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Inhibition of Prothrombinase at Macroscopic Lipid Membranes: Competition between Antithrombin and Prothrombin

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ABSTRACT: The kinetics of inhibition of prothrombinase during prothrombin conversion by antithrombin and antithrombin–heparin complexes was studied in a tubular flow reactor. Prothrombinase was assembled at a macroscopic phospholipid membrane, composed of 25 mol % phosphatidylserine and 75 mol % phosphatidylcholine, deposited on the inner wall of a glass capillary, by perfusion with a factor Xa–factor Va mixture. Measurement of thrombin production allowed estimation of the amount of prothrombinase present at the capillary wall. Perfusion with a mixture of prothrombin and antithrombin or antithrombin–heparin complexes caused a progressive decline of the prothrombinase activity. The rate of inactivation steeply decreased with increasing prothrombin concentrations, indicating competitive inhibition. Analysis of competitive inhibition data requires estimation of the time-dependent substrate concentration, C, near the prothrombin converting surface using earlier developed transport theory [Billy, D., et al. (1995) J. Biol. Chem. 270, 1029–1034]. It appears that the inhibition rate is proportional to the fraction of enzyme, K/(K + C), not occupied by substrate. The value of K of prothrombinase estimated from the dependence of the inhibition rate on the prothrombin concentration (K = 2–3 nM) is in excellent agreement with the value estimated from the substrate conversion rate (K = 3 nM). Therefore inhibition of prothrombinase by antithrombin and antithrombin–heparin complexes is fully competitive with the substrate: prothrombin. Our results show that prothrombinase assembled on macroscopic lipid surfaces by virtue of its low K value is protected for inhibition due to highly effective competition of prothrombin with antithrombin for the active site of factor Xa.

The activation of prothrombin to thrombin is a central reaction of the blood coagulation that finally results in the formation of a hemostatic plug (Jackson & Nemerson, 1980; Mann et al., 1990). Effective activation of prothrombin requires prothrombinase, the complex of the serine protease factor Xa and cofactor Va assembled on a phosphatidylserine-containing phospholipid membrane (Hemker et al., 1967; Nesheim et al., 1979; Rosing et al., 1980). The spatial propagation of the coagulation activation reactions is restricted by the requirement of phosphatidylserine-containing membranes, in vivo presumably provided by activated platelets and vascular lesions (Rosing et al., 1985; Mann et al., 1990; Zwaal et al., 1992, Comfurius et al., 1994). Furthermore, the coagulation is down-regulated in time by several inactivation reactions. The cofactors Va and VIIIa are eliminated by activated protein C (Esmon, 1989) that becomes available during thrombin generation. The extrinsic pathway is inhibited by tissue factor pathway inhibitor (Rapaport, 1989). The serine proteases of the blood coagulation are primarily inhibited by antithrombin (Egeberg, 1965; Abilgaard, 1969). This protein, with a plasma concentration of about 2 μM, forms equimolar complexes with serine proteases. The principal targets of antithrombin are factor Xa and thrombin. Heparin, widely in use as anticoagulant drug, up to 1000-fold enhances the rate of inactivation of these enzymes by antithrombin (Rosenberg & Damus, 1973; Danielsson et al., 1986; Olson, 1988; Craig et al., 1989).

Kinetics of prothrombin conversion by prothrombinase bound to macroscopic phospholipid surfaces has recently been studied in our laboratory both on uniformly accessible surfaces, i.e., the rotating disc, and in the tubular flow reactor (Schoen et al., 1990; Giesen et al., 1991; Willems et al., 1993; Billy et al., 1995). It was shown that analysis of kinetics requires a careful account of mass transfer to the catalytic surface. The most important observation was the huge catalytic efficiency of prothrombinase bound to planar bilayers because of the extremely low value of the Michaelis constant (K = 3 nM), which is nearly two orders lower than found for prothrombinase bound to small vesicles (Rosing et al., 1980; Giesen et al., 1991). This could imply that inhibition of prothrombinase bound to macroscopic lipid membranes by pseudo-substrates like antithrombin can be easily counteracted by the presence of prothrombin. Indeed a recent study demonstrated a nearly complete protection from antithrombin-mediated inhibition by prothrombin (Speijer et al., 1995).

