The Kinetics of Inhibition of Thrombin by Antithrombin in the Presence of Components of the Hemostatic System

By Jolyon Jesty

The inhibition of human thrombin by antithrombin has been measured in pure systems in the presence of other components of the hemostatic system that might affect the kinetics of the reaction. These included fibrinogen, calcium ions, phospholipid, prothrombin, platelets (both adenosine diphosphate [ADP]-stimulated and -unstimulated), and platelet extracts. Inhibition rates were measured in each case by a discontinuous amidolytic assay over a range of antithrombin concentrations, from 0 to 4.5 μmol/L. Under all conditions, rates of inhibition were proportional to antithrombin concentration. Calcium ions at 5 mmol/L caused a small (20%) reduction in rate, but phospholipid and prothrombin had no additional effect. In contrast, both fibrinogen and platelets significantly changed the rate of inhibition. In the presence of calcium, fibrinogen at concentrations from 0 to 12 μmol/L reduced the rate of inhibition in a competitive manner, giving an apparent Kₐ for fibrinogen of 6.0 μmol/L. As the plasma fibrinogen level is about 8 μmol/L, one may therefore predict that variations in fibrinogen level will have a significant effect on the rate of thrombin inhibition in plasma. More unexpected was the observation that platelets increase the rate of inhibition: unstimulated platelets increased the rate constant by 40%, and ADP-stimulated platelets increased it by 55%. However, this acceleratory effect could not be mimicked with either a KCl extract or a Triton extract of platelets, and its cause remains unknown. In sum, it has been shown that the rate of inhibition of thrombin can be modulated in at least three ways—antithrombin concentration, fibrinogen concentration, and platelets; each of which can vary independently in vivo. It is well known that defects of the first lead to an increased risk of thrombosis, and it is proposed that this may be substantially caused by changes in the kinetics of inhibition such as those described. Additionally, it is suggested that changes in inhibition rate caused by other components may also be significant, for the same reason, in modulating the clotting system in vivo.

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Supported in part by National Institutes of Health (NIH) Grant No. HL 21113. During part of this work, the author was the recipient of NIH Research Career Development Award No. KO4 HL 00477.

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that may affect the kinetics of inhibition in a less specific way.
The present paper approaches some of these questions, and
describes studies of the inhibition of thrombin in a plasma-
and heparin-free system, and the effect of Ca\(^{2+}\) ions,
phospholipid, prothrombin, platelets, and fibrinogen on the
kinetics of inhibition. In two of these cases—platelets and
fibrinogen—a significant effect is indeed observed.

MATERIALS AND METHODS

Hydroxyethylpiperazineethanesulfonic acid (HEPES) and morpholino-
ethanesulfonic acid (MES) were from Calbiochem-Behring, La
Jolla, Calif. Bovine serum albumin [BSA] (fatty acid-free), Lubrol-
PX, Triton X-100, N-ethylmaleimide, and apyrase (grade 5 from
potato) were products of Sigma Chemical Co, St Louis. The
thrombin substrate Chromozym-TM (tosyl-Gly-Pro-Arg-p-nitroan-
ilide) was from Boehringer Mannheim, Indianapolis. Phosphatidyl-
seline (PS) and phosphatidylycholine (PC) were products of Supelco,
Belafonte, Pa, and were prepared as a sonicated equimolar mixture
at a stock concentration of 2 mg/mL in 0.1 mol/L NaCl/0.05 mol/L
Tris/HCl, pH 7.5. CM-Sephadex C50 is a product of Pharmacia,
Piscataway, NJ. Other reagents used were at least analytical grade,
from various suppliers.

The standard buffer used throughout the study was a further
modification of the HEPES-modified Ca\(^{2+}\)-free Tyrode’s solution of
Peerschke et al,\(^{18}\) containing 0.1% BSA/0.1% glucose/117 mmol/L
NaCl/2.7 mmol/L KCl/0.47 mmol/L NaH\(_2\)PO\(_4\)/1 mmol/L
MgCl\(_2\)/50 mmol/L HEPES/NaOH, pH 7.4. It is referred to as
high-HEPES buffer (HHB). In experiments involving unstimulated
platelets, this buffer was supplemented with apyrase (0.1 mg/mL).

Fibrinogen. Fibrinogen (-95% clottable) was from Kabi (Hel-
ena Laboratories, Beaumont, Tex). A stock solution of 25 mg
of protein per milliliter was prepared in HHB lacking BSA and
dialyzed twice against 100 vol of the same buffer. The concentration
was then adjusted to 20 mg/mL (\(A_{\text{280}} = 1.36\)).

Platelets. Platelets were prepared as follows: Blood (40 mL)
collected into 0.4 mL 40% sodium citrate and 0.2 mL 0.1 mol/L
sodium EDTA, pH 6.5, was centrifuged for 45 minutes at 650 g.
The supernatant platelet-rich plasma was carefully titrated to pH
6.5 with 1 mol/L acetic acid, and then centrifuged at 1,500 g for
ten minutes. The platelets were then washed twice with a solution
containing 0.1% BSA and 2 mg/mL apyrase in 5 mmol/L MES/0.15
mol/L NaCl/0.1 mmol/L EDTA/NaOH, pH 6.5; the platelet pellet
was suspended in ~0.5 mL of wash solution, diluted to 25 mL
with same, and then centrifuged. The resulting washed platelets
were suspended in 2 mL of wash solution, counted with an electronic
counter (Particle Data, Elmhurst, Ill), and finally adjusted to a
count of \(10^8\) per microliter with the same solution.

Platelet extracts. A KCl extract of platelets was a generous gift
of Efstatia Kalomiris of this department, and was made by
extraction (3 mol/L KCl) of 10 units of outdated platelets. By gel
electrophoresis and periodic acid-Schiff (PAS) staining, this mate-
rial contained glycolcacin at a concentration at least equivalent
to the concentration of glycolcacin/glycoprotein Ib in whole platelets
at \(5 \times 10^9\) per microliter. The Triton X-100 extract of platelets
was prepared as follows: Platelets from 100 mL of freshly drawn blood
were washed as described above, except that the wash buffer
contained 5 mmol/L N-ethylmaleimide and 10 mmol/L EDTA.
The washed platelets were centrifuged and suspended in 1 mL of wash
buffer containing 1% Triton X-100, rocked for one hour at 4°C, and
then centrifuged for 30 minutes at 90,000 g at 4°C in a Beckman
Airfuge (Irvine, Calif). Both platelet extracts were used in inhibition
experiments at a concentration of 10% (vol/vol).

\(a\)-Thrombin. Human \(a\)-thrombin was prepared from citrated
plasma by a modification of the method of Fenton et al,\(^{17}\) using
chromatography on CM-Sephadex C50. The specific activity of this
preparation is 3,500 NIH U/mg, and it exceeds 95% activity by
active-site titration with \(p\)-nitrophenylglycinodibenzolate.

Antithrombin. Human antithrombin was prepared from bar-
ium-citrate-adsorbed plasma by the method of Jesty,\(^{18}\) using hepa-
rin–agarose prepared by the method of Miller-Andersson et al,\(^{18}\)
followed by chromatography on diethyiaminoethyl (DEAE)–
Sephadex to remove traces of heparin. It was measured by absor-
bancy \(-A_{\text{280}} = 5.7\).\(^{19}\)

Amidolytic assay of thrombin inhibition. The assay of thrombin
inhibition was done by a discontinuous two-stage assay. In the first
stage, thrombin was incubated under the appropriate conditions, and
samples were then removed at timed intervals into the second
stage—amidolytic assay of remaining free thrombin in the spectro-
photometer. The first stage was done at 37°C in HHB buffer, pH
7.4. Samples (0.1 mL) were removed into polystyrene cuvettes
containing 0.1 mL 1 mmol/L Chromozym-TH and 0.2 mL of a
solution containing 0.1 mol/L NaCl/0.05 mol/L Tris/0.01 mol/L
EDTA/NaOH/2% Lubrol-PX, pH 7.5. The detergent, Lubrol-PX,
was essential in all experiments involving measurement of inhibition
in the presence of platelets (see Results). Although it causes a
significant reduction in the sensitivity of the thrombin assay, it does
not affect the stability of the thrombin–antithrombin complex; rates
of inhibition in simple mixtures containing only thrombin and
antithrombin were measured with and without Lubrol in the cuvette
and were found to be identical.

