Assembly of the Prothrombinase Complex Enhances the Inhibition of Bovine Factor Xa by Tick Anticoagulant Peptide†

Sriram Krishnaswamy,* George P. Vlasuk,‡ and Peter W. Bergum‡

Hematology/Oncology Department of Medicine, Emory University, Atlanta, Georgia 30322, and Molecular Pharmacology CORVAS International Inc., 3030 Science Park Road, San Diego, California 92121

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ABSTRACT: The interaction of factor Xa with factor Va on a membrane surface results in the assembly of the prothrombinase complex. The highly specific and multistep interaction between recombinant tick anticoagulant peptide (rTAP) and factor Xa was used to probe perturbations in the macromolecular interaction sites of factor Xa that accompany prothrombinase assembly. Steady-state kinetic studies indicated that the incorporation of factor Xa into prothrombinase resulted in a modest 3-fold increase in the rate constant for inhibition by rTAP. However, the overall dissociation constant for the enzyme-inhibitor interaction (K_i*) was decreased ~30-fold to 5.3 pM. This finding was verified by fluorescence stopped-flow studies of the multistep reaction between rTAP and solution-phase factor Xa or prothrombinase by using 4-aminobenzamidine. The second-order rate constant for the binding or rTAP to the protease (k_{+1} = 3.35 × 10^6 $M^{-1}\cdot s^{-1}$) was increased ~2-fold (k_{+1} = 6.6 × 10^6 $M^{-1}\cdot s^{-1}$) following the assembly of prothrombinase, while the rate constant for the subsequent slow displacement of the fluorophore from the active site of factor Xa was decreased by 20-fold. Therefore, factor Va alters macromolecular interaction sites on factor Xa, which leads to the stabilization of intermediates in the reaction of the protease with rTAP and an increased overall affinity for the inhibition of factor Xa. Fluorescence measurements of prothrombinase assembly using factor Xa modified with dansylglutamylglycinylarginine chloromethyl ketone (DEGR-Xa) indicated that the preformed rTAP–Xa binary complex bound to factor Va more tightly (K_d = 30.7 ± 6.2 pM) than factor Xa alone (K_d = 1.25 ± 0.29 nM). The 30-fold higher affinity of the rTAP–Xa complex for factor Va can completely account for the increased affinity of rTAP for prothrombinase and implies adequate thermodynamic description of the reactions involved. Collectively, the data suggest that the interaction of factor Xa with factor Va on a membrane surface alters macromolecular recognition sites on factor Xa involved in binding rTAP. As a result of this conformational change, the inhibition of factor Xa by rTAP is thermodynamically favored when the enzyme is assembled in the prothrombinase complex.

The coagulation serine protease, factor Xa, can interact reversibly but tightly with factor Va on a membrane surface in the presence of Ca^{2+} ions (Mann et al., 1988). The resultant macromolecular complex, referred to as prothrombinase, catalyzes the proteolytic activation of prothrombin with a significantly increased catalytic efficiency (~10^5-fold greater) when compared to the reaction catalyzed by factor Xa alone (Mann et al., 1988; Nesheim et al., 1979; Rosing et al., 1980). Thus, the assembly of the prothrombinase complex is an important requisite for rapid thrombin formation following activation of the coagulation cascade.

The prothrombinase complex is stabilized through reversible interactions between factor Va and membranes (K_d ~ 10^{-9} M), between factor Xa and membranes (K_d ~ 10^{-7} M), and between membrane-bound factors Xa and Va (K_d ~ 10^{-9} M) (Krishnaswamy & Mann, 1988; Krishnaswamy et al., 1988; Krishnaswamy, 1990). The substrate, prothrombin, binds reversibly both to membranes (K_d ~ 10^{-6} M) and also to factor Va (K_d ~ 10^{-6} M) (Nelsestuen & Broderius, 1977; Luckow et al., 1989). The substrate–membrane interaction is considered important for the co-condensation of enzyme and substrate on the surface (Nesheim et al., 1984; Nesheim et al., 1992) or the delivery of substrate to membrane-assembled prothrombinase (Giesen et al., 1991), whereas the substrate–cofactor interaction is considered important for the formation of the ternary complex between protease, cofactor, and substrate (Boskovic et al., 1990). Thus, several protein–protein and protein–membrane interactions are required for the function of the prothrombinase complex, any or all of which could play a role in the increased catalytic efficiency of prothrombinase relative to solution-phase factor Xa.

Steady-state kinetic measurements of prothrombin activation catalyzed by factor Xa alone *versus* the reaction catalyzed by prothrombinase have indicated that the increased catalytic efficiency of prothrombinase derives from a 100-fold decrease in the K_m for substrate and a 3000-fold increase in the k_{cat} for product formation (Rosing et al., 1980). The mechanism(s) underlying these kinetic changes has been the subject of several studies. Since catalysis by prothrombinase involves a membrane-bound enzyme acting on a substrate which can also bind to the same surface, the physical significance of the observed Michaelis constant for prothrombin is unclear. Although several models have been proposed, the basis for the reduced K_m for prothrombin activation by prothrombinase remains controversial (Pusey & Nelsestuen, 1983; van Rijn et al., 1984; Nesheim et al., 1984; Nesheim et al., 1981a). The large increase in the k_{cat} for the reaction that accompanies the incorporation of factor Xa into prothrombinase has implied that the active site of factor Xa may be altered by virtue of

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* To whom correspondence should be addressed at the Division of Hematology/Oncology, Department of Medicine, Drawer AR, Emory University, Atlanta, GA 30322. Telephone: (404) 727-3806; Fax: (404) 727-3404.

‡ Molecular Pharmacology CORVAS International Inc.

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its interaction with the cofactor (Nesheim et al., 1984). This suggestion is based on the data available for other analogous enzyme complexes of coagulation and is primarily supported by spectral changes in a fluorescent reporter group covalently incorporated into the active site of factor Xa (Higgins et al., 1985; Husten et al., 1987). However, measurements of the reaction of a fluorescent peptidyl chloromethyl ketone with factor Xa in the presence or absence of other constituents of prothrombinase failed to yield evidence for detectable alterations in the catalytic residues of factor Xa (Walker & Krishnaswamy, 1993). Modest changes in the association rate constant for this inhibitor did suggest the possibility of alterations in the extended macromolecular recognition sites of the protease as a consequence of its interaction with factor Va. Thus, one possible mechanism through which the cofactor exerts its effect on prothrombin activation is to alter binding interactions at the extended macromolecular recognition sites on factor Xa. However, the available experimental evidence supporting this possibility is not compelling. The initial evaluation of this hypothesis would require the demonstration of factor Va-induced changes in the macromolecular interactions of the protease using reagents that interact with sites on factor Xa removed from both the catalytic residues and the binding sites already probed by tripeptidyl inhibitors or substrates.

Tick anticoagulant peptide (TAP)[1] is one of several slow, tight-binding inhibitors of factor Xa that have been described. TAP is a 60-residue polypeptide isolated from the hematophagous organism *Ornithodoros moubata* and is highly specific for factor Xa (Waxman et al., 1990). Although limited sequence homology exists between recombinant TAP (rTAP) and proteinase inhibitors of the Kunitz family, rTAP is not cleaved by factor Xa during the inhibition reaction (Sardana et al., 1991; Jordan et al., 1990). At present, the most favorable functional comparisons of the action of rTAP on factor Xa can be made with the specific and high-affinity inhibition of the coagulation protease thrombin by hirudin (Stone & Maraganore, 1993). The kinetic mechanism of the inhibition of human factor Xa by rTAP has been well-characterized by Jordan et al. (1992) with good evidence for a multistep reaction involving interactions of rTAP at "exosite(s)" (sites removed from the active site) on factor Xa. Using stopped-flow measurements, these workers resolved and assigned rate and/or equilibrium constants to at least three reversible steps in the reaction which led to the observed tight-binding inhibition of factor Xa by rTAP (K_i^* = 180 pM) (Jordan et al., 1992). Thus, the specific and high-affinity interaction between rTAP and factor Xa is achieved through stepwise interactions between the inhibitor and multiple sites on the protease (Jordan et al., 1992).

Kinetic and thermodynamic information regarding the action of rTAP on the prothrombinase complex is not available. Since prothrombinase, rather than free factor Xa, is considered the physiologically relevant catalyst for prothrombin activation (Mann et al., 1988; Mann et al., 1990), information pertaining to the reaction between rTAP and factor Xa in the presence of the other constituents of prothrombinase may prove useful in predicting the properties of rTAP administered therapeutically to modulate coagulation (Schaffer et al., 1991).

Studies of the reaction between rTAP and factor Xa or prothrombinase also have the potential for providing unique information relating to perturbations in or the participation of exosite(s) in factor Xa function. This potential is exemplified by work in the thrombin–hirudin system which has led to an increase in the understanding of structure–function relationships and the role of exosites in the reactions of thrombin with its macromolecular substrates and the cofactor thrombomodulin (Stone & Maraganore, 1993).

In the present work, we have used rTAP as a probe of the macromolecular recognition sites of factor Xa. We have examined the kinetics and thermodynamics of the reaction between rTAP and factor Xa in the presence of the other constituents of the prothrombinase complex in order to detect perturbations in factor Xa that result from its interaction with factor Va on the membrane surface.

EXPERIMENTAL PROCEDURES

Materials. Hepes, L-α-phosphatidylcholine (hen egg), L-α-phosphatidylserine (bovine brain), and t-Boc isoleucylglutamylglycylarginine 7-amido-4-methylcoumarin (IEGR-AMC) were purchased from Sigma. Cyclohexylglycylglycylarginine *p*-nitroanilide (Spectrozyme fXa, SpXa) was obtained from American Diagnostica. Stock solutions (4–7 mM) of either substrate were prepared in water and concentrations verified using E^{M}_{342} = 8270 $M^{-1}\cdot cm^{-1}$ for SpXa or E^{M}_{325} = 17 200 $M^{-1}\cdot cm^{-1}$ for IEGR-AMC (Lottenberg & Jackson, 1983; Jordan et al., 1992). The fluorophore 4-aminobenzamidine (PAB) was from Aldrich. The concentrations of solutions of PAB in water were determined using E^{M}_{293} = 15 000 $M^{-1}\cdot cm^{-1}$ (Evans et al., 1982). Dansylglutamylglycylarginine chloromethyl ketone and glutamylglycylarginine chloromethyl ketone were obtained from Calbiochem. Phospholipid vesicles (PCPS), composed of 75% (w/w) phosphatidylcholine and 25% (w/w) phosphatidylserine, were prepared by sonication and differential centrifugation as described (Higgins & Mann, 1983). The concentrations of phospholipids are expressed as the concentration of the monomeric lipid species determined by a colorimetric phosphate assay. Unless otherwise noted, all kinetic and binding measurements were conducted in 20 mM Hepes, 0.15 M NaCl, 2 mM $CaCl_2$, and 0.1% (w/v) polyethylene glycol 8000 at pH 7.4 (assay buffer).

Proteins. Recombinant TAP was expressed in the yeast Pichia Pastoris,[2] purified as previously described and stored as the lyophilized TFA salt (Lehman et al., 1993). This material gave a single peak upon analysis by reversed-phase HPLC (Aquapore C8) and yielded the expected amino acid composition (Waxman et al., 1990). Initial stock solutions of rTAP (200–900 μM) were prepared on the basis of lyophilized weight, and concentrations were verified using a calculated E_{280} = 17 880 $M^{-1}\cdot cm^{-1}$ and F_{wt} = 6980 Da or by amino acid analysis (Cantor & Schimmel, 1980). The discrepancy between concentrations determined by the two methods was within experimental error (<8%). Bovine factor Va was isolated as previously described with additional purification by chromatography on fast flow S-sepharose (Kalafatis et al., 1993). Bovine factor Xa was prepared by the activation of factor X by the purified factor X activator from Russell's viper venom followed by affinity chromatography on benzamidine sepharose as previously described (Krishnaswamy et al., 1987; Jesty & Nemerson, 1976). The material prepared by this method contained approximately

[1] Abbreviations: DEGR-Xa, factor Xa modified with dansylglutamylglycylarginine chloromethyl ketone; EGR-Xa, factor Xa modified with glutamylglycylarginine chloromethyl ketone; IEGR-AMC, t-Boc isoleucylglutamylglycinylarginine 7-amido-4-methylcoumarin; PAB, 4-aminobenzamidine; PCPS, small unilamellar vesicles composed of 75% (w/w) L-α-phosphatidylcholine and 25% (w/w) L-α-phosphatidylserine; rTAP, recombinant tick anticoagulant peptide; SpXa, cyclohexylglycylglycylarginine *p*-nitroanilide.

[2] Y. LaRoche, M. Lauwereys, and G. P. Vlasuk, manuscript in preparation.

equal amounts of the α and β forms of Xa. Titration of several such preparations using *p*-nitrophenol-*p'*-guanidino benzoate (Chase, Jr. & Shaw, 1967) consistently yielded 1.08–1.15 mol active sites/mol Xa. Factor Xa was modified with either dansylglutamylglycinylarginine chloromethyl ketone to yield DEGR-Xa or glutamylglycinylarginine chloromethyl ketone to yield EGR-Xa by described procedures (Krishnaswamy et al., 1993). The protein preparations were judged homogeneous following analysis by SDS-PAGE with and without disulphide bond reduction and staining with Coomassie Brilliant Blue (Laemmli, 1970). Protein concentrations were calculated using the following molecular weights and extinction coefficients ($E^{0.1\%}_{280}$): factor Va, 168 000, 1.74 (Laue et al., 1984; Krishnaswamy & Mann, 1988); factor X, 56 500, 1.24; and factor Xa, 45 300, 1.24 (Jackson et al., 1968; Fujikawa et al., 1974).

Measurements of the Rate of Factor Xa Inhibition. The inhibition of factor Xa was inferred from exponential decays in the rate of SpXa hydrolysis in the presence of rTAP. Reaction mixtures (150 μL) were prepared in 96-well plates (Corning Assay Plate) containing SpXa (200 μM final) and increasing concentrations of rTAP in assay buffer. Reactions were initiated with a 50-μL enzyme solution in the same buffer to yield final concentrations of 0.5 nM Xa, 0.5 nM Xa *plus* 60 μM PCPS or 0.5 nM Xa and 60 μM PCPS *plus* 20 nM Va. Following mixing by brief vibration, SpXa hydrolysis was monitored by continuously measuring absorbance at 405 nm at ambient temperature (estimated at 23 ± 2 °C) using a Vmax kinetic plate reader (Molecular Devices). Data acquired over 30 min were corrected for the measured 25-s interval between the initiation of reactions and the onset of absorbance measurements.

Measurement of the Overall Equilibrium Constant (K_i) for the Inhibition of Factor Xa.* Solutions (175 μL) contained 0.32 or 0.57 nM factor Xa with or without 60 μM PCPS and increasing concentrations of rTAP in assay buffer. Following incubation for 120 min at ambient temperature, the concentration of free (uninhibited) factor Xa was determined from initial steady-state rates of SpXa hydrolysis measured over a 3-min period. The initial velocity measurements were conducted at ambient temperature by the addition of 25 μL of SpXa solution (100 μM final) and continuous measurement of absorbance at 405 nm. The assumption necessary for this approach is that the new equilibrium between factor Xa or Xa-PCPS and rTAP perturbed due to dilution or the addition of SpXa is slow to reestablish in relation to the time of the initial velocity measurement (see below). Thus, all ligand concentrations for the calculation of K_i* are expressed as the concentrations present in the initial 175-μL incubation mixture.

The K_i* for the inhibition of prothrombinase (Xa-Va-PCPS) by rTAP was assessed by measurements of the hydrolysis of the fluorescent substrate IEGR-AMC. Reaction mixtures (2.5 mL) containing 4 pM Xa, 50 μM PCPS, 20 nM Va, and varying concentrations of rTAP were prepared in Assay Buffer using Minisorp (NUNC, Denmark) tubes. Following incubation for 120 min at ambient temperature, 2.0 mL of each reaction mixture was transferred to a 1 × 1 cm quartz cuvette and initiated by the addition of 20 μL of the fluorescent substrate (71 μM final) with gentle mixing. Substrate hydrolysis was monitored in a SLM-8000C fluorescence spectrophotometer (SLM, Urbana, IL) with modified hardware and software (OLIS, Bogart, GA) by using λ_{ex} = 380 nm and measuring broadband fluorescence (≥408 nm) with a KV-408 long-pass filter (Schott, Duyrea, PA) in the emission beam. Initial, steady-state velocities were determined at high sensitivity by acquiring 100 points over 200 s with 32 readings averaged per datum and expressed as the change in relative fluorescence per unit time without any normalization. It was therefore assumed that instrument drift was small during the experiment. This was verified by the lack of obvious systematic changes in rate in assays of IEGR-AMC hydrolysis by 4 pM factor Xa randomly repeated during the experiment.

Stopped-Flow Measurement Using 4-Aminobenzamidine. Fluorescence stopped-flow measurements were conducted using a two-syringe apparatus (Kinetic Instruments, Ann Arbor, MI) attached to the SLM-8000C spectrophotometer as previously described (Krishnaswamy et al., 1988; Walker & Krishnaswamy, 1993). Measurements were conducted at 25 °C by using λ_{ex} = 320 nm and measuring broadband fluorescence ($\lambda_{em} \geq$ 345 nm) with a long-pass filter in the emission beam. For measurements of the dependence of the observed rate constants on the concentration of rTAP, Syringe A contained enzyme solution (Xa, 1 μM Xa; Xa-PCPS, 1 μM Xa *plus* 200 μM PCPS; or Xa-Va-PCPS, 1 μM Xa, 1.5 μM Va *plus* 200 μM PCPS) equilibrated with 25 μM PAB in Assay Buffer. Syringe B contained increasing concentrations of rTAP also equilibrated with 25 μM PAB in the same buffer. Reactions were initiated by mixing equal volumes of the contents of both syringes. Fluorescence intensity was measured using two collection intervals (e.g.: 200 points in 0–400 ms, datum interval = 2 ms followed by 200 points in 0.7–60 s, datum interval = 300 ms) to permit adequate description of the initial rapid rise in fluorescence followed by the slow decay phase.

In experiments where the concentration of PAB was varied, the same experimental design was followed except that the concentration of PAB was varied in both syringes and the final concentration of rTAP was 17.5 μM.

Fluorescence Measurements of Prothrombinase Assembly in the Presence of rTAP. The ability of factor Xa or the Xa–rTAP binary complex to bind to factor Va was assessed by competitive binding experiments using DEGR-Xa and measurements of fluorescence intensity either by using increasing amounts of competitor to displace the preassembled DEGR-Xa–Va-PCPS complex or by examining the ability of DEGR-Xa to bind to variable concentrations of factor Va in the presence of a fixed concentration of the competitor.

Fluorescence titrations were conducted in 1 × 1 cm stirred quartz cuvettes maintained at 25 °C by using λ_{ex} = 280 nm and measuring broadband fluorescence with a long-pass filter (Schott KV-500) in the emission beam. The reaction mixture (2.0 mL) contained 50 nM DEGR-Xa, 70 nM Va, and 100 μM PCPS in assay buffer. Titrations were performed by making microliter additions of a concentrated stock solution of Xa (10.05 μM) or the Xa–rTAP binary complex (10.05 μM Xa plus 12.3 μM rTAP preincubated in assay buffer for 15 min at room temperature). Fluorescence intensity was measured approximately 2 min after each addition by integrating the signal over 5 s and recording the average of 8–10 separate measurements (SD <0.2%). Measurements from three separate reaction mixtures were used for each titration: (1) 50 nM DEGR-Xa, 70 nM Va, and 100 μM PCPS with varying amounts of competitor; (2) 50 nM DEGR-Xa, 70 nM Va, and 100 μM PCPS with equivalent volumes of buffer added; (3) 50 nM EGR-Xa, 70 nM Va, and 100 μM PCPS with varying amounts of competitor. The experimental signal (reaction 1) was corrected for the scattering contribution (reaction 3) and for dilution effects (reaction 2) and expressed as the fraction of the intensity observed in the absence of factor Va. Both corrections were small and maximally corresponded to 3–5% of the observed signal. When the Xa–rTAP binary complex was used as the competitor, control

experiments indicated that the addition of equivalent concentrations of free rTAP had no measurable effect on fluorescence intensity beyond effects due to dilution.

For experiments in the second configuration, reaction mixtures prepared in Assay Buffer contained 20 nM DEGR-Xa, 50 μM PCPS, and 0 or 20 nM unmodified Xa in the presence or absence of 100 nM rTAP. Titrations were performed by the addition of microliter amounts of factor Va, and fluorescence intensity was recorded and corrected for dilution and scattering effects using reaction mixtures equivalent to those described above.

Data Analysis. Data sets were fitted to the indicated expressions by unweighted nonlinear least squares analysis using the Marquardt algorithm (Bevington, 1969) to extract the appropriate constants and their 95% confidence limits. The quality of the fits was evaluated by considerations described previously (Straume & Johnson, 1992). Linear regression of replicated measurements was performed by weighting the data using the reciprocal of one standard deviation of the dependent variable (Bevington, 1969).

Kinetics of Enzyme Inhibition. Progress curves for the inhibition of factor Xa by rTAP during SpXa hydrolysis were analyzed according to eqs 4–6 of Williams et al. (1979) describing the pseudo-first-order decrease in velocity to a steady-state value (v_{ss}) as a function of time and incorporating corrections for the depletion of inhibitor and the effects of the indicator substrate. Given the multistep reaction (Scheme 1) previously determined (Jordan et al., 1992) for the inhibition of human Xa by rTAP, when [I] $\ll K_1$, the concentration of EI is negligible during the reaction time course. In this special case, the dependence of k_{obs} on the concentration of rTAP is given by eqs 1 and 2 (Williams et al., 1979):

$$k_{obs} = k_{inh} I_{eff} \tag{1}$$

$$I_{eff} = \frac{\sqrt{\left(K_i^*\left(1+\frac{S}{K_m}\right)+E+I\right)^2-(4EI)}}{\left(1+\frac{S}{K_m}\right)} \tag{2}$$

where S and K_m refer to the concentration of SpXa and the Michaelis constant for SpXa, respectively. E and I refer to total concentrations of enzyme and inhibitor, K_i^* is the overall equilibrium constant for the E–I interaction (below), and I_{eff} is the effective inhibitor concentration, corrected for changes in the free concentration of inhibitor and the effects of SpXa. Replicate traces at each inhibitor concentration were fitted to eqs 4–6 of Williams et al. (1979), and the fitted values of k_{obs} were analyzed according to eqs 1 and 2 to obtain estimates of k_{inh}. The Michaelis constants used to correct for the effective inhibitor concentration were determined from initial velocity measurements of SpXa hydrolysis using factor Xa (K_m = 67.6 ± 9.5 μM) and Xa *plus* PCPS (K_m = 67.0 ± 6.8 μM) or Xa and Va *plus* PCPS (K_m = 83.4 ± 4.4 μM) under the present experimental conditions. K_i^* used for these analyses were determined from the experiments described below.

Determination of the Overall Equilibrium Constant for Inhibition by rTAP. The overall equilibrium constant (K_i^*) was determined from initial velocity measurements of synthetic substrate hydrolysis using eqs 3 and 4 and the assumptions described above:

$$E_i = \frac{(nI+E+K_i^*)-\sqrt{(nI+E+K_i^*)^2-4nIE}}{2} \tag{3}$$

$$V_{obs} = (V_\infty E) + \left(V_0 E\left(1-\frac{E_i}{E}\right)\right) \tag{4}$$

where n moles of I reversibly combine per mole of E to yield the inhibited species E_i, governed by the overall equilibrium constant K_i^*. E and I refer to total concentrations of enzyme and inhibitor, and V_{obs} is the observed initial velocity. The terms V_0 and V_∞ represent the specific activities (initial velocity/unit concentration of enzyme) in the absence of added inhibitor and at infinite inhibitor concentrations, respectively.

Initial velocity data obtained using two or more fixed concentrations of factor Xa and Xa *plus* PCPS or Xa and Va *plus* PCPS and increasing concentrations of rTAP were analyzed by eqs 3 and 4 to extract fitted values of n, K_i^*, V_0, and V_∞. In some cases, where only one concentration of enzyme was used, n was fixed at 1 to extract fitted values for the other three terms.

Stopped-Flow Measurements. The biphasic rise and fall in fluorescence intensity observed upon reacting rTAP with factor Xa in the presence of PAB was analyzed according to consecutive (pseudo) first-order reactions given by

$$F_t = \text{offset} + F_1 e^{-k_{1,obs}t} + F_2\left[\frac{k_{1,obs}}{k_{2,obs}-k_{1,obs}}\left(e^{-k_{1,obs}t}-e^{-k_{2,obs}t}\right)\right] \tag{5}$$

where the observed signal (F_t) at time t is related to the starting signal due to the Xa–PAB binary complex (F_1) and the enhanced fluorescence intensity of the ternary Xa–PAB–rTAP complex (F_2) which occurs as an intermediate in the decay of fluorescence to a limiting value equivalent to that of free PAB (offset). The constants $k_{1,obs}$ and $k_{2,obs}$ reflect the observed (pseudo) first-order constants for the rise and fall, respectively. Stopped-flow tracings were analyzed by this equation to obtain fitted values for offset, F_1, F_2, $k_{1,obs}$, and $k_{2,obs}$.

Competitive Binding Measurements. Equilibrium constants were extracted from fluorescence intensity measurements, where the titrant was factor Va with a fixed concentration of DEGR-Xa, a saturating concentration of PCPS and different fixed concentrations of competitor. At zero competitor concentration, the binding isotherm is adequately described by a quadratic expression (equivalent to eq 3 above) which yields equilibrium parameters (K_{dXa-Va}, n_{Xa-Va}) for the binding of DEGR-Xa and Va on the membrane surface. Experimental considerations and the development of the model for this situation have been previously described in detail (Krishnaswamy, 1990). The general case solution for binding curves in the presence of competitor is given by the cubic equation previously developed by Olson et al. (1991). This equation does not require any assumptions regarding the equivalence of the stoichiometries or the near equivalence of the K_ds for the indicator or competitor interactions. Thus, complete data sets were analyzed according to the the quadratic expression (when competitor = 0) and the cubic equation 17 described by Olson et al. (1991) (when competitor > 0) to solve for the concentration of DEGR-Xa bound to factor Va. The fractional saturation of DEGR-Xa was related to the observed signal by

$$\frac{F}{F_0} = F_0 + \left[F_{max}\frac{Xa_B}{Xa_T}\right] \tag{6}$$

where F/F_0 is the normalized fluorescence intensity, F_0 is the fluorescence intensity in the absence of factor Va, F_{max} is the fluorescence change at saturating concentrations of factor Va, and Xa_B and Xa_T refer to the bound and total concentrations of DEGR-Xa, respectively. Analysis yielded fitted equilibrium parameters for the interaction of DEGR-Xa with factor Va (K_{dXa-Va}, n_{Xa-Va}), for the competitor with factor Va

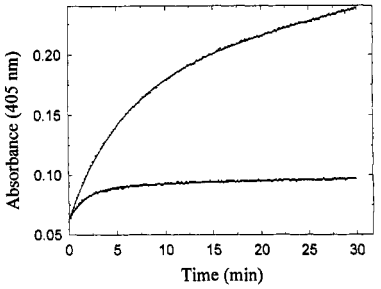


FIGURE 1: Kinetics of inhibition of factor Xa by TAP in the presence or absence of factor Va and membranes. Reaction mixtures containing 200 μM Spectrozyme fXa and 3.0 nM TAP were initiated by the addition of 0.5 nM factor Xa (upper trace) or preassembled prothrombinase: 0.5 nM Xa, 20 nM Va, 60 μM PCPS (lower trace). Product formation was measured at ambient temperature and analyzed as described under Data Analysis to yield k_{obs} = (2.95 ± 0.03) × 10^{-3} s^{-1}, v_{ss} = (3.34 ± 0.04) × 10^{-5} A_{405}·s^{-1} for factor Xa and k_{obs} = (8.97 ± 0.20) × 10^{-3} s^{-1}, v_{ss} = (0.37 ± 0.01) × 10^{-5} A_{405}·s^{-1} for prothrombinase.

Scheme 1

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} EI^* \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} EI^{**}$$

($K_{d_{Comp-Va}}$, $n_{Comp-Va}$) and the limits of the fluorescence signal (F_{max}, F_0).

RESULTS

Kinetic Model for the Reaction of rTAP with Factor Xa. A multistep reaction scheme for the inhibition of human factor Xa by rTAP determined from stopped-flow measurements by Jordan et al. (1992) is illustrated in Scheme 1 where the reversible bimolecular combination of enzyme and inhibitor is further stabilized by two additional unimolecular equilibria. The subscripted rate constants represent the forward and reverse rate constants for steps 1–3 with equilibrium dissociation constants given by K_1, K_2, and K_3, respectively. Evidence for the first two steps was obtained from stopped-flow measurements of the inhibition of factor Xa by rTAP and evidence for all three steps was obtained from stopped-flow measurements of the displacement of PAB from the active site of human factor Xa (Jordan et al., 1992). Scheme 1 was assumed to adequately describe the reaction between bovine factor Xa and rTAP in the present work. This assumption was based on the kinetic evidence provided by Jordan et al. (1992) and on the basis of the high degree of structural and functional homology between bovine and human factor Xa (Jackson, 1984). In addition, there is reasonable agreement between our data (below) and this kinetic scheme.

The species EI, EI*, and EI** represent substantially or completely inhibited enzyme–inhibitor complexes. Thus, measurements of rates of inhibition of Xa by rTAP only provide information relating to the rate constants of the first two steps in the pathway. In contrast, the extent of inhibition of factor Xa by rTAP is determined by the equilibrium constants for all three steps.

Effect of Prothrombinase Assembly on the Rate of Inhibition of Factor Xa by rTAP. The inhibition of factor Xa by rTAP was monitored by measuring the exponential decay in progress curves of SpXa hydrolysis following the addition of factor Xa to reaction mixtures containing substrate and inhibitor. Progress curves for the inhibition of factor Xa alone or Xa assembled in the prothrombinase complex are illustrated in Figure 1. The inhibition of the amidolytic activity of factor Xa proceeded slowly under these conditions, reaching a limiting steady-state velocity in approximately 20 min. The presence of saturating concentrations of PCPS had little effect on the progress curve for the inhibition of factor Xa (not shown). However, the amplitude of the progress curve was substantially smaller when the same concentration of factor Xa was incorporated into prothrombinase by the addition of saturating concentrations of factor Va and PCPS. The decreased amplitude resulted from a 3-fold increase in the k_{obs} for inhibition and an approximate 10-fold decrease in the limiting steady-state velocity. These data indicate that the assembly of prothrombinase alters both the rate and extent of factor Xa inhibition by rTAP.

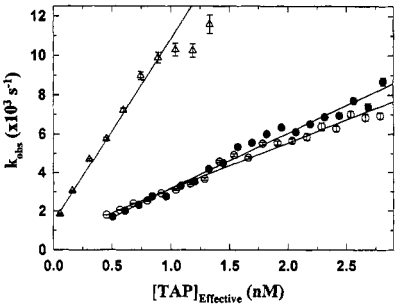


FIGURE 2: Dependence of k_{obs} for inhibition on the effective concentration of TAP. The observed rate constant for inhibition was determined from progress curves as illustrated in Figure 1, using increasing concentrations of TAP and 0.5 nM Xa (○), 0.5 nM Xa plus 60 μM PCPS (●) or 0.5 nM Xa, 20 nM Va, and 60 μM PCPS (△). Error bars denote 95% confidence limits from duplicate experiments, and the lines were drawn by weighted least squares regression analysis. The dependence of k_{obs} on the effective concentration of TAP calculated according to eqs 1 and 2 was used to extract the rate constants listed in Table 1.

The influence of prothrombinase constituents on the rate of inhibition of factor Xa by rTAP was assessed by studies of the dependence of k_{obs} on the concentration of rTAP (Figure 2) over a range that was substantially below the published values of K_1 (6–68 μM) determined for the reaction with human factor Xa (Jordan et al., 1992). Under these conditions, the concentration of EI is negligible and, thus, k_{obs} is expected to be linearly dependent on the concentration of rTAP (Morrison & Walsh, 1988). The observed data (Figure 2) are consistent with this prediction. Following corrections for inhibitor depletion and the effects of the indicator substrate (eqs 2 and 3), the slope of the plots yields the second-order rate constant k_{+2}/K_1, provided the rapid equilibrium assumptions apply ($k_{+1}I + k_{-1} \gg k_{+2} + k_{-2}$) (Morrison & Walsh, 1988). However, when these assumptions do not apply, the observed slope approaches a limiting value equal to k_{+1} (Hiromi, 1979). Since the adequacy of the rapid equilibrium assumptions cannot be verified in this experimental system, we have used the second-order rate constant (k_{inh}) derived from the slopes in Figure 2 as an index of the rate constants for the initial portion of the inhibition reaction without ascribing any physical meaning to the values. Interestingly, the second-order rate constants determined in the present work are in good agreement with those determined in comparable experiments with human factor Xa which were interpreted to reflect the term k_{+2}/K_1 (Jordan et al., 1992).

The second-order rate constants obtained for the inhibition of factor Xa alone or the Xa–PCPS binary complex by rTAP were nearly equivalent (Table 1), indicating that the binding of factor Xa to the membrane surface does not significantly perturb the initial binding interactions between enzyme and inhibitor. The rate constant was increased approximately 3-fold when saturating concentrations of factor Va and PCPS were present (Table 1). Thus, the incorporation of factor Xa into the prothrombinase complex results in a modest but detectable change in the rate constants for the initial steps of

Table 1: Inhibition of Factor Xa by rTAP in the Presence of Prothrombinase Constituents

enzyme species[a]	$k_{INH} \pm SE \times 10^{-6}$ [b] ($M^{-1}\cdot s^{-1}$)	$K_i^* \pm SE$[c] (pM)	$n \pm SE$[d] (mol TAP/mol Xa)	inhibition$_{Max}$[e] (%)
Xa	2.4 ± 0.1	120 ± 7.2	0.98 ± 0.02	96.1
Xa–PCPS	2.8 ± 0.2	165 ± 18	1.11 ± 0.06	96.7
Xa–Va–PCPS	9.8 ± 1.3	5.3 ± 0.76	*f*	100

[a] The enzyme species correspond to solution-phase factor Xa (Xa), factor Xa saturated with PCPS (Xa–PCPS), and factor Xa saturated with both factor Va and PCPS (Xa–Va–PCPS). [b] Overall second-order rate constant calculated by weighted linear regression of the data illustrated in Figure 2. [c] Overall equilibrium constant for the inhibition of enzyme by rTAP. [d] Stoichiometry of the inhibition reaction at saturation. [e] Maximum inhibition at infinite rTAP calculated from fitted values of V_0 and V_∞ in eq 4. [f] Stoichiometry was fixed at 1.

the reaction between protease and inhibitor which lead to enzyme inhibition.

Effect of Factor Va on the Overall Stability of the Xa–rTAP Interaction. The progress curves for the inhibition of factor Xa by rTAP provided initial indications that the presence of factor Va and PCPS had a large effect on the limiting steady-state velocity and hence the extent of the inhibition by rTAP. This possibility was directly assessed by measuring the overall equilibrium constant (K_i^*) for the Xa–rTAP interaction in the presence or absence of prothrombinase constituents.

Titrations were performed by permitting reaction mixtures containing enzyme and increasing concentrations of rTAP to achieve equilibrium followed by the assessment of the free protease concentration by initial velocity measurements. For these experiments, it was assumed that the initial equilibrium was not significantly perturbed by substrate during the initial velocity measurements. This assumption seems reasonable since the measurements were performed on a short time scale (4 min) relative to the measured $t_{1/2}$ of 16 min for the unimolecular regain of Xa activity following dissociation of the enzyme–inhibitor complex (Jordan et al., 1990).

Titration curves obtained at two fixed concentrations of factor Xa and increasing concentrations of rTAP are illustrated in Figure 3A. The data could be adequately described by eqs 3 and 4 to infer overall equilibrium parameters (Table 1) that are in good agreement with values that have been previously reported with human factor Xa (Jordan et al., 1992). The fitted limiting velocity at infinite inhibitor concentration corresponded to 96% inhibition. Analysis of these and other similar data sets by setting the velocity at infinite inhibitor concentration to zero (eq 4) yielded inadequate fits with poorly determined stoichiometries that were significantly less than 1. The stoichiometry for the Xa–rTAP interaction was therefore directly assessed by titrations performed using a high fixed concentration of Xa ([Xa] $\gg K_i^*$, Figure 3A, inset). In these experiments, activity decreased to a limiting value corresponding to approximately 5% of the initial activity. The initial portion of the inhibition curve could be extrapolated to yield a stoichiometry of 1.08 ± 0.03 mol rTAP/mol Xa at saturation.

Stopped-flow absorbance measurements were conducted to investigate the basis for the residual activity in the presence of saturating concentrations of rTAP (data not shown). Preincubated mixtures containing 0.5 μM Xa and 1.5 μM rTAP in assay buffer were reacted with an equal volume of 200 μM SpXa, and product release was monitored at 405 nm. The resultant progress curves were characterized by a lag phase on the order of 5 s before a limiting steady-state velocity was achieved. Analysis indicated that the increase in velocity occurred with a rate constant of ~0.3 s^{-1} which was independent of the concentration of rTAP between 0.8 and 4.0 μM. These data are consistent with the possibility that while species EI** is inactive, the intermediates in the inhibition pathway (EI and EI*, Scheme 1) can bind and hydrolyze SpXa slowly, leading to the small but detectable

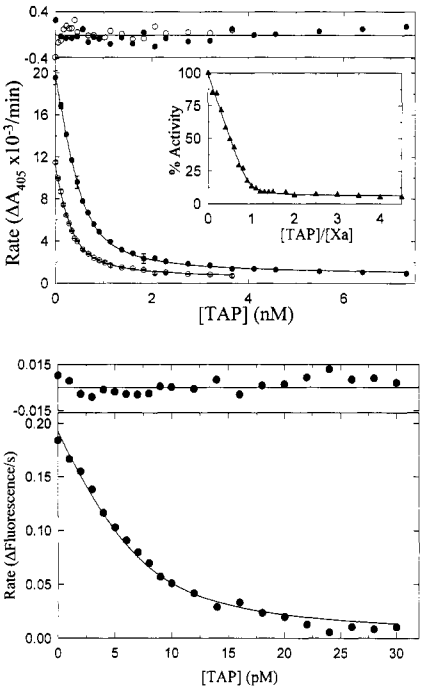


FIGURE 3: Influence of factor Va on the stability of the TAP–Xa complex. (A, Top) Determination of K_i^* for the inhibition of factor Xa by TAP. Reaction mixtures containing 0.32 nM (○) or 0.57 nM (●) factor Xa were incubated with the indicated concentrations of TAP for 60 min in assay buffer. Remaining factor Xa was assessed by initial velocity measurements following the addition of 100 μM SpXa. The curves are drawn by fitting to eqs 3 and 4 to yield the parameters K_i^* = 120 ± 7.2 pM, n = 0.98 ± 0.02 mol TAP/mol Xa at saturation, V_0 = 33.38 ± 0.19 mOD/min/nM Xa and V_∞ = 1.32 ± 0.09 mOD/min/nM Xa. The residuals to the fitted lines are illustrated in the upper portion of the figure. Inset: The stoichiometry for the interaction was independently assessed by determining the Xa activity remaining after the incubation of 100 nM Xa (>500 K_i^*) with increasing concentrations of TAP. (B, Bottom) Determination of K_i^* for the inhibition of prothrombinase by TAP. Reaction mixtures (2.0 mL) containing 4.0 pM Xa, 20 nM Va, and 50 μM PCPS in assay buffer were incubated with the indicated concentrations of TAP for 120 min at room temperature. Initial velocities were determined by fluorescence measurements following the addition of 71 μM IEGR-AMC using λ_{ex} = 380 nm and λ_{em} > 408 nm. The curve is drawn according to eqs 3 and 4, assuming a stoichiometry of 1.0 to yield K_i^* = 5.3 ± 0.76 pM. The residuals to the fitted line are shown in the upper portion of the figure.

rates of SpXa hydrolysis even at saturating concentrations of rTAP. This idea is consistent with the demonstration that rTAP can bind to DEGR-Xa, albeit weakly (Jordan et al., 1992), the interpretations of fluorescence changes in the presence of PAB (Scheme 2), and the agreement between the observed rate constant for the lag phase and the determined rate constant for the interconversion between EI* and EI** ($k_{+3} + k_{-3}$, below).

The determined K_i^* for the inhibition of the Xa–PCPS binary complex was essentially equivalent to that observed with solution-phase factor Xa (Table 1). In contrast, when factor Xa was incorporated into prothrombinase, titration curves obtained at equivalent concentrations to those illustrated

in Figure 3A indicated a large decrease in K_i* with an upper limit estimate of 7–10 pM (data not shown). A more reliable estimate of K_i* under these conditions was determined from initial velocity studies using the fluorescent substrate IEGR-AMC. By this method, reproducible initial rates could be obtained at 10^{-12} M Xa in a 2-min period. A titration curve illustrating the inhibition of 4 pM prothrombinase (4 pM Xa, 20 nM Va, 50 μM PCPS) by increasing concentrations of rTAP is presented in Figure 3B. Analysis according to eqs 3 and 4, but assuming a unit stoichiometry, yielded a K_i* of 5.3 pM. Thus, assembly of prothrombinase leads to a 25–30-fold increase in the overall stability of the protease–inhibitor complex.

The values for k_{inh} and K_i* obtained using solution-phase factor Xa or Factor Xa saturated with PCPS were essentially identical (Table 1). In the latter case, concentrations were chosen on the basis of the published K_d and stoichiometry such that greater than 90% of the added Xa could be assumed to be bound to the membrane surface (Krishnaswamy et al., 1988). Since the binding of factor Xa to membranes has no detectable effect on its reaction with rTAP, it follows that the modest increase in the second-order rate constant and the large decrease in the equilibrium constant for the inhibition reaction in the presence of factor Va *plus* PCPS arise as a consequence of the interaction between factors Xa and Va on the membrane surface. The 3-fold increase in k_{inh} *versus* the 30-fold decrease in K_i* observed upon prothrombinase assembly suggests that the enzyme–cofactor interaction leads to either significant changes in K_3 (Scheme 1) or changes in other rate constants that are not reflected by k_{inh}.

Rapid Kinetic Measurement of the Xa–rTAP Interaction. Stopped-flow measurements of the Xa–rTAP interaction were undertaken to further evaluate the effect of prothrombinase assembly on reaction steps in the inhibition pathway that were inaccessible to measurements of enzyme inhibition. The reaction between Xa and rTAP was measured by fluorescence stopped-flow, using the reversible serine protease inhibitor PAB, which exhibits enhanced fluorescence when bound to the active site of factor Xa (Craig et al., 1989).

Reactions initiated by rapid mixing of rTAP with the preformed Xa–PAB binary complex yielded a biphasic change in fluorescence (Figure 4A). The fluorescence intensity increased rapidly with a $t_{1/2} \cong 20$ ms to a value substantially greater than that of the Xa–PAB complex, followed by a slow decay ($t_{1/2} \cong 5.7$ s) to a value in the range of the same concentration of free PAB. Equivalent findings have been previously described in the detailed stopped-flow studies of Jordan et al. (1992) using human factor Xa and have been interpreted according to Scheme 2, where the subscripted rate constants are equivalent to those described in Scheme 1 and P denotes PAB. E and P are assumed to be in rapid equilibrium, governed by the dissociation constant $K_{E,P}$. The starting fluorescence signal is determined by the concentration of EP. The rapid increase in fluorescence following mixing of I with EP results from the formation of the ternary EPI and EPI* species. The subsequent slow decrease in the signal to a value comparable to that of free PAB results from the dissociation of P from EI* followed by the reversible, unimolecular formation of EI**.

Under pseudo-first-order conditions ($I > E$ and $I \gg K_i$*), the fluorescence change would result from two consecutive first-order reactions that are far from equilibrium, described by eq 5 in Data Analysis. The observed signal was adequately described by this expression (Figure 4A) and yielded the rate constants ($k_{1,obs}$ and $k_{2,obs}$) describing the rise and fall phases, respectively. The inclusion of saturating concentrations of

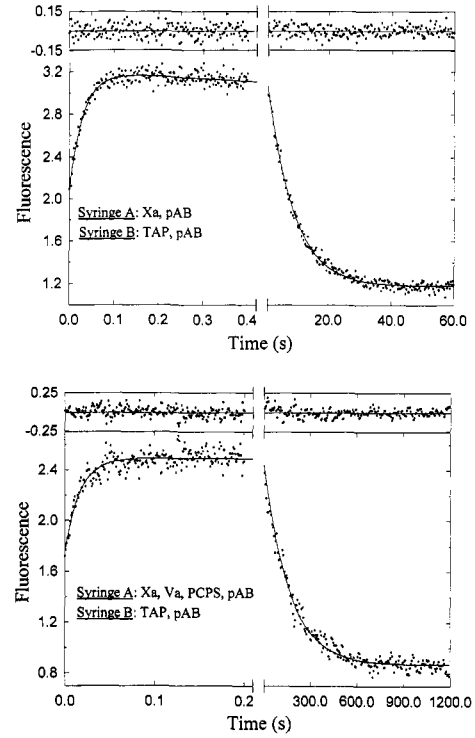


FIGURE 4: Stopped-flow measurements of the reaction between Xa and TAP. (A, Top) Reaction of TAP with solution-phase factor Xa. The reaction between Xa and TAP was monitored following rapid mixing of equal volumes of Syringe A (1.0 μM Xa, 25 μM pAB in assay buffer) with Syringe B (20 μM TAP, 25 μM pAB in the same buffer). Reaction progress was monitored using λ_{ex} = 320 nm and $\lambda_{em} \geq 345$ nm by the acquisition of 200 points in 0.4 s, followed by 200 points in 59.6 s. The curve is drawn according to eq 5, using the fitted parameters $k_{1,obs} = 32.35 \pm 1.0$ s^{-1}, $k_{2,obs} = 0.123 \pm 0.001$ s^{-1}, $F_1 = 0.897 \pm 0.022$, $F_2 = 2.02 \pm 0.006$, and offset = 1.18 ± 0.005. (B, Bottom) Reaction of TAP with preassembled prothrombinase. The contents of the driving syringes were as follows: Syringe A, 1.0 μM Xa, 1.5 μM Va, 200 μM PCPS, 25 μM pAB; Syringe B, 20 μM TAP, 25 μM pAB. Data were acquired over two time scales (200 points in 0.2 s and 200 points in 1199.8 s). The curve is drawn according to eq 5, using the fitted parameters $k_{1,obs} = 65.67 \pm 5.6$ s^{-1}, $k_{2,obs} = 0.0063 \pm 0.0001$ s^{-1}, $F_1 = 0.869 \pm 0.041$, $F_2 = 1.62 \pm 0.01$, and offset = 0.869 ± 0.008. The residuals to the fitted line are illustrated in the top portion of each panel.

Scheme 2

$$E + P \overset{K_{E,P}}{\rightleftharpoons} EP + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EPI \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} EPI^* \overset{K_{EI^*,P}}{\rightleftharpoons} EI^* + P \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} EI^{**}$$

PCPS to preform the Xa–PCPS binary complex had no detectable effect on the stopped-flow results (data not shown). These findings are consistent with the identical results obtained in the inhibition measurements (above) using either solution-phase factor Xa or the Xa–PCPS binary complex.

A stopped-flow trace obtained by reacting rTAP with factor Xa saturably incorporated into the prothrombinase complex is illustrated in Figure 4B. Although the fluorescence trace obtained with prothrombinase exhibited the same rise and fall behavior observed in the reaction between solution-phase Xa and rTAP, the rate constant for the rapid rise in fluorescence ($k_{1,obs}$) was increased approximately 2-fold. In addition, the decay phase in the reaction between rTAP and prothrombinase was approximately 20-fold slower, requiring measurement over 20 min instead of the 1-min observation period necessary for the reaction between rTAP and solution-phase factor Xa.

Control experiments were performed to verify that the observed changes were related to a specific effect of factor Va

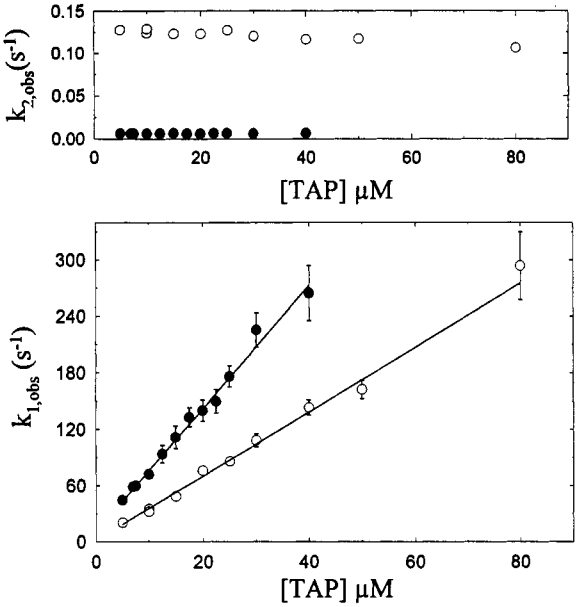


FIGURE 5: Dependence of the observed rate constants on the concentration of TAP. Stopped-flow measurements were conducted by reacting increasing concentrations of TAP with either 0.5 μM solution-phase factor Xa (○) or 0.5 μM prothrombinase, 0.5 μM Xa, 0.75 μM Va, and 100 μM PCPS (●), and measured with 25 μM pAB as illustrated in Figure 4. The linear increase in the rate constant for the rapid increase in fluorescence ($k_{1,obs}$, lower panel) was used to extract the intrinsic values of k_{+1} = (3.35 ± 0.11) × 10^6 $M^{-1}·s^{-1}$ for the reaction with solution-phase factor Xa and k_{+1} = (6.58 ± 0.32) × 10^6 $M^{-1}·s^{-1}$ for prothrombinase. The rate constant for the slow decay of fluorescence ($k_{2,obs}$, upper panel) was independent of TAP and yielded mean values of 0.123 ± 0.011 s^{-1} for the reaction with factor Xa and (6.3 ± 0.07) × 10^{-3} s^{-1} for prothrombinase. The mean ±1 SD from 6–10 replicate measurements is illustrated for each data point.

on the reaction between the protease and rTAP and not due to effects on the indicator reaction (data not shown): (i) Equilibrium titrations of Xa, Xa–PCPS, or Xa–Va–PCPS with PAB yielded equivalent dissociation constants ($K_{E,P}$, Scheme 2). The observed changes are therefore not related to altered PAB binding by Xa in the presence of prothrombinase constitutents. (ii) Fluorescence stopped-flow measurements of the displacement of PAB from Xa or the Xa–Va–PCPS complex with benzamidine indicated that the dissociation reaction proceeded with a rate constant in excess of 200 s^{-1}. The intrinsic dissociation rate constant of PAB from the enzyme therefore does not appear to limit the fluorescence changes, and (iii) essentially superimposable stopped-flow traces with and without Va and PCPS were obtained when reactions were conducted in buffer containing 2 mM EDTA instead of 2 mM Ca^{2+}. Thus, the altered values of $k_{1,obs}$ and $k_{2,obs}$ in the presence of factor Va and PCPS result from the Ca^{2+}-dependent assembly of the prothrombinase complex on the membrane surface.

The dependence of the observed rate constants on increasing concentrations of rTAP for the reaction using solution-phase factor Xa or the Xa–Va–PCPS complex is illustrated in Figure 5. In both cases, the rate constant for the rapid rise in fluorescence ($k_{1,obs}$) increased linearly with increasing concentrations of the inhibitor. No indications of saturation were obtained even at values of $k_{1,obs}$ as high as ~300 s^{-1}, which represents the upper limit for reliable measurements with the present instrumentation. This rate constant was independent of the concentration of PAB (20-200 μM, below) and the fixed concentration of factor Xa. Thus, as previously reported (Jordan et al., 1992), the rapid phase of the stopped-flow measurements is first order in I and zero order in both E and

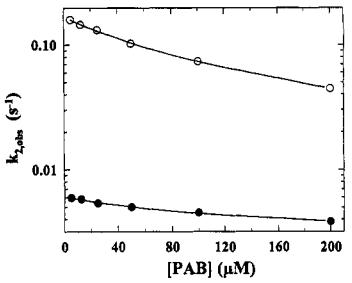


FIGURE 6: Dependence of $k_{2,obs}$ on the concentration of PAB. The rate constant for the slow decay in fluorescence ($k_{2,obs}$) was measured at increasing concentrations of pAB by measuring the reaction between 17.5 μM TAP and 0.5 μM factor Xa (○) or 0.5 μM prothrombinase: 0.5 μM Xa, 0.75 μM Va, and 100 μM PCPS (●). The curves are drawn following analysis according to eq 7 to yield $K_{EI^*,P}$ = 86.68 ± 9.08 μM and $k_{+3} + k_{-3}$ = 0.17 ± 0.007 s^{-1} for the reaction with factor Xa or $K_{EI^*,P}$ = 116.3 ± 25.6 μM and $k_{+3} + k_{-3}$ = 6.09 × 10^{-3} ± 3.2 × 10^{-4} s^{-1} for the reaction with prothrombinase.

P, eactly as would be expected if the increase in fluorescence reflected the formation of the ternary species (EPI + EPI*, Scheme 2). The intrinsic second-order rate constant (k_{+1},- Scheme 2) obtained from the slope of the plots was (3.35 ± 0.11) × 10^6 $M^{-1}·s^{-1}$ for the reaction of rTAP with solution-phase factor Xa and (6.58 ± 0.32) × 10^6 $M^{-1}·s^{-1}$ for the reaction with factor Xa incorporated into prothrombinase. These values compare favorably with the second-order rate constants determined from inhibition measurements (Table 1) and are consistent with the conclusion that one consequence of the interaction between factors Va and Xa is a modest increase in the rate at which rTAP can bind to factor Xa.

The rate constant for the slow decrease in fluorescence ($k_{2,obs}$) was independent of the concentration of rTAP (Figure 5). However, over the range of rTAP concentrations studied, the values of $k_{2,obs}$ determined for the reaction of rTAP with solution-phase factor Xa were consistently ~20-fold greater than those obtained with the fully assembled prothrombinase complex. One conclusion from these measurements is that the interaction of Xa with factor Va on the membrane surface has a large effect on the rate constant(s) for steps that follow the initial binding of rTAP to the protease.

The significantly lower values for $k_{2,obs}$ obtained with prothrombinase could also arise from large differences in K_{P,EI^*} for solution-phase factor Xa *versus* the enzyme complex. This possibility was evaluated by studying the dependence of $k_{2,obs}$ on the concentration of PAB in the reaction of rTAP with factor Xa or the Xa–Va–PCPS ternary complex (Figure 6). In both cases, $k_{2,obs}$ decreased with increasing concentrations of PAB. The data were analyzed according to eq 7 to obtain estimates for K_{P,EI^*} and the intrinsic rate constants for the isomerization of EI* to EI**.

$$k_{2,obs} = k_{-3} + \frac{k_{+3}K_{P,EI^*}}{(K_{P,EI^*} + PAB)} \quad (7)$$

Although k_{-3} was poorly determined in either case, values of $k_{+3} + k_3$ determined by extrapolation to zero PAB were approximately 27-fold lower for the reaction of rTAP with prothrombinase than with solution-phase factor Xa (Figure 6). In contrast, fitted values of K_{P,EI^*} determined for the two enzyme species were comparable to each other. These data support the conclusion that the large decrease in $k_{2,obs}$ evident in the reaction of rTAP with prothrombinase does not appear to be related to a decrease in K_{P,EI^*} or other features of the indicator reaction influenced by the Xa–Va interaction.

The findings of the stopped-flow studies are consistent with the conclusions derived from measurements of the inhibition of factor Xa *versus* Xa–Va–PCPS by rTAP. The results

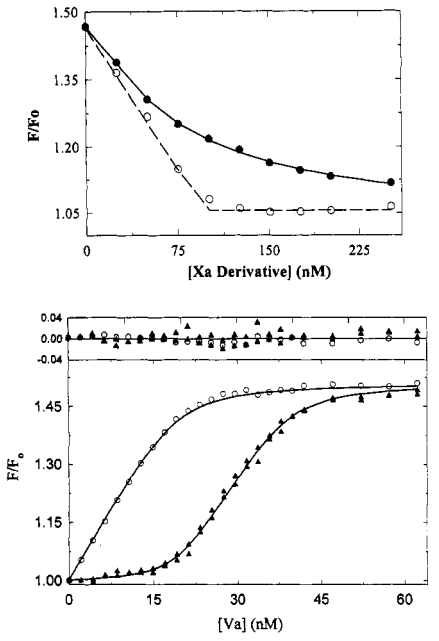


FIGURE 7: Effect of TAP on the interaction between factors Xa and Va. (A, Top) Fluorescence measurements were performed as described under Experimental Procedures using reaction mixtures containing 50 nM DEGR-Xa, 70 nM Va, and 100 μM PCPS in assay buffer with increasing concentrations of factor Xa (●) or the preformed Xa–TAP binary complex (○). The solid curve was drawn using fitted values of $K_{d_{Comp\text{-}Va}}$ = 1.25 ● 0.29 nM, $n_{Comp\text{-}Va}$ = 1.08 ± 0.08 mol competitor/mol Va at saturation, F_0 = 1.00 ± 0.02, and F_{max} = 0.5 ● 0.02, assuming the values of $K_{d_{Xa\text{-}Va}}$ and $n_{Xa\text{-}Va}$ determined in part B. The dashed lines were arbitrarily drawn. (B, Bottom) Reaction mixtures contained 20 nM DEGR-Xa , 50 μM PCPS, and 100 nM TAP (○) or 20 nM DEGR-Xa, 50 μM PCPS, and 100 nM TAP *plus* 20 nM unmodified Xa (▲) in assay buffer at 25 °C. The incremental increase in fluorescence intensity was determined as described under Experimental Procedures following titration with increasing concentrations of factor Va. The curves are drawn using the fitted values of $K_{d_{Xa\text{-}Va}}$ = 1.16 ● 0.15 nM, $n_{Xa\text{-}Va}$ = 0.89 ± 0.02 mol Xa/mol Va at saturation, $K_{d_{Comp\text{-}Va}}$ = 30.7 ± 6.2 pM, $n_{Comp\text{-}Va}$ = 1.01 ± 0.01 mol competitor/mol Va at saturation, F_0 = 1.0 ● 0.003, and F_{max} = 0.53 ± 0.02.

collectively suggest that the incorporation of factor Xa into the prothrombinase complex leads to altered rate constants for at least two steps in the reaction pathway (k_{+1} and k_{+3} + k_{-3}) and a significant increase (~30-fold) in the overall stability of the protease–rTAP complex. Since these effects result from the interaction between factors Xa and Va on the membrane surface, the data are consistent with the interpretation that the cofactor elicits a conformational change in factor Xa that leads to a more favorable interaction between the inhibitor and protease. If all reaction steps have been adequately considered, these conclusions predict that the binding of rTAP to factor Xa should, in turn, significantly increase the affinity for the protease–cofactor interaction.

Effect of rTAP on the Interaction between Factors Xa and Va. The effect of rTAP on the interaction between factors Xa and Va on the membrane surface was assessed by using DEGR-Xa and measuring the fluorescence change that accompanies the incorporation of this fluorescent Xa derivative into prothrombinase (Nesheim et al., 1981b).

In initial experiments, the affinity of the Xa–rTAP binary complex for factor Va was assessed by examining the ability of either factor Xa or the Xa–rTAP binary complex to displace DEGR-Xa from the preformed DEGR–Xa–Va–PCPS ternary complex (Figure 7A). The saturable incorporation of DEGR-Xa into prothombinase results in a large increase in fluorescence intensity (52–55%) of this reporter group (Krishnaswamy et al., 1988). When the concentration of PCPS is in excess, the individual protein-membrane binding steps are saturated, leading to the simplification of the interpretation of the fluorescence change to represent the interaction of DEGR-Xa and Va on the membrane surface (Krishnaswamy, 1990).

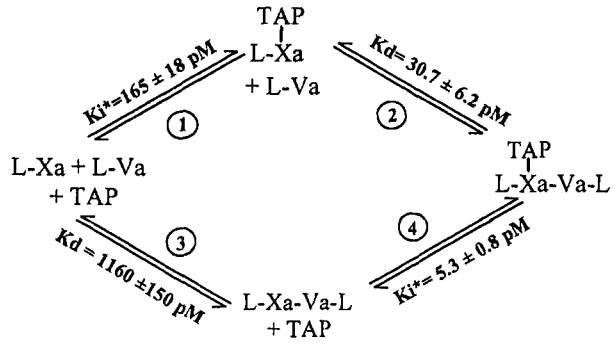
In these experiments, increasing concentrations of unmodified factor Xa decreased the fluorescence intensity monotonically, reflecting the ability of Xa to compete with DEGR-Xa for the interaction with factor Va. Equilibrium constants determined from this experiment indicated that Xa and DEGR-Xa interact with factor Va with near identical affinities as has previously been described (Nesheim et al., 1981b; Krishnaswamy, 1990). In contrast, incremental additions of the preformed Xa–rTAP binary complex yielded a greater reduction in the fluorescence signal at lower concentrations (Figure 7A). The resultant titration curve was characterized by a sharp break, implying that Xa bound to rTAP has a significantly increased affinity for factor Va. Control experiments established that equivalent concentrations of rTAP had no significant effect on the fluorescence of DEGR-Xa. This observation is consistent with a relatively weak interaction between rTAP and human DEGR-Xa (K_d ~ 6 μM) determined by Jordan et al. (1992). However, the interpretation of these experiments is complicated by the fact that the concentration of Xa and rTAP were simultaneously varied in these experiments.

Competition experiments were therefore conducted to examine the ability of DEGR-Xa and Xa–rTAP to compete for added factor Va in the presence of saturating concentrations of PCPS (Figure 7B). Factor Va was the varied ligand in the presence of a fixed concentration of DEGR-Xa, PCPS, and rTAP using different fixed concentrations of unmodified Xa. In the absence of factor Xa, the increase in fluorescence intensity saturated monotonically. This curve was essentially identical to one obtained in the absence of added rTAP (data not shown). Thus, 100 nM rTAP has no detectable effect on the binding of DEGR-Xa to factor Va on the membrane surface. In the presence of both Xa and rTAP, a sigmoid titration curve was obtained. Such a curve shape is expected if the competitor binds to the varied ligand with much greater affinity than does DEGR-Xa. The data were analyzed as described in the Data Analysis to simultaneously extract the equilibrium parameters for the DEGR–Xa–Va interaction and the rTAP–Xa–Va interaction along with the limits of the fluorescence data (Figure 7B). The fitted parameters for the interactions of DEGR-Xa ($K_{d_{Xa\text{-}Va}}$, $n_{Xa\text{-}Va}$, and F_{max}) are comparable to those determined previously in a variety of different types of experiments (Krishnaswamy, 1990). The parameters obtained for the interaction of Xa–rTAP with factor Va indicate that approximately 1 mol of inhibitor–protease complex also binds to factor Va at saturation but with a dissociation constant ($K_{d_{Comp\text{-}Va}}$ = 30.7 ± 6.2 pM) that is approximately 35-fold lower. Thus, the binding of rTAP to factor Xa substantially alters the affinity of the Xa–Va interaction on the membrane surface.

DISCUSSION

The reactions between rTAP and factor Xa or factor Xa saturably incorporated into prothrombinase have been examined by kinetic studies of enzyme inhibition, by stopped-flow measurements designed to resolve several steps in the inhibition pathway, and by direct binding studies of prothrombinase assembly. The results from all three types of measurements are internally consistent and indicate that the interaction between factors Va and Xa on the membrane surface leads to a ~30-fold increase in the overall affinity of

Scheme 3



the protease for the inhibitor. The increased stability of the protease–inhibitor complex is achieved through a modest change in the bimolecular reaction which yields the EI binary complex (step 1, Scheme 1) and through a much larger change in the rate constant(s) for a following step (step 3, Scheme 1) that involves the reversible isomerization of the binary complex. One interpretation of these observations is that factor Va elicits changes in sites on the protease (a conformational change) that lead to altered interactions between factor Xa and rTAP. Additional support for this hypothesis is provided by the finding that the binding of rTAP to factor Xa also enhances the ability of the protease to interact with the cofactor. The inferred and measured thermodynamic parameters are illustrated in Scheme 3, where L represents a site for either Xa or Va on the PCPS surface, and prothrombinase is represented as the L–Xa–Va–L complex on the basis of previous work (Krishnaswamy, 1990). rTAP can bind either to the Xa–L binary complex or to preassembled prothrombinase governed by the overall equilibrium dissociation constants (K_i*, Table 1). The values for K_i* (steps 1 and 4) determined from kinetic studies of enzyme inhibition indicate a ~30-fold increase in the affinity of rTAP for factor Xa when the protease is incorporated into prothrombinase. The dissociation constants for prothrombinase assembly and for the binding of the Xa–rTAP complex to factor Va were derived from fluorescence measurements (steps 2 and 4). In each case, the use of saturating concentrations of PCPS permitted experimental simplification to yield the K_d for the interaction between membrane-bound proteins, as previously described (Krishnaswamy, 1990). The Xa–rTAP binary complex bound to factor Va on the membrane surface with a K_d that was ~37-fold lower.

The equilibrium dissociation constants illustrated in Scheme 3 indicate that the decreased K_i* for the inhibition of prothrombinase by rTAP can be accounted for by a proportional decrease in the K_d for the binding of the rTAP–Xa binary complex to factor Va (*i.e.* $\Delta G°_1 + \Delta G°_2 \approx \Delta G°_3 + \Delta G°_4$). Therefore, within experimental uncertainty, Scheme 3 implies an adequate thermodynamic description of the reactions involved and supports the interpretation that changes in the reaction of rTAP with prothrombinase result from factor Va-induced perturbations in the extended macromolecular recognition sites on factor Xa.

Two alternative explanations could also account for the observations: (i) rTAP could, itself, bind to membranes or indirectly induce changes in the factor Xa–membrane interaction, and (ii) rTAP could simultaneously bind to factor Va and factor Xa. The first possibility is not consistent with the minimal effects of the Xa–membrane interaction on the observed kinetic and equilibrium constants (Table 1). In addition, the fluorescence binding measurements conducted at saturating concentrations of PCPS would be insensitive to any increase in the affinity of Xa–rTAP for membranes. The second possibility would imply that the increased affinity of rTAP for prothrombinase or of Xa–rTAP for factor Va is not related to changes in the factor Xa molecule but arises from an additional but weak interaction ($K_d \sim 10^{-3}$–10^{-2} M) between rTAP and factor Va. While this possibility may seem unlikely and is not presently favored, we are unable to rule it out.

The kinetic measurements of the reaction between rTAP and bovine factor Xa are generally in good agreement with data previously obtained in studies using human factor Xa (Jordan et al., 1992). The only area of discrepancy relates to the results of the stopped-flow measurements using PAB. A hyperbolic dependence of the rate constant for the rapid increase in fluorescence ($k_{1,obs}$ in the present work) on the concentration of rTAP was previously observed with human factor Xa in the absence of added Ca^{2+} (Jordan et al., 1992). In contrast, $k_{1,obs}$ was found to increase linearly with increasing rTAP in the present work (Figure 5). Selected experiments repeated in assay buffer lacking Ca^{2+} indicated that this was not a reason for the observed discrepancy (not shown). There may be significant kinetic differences in the reaction of rTAP with human and bovine Xa. However, the close agreement between the second-order rate constant (k_{inh}) determined from inhibition measurements (Table 1) and the values of k_{+1} determined from the stopped-flow studies (Figure 5), along with the lack of obvious saturation at rate constants as high as ~300 s^{-1}, all suggest that a reasonable explanation may be related to the inadequacy of the rapid equilibrium assumption in describing the binding of rTAP to bovine factor Xa (*i.e.* $k_{+1}I + k_{-1}$ not much greater than $k_{+2} + k_{-2}$). We have therefore interpreted the measured rate constants accordingly. Consequently, the three-step kinetic mechanism (Scheme 1) determined for human factor Xa (Jordan et al., 1992) has been retained for the sake of consistency even though a two-step reaction would be sufficient to interpret the data obtained with bovine factor Xa.

Other workers have previously compared the inhibition of solution-phase factor Xa with that of prothrombinase using several other plasma proteinase inhibitors (Walker & Esmon, 1979: Ellis et al., 1984). In all cases, the incorporation of factor Xa into prothrombinase either reduced the rate of inhibition or had no effect on the reaction at plasma concentrations of the inhibitors tested. Thus, rTAP represents a unique inhibitor for factor Xa in that the inhibition of factor Xa incorporated into prothrombinase is the thermodynamically favored reaction. This may result from the highly specific nature of the Xa–rTAP interaction involving exosite(s) on factor Xa that are not involved in the inhibition reactions of the other less-selective plasma proteinase inhibitors. Interestingly, recent data obtained with tissue factor pathway inhibitor also indicate that inhibition of factor Xa is enhanced in the presence of the other constituents of prothrombinase (Huang et al., 1993).

Previous studies of the reaction of the irreversible transition-state inhibitor DEGRck failed to provide any indication of changes in the catalytic residues of factor Xa following its incorporation into the prothrombinase complex (Walker & Krishnaswamy, 1993). Thus, the mechanism through which factor Va alters the k_{cat} for prothrombin activation does not apparently involve changes in the catalytic residues but many involve altered interactions at sites removed from the catalytic residues on factor Xa. The use of rTAP as a probe of the extended macromolecular interaction sites of factor Xa in the present study has provided evidence that the surface of factor Xa is in fact altered as a result of its interaction with factor Va and that these perturbations can lead to changes in

macromolecular interactions with the protease. Taken together, these observations imply that changes in the exosite(s) of the protease are one consequence of the incorporation of factor Xa into the prothrombinase complex. It remains to be established whether these changes can influence the interaction between factor Xa and prothrombin, and they are thus at least partly responsible for the increased catalytic efficiency of the prothrombinase complex.

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