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The inhibition equilibrium and kinetics of association and dissociation of the binding of three types of recombinant tissue factor pathway inhibitor (TFPI), namely full-length TFPI, C-terminal-truncated TFPI, and TFPI without the third Kunitz domain ($TFPI_{1-161}$), to factor Xa have been measured. Formation and dissociation of the complexes were monitored by continuous measurement of the changes in the rate of hydrolysis of a peptidyl-*p*-nitroanilide substrate. Progress curves of product formation were fitted to a set of equations describing a one-step bimolecular inhibitory reaction in the presence of a competing substrate. For full-length TFPI the rate constants of association (k_{on}) and dissociation (k_{off}) were $(5.1 \pm 0.7) \times 10^6$ $M^{-1} \cdot s^{-1}$ and $(2.6 \pm 0.9) \times 10^{-4}$ s^{-1} respectively. Thus, although the inhibition constant (50 pM) is far below the plasma concentration (2.5 nM) of TFPI, the half-time for transition to equilibrium in plasma is rather long (66 s). The truncated forms of TFPI differ in that they have a 4-fold lower k_{on} value but a similar dissociation rate constant. Therefore the inhibition constant, K_i, is 4-fold higher (0.2 nM) and the half-time to achieve equilibrium is prolonged to 250 s. The k_{on} values of full-length and C-terminal-truncated TFPI, but not that of $TFPI_{1-161}$, were found to decrease with increasing ionic strength.

INTRODUCTION

The enzymic machinery involved in the blood-coagulation process is controlled by a number of positive- and negative-feedback systems. As a result, blood coagulation is a system that, on the one hand, responds rapidly to small triggering signals and, on the other hand, is rapidly shut down so that it does not propagate beyond the site of injury. The key steps in the regulation of this process are the activation and inactivation of membrane-bound proteins which function as essential cofactors of membrane-bound enzymes, e.g. activation of factors V and VIII by thrombin and their inactivation by activated protein C (for a review see [1]).

The enzymic complex that consists of the cofactor tissue factor (TF, an integral membrane glycoprotein) and the serine protease factor VIIa is thought to be the major initiator of the blood-coagulation process *in vivo* [2]. The neutralization of the enzymic activity of this complex by tissue factor pathway inhibitor (TFPI), a 42 kDa glycoprotein consisting of three tandem Kunitz-type inhibitor domains, a negatively charged N-terminus and a positively charged C-terminus [3], is assumed to cause an essential shift in the coagulation pathways [4–6]. That is, an initial burst of TF–factor VIIa-dependent factor Xa generation is turned off by TFPI, and is then followed by a maintenance dose of factor Xa produced by the classic 'intrinsic' pathway. Factor Xa seems to play an essential role in shutting off its own generation [7–9], because the inhibition of the TF/VIIa activity by plasma concentrations of TFPI is only significant when factor Xa is also present. The final product of the inhibition reaction is a dissociable quaternary Xa/TFPI/VIIa/TF complex [5].

In order to understand the importance of this regulatory process and the potential therapeutic role of TFPI as an anticoagulant drug, knowledge of the kinetics of the formation of the quaternary complex is essential. It emerged from studies reported so far that the ability of TFPI to inhibit TF/VIIa-induced coagulation seems to depend on the rate of association between factor Xa and TFPI, the first step in the formation of the inactive Xa/TFPI/VIIa/TF complex [10,11]. It has been postulated that TFPI is a slow, tight-binding, competitive and reversible inhibitor of factor Xa [5], but so far no data have been reported on the association and dissociation rate constants. In this paper we report on the rates of association and dissociation of complex-formation between human factor Xa and TFPI. We have examined three variants of recombinant TFPI, namely full-length TFPI, the C-terminal truncated TFPI lacking the highly basic sequence of residues 254–265, and TFPI truncated between the second and third Kunitz domains ($TFPI_{1-161}$). The role of the basic C-terminus in the interaction between factor Xa and TFPI was also examined by varying the ionic strength.

EXPERIMENTAL

Materials

The chromogenic substrate for factor Xa, methoxycarbonyl-D-cyclohexylglycyl-glycyl-arginine *p*-nitroanilide (Spectrozyme FXa) was from Pentapharm (Basel, Switzerland). BSA (fatty-acid-free) was purchased from Sigma (St. Louis, Mo, U.S.A.). All reactions were carried out at 37 °C in 50 mM Tris/HCl, containing 175 mM NaCl and 0.5 mg of BSA/ml, pH 7.9 (TBSA).