To obtain inhibition rates, the amidolytic rates in the timed
samples were fitted in unweighted fashion to an exponential decay to
obtain the pseudo-first-order rate constant. Because first-order
kinetics does not require knowledge of the unitage, amidolytic
activities were not converted to absolute thrombin activities before
filing. Apparent second-order rate constants were obtained by
unweighted linear regression from plots of first-order rate against
antithrombin concentration. The hyperbola described in the text was
fitted by weighted nonlinear regression. Where standard deviations
are given, these were derived from analysis of variance of the
regression procedure,\(^{20}\) but it should be pointed out that, in general,
such estimates of SD are somewhat optimistic.

RESULTS

The general approach to the question of the effect of other
components on the inhibition of thrombin by antithrombin
was the determination of the second-order rate constant in
each case, and the use of that parameter to define the kinetics
of inhibition. In all cases except one (Triton platelet
extract—see below), the first-order rate was proportional to
inhibitor concentration over the range of antithrombin con-
centrations used (0 to 4.5 mmol/L), ie, there was no evidence
of complications arising from more complex kinetics, such as
saturating behavior caused by a rapid reversible first step.\(^6\)

Effect of Ca\(^{2+}\), phospholipid, and prothrombin. The
experimental method used in these studies is shown by
consideration of the first conditions studied—inhibition in
the absence of Ca\(^{2+}\) or other possible effectors. Figure 1
shows a typical set of inhibition curves plotted as first-order
decays directly from the amidolytic rates. It is clear that over
the time scales used, there is no significant deviation from
first-order behavior, such as might be apparent if inhibition
were approaching equilibrium. The rate constants obtained
from the lines in Fig 1, and from a second identical set of
 experiments, are plotted as a function of antithrombin concentration in Fig 2 (line A). It is clear from the linearity (P > .5) and from the near-zero value of the fitted intercept (0.035 ± 0.029 min⁻¹) that inhibition under these conditions is characterized by simple second-order behavior. The apparent second-order rate constant is $4.6 \pm 0.1 \times 10^3$ (mol/L⁻¹) · min⁻¹. (This and other rate constants are collected in Table I.)

Line B in Fig 2 shows the inhibition of thrombin in duplicate experiments in the presence of 5 mmol/L CaCl₂. Second-order behavior is again seen, giving an apparent rate constant of $3.8 \pm 0.2 \times 10^3$ (mol/L⁻¹) · min⁻¹. Given the error limits (for estimates of SD, see Materials and Methods), this difference is probably significant, showing a reduction in the rate of inhibition in the presence of 5 mmol/L Ca²⁺ of nearly 20%. Figure 2 also shows the effect of a relatively low level of negatively charged phospholipid (equimolar PS:PC at 20 μg/mL) on inhibition in the presence of 5 mmol/L Ca²⁺ (line C). By comparison with the rate with Ca²⁺ alone, phospholipid causes a small but insignificant increase in the apparent second-order rate constant to a value of $4.1 \pm 0.1 \times 10^3$ (mol/L⁻¹) · min⁻¹.

The final line in Fig 2 (D) concerns prothrombin. From the work of many investigators, it is clear that prothrombin is, under certain conditions, a major substrate of thrombin, the products of the reaction being prothrombin I and fragment I. Prothrombin, in the presence of Ca²⁺ and phospholipid, was therefore included in the inhibition incubation at a concentration of 0.2 mg/mL (prothrombin in human plasma is about 0.12 mg/mL). As can be seen from comparison of lines C and D, prothrombin has no significant effect at this concentration, the rate constant being $4.0 \pm 0.1 \times 10^3$ (mol/L⁻¹) · min⁻¹.

Effect of fibrinogen on inhibition. The major substrate of thrombin in plasma is fibrinogen, but the study of its effect on the inhibition of thrombin clearly requires care to ensure that fibrin production does not interfere with the assay of inhibition. This was surmountable because the chromogenic substrates now available for thrombin possess sufficient sensitivity at levels of thrombin where fibrin production is minimal. The concentration used in these experiments was 0.54 nmol/L (20 ng/mL, 0.07 U/mL), and the time courses followed were limited to two minutes. It was shown that not only was there no clot formation in the first incubation stage

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**Fig 1.** Assay of thrombin inhibition. Thrombin (0.54 nmol/L) was incubated at 37°C in HHB, pH 7.4, with antithrombin at 0 (C), 0.89 (Θ), 1.79 (□), 2.68 (■), 3.57 (Δ), and 4.46 (▲) μmol/L. At the times indicated, 0.1-ml samples were removed into cuvettes containing 0.1 ml Chromozym-TH and 0.2 ml 2% Lubrol-PX/0.1 mol/L NaCl/0.05 mol/L Tris/0.01 mol/L EDTA/HCl, pH 7.5. Enzyme activities in each sample, measured as rates of absorbance change at 405 nm (arbitrary units; see Materials and Methods) are shown (points), and were fitted to an exponential decay (lines) to obtain the first-order rate constants for inhibition.

**Fig 2.** Effect of Ca²⁺, phospholipid, and prothrombin. Duplicate assays of thrombin inhibition rate were done under each condition as described for Fig 1, and contained (A) no other additions (O); (B) 5 mmol/L CaCl₂; (C) 5 mmol/L CaCl₂ + PS:PC (20 μg/mL); (D) 5 mmol/L CaCl₂ + PS:PC (20 μg/mL) + prothrombin (0.2 mg/mL) (B). The resulting mean first-order rate constants (points) are plotted against antithrombin concentration. Lines were fitted by linear regression.

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**Table 1.** Apparent Second-Order Rate Constants of Thrombin Inhibition by Antithrombin

<table>
<thead>
<tr>
<th>Conditions</th>
<th>$10^4 \times$ Rate Constant ± SD (mol/L⁻¹) · min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>HHB buffer only</td>
<td>4.6 ± 0.1</td>
</tr>
<tr>
<td>HHB + 5 mmol/L CaCl₂</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td>HHB + Ca²⁺ + PS:PC (20 μg/mL)*</td>
<td>4.1 ± 0.1</td>
</tr>
<tr>
<td>HHB + Ca²⁺ + PS:PC + prothrombin (0.2 mg/mL)</td>
<td>4.0 ± 0.1</td>
</tr>
<tr>
<td>HHB + Ca²⁺ + platelets (2 × 10⁵/μL) + ADP (0.1 μmol/L, 2 min)</td>
<td>5.7 ± 0.2</td>
</tr>
<tr>
<td>HHB + Ca²⁺ + platelets + ADP (10 μmol/L, 2 min)</td>
<td>6.3 ± 0.3</td>
</tr>
<tr>
<td>HHB + Ca²⁺ + KCl platelet extract</td>
<td>4.1 ± 0.2</td>
</tr>
<tr>
<td>HHB + Ca²⁺ + Triton X-100 extract buffer</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td>HHB + Ca²⁺ + Triton X-100 platelet extract</td>
<td>2.9 ± 0.1</td>
</tr>
</tbody>
</table>

Rates obtained in the presence of fibrinogen are shown in Fig 4. SD was derived from regression analysis (see Materials and Methods).