This paper reports a detailed kinetic study on the competitive effect of prothrombin on the inactivation of prothrombinase by antithrombin. Our main goal was elucidate to what extent the protection of prothrombinase could be attributed to competition between prothrombin and antithrombin for the active site of factor Xa. By restriction of our measurements to low densities of prothrombinase at the capillary wall, we could use the earlier developed transport theory to estimate the prothrombin concentration at the catalytic
for antithrombin, as determined by stoichiometric titration with antithrombin (Schoen et al., 1990b), was 35 pmol g⁻¹.

The molar concentration of the heparin with high affinity for antithrombin was from Brand AG (Wertheim, Germany). An XYZ translation table (Isel, Eiterfelt, Germany). An international standard for Heparin (UFH) was a gift from the National Institute for Biological Standards and Control (Potters Bar, U.K.). Its potency was stated as 193 units mg⁻¹. Bovine factor Va, human prothrombin, human factor Xa, and heparin: Tris, tris(hydroxymethyl) aminomethane, EDTA, ethylenediaminetetraacetic acid.

Materials. Glass capillaries with an inner diameter of 0.65 mm and a length of 127 mm (volume of 42 μL) were obtained from Brand AG (Wertheim, Germany). 1,2-Dioleoyl-sn-glycerol-3-phosphatidylcholine (DOPC) and 1,2-dioleoyl-sn-glycerol-3-phosphatidylserine (DOPS) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). S2238, chromogenic substrate for thrombin, was obtained from Chromogenix (Möndal, Sweden). Bovine serum albumin was from Sigma (St. Louis, MO). The 4th International Standard for Heparin (UFH) was a gift from the National Institute for Biological Standards and Control (Potters Bar, U.K.). Its potency was stated as 193 units mg⁻¹. Bovine factor Va, human prothrombin, human factor Xa, and human antithrombin were purified and quantified as described (Lindhout et al., 1982; Schoen et al., 1989).

The Flow Reactor. The glass capillaries were cleaned and rendered hydrophilic as described (Billy et al., 1995). In order to deposit a phospholipid bilayer on the capillary wall, the glass capillary was filled and incubated for 20 min with a suspension of unilamellar phospholipid vesicles (25 mol % DOPS/75 mol % DOPC) in Tris buffer (50 mM Tris-HCl, 175 mM NaCl, pH 7.9). To remove nonbound phospholipid, the capillary was rinsed at a flow rate of 1.2 mL min⁻¹ with Tris buffer containing 3 mM CaCl₂ and 0.5 mg mL⁻¹ bovine serum albumin. The phospholipid-coated capillary was connected to a syringe pump (Harvard Apparatus Co., South Natick, MA). An XYZ translation table (Isel, Eiterfelt, Germany) was used to collect samples from the tip of the flow reactor into disposable cuvettes (Sarstedt, Numbrecht, Germany). All procedures were performed at 37 °C.

Inhibition of Phospholipid-Bound Prothrombinase. All perfusions were performed at 37 °C and pH 7.9 in Tris buffer containing 50 mM Tris-HCl, 175 mM NaCl, 3 mM CaCl₂, and 0.5 mg mL⁻¹ bovine serum albumin. Prothrombinase was assembled at the capillary wall by perfusion with a solution containing 1 mM factor Xa and 1 mM factor Va for 10 min at a flow rate of 30 μL min⁻¹ (wall shear rate 20 s⁻¹). Then the thrombin production was measured by perfusion with a mixture containing 1 mM factor Va and prothrombin. Samples of 60 μL of effluent were collected, each 2 min, into cuvettes containing 500 μL of Tris buffer containing 20 mM EDTA. The amounts of thrombin in the sample were measured spectrophotometrically after addition of 60 μL of the chromogenic substrate S2238 (2.5 mM). The inhibition of prothrombinase was studied by perfusion with a mixture containing antithrombin or antithrombin–heparin, 1 mM factor Va, and various concentrations of prothrombin.

