Selective Inhibition of the Prothrombinase Complex: Factor Va Alters Macromolecular Recognition of a Tick Anticoagulant Peptide Mutant by Factor Xa[†,‡]

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ABSTRACT: The prothrombinase complex assembles through reversible interactions between the protease, factor Xa, the cofactor, factor Va, and acidic phospholipid membranes in the presence of calcium ions. Changes in macromolecular recognition by factor Xa which may result from its interaction with factor Va in the prothrombinase complex have been probed using a recombinant derivative of tick anticoagulant peptide where Arg^3 has been replaced with Ala (R3A-TAP). In contrast to the wild type inhibitor, R3A-TAP was a weak competitive inhibitor of factor Xa (K_i = 794 nM). The inhibition of the prothrombinase complex by R3A-TAP was characterized by slow, tight-binding kinetics with an increased affinity of ∼4000-fold (K_i^* = 0.195 nM) relative to that of solution-phase factor Xa. Stopped-flow measurements using *p*-aminobenzamidine (PAB) demonstrated that the reaction between solution-phase factor Xa and R3A-TAP could be adequately described by a single reversible step with rate constants that were consistent with equilibrium binding measurements. The rate-limiting bimolecular combination of R3A-TAP and factor Xa was competitive with PAB binding to the protease. In contrast, the reaction of R3A-TAP with prothrombinase measured using PAB yielded biphasic stopped-flow traces, indicating a multistep pathway for the reaction of the inhibitor with the enzyme complex. The kinetic measurements were consistent with the initial formation of a ternary complex between R3A-TAP, prothrombinase, and PAB followed by two unimolecular steps which lead to PAB dissociation from the enzyme. In this case, prior occupation of the active site by PAB had no effect on the bimolecular reaction between R3A-TAP and prothrombinase. Thus, the interaction of factor Xa with factor Va on the membrane surface alters recognition of R3A-TAP by the protease, leading to changes in the thermodynamics as well as in the observed kinetic mechanism for the reaction. Therefore, a single amino acid substitution in TAP reveals large changes in macromolecular recognition by factor Xa as a consequence of its interaction with the cofactor within the prothrombinase complex.

The prothrombinase complex is formed by association of the protease factor Xa with factor Va on appropriate phospholipid surfaces in the presence of calcium ions (Jackson & Nemerson, 1980; Mann et al., 1988, 1990). This enzyme complex converts prothrombin to the clot forming protease thrombin by two successive cleavages (Jackson & Nemerson, 1980; Mann et al., 1988, 1990). Although factor Xa, the catalytic component of prothrombinase, is also capable of activating prothrombin in solution, incorporation into prothrombinase amplifies its catalytic activity by a factor of $\sim 10^5$ (Mann et al., 1988; Nesheim et al., 1979; Rosing et al., 1980). The full catalytic activity of prothrombinase may result from a cumulative effect of the interaction of all accessory components with factor Xa. However, kinetic studies suggest that factor Xa in solution saturated with high concentrations of factor Va approximates the catalytic efficiency of the membrane-assembled prothrombinase (Boskovic et al., 1990). Hence, the enhanced activity of factor Xa within prothrombinase can largely be attributed to the interaction with factor Va, while the ability of factor Va, factor Xa, and prothrombin to bind to phospholipid membranes promotes the formation of the protease−cofactor complex and subsequently the delivery of the substrate to membrane-bound enzyme (Mann et al., 1990; Boskovic et al., 1990; Krishnaswamy et al., 1988; Giesen et al., 1991a,b; Walker & Krishnaswamy, 1994).

The effect of factor Va on prothrombin activation may result from alterations induced in the protease and/or the substrate as both prothrombin and factor Xa can bind to the cofactor (Jackson & Nemerson, 1980; Mann et al., 1988; Esmon & Jackson, 1974; Luckow et al., 1989). Alterations in factor Xa resulting from its interaction with factor Va have been inferred from changes in fluorescence intensity and anisotropy of a fluorophore covalently tethered to the active site of factor Xa using the active site label dansylglutamylglycylarginyl chloromethyl ketone (Nesheim et al., 1981; Higgins et al., 1985; Husten et al., 1987). Since, in contrast to prothrombin, dansylglutamylglycylarginyl chloromethyl

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ketone is a substrate analog that lacks the ability to bind to the cofactor, it has been used to compare the reactivities of the active site of prothrombinase with that of factor Xa (Walker & Krishnaswamy, 1993). The kinetics of inactivation of factor Xa by this reagent were not significantly affected by incorporation of the protease into prothrombinase (Walker & Krishnaswamy, 1993). These lines of evidence have suggested the possibility that factor Va induces alterations at sites of factor Xa remote from the catalytic residues, referred to as exosites, which could contribute to the acceleration of prothrombin activation (Walker & Krishnaswamy, 1993). Detection of these altered sites in factor Xa incorporated into the prothrombinase complex requires specific macromolecular ligands of factor Xa which lack interactions with factor Va and membranes.

Tick anticoagulant peptide originally isolated from *Ornithodoros moubata* (wt-TAP[1]) has been shown to be a tight and specific inhibitor of factor Xa ($K_i^* = 180$ pM) (Waxman et al., 1990). The 60 residues of wt-TAP form a globular structure linked by three disulfide bonds with the first four N-terminal residues extending into solution (Sardana et al., 1991; Lim-Wilby et al., 1995; Antuch et al., 1994). Mutagenesis studies have been employed to localize sites responsible for interacting with factor Xa (Dunwiddie et al., 1992; Mao et al., 1995). A substantial loss of binding energy was observed after substitution of Arg^3 by Asn, indicating an essential role for this residue in the inhibition of factor Xa (Dunwiddie et al., 1992). In addition, a synthetic peptide containing the first nine N-terminal residues of TAP was also found to inhibit the hydrolytic activity of factor Xa, further substantiating the role of Arg^3 in contributing to binding interactions within the active site cleft (Mao et al., 1995). Mutagenesis studies with TAP have also provided some evidence for additional interactions remote from the active site of factor Xa at putative exosites (Dunwiddie et al., 1992). These types of interactions were also invoked in the interpretation of the multistep mechanism observed for the inhibition of factor Xa by wt-TAP (Jordan et al., 1992). In contrast, other macromolecular inhibitors of factor Xa, in particular antithrombin, interact predominantly with the active site region of factor Xa (Olson & Bjork, 1992). Therefore, the unique specificity and affinity of wt-TAP for factor Xa appear to be related, in some way, to extended macromolecular interactions between protease and inhibitor.

Unlike most other inhibitors of factor Xa (Ellis et al., 1984; Walker & Esmon, 1979), recent studies have demonstrated that the affinity of factor Xa for wt-TAP is increased by a factor of ~30 following the assembly of the prothrombinase complex (Krishnaswamy et al., 1994). In stopped-flow measurements comparing the binding of wt-TAP to factor Xa and to prothrombinase, a ternary complex consisting of wt-TAP, protease, and the active site probe, *p*-aminobenzamidine, was found to be stabilized as a result of the Xa−Va interaction on membranes (Krishnaswamy et al., 1994). The higher stability of the wt-TAP−prothrombinase interaction was attributed to a modulation of exosite interactions by factor Va (Krishnaswamy et al., 1994). Similarly, modulation of the recognition of the physiologic substrate prothrombin, at possibly these same exosites of factor Xa by the cofactor, might account not only for the higher efficiency of activation of prothrombin by prothrombinase *versus* factor Xa in solution but also for the associated differences in the recognition of the two cleavage sites in prothrombin despite their identical P3−P1[2] residues (Nesheim & Mann, 1983).

Since these favorable alterations in the interaction of factor Xa and wt-TAP at exosites should be more pronounced after abolishment of the active site interactions, the mutant R3A-TAP was prepared and characterized with the intent of developing a more selective probe for alterations in factor Xa structure induced by factor Va in the prothrombinase complex.

EXPERIMENTAL PROCEDURES

Materials

Hepes, L-α-phosphatidylcholine (hen egg), and L-α-phosphatidylserine (bovine brain) were obtained from Sigma (St. Louis, MO). Polyethylene glycol 8000 (PEG) was from J. T. Baker. Cyclohexylglycylglycylarginine *p*-nitroanilide (Spectrozyme Xa, SpXa) was purchased from American Diagnostica (Greenwich, CT), and *p*-aminobenzamidine (PAB) was purchased from Aldrich (Milwaukee, WI). Stock solutions were prepared in water, and the concentrations were determined spectrophotometrically using $E^{M}_{342} = 8270\ M^{-1}\ cm^{-1}$ for SpXa and $E^{M}_{293} = 15\,000\ M^{-1}\ cm^{-1}$ for PAB (Lottenberg & Jackson, 1983; Evans et al., 1982). Phospholipid vesicles (PCPS) were prepared by sonication of an emulsion of 75% L-α-phosphatidylcholine and 25% L-α-phosphatidylserine and differential centrifugation as described (Higgins & Mann, 1983; Krishnaswamy et al., 1993). The concentration of phospholipid is stated in terms of the concentration of inorganic phosphorus determined after oxidation by a phosphomolybdate assay (Gomori, 1942). Unless otherwise specified, all kinetic and binding studies were performed in 20 mM Hepes (pH 7.4), 150 mM NaCl, 2 mM $CaCl_2$, and 0.1% (w/v) PEG (referred to as assay buffer).

Proteins

Bovine factor X was purified from bovine plasma and converted to Xa by proteolytic cleavage by the purified factor X activator isolated from Russell's Viper Venom (Jesty & Nemerson, 1976). The resulting factor Xa was separated from the activation peptide and venom by affinity chromatography on benzamidine-Sepharose (Krishnaswamy et al., 1987, 1993). The preparations contained approximately equal amounts of α- and β-forms. Active site titration with *p*-nitrophenol *p*′-guanidinobenzoate (ICN Pharmaceuticals, Cleveland, OH) (Chase & Shaw, 1967) yielded a range of 1.08−1.15 mol of active sites/(mol of factor Xa) in several different preparations, implying a small systematic underestimate in the extinction coefficient for the protease (below). Bovine factor Va was prepared as described previously with an additional purification step on fast flow S-Sepharose (Krishnaswamy & Mann, 1988; Kalafatis et al., 1993). The homogeneity and purity of the protein preparations were established by SDS−PAGE (Laemmli, 1970).

[1] Abbreviations: PAB, *p*-aminobenzamidine; PEG, polyethylene glycol 8000; PCPS, small unilamellar vesicles composed of 25% (w/w) phosphatidylserine and 75% (w/w) phosphatidylcholine; SpXa, Spectrozyme Xa, cyclohexylglycylglycylarginine *p*-nitroanilide; R3A-TAP, recombinant mutant of tick anticoagulant peptide with alanine substituted for arginine at position 3; wt-TAP, wild type recombinant tick anticoagulant peptide.

[2] Nomenclature of Schechter and Berger (1967).

The mutation Arg[3]Ala was introduced into a synthetic gene encoding wt-TAP by using oligonucleotide-directed mutagenesis as described (Stanssens et al., 1989), and the protein was expressed in the yeast *Pichia pastoris* as described previously for wt-TAP (Laroche et al., 1994). The secreted product was purified from the culture medium by cation exchange chromatography and reversed-phase HPLC using procedures developed for wt-TAP and stored lyophilized as the trifluoroacetate (Laroche et al., 1994). The purified product was characterized by microbore reversed-phase HPLC, amino acid analysis, and N-terminal sequencing of the intact protein as well as of the three fragments derived from a lysyl endopeptidase (Wako, Richmond, VA) digest of reduced and carboxymethylated material (Sardana et al., 1991). Further characterization was performed by electrospray mass spectrometry of R3A-TAP as well as of its proteolytic fragments. Stock solutions of R3A-TAP (~600 μM) were prepared in water and stored at 4 °C.

The concentrations of the protein solutions were determined 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 (Jackson et al., 1968; Fujikawa et al., 1974); and factor Xa, 45 300, 1.24 (Jackson et al., 1968; Fujikawa et al., 1974). The molecular weight for wt-TAP was 6980 and for R3A-TAP was 6894. The calculated extinction coefficient (Gill & von Hippel, 1989) for both proteins was 17 900 M^{-1} cm^{-1} which was confirmed by amino acid analysis.

Inhibition Measurements

Dissociation constants for R3A-TAP binding to factor Xa were inferred from measurements of residual factor Xa amidolytic activity following incubation of the protease with increasing concentrations of the inhibitor (Krishnaswamy et al., 1994). The assays were performed at room temperature (23 ± 2 °C) in assay buffer. When calcium was omitted, assay buffer contained 5 mM EDTA instead of 2 mM $CaCl_2$. For measurements of the interaction of R3A-TAP with factor Xa alone, reaction mixtures (150 μL) prepared in a 96-well plate contained 0.5 nM factor Xa and different fixed concentrations of R3A-TAP. Following incubation at room temperature, the reactions were initiated with 50 μL containing increasing concentrations of SpXa prepared in assay buffer. Initial velocities for substrate hydrolysis were determined by monitoring absorbance at 405 nm using a V_{max} kinetic plate reader (Molecular Devices, Menlo Park, CA).

For the determination of the effect of the accessory components of prothrombinase on the inhibition of factor Xa by R3A-TAP, increasing concentrations of inhibitor were incubated in reaction mixtures (150 μL) containing 0.5 nM factor Xa plus 40 μM PCPS or the indicated concentrations of factor Xa plus 40 μM PCPS and 40 nM factor Va in either assay buffer or buffer containing EDTA. Residual enzyme activity was determined after a 90 min incubation at room temperature as described above following the addition of a 50 μL solution of 800 μM SpXa prepared in the appropriate buffer. For the measurements with the complete prothrombinase complex, the concentrations of R3A-TAP reflect those present in the initial 150 μL incubation mixture. For all other combinations, calculations used inhibitor and substrate concentrations present in the final 200 μL reaction mixture.

Steady State Fluorescence Measurements

Steady state fluorescence measurements of the competitive binding of R3A-TAP and PAB to factor Xa were performed using a SLM 8000C fluorescence spectrophotometer (SLM Instruments, Urbana, IL) with adapted hardware and software (OLIS, Bogart, GA) as previously described (Krishnaswamy & Mann, 1988). Measurements were performed in 1 cm^2 stirred quartz cuvettes maintained at 25 °C. The excitation wavelength was 320 nm, and ratiometric fluorescence intensity ($\lambda_{em} \geq 345$ nm) was measured using a long-pass filter (Schott KV 345) in the emission beam.

All fluorescence titrations were performed in assay buffer, and four reaction mixtures (2.0 mL each) were prepared for each fixed concentration of R3A-TAP used: (A) 0.5 μM factor Xa, (B) 0.5 μM factor Xa plus R3A-TAP, (C) R3A-TAP, and (D) buffer alone. Microliter additions of PAB to achieve the indicated final concentration were made into each reaction mixture, and fluorescence intensity was recorded approximately 3 min after each addition. Periodically, fluorescence measurements were repeated after ~10 min to ensure the recording of a limiting fluorescence signal. The signals from reactions C and D were used to determine the baseline fluorescence intensity and the required correction for inner filter effects (Data Analysis). The effects of R3A-TAP alone on the fluorescence of PAB were negligible even at the highest concentrations of R3A-TAP. Inner filter corrections calculated from mixtures C and D were then applied to all four sets of data to yield the corrected fluorescence signal (F_{Acorr}, F_{Bcorr}, F_{Ccorr}, and F_{Dcorr}). The binding curve describing the interaction of PAB with factor Xa was calculated from $F_{Acorr} - F_{Dcorr}$, and the binding isotherm for the interaction of PAB with factor Xa in the presence of R3A-TAP was calculated as $F_{Bcorr} - F_{Ccorr}$.

Rapid Kinetic Measurements

Fluorescence stopped-flow experiments were performed with a two-syringe apparatus (Kinetic Instruments, Ann Arbor, MI) inserted into the SLM-8000C spectrophotometer (Krishnaswamy et al., 1988). The excitation wavelength was 320 nm, and the time course of the change in fluorescence intensity was monitored using a long-pass filter (Schott KV 345, $\lambda_{em} \geq 345$ nm) in the emission beam. All reactants were dissolved in assay buffer at 25 °C.

Rapid Kinetic Studies with Factor Xa. The reactions were initiated by rapid mixing of equal volumes of 1 μM factor Xa plus PAB (syringe A) with an inhibitor solution containing R3A-TAP or wt-TAP and the same concentration of PAB (syringe B). The final concentrations of factor Xa and inhibitor were $^1/_2$ of those present in the driving syringes. Ratiometric fluorescence traces were collected following mixing, by the acquisition of 400 data points over a period corresponding to eight to ten half-lives of the observed decay (5−80 s). Rate constants were derived from the analysis of between five and nine replicate traces. When the concentration of R3A-TAP was varied, the concentration of PAB in both syringes was fixed at 25 μM. Alternatively, the dependence of k_{obs} on the concentration of PAB was determined by varying the concentration of this ligand equally in both syringes using final concentrations of 7.5 or 15 μM R3A-TAP.

Rapid Kinetic Studies with Prothrombinase. The experimental approach for the rapid kinetic measurements on prothrombinase was similar to those used for factor Xa alone,

except that the prothrombinase complex was preformed in syringe A by the incubation of 1 μM factor Xa with saturating concentrations of factor Va (1.5 μM) and PCPS (200 μM). Therefore, the concentration of prothrombinase (enzyme) was determined by the limiting concentration of factor Xa. Since the fluorescence decays were biphasic, data were collected using two time scales (200 points, 0−300 ms, datum interval of 1.5 ms followed by 200 points, 0.3−40 s, datum interval of 0.2 s) to completely describe the rapid and slow phases.

Data Analysis

Constants were calculated from the data using nonlinear least-squares analysis according to the appropriate equations using the Marquardt algorithm (Bevington, 1969). The quality of the fits was assessed as described previously (Straume & Johnson, 1992; Durbin & Watson, 1951), and the fitted constants are listed at ±95% confidence limits.

Inhibition of Factor Xa by R3A-TAP. Initial velocity data obtained with solution-phase factor Xa or with the incompletely assembled prothrombinase complex were analyzed according to the rate expression for simple, complete competitive inhibition (Segel, 1975), to obtain fitted values of V_{max}, K_m, and K_i.

Inhibition of Prothrombinase by R3A-TAP. Initial velocity data obtained following incubation of increasing concentrations of R3A-TAP with two fixed concentrations of prothrombinase were analyzed according to eqs 1 and 2:

$$E_i = \frac{nI + E + K_i^* - \sqrt{(nI + E + K_i^*)^2 - 4nIE}}{2} \quad (1)$$

$$v_{obs} = v_\infty E + v_o E\left(1 - \frac{E_i}{E}\right) \quad (2)$$

where E and I are the total concentrations of enzyme (prothrombinase) and inhibitor (R3A-TAP), respectively, E_i is the concentration of inhibited enzyme, K_i^* is the overall dissociation constant for the binding interaction, and n represents the moles of I bound per mole of E at saturation. Equation 2 relates the concentration terms to the observed initial velocity (v_{obs}), where v_o and v_∞ are initial velocities at zero and infinite concentrations of I, respectively, normalized to the concentration of E. Analysis according to eqs 1 and 2 yielded fitted values for K_i^*, n, v_o, and v_∞.

Fluorescence Binding Measurements in the Presence of PAB and R3A-TAP. Because of the relatively weak interaction between PAB and factor Xa, high concentrations of PAB are necessary to observe the binding interaction which leads to a significant absorption of the exciting light. The data therefore require corrections for the inner filter effect (Lakowicz, 1983).

The nonlinear dependence of fluorescence on PAB titrated into buffer alone was analyzed according to eq 3:

$$F_{obs} = \frac{Pm + c}{10^{P\epsilon L}} \quad (3)$$

where P is the concentration of PAB, m and c are the slope and intercept of the expected linear dependence of corrected fluorescence on the concentration of PAB in buffer, respectively, ϵ is the extinction coefficient of PAB at the excitation wavelength (determined to be 6320 M^{-1} cm^{-1}), and L is the effective path length for the fluorescence cell. The fitted value of L determined by analysis according to eq 3 ranged from 0.43 to 0.46 cm and was used along with the measured value of ϵ to derive a corrected fluorescence signal (F_{corr}) using

$$F_{corr} = F_{obs} 10^{P\epsilon L} \quad (4)$$

where all the terms have the same meaning as in eq 3. Following corrections for the fluorescence of free probe (above), the resulting relationship between F_{corr} and concentration terms is given by eq 5:

$$F_{corr} = F_o + F_\infty \frac{Xa{-}P}{Xa} \quad (5)$$

where F_o is the fluorescence in the absence of PAB, F_∞ is the fluorescence at infinite concentrations of PAB, Xa represents the total concentration of enzyme, and Xa−P is the concentration of PAB bound to factor Xa.

The data were analyzed according to Scheme 1 (Results). In the absence of R3A-TAP, the dependence of Xa−P on the total concentration of PAB is given by a quadratic expression, similar to eq 1. In the presence of R3A-TAP, the isotherm is described by a cubic equation (Olson et al., 1991), which has been derived without any assumptions regarding the binding stoichiometries or dissociation constants of both ligands. Assuming a unit stoichiometry for the binding of PAB to Xa ($n_{Xa,P} = 1$), nonlinear regression analysis employing the Newton−Raphson method (Bevington, 1969) yielded fitted values of F_o, F_∞, $K_{Xa,P}$, $K_{Xa,I}$, and $n_{Xa,I}$.

Stopped-Flow Measurements with Factor Xa and R3A-TAP. The decay of fluorescence observed following mixing of R3A-TAP with factor Xa in presence of PAB was analyzed using

$$F_t = \text{offset} + Qe^{-k_{obs}t} \quad (6)$$

where F_t is the fluorescence at time t, offset is the fluorescence at infinite time, and Q is the amplitude of the fluorescence change. Since PAB and R3A-TAP bind competitively to factor Xa (Scheme 1), the relationship between the observed rate constant (k_{obs}) and the intrinsic rate constants for R3A-TAP (I) binding depends on the concentration of PAB (Olson & Shore, 1986):

$$k_{obs} = \frac{k_{+1}I}{1 + \frac{P}{K_{Xa,P}}} + k_{-1} \quad (7)$$

where P is the concentration of PAB and $K_{Xa,P}$ is the equilibrium dissociation constant for P binding to factor Xa. Equation 7 predicts a linear relationship between k_{obs} and the effective concentration of R3A-TAP, defined as [I]/(1 + [P]/$K_{Xa,P}$). Data sets of the dependence of k_{obs} on the concentration of R3A-TAP at fixed PAB concentrations or the concentration of PAB at fixed concentrations of R3A-TAP were globally analyzed according to eq 7 to extract k_{+1}, k_{-1}, and $K_{Xa,P}$.

Stopped-Flow Measurements with Prothrombinase. The biphasic fluorescence decay observed for the interaction of R3A-TAP with prothrombinase in the presence of PAB was analyzed as two consecutive first-order reactions (Moore &

Pearson, 1981):

$$F_t = \text{offset} + Q_1 e^{-k_{1,obs}t} + Q_2 \frac{k_{1,obs}}{k_{2,obs} - k_{1,obs}} (e^{-k_{1,obs}t} - e^{-k_{2,obs}t}) \quad (8)$$

where the observed fluorescence at time t (F_t) is related to the observed rate constants as well as the amplitude terms (Q_1 and Q_2) for the fast and slow phases. Because of the "ill-conditioned" nature of multiexponential decays (Cornish-Bowden, 1979), the reliability of the fitted terms was improved by averaging replicate traces prior to fitting the data.

RESULTS

Inhibition of Factor Xa by R3A-TAP. The interaction of R3A-TAP with factor Xa was inferred from the residual rate of SpXa hydrolysis after preincubation of the protease with increasing concentrations of inhibitor. Variation of the preincubation time between ~1 and 120 min was without effect on the inhibition by R3A-TAP, but inhibition was dependent on the concentration of SpXa used to assess enzyme activity. These data indicate that the binding of R3A-TAP to factor Xa occurs rapidly relative to the time required to measure residual rates.

Initial velocity measurements were conducted using increasing concentrations of SpXa in the presence of several fixed concentrations of R3A-TAP (Figure 1A). The data could be adequately described by the rate expression for linear competitive inhibition. Analysis according to this type of inhibition with an assumed stoichiometry of 1 mol of R3A-TAP bound per mole of Xa at saturation yielded a K_i of 794 ± 30 nM. The quality of the fit did not improve significantly when the data were analyzed according to more complex hyperbolic inhibition mechanisms (Segel, 1975). Fitting of the data to the rate expressions for other simple inhibition schemes, however, yielded much poorer fits.

R3A-TAP therefore appears to be a simple competitive inhibitor of factor Xa of moderate affinity that binds to the enzyme through steps that are in rapid equilibrium with substrate hydrolysis. In contrast, wt-TAP is a slow, tight-binding inhibitor of factor Xa that reacts by a multistep mechanism which is not consistent with linear competitive inhibition (Jordan et al., 1992; Krishnaswamy et al., 1994). The resulting complex between wt-TAP and factor Xa is very tight ($K_i^* = 0.18$ nM) (Jordan et al., 1990). Thus, substitution of Arg^3 in wt-TAP with Ala markedly alters the kinetics and energetics of the interaction between factor Xa and the inhibitor.

Inhibition of Prothrombinase by R3A-TAP. The ability of R3A-TAP to inhibit the prothrombinase complex was assessed using saturating concentrations of PCPS and factor Va to assemble solution-phase factor Xa into the ternary Xa−Va−PCPS complex. In contrast to the observations of the weak and relatively rapid inhibition of solution-phase factor Xa, inhibition of the prothrombinase complex required much lower concentrations of R3A-TAP and prolonged incubation (>30 min) to reach a limiting value (not shown). Thus, R3A-TAP exhibits the features of a slow, tight-binding inhibitor of prothrombinase.

The overall dissociation constant (K_i^*) for the inhibition of prothrombinase by R3A-TAP was therefore determined by incubating increasing concentrations of R3A-TAP with two fixed concentrations of the prothrombinase complex for 90 min, prior to the determination of residual enzymatic activity (Figure 1B). Variation of the incubation time and SpXa concentration was without effect (not shown). This indicates that the initial incubation period is sufficient to allow the reactants to reach equilibrium which is not significantly perturbed during the 5 min required for the initial velocity measurement. Analysis of the inhibition data (Figure 1B) using eqs 1 and 2 (Data Analysis) yielded a K_i^* of 195 ± 35 pM for the inhibition of prothrombinase and a stoichiometry of 0.9 ± 0.05 mol of R3A-TAP bound per mole of enzyme at saturation. The measured K_i^* indicates that the affinity of R3A-TAP for the prothrombinase complex is 4000-fold greater than the affinity of the inhibitor for

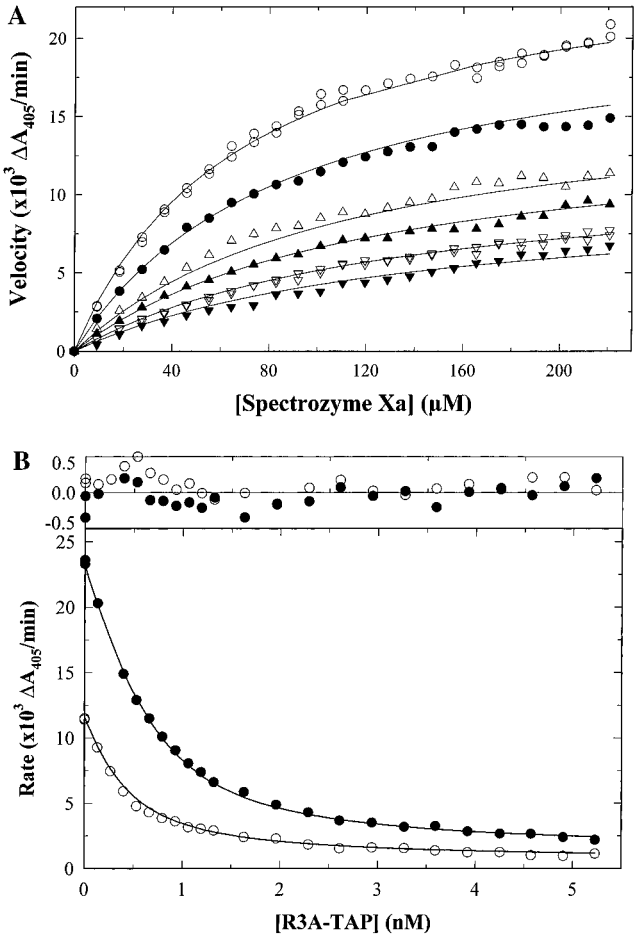


FIGURE 1: Effect of prothrombinase assembly on inhibition by R3A-TAP. (A) Steady state measurements for the inhibition of factor Xa in solution. Initial, steady state velocities were determined using the indicated concentrations of Spectrozyme Xa and 0.5 nM Xa in assay buffer and 0 (○), 0.5 μM (●), 2.0 μM (△), 3.0 μM (▲), 5.0 μM (▽), or 7.5 μM (▼) R3A-TAP. The lines are drawn according to the rate equation for simple competitive inhibition using the following constants: $K_m = 58.3 \pm 2.8$ μM, $V_{max} = (48.3 \pm 0.8) \times 10^{-3}$ A_{405} min^{-1} (nM Xa)$^{-1}$, and $K_i = 794 \pm 29.6$ nM. (B) Inhibition of the prothrombinase complex at equilibrium. Reaction mixtures containing the indicated concentrations of R3A-TAP, 40 nM factor Va, 40 μM PCPS, and either 0.3 nM (○) or 0.6 nM (●) factor Xa in assay buffer were incubated for 90 min at room temperature. Residual enzyme activity was determined in duplicate from initial velocity measurements following initiation with 200 μM Spectrozyme Xa in the same buffer. The lines are drawn following analysis with eqs 1 and 2 using the following fitted terms: $v_o = (36.5 \pm 0.3) \times 10^{-3}$ A_{405} min^{-1} (nM prothrombinase)$^{-1}$, $v_\infty = (2.1 \pm 0.22) \times 10^{-3}$ A_{405} min^{-1} (nM prothrombinase)$^{-1}$, $K_i^* = 195 \pm 35$ pM, and $n = 0.9 \pm 0.05$ mol of R3A-TAP/(mol of prothrombinase) at saturation. The residuals to the fitted lines are illustrated in the upper panel.

Table 1: Dissociation Constants for the Inhibition of Factor Xa by R3A-TAP or wt-TAP in the Presence of the Components of the Prothrombinase Complex

	$K_i \pm$ SE[c] (nM)	$K_i^* \pm$ SE[d] (nM)
enzyme mixture[b]	R3A-TAP	wt-TAP[a]
Xa/Ca^{2+}	794 ± 30	0.120 ± 0.007
Xa/Ca^{2+}/PCPS	791 ± 23	0.165 ± 0.018
Xa/EDTA/PCPS	915 ± 70	ND[e]
Xa/Ca^{2+}/PCPS/Va	0.195 ± 0.035[d]	0.0053 ± 0.0008
Xa/EDTA/PCPS/Va	903 ± 20	ND

[a] The values for wt-TAP were taken from Krishnaswamy et al. (1994). [b] The concentrations of the components in each mixture are listed in Experimental Procedures. The enzyme components were mixed in assay buffer containing either 2 mM Ca^{2+} or 5 mM EDTA. [c] Equilibrium constant ±95% confidence limits inferred from steady state measurements assuming competitive inhibition. [d] Overall equilibrium dissociation constant determined from initial velocity measurements following prolonged incubation of enzyme and inhibitor. [e] ND = not determined.

solution-phase factor Xa. The near unity stoichiometry determined for the inhibition reaction is consistent with measurements using wt-TAP (Krishnaswamy et al., 1994; Jordan et al., 1990) and also excludes the possibility of selective inhibition of prothrombinase relative to factor Xa by an unidentified trace contaminant in the recombinant protein preparation.

The inhibition of factor Xa was studied in the presence of various combinations of prothrombinase constituents (Table 1). The relatively weak inhibition of solution-phase factor Xa by R3A-TAP was not affected by the presence of calcium ions or by the saturable incorporation of the protease into the Xa−PCPS binary complex. Conversely, tight binding of R3A-TAP to the Xa−Va−PCPS ternary complex could be eliminated by EDTA added to chelate calcium ions required for the high-affinity interaction between factors Xa and Va on membranes (Krishnaswamy et al., 1993). Since tight inhibition of factor Xa is only observed in the presence of all constituents of prothrombinase, it follows that the interaction between the protease and the cofactor on the membrane surface, in some way, alters the recognition of R3A-TAP by factor Xa and leads to a 4000-fold decrease in K_i^*.

Rapid Kinetic Studies of the Reaction of R3A-TAP with Factor Xa. The reaction of factor Xa with R3A-TAP was further studied by stopped-flow measurements using the fluorescent serine protease inhibitor, PAB, which has been successfully used to investigate the reaction pathway of wt-TAP with factor Xa or prothrombinase (Jordan et al., 1992; Krishnaswamy et al., 1994).

A stopped-flow trace of the fluorescence change observed following rapid mixing of R3A-TAP with factor Xa preincubated with PAB is illustrated in Figure 2. The signal decayed as a single exponential ($t_{1/2} \sim 250$ ms) from a starting value corresponding to the enhanced fluorescence intensity of PAB bound reversibly to factor Xa to a final value approximately equal to that expected for the same concentration of free PAB (Figure 2). Equivalent stopped-flow measurements with wt-TAP yield a biphasic fluorescence trace which results from the initial, rapid reaction of wt-TAP to form an intermediate ternary complex followed by slower isomerization with displacement of PAB from the enzyme (Krishnaswamy et al., 1994).

In order to investigate the mechanism of the inhibition of factor Xa by R3A-TAP, stopped-flow measurements were

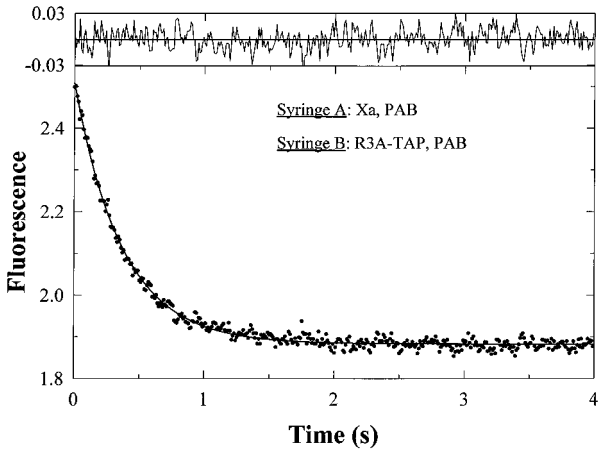


FIGURE 2: Reaction of factor Xa with R3A-TAP. Fluorescence was measured following rapid mixing of R3A-TAP with factor Xa in the presence of PAB as described in Experimental Procedures. The final concentrations of reactants were 0.48 μM factor Xa, 14.4 μM R3A-TAP, and 19.5 μM PAB. The data were analyzed using eq 6 to yield the following fitted terms: offset $= 1.88 \pm 0.004$, $Q = 0.625 \pm 0.004$, and $k_{obs} = 2.7 \pm 0.03$ s^{-1}. The residuals to the fitted lines are illustrated in the upper panel.

used to examine the dependence of k_{obs} on the concentration of R3A-TAP at a fixed concentration of PAB (25 μM). The experiments were performed under pseudo-first-order conditions ([R3A-TAP] ≫ [Xa]) which was verified by the independence of k_{obs} of the concentration of factor Xa. In all experiments, single-exponential fluorescence decays were observed with amplitudes corresponding to the complete dissociation of PAB from factor Xa. The pseudo-first-order rate constant (k_{obs}) for the fluorescence decay increased linearly with increasing concentrations of R3A-TAP (Figure 3A). Accordingly, the rate-limiting step corresponds to a bimolecular reaction between R3A-TAP and factor Xa.

The dependence of the pseudo-first-order rate constant on the concentration of PAB at two fixed concentrations of R3A-TAP is illustrated in Figure 3B. The observed rate constant for the fluorescence decay decreased hyperbolically with increasing concentrations of PAB. All these results can be accommodated in the following minimal mechanism for the reaction of factor Xa with R3A-TAP (I) in the presence of PAB (P) (Scheme 1).

Assuming that factor Xa and PAB (P) are in rapid equilibrium, determined by $K_{Xa,P}$, the relationship between the intrinsic rate constants and the k_{obs} therefore contains I, P, and $K_{Xa,P}$ terms (eq 7, Data Analysis). Global analysis of all stopped-flow data using eq 7 yielded fitted values for $K_{Xa,P}$ of 48 ± 7 μM, k_{+1} of 0.23 ± 0.01 μM^{-1} s^{-1}, and k_{-1} of 0.20 ± 0.04 s^{-1}. The adequacy of eq 7 in describing the reaction of R3A-TAP with factor Xa in the presence of PAB is illustrated by the linear dependence of k_{obs} on the effective concentration of R3A-TAP obtained under a wide range of conditions (Figure 3B, inset).

According to Scheme 1, combination of the association (k_{+1}) and dissociation (k_{-1}) rate constants of the reaction between R3A-TAP and factor Xa yields a $K_{Xa,I}$ of 870 ± 90 nM. The good agreement with the K_i term determined from steady state measurements (Table 1) supports the conclusion that PAB does not alter the reaction of R3A-TAP with factor Xa and reduces the likelihood that reaction steps affecting the equilibrium have eluded observation. Further, the fitted value of $K_{Xa,P}$ and that directly determined in the present work are in good agreement with previous measurements

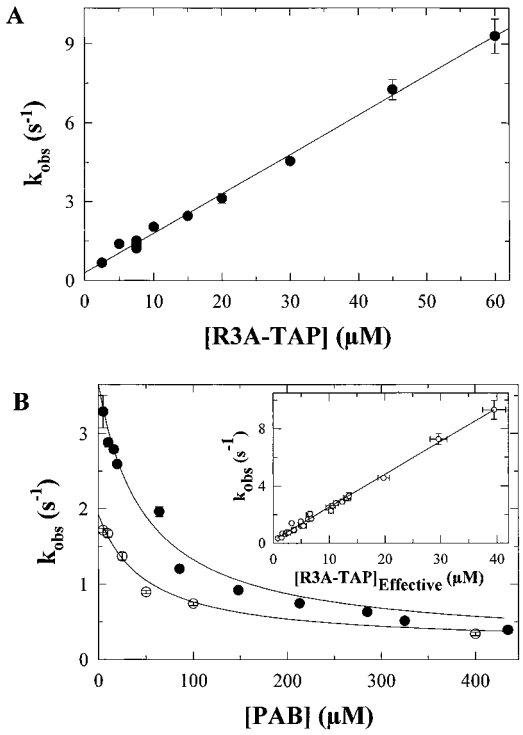
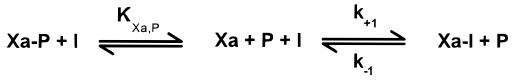


FIGURE 3: Influence of reactant concentrations on the reaction of R3A-TAP with factor Xa. (A) Dependence of k_{obs} on the concentration of R3A-TAP using 0.5 μM factor Xa and 25 μM PAB. (B) Dependence of k_{obs} on the concentration of PAB at 0.5 μM Xa and either 7.5 μM (○) or 15 μM (●) R3A-TAP. In either panel, mean values of k_{obs} from between five and nine replicate traces are presented along with error bars indicating 1 standard deviation. The lines are drawn following fitting to eq 7 with the following fitted terms: $k_{+1} = (2.3 \pm 0.1) \times 10^5$ M^{-1} s^{-1}, $k_{-1} = 0.2 \pm 0.04$ s^{-1}, and $K_{Xa,P} = 48.2 \pm 6.6$ μM. The inset illustrates the linear dependence of k_{obs} on the effective concentration of R3A-TAP over a wide range of reactant concentrations predicted by eq 7.

Scheme 1



(Krishnaswamy et al., 1994). Thus, Scheme 1 appears to adequately describe this reaction.

Equilibrium Titration with p-Aminobenzamidine. Agreement between the data and Scheme 1 notwithstanding, it is possible that additional steps are important in the reaction pathway but have escaped detection because of a fortuitous relationship between the rate constants and/or the fluorescence quantum yields of the possible species. In particular, the restricted range of concentrations of R3A-TAP used in the kinetic measurements might have precluded the detection of additional isomerization steps of an initially formed factor Xa−R3A-TAP complex. This possibility was investigated by direct equilibrium binding measurements of the competitive interactions of PAB and R3A-TAP with factor Xa.

Factor Xa was titrated by incremental addition of PAB in the presence of various fixed concentrations of R3A-TAP (Figure 4). Competition between both ligands for factor Xa results in the displacement of R3A-TAP from factor Xa by PAB (Figure 4). Following corrections for the inner filter effect and the background signal (Experimental Procedures), the data were simultaneously analyzed according to Scheme 1 as described in Data Analysis. The fitted values for $K_{Xa,P}$ of 29.1 ± 0.9 μM and $K_{Xa,I}$ of 556 ± 18 nM are in excellent

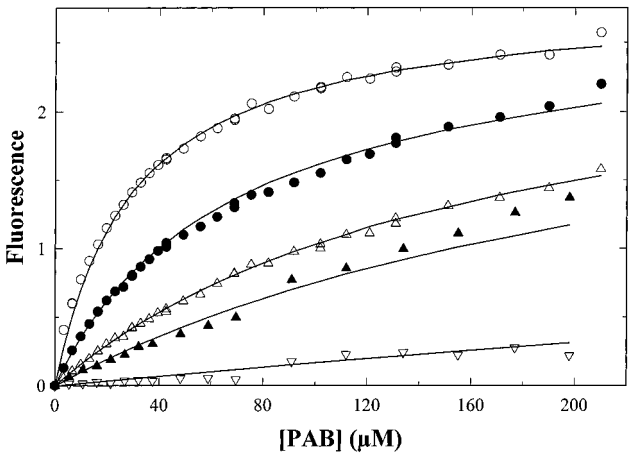


FIGURE 4: Equilibrium binding of PAB to factor Xa in the presence of R3A-TAP. Reaction mixtures containing 0.5 μM factor Xa and 0 (○), 1.0 μM (●), 3.0 μM (△), 5.0 μM (▲), and 30.0 μM (▽) R3A-TAP were titrated with increasing concentrations of PAB. The data represent values corrected for the inner filter effect and the fluorescence contribution of the free probe. The lines were drawn following analysis according to Scheme 1 as described in Data Analysis assuming $n_{Xa,P} = 1$ and using the following fitted terms: $K_{Xa,P} = 29.1 \pm 0.87$ μM, $K_{Xa,I} = 556 \pm 18$ nM, $n_{Xa,I} = 0.99 \pm 0.02$ mol of R3A-TAP/(mol of Xa), $F_o = 0.004 \pm 0.009$, and $F_\infty = 2.82 \pm 0.05$.

agreement with the corresponding values determined by two other kinetic techniques. In addition, a stoichiometry of ~1 mol of R3A-TAP bound per mole of factor Xa at saturation could be determined. Since the dissociation constant for probe binding ($K_{Xa,P}$) compares well with that determined from the stopped-flow measurements, the rapid equilibrium assumption for PAB binding to factor Xa appears to be valid. Further, since dissociation constants obtained in the static titration are overall dissociation constants, they are reflective of all equilibria involved. Thus, the close agreement between the values for $K_{Xa,I}$ determined by titration and the same term calculated from the rate constants or initial velocity measurements further suggests that the reaction between R3A-TAP and factor Xa can be adequately described by a single reversible step as illustrated in Scheme 1 and is exclusive of the binding of active site-directed ligands such as PAB or synthetic peptidyl substrates.

Kinetic Characterization of the Reaction of R3A-TAP with Prothrombinase. The kinetic basis for the factor Va-dependent enhancement of the inhibition of the prothrombinase complex by R3A-TAP (Table 1) was investigated by rapid kinetic studies.

A typical trace for the reaction of prothrombinase with R3A-TAP is depicted in Figure 5, at identical concentrations of R3A-TAP, enzyme, and PAB as used in Figure 2. In contrast to the monoexponential fluorescence decay observed with factor Xa, the rapid mixing of prothrombinase with R3A-TAP in presence of PAB yielded a biphasic trace (Figure 5). Approximately 70% of the fluorescence change occurred rapidly ($t_{1/2} \approx 25$ ms), with a much slower ($t_{1/2} \approx$ 2s) decrease in the remaining 30% of the signal. The net reaction, however, observed with prothrombinase appears to be the displacement of PAB from the enzyme by R3A-TAP, since the limiting signal is equal to that expected for free PAB and the overall amplitude is equivalent to that obtained in the reaction with factor Xa.

While these data suggest a multistep reaction, with kinetically resolvable intermediates, alternative explanations could also account for the data. Potentially trivial explana-

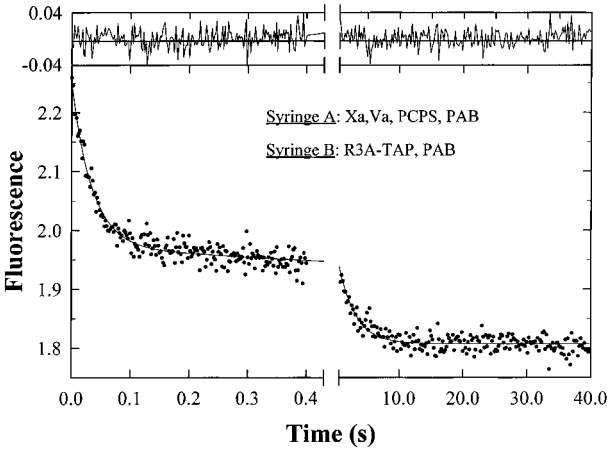


FIGURE 5: Reaction of R3A-TAP with prothrombinase. Fluorescence was measured following rapid mixing of R3A-TAP with the preassembled prothrombinase complex in the presence of PAB as denoted in the figure and described in Experimental Procedures. The final concentrations of reactants were 0.48 μM factor Xa, 0.75 μM factor Va, 100 μM PCPS, 14.4 μM R3A-TAP, and 19.5 μM PAB. The lines are drawn following analysis according to eq 8 using the following fitted terms: $k_{1,obs} = 28.5 \pm 1.1$ s^{-1}, $k_{2,obs} = 0.38 \pm 0.02$ s^{-1}, $Q_1 = 0.44 \pm 0.006$, $Q_2 = 0.16 \pm 0.002$, and offset $= 1.817 \pm 0.001$. The residuals to the fitted line are illustrated in the upper panel.

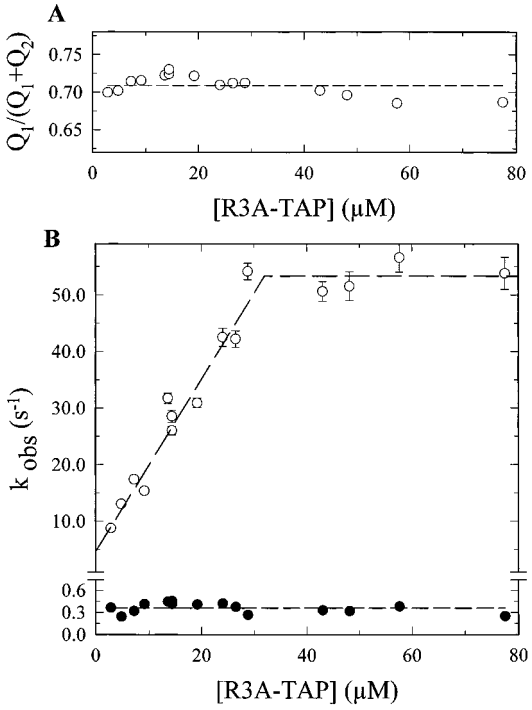


FIGURE 6: Effect of the concentration of R3A-TAP on the reaction with prothrombinase. Stopped-flow measurements were conducted using preassembled prothrombinase and increasing concentrations of R3A-TAP in the presence of PAB. The final concentrations of reactants were 0.48 μM factor Xa, 0.75 μM factor Va, 100 μM PCPS, 19.5 μM PAB, and the indicated concentrations of R3A-TAP. The data were analyzed according to eq 8, and error bars indicate 95% confidence limits of the fitted terms. (A) Dependence of the normalized amplitude of the fast phase on the concentration of R3A-TAP. The dashed line denotes the mean of all measured values. (B) Dependence of $k_{1,obs}$ (○) and $k_{2,obs}$ (●) on the concentration of R3A-TAP is illustrated using a broken *y*-axis. The dashed lines are drawn following analysis according to Scheme 2 to estimate the following terms: $k_{+1} \leq (1.52 \pm 0.03) \times 10^6$ M^{-1} s^{-1}, $k_{+2} + k_{-2} = 53.3 \pm 2.3$ s^{-1}, $k_{-2}k_{-1}/(k_{-2} + k_{-1}) = 4.6 \pm 0.3$ s^{-1}, and $k_{+3} + k_{-3} = 0.3$ s^{-1}.

tions include (1) an altered interaction of PAB with prothrombinase relative to solution-phase factor Xa, (2) unsuccessful kinetic isolation of pseudo-first-order conditions, and (3) heterogeneity at the level of enzyme or inhibitor yielding fast- and slow-reacting forms of prothrombinase or R3A-TAP.

The results of fluorescence measurements and steady state inhibition studies of prothrombinase (not shown) indicated that the incorporation of factor Xa into prothrombinase did not alter the fluorescence of bound PAB or the K_i for inhibition of synthetic substrate hydrolysis. Superimposable traces were obtained at different concentrations of prothrombinase (not shown), consistent with the establishment of pseudo-first-order conditions ([E] $\ll$ [I]).

Stopped-flow measurements were conducted at increasing concentrations of R3A-TAP (I) using constant concentrations of prothrombinase (E) and PAB (P) (Figure 6). Biphasic traces were obtained at inhibitor concentrations as high as 80 μM and were characterized by a constant ratio of the amplitude of the fast phase to the total amplitude (Figure 6A). The traces were adequately described by eq 8, which yields $k_{1,obs}$ and $k_{2,obs}$ (Data Analysis). The rate constants for the two phases were found to vary differentially with increasing concentrations of R3A-TAP (Figure 6B). This observation excludes kinetic heterogeneity of either R3A-TAP or prothrombinase as an explanation for the multiphasic decay and suggests a sequential multistep mechanism for the binding of R3A-TAP to prothrombinase.

The pseudo-first-order rate constant for the rapid phase ($k_{1,obs}$) increased linearly with increasing concentrations of R3A-TAP and saturated at $\sim$50 s^{-1} at concentrations above 30 μM (Figure 6B). Since the amplitude of the fast phase does not account for the release of P from E and remains constant over the whole range of concentrations used, it cannot be related to the displacement of PAB from prothrombinase. Thus, the initial rapid fluorescence change probably represents the formation of a ternary E−I−P complex. This interpretation is consistent with the dependence of $k_{1,obs}$ on the concentration of R3A-TAP. Selected experiments indicated that $k_{1,obs}$ was independent of the concentration of prothrombinase. Thus, at least two sequential steps are responsible for the initial rapid fluorescence change. The saturable increase in $k_{1,obs}$ indicates a bimolecular reaction between the PAB−prothrombinase binary complex (E−P) and R3A-TAP (I) to form a ternary complex (E−P−I) followed by an unimolecular isomerization step which is solely rate-determining when the encounter complex is saturated with inhibitor. This isomerization step must generate the pertinent fluorescence change to account for the data. These steps are indicated by the rate constants k_{+1}, k_{-1}, k_{+2}, and k_{-2} in Scheme 2.

Scheme 2

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

In contrast, $k_{2,obs}$ was independent of the concentration of R3A-TAP (Figure 6B). Since the limiting signal agrees with the expected fluorescence intensity of free P, the second slow fluorescence change must result from the displacement of P from the ternary complex formed in the initial part of the reaction. Thus, $k_{2,obs}$ would represent the step(s) leading to P dissociation and would be expected to be independent of the R3A-TAP concentration. This process is illustrated by the steps governed by k_{+3}, k_{-3}, and $K_{EI^*,P}$ in Scheme 2.

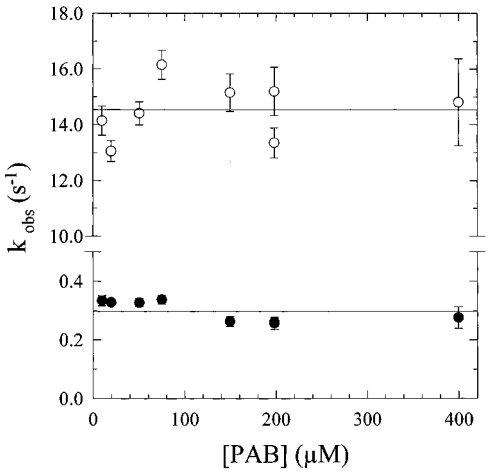


FIGURE 7: Effect of the concentration of PAB on the reaction with prothrombinase. Stopped-flow measurements were conducted using preassembled prothrombinase and increasing concentrations of PAB in the presence of R3A-TAP. The final concentrations of reactants were 0.48 μM factor Xa, 0.75 μM factor Va, 100 μM PCPS, 4.8 μM R3A-TAP, and the indicated concentrations of PAB. The data were analyzed according to eq 8 to obtain fitted values for $k_{1,obs}$ (○) and $k_{2,obs}$ (●). The error bars indicate 95% confidence limits of the fitted terms.

To further test the ability of Scheme 2 to describe the data, stopped-flow measurements were carried out using increasing concentrations of PAB at fixed concentrations of R3A-TAP (I) and prothrombinase (E). No obvious systematic change in either $k_{1,obs}$ or $k_{2,obs}$ was seen with increasing concentrations of PAB (Figure 7). The normalized ratio of the two amplitude terms was also invariant over this concentration range (not shown). Since the probe is expected to bind to prothrombinase by steps that are rapid relative to the other steps in the pathway, the formation of the two ternary complexes (E−I−P and E−I−P*, Scheme 2), given by $k_{1,obs}$, would be expected to be independent of the concentration of PAB. Further, the invariance of $k_{1,obs}$ at concentrations of PAB ranging from $\sim 0.5K_{E,P}$ to $\sim 16K_{E,P}$ allows the conclusion that P binding to E does not effect the initial interactions between I and E. Thus, the initial binding of R3A-TAP to factor Xa within the prothrombinase complex is not influenced by prior occupation of the primary specificity pocket of the protease by PAB.

The rate constant for the second phase ($k_{2,obs}$), representing the dissociation of PAB from the ternary complex, was also independent of the concentration of PAB in the range of concentrations (Figure 7). Provided PAB binding and dissociation from the ternary complex remains rapid, this step can be accommodated (Scheme 2) by the rapid dissociation of PAB from EI* in a step that precedes its isomerization to EI**. The fact that $k_{2,obs}$ does not depend on the concentration of PAB implies that the species EI* has very little or no affinity for this active site probe (*i.e.* $K_{EI^*,P} \gg K_{E,P}$). Thus, the dissociation of PAB from the ternary complex formed in the rapid phase of the reaction is followed by an additional slow conformational change. Alternatively, a model with a slow isomerization between two ternary complexes EPI* to EPI** followed by P dissociation could also account for the data. This and other possibilities cannot be eliminated with the available data. Despite this ambiguity in the last step of the reaction scheme, we have retained Scheme 2 as an adequate description of the reaction pathway between R3A-TAP and prothrombinase because it corresponds directly with the determined pathway for the inhibition of factor Xa or prothrombinase by wt-TAP. The difference in the sign of the fluorescence change for the initial step observed with R3A-TAP relative to previous measurements (Krishnaswamy et al., 1994) indicates that the mutant inhibitor perturbs the active site environment differently than wt-TAP.

Analysis of the stopped-flow data (Figures 6 and 7) according to Scheme 2 yields estimates for some of the intrinsic rate constants illustrated in this multistep pathway (Hiromi, 1979). The sharp saturation of $k_{1,obs}$ with increasing R3A-TAP suggests that the application of the rapid equilibrium assumption (*i.e.* $k_{+1}I, k_{-1} \gg k_{+2} + k_{-2}$) to the initial binding of I to EP is not valid. This is further supported by the relationship of the midpoint of the saturation function to the various rate constants estimated below. Thus, the slope of the initial portion of the dependence of $k_{1,obs}$ on R3A-TAP (Figure 6B) yields an upper limit estimate for k_{+1} of $\leq 1.52 \times 10^6$ M^{-1} s^{-1}. The intercept yields $k_{-2}k_{-1}/(k_{-2} + k_{-1}) = 4.6$ s^{-1}, and the limiting value for $k_{1,obs}$ at saturating R3A-TAP concentrations yields $k_{+2} + k_{-2} = 53.3$ s^{-1}. According to Scheme 2, the mean value for $k_{2,obs}$ yields $k_{+3} + k_{-3} = 0.3$ s^{-1}.

The rapid kinetic measurements demonstrate that the reaction of R3A-TAP with prothrombinase is a multistep reaction with initial interactions that are independent of the occupation of the primary specificity pocket of the protease. The data indicate that the large increase in the inhibitory potency of R3A-TAP following the incorporation of factor Xa into the prothrombinase complex is also accompanied by changes in the observed kinetic mechanism.

DISCUSSION

Prior mutagenesis studies have suggested an important role for Arg^3 in active site-dependent reactions with factor Xa along with additional contacts between the inhibitor and site(s) on the protease distant from the active site (exosites) (Dunwiddie et al., 1992). The intent of the present studies was therefore to use R3A-TAP as a selective probe of perturbations in the exosites of factor Xa. Although a loss of active site interactions with factor Xa after mutation of Arg^3 to Ala would provide an appropriate explanation of the observed thermodynamic effects of the mutation, this simple model cannot fully account for all the observations. A loss of active site interactions might be expected to facilitate the simultaneous binding of R3A-TAP and PAB to factor Xa, yet evidence for a ternary complex of this type was not obtained. It is possible that competition between R3A-TAP and substrates or active site ligands does not require a direct interaction between inhibitor and the active site of factor Xa. On the other hand, the apparently different kinetic mechanism of the reaction of R3A-TAP with factor Xa relative to the previously described mechanism for wt-TAP raises the possibility that the two reactions may yield different final complexes. Thus, although mutagenesis indicates an essential role of Arg^3 for the stability of the factor Xa−TAP complex, a specific functional role of this residue in the binding of wt-TAP to factor Xa cannot be unequivocally assigned.

Substitution of Ala for Arg^3 in wt-TAP reproduced the deleterious effect on the inhibition of solution-phase factor Xa previously observed upon replacing this residue with Asn (Dunwiddie et al., 1992). Surprisingly, the affinity of the inhibitor for prothrombinase was less extensively affected

by the mutation. Compared to the 3000-fold difference in the catalytic efficiency of prothrombin activation by factor Xa and by prothrombinase, relatively small changes, if at all, have been observed in the reaction of other substrates and inhibitors of factor Xa upon assembly of the prothrombinase complex (Ellis et al., 1984; Walker & Krishnaswamy, 1993; Krishnaswamy et al., 1994; Huang et al., 1993; Mast & Broze, 1996). The present study with R3A-TAP demonstrates that cofactor-induced alterations in macromolecular recognition can in fact lead to binding changes of sufficient magnitude to explain the improved catalytic efficiency of prothrombinase relative to factor Xa. Accordingly, if the recognition sites on factor Xa occupied by R3A-TAP are relevant to those involved in the recognition of the natural substrate, prothrombin, the interaction of factor Va with factor Xa on membranes could lead to large changes in substrate affinity irrespective of additional interactions between the substrate and the cofactor and/or membranes.

The data indicate that the formation of the prothrombinase complex not only leads to an increase in the affinity for R3A-TAP but also alters the observed kinetic mechanism for the reaction between inhibitor and protease. As a result, the Arg^3Ala mutation leads to a functional dissociation of the inhibition of factor Xa from the inhibition of prothrombinase. Although three independent approaches have been used to establish the single-step mechanism for the reaction of factor Xa with R3A-TAP (Scheme 1), it is possible that very weak additional isomerization steps following the bimolecular combination of enzyme and inhibitor have escaped detection. Thus, both solution-phase factor Xa as well as prothrombinase may well react with the inhibitor through multistep reaction pathways. However, the two reactions differ unambiguously from each other in that prior occupation of the protease active site by ligands precludes the binding of R3A-TAP to solution-phase factor Xa but has no effect on the bimolecular combination of the inhibitor with prothrombinase. In either case, the relevant rate-limiting step that is affected corresponds to the bimolecular combination of the enzyme with inhibitor. Consequently, it is not possible to rationalize these kinetic differences in terms of factor Va-induced changes in the rate-limiting step of a common kinetic scheme.

Nevertheless, these kinetic effects could be interpreted in terms of subtle differences in the kinetics or energetics of the same molecular contacts. For example, it is possible that substitution of Arg^3 with Ala in wt-TAP simply weakens selected interactions with factor Xa which can be overcome upon assembly of the prothrombinase complex. In this case, it is possible that the reaction between R3A-TAP with factor Xa, which results from a bimolecular step exclusive with PAB binding, involves the same interactions as a unimolecular step observed late in the multistep kinetic pathway with wt-TAP. The effects of prothrombinase assembly on reaction kinetics might be explained by an enhancement of these defective interactions as a result of factor Va-induced changes in factor Xa which makes additional steps earlier in the reaction sequence evident. These and other possibilities cannot be resolved with the present data. However, it is clear that factor Va modulates factor Xa within the prothrombinase complex in such a way so as to yield large changes in the thermodynamics of the recognition of R3A-TAP by the protease which either results from and/or explains changes in the observed kinetic mechanism. It is possible that such effects on macromolecular recognition by factor Xa may be a general consequence of the assembly of the prothrombinase complex.

In the multistep reaction observed with prothrombinase, prior occupation of the active site by PAB has no effect on the initial binding interactions with R3A-TAP. It therefore appears reasonable to speculate that the formation of this ternary complex results from enhanced interactions between R3A-TAP and macromolecular recognition elements present on exosites. This is consistent with the proposal of perturbed exosite interactions as the basis for the enhanced binding of wt-TAP to prothrombinase (Krishnaswamy et al., 1994). It is possible that cofactor-induced alterations in exosites of factor Xa might constitute a major effect of factor Va on protease function within the prothrombinase complex. This possibility may provide an explanation for the differential effects of factor Va on the two cleavages required for prothrombin activation (Nesheim & Mann, 1983; Krishnaswamy et al., 1987) and the altered order of bond cleavages in prothrombin activation by prothrombinase relative to factor Xa (Krishnaswamy et al., 1986, 1987; Walker & Krishnaswamy, 1994) as well as a potential explanation for the lack of observed differences in the reaction of factor Xa and prothrombinase with several active site-directed reagents (Walker & Krishnaswamy, 1993; Ellis et al., 1984).

The significance of the differences in the reaction of R3A-TAP with prothrombinase and with factor Xa could be compromised if the reporter PAB introduced artifacts in the measurements. Recent studies have questioned the extrapolation of kinetic conclusions derived from studies in the presence of PAB to the reaction pathway in the absence of the probe (Mao et al., 1995). The equivalence of the dissociation constants of PAB for prothrombinase and factor Xa rules out differences in probe binding as an explanation for the altered kinetic mechanism (Krishnaswamy et al., 1994). For the reaction of R3A-TAP with factor Xa in solution, measurements of the reaction using peptidyl substrates or PAB yielded consistent values. In the case of prothrombinase, all rate-limiting steps were independent of the concentration of PAB. These facts all indicate that the observed exclusive binding between PAB and R3A-TAP for binding to factor Xa *versus* the lack of effect of PAB on the initial binding of the inhibitor to prothrombinase reflect the intrinsic properties of both proteases. Nevertheless, as suggested (Mao et al., 1995), we cannot unequivocally exclude the possibility that PAB exerts some effect on the later steps in the multistep pathway with prothrombinase. This point may have some bearing on the additional differences noted between the two kinetic mechanisms.

In the present study, replacement of Arg^3 in wt-TAP with Ala not only resulted in a loss of inhibitory potency but also produced an inhibitor with enhanced selectivity for prothrombinase relative to factor Xa. With a sub-nanomolar dissociation constant for prothrombinase and a micromolar inhibition constant for factor Xa, R3A-TAP exhibits unprecedented specificity for the enzyme complex. The significantly tighter binding of R3A-TAP to prothrombinase is associated with a change in the observed kinetic mechanism for the reaction. The difference between both mechanisms is most evident in the fact that R3A-TAP and PAB bind mutually exclusively to factor Xa while none of the rate-limiting steps in the inhibition of prothrombinase involves competition with active site ligands. Thus, the single mutation of Arg^3Ala introduces a molecular switch that reveals differential recognition of the inhibitor by prothrom-

binase and by factor Xa, yielding inhibited complexes with large differences in stability. It remains to be established if comparable effects play a significant role in the enhanced catalytic efficiency of the prothrombinase complex in prothrombin activation.

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