_e$ reaction.

To determine whether the saturable formation of an intermediate X$_e$-AT encounter complex could be observed on the X$_e$/AT reaction pathway (Scheme 1), kinetic measurements of the reaction were made in the stopped-flow fluorimeter using the p-aminobenzamidine displacement technique over an effective antithrombin concentration range extending as high as $300 \mu M$ and under pseudo-first order conditions. In all cases, single exponential reactions were observed which showed no significant loss in reaction amplitude due to displacement of the probe in the instrument dead time and which yielded pseudo-first order rate constants that increased linearly with the effective antithrombin concentration (Fig. 4). The slope of the linear kinetic plot provided a bimolecular rate constant, $k/K_{X,AT}$, of $2.1 \pm 0.1 \times 10^8$ M$^{-1}$ s$^{-1}$.

Kinetics of Factor X$_e$ Inhibition by Antithrombin-Heparin Complex—Fig. 2 compares reactions of factor X$_e$ with either antithrombin or antithrombin-heparin complex at identical concentrations. Pseudo-first order conditions resulting in only a single heparin turnover were achieved for the latter reactions by employing a large molar excess of AT-H complex over the factor X$_e$ concentration (15–17). Moreover, a 1.1–2-fold molar excess of antithrombin over heparin insured that nearly all heparin molecules were complexed with the inhibitor, based on the dissociation constant of $0.23 \pm 0.02$ $\mu M$ determined for this interaction under these reaction conditions (16, 17). As can be seen in Fig. 2, these conditions resulted in a single exponential decay of bound p-aminobenzamidine fluorescence at all AT-H complex concentrations with reaction amplitudes equal to the initial bound probe fluorescence. Indistinguishable reaction curves were obtained whether heparin was premixed with antithrombin or with factor X$_e$. In contrast to the reaction with antithrombin, antithrombin-heparin complex inhibited factor X$_e$ over a time frame that was about 300-fold shorter at comparable inhibitor concentrations. Like the reaction with antithrombin, however, the pseudo-first order rate constant increased linearly with the effective AT-H complex concentration at least as high as $25 \mu M$ (Fig. 5). Over this concentration range, the reaction thus appeared to be a simple bimolecular process with antithrombin-heparin complex rather than antithrombin acting as the inhibitor in Scheme 1. A bimolecular rate constant 300-fold greater than that found in the absence of heparin of $6.4 \pm 0.1 \times 10^8$ M$^{-1}$ s$^{-1}$ was obtained from the slope of this linear dependence. Similar experiments conducted at approximately physiological ionic strength (0.15, achieved by reducing the NaCl in the reaction buffer to $0.10 M$) also yielded a linear dependence of $k_{obs}$ on effective AT-H complex concentration (using a measured $K_D$ of $9.015 \pm 0.002 \mu M$ to

**Fig. 4.** Dependence of pseudo-first order rate constants and amplitudes for the AT/factor X$_e$ reaction on antithrombin concentration. Pseudo-first order rate constants and fluorescence amplitude changes were measured for reactions of antithrombin with 0.4–1 $\mu M$ factor X$_e$ and 40 or 80 $\mu M$ p-aminobenzamidine by fitting probe displacement curves to a single exponential decay function, as described under “Materials and Methods” and plotted versus the antithrombin concentration, corrected for probe competition, as shown. Average values for reaction amplitudes (in volts) were corrected (<16%) for the small inner filter effect due to end absorption of antithrombin at higher inhibitor concentrations, as described previously (16). Rate constant data were fit by linear regression analysis while an average value was calculated for the experimentally indistinguishable amplitudes (solid lines).

**Fig. 5.** Dependence of pseudo-first order rate constants and amplitudes for the heparin-catalyzed AT/factor X$_e$ reaction on the concentration of antithrombin-heparin complex. Varying concentrations of heparin nearly saturated with antithrombin (>80%) by employing a 1.1–2-fold molar excess of antithrombin were reacted with 0.1–1 $\mu M$ factor X$_e$ and 80 $\mu M$ p-aminobenzamidine in the stopped-flow fluorimeter and $k_{obs}$ and reaction amplitudes (normalized to the extrapolated amplitude at [AT-H] = 0) determined from exponential fits of probe displacement curves as in Fig. 4. The contribution of the uncatalyzed reaction to $k_{obs}$ due to the excess free antithrombin was calculated to be negligible (<0.5%). Solid lines represent nonlinear least squares fits to Equations 1 and 2 (with AT-H complex replacing AT). The inset compares $k_{obs}$ values measured by p-aminobenzamidine displacement (circles) at lower AT-H complex concentrations with those measured by quenching of intrinsic protein fluorescence (triangles). The latter data were obtained from reactions of 0.1–0.5 $\mu M$ factor X$_e$ with 1–6 $\mu M$ AT and a 1.3–3-fold molar excess of heparin as described under “Materials and Methods.”
calculate \([AT\cdot H]\) up to 6 \(\mu M\) with a similar bimolecular rate constant of 7.4 \(\pm 0.1 \times 10^6 \text{M}^{-1} \text{s}^{-1}\).

To confirm the proportionality between \(k_{\text{obs}}\) and the AT-H complex concentration, the heparin concentration was fixed at 3 \(\mu M\) and the antithrombin concentration varied, again under conditions where the calculated AT-H complex concentration was always in large molar excess over the \(X_a\) concentration. This resulted in a saturable dependence of \(k_{\text{obs}}\) on antithrombin concentration (not shown) which, when fit to the appropriate form of Equation 1 (16), indicated the titration of a protein-heparin complex having a \(K_a\) of 0.26 \(\pm 0.09 \mu M\) that was indistinguishable from the value determined for the AT-H interaction by direct binding experiments (16, 17).