Data Analysis. In a previous study (Billy et al., 1995), we showed that the thrombin production Jثل (pmol min⁻¹)
in a capillary flow reactor containing an amount E (pmol) of prothrombinase can adequately be described by Michaelis–Menten kinetics in terms of kcat (3600 min⁻¹), Km (3 nM), and the prothrombin concentration C₀ (nM) near the lipid membrane at the capillary wall:

\[ J_{ثل} = \frac{k_{cat} E C_0}{(C_0 + K_m)} \]  \hspace{1cm} (1)

The concentration C₀ is lower than the concentration Cₙ in perfusion buffer at the inlet of the capillary due to the limited rate of mass transfer of protein from solution to the capillary wall:

\[ C_0 = C_n - \frac{J_{ثل}}{\Delta} \]  \hspace{1cm} (2)

with \( \Delta \) the mass transfer coefficient (L min⁻¹), which depends on the capillary dimensions, the fluid flow rate, the kinematic viscosity of the fluid, and the diffusion constant of the protein (Willems et al., 1993; Billy et al., 1995). For the experimental conditions used in this study a theoretical value of \( \Delta = 10.8 \mu \text{L min}^{-1} \) can be calculated (Billy et al., 1995). For high densities of prothrombinase on the capillary wall the depletion of prothrombin is nearly complete, i.e., \( C_0 \approx 0 \), and thrombin production becomes entirely transport rate limited \( J_{ثل} = \Delta C_0 \). Using this notion, we determined the value of the mass transfer from measurements of the thrombin production at high surface density of prothrombinase (0.75 fmol), which is attained after a 60 min perfusion with 2 pM factor Xa, 50 pM factor Va, and 50–200 nM prothrombin. This resulted in a value 9.8 ± 0.25 μL min⁻¹ (mean ± SEM, n = 10) for the mass transfer coefficient \( \Delta \).

Equations 1–2 can be used to predict the thrombin production for a given amount \( E \) of prothrombinase and a given prothrombin concentration \( C_n \). Alternatively, one can estimate the amount of prothrombinase present in the capillary from the steady-state thrombin production:

\[ E = \left( \frac{J_{ثل}/k_{cat}}{K_m + C_0} \right) \frac{C_0}{C_n} \]  \hspace{1cm} (3)

The applicability of eqs 2–3 for the assessment of the amount of prothrombinase is limited to low densities, i.e., situations with a thrombin production below 90% of the transport limit. The calculation of \( C_n \), near the transport limit involves large relative errors, because \( J_{ثل}/\Delta \) approaches \( C_n \).

Equations 2–3 were used to measure the decline of prothrombinase bound to the capillary by assessment of the steady-state rate of thrombin production before and after exposure to antithrombin–prothrombin mixtures.

Analysis of competitive inhibition of prothrombinase in the flow reactor is complicated by the depletion of the substrate near the capillary wall. For purely competitive inhibition only the fraction of the enzyme not occupied by the substrate is susceptible to inhibition. According to eq 1 the fraction of enzyme not occupied by substrate depends on the substrate concentration, \( C_n \), near the wall and is equal to \( K_m/(C_0 + K_m) \). The disappearance rate of the enzyme thus is given by

\[ \frac{d}{dt} E = -k_{\text{inh}}JEK_m/(C_0 + K_m) \]  \hspace{1cm} (4)

with \( k_{\text{inh}} \) the second-order rate constant of inhibition of the unprotected enzyme and \( J \) the concentration of the inhibitor. As consequence of the progressive inhibition of the enzyme, the concentration \( C_0 \), however, is not constant but increases.

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1 Abbreviations: DOPS, 1,2-dioleoyl-sn-glycerol-3-phosphatidylserine; DOPC, 1,2-dioleoyl-sn-glycerol-3-phosphatidylcholine; S2238, H-D-phenylalanyl-l-pipecolyl-l-arginine-p-nitroanilide; UFH, unfractionated heparin; Tris, tris(hydroxymethyl) aminomethane, EDTA, ethylenediaminetetraacetic acid.
In time, because a lower thrombin production results in a higher value of $C_0$, according to eq 2. Therefore, the term $k_{inh}K_m/(C_0 + K_m)$ is not constant, and the decay of enzyme is not exponential. Formal integration of eq 4 results in the expression

