Contribution of the Prothrombin Fragment 2 Domain to the Function of Factor Va in the Prothrombinase Complex†

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ABSTRACT: The prothrombinase complex assembles through reversible interactions between factor Xa, factor Va and acidic phospholipid-containing membranes in the presence of calcium ions. This complex catalyses the conversion of prothrombin to thrombin through two proteolytic steps. We have used prethrombin 2 as a substrate analog for the first cleavage reaction of prothrombin activation (cleavage at $Arg^{323}-Ile^{324}$) catalyzed by the prothrombinase complex and have also relied on the known ability of prethrombin 2 to interact tightly but reversibly with fragment 2 or fragment 1.2. The kinetics of cleavage at $Arg^{323}-Ile^{324}$ have been assessed with these substrate analogs to investigate the contribution of cofactor−substrate interactions mediated by the fragment 2 domain to the ability of factor Va to enhance the catalytic efficiency of factor Xa within the prothrombinase complex. Initial velocity measurements indicated that the rate of prethrombin 2 cleavage by the factor Xa−PCPS binary complex was increased by a factor of ∼1300 upon the addition of saturating concentrations of factor Va to assemble prothrombinase. Although the measured initial velocity was higher when either fragment 2 or fragment 1.2 was present, the factor Va-dependent enhancement in initial rate (2600- and 1500-fold) was comparable in each case. Steady state kinetic constants were obtained using prethrombin 2, prethrombin 2 *plus* fragment 2, and prethrombin 2 *plus* fragment 1.2 as substrates. For each substrate, the addition of saturating concentrations of factor Va to the Xa−PCPS binary complex led to increases in catalytic efficiency of between 1000 and 9000-fold. The kcat/Km for prethrombin 2 cleavage by prothrombinase was essentially identical to that obtained for prethrombin 2 saturated with fragment 2. Thus, comparable accelerating effects of factor Va are observed independent of the presence of the fragment 2 domain in the substrate. The results indicate that interactions between factor Va and the substrate mediated by the fragment 2 domain do not contribute in a dominant way to the ability of factor Va to enhance the catalytic efficiency of factor Xa within the prothrombinase complex.

The proteolytic conversion of prothrombin to thrombin is a requisite step of the blood coagulation cascade (Jackson & Nemerson, 1980; Mann et al., 1988). This reaction is catalyzed by a multicomponent complex, composed of a serine protease, factor Xa, a protein cofactor, factor Va, and membranes containing acidic phospholipids (Jackson & Nemerson, 1980; Mann et al., 1988, 1990). The reversible interactions between these macromolecules in the presence of calcium ions yields the prothrombinase complex (Mann et al., 1988, 1990). The functional properties of factor Xa are significantly altered as a result of macromolecular interactions within the prothrombinase complex. At the physiological concentration of prothrombin, the rate of thrombin formation by factor Xa alone is increased by a factor of ∼100 000 when the protease is assembled into the prothrombinase complex (Mann et al., 1988, 1990).

Explanations for the enhanced activity of the prothrombinase complex have largely been sought from steady state kinetic measurements of thrombin formation comparing the activity of factor Xa alone with that observed using binary and ternary combinations of the protease with factor Va and model membranes containing acidic/amino phospholipids (Rosing et al., 1980; Nesheim et al., 1979, 1984; van Rijn et al., 1984). It is generally observed that membranes decrease the Km for prothrombin by a factor of ∼100 and factor Va increases the kcat by a factor of ∼3000 (Rosing et al., 1980; Nesheim et al., 1979; van Rijn et al., 1984). These individual effects seem to adequately explain the $\sim 10^5$-fold increase in catalytic efficiency observed upon assembly of the prothrombinase complex (Rosing et al., 1980; Nesheim et al., 1981, 1984).

The increased kcat for prothrombin activation, commonly attributed to the effect of factor Va on the reaction, implies that the cofactor either allosterically modulates the active site of factor Xa so that it is more complementary to the transition state or that it binds prothrombin and alters structures surrounding the scissile bond(s) (Jackson & Nemerson, 1980; Walker & Krishnaswamy, 1993). Studies have focused on distinguishing between the contributions of

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these two possible effects by using reagents directed to the active site or macromolecular recognition sites of factor Xa with no known interactions with factor Va or membranes (Walker & Krishnaswamy, 1993; Krishnaswamy et al., 1994). These approaches indicate that the incorporation of factor Xa into the prothrombinase complex does not lead to detectable changes in the reactivity of the catalytic residues but may perturb extended macromolecular recognition sites on the protease. Recent studies with a recombinant derivative of the specific factor Xa inhibitor, tick anticoagulant peptide, suggest that the interaction of factor Xa with factor Va can lead to a large enhancement ($\sim 10^3$-fold) in the affinity of macromolecular interactions with the protease (Betz et al., 1997). However, the significance of these types of changes with respect to recognition of prothrombin by prothrombinase are unknown since previous observations of factor Va-induced changes in substrate affinity have generally been attributed to result from the ability of factor Va to directly bind prothrombin (van Rijn et al., 1984; van de Waart et al., 1984; Boskovic et al., 1990).

The potential for factor Va to influence the kcat for prothrombin activation by binding and altering the substrate derives from evidence demonstrating that prothrombin can interact reversibly with factor Va (Kd $\approx 8\ \mu$M) through the fragment 2 domain of the substrate (Esmon et al., 1973; Esmon & Jackson, 1974; Luckow et al., 1989; Guinto & Esmon, 1984; Boskovic et al., 1990). Studies with factors Xa and Va in solution have indicated a dominant contribution of the substrate−cofactor interaction to prothrombin activation (Boskovic et al., 1990). Previous studies have also demonstrated a near absolute requirement of fragment 2 for factor Va to significantly accelerate the cleavage of prethrombin 2 by factor Xa (Esmon & Jackson, 1974). However, these lines of evidence have been derived from solution-phase studies with factor Xa only fractionally saturated with factor Va. Therefore, the significance and quantitative contribution of the substrate−cofactor interaction to the function of factor Va in the membrane assembled prothrombinase complex is not clear.

The interpretation of the steady state kinetic studies of prothrombin activation is complicated by the fact that the conversion of the substrate to thrombin requires the cleavage of two peptide bonds (Figure 1) (Jackson & Nemerson, 1980; Mann et al., 1988). Product formation catalyzed by prothrombinase predominantly results from cleavage at Arg^{323}−Ile^{324} followed by cleavage at Arg^{274}−Thr^{275}, yielding the protease meizothrombin as an intermediate (Krishnaswamy et al., 1986, 1987; Rosing et al., 1986). Cleavage of the bonds in the opposite order yielding prethrombin 2 as an intermediate accounts for most of the thrombin formed in the absence of factor Va (Esmon et al., 1974a; Krishnaswamy et al., 1987; Rosing et al., 1986). Since prethrombin 2 is inactive and meizothrombin differs from thrombin in its reaction with a variety of substrates and inhibitors, initial velocity measurements using prothrombin reflect complex contributions of the products of two enzymatic reactions as well as two possible kinetic pathways (Walker & Krishnaswamy, 1994). Consequently, even though initial rates of thrombin formation determined at varying concentrations of prothrombin are adequately described by a rectangular hyperbola (Rosing et al., 1980; Nesheim et al., 1979; Krishnaswamy et al., 1986, 1987), it is unlikely that the derived Km and kcat values or changes in these terms attributed to specific macromolecular interactions within the prothrombinase complex can be interpreted in the traditional way.

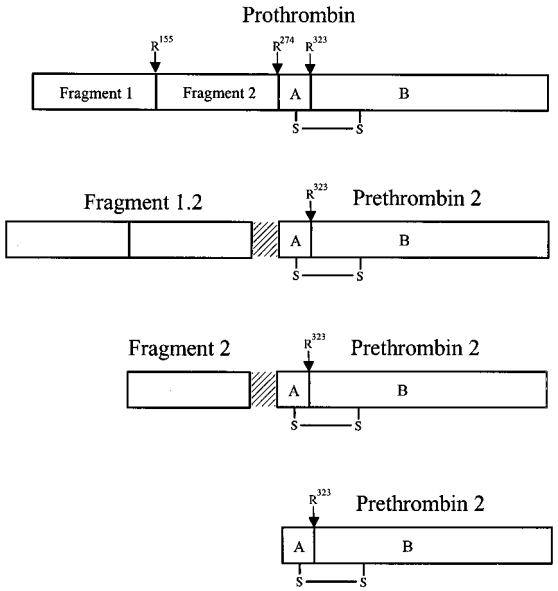

FIGURE 1: Schematic illustration of cleavage sites in prothrombin and derivatives. Thrombin formation requires cleavage of prothrombin at Arg^{323}−Ile^{324} (R^{323}) followed by Arg^{274}−Thr^{275} (R^{274}) by prothrombinase. Prior cleavage at R^{274} yields prethrombin 2 plus fragment 1.2. The fragment 1 domain mediates the high-affinity interaction of prothrombin with membranes, and fragment 2 is responsible for binding factor Va. The site denoted by R^{155} is subject to cleavage by thrombin which liberates fragment 1 from either prothrombin or fragment 1.2. The diagonal lines denote the ability of prethrombin 2 to interact tightly with the fragment 2 domain. The prethrombin 2 derivatives require further cleavage at R^{323} to form thrombin and represent substrates which cannot interact with membranes or factor Va (Prethrombin 2), which can bind to factor Va but not membranes (Prethrombin 2 plus Fragment 2) and which can bind both factor Va and membranes (Prethrombin 2 plus Fragment 1.2).

Prior cleavage of prothrombin at Arg^{274}−Thr^{275} yields prethrombin 2 (Figure 1), which interacts tightly but noncovalently with the fragment 1.2 activation peptide (Esmon et al., 1974a; Myrmel et al., 1976). The kinetics of cleavage of the intact Arg^{323}−Ile^{324} bond in this prothrombin derivative either by factor Xa or by prothrombinase has been shown to be indistinguishable from the cleavage of the same bond in prothrombin by either enzyme (Walker & Krishnaswamy, 1994; Carlisle et al., 1990). Prethrombin 2 is therefore an appropriate substrate analog for studies of the first cleavage reaction in the activation of prothrombin which is also the step most profoundly influenced by factor Va and membranes (Nesheim & Mann, 1983; Krishnaswamy et al., 1986, 1987; Walker & Krishnaswamy, 1994). We have relied on these features of prethrombin 2 as well as its established ability to interact tightly with fragment 2 or fragment 1.2 (Figure 1) to provide a family of substrates with a single cleavage site without or noncovalently associated with the cofactor binding domain. In combination with the directly measured equilibrium constants for the binary and ternary interactions within the prothrombinase complex we have used these substrates to assess the requirement of the fragment 2 domain for the ability of factor Va to accelerate bond cleavage by factor Xa within the prothrombinase complex.

EXPERIMENTAL PROCEDURES

Materials

Hepes, L-α-phosphatidylserine (bovine brain) and L-α-phosphatidylcholine (hen egg) were from Sigma (St. Louis, MO). The thrombin substrate, S2238,[1] was from Kabi-Pharmacia (Franklin, OH), and polyethylene glycol with an average molecular weight of 8000 was from J. T. Baker (Danvers, MA). Stock solutions of S2238 were prepared in water and substrate concentrations were determined using $E^{M}_{342} = 8270\ M^{-1}\ cm^{-1}$ (Lottenberg & Jackson, