An Automated Approach to Characterize, in Simple Kinetic Terms, the Generation and Inactivation of Thrombin and the Interaction of Fibrinogen with Thrombin

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Here, we used a fully automated, computer-directed centrifugal analyzer (which permitted simultaneous turbidimetry and calculation of results) and purified thrombin, fibrinogen, and various inhibitors to study clot formation. The Km and Vm for these reactions were useful in detecting and partly characterizing anticoagulants. We also explored the generation and inactivation of thrombin, using the two-stage prothrombin time and antithrombin activity tests. The amount of thrombin instantaneously generated and inactivated was monitored under artificially created pathological conditions. The pseudo-first-order rate constant for thrombin generation and inactivation and the instantaneous concentration of enzymatically active and inactive thrombin were used in the characterization of these conditions. We believe this approach is suitable for routine clinical use.

The generation of enzymatically active thrombin (EC 3.4.21.5) and its transformation to an enzymatically inactive form are closely related steps in the complex network of enzymatic reactions that constitute the biochemical mechanism of coagulation. Once enzymatically active thrombin persists in blood, it will interact with fibrinogen to produce clotting.

Our aim here was to develop a simple approach that would enable us to evaluate these processes and how they might be affected in disease. Biggs (1) characterized the generation and concomitant inactivation of thrombin during the two-stage prothrombin time test as a function of two consecutive first-order enzymatic reactions.

In the present work, the concentration of active thrombin at any given instant is determined experimentally, and the inactivated fraction is calculated. The results are expressed as rate constants for generation and inactivation and as concentrations of the enzymatically active and inactive thrombin at any given time during the reaction. We have used these kinetic parameters in an attempt to evaluate how these complex enzymatic interactions may be affected under conditions in which we simulated disease states.

Materials and Methods

Apparatus

For most of this work we used the "Multistat Plus III" centrifugal analyzer (Instrumentation Laboratory, Lexington, MA). In this instrument (2, 3) a 20-cuvette disposable rotor is used, which permits 19 different reactions to be measured simultaneously. The Model 557 double-beam spectrophotometer (Hitachi, Tokyo, Japan) and the Model ICA1 calcium-ion-selective electrode (Radiometer of America, Westlake, OH) were used for determining protein and free calcium.

Software

We developed an algorithm to organize our equations with the spreadsheet "MathPlan" (Word Perfect Corp., Orem, UT). Next, we wrote a computer program for the analyzer, with this algorithm, that permitted on-line calculation of results.

Reagents

Tris-buffered saline (TBS, 50 mmol/L, pH 7.4) was prepared by mixing 5.72 g of Tris HCl and 1.66 g of Tris base with 1 L of isotonic saline. Lyophilized human fibrinogen, 1 g per vial, and S-2238 (H-p-Phe-Pip-Arg-pNA) were purchased from Kabi via Helena Laboratories, Beaumont, TX. Clottable fibrinogen concentration was determined as described by Ratnoff and Menzies (4). Bovine thrombin, 100 NIH units per vial, was purchased from American Dade, Miami, FL. Heparin, 3 USP units/mL, and purified antithrombin III, 54 mg per vial, was purchased from Sigma Chemical Co., St. Louis, MO. Bovine brain thromboplastin was from Ortho Diagnostics, S. Raritan, NJ. Rabbit antobody to human antithrombin III, 0.63 g/L, was from Calbiochem, Behring Diagnostics, La Jolla, CA. Plasma containing 110 mmol of sodium citrate per liter was obtained from our hematology department after all clinical testing had been performed.

Pooled normal plasma was defibrinated (ADFP) with 50 μL Sigma’s "Atroxin" (venom from Barthaops atrox) per milliliter of plasma, which clotted the fibrinogen. After 5 min of incubation at 25 °C, the fibrin clot was removed. The retention of prothrombin in ADFP was verified by conventional clotting and immunological procedures (5, 6).