$$-\ln(E/E_o) = k_{inh}t/\ln(1/t_o) \int_0^t K_m/(C_o(t) + K_m)$$

for the residual enzyme activity $E_r$ after exposure time $t_o$ to the inhibitor. The term in square brackets represents the mean value of the protection factor, $K_m/(C_o + K_m)$, during the inhibition step. Evaluation of this term would require intermediate values of $C_o(t)$, which are difficult to obtain because inhibition measurements only provide values of $E$ and $C_o$ at the start, $t = 0$, and the end, $t = t_o$, of the perfusion with inhibitor. It appears, however, that the approximation $K_m/(C_o(0) + C_o(t_o))/2 + K_m$ of the mean value of the protection factor provides sufficient accuracy (see below). Insertion of this approximation in eq 5 results in the following simple modification of the classical formula for competitive inhibition:

$$-\ln(E/E_o) = k_{inh}t/(1/t_o) \int_0^t K_m/(C_o(t) + C_o(t_o))/2 + K_m)$$

Rearrangement of this equation results in

$$-\ln(E/E_o) = 1/(K_mk_{inh})(C_o(0) + C_o(t_o))/2 + 1/k_{inh}$$

Data thus can be analyzed in a plot of $-\ln(E/E_o)$ versus $[C_o(0) + C_o(t_o))/2$. The validity of the approximation of eq 6 was assessed by numerical simulations of eq 4.

For known values of the rate constants, $\Delta, k_{cat}, K_m$, and $k_{inh}$, and the prothrombinase density $E$, the value of $C_o$ can be calculated using eqs 1-2:

$$C_o = 0.5[C_b - K_m - k_{cat}E/\Delta + \sqrt{(C_b - K_m - k_{cat}E/\Delta)^2 + 4C_bK_m}]$$

Equation 4, therefore, can be solved numerically for given values of the rate constants and a given prothrombinase density $E$, at the start of the exposure to the inhibitor. Such numerical simulations showed that the error in the parameters $K_m$ and $k_{inh}$ caused by the approximation used in eq 6 amounted to less than 4% of the estimated values.

**RESULTS**

**Inhibition of Prothrombin Activation by Antithrombin.** Our experimental approach requires the estimation of $C_o$, the concentration of prothrombin near the capillary wall. This is only possible for thrombin productions below 90% of the transport limit. It was calculated using eqs 1-2 that this condition is satisfied for all values of the prothrombin concentrations in the perfusate if the amount of prothrombinase bound to the capillary remains below 0.09 fmol. We choose to target at a prothrombinase content of 0.08 fmol. This amount of prothrombinase can be assembled by perfusion (30 $\mu$L min$^{-1}$) for 10 min with mixture containing 1 pM factor Xa and 1 nM factor Va.

After this perfusion, the prothrombinase activity in the capillary was measured from the steady-state thrombin production upon perfusion with prothrombin (50 nM) and factor Va (1 nM) for 20 min. Figure 1 shows that the thrombin production reached within 10 min a stable level of 0.27 ± 0.001 pmol min$^{-1}$ (mean ± SD, n = 7). Thus a reproducible amount of 0.08 ± 0.0004 fmol of prothrombinase, calculated according to eqs 2-3, is assembled at the capillary wall. The measured thrombin formation is about 50% of the transport limit, 0.49 pmol min$^{-1}$ at 50 nM prothrombin. Next, the prothrombinase was exposed to antithrombin for various times by continuation of the perfusion with a mixture containing antithrombin (2 $\mu$M), prothrombin (50 nM), and factor Va (1 nM). The thrombin concentration, measured at the outlet of the capillary, dropped to a low level, presumably because most of the thrombin generated is inactivated by antithrombin during the residence time of more than 1 min in the capillary. Finally, residual prothrombinase activity was measured during a second perfusion with prothrombin (50 nM) and factor Va (1 nM). The thrombin generation was restored to a new steady-state level of thrombin production lower than the initial rate of thrombin production. It is apparent that the residual prothrombinase activity decreases with increasing duration of the antithrombin perfusion. Residual thrombin production after 6 min exposure was 90% and decreased to 58% after 40 min exposure. Control experiments showed that in absence of antithrombin the thrombin production remained stable during 50 min (residual production > 99% of the initial steady-state production).

The thrombin production rates presented in Figure 1 permit the quantification of the amount of prothrombinase remaining in the capillary after inhibition using eqs 2-3. Figure 2 shows a plot of the residual prothrombinase activity as function of the inhibition time. An exponential fit to these data results in an apparent first-order rate constant of prothrombinase inhibition $k_{app} = 0.017$ min$^{-1}$, which corresponds to a second-order rate constant of 0.008 × 10$^6$ M$^{-1}$ min$^{-1}$. In contrast, a fit of the model for competitive inhibition to these data, according to eqs 4 and 7, using a fixed value for $K_m = 3$ nM (Billy et al., 1995), resulted in a value $k_{inh} = 0.085 × 10^6$ M$^{-1}$ min$^{-1}$ for the second-order rate constant of inhibition. This 10-fold higher value, which

![Figure 1: Inhibition of prothrombinase activity by antithrombin. Fixed amounts of prothrombinase were assembled on the capillary wall in a perfusion with 1 pM factor Xa and 1 nM factor Va for 10 min. Prothrombinase activity was measured upon perfusion with 50 nM prothrombin and 1 nM factor Va. After 20 min the perfusion mixture was changed to a solution containing 2 $\mu$M antithrombin, 50 nM prothrombin, and 1 nM factor Va, and the perfusion was continued for 6, 10, 16, 20, 30, and 40 min. Following this inhibition step, remaining prothrombinase activity was determined by a second perfusion with 50 nM prothrombin and 1 nM factor Va for 20 min. Volumetric flow of the perfusions was 30 $\mu$L min$^{-1}$ (wall shear rate of 20 s$^{-1}$). Experiments were performed at 37 °C and pH 7.9 in buffer containing 50 mM Tris-HCl, 175 mM NaCl, 0.5 mg mL$^{-1}$ BSA, and 3 mM CaCl$_2$.](image-url)
Antithrombin. In order to obtain quantitative data on the competitive inhibition model (eq 4) with fixed value of $K_m = 3$ nM.

is in excellent agreement with the value $0.085 \times 10^6$ M$^{-1}$ min$^{-1}$ found for inhibition by antithrombin in absence of prothrombin (Speijer et al., 1995), reflects the effective protection of prothrombinase by prothrombin: the prothrombin concentration $C_o$ at the capillary wall, calculated according eq 2, in these experiments varied during the antithrombin perfusion between 23, at the start, and 34 nM, at the end. This corresponds to an average fractional occupation, $C_f/(K_m + C_o)$, of the prothrombinase of about 90%, resulting in a 90% protection of the prothrombinase for inhibition by antithrombin in the model for competitive inhibition, cf. eq 4.

Analysis of Competitive Inhibition of Prothrombinase by Antithrombin. In order to obtain quantitative data on the effect of prothrombin on prothrombinase inhibition in the flow reactor, experiments as shown in Figure 1 were performed for several prothrombin concentrations (0–200 nM). The inhibition step consisted of a perfusion during 10 min with a mixture containing factor Va (1 nM), prothrombin, and antithrombin (0–4 $\mu$M). The initial and residual amount of prothrombinase bound to the capillary, $E_o$ and $E_r$, were quantified by using eqs 2–3 from the initial and final steady-state level of thrombin production, respectively. These experiments unequivocally showed that the inhibition kinetics of prothrombinase depends on the prothrombin concentration present in the perfusion mixture. For 10 nM prothrombin, the apparent second-order rate constant of inhibition was $0.061 \times 10^6$ M$^{-1}$ min$^{-1}$, while at 200 nM prothrombin the apparent second-order rate constant was 30-fold lower ($0.002 \times 10^6$ M$^{-1}$ min$^{-1}$). A fit with the model for competitive inhibition to these data with a fixed $K_m$ value of 3 nM resulted in a value $0.079 \times 10^6$ M$^{-1}$ min$^{-1}$ for the true second-order inhibition constant $k_{inh}$.

The previous results indicate competition between antithrombin and prothrombin for the active site of the prothrombinase complex. In the analysis of these data with the competitive binding model, we, however, used the $K_m$ value as obtained from the kinetics of prothrombin conversion. In order to analyze the data with the model for competitive inhibition and to identify the $K_m$ value independently using eq 6, we performed additional experiments at low prothrombin concentrations (0–30 nM) and an antithrombin concentration of 4 $\mu$M during the 10 min lasting inhibition perfusion. The results are presented in Figure 3 as the plot of $-h_t / \ln(E/E_o)$ versus the average concentration $C_o$ at the capillary wall, cf. eq 6. According to this equation, data should represent a straight line with a slope $1/(K_m k_{inh})$ and an intercept at the vertical axis of $1/k_{inh}$ with $K_m$ the Michaelis constant and $k_{inh}$ the true second-order rate constant of inhibition. The good agreement between data and the model of competitive inhibition in eq 6 is apparent. The best fitting straight line results in values $K_m = 2.5$ nM and $k_{inh} = 0.055 \times 10^6$ M$^{-1}$ min$^{-1}$. A fit of eq 1 to the steady-state thrombin generation measured prior to the inhibition step as function of the prothrombin concentration resulted in a $K_m$ value of 3.2 nM. This excellent agreement between these independently determined $K_m$ values corroborates the validity of the competitive inhibition model.

We further addressed the question whether inhibition of prothrombinase by antithrombin–heparin complexes also is fully competitive. In perfusion experiments the inhibition step was performed with a mixture containing antithrombin (0.4 $\mu$M) and unfractionated heparin (0.05 $\mu$g mL$^{-1}$). The antithrombin concentration was chosen 10-fold lower than in the previous experiments in order to minimize the inhibitory effect of antithrombin alone. Yet, the concentration of 0.4 $\mu$M is sufficiently high to justify the assumption that all heparin is associated with antithrombin (Schoen et al., 1990b). The concentration of heparin–antithrombin complexes is 1.75 nM. The results, presented in Figure 4, show a good agreement between the data and the model of competitive inhibition. The best fitting straight line corresponds to the values $K_m = 2.0$ nM and $k_{inh} = 128 \times 10^6$ M$^{-1}$ min$^{-1}$. Thus, the heparin-dependent inhibition of prothrombinase in the presence of prothrombin can also be described by a simple competition model.

**DISCUSSION**

The present study demonstrates that the nearly complete protection by prothrombin of prothrombinase for inhibition by antithrombin and antithrombin–heparin, reported earlier (Schoen et al., 1991; Speijer et al., 1995), is completely explained by the competition of both proteins for the active site of factor Xa. This finding stresses the significance of the extremely high catalytic efficiency of prothrombinase.
Competitive Inhibition of Prothrombinase

FIGURE 4: Competitive prothrombinase inhibition by antithrombin-heparin complexes. Inhibition experiments were performed as described in the legend of Figure 3 with mixture containing antithrombin (0.4 mM) and heparin (0.05 mg L\(^{-1}\)). This corresponds to a concentration of \(I = 1.75\) nM of heparin-antithrombin complexes and 398 nM antithrombin. Shown is a plot of \(-\ln(1 - K_{inh}/K_m)\) versus the mean prothrombin concentration, \(C_{(0)/2 + C_{yr}}\), at the capillary wall during the inhibition step. The straight line is the best fit of the model (eq 6) to these data with estimated values \(k_{inh} = 128 \times 10^6\) M\(^{-1}\) min\(^{-1}\) and \(K_m = 2.0\) nM.

It is calculated that the antithrombin concentration at the capillary wall is at most 31 nM (<1.5%) below the bulk concentration of 2–4 \(\mu\)M. For the antithrombin–heparin complexes (concentration 1.75 nM) the same reasoning would erroneously suggest a massive depletion. In the steady state, however, no net heparin consumption takes place at the capillary wall as heparin from the antithrombin–heparin–thrombin complexes is released and reassociates with antithrombin. Therefore, thrombin production only results in a small (<7%) at 0.4 \(\mu\)M antithrombin) depletion of antithrombin at the capillary wall.

Limited protective effects of phospholipids and factor Va on the inhibition of factor Xa by antithrombin and antithrombin–heparin have been reported earlier (Marciniak, 1973; Teitel, 1983; Ellis et al., 1986; Barrowcliffe et al., 1987). A somewhat larger reduction of the inhibition rate, namely, 4–8-fold compared to factor Xa, was reported for factor Xa assembled in prothrombinase during thrombin generation (Lindhout et al., 1986; Schoen et al., 1989). These experiments were performed with prothrombinase assembled on small unilamellar vesicles in the presence of 0.5–1.5 \(\mu\)M prothrombin. The limited protection observed in these earlier studies is compatible with competitive inhibition, because of the relatively high value of \(K_m\) (0.1–0.2 \(\mu\)M) found for small vesicles (Rosing et al., 1980; Giesen et al., 1991). Also in clotting plasma the inhibition rate of prothrombinase is significantly lower than that of free factor Xa (Béguin et al., 1988).

Interestingly, the inhibition rate found in the present study for prothrombinase not occupied by prothrombin is comparable to the inhibition rate reported for free factor Xa in the presence of Ca\(^{2+}\). For inhibition by antithrombin, we observed a true second-order rate constant of \(0.06 \times 10^6\) M\(^{-1}\) min\(^{-1}\), which is only 50% lower than the value, 0.12 \(\times 10^6\) M\(^{-1}\) min\(^{-1}\), reported for free factor Xa (Olson, 1992). The value \(k_{inh} = 130 \times 10^6\) M\(^{-1}\) min\(^{-1}\) for antithrombin–heparin (UFH) is even closer to the value \(k_{inh} = 180 \times 10^6\) M\(^{-1}\) min\(^{-1}\) calculated by interpolation from the rate constants \(k_{inh} = 260 \times 10^6\) M\(^{-1}\) min\(^{-1}\) at 1.5 mM CaCl\(_2\) and 120 \(\times 10^6\) M\(^{-1}\) min\(^{-1}\) at 4 mM CaCl\(_2\) found for free factor Xa (Schoen et al., 1992).

Prothrombin activation is a more intricate process than most enzymatic reactions analyzed in studies on competitive inhibition. First, the extremely high catalytic efficiency (\(k_{cat}/K_m = 2 \times 10^{10}\) M\(^{-1}\) s\(^{-1}\)) of prothrombin activation by prothrombinase bound to the macroscopic lipid membrane (Billy et al., 1995) is considerably higher than the diffusion limited [about 2–3 \(\times 10^8\) M\(^{-1}\) s\(^{-1}\) (Giesen et al., 1991)] for solution-mediated substrate supply. By comparison of the kinetics of prothrombin activation on small and large vesicles and on planar membranes in this earlier study, we demonstrated that the value of \(K_m\) decreases with increasing membrane area surrounding the prothrombinase. It was concluded that the fluxes of prothrombin from solution to the membrane and on the membrane toward the prothrombin converting complex, both regulated by the resulting prothrombin concentration in solution near the membrane, govern the kinetics of prothrombin conversion. The membrane acts as a funnel that facilitates the collection of prothrombin that once bound to the membrane is efficiently delivered to the prothrombinase by lateral transport (Giesen et al., 1991). Secondly, processing of prothrombin by prothrombinase involves two successive proteolytic cleavages
(Rosing et al., 1986; Krishnaswamy et al., 1986). Despite the gross simplification associated with the application of the simple Michaelis–Menten model, several studies have shown a good agreement between model and measured activation kinetics both on vesicles (Nesheim et al., 1979; Rosing et al., 1980; Giesen et al., 1991) and on planar bilayers (Giesen et al., 1991; Willems et al., 1993; Billy et al., 1995). The present study extends this observation: identical values for \( K_m \) were obtained from kinetics of prothrombin conversion and from the prothrombin dependent kinetics of prothrombinase inhibition. This confirms the notion that the Michaelis constant reflects the binding of prothrombin to factor Xa in the prothrombinase complex. Comparison of pentasaccharide and UFH revealed that the latter heparin on molecular basis is about 30-fold more effective as an inhibitor of prothrombinase (Speijer et al., 1995). This suggests a role of heparin–prothrombinase interaction in the enhanced inhibition in addition to the pentasaccharide. Despite this conjectured interaction, our data show that inhibition of prothrombinase by heparin–antithrombin complexes is also fully competitive, with a similar value of the Michaelis constant, \( K_m = 2 \text{nM} \), estimated from the inhibition kinetics. The observation that inhibition of prothrombinase by antithrombin is fully competitive with prothrombin conversion is probably physiologically relevant. With a value of the Michaelis constant \( K_m = 3 \text{nM} \) nearly 700–1000-fold below the plasma concentration, our observation indicates a complete protection of prothrombinase during the initial phase of activation of the coagulation cascade.

REFERENCES


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