At effective antithrombin-heparin complex concentrations greater than 25 \(\mu M\), a significant downward curvature from the initial proportional increase in \(k_{\text{obs}}\) with AT-H complex concentration was found up to the highest experimentally feasible concentrations of about 150 \(\mu M\) (Fig. 5), suggesting the progressive saturation of an intermediate \(X_a\)-AT-H ternary complex. Consistent with this conclusion, a concomitant increasing loss of reaction amplitude was observed over this range of AT-H complex concentration due to a fraction of the bound \(p\)-aminobenzamidine being displaced in the 2-ms dead time of the stopped-flow instrument (Fig. 5). These results were thus consistent with antithrombin-heparin complex inhibition of factor \(X_a\) proceeding by the two-step reaction of Scheme 2.

\[
K_{X_aP} \quad \frac{K_{X_aAT}}{X_a \cdot P \rightleftharpoons P + X_a \cdot AT \cdot H \rightleftharpoons X_a \cdot AT \cdot H \rightarrow X_a \cdot AT + H}
\]

**SCHEME 2**

From the nonlinear least squares fit of rate constant data to the predicted hyperbolic dependence of Equation 1 (with AT-H replacing AT), a dissociation constant for the \(X_a\) interaction with AT-H complex, \(K_{X_aAT}\), of 210 \(\pm 60 \mu M\) was determined, and a unimolecular rate constant for conversion of the intermediate ternary complex to the covalent complex, \(k_{\text{on}}\), of 140 \(\pm 30 \text{ s}^{-1}\) was obtained. A reciprocal plot of the amplitudes, according to Equation 2, is given in Fig. 5 which shows the predicted linear dependence on effective antithrombin-heparin complex concentration (15). From the intercept/slope of the weighted linear regression line, a dissociation constant of 140 \(\pm 40 \mu M\) was calculated, in reasonable agreement with the kinetic value, considering the experimental error.

**Characterization of Protein Fluorescence Changes Accompanying the Reaction of Antithrombin with Factor \(X_a\) in the Absence and Presence of Heparin**—Although the previous single turnover experiments were consistent with the irreversible formation of the covalent \(X_a\)-AT complex from an intermediate \(X_a\)-AT-H ternary complex, they provided no indication whether heparin was released concomitant with the formation of the covalent or in a subsequent slower step (Scheme 3).

\[
X_a \cdot AT \cdot H \rightleftharpoons X_a \cdot AT \cdot H \rightarrow X_a \cdot AT \cdot H \rightarrow X_a \cdot AT + H
\]

**SCHEME 3**

The latter possibility would result in heparin dissociation limiting the rate at which heparin can complete a catalytic cycle. To investigate these alternatives, we monitored the loss of the 40% enhanced protein fluorescence of the antithrombin-heparin complex as a signal for heparin dissociation during the reaction with factor \(X_a\) (17).

Before we could do this, it was necessary to determine whether the heparin-independent reaction of antithrombin with factor \(X_a\) was accompanied by any changes in protein fluorescence as were previously found to accompany the AT-thrombin reaction (17, 33). Fig. 6 compares the protein fluorescence changes accompanying the reaction of a 2-fold molar excess of antithrombin with factor \(X_a\) in the absence and presence of heparin concentrations which saturated the inhibitor. In the absence of heparin, the total protein fluorescence following complete conversion to the product \(X_a\)-AT complex was unchanged from the summed fluorescence contributions due to factor \(X_a\) and antithrombin, indicating that covalent complex formation did not alter the protein fluorescence of either the inhibitor or the protease. In the presence of heparin levels which saturated antithrombin, the summed protein fluorescence of antithrombin-heparin complex and factor \(X_a\) was greater than that of equivalent concentrations of antithrombin and factor \(X_a\) by an amount equal to the approximately 40% enhanced fluorescence of the AT-H complex. A stoichiometric quenching of this enhanced fluorescence occurred upon reaction with factor \(X_a\) resulting in a final level of protein fluorescence due to the \(X_a\)-AT complex that was indistinguishable from that found for the \(X_a\)-AT complex produced in the absence of heparin (after correction for the excess AT-H complex employed in these experiments). Heparin at these levels was found to have no detectable effect on the protein fluorescence of either factor \(X_a\) or the covalent \(X_a\)-AT complex. Moreover, the amounts of reactive site-cleaved antithrombin (AT\(_{\text{M}}\)) formed in this reaction (15–20% as judged from inhibitor/protease titrations; see "Materials and Methods" and Ref. 5), together with the similar fluorescence yields of antithrombin and AT\(_{\text{M}}\) (17), indicated that AT\(_{\text{M}}\) made no significant contribution to these protein fluorescence changes. Analysis of reaction products formed in these experiments by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis confirmed the quantitative conversion of factor \(X_a\) to the covalent \(X_a\)-AT complex with insignificant complex degradation. These results thus indicated that protein fluorescence changes could be used to follow the rate of heparin dissociation during a single turnover reaction of antithrombin-heparin complex with factor \(X_a\).

When the reaction between AT-H complex and factor \(X_a\)
was examined in the stopped-flow fluorimeter using protein fluorescence detection under pseudo-first order conditions, protein fluorescence was found to decay exponentially with no evidence for lags as would be expected if heparin dissociation occurred as a distinct rate-limiting step subsequent to the covalent complex formation step (i.e. $k_{\text{diss}} \ll k_{\text{h}}$ in Scheme 3) (17). Observed pseudo-first order rate constants determined for these reactions increased linearly with antithrombin-heparin complex concentration over the concentration range examined and were indistinguishable from factor X$_a$ inactivation rate constants obtained using the $p$-aminobenzamidine signal (when compared at the same effective AT-H complex concentrations) (Fig. 5, inset). A bimolecular rate constant of $6.6 \pm 0.1 \times 10^{6}$ M$^{-1}$s$^{-1}$ indistinguishable from that determined by probe displacement was thus obtained from the slope of this linear plot. These results indicated that heparin was released either concomitant with covalent complex formation or in a subsequent more rapid step ($k_{\text{diss}} \gg k_{\text{h}}$ in Scheme 3).

Correlation between Rate Constants for Single and Multiple Heparin Turnover Reactions—To confirm that the reaction model for heparin catalysis deduced from single heparin turnover kinetic studies accurately predicted the behavior of heparin under conditions where multiple heparin turnovers occurred, the kinetics of the heparin-catalyzed AT/X$_a$ reaction were investigated at heparin concentrations well below those of antithrombin and factor X$_a$. Under these conditions the reaction can be treated by classical Michaelis-Menten kinetics since it is formally equivalent to a two-substrate/enzyme reaction where factor X$_a$ and antithrombin are the substrates and heparin is the enzyme (i.e. catalyst) (17, 34, 35). Thus, at catalytic levels of heparin (i.e. $[H]_0 \ll [X_a]_0, [AT]_0$) the rate at which factor X$_a$ is inactivated by antithrombin should be equal to a Michaelis-Menten equation term plus an uncatalyzed reaction term (17):

$$\frac{-d[X_a]}{dt} = \frac{k_{\text{cat,app}}[H]_0[X_a]_0}{K_{\text{M,app}} + [X_a] + h_{\text{uncat}}[X_a]},$$

where $k_{\text{cat,app}}, K_{\text{M,app}},$ and $h_{\text{uncat}}$ are functions of $[AT]_0$ (and $[P]_0$ when present). This equation indicates that the initial velocity of factor X$_a$ inactivation will increase linearly with $[H]_0$, but exhibit a hyperbolic dependence on the initial factor X$_a$ concentration, $[X_a]_0$, when the concentration of heparin or X$_a$, respectively, is varied at fixed concentrations of the other reaction components. Fig. 7A shows that initial velocities measured by $p$-aminobenzamidine displacement (and corrected for probe competition) were linearly dependent on $[H]_0$ up to a heparin concentration of 100 nM when fixed AT, X$_a$, and P concentrations of 5, 0.5, and 80 nM, respectively, were used. The linearity of this plot at heparin concentrations only 5-fold less than $[X_a]_0$ is consistent with the negligible concentrations of ternary complex produced at these X$_a$, AT, and heparin concentrations, as predicted from the weak affinity of X$_a$ for the AT-H complex (Fig. 5). The ordinate intercept yielded a bimolecular rate constant of $2.0 \pm 0.4 \times 10^{5}$ M$^{-1}$s$^{-1}$ for the uncatalyzed reaction, indistinguishable from the value determined in Fig. 4.

To determine the $K_m$ and $k_{\text{cat}}$ for factor X$_a$ as a substrate of the heparin-catalyzed reaction, initial velocities were determined as a function of $[X_a]_0$ at several fixed antithrombin concentrations and a heparin concentration of 20 nM (Fig. 7B). An indistinguishable linear dependence of the initial reaction velocity (corrected for the contribution of the uncatalyzed reaction) on $[X_a]_0$ was observed at fixed antithrombin concentrations of 1–3.5 µM, consistent with these X$_a$ concentrations being well below catalyst saturation (i.e. $<< K_{\text{M,app}}$), but with the antithrombin concentrations being saturating. The linear dependence of the initial velocity on $[X_a]_0$ was confirmed by showing that progress curves for reactions where antithrombin and $p$-aminobenzamidine concentrations did

![Fig. 7. Dependence of steady-state heparin turnover rates on heparin, factor X$_a$ and antithrombin concentrations. Initial rates of heparin-catalyzed factor X$_a$ inactivation by antithrombin were determined by $p$-aminobenzamidine displacement as described under "Materials and Methods" in A, as a function of heparin concentration at 5 µM AT, 0.5 µM factor X$_a$, and 80 µM $p$-aminobenzamidine; in B, as a function of the factor X$_a$ concentration at 20 nM heparin, 40 or 80 µM $p$-aminobenzamidine, and antithrombin concentrations of 1.0 (□), 2.5 (△), or 3.5 µM (○); and in C, as a function of the antithrombin concentration at 0.5 µM factor X$_a$, 20 nM heparin, and 80 µM $p$-aminobenzamidine. Reaction rates were corrected in B and C for the uncatalyzed reaction and in all cases for probe competition as described under "Materials and Methods." Solid lines represent linear regression fits (panels A and B) or a nonlinear least squares fit to the Michaelis-Menten equation with antithrombin as the varied substrate (panel C).]
not significantly change (i.e. [AT]o, [P]o > [Xa]o) corresponded to single exponential decays as predicted from the integrated velocity equation under these conditions (36). In these cases, initial velocities could be more accurately determined from the exponential rate constant (see ”Materials and Methods”). Because the antithrombin concentrations employed in the experiments of Fig. 7, A and B, were saturating, the true kcat/Km for factor Xa could be obtained from the slopes of the linear plots of these figures after dividing by the fixed Xa or heparin concentrations employed, respectively (Equation 3) (17). The respective values obtained, 6.7 ± 0.2 × 10⁸ and 6.8 ± 0.4 × 10⁸ m⁻¹ s⁻¹, were indistinguishable from the bimolecular rate constant determined for antithrombin-heparin complex inactivation of factor Xa in a single heparin turnover (Fig. 5).

Under conditions where the factor Xa concentration is well below its K, the two-substrate/enzyme reaction model predicts that the apparent K, for antithrombin will be identical to the Kd for its interaction with the catalyst, assuming that binding steps are rapidly equilibrated (36). Fig. 7C shows initial velocity data obtained as a function of [AT]o at fixed Xa and heparin concentrations of 0.3 μM and 20 nM, respectively, to determine the apparent K, for this substrate. A saturable dependence of initial velocities on [AT]o, with a limiting value similar to that measured in Fig. 7B at 0.3 μM factor Xa, was observed, after correction for the uncatalyzed rate. The data were satisfactorily fit by the Michaelis-Menten equation with antithrombin as the varied substrate (solid line) which provided a K, of 0.23 ± 0.09 μM that was in direct agreement with the Kd determined for the antithrombin-heparin interaction (see above).

**DISCUSSION**

A combination of rapid kinetic and steady-state kinetic approaches employing both intrinsic and extrinsic fluorescence probes has been used to characterize the heparin-catalyzed reaction of antithrombin with factor Xa as a basis for comparison with previous studies conducted with thrombin as the protease. The 200-fold fluorescence enhancement of p-aminobenzamidine when bound at the active-site of factor Xa provided one signal for continuously monitoring the progress of factor Xa inhibition by antithrombin in the absence and presence of heparin from the decreased fluorescence accompanying the competitive displacement of the bound probe. The Kd of ~80 μM determined for the Xa-P interaction in this study is somewhat lower than the value of 115 μM recently reported (37). This may be due to the failure to correct for the weaker non-active site binding component we observed in this study which could have the effect of increasing the apparent Kd. This weak binding component could possibly reflect an interaction of the positively charged p-aminobenzamidine molecule with the anionic γ-carboxyglutamic acid region of factor Xa, since in the case of trypsin and thrombin, only active site binding of p-aminobenzamidine was observed (28). Because this secondary binding interaction does not involve the active site and contributes negligibly to the enhanced fluorescence of p-aminobenzamidine at concentrations required to saturate the active site interaction, it is not likely to interfere with the use of p-aminobenzamidine as a probe of factor Xa active site interactions.

Examination of the reaction of antithrombin with factor Xa in the absence of heparin over a wide range of effective inhibitor concentrations (i.e. corrected for probe competition) extending as high as 300 μM has shown no evidence for an intermediate Xa-AT noncovalent complex. That an intermediate complex exists in this reaction, however, is suggested from the slow bimolecular association rate constant which is several orders of magnitude below that expected for a diffusion-limited association (38). The linear dependence of kobs and independence of reaction amplitudes on antithrombin concentration contrasts with the hyperbolic dependence of rate constants and amplitudes on inhibitor concentration previously observed over the same effective antithrombin concentration range for the thrombin reaction (15). This would imply that the Kd for an intermediate Xa-AT complex must greatly exceed that of the intermediate thrombin-AT complex (Kd ~1 nM) (Table 1). Likewise, the unimolecular rate constant for conversion of the intermediate Xa-AT complex to the product Xa-AT complex must be considerably greater than the highest measured rate constant for this reaction of 0.6 s⁻¹. The failure to observe saturation behavior in this reaction means that only a bimolecular rate constant equal to the ratio, k/Kd(AT) (Scheme 1), can be determined from our data (15). The value we obtained of 2.1 × 10⁶ m⁻¹ s⁻¹ compares favorably with other reported values for this rate constant in the literature measured under somewhat different experimental conditions (6, 37, 39, 40) and is ~4–5 fold lower than the value previously measured for the thrombin/AT reaction (Table 1).

A linear dependence of kobs on the antithrombin-heparin complex concentration was also observed at effective concentrations up to 25 μM, consistent with the heparin-accelerated reaction proceeding as a simple bimolecular association between free factor Xa and AT-H complex under these conditions. Such kinetic behavior does not preclude a reaction pathway in which factor Xa initially binds heparin followed by reaction with free antithrombin as would occur in the random addition model of Griffith (35). Thus, the latter pathway would not be evident if AT-H binary complexes predominated over Xa-H binary complexes under the conditions of our experiments. This would be in keeping with 1) reported binding constants for the factor Xa-heparin binary complex interaction which are about 2 orders of magnitude weaker than the antithrombin-heparin binary complex interaction (41), 2) the considerably weaker affinity of factor Xa compared to antithrombin for heparin-Sepharose (22, 42), and 3) the anionic charge of factor Xa (43) and heparin. It follows, however, that a reaction pathway in which factor Xa binds to heparin before antithrombin can make only a minor

**Table 1**

Comparison of kinetic parameters for factor Xa and thrombin inhibition by antithrombin or antithrombin-heparin complex

| Inhibitor | Antithrombin | Antithrombin-heparin complex |
|-----------|--------------|-----------------------------|
| Enzyme | Factor Xa | Thrombin | Factor Xa | Thrombin |
| k/Kd (M⁻¹ s⁻¹) | 2 × 10⁷ | 8 × 10⁻²⁴ | 7 × 10⁸ | 7 × 10⁻⁶⁰ |
| Kd (M) | 1 × 10⁻⁹ | 2 × 10⁻⁴ | 10⁻⁸ | 140 |
| k (s⁻¹) | 5.4⁸ | 8.4⁻⁵⁰ |

*These values which were obtained with the same high affinity heparin preparation used in the present study, differ somewhat in Kd and thereby in k/Kd, but not in k, from previously reported values (15–17). This may reflect a difference in charge density between heparin preparations used in this and previous studies (46).
Heparin-Antithrombin-Factor $X_a$ Transient Kinetics

Contribution to the reaction mechanism over a wide range of antithrombin, factor $X_a$, and heparin concentrations, including those likely to occur physiologically (16).