*Equimolar phosphatidylsersine/phosphatidylycholine.
under these conditions; there was also none in the second stage in the spectrophotometer cuvettes. This was tested by removing samples from incubations into cuvettes containing the cuvette buffer (with Lubrol) but no p-nitroanilide substrate. Under these conditions, A$_{540}$ remains unchanged over at least three minutes. Only in the presence of substrate was there any absorbency change, and this was linear with time.

As in the determination of rate constants described above, inhibition at each fibrinogen concentration (0 to 12 μmol/L) was measured at six antithrombin concentrations (0 to 4.5 μmol/L) in two duplicate sets. In all cases, Ca$^{2+}$ was present at a concentration of 5 mmol/L. The first-order plots (not shown) remained linear throughout, even at the highest concentrations of fibrinogen. The results of these 60 experiments are shown in Fig 3, and it can be seen that fibrinogen has a significant effect on the rate of inhibition of thrombin by antithrombin.

The simplest model of the effect of an enzyme substrate on the inhibition of an enzyme by an irreversible inhibitor assumes that the substrate behaves as a competitive inhibitor of the enzyme, binding rapidly and reversibly to the active site. The kinetics of enzyme inhibition in this model remain pseudo-first-order and follow this equation:

$$E = E_0e^{-kt} = E_0e^{-k_m(S + K_m)t}$$

where $E_t$ is the enzyme concentration at time $t$, $k$ is the pseudo-first-order rate constant for inhibition, $k_m$ is the apparent Michaelis binding constant for enzyme and substrate, and $S$ is the substrate concentration. Thus, the effect of a substrate is a reduction of the rate of inhibition by the familiar factor $K_m/(S + K_m)$, and a plot of observed rate against substrate concentration should be a hyperbola. The apparent second-order rate constants for inhibition are plotted as a function of fibrinogen concentration in Fig 4, and were fitted to a hyperbola ($P > .5$). The value of apparent $K_m$ obtained by nonlinear regression is 6.0 ± 1.3 μmol/L fibrinogen, and the intercept (the rate constant at 0 substrate) is $4.0 \pm 0.5 \times 10^2$ (mol/L$^{-1}$) · min$^{-1}$. The latter fitted parameter is in excellent agreement with the rate constant obtained previously (Fig 1, line B). These data show that fibrinogen in a pure system at its normal plasma concentration (∼8 μmol/L) causes a significant reduction in the rate of inhibition of thrombin by inhibitors. This is considered further under Discussion.

**Effect of platelets on inhibition.** The marked effect of fibrinogen on the inhibition of thrombin by antithrombin confirmed that competition for the active site can play a role in modifying the kinetics of control of thrombin. Platelets too are subject to the action of thrombin in a process that requires the active site, and platelets—both unstimulated and ADP-stimulated—were thus considered as potential modifiers of the inhibition reaction.

Inhibition of thrombin by antithrombin was measured in a manner similar to that already described. Two features, however, bear further notice. First, because of the necessity to have both thrombin and platelets present, it is likely that the platelets suffered some small stimulation by thrombin (0.07 U/mL) during these experiments. It may be emphasized, however, that this thrombin level is too low for maximal platelet aggregation over the short time courses used, and that in the experiments with unstimulated platelets in the presence of apyrase, aggregation was not seen in the two-minute incubations. Second, it should be noted that the second stage of the inhibition assay—the measurement of thrombin activity in the presence of 1% Lubrol-PX—totally dissolves the platelets and removes the source of any possible interference in the amidolytic assay. Members of the Brij, Tween, and Triton families of nonionic detergents do not work well for this purpose.

Figure 5 shows the second-order plots obtained in the
presence of Ca\(^{2+}\) (1) with no additions; (2) with unstimulated platelets; and (3) with platelets stimulated with 10 \(\mu\)mol/L ADP. As before, each point represents data from two determinations of the first-order rate constant. It is clear that under both sets of conditions, platelets have a significant activating effect on the inhibition of thrombin by antithrombin in the presence of Ca\(^{2+}\). The apparent second-order rate constants obtained were (1) \(3.7 \pm 0.2 \times 10^5\) (mol/L\(^{-1}\)) \cdot \text{min}^{-1}; (2) 5.8 \pm 0.2 \times 10^5 (mol/L\(^{-1}\)) \cdot \text{min}^{-1}; and (3) 6.4 \pm 0.3 \times 10^5 (mol/L\(^{-1}\)) \cdot \text{min}^{-1}. This acceleratory effect of platelets was reproducible over two other similar experiments (data not shown). Although the acceleratory effect of the platelets (lines B and C compared to line A) is significant, given the standard errors, the difference between stimulated and unstimulated is probably not.

The experiments on platelets just described were done at a platelet count of \(2 \times 10^9\) per microliter, which is somewhat lower than the normal level in platelet-rich plasma (~3.5 \(\times 10^9\) per microliter). However, attempts to study inhibition in the presence of higher concentrations of platelets (\(5 \times 10^9\) per microliter) were not very successful; although inhibition rates were measurable, the higher platelet concentration led to increased noise in the chromogenic assay and poorer reproducibility. From the results obtained (data not shown), it seemed that a \(2/3\)-fold increase in platelet count has little further effect upon the rate of inhibition beyond that observed at a count of \(2 \times 10^9\) per microliter. This result, however, is provisional.

**Effect of platelet extracts.** The acceleratory effect of platelets on thrombin inhibition was further examined with two platelet extracts in an attempt to define what platelet component(s) might be responsible. First, a KCl extract of platelets (containing, in particular, very high levels of glycodecalin) was investigated. Although it is difficult to quantify the glycodecalin concentration, it is estimated from PAS staining intensities of electrophoresis gels that the final concentration in the inhibition incubations exceeded the normal platelet glycoprotein I b content by about fivefold. No change in the second-order rate constant for inhibition was observed, however, suggesting that acceleration is not a result of thrombin binding to this material (Fig 5, line D).

A further attempt was made to mimic the acceleratory effect with a Triton extract of fresh platelets. Although 0.1% Triton reduces the rate of inhibition (Fig 5, line E), the platelet extract has no additional effect (Fig 5, line F). It should be noted, however, that the platelet extract interfered quite severely with the amidolytic thrombin assay, causing time-dependent changes in light scattering. This was a particular problem at the higher concentrations of antithrombin, at which the inhibition rate exceeds 1 min\(^{-1}\). These data points were therefore omitted from the determination of \(k_{\text{app}}\) (line F).

Combined results from these studies, excluding the fibrinogen data (Fig 4), are shown in Table I. Of the possible effectors studied, both fibrinogen and platelets had a significant effect on the kinetics of inhibition by antithrombin—the first, a significant reduction in rate, and the second, a smaller increase in rate.

**DISCUSSION**

The present studies have focused on the effect of several components of the hemostatic system on the inhibition of thrombin by antithrombin. Previous studies had already shown the kinetics of this reaction in pure systems to follow second-order behavior over the range of antithrombin concentrations that are found under normal conditions, and the results presented here confirm that this rule still holds when other components are present. The importance of this is considered further below.

**Effect of Ca\(^{2+}\), phospholipid, and prothrombin.** Calcium ions and phospholipid were studied as possible effectors of the inhibition of thrombin because they are major components of the hemostatic system, particularly at sites of vessel damage and the formation of a platelet plug. The Ca\(^{2+}\) concentration used (5 mmol/L) was slightly higher than the estimated free Ca\(^{2+}\) concentration in plasma (~3.5 mmol/L), and had a small but apparently significant effect, reducing the rate of inhibition by some 20% (Fig 2, lines A and B). As might be expected from the fact that thrombin does not have \(\gamma\)-carboxyglutamate residues, negatively charged phospholipid micelles had no additional effect over Ca\(^{2+}\) alone (Fig 2, line C).

