Prothrombinase is protected from inactivation by tissue factor pathway inhibitor: competition between prothrombin and inhibitor*

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The inhibition of prothrombinase by tissue factor pathway inhibitor (TFPI) has been studied in the presence and absence of prothrombin. The rate constant of association of prothrombinase with full-length TFPI was 2.1×10^7 $M^{-1} \cdot s^{-1}$ and 0.05×10^7 $M^{-1} \cdot s^{-1}$ for the reaction with C-terminus truncated TFPI ($TFPI_{1\text{-}161}$). The rate constant of dissociation was 0.65×10^{-4} s^{-1} in both cases. The rate constant of inhibition of prothrombinase by $TFPI_{1\text{-}161}$ was similar to that of solution-phase factor Xa. In contrast, phospholipids and factor Va enhanced the association rate of the reaction between factor Xa and full-length TFPI by approx. 20-fold. Although TFPI, and in particular the full-length variant of the molecule, is a potent inhibitor of prothrombinase (overall inhibition constant of 3 pM), we also found that prothrombin competed very effectively with TFPI for the active site of factor Xa in the prothrombinase complex. A 50 % reduction of the rate constant of inhibition was measured in the presence of 4 nM prothrombin, i.e. 0.2 % of the plasma concentration of prothrombin. The physiological significance of TFPI as an inhibitor of prothrombinase activity is thus questionable.

INTRODUCTION

Factor Xa-catalysed prothrombin activation is dramatically enhanced by the cofactor factor Va, calcium ions and negatively charged phospholipid surfaces. Factor Va causes a 3000-fold increase in the k_{cat} for thrombin formation and the assembly of the factor Xa–factor Va complex at phospholipid membranes results in a large reduction of the K_m for prothrombin [1,2]. A number of studies have demonstrated that phospholipids and factor Va protect factor Xa from inhibition by antithrombin [3–6]. Recent work from our laboratory [7,8] showed that prothrombinase, assembled on macroscopic lipid surfaces, by virtue of its low K_m value (3 nM) is protected from inhibition by antithrombin and antithrombin–heparin. This is due to the highly effective competition of prothrombin with antithrombin for the active site of factor Xa.

Tissue factor pathway inhibitor (TFPI), a 42 kDa glycoprotein consisting of three tandem Kunitz-type inhibitor domains, a negatively charged N-terminus and a carboxy-terminus that contains a dense cationic region [9], is another major inhibitor of factor Xa. The half-life of factor Xa in the presence of the assumed plasma concentration of TFPI (i.e. 2.5 nM) is about 65 s [10]. In contrast with the inhibition of factor Xa by antithrombin, in which incorporation of factor Xa in the prothrombinase complex resulted in a diminished rate of inhibition, factor Xa inhibition by TFPI was enhanced in the presence of factor Va and phospholipid [11,12]. Huang et al. [11] also tested the ability of TFPI to inhibit prothrombinase in the presence of prothrombin. They concluded that the observed reduction of the initial rate of thrombin generation was in good agreement with their model of inhibition, i.e. an inhibition constant of 2 nM for the initial encounter complex between factor Xa and TFPI and a K_m of 1 μM for prothrombin in the prothrombinase complex. In a recent paper the same group [12] confirmed their earlier finding that the prothrombinase complex is relatively protected from TFPI inhibition when its physiological substrate, prothrombin, is present. Unfortunately, the quantitative kinetics of prothrombinase inhibition, both in the absence and presence of prothrombin, remained unknown.

In the present work, we have examined the kinetics of the reaction between prothrombinase and purified recombinant, full-length (FL) TFPI produced in *Escherichia coli*, and the effect of prothrombin on the rate constant of inhibition. We have previously reported [13] that truncation of TFPI between the second and third Kunitz domains ($TFPI_{1\text{-}161}$) results in a 10-fold reduction in the rate constant of inhibition of factor Xa when phospholipids are present. In order to investigate the contribution of the supposed [13,14] phospholipid binding site of FL-TFPI to the inhibitory action of TFPI on factor Xa in the prothrombinase complex, we conducted the same experiments with $TFPI_{1\text{-}161}$.

MATERIALS AND METHODS

Materials

1,2-Dioleoyl-*sn*-glycero-3-phosphatidylserine (DOPS) and 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, U.S.A.). Small unilamellar vesicles of mixed phospholipids composed of 25 mol % DOPS and 75 mol % DOPC were prepared as described before [15]. S2238, the chromogenic substrate for thrombin, was obtained from Chromogenix (Mölndal, Sweden). BSA was from Sigma (St. Louis, MO, U.S.A.). All reactions were carried out at 37 °C in Tris buffer [50 mM Tris/HCl/175 mM NaCl/3 mM $CaCl_2$/BSA (0.5 mg/ml), pH 7.9].

Proteins

Bovine factor Va was prepared and quantified as described previously [16]. Human prothrombin was purified as described [17] and the molar concentration was determined after complete

Abbreviations used: TFPI, tissue factor pathway inhibitor; $TFPI_{1\text{-}161}$, C-terminus truncated TFPI; FL, full-length; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylserine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine; pNPGB, *p*-nitrophenyl *p*′-guanidinobenzoate hydrochloride.

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activation with *Echis carinatus* venom (Sigma, St. Louis, MO, U.S.A.) by active site titration with *p*-nitrophenyl *p'*-guanidinobenzoate hydrochloride (pNPGB) [18]. Human factor Xa was prepared by activation of purified factor X [19] with the factor X activating protein from Russell's-viper venom (Sigma) and isolated as was described for bovine factor Xa [20]. The molar concentration was determined by active site titration with pNPGB [21]. Recombinant FL-TFPI produced in *E. coli* was supplied by Searle/Chiron (Chesterfield, MO, U.S.A.) and the carboxy-terminal truncated variant of TFPI, lacking the third Kunitz-type domain and the C-terminal region ($TFPI_{1\text{-}161}$), was kindly provided by Dr. Ole Nordfang (Novo Nordisk, Gentofte, Denmark). The molar concentrations of the TFPI preparations were determined by titration with known amounts of factor Xa [10].

Inhibition of prothrombinase activity in the absence of prothrombin

$TFPI_{1\text{-}161}$ (0–50 nM) and FL-TFPI (0–1 nM) were incubated with a mixture containing factor Xa (0.1 pM), factor Va (0.5 nM) and phospholipids (10 μM) in flat-bottomed microwells. After timed intervals, prothrombin (200 nM) and S2238 (0.2 mM) were added to determine the residual prothrombinase activity. The change in absorbance (A) as a function of time (t) was recorded at 405 nm using a thermostatically controlled microplate reader (SLT Lab Instruments, model 340 ATTC). A least-squares fit of the quadratic function $A(t) = A + Bt + Ct^2$ to these data allowed estimation of constant C, which is proportional to the prothrombinase concentration.

Inhibition of prothrombinase activity in the presence of prothrombin

A mixture (300 μl) containing factor Xa (0.66 pM), factor Va (0.66 nM) and phospholipids (13.3 μM) was added to 100 μl of a mixture containing prothrombin in the absence or presence of TFPI. At timed intervals, samples were removed and assayed for thrombin using S2238 as described previously [7].

Recovery of prothrombinase activity

Mixtures of 5 pM factor Xa and 5 nM factor Va and mixtures of 10 pM factor Xa and 10 nM factor Va were incubated with 100 μM phospholipid and either 0.5 nM FL-TFPI or 15 nM $TFPI_{1\text{-}161}$ for 30 min at 37 °C. Aliquots of the incubation mixtures were diluted (10- or 20-fold) in a solution of prothrombin (200 nM) to a final prothrombinase concentration of 0.5 pM. At timed intervals, samples were removed for thrombin measurement.

Analysis of prothrombinase inhibition

Throughout this study we used inhibitor concentrations that were at least 1000-fold higher than the prothrombinase concentration. The inhibition of prothrombinase (PTase) is modelled as a simple bimolecular association reaction:

$$\text{PTase} + \text{TFPI} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{PTase—TFPI}$$

When consumption of inhibitor is neglected, the kinetics of the inhibition of prothrombinase is described by the mono-exponential function:

$$E(t) = E_{eq} + (E_0 - E_{eq})\, e^{-k_{obs} \cdot t} \quad (1)$$

where:

$$E_{eq} = E_0 k_{-1} / (k_{+1}[\text{TFPI}] + k_{-1}) \quad (2)$$

and:

$$k_{obs} = k_{+1}[\text{TFPI}] + k_{-1} \quad (3)$$

where [TFPI] is the initial concentration of TFPI, E_0 the prothrombinase concentration at $t = 0$, E_{eq} the final equilibrium concentration of uninhibited prothrombinase, k_{+1} the rate constant of association and k_{-1} the rate constant of dissociation.

Inhibition of prothrombinase in the presence of prothrombin was analysed from the time course of thrombin generation by a discontinuous chromogenic assay of timed samples. These experiments were performed with prothrombin concentrations ranging from values far below the Michaelis constant, K_m, to values exceeding the K_m. The analysis must therefore account for the consumption of prothrombin during the experiment. The kinetics of thrombin production is given by:

$$d/dt\ T_t = E\, k_{cat}(PT_i - T_t)/(K_m + PT_i - T_t) \quad (4)$$

where PT_i is the initial prothrombin concentration, T_t the thrombin concentration at time t, E the prothrombinase concentration at time t as given by eqn. (1) and K_m the apparent Michaelis constant for prothrombin activation by prothrombinase. The K_m of the reaction determined from initial rates of thrombin formation was 70 nM of prothrombin. Equation (4) was solved numerically and data were analysed by a least-squares fit, adjusting the parameters k_{cat} and k_{obs}, of the numerical solution of eqn. (4) to the experimental data.

The value of k_{-1} in equation (3) can be determined from a plot of k_{obs} versus the TFPI concentration. In our case it appeared that k_{-1} is too low to allow an accurate estimation by this procedure. Therefore we performed experiments in which prothrombinase and TFPI were incubated at a high concentration. Hence virtually all prothrombinase was complexed with TFPI. Then, after dilution into a prothrombin-containing solution, the recovery of prothrombinase activity was followed with time. Under these conditions the recovery of prothrombinase activity, $E(t)$, is given by:

$$E(t) = E_0(1 - e^{-k \cdot t}) \quad (5)$$

with E_0 the total prothrombinase concentration and k the rate constant of dissociation of the prothrombinase–TFPI complex. The experimental data were analysed by fitting the numerical solution of eqn. (4), with $E(t)$ as given by eqn. (5), to the thrombin generation curves.

RESULTS

Initial experiments on prothrombin activation by prothrombinase in the presence of TFPI were performed to find suitable conditions under which to perform the inhibition studies. Figure 1 shows that prothrombinase is much less susceptible to inhibition by FL-TFPI (0.5 nM) in the presence of 100 nM prothrombin than in the presence of 5 nM prothrombin. In the latter case, thrombin formation appeared to be completely inhibited 10 min after the addition of prothrombin and TFPI. In contrast, thrombin formation was only slightly diminished when prothrombinase was incubated with FL-TFPI (0.5 nM) and 100 nM prothrombin. The results of this experiment indicate that prothrombin is able to prevent the inhibition of prothrombinase by TFPI. We made use of this finding by designing experiments in which high prothrombin concentrations (200 nM) were used to measure residual prothrombinase activity in the incubation mixtures of prothrombinase and TFPI.

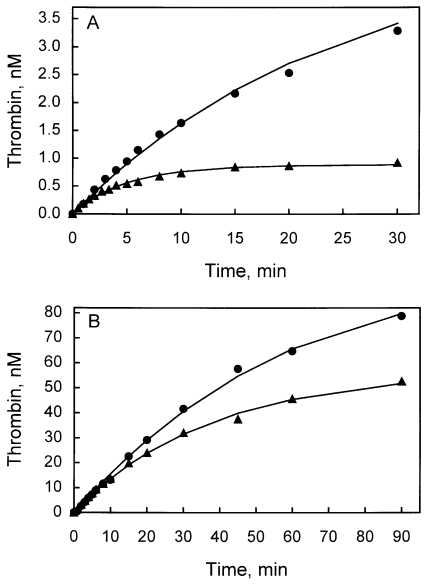

Figure 1 Prothrombin activation in the presence of FL-TFPI

Prothrombin activation was started by the addition of prothrombin (●) and prothrombin and FL-TFPI (▲) to a mixture containing factor Xa (0.5 pM), factor Va (0.5 nM), phospholipid (10 μM) and Ca^{2+} (3 mM) in Tris buffer. The FL-TFPI concentration was 0.5 nM and the prothrombin concentration was 5 nM (**A**) or 100 nM (**B**). Equation (2) was fitted to the data sets to obtain k_{obs} as described under the Materials and methods section.

Prothrombinase inhibition by TFPI in the absence of prothrombin

To obtain the kinetics of inhibition of prothrombinase by TFPI in the absence of prothrombin, prothrombinase was incubated during varying time intervals with various concentrations of FL-TFPI and $TFPI_{1\text{-}161}$. The residual prothrombinase activity was measured as described under the Materials and methods section. Within the time course of the assay (5–10 min) no deviations in the fits were observed, indicating no ongoing TFPI inhibition of prothrombinase. The plots of residual prothrombinase activities versus reaction time with TFPI were analysed to determine the rate constant of inactivation, k_{obs} (eqn. 1, the Materials and methods section).

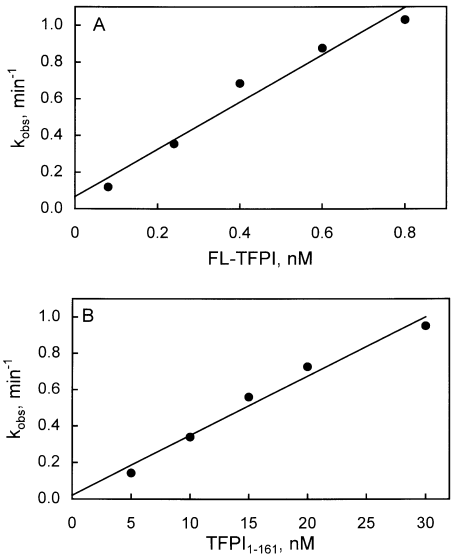

Figure 2 Dependence of the observed rate constants on the concentration of TFPI

Prothrombinase (0.1 pM) was incubated with increasing concentrations of FL-TFPI (**A**) and $TFPI_{1\text{-}161}$ (**B**). Residual prothrombinase activity was determined from the initial rate of thrombin generation after adding 200 nM prothrombin to the incubation mixture as described under the Materials and methods section.

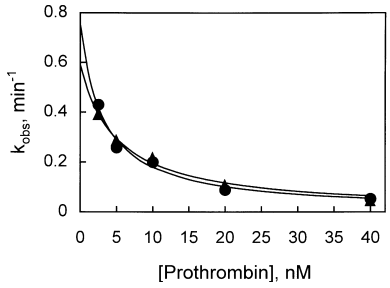

Figure 3 Dependence of the observed rate constants of inhibition on the concentration of prothrombin

The k_{obs} values were inferred from thrombin generation curves obtained from incubations of prothrombinase in the presence of varying amounts of prothrombin and 0.5 nM FL-TFPI (●) and 30 nM $TFPI_{1\text{-}161}$ (▲). The lines represent the best fit of eqn. (6) to the data.

Figure 2 shows k_{obs} as a function of the TFPI concentration. The data were fitted to a straight line yielding a slope ($\pm$S.E.) of $(2.1 \pm 0.2) \times 10^7$ $M^{-1} \cdot s^{-1}$ and $(0.54 \pm 0.05) \times 10^6$ $M^{-1} \cdot s^{-1}$ for FL-TFPI and $TFPI_{1\text{-}161}$ respectively. It is apparent that under the conditions of this experiment, the rate of inactivation in the presence of FL-TFPI is 40-fold faster than in the presence of the C-terminus-truncated TFPI, $TFPI_{1\text{-}161}$. The rate constant of dissociation, k_{-1}, of the prothrombinase–TFPI complex was calculated from the intercept of the line with the vertical axis (eqn. 3). The estimated values ($\pm$S.E.) were $(1.12 \pm 1.15) \times 10^{-3}$ s^{-1} and $(0.38 \pm 0.88) \times 10^{-3}$ s^{-1} for FL-TFPI and $TFPI_{1\text{-}161}$ respectively. The large error in these estimated parameters does not allow a conclusion to be drawn with respect to their significance.

Effect of prothrombin on the kinetics of TFPI inhibition of prothrombinase

Prothrombinase was incubated with varying prothrombin concentrations in the presence of 0.5 nM FL-TFPI or 30 nM $TFPI_{1\text{-}161}$. The thrombin generation curves were analysed according to the model described under the Materials and methods section (eqn. 4). A typical example of such a thrombin generation curve (symbols) and the best fit (line) is shown in Figure 1. The estimated first-order rate constants of inhibition of prothrombinase, k_{obs}, were plotted versus the prothrombin concentration (Figure 3). It is shown that the k_{obs} of the inhibition reaction between prothrombinase and either FL-TFPI or $TFPI_{1\text{-}161}$ decreases dramatically with the prothrombin concentration.

The relationship between the k_{obs} and prothrombin concentration ([PT]) was analysed using a model for competitive inhibition:

$$k_{obs,app} = k_{obs}\, K_i/(K_i + [PT]) \quad (6)$$

with K_i the concentration of prothrombin that results in half-maximal reduction of k_{obs}. Both K_i and k_{obs} (the rate constant in the absence of prothrombin) were estimated by a least-squares fit of equation (6) to the experimentally determined rate constants,

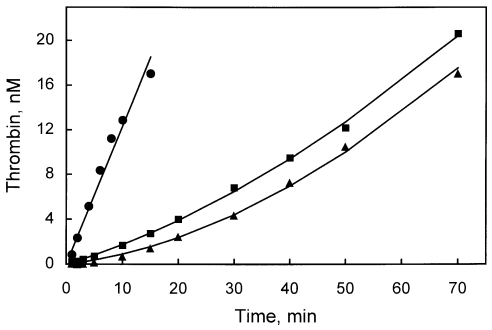

Figure 4 Recovery of prothrombinase activity

Prothrombinase (5 pM) was incubated for 30 min at 37 °C with (●) no TFPI; (▲) 0.5 nM FL-TFPI and (■) 15 nM $TFPI_{1-161}$. The reaction mixture was diluted 10-fold in 200 nM prothrombin. See the Materials and methods section for further details.

$k_{obs,app}$, as a function of the prothrombin concentration. The results of such a fit are indicated by the solid lines in Figure 3. The estimated K_i value was 3.1 ± 0.9 nM (estimate $\pm$ S.E.E.) for FL-TFPI and 4.9 ± 0.9 nM (estimate $\pm$ S.E.E.) for $TFPI_{1-161}$. Thus prothrombin protects prothrombinase from inhibition by FL-TFPI and $TFPI_{1-161}$ equally well. An intriguing finding is the low prothrombin concentration (about 4 nM) at which half-maximal protection was observed. This value is about 20-fold smaller than the apparent K_m (70 nM) of prothrombin activation under these conditions. The true rate constants of inhibition, estimated by the fit procedure, were 0.75 (estimate $\pm$ S.E.E.) 0.16 min^{-1} (estimate $\pm$ S.E.E.) and 0.58 ± 0.05 min^{-1} (estimate $\pm$ S.E.E.) for FL-TFPI (0.5 nM) and $TFPI_{1-161}$ (30 nM) respectively. The respective second-order rate constants are 2.5×10^7 $M^{-1} \cdot s^{-1}$ and 0.32×10^6 $M^{-1} \cdot s^{-1}$. These values are in good agreement with the earlier determined values from direct inhibition studies in the absence of prothrombin, namely 2.1×10^7 $M^{-1} \cdot s^{-1}$ and 0.54×10^6 $M^{-1} \cdot s^{-1}$ for FL-TFPI and $TFPI_{1-161}$ respectively.

Protecting effect of prothrombin and phospholipid concentration

To confirm our notion that TFPI has to compete with prothrombin for the active site of the prothrombinase complex, we performed inhibition experiments in the presence of varying concentrations of phospholipid. Prothrombinase (0.5 pM factor Xa, 0.5 nM factor Va, and 1, 10 or 100 μM phospholipid) was incubated with prothrombin (50 nM) and FL-TFPI (0.5 nM). The thrombin generation curves were analysed to yield the rate constant of inhibition of prothrombinase activity. For the respective phospholipid concentrations, we found rate constants of 0.08 min^{-1}, 0.15 min^{-1} and 0.26 min^{-1}. Thus the observed rate constant of inhibition increased markedly with the phospholipid concentration. This observation is compatible with the notion that when phospholipid vesicles, bearing no prothrombinase, bind prothrombin, the effective prothrombin concentration for competition with TFPI will decrease and thus the rate constant of inactivation will increase.

Dissociation of the prothrombinase–TFPI complex

The observed competition between TFPI and prothrombin for prothrombinase is indicative of the formation of a dissociable prothrombinase–TFPI complex. In an attempt to quantify the rate of dissociation, we diluted preformed prothrombinase–TFPI complexes in a solution containing prothrombin (see the Materials and methods section). Figure 4 shows the thrombin formation in a 10-fold diluted prothrombinase/TFPI reaction mixture. The best fit (line) to eqn. (5) (see the Materials and methods section) is in accordance with an exponential increase in prothrombinase activity with time.

Table 1 Dissociation of the prothrombinase–TFPI complex

Abbreviations: fXa, factor Xa; fVa, factor Va; PSPC, 25 mol% DOPS/75 mol% DOPC.

Prothrombinase	Inhibitor	Conc. (nM)	Dilution (fold)	$10^4 \times k_{-1}$ (s^{-1})
5 pM fXa, 5 nM fVa, 100 μM PSPC	FL-TFPI	0.5	10	0.58
5 pM fXa, 5 nM fVa, 100 μM PSPC	$TFPI_{1-161}$	15	10	0.82
10 pM fXa, 10 nM fVa, 100 μM PSPC	FL-TFPI	0.5	20	0.52
10 pM fXa, 10 nM fVa, 100 μM PSPC	$TFPI_{1-161}$	15	20	0.65

The rate constants of dissociation for FL-TFPI and $TFPI_{1-161}$ complexes with prothrombinase, obtained by the fit procedure, are listed in Table 1. The rate constant of dissociation was approx. 0.6×10^{-4} s^{-1} in all cases. These values are about 3-fold lower than those reported previously for the interaction between factor Xa and TFPI ($k_{-1} = 2.5 \times 10^{-4}$ s^{-1}) [10]. From the second-order rate constants of association (2.1×10^7 $M^{-1} \cdot s^{-1}$ and 0.54×10^6 $M^{-1} \cdot s^{-1}$ for FL-TFPI and $TFPI_{1-161}$ respectively) and a rate constant of dissociation of 0.6×10^{-4} s^{-1} we calculated an overall inhibition constant, $K_i = k_{-1}/k_{+1}$, of 3.5 pM for FL-TFPI and 140 pM for $TFPI_{1-161}$.

DISCUSSION

The reaction between TFPI variants and prothrombinase, the complex of factor Xa and factor Va assembled on a phospholipid membrane composed of 25 mol% DOPS and 75 mol% DOPC, has been examined by kinetic studies of enzyme inhibition both in the absence and the presence of varying concentrations of prothrombin. Inhibition kinetics in the absence of prothrombin were determined from the residual prothrombinase activity as measured with a prothrombin concentration (200 nM) that stopped further inactivation of prothrombinase during the assay. Inhibition kinetics in the presence of prothrombin were derived from the thrombin progress curves.

TFPI inhibition of prothrombinase

Our results indicate that the inhibition kinetics of factor Xa associated with factor Va at a phospholipid surface are comparable to those reported for phospholipid-bound factor Xa in the absence of factor Va. The rate constant of association of FL-TFPI with prothrombinase was 2.1×10^7 $M^{-1} \cdot s^{-1}$, whereas the rate constant reported for the association of the factor Xa–FL-TFPI complex in the presence of phospholipids was between 0.34×10^7 $M^{-1} \cdot s^{-1}$ and 1.6×10^7 $M^{-1} \cdot s^{-1}$ [13,22]. Interestingly, the rate constant of association of $TFPI_{1-161}$ with prothrombinase (0.5×10^6 $M^{-1} \cdot s^{-1}$) is about 40-fold lower than found for FL-TFPI, but comparable to the constant of 0.31×10^6 $M^{-1} \cdot s^{-1}$ reported for the complex between free factor Xa and $TFPI_{1-161}$ [10].

Extending the work of Huang et al. [11], who reported that the kinetics of inhibition of prothrombinase differ from that of free factor Xa, we demonstrate here that this is only the case for FL-TFPI. It is apparent that the simultaneous interaction of the C-terminus of TFPI with factor Xa is involved in the factor Va and phospholipid-dependent rate-enhancing effect. Interestingly,

cofactor-induced alterations have also been suggested for reactions of factor Xa with other inhibitors. The assembly of factor Xa with factor Va was reported to increase the rate of the inhibition in the case of Tick Anticoagulant Peptide [23], but to decrease the rate of inhibition of factor Xa by antithrombin [5,24].

Further analysis of the data (Figure 2) failed to yield quantitative data about the reversibility of complex formation: the estimated values for k_{-1} were too 'noisy'. A set of experiments was therefore performed, in which TFPI-inhibited prothrombinase was incubated with prothrombin (200 nM). The rate constants of dissociation estimated from the thrombin progress curves varied at approx. 0.65×10^{-4} s^{-1} for the two variants of TFPI. The half-life of the complex is thus about 3 h.

Effect of prothrombin on prothrombinase inhibition

Work by Broze and co-workers has indicated that prothrombin makes prothrombinase resistant to inhibition by TFPI [11,12]. We have extended these studies in order to clarify the precise nature of this protecting effect. A single-step reversible model, as described in the Materials and methods section, was fitted to thrombin progress curves, resulting from prothrombin activation in the presence of TFPI. This model is a simplification of a more complex mechanism of inhibition in which factor Xa and TFPI react immediately to form an initial collision complex followed by a slow isomerization to a tightened enzyme–inhibitor complex [11]. However, studies by our group [10] and others [22] have shown that at TFPI concentrations higher than 0.1 nM, the model can indeed be simplified to a single reversible step with enzyme–inhibitor complex as the final product. Excellent fits, as illustrated in Figure 1, indicate that the kinetics of inhibition in the presence of different prothrombin concentrations are indeed adequately described by the assumed model.

From a plot of the observed rate constants of inhibition as a function of the prothrombin concentration (Figure 3) and data analysis according to a simple competition model (eqn. 6), the following conclusions can be drawn: (1) prothrombin protects the inhibition of prothrombinase by FL-TFPI and $TFPI_{1\text{-}161}$ equally well and (2) half-maximal effect was obtained at a prothrombin concentration of about 4 nM, which is far below the plasma concentration of prothrombin (2 μM) but also below the observed apparent K_m value of prothrombin activation (70 nM). We also found that the extent of protection varied with the phospholipid concentration. This observation is compatible with the notion that saturation of prothrombinase with prothrombin requires increasing amounts of prothrombin with increasing amounts of phospholipid vesicles [1,2,25].

At present we have no explanation for the low prothrombin concentration at which half-maximal inhibition is achieved. One expects a half-maximal inhibition at a prothrombin concentration close to the apparent K_m value of 70 nM of prothrombin. Once more, this finding illustrates that the meaning of the K_m value in this case is poorly understood. Interestingly, the observed K_i (approx. 4 nM) is similar to the prothrombin concentration near the phospholipid membrane that is required to achieve half-saturation of prothrombinase when assembled at a planar phospholipid bilayer [7,26]. Moreover, studies of the effect of prothrombin on the inhibition of prothrombinase by antithrombin in a tubular flow reactor demonstrated that prothrombin competed with antithrombin for the active site of factor Xa with a K_i value identical with the true K_m value [8]. It is, therefore, tempting to speculate that the K_i for the reduction of the TFPI-dependent prothrombinase inhibition rate in the presence of prothrombin is also a reflection of the true K_m value for prothrombin activation at the membrane of a small unilamellar phospholipid vesicle.

The results of this study suggest that FL-TFPI is a very potent inhibitor of prothrombinase. The half-life of prothrombinase is 20 s at an assumed plasma concentration of 2 nM for TFPI. However, prothrombin competes very strongly with TFPI for the active site of prothrombinase. The rate of inhibition is 500-fold reduced at the plasma concentration of prothrombin. Thus only when prothrombin becomes severely depleted near the membrane-bound prothrombinase as a result of transport-limited catalysis [27] could one expect an inhibitory effect of TFPI on prothrombin activation. Yet, in that case, inhibition will not result in reduced thrombin formation. The physiological significance of TFPI as an inhibitor of prothrombinase activity is thus questionable.

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