Deviations from a linear dependence of $k_{\text{act}}$ on antithrombin-heparin complex concentrations were observed at effective AT-H complex concentrations above 25 $\mu$M. That these deviations were due to the progressive saturation of a ternary $X_a$-AT-H complex rather than some other effect of high AT-H complex concentrations (e.g. AT self-association) was indicated from the dead time displacement of p-aminobenzamidine from factor $X_a$ which accompanied these deviations. The latter observations thus independently confirmed that an active site-dependent interaction between factor $X_a$ and AT-H complex with a $K_a$ similar to that determined from the rate constant data was rapidly established prior to product $X_a$-AT complex formation. The insignificant binding of p-aminobenzamidine to heparin or antithrombin documented in previous studies (15) indicates that probe interactions with the polysaccharide or the inhibitor cannot be responsible for the observed nonlinear dependence of $k_{\text{act}}$ on reaction amplitude on AT-H complex concentration. Formation of the intermediate ternary complex from factor $X_a$ and AT-H complex was characterized by a relatively weak dissociation constant of 140-210 $\mu$M, in marked contrast to the approximately 25-fold lower $K_d$ for this interaction found with thrombin as the protease under identical experimental conditions (Table I). The limiting rate constant of 140 s$^{-1}$ for the conversion of the intermediate ternary complex to the product $X_a$-AT was comparable to the range of inhibitor concentrations indicating that protease reactions are similar under these experimental conditions (Table I). Since the faster limiting rate constant for the heparin-enhanced reaction of factor $X_a$ relative to thrombin compensates for its weaker initial binding interaction, the bimolecular rate constants for these two protease reactions are similar under these experimental conditions (Table I). The evidence for an intermediate binding step in the presence, but not the absence, of heparin over a comparable range of inhibitor concentrations indicates that at least part (i.e. minimally 10-fold) of the 300-fold heparin enhancement of the $X_a$-AT bimolecular rate constant is due to heparin increasing the binding affinity of factor $X_a$ for antithrombin in the intermediate complex. The full extent to which heparin enhances this binding affinity and whether the 140 s$^{-1}$ limiting rate constant represents a heparin-enhanced value cannot, however, be determined from our data due to our inability to quantitate the two reaction steps in the absence of heparin.

Assuming a rapid equilibrium addition of antithrombin and factor $X_a$ to the heparin catalyst, $K_m$ and $k_{\text{act}}$ values for factor $X_a$ obtained in multiple heparin turnover studies should correspond to the ternary complex $K_p$ and the limiting product formation rate constant, respectively, determined in single turnover studies (17, 36). $K_m$ values for factor $X_a$ of 160 and 100 nM and $k_{\text{act}}$ values of $\approx 1$ s and 0.7 s$^{-1}$ at saturating antithrombin concentrations were reported by Griffith (35) and Pletcher and Nelsetuen (34), respectively, for the heparin-catalyzed $X_a$/AT reaction. These values are clearly several orders of magnitude lower than those predicted by our rapid kinetic studies. Since this discrepancy could be due to a slow release of heparin from the product of the reaction which would not have been detected by p-aminobenzamidine displacement in a single heparin turnover, we used protein fluorescence changes to follow the rate of heparin dissociation during the reaction.

Quenching of the enhanced protein fluorescence of the antithrombin-heparin complex was found to accompany its reaction with factor $X_a$ as was previously found with the thrombin reaction (17), indicating that the antithrombin conformation responsible for tight binding of heparin (44) is lost upon formation of the covalent $X_a$-AT complex, thereby facilitating rapid heparin dissociation from the reaction product (2, 6, 17). Indistinguishable bimolecular rate constants were measured when single turnover reactions of AT-H complex with factor $X_a$ were monitored by protein fluorescence quenching or p-aminobenzamidine displacement, implying that heparin must dissociate either concomitantly with the 140 s$^{-1}$ step or in a much faster subsequent reaction step (Scheme 3). Heparin dissociation thus cannot be rate-limiting during catalytic turnover. To confirm the conclusions of our rapid kinetic studies, we examined the reaction kinetics under conditions similar to past kinetic studies (34, 35), i.e. at catalytic heparin concentrations where multiple heparin turnovers would occur. These kinetic data were consistent with the prediction of our rapid reaction data of an extremely weak $K_m$ for factor $X_a$ and showed no evidence for saturation of the reaction at factor $X_a$ concentrations previously reported to be saturating. The higherionic strength conditions employed in our experiments cannot account for the discrepancies with past studies since the bimolecular rate constant for this reaction, $k_{\text{act}}/K_m$, was found to be largely independent of ionic strength. The saturation behavior with respect to factor $X_a$ reported by Pletcher and Nelsetuen (34) may be due to experimental error since the highest factor $X_a$ concentration they employed was less than one-fifth of $K_m$. In contrast, Griffith’s study (35) employed factor $X_a$ concentrations nearly equivalent to his reported $K_m$ for factor $X_a$. It is possible that systematic errors in measuring true initial velocities at higher factor $X_a$ concentrations could have contributed to the apparent saturation behavior observed in the latter study.

In summary, our results indicate that the heparin-catalyzed AT/$X_a$ reaction can be described by the same sequence of reactions previously demonstrated for the heparin-catalyzed AT/thrombin reaction (Scheme 4). Thus, heparin initially binds to antithrombin and induces a favorable conformational change in the inhibitor which shifts the binding equilibrium in favor of complex formation (17, 29, 44). A ternary $X_a$-AT-H complex is then formed in which the reactive site bond of antithrombin is reversibly bound at the protease active site. This binding interaction, although weak relative to the analogous interaction with thrombin, is nevertheless enhanced by

---

**SCHEME 4**

![Scheme 4](attachment:image.png)

---

2 Chromatography of antithrombin on Sephacryl S-200 (2.5 x 100) at initial loading concentrations of 1, 0.1, and 0.01 mM (in ~5 ml) resulted in indistinguishable symmetrical elution bands at equivalent elution volumes, consistent with no detectable self-association occurring in the absence of heparin at the antithrombin concentrations employed in this study.

3 This is not true at physiological ionic strength (I 0.15) where a further decrease in $K_p$ for the binding step of the heparin-enhanced thrombin/AT reaction (unpublished data) results in thrombin being inhibited by AT-H complex with a 30-fold faster bimolecular rate constant than factor $X_a$ (45).
heparin by at least an order of magnitude. Factor \( X_a \) and antithrombin subsequently react in the ternary complex in an irreversible reaction step at 140 s\(^{-1} \) to form the covalent complex. This reaction induces antithrombin to undergo an additional conformational change either concomitant with this step or in a subsequent faster reaction step that results in the \( X_a \)-AT product complex binding heparin with an affinity several orders of magnitude weaker than that of antithrombin (6, 17). Rapid heparin dissociation from the product and retarding to unreacted antithrombin to begin another catalytic cycle is thus favored, thereby preventing the accumulation of nonproductive product-heparin complexes.

While the reaction pathways for heparin-catalyzed reactions of antithrombin with factor \( X_a \) and thrombin are similar, the mechanism by which heparin accelerates these two reactions may still differ significantly. Thus, the ability of heparin oligosaccharides as small as the unique pentasaccharide, which binds antithrombin to produce an accelerating effect on the AT/\( X_a \) reaction comparable to that of larger heparin chains, whereas heparin chains of at least 18 saccharide units are required to accelerate the AT/thrombin reaction, has implied a fundamental mechanistic difference between these reactions (1, 2). These observations have suggested that the antithrombin conformational change plays a primary role in heparin's ability to accelerate the AT/\( X_a \) reaction whereas the binding of both antithrombin and protease to the same heparin molecule is largely responsible for heparin's ability to accelerate the AT/thrombin reaction. In the context of these hypotheses, our current results would suggest that the heparin-induced antithrombin conformational change functions in part by making antithrombin more complementary to the factor \( X_a \) active site. The role of this conformational change in enhancing the rate of covalent complex formation as well as the contribution of a factor \( X_a \)-heparin interaction to heparin's accelerating effects, however, remain to be elucidated.

Acknowledgments—We wish to thank Dr. Paul Bock of the American Red Cross, Detroit, for his critique of the manuscript and for suggesting that kinetic data be plotted as a function of effective inhibitor concentration.