Although thrombin does not bind to phospholipid, it is known to act on prothrombin, producing prothrombin I and fragment I. Silverberg\(^{11}\) studied the effect of Ca\(^{2+}\) on this
reaction; although in the absence of calcium ions \( k_{\text{cat}}/K_m \) is \( 4 \times 10^8 \text{ (mol/L)}^{-1} \cdot \text{s}^{-1} \), the rate of cleavage is reduced at least 20-fold in the presence of 1 mmol/L \( \text{Ca}^{2+} \), and more at higher concentrations, at least partly through an effect on \( K_m \). With these data in mind, the lack of effect of prothrombin (200 \( \mu \text{g/mL} \)) on the rate of thrombin inhibition in the presence of \( \text{Ca}^{2+} \) and PS:PC (Fig 2, line D) was unsurprising.

**Effect of fibrinogen.** In contrast with prothrombin, fibrinogen is an excellent substrate of thrombin. In a human system, the \( K_m \) for fibrinopeptide A release under the same conditions of pH and ionic strength as those used here, but at a lower \( \text{Ca}^{2+} \) concentration, is 7.2 \( \mu \text{mol/L} \). In human plasma, the concentration of fibrinogen is normally of the order of 8 \( \mu \text{mol/L} \), and it was therefore expected that fibrinogen at its plasma concentration would have a significant effect on thrombin inhibition by antithrombin. Furthermore, since the cleavage of fibrinogen by thrombin follows Michaelis-Menten kinetics, such an effect should be characterized by competitive behavior, reducing the rate of inhibition by a factor \( K_m/(K_m + S) \), where \( K_m \) is an apparent Michaelis constant. This was indeed found to be the case, and the apparent \( K_m \) obtained from the effect of fibrinogen on inhibition was 6.0 \( \pm 1.3 \mu \text{mol/L} \), in excellent agreement with the \( K_m \) reported.

Although it is well known that values of \( K_m \) for proteolytic reactions are not identical to the true Michaelis binding constant \((C + S = \text{ES-equilibrium})\), particularly when \( k_{\text{cat}}/K_m \) is high, the apparent binding constant \((C + S = \text{ES-complexes})\) should correspond with the \( K_m \) as found in this study. At the normal plasma level of fibrinogen \((\pm 8 \mu \text{mol/L})\), the reduction in inhibition rate is about 55%.

Because thrombin catalyzes other reactions during clotting, similar arguments can be advanced for the effect of such other substrates on thrombin inhibition. Examples are factor V, factor VIII, and protein C, but their effect has not been studied in detail.

**Effect of platelets.** Because platelets are known to be activated by thrombin in a reaction requiring the active site, they too are possible modifiers of the kinetics of thrombin inhibition. Predictions, however, were not so simple as in the case of prothrombin and fibrinogen; although it is clear that active thrombin is needed to activate platelets, the active site is not apparently required for simple binding. In addition to the putative agonist-binding site, which may be glycoprotein V, \(^{12}\) it is well known that platelets contain substantial amounts of two other proteins that interact with thrombin, glycoprotein Ib and coagulation factor V, \(^{12}\) the latter being a thrombin substrate during thrombin-induced platelet activation. In all, it was expected that platelets would cause a reduction in the rate of thrombin inhibition.

Such a reduction, however, was not observed; in fact, a significant increase in rate of about 50% was seen, and this did not seem to depend on whether the platelets were activated. The first possible cause that came to mind was that platelets provide a heparin-like activity, causing an increased rate of antithrombin action. But one may also argue that this is unlikely because platelet factor 4 is probably released to some extent when thrombin at this level is incubated with the platelets, and is almost certainly released during stimulation with ADP.

Because the experimental system required thrombin, it was obviously impossible to study the effect of completely unstimulated platelets on the rate of thrombin inhibition. However, two experimental observations suggest that the effect of thrombin under these conditions is small. First, there was no significant difference between the effect of unstimulated platelets and those stimulated with ADP. Second, in the experiments with unstimulated platelets, there was no evidence of thrombin decay not following first-order kinetics, ie, incubation of thrombin at 0.54 nmol/L with the platelets did not change the rate of inhibition during the two-minute course of the incubations.

**Effect of platelet extracts.** Because it seemed possible that a platelet component(s) might interact with thrombin and/or antithrombin in a heparin-like fashion, two types of platelet extract were investigated to determine whether the acceleratory effect could be mimicked in a soluble system, thus allowing further analysis of the observation. The first, a KCl extract of outdated platelets, represents those membrane proteins that are released from the platelets at high ionic strength, and include glycocalcin as a major component, a fact confirmed by PAS staining of electrophoresis gels. The second was a Triton X-100 extract of fresh platelets made in the presence of calpain inhibitors, and includes most of the total platelet protein, with the exception of material associated with the cytoskeleton. However, in the presence of either platelet extract, the second-order rate constants were identical to those in the respective controls. Other means of duplicating the effect of whole platelets are at present being investigated.

The present study has shown that the inhibition of thrombin by antithrombin in pure systems is subject to significant modulation by some other components of the hemostatic system. Of those studied, the largest effect was observed with fibrinogen; at its normal plasma concentration, the rate of inhibition fell >50%. It should be emphasized, however, that this observation can be quantitatively related to the situation in plasma only with regard to the inhibition of thrombin that has escaped from the site of a clot and is free in solution; the effect of fibrin on thrombin inhibition is not easily studied, although the studies of Liu et al\(^{14}\) suggest that fibrin may offer some protection against inhibition, even though the active site of thrombin is apparently not directly involved in binding.\(^{24,25}\) The present study has not included studies of the effect of fibrin because the experimental system did not permit measurement of total free thrombin levels after clot formation (non-crosslinked fibrin is insoluble in the Lubrol-PC used in the amidolytic assay). One may note, however, that a fibrin clot will usually also contain substantial levels of fibrinogen, which will itself cause protection.

The results of the present study are an early step in quantifying the role of thrombin protection, but it is clear that this is an important facet of control. Clinical data clearly show that effective control of clotting in vivo is tightly linked to the plasma antithrombin concentration. Even at plasma levels as high as 2 to 2.5 \( \mu \text{mol/L} \) (50% normal), antithrombin would seem to be in considerable excess over even the maximum theoretical levels of the target enzymes that could
be generated in the plasma (e.g., prothrombin is ~1.7 μmol/L), let alone the much lower levels that would be generated at a site of thrombus formation. The system, therefore, has ample capacity in terms of the amount of antithrombin available. However, because of the second-order character of inhibition, the 50% reduction in inhibition rate at this inhibitor level will severely compromise the kinetics of enzyme removal, regardless of whether small amounts of thrombin are involved or large amounts. Thus, the thrombotic defects associated with partial antithrombin deficiency may be rooted in essentially a kinetic defect rather than a defect of capacity.

If this is the case, it is reasonable to examine other components of the system that are capable of affecting the kinetics of inhibition, since one may predict that anything that changes the normal kinetics of inhibition may be expected to mimic the effect of changes in antithrombin concentration. Thus, one might expect to find thrombin inhibition significantly accelerated in the fibrinogen-deficient patient, although clinically this would clearly be impossible to assess. Conversely, given the present data on the effect of platelets in a pure system, one may expect thrombocytopenic patients to have slightly compromised inhibition—again, probably not clinically significant. In the general population, however, it is quite easy to imagine situations in which combined small changes in such modulators of inhibition could in sum amount to a significant (~50%) change in the kinetics of inhibition, and perhaps lead to a defect in clinical importance.

Finally, it is important to stress that, although the present studies were done in the absence of heparin, the general conclusion of kinetic control by antithrombin concentration may be expected to hold in the presence of heparin. In all of the various models proposed for the mechanism of action of heparin, the rate of inhibition is still a function of antithrombin concentration.26,27

ACKNOWLEDGMENT

I wish to express my thanks to Ginger Stein for excellent technical assistance in this study, and to Effie Kalomiris for her generous gift of a KCl extract of platelets.

REFERENCES

The kinetics of inhibition of thrombin by antithrombin in the presence of components of the hemostatic system

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