Procedures

Each cuvette, of the cuvette rotor, has an inner sample chamber and an outer reagent chamber. In each experiment, 0.1 mL of fibrinogen solution was placed in the outer chamber and 0.1 mL of thrombin, or thrombin generated from ADFP, was placed in the inner chamber. A contoured dam separates these chambers, preventing premature mixing.

Once the cuvette rotor has been filled it is placed inside the analyzer, where it is accelerated, mixing the reaction constituents. Reaction measurements are recorded as absorbance vs time. All experiments were performed at 37 °C, 340 nm for 126 s.

When we studied the effects of ADFP, heparin, or S-2238 on the interaction of fibrinogen and thrombin, these inhibitors were incorporated into the outer cuvette chamber. When the effects of plasma deficient in factor II, of anti-antithrombin III, or of antithrombin III on the generation and inactivation of thrombin were explored, these inhibitors were placed in

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the inner cuvette chamber. To study the generation and inactivation of thrombin, we constructed a standard curve of thrombin concentration vs the reciprocal of twice the clotting time. Clotting time was calculated from the change in absorbance as described previously (2).

The rate of thrombin generation was investigated after mixing 50 μL of ADP with 50 μL of thromboplastin. These mixtures were incubated from 0.5 to 10 min at 37 °C. The rate of thrombin inactivation was explored after mixing 50 μL of ADP with 50 μL of purified thrombin. These mixtures were incubated for 0–10 min at 37 °C.

We found the optimal free calcium concentration for our experiments to be between 10 and 60 mmol/L.

**Results**

Figure 1 shows the effect of 10 NIH units of thrombin per milliliter on the absorbance increase produced at various fibrinogen concentrations. Addition of ADP to purified fibrinogen depressed the maximum absorbance at every concentration, but did not affect the linearity of the reaction.

Figure 2 shows the effect on the fibrinogen interaction of thrombin in various concentrations. When the thrombin concentration was between 5 and 20 NIH units/mL, direct proportionality between [FBG] and the maximum absorbance was maintained. Comparative analysis, based on 10 determinations of the data in Figures 1 and 2, produced a CV <3%. Once a sensitive, reproducible methodology was established, we calculated the initial velocity and order of the reaction by measuring the change of substrate concentration, [FBG], as a function of time, with the "MathPlan" spreadsheet.

The input data consisted of (a) the concentration and

![Graph](image1)

**Fig. 1. Effect of fibrinogen concentration on the extent of absorbance increase during clotting**

The open symbols—○, △, and □—represent pure fibrinogen only at 19.1, 9.5, 3.8, and 1.9 mmol/L, respectively. The closed symbols—●, ■, and ▣—represent the addition of ADP, diluted 10-fold, to each of the fibrinogen concentrations listed above. The thrombin concentration was kept constant at 10 NIH units/mL. All dilutions were made with Tris-buffered saline.

![Graph](image2)

**Fig. 2. Effect of thrombin concentration on the rate and extent of absorbance increase during clotting**

Fibrinogen concentration was kept constant at 9.1 μmol/L whereas the thrombin concentration was varied: 10 (□), 5 (○), 1 (△), 0.5 (▲), and 0.2 (●) NIH units/mL. All dilutions were made with Tris-buffered saline.

The absorbance of the fibrinogen standards, (b) the time and absorbance at specific time intervals during the reaction, and (c) the initial [FBG]. To simplify calculations, we introduced the following terms into the spreadsheet:

(a) the velocity of the reaction at any time (t), \( V_t = (S_n - S_{n-1})/(T_n - T_{n-1}) \), where \( S_t \) to \( S_n \) is the substrate concentration at any \( T_t \) to \( T_n \) time;

(b) the average substrate concentration at any \( T_n - T_{n-1} \) interval, \( S_\text{average} = (S_n - S_{n-1})/\ln (S_n - S_{n-1}) \);

and (c) the initial velocity (7) calculated from the equation

\[
V_0 = \{4[(P_3 - P_1)/(T_3 - T_1)] - ([P_4 - P_0]/[T(T_4 - T_0)])\}^{1/4} \tag{1}
\]

where \( P_1 \) to \( P_n \) is the product concentration at any time \( T_1 \) to \( T_n \).

The order of the reaction was obtained from a plot of [FBG] or, alternatively, \( \ln([FBG]/[FBG]_0) \) vs time. We deter-

![Graph](image3)

**Fig. 3. Lineweaver-Burk plots of the thrombin-fibrinogen interaction in the presence of various inhibitors**

Thrombin concentration was kept constant at 10 NIH units/mL whereas the fibrinogen (substrate) concentration was varied in the presence of: no additions (△), 10-fold diluted ADP (○), 10-fold diluted ADP + 1 USP unit heparin per milliliter (■), and 1.875 mmol/L S-2238 (●).
Table 1. Kinetics of Thrombin–Fibrinogen Interaction in the Presence of Inhibitors

<table>
<thead>
<tr>
<th>Inhibitors added</th>
<th>$K_m$ μmol x $V_m$ μmol x $[THB]$^{-1} $\times L^{-1}$</th>
<th>$[THB]$^{-1} $\times min^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None*</td>
<td>7.88</td>
<td>62.00</td>
</tr>
<tr>
<td>ADP, diluted 10-fold</td>
<td>6.85</td>
<td>44.44</td>
</tr>
<tr>
<td>ADP (1:10) + 1 USP unit</td>
<td>5.25</td>
<td>23.52</td>
</tr>
<tr>
<td>S-2236, 1.875 mmol/L</td>
<td>26.53</td>
<td>27.39</td>
</tr>
</tbody>
</table>

*Fibrinogen concentrations, 1.91–45 μmol/L; thrombin concentration, 10 NIH units/mL.

We determined the $K_m$ and $V_m$ of the reactions, with a Lineweaver–Burk plot. Figure 3 and Table 1 show results, in the presence and absence of inhibitors, for such plots. Heparin and ADP significantly decreased the $V_m$ and had only a minor effect on the $K_m$. Addition of S-2236 increased the $K_m$ by twofold and decreased the $V_m$ by threefold.

During the incubation stage of the two-stage prothrombin test, where thromboplastin was mixed with ADP, thrombin was continuously generated and inactivated. To assess the total amount of thrombin generated and inactivated, it is essential to have an approach that determines the active fraction and calculates the inactive fraction of thrombin. If the concentration of active thrombin and the rate of inactivation are known, the concentration of inactivated thrombin can be calculated. We found that with the antithrombin activity test, the pseudo-first-order rate constant for thrombin inactivation could be calculated from the equation

$$k_2 = \frac{1}{t} \ln \frac{[\text{THB}]_0}{[\text{THB}]_t}$$

To ascertain the accuracy of the antithrombin activity test and the relative merit of equation 2, we calculated $k_2$ with purified antithrombin III as the source of antithrombin activity, and these results are shown in Figure 4. When the antithrombin III concentration was increased from 0.3 to 2.66 nmol/L, $k_2$ increased from 0.18 to 0.50. A plot of $k_2$ vs antithrombin III concentration was linear. The slope of this line was equal to the second-order rate constant, $1 \times 10^5$ mol$^{-1}$ min$^{-1}$ (Figure 5). The concentration of inactivated thrombin present during the two-stage prothrombin test, at any time, $t$, was calculated from the equation

$$[\text{THB}]_{\text{inact}} = k_2 \int_0^t [\text{THB}]_{\text{assay}} \cdot dt$$

The total amount of thrombin generated at any time $t$ is the sum of the active and inactive fractions and can be calculated from the equation

$$[\text{THB}]_{\text{total}} = [\text{THB}]_{\text{assay}} + k_2 \int_0^t [\text{THB}]_{\text{assay}} \cdot dt$$

Because the [prot] present at 0 time was the source of both fractions of thrombin, the sum of these two thrombin fractions, $[\text{THB}]_{\text{total}}$, at the end of the reaction is equal to the concentration of prothrombin at 0 time, according to the equation

$$[\text{THB}]_{\text{total}} = [\text{prot}]_0$$

The rate constant for thrombin generation, $k_1$, was calculated from the equation

$$k_1 = \frac{1}{t} \ln \frac{[\text{prot}]_0}{[\text{prot}]_t} = \frac{1}{t} \ln \frac{[\text{THB}]_{\text{total}} - [\text{THB}]_0}{[\text{THB}]_{\text{total}} - [\text{THB}]_0}$$

To calculate $[\text{THB}]_{\text{inact}}$, $[\text{THB}]_{\text{total}}$, $k_1$, and $k_2$ automatically, we introduced equations 2 through 6 into MathPlan and prepared a second worksheet. The experimental data needed for the calculations were the thrombin activity at 0 time and at $t$ time during the antithrombin activity test and during the two-stage prothrombin time test.

To explore the potential clinical usefulness of these calculations, we simulated disease conditions, using mixtures where the concentrations of prothrombin and antithrombin were varied independently. A 1:2 and a 1:4 mixture of normal and factor-II-deficient ADP(s) decreased [prot] without significantly changing the antithrombin concentration. The addition of pure antithrombin III or its removal by a monospecific antibody yielded mixtures in which the antithrombin III was increased or decreased, respectively, without significantly altering [prot]. Table 2 summarizes the results obtained from these simulations. The most conspicuous difference seen between the normal and prothrombin-poor ADP(s) was the amount of total thrombin generated (Table 2, c and d). The rate of thrombin inactivation was increased in the mixture enriched with antithrombin III (Table 2e); it was decreased when the antithrombin III was removed (Table 2f).

Fig. 4. Pseudo-first-order rate constant of the thrombin–antithrombin III interaction

Thrombin and fibrinogen concentrations were kept constant (5 NIH units/mL and 4.7 μmol/L, respectively), whereas the concentration of antithrombin III was varied: 0.035 (□), 0.070 (●), 1.4 (△), 2.8 (○) mmol/L.

Fig. 5. Calculation of the second-order rate constant

The pseudo-first-order rate constant was calculated from the data shown in Figure 4, $k$(min$^{-1}$), and plotted vs antithrombin III concentration.
Table 2. Effect of Artificially Created Pathological Conditions on Thrombin Generation and Inactivation

<table>
<thead>
<tr>
<th>Mixture</th>
<th>$k_1 \times \text{min}^{-1}$ for thrombin generation</th>
<th>$k_2 \times \text{min}^{-1}$ for thrombin inact.</th>
<th>Detd. by</th>
<th>Incubation time, min</th>
<th>Detd. by</th>
<th>[THB]<em>{final} = [\text{prot}]</em>{\text{a}}^a \times \text{min}^{-1}</th>
<th>[THB]<em>{inact} = [\text{prot}]</em>{\text{a}}^a \times \text{min}^{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADFP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c</td>
<td>1.14</td>
<td>0.27</td>
<td>Assay</td>
<td>1.0</td>
<td>3.0</td>
<td>4.5</td>
<td>3</td>
</tr>
<tr>
<td>d</td>
<td>0.81</td>
<td>0.27</td>
<td>Assay</td>
<td>1.0</td>
<td>3.0</td>
<td>4.5</td>
<td>3</td>
</tr>
<tr>
<td>e</td>
<td>0.70</td>
<td>0.25</td>
<td>Assay</td>
<td>1.0</td>
<td>3.0</td>
<td>4.5</td>
<td>3</td>
</tr>
<tr>
<td>f</td>
<td>2.04</td>
<td>1.14</td>
<td>Assay</td>
<td>1.0</td>
<td>3.0</td>
<td>4.5</td>
<td>3</td>
</tr>
<tr>
<td>g</td>
<td>2.01</td>
<td>0.21</td>
<td>Assay</td>
<td>1.0</td>
<td>3.0</td>
<td>4.5</td>
<td>3</td>
</tr>
</tbody>
</table>

*Thrombin, NIH units/mL.

1. Calculated with equation 4.

2. Normal ADFP + an equal volume of ADFP prepared from FII-deficient plasma.

3. Normal ADFP + three times the volume of ADFP prepared from FII-deficient plasma.

4. Normal ADFP + purified antithrombin III.

5. Normal ADFP mixed with antibody prepared against antithrombin III.

Discussion

Previous workers (8–12) showed that the extent of abortus increase during clotting is a reliable measure of fibrinogen concentration. However, the presence of ADFP causes proteins entrapped in the fibrin network to change the scattering properties of these polymers. The colored particles introduced by ADFP absorbed light, but the lipid particles scattered light. We compensated for these nonspecific, optics-affecting events by adding ADFP, derived from the patient’s plasma, to the fibrinogen solution used to prepare the standard curves. Under these conditions, our results were both accurate and reproducible.

To test accuracy, we compared our kinetic constants obtained in this research with those published by others. We found the $K_m$ for the thrombin–fibrinogen interaction to be $7.8 \times 10^{-6}$ mol/L, well within the reported (13–18) range, 1.79 $\times 10^{-6}$ to $3.3 \times 10^{-6}$ mol/L.

With respect to the thrombin–antithrombin interaction, we obtained pseudo-first- and second-order rate constants of $0.20 \times \text{min}^{-1}$ and $1.3 \times 10^6 \text{mol}^{-1}\text{min}^{-1}$, respectively (Figures 4 and 5). Our pseudo-first-order rate constant was close to those published by Jesty (19, 20), and our second-order rate constant was similar to that reported by Downing et al. (21). This favorable agreement confirmed the quantitative suitability of this approach.

Clinical applicability was evaluated by the following experiments. The thrombin–fibrinogen interaction was measured in the presence of ADFP, ADFP and heparin, or S-2238 (Figure 3 and Table 1) to simulate conditions that could be caused by the presence of coagulation inhibitors such as therapy with heparin. Depression of the $V_m$ provided the measure of inhibition while alteration of $K_m$ or lack of it, was indicative of the mechanism of inhibition. In the presence of absence of heparin, ADFP depressed the $V_m$ but did not alter the $K_m$ indicating a noncompetitive mechanism. In contrast, S-2238 depressed the $V_m$ and significantly increased the $K_m$ (Figure 3 and Table 1). This is consistent with a partly competitive mechanism operating in the presence of a large concentration of inhibitor.

We studied the rate of thrombin generation and inactivation, with the two-stage prothrombin time and antithrombin activity test(s). With mixtures of normal ADFP and prothrombin-deficient ADFP, we simulated a condition of impaired coagulation often seen in severe hepatic disorders or disseminated intravascular coagulation. The results show a decrease in total thrombin generated without any significant change in the rate of its generation or inactivation (Table 2, c and d).

In other mixtures we deprived ADFP of antithrombin with a monospecific antibody, to mimic conditions seen in the use of oral contraceptives, major surgery, disseminated intravascular coagulation, and hereditary antithrombin deficiency. Our results (Table 2f) show that, as compared with normal ADFP, the antithrombin-III-deprived ADFP produced a greater $[\text{THB}]_{inact}/[\text{THB}]_{final}$ ratio during the first 4.5 min and a decreased rate of thrombin inactivation ($k_2$). Conversely, when normal ADFP was enriched with purified antithrombin III, the $[\text{THB}]_{inact}/[\text{THB}]_{final}$ ratio was decreased, and the rate constant of thrombin inactivation ($k_2$) was increased (Table 2e). In each mixture, the total amount of thrombin formed was the same.

In conclusion: we showed that this simple, automated, clot-based approach can be used to evaluate conditions that may impede the normal function of hemostasis. We consider the use of kinetic constants and real concentrations of substrate and product to characterize clinical conditions to be a correct approach.

References

10. Yuji I, Okamoto H, Kanai S, Tamaura J. Faster determination...