REFERENCES

1. Björk, I., and Lindahl, U. (1982) Mol. Cell. Biochem. 48, 161–182
2. Björk, I., Olson, S. T., and Shore, J. D. (1988) in Heparin (Lane, D. L., and Lindahl, U., eds) Edward Arnold Ltd., London, in press
3. Oosta, G. M., Gardner, W. T., Beeler, D. L., and Rosenberg, R. D. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 829–833
4. Lane, D. L., Denton, J., Flynn, A. M., Thunberg, L., and Lindahl, U. (1984) Biochem. J. 218, 725–732
5. Danielson, Å., Raeb, E., Lindahl, U., and Björk, I. (1986) J. Biol. Chem. 261, 15467–15473
6. Jordan, R. E., Oosta, G. M., Gardner, W. T., and Rosenberg, R. D. (1980) J. Biol. Chem. 255, 10081–10090
7. Griffith, M. J. (1982) J. Biol. Chem. 257, 7360–7365
8. Nesheim, M. E. (1983) J. Biol. Chem. 258, 1708–1717
9. Hoylaerts, M., Owen, W. G., and Collen, D. (1984) J. Biol. Chem. 259, 5670–5677
10. Pomerantz, M. W., and Owen, W. G. (1978) Biochim. Biophys. Acta 535, 66–77
11. Blackburn, M. N., Smith, R. L., Carson, J., and Sibley, C. C. (1984) J. Biol. Chem. 259, 939–941
12. Koida, T., Odani, S., Takahashi, K., Ono, T., and Sakuragawa, N. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 289–293
13. Chang, J.-Y., and Tran, T. H. (1986) J. Biol. Chem. 261, 1174–1176
14. Peterson, C. B., Noyes, C. M., Poon, M.-C., and Griffith, M. J. (1987) J. Biol. Chem. 262, 8061–8065
15. Olson, S. T., and Shore, J. D. (1982) J. Biol. Chem. 257, 14981–14985
16. Olson, S. T. (1988) J. Biol. Chem. 263, 1698–1708
17. Olson, S. T., and Shore, J. D. (1986) J. Biol. Chem. 261, 13151–13159
18. Choisy, J., Petitou, M., Lormeau, J.-C., Sinay, P., Casu, B., and Gatti, G. (1993) Biochem. Biophys. Res. Commun. 116, 492–499
19. Thunberg, L., Lindahl, U., Tengblad, A., Laurent, T. C., and Jackson, C. M. (1979) Biochem. J. 181, 241–243
20. Melhado, L., Peltz, S. W., Leytus, S. P., and Mangel, W. F. (1982) J. Am. Chem. Soc. 104, 7299–7306
21. March, S. C., Parikh, I., and Castrecassas, P. (1974) Anal. Biochem. 60, 149–152
22. Miller, J. P., Brooze, G. J., Jr., and Majerus, P. (1984) Biochemistry 23, 304–310
23. Deleted in proof
24. Laemmli, U. K. (1970) Nature 227, 680–685
25. DiCicco, R. G., Hermodson, M. A., and Davie, E. W. (1977) Biochemistry 16, 5255–5260
26. Laura, R., Robinson, D. F., and Bing, D. H. (1980) Biochemistry 19, 4859–4864
27. Nordenman, B., Nyström, C., and Björk, I. (1977) Eur. J. Biochem. 78, 195–203
28. Evans, S. A., Olson, S. T., and Shore, J. D. (1982) J. Biol. Chem. 257, 3014–3017
29. Olson, S. T., and Shore, J. D. (1981) J. Biol. Chem. 256, 11065–11072
30. Olson, S. T., and Shore, J. D. (1983) J. Biol. Chem. 258, 11065–11072
31. Orsi, B. A., and Tipton, K. F. (1979) Methods Enzymol. 63, 159–183
32. Duggleby, R. G. (1984) Comp. Biol. Med. 14, 447–455
33. Dixon, M. (1953) Biochem. J. 55, 170–171
34. Wong, R. F., Windser, S. R., and Feinman, R. D. (1983) Biochemistry 22, 3994–3999
35. Fletcher, C. H., and Nelsestuen, G. L. (1983) J. Biol. Chem. 258, 1096–1091
36. Griffith, M. J. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 5460–5464
37. Dixon, M., and Webb, E. C. (1979) Enzymes, 3rd Ed., pp. 82–86, Academic Press, New York
38. Atha, D. H., Lormeau, J.-C., Petitou, M., Rosenberg, R. D., and Choay, J. (1987) Biochemistry 26, 6454–6461
39. Ferre, A. (1977) Enzyme Structure and Mechanism, pp. 126–133, W. H. Freeman & Co., New York
40. Ellory, J. V., Scully, M., MacGregor, I., and Kakkar, V. K. (1982) Biochim. Biophys. Acta 701, 24–31
41. Barrowcliffe, T. W., Hawcroft, S. J., Kemball-Cook, G., and Lindahl, U. (1987) Biochem. J. 245, 31–37
42. Jordan, R. E., Oosta, G. M., Gardner, W. T., and Rosenberg, R. D. (1980) J. Biol. Chem. 255, 10070–10080
43. Miller-Anderson, M., Borg, H., and Andersson, L.-G. (1974) Thromb. Res. 6, 439–452
44. Jackson, C. M. (1984) Prog. Hemostasis Thromb. 7, 55–109
45. Olson, S. T., Srinivasan, R. K., Björk, I., and Shore, J. D. (1981) J. Biol. Chem. 256, 11073–11079
46. Olson, S. T., Björk, I., Craig, P. A., Shore, J. D., and Choay, J. (1987) Thromb. Haemostasis 58, 8
47. Hurst, R. E., Poon, M.-C., and Griffith, M. J. (1983) J. Clin. Invest. 72, 1042–1045

Continued on next page.
**Heparin-Antithrombin-Factor Xa Transient Kinetics**

### MATERIALS AND METHODS

**CHEMICALS**
- p-Aminobenzamidine (2HCl) and polybrene were from Aldrich, benzamidine-HCl and heparin (porcine intestinal mucosal) were from Sigma Chemical Co. and p-aminoethylmethylenemalonodinitrile was from American Biochemical. Gel filtration media and heparin-Sepharose were from Pharmacia. Alternately, heparin-Sepharose was prepared by linking the stroma heparin product at 40 mg/ml to an equal volume of Sepharose 4B. Following activation of the gel with CNBr essentially according to March et al., [25]. All other chemicals were of the highest grade commercially available.

**PROTEINS**
- Factor Xa was prepared from a plasma sample essentially as described in [26], except that thrombin was used in place of factor Xa. Solutions of 0.6-0.3 M NaCl, 0.1 M EDTA, 0.5 M benzamidine, pH 6.5, prior to use, benzamidine was removed and the buffer exchanged by the gel filtration of the sample through a Sephadex G-25 column (gel volume < 10 μL) equilibrated with 20 mM Tris, 0.25 M NaCl, 50 μM EDTA, and 0.1 M FEG buffer, pH 6.0 (5 μL). No activity was observed when stored at 2°C over the course of a day when if no factor X was kept in this buffer. For factor Xa, solutions in this buffer were extensively dialyzed just prior to use in a gel filtration plate using a 0.2 mL buffer mixture of 20 mM Tris, 0.1 M NaCl, and 10% polybrene in a polysulfone reemulor (polybrene). Factor Xa preparations were primarily > 80% active based on the concentration data obtained by absorbance at 280 nm, assuming a 1:1 binding stoichiometry.

**Subtraction Procedure**
- p-Aminobenzamidine excitation spectra were obtained by subtracting the UV absorbance data from the visible absorbance data obtained by monitoring of the reaction at the characteristic absorbance of the factor Xa and 1.3-fold molar excess of heparin over antithrombin was employed [27]. Reactions monitored by p-aminoethylmethylenemalonodinitrile in the absence of heparin with half-times > 10 s were done with a Perkin-Elmer 550-10S spectrophotometer using emission and excitation wavelengths of 340 nm (nm bandpass) and 370 nm (10 nm bandpass), respectively. We observed photodecomposition problems observed to accompany these slower reactions in the stopped-flow instrument. The rapid time frame of reaction monitored by protein fluorescence (λex < λem) was disturbed by protein photodecomposition [28]. Stopped-flow records were fit to single exponential as described above (15-17) with observed reaction constants derived from fitting of 4-7 reaction traces averaged for each set of reaction conditions. Exponential traces derived from Perkin-Elmer experiments were analyzed by sampling of τs at τintervals of 2τ, 5τ, and 10τ, and the fluorescence data (time, and the endpoint fluorescence, respectively) to obtain τs. Rates constants were measured in the presence of heparin by the procedure described in [26] using a stopped-flow process.

### RESULTS

- The kinetic curves of factor Xa reactions with antithrombin in the presence of various concentrations of heparin were monitored by stopped-flow experiments (16). The instrument excitation wavelength and bandpasses, and emission wavelengths of 325 nm (4 nm bandpass) and 370 nm (10 nm bandpass) were used. Rate constants for factor Xa-antithrombin and p-aminoethylmethylenemalonodinitrile were calculated in reaction monitored by protein fluorescence (λex < λem) was disturbed by protein photodecomposition [28]. Stopped-flow curves were fit to single exponential as described above (15-17) with observed reaction constants derived from fitting of 4-7 reaction traces averaged for each set of reaction conditions. Exponential traces derived from Perkin-Elmer experiments were analyzed by sampling of τs at τintervals of 2τ, 5τ, and 10τ, and the fluorescence data (time, and the endpoint fluorescence, respectively) to obtain τs. Rates constants were measured in the presence of heparin by the procedure described in [26] using a stopped-flow process.

### CHARACTERIZATION OF p-AMINOBENZAMIDINE AS A FLUORESCENCE PROBE FOR THE ACTIVE SITE OF FACTOR XA
- The fluorescence intensity of factor Xa was measured at 25°C with an instrument equipped with a 325 nm excitation filter (100 nm bandpass) and 370 nm (10 nm bandpass) emission filter in the absence of heparin. The increase in fluorescence was monitored by a stopped-flow instrument. The instrument excitation wavelength and bandpasses, and emission wavelengths of 325 nm (4 nm bandpass) and 370 nm (10 nm bandpass) were used. The fluorescence data was fit to a single exponential as described above (15-17) with observed reaction constants derived from fitting of 4-7 reaction traces averaged for each set of reaction conditions. Exponential traces derived from Perkin-Elmer experiments were analyzed by sampling of τs at τintervals of 2τ, 5τ, and 10τ, and the fluorescence data (time, and the endpoint fluorescence, respectively) to obtain τs. Rates constants were measured in the presence of heparin by the procedure described in [26] using a stopped-flow process.

### DATA ANALYSIS
- All data were expressed as means ± SEM. The data were analyzed by using a Student's t-test for unpaired samples. The data were analyzed by using a Student's t-test for unpaired samples.

### REFERENCES

6. P.E. Bock, P.A. Craig, S.T. Olson, and P. Singh, submitted for publication to Arch. Biochem. Biophys.
Heparin-Antithrombin-Factor X Transient Kinetics

**FIGURE 1** CHARACTERIZATION OF p-AMINOBENZAMIDINE BINDING TO FACTOR X. A. Emission spectra ($\lambda_{em}$ 330 nm) of free p-aminobenzamidine (dotted line) and p-aminobenzamidine bound to the factor X active-site (solid line) were obtained as described in Materials and Methods and normalized to the same concentration. B. Active-site binding of p-aminobenzamidine to factor X, analyzed from the enhancement of p-aminobenzamidine fluorescence accompanying the inhibition of 0.5 nM factor X with the probe as described in Materials and Methods after subtraction of the enhanced fluorescence observed upon inhibition of 0.5 nM active-site blocked factor X with probe. The solid line shows the nonlinear least-squares fit to a single binding site model. C. p-aminobenzamidine inhibition of the initial rate of hydrolysis of 49 nM (○), 98 nM (●), or 196 nM (▲) Spectrozyme FXa by 5 nM factor X was analyzed according to Dixon (32). Solid lines represent a global nonlinear least-squares fit of the data.

**FIGURE 2** COMPETITIVE EFFECT OF p-AMINOBENZAMIDINE ON THE ANTIITHROMBIN/FACTOR X REACTION. Reaction of 0.5 nM factor X with 5 nM antithrombin in the presence of varying concentrations of p-aminobenzamidine was monitored by p-aminobenzamidine displacement and pseudo-first order rate constants determined as described in Materials and Methods. The solid line is a nonlinear least-squares fit to the equation $k_{obs} = k/(K_{AT} + [AT]) + (1 + [AT]K_{AT}p)$ with $k$, $K_{AT}$, and $K_{AT}p$ the fitted parameters (19).