Proteins

The human coagulation factor Xa was purified to homogeneity and quantified as previously described [12]. The recombinant TFPI preparations were kindly supplied by Dr. Ole Nordfang (Novo Nordisk, Gentofte, Denmark). Full-length recombinant human TFPI (FL-TFPI) and the C-terminal-truncated variant of TFPI lacking the C-terminal basic region were obtained from transfected baby-hamster kidney cells as described previously [13] and were separated by cation-exchange chromatography [10]. The preparation of truncated TFPI consisted of a mixture

Abbreviations used: TF, tissue factor; TFPI, tissue factor pathway inhibitor; $TFPI_{1-161}$, recombinant C-terminal-truncated TFPI containing residues 1–161; TBSA, 50 mM Tris/HCl (pH 7.9) containing 175 mM NaCl and 0.5 mg of BSA/ml; pNA, *p*-nitroanilide.

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of variants ending at amino acids Ile-247, Ser-248, Gly-250 and Thr-255 [10]. The C-terminal-truncated variant of TFPI lacking the third Kunitz-type domain and the C-terminal region ($TFPI_{1-161}$) was expressed in *Saccharomyces cerevisiae* and purified as described previously [14]. All preparations of TFPI were more than 95% pure, as judged from Coomassie-Blue-staining after SDS/PAGE.

Factor Xa assay

The assay was performed in polystyrene cuvettes at 37 °C in 0.5 ml of TBSA containing 300 μM Spectrozyme FXa. Hydrolysis of the peptidyl *p*-nitroanilide substrate was initiated by addition of factor Xa. The A_{405} was monitored and the amount of product formed was calculated by using an absorption coefficient of 9900 $M^{-1} \cdot cm^{-1}$. The K_m and $k_{cat.}$ values of the reaction were estimated from initial-rate measurements at different substrate concentrations, and were 310 μM and 250 s^{-1} respectively.

Determinations of the TFPI molar concentrations

TFPI (10 nM) was incubated in 470 μl of TBSA with increasing amounts of factor Xa. After 30 min at 37 °C, 30 μl of 5 mM Spectrozyme FXa was added and the residual amount of factor Xa was calculated from its known specific activity towards Spectrozyme FXa. The linear part of the titration curve was extrapolated, and the intercept with the axis representing the total factor Xa concentration was taken as the molar concentration of TFPI in the reaction mixture. It was assumed that factor Xa forms an equimolar complex with TFPI.

Measurement of K_i

Factor Xa was titrated with increasing amounts of TFPI (0–10 nM) in a cuvette with 470 μl of TBSA for 40 min at 37 °C. The residual factor Xa activity was determined from the initial rate of *p*-nitroanilide (pNA) production as described. A series of three such titrations were done for each form of TFPI: 0.75–1.5–3.0 nM factor Xa for $TFPI_{1-161}$, 0.2–0.4–0.8 nM factor Xa for C-terminal-truncated TFPI, and 0.1–0.2–0.5 nM factor Xa for full-length TFPI. The data were expressed as the ratio of inhibited velocities (v_s) to the control velocity in the absence of TFPI (v_0).

Progress curves

Progress curves of pNA production in the presence of factor Xa (0.4 nM) and varying amounts of the different forms of TFPI were conducted in cuvettes at 37 °C in 0.5 ml of TBSA. The hydrolysis of the peptidyl substrate by factor Xa was monitored at 405 nm during 2 min. Then a small sample (1–20 μl) of TFPI was added. The reaction was monitored until factor Xa, TFPI and substrate attained a steady-state equilibrium. Data sampling was performed every 1 s. Progress curves were also obtained by preincubation of factor Xa with different concentrations of TFPI for at least 40 min at 37 °C and starting the reaction by the addition of a portion (50 μl) of the reaction mixture to a cuvette with 450 μl of TBSA containing 300 μM Spectrozyme FXa.

Theory and data analysis

The inhibition of factor Xa (E) by TFPI (I) in the presence of a peptidyl pNA substrate (S) is analysed by using the simplest scheme for competitive inhibition (Scheme 1):

$$\begin{array}{ccccc} E + S & \overset{K_m}{\rightleftharpoons} & ES & \xrightarrow{k_{cat}} & P \\ + & & & & \\ I & & & & \\ k_{on} \uparrow\downarrow k_{off} & & & & \\ EI & & & & \end{array}$$

Scheme 1

It was further assumed that the steady-state equilibrium between factor Xa and the substrate was attained instantaneously.

Equilibrium studies

The inhibitor constant K_i can be calculated from

$$v_s/v_0 = ([E]_0 - [I]_0 - K_i + \sqrt{\{([E]_0 + [I]_0 + K_i)^2 - 4[E]_0[I]_0\}})/2[E]_0 \quad (1)$$

where v_s/v_0 is the ratio of inhibited velocity to the control velocity in the absence of inhibitor and $[E]_0$ and $[I]_0$ are the enzyme and inhibitor concentration respectively [15,16]. The observed v_s/v_0 values of a set of three TFPI titration curves at different fixed amounts of factor Xa were fitted simultaneously by a non-linear least-squares regression algorithm (BMDP Statistical Software Inc., Los Angeles, CA, U.S.A.) to eqn. [1] to obtain an estimate of the inhibitor constant K_i.

Progress curves

Data from progress curves of pNA production in the presence of TFPI were fitted by the non-linear least-squares regression algorithm to the following coupled system of differential equations:

$$d(P)/dt = k_{cat.}([E]_0 - [EI])\{([S]_0 - [P])/(K_m + [S]_0 - [P])\} \quad (2)$$

$$d(EI)/dt = k_{on}^{app.}([E]_0 - [EI])([I]_0 - [EI]) - k_{off}[EI] \quad (3)$$

where

$$k_{on}^{app.} = k_{on} K_m/(S + K_m)$$

with addition of TFPI to the factor Xa–substrate mixture at zero time. Thus the initial conditions are: $[P]_0 = 0$ and $[EI]_0 = 0$.

Data from progress curves in the absence of TFPI were fitted to the following equation:

$$d(P)/dt = k_{cat.}[E]_0(1 - e^{-kt})\{([S]_0 - [P])/(K_m + [S]_0 - [P])\} \quad (4)$$

Since no inhibitor of factor Xa was added, the pseudo-first-order rate constant k reflects non-specific loss of factor Xa activity.

When preincubated mixtures of factor Xa and TFPI were added to a solution containing the substrate for factor Xa, the initial concentration of the factor Xa–TFPI complex (EI) in the cuvette is given by eqn. (5):

$$[EI]_0 = ([E]_0 + [I]_0 + K_i/d - \sqrt{\{([E]_0 + [I]_0 + K_i/d)^2 - 4[E]_0[I]_0\}})/2 \quad (5)$$

where $[E]_0$ and $[I]_0$ are the total concentrations of factor Xa and TFPI in the cuvette and d is the ratio of the final volume of the reaction mixture to the sample volume of the preincubation mixture. The progress curves were fitted to the system of coupled differential equations (2) and (3), with the boundary conditions given by equation (5) and $[P]_0 = 0$. The estimated parameters were $k_{on}^{app.}$ and k_{off}. The value for $k_{cat.}$ was held constant at the independently determined value of 250 s^{-1}.

RESULTS

Inhibition kinetics as derived from progress curves stated with factor Xa

The kinetics of factor Xa–TFPI complex-formation were derived from progress curves of factor Xa-catalysed pNA formation in

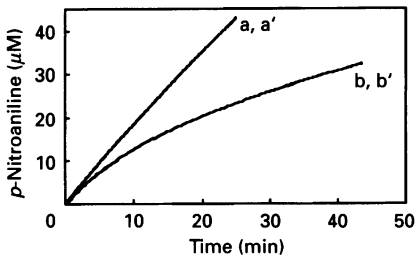

Figure 1 Time course of inhibition of human factor Xa by full-length TFPI

Spectrophotometer tracings showing hydrolysis of peptidyl-pNA substrate (300 μM) by human factor Xa (0.4 nM) in the absence (**a**) or presence (**b**) of full-length TFPI (0.4 nM). Lines **a'** and **b'** are the results of the fitting procedures as described in the Experimental section.

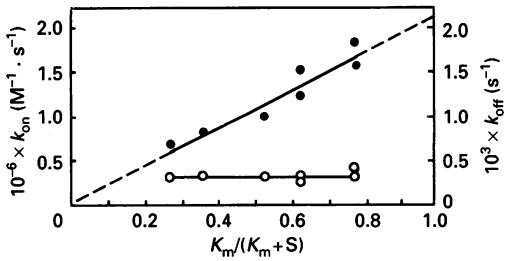

Figure 2 Effect of factor Xa substrate concentration on k_{on} and k_{off}

Progress curves of the hydrolysis of peptidyl-pNA substrate (100–900 μM) by factor Xa (0.4 nM) in the presence of C-terminal-truncated TFPI (1 nM) were analysed as described in the Experimental section to yield apparent k_{on} (●) and apparent k_{off} (○) values.

the presence of TFPI. The factor Xa concentrations and those of the recombinant TFPI variants used in these experiments were chosen such that the progress curves of product formation revealed both the progress of inhibition and the steady-state velocity. Consequently, the TFPI concentration had to be chosen rather close to the factor Xa concentration, causing a significant depletion of free TFPI during the course of the reaction. The rather long duration of the experiments also caused in some cases a significant depletion of substrate. In our analysis, however, we corrected for both the depletion of substrate and free inhibitor (see the Experimental section; cf. eqns. 2 and 3, respectively). These equations cannot be solved in closed form, and therefore a numerical solution was used to analyse the experimental data.

A representative example of a plot of pNA versus time for the factor Xa-catalysed reaction in the absence of TFPI (curve a) is shown in Figure 1. The simulated curve a', obtained by fitting eqns. (2) and (3) to the experimental data, is superimposed on the experimental curve. The residuals, calculated as observed amount of product minus the predicted value, varied around zero and were less than 0.05 μM. The estimated $k_{cat.}$ and k values were 240 s^{-1} and 0.005 min^{-1} respectively. This value of $k_{cat.}$ as estimated from the progress curve is very close to the value (250 s^{-1}) determined from initial-rate experiments. The half-life time of the factor Xa activity is 0.693/0.005 = 150 min, i.e. about 6 times the duration of the experiment. We conclude that the progress curves in the absence of TFPI are adequately described by the model. Thus, factor Xa activity was found to be sufficiently stable, and no signs of product inhibition were observed.

Figure 1 also shows a typical example of a progress curve in the presence of 0.4 nM full-length TFPI (curve b). The simulated curve b', obtained by fitting the experimental data to eqns. (2) and (3), is superimposed on the experimental curve. Again, the residuals of the observed minus simulated data were very small (less than 0.02 μM) and varying around zero. The standard errors of the estimated rate constant of association (k_{on}), the rate constant of dissociation (k_{off}) and the catalytic rate constant of the factor Xa–substrate reaction ($k_{cat.}$) were less than 0.1 % of the value. Thus the progress curves in the presence of TFPI are also adequately described by the model.

We next verified our assumption that the peptidyl substrate competes with TFPI for the active site of factor Xa. Progress curves were performed in the presence of a variety of substrate concentrations (100–900 μM). The reactions were started by the addition of factor Xa (0.25 nM), and after 2 min C-terminal-truncated TFPI (1 nM) was added. The progress curves in the presence of TFPI were fitted to eqns. (2) and (3). Figure 2 shows a replot of the estimated parameters, apparent k_{on} and apparent k_{off}, versus $K_m/(K_m+S)$. It is apparent that k_{off} does not vary with the substrate concentration, whereas the apparent k_{on} value is a linear function of $K_m/(K_m+S)$. Extrapolation to zero substrate concentration gives a true k_{on} of 2.1×10^6 $M^{-1} \cdot s^{-1}$. The mean ± S.D. of the estimated $k_{cat.}$ values was 241 ± 8 s^{-1}. This value closely agreed with the experimental value (250 s^{-1}) as determined from the rates of pNA production at the different substrate concentrations in the absence of TFPI. These data show that the peptidyl substrate indeed competes with TFPI for the active site. The true k_{on} values can be calculated from the observed ones by application of the expression

$$k_{on} = k_{on}^{app.} \cdot (1+S/K_m)$$

Progress curves were then performed in the presence of varying amounts of the three different forms of recombinant TFPI. The k_{on} values corrected for substrate competition and the k_{off} values as obtained by the fit procedure are listed together with their standard errors in Table 1. The second-order rate constant of association of the reaction between full-length TFPI and factor Xa [$(5.08 \pm 0.68) \times 10^6$ $M^{-1} \cdot s^{-1}$] is about 4-fold higher than the rate constant found for the reactions of factor Xa with C-terminal-truncated TFPI and $TFPI_{1-161}$ [$(1.46 \pm 0.08) \times 10^6$ $M^{-1} \cdot s^{-1}$ and $(1.14 \pm 0.15) \times 10^6$ $M^{-1} \cdot s^{-1}$ respectively]. The rate constant of dissociation of the TFPI–factor Xa complex was found to be the same for all forms of recombinant TFPI tested [$(0.26 \pm 0.05) \times 10^{-3}$ s^{-1}]. Progress-curve analysis at a variety of TFPI concentrations yielded similar $k_{cat.}$ values very close to the experimentally determined value of 250 s^{-1} (Table 1). The excellent agreement between model (Scheme 1) and experimental data suggest that the inhibitory reaction of factor Xa with TFPI indeed can be described as a simple bimolecular reaction. However, we have to emphasize that the conditions under which these observations were made might not discriminate between a slow bimolecular reaction and a rapid association to form the E·I complex, followed by a slow unimolecular change to a stable and inactive complex [17].

Inhibition kinetics derived from progress curves started with the peptidyl substrate

To verify the reversibility of the binding of TFPI to factor Xa, rate constants were also derived from progress curves of product formation that were started with the peptidyl substrate. Factor Xa and TFPI were preincubated at concentrations 10 times those used in the progress curves started with factor Xa. Recovery of the factor Xa activity was then continuously monitored after a

Table 1 Kinetic and equilibrium constants for TFPI–factor Xa complex formation from progress curves started with factor Xa

Details of the procedures by which the constants were measured are given in the text.

Inhibitor	Concn. (nM)	$k_{cat.}$ (s^{-1})	$10^{-6} \times k_{on}$ ($M^{-1} \cdot s^{-1}$)	$10^{3} \times k_{off}$ (s^{-1})	$10^{9} \times K_i^*$ (M)
Full-length TFPI	0.2	238.2±0.1	5.51±0.03	0.141±0.018	0.026
	0.4	232.4±0.1	4.33±0.04	0.298±0.003	0.068
	0.6	215.0±0.2	5.40±0.02	0.300±0.001	0.056
Truncated TFPI	0.5	238.3±0.1	1.53±0.01	0.283±0.001	0.18
	1.0	248.0±0.2	1.49±0.01	0.275±0.001	0.18
	1.5	247.5±0.2	1.37±0.01	0.271±0.001	0.19
$TFPI_{1-161}$	0.5	247.6±0.3	1.32±0.01	0.265±0.005	0.20
	1.0	248.1±0.3	1.09±0.01	0.216±0.003	0.20
	2.0	247.3±0.5	1.02±0.01	0.320±0.003	0.32

* Calculated from k_{off}/k_{on}.

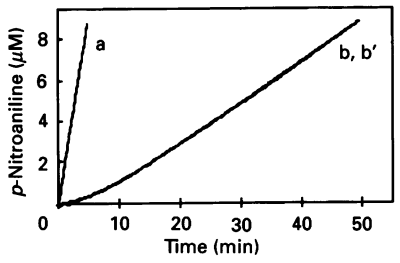

Figure 3 Progress curve of the recovery of factor Xa activity

Spectrophotometer tracings showing hydrolysis of peptidyl-pNA substrate (300 μM) after addition of a sample of human factor Xa (4 nM) incubated for 40 min in the absence (**a**) and presence (**b**) of full-length TFPI (4 nM). The final concentrations in the cuvette were 0.4 nM factor Xa and 0.4 nM TFPI. Line (**b'**) is the result of the fitting procedure as described in the Experimental section.

10-fold dilution of the preincubated mixture into a cuvette containing the peptidyl substrate. A typical example of a progress curve of the recovery of factor Xa activity is shown in Figure 3. The data from the progress curves were fitted to the differential equations (2) and (3) with the initial amount of TFPI–factor Xa complex given by equation (5). The $k_{cat.}$ value was held constant at 250 s^{-1}. The predicted data were in excellent agreement with the experimental data. The values of k_{on} and k_{off} as derived from the fitting procedure are listed together with their standard errors in Table 2, and are in good agreement with the estimates given in Table 1.

Determination of the inhibitor constant

The inhibitor constants (K_i) of the reactions of factor Xa with the different forms of recombinant TFPI were determined under equilibrium conditions in the absence of substrate. K_i values were estimated with different fixed factor Xa concentrations and varying TFPI concentrations as described in the Experimental section. The fitting procedure resulted in inhibitor constants of 0.036±0.004 nM, 0.311±0.009 nM and 0.42±0.03 nM for the full-length TFPI, C-terminal-truncated TFPI and $TFPI_{1-161}$ respectively. We note that an equilibrium between factor Xa and TFPI was established before addition of the substrate. Prolonged incubation times (60 min) resulted in identical residual factor Xa activities. Because the residual factor Xa activity was determined within 1 min after the addition of substrate, and in view of the slow dissociation of the TFPI–factor Xa complex (2×10^{-4} s^{-1}), it is assumed that these K_i values represent the true inhibitor constant. We further note a good agreement between these experimentally determined inhibitor constants and those calculated from the rate constants k_{off} and true k_{on} (Tables 1 and 2). This agreement between kinetic and equilibrium data again

Table 2 Kinetic and equilibrium constants for TFPI–factor Xa complex formation from progress curves started with substrate

Inhibitor	Concn.* (nM)	$10^{-6} \times k_{on}$ ($M^{-1} \cdot s^{-1}$)	$10^{3} \times k_{off}$ (s^{-1})	$10^{9} \times K_i$† (M)
Full-length TFPI	0.2	4.19±0.03	0.167±0.001	0.039
	0.4	4.83±0.02	0.373±0.001	0.077
	0.6	6.53±0.01	0.335±0.003	0.051
Truncated TFPI	0.5	0.987±0.002	0.181±0.003	0.18
	1.0	1.43±0.01	0.275±0.001	0.19
	1.5	1.59±0.01	0.325±0.013	0.20
$TFPI_{1-161}$	0.5	1.110±0.003	0.283±0.001	0.25
	1.0	0.912±0.005	0.263±0.007	0.29
	2.0	0.51±0.06	0.206±0.001	0.40

* Final concentration in the assay.
† Calculated from k_{off}/k_{on}.

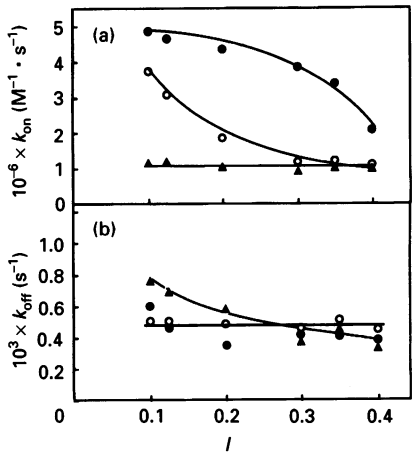

Figure 4 Effect of ionic strength on k_{on} and k_{off} of the TFPI–factor Xa reaction

The k_{on} values (**a**) and k_{off} values (**b**) were obtained from progress curves of the hydrolysis of peptidyl-pNA substrate (300 μM) by factor Xa (0.4 nM) in the presence of 1 nM $TFPI_{1-161}$ (▲), 1 nM C-terminal-truncated TFPI (○) and 0.4 nM full-length TFPI (●).

reinforces our conclusion that the simplified model allows an adequate description of the experimental data.

Effect of ionic strength on the factor Xa-TFPI inhibitory reaction

Among the three different forms of TFPI, the full-length TFPI showed the highest k_{on} and lowest K_i value in the interaction with human factor Xa. Since only the full-length TFPI contains a highly positively charged C-terminus, it was expected that electrostatic interactions between factor Xa and TFPI determines to some extent the binding kinetics. We therefore examined the effect of ionic strength (I) of the reaction medium on the kinetic constants, k_{on} and k_{off}, of the reaction of factor Xa with the three recombinant forms of TFPI.

We first determined at each ionic strength the $k_{cat.}$ and K_m values of the reaction between factor Xa and the peptidyl substrate Spectrozyme FXa. We found that the K_m varied from 150 μM at $I = 0.1$ to 400 μM at $I = 0.4$. The I had no effect on the $k_{cat.}$. Progress curves in the presence of a fixed amount of TFPI were then performed in TBSA buffer containing varying amounts of NaCl. The value of K_m as determined at each I value was held fixed at this value for the analysis of the progress-curve data according to eqns. (2) and (3). Figure 4 shows the effect of I on the kinetic constants of the reactions between factor Xa and full-length TFPI, C-terminal-truncated TFPI and $TFPI_{1-161}$. The k_{on} values were corrected for substrate competition by application of the expression $k_{on} = k_{on}^{app.}(1 + S/K_m)$. The standard errors of the estimates were less than 1 % of the value. It is apparent that I hardly affected the binding kinetics of $TFPI_{1-161}$ to human factor Xa. Interestingly, the C-terminal-truncated TFPI showed the strongest dependency on I in its reaction with factor Xa. Moreover, the differences between truncated TFPI and full-length TFPI with respect to their k_{on} values almost disappeared at low I.

DISCUSSION

The much higher anticoagulant activity of full-length TFPI, as compared with a C-terminal-truncated TFPI, is attributed to a C-terminal-mediated inhibitory activity of the full-length TFPI against factor Xa [10,11]. However, no differences were detected between the two forms of TFPI in the initial rates of tissue factor/factor VIIa-catalysed factor X activation in a buffer system [11]. To understand the structure–function relationships, it thus becomes increasingly important to derive the kinetic constants of the factor Xa–TFPI reaction on the basis of an appropriate kinetic model of that interaction.

We based our kinetic model on the assumption that the association process between factor Xa and the different recombinant forms of TFPI is a reversible bimolecular reaction (Scheme 1). It is very likely that we over-simplified the mechanism of action of the inhibitor. Laskowski and Kato [18] proposed a standard mechanism of action of Kunitz-type inhibitors in which the stable protease–inhibitor complex is preceded by a loose pre-equilibrium complex. Consequently, in the latter case the estimated kinetic constants obtained with the model as depicted in Scheme 1 describe the overall process of inhibition, including all steps before equilibrium is attained.

Three different approaches were used to generate the data from which the association rate constants (k_{on}) and disssociation rate constants (k_{off}) as well as the inhibition constant, K_i, were obtained: (1) the formation of the inactive factor Xa–TFPI complex was monitored by continuous measurement of the changes in the rate of pNA formation, (2) the rate of pNA formation was continuously monitored after preincubating factor Xa with TFPI in the absence of substrate and diluting the factor Xa–TFPI complex into a reaction mixture containing the peptidyl substrate, and (3) the residual factor Xa activity after preincubating factor Xa and TFPI was measured from the initial rate of pNA formation after the addition of peptidyl substrate to the reaction mixture.

The present data show that the different experimental settings yielded virtually identical values for the rate constants k_{on} and k_{off} (Tables 1 and 2). In addition, the K_i values calculated from the rate constants were comparable with those determined from equilibrium experiments. The data thus strongly indicated that the kinetics of the TFPI–factor Xa reaction could be adequately described by Scheme 1. Our observation that the estimated $k_{cat.}$ value did not vary significantly with the TFPI concentration (Tables 1 and 2) is also consistent with a mechanism of inhibition in which factor Xa and TFPI slowly interact. However, it should be pointed out that these observations did not exclude a two-step mechanism. We wish to emphasize that the time-dependence of the reaction between factor Xa and the TFPI variants was such that at TFPI concentrations higher than 1 nM for full-length TFPI, and higher than 2 nM for C-terminal-truncated TFPI and $TFPI_{1-161}$, the duration of pre-steady state became too short to be analysed. Thus we were unable to vary the TFPI concentration over a sufficiently wide range to ensure that a kinetically relevant concentration of an initial weakly associated intermediate TFPI–factor Xa complex was not generated. Indeed, a numerical simulation of the two-stage model revealed that for TFPI concentrations that are 10–20 % of the dissociation constant of the rapidly dissociable intermediate TFPI–factor Xa complex, and for association rates exceeding 4–5 times the observed rate, the kinetics of the one- and two-stage models are indistinguishable (results not shown). At higher TFPI concentrations the simulations predicted a discrepancy between the values of the inhibitor constant obtained from equilibrium experiments and those estimated from the progress curves.

The model, as depicted in Scheme 1, predicts that the peptidyl substrate for factor Xa competes with the binding of TFPI to the active site of factor Xa. Indeed, the association rate constant is a linear function of $K_m/(K_m + S)$. As was expected for a reversible binding interaction, the dissociation rate constant did

Table 3 Kinetic constants (mean ± S.D.) and half-time for transition to equilibrium

Inhibitor	$10^{-6} \times k_{on}$ ($M^{-1} \cdot s^{-1}$)	$10^{4} \times k_{off}$ (s^{-1})	$t_{\frac{1}{2}}$* (s)
Full-length TFPI	5.13 ± 0.87	2.56 ± 0.93	66
Truncated TFPI	1.40 ± 0.21	2.68 ± 0.46	226
$TFPI_{1-161}$	1.08 ± 0.14	2.82 ± 0.50	283

* Half-time for transition to equilibrium.

not vary with the substrate concentration (Figure 3). However, the extent of dissociation is affected by a competing substrate; $K_{i(app.)}$ increased with substrate concentration.

The average k_{on} and k_{off} values (±S.D.) of the different forms of TFPI calculated from the data shown in Tables 1 and 2 are summarized in Table 3. The rate constant of association of full-length TFPI is about 4 times the rate constants of association found for C-terminal-truncated TFPI and $TFPI_{1-161}$. Apparently, the positively charged C-terminal region of TFPI is involved in the association process with factor Xa, which then points to an ionic interaction being important in the binding of full-length TFPI to factor Xa. Our data on the association kinetics as obtained at different ionic strength, however, suggest that ionic interaction is also important in the reaction between C-terminal-truncated TFPI and factor Xa. The binding kinetics of TFPI lacking the third Kunitz domain ($TFPI_{1-161}$) were not sensitive to variations in ionic strength. The data further suggest that the differences between the kinetics of full-length TFPI and C-terminal-truncated TFPI diminish at low ionic strength.

While our investigation was in progress, Wesselschmidt et al. [11] reported K_i values of 3 and 20 pM for factor Xa inhibition by full-length and C-terminal-truncated TFPI respectively. The values reported in the present paper were 36 pM and 310 pM respectively. Thus, despite the similarities in experimental conditions (buffer, concentrations of reactants), our values are more than 10-fold higher than as reported by Wesselschmidt et al. [11]. Whether differences in experimental design, data analysis and the types of cells used to express the proteins caused this disparity remains to be seen. Interestingly, 30-fold greater K_i values were reported by the same group for TFPI produced by HepG2 [5].

If we assume that under physiological conditions the TFPI concentration in plasma (2 nM) exceeds that of factor Xa and that competing ligands can be ignored, the half-times for transition to equilibrium can be estimated from the expression:

$$t_{\frac{1}{2}} = 0.693/(k_{on}[\text{TFPI}] + k_{off})$$

When the data as presented in Table 3 are put in a physiological context, it is evident that, of the different variants of TFPI, full-length TFPI is the better inhibitor of factor Xa. Both its equilibrium position ($[\text{TFPI}]/K_i = 70$) and the time required to produce half-saturation of factor Xa (66 s) point to a greater potency than the other forms of TFPI ($[\text{TFPI}]/K_i = 13$ and a $t_{\frac{1}{2}}$ of complex-formation of 250 s). In considering the speed of reaction, the rate of dissociation (reversibility of the complex formation) can be ignored for full-length TFPI, but also for the truncated forms: the half-lifetime of the TFPI–factor Xa complexes is 3500 s.

When compared with other (natural) inhibitors of factor Xa, such as antithrombin III–heparin, full-length TFPI is a faster-reacting inhibitor: the bimolecular rate constants are $6.4 \times 10^5\ M^{-1} \cdot s^{-1}$ [19] and $5.1 \times 10^6\ M^{-1} \cdot s^{-1}$ (Table 3) respectively. However, when we take into account a 1000-fold difference in plasma concentration, antithrombin III (even in the absence of heparin) becomes as important as TFPI as a factor Xa inhibitor (under the assumption that depletion of TFPI does not occur). That is, the half-lifetime of factor Xa activity in plasma is 84 s [20] and that of factor Xa in the presence of 2 nM of the native full-length TFPI is 66 s (Table 3). Therefore, it may not be excluded that TFPI, in addition to its specific function in inhibiting tissue-factor-dependent factor Xa generation, also acts as a physiologically important inhibitor of factor Xa.

As yet, it remains to be seen whether the kinetic data of the bimolecular reaction between TFPI and factor Xa reflect the kinetics of the inhibitory reaction between TFPI and TF/factor VIIa-catalysed factor Xa generation. If so, then one could speculate that TFPI is a rather inefficient inhibitor, because, due to its relative long half-time of complex-formation with factor Xa (66 s) and the high catalytic efficiency of the initiator of blood coagulation (TF/factor VIIa), a large amount of factor Xa can be produced before the activator is neutralized.

We thank Dr. Ole Nordfang (Novo Nordisk, Gentofte, Denmark) for providing the recombinant TFPIs used in this study.

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Received 11 June 1993/29 July 1993; accepted 16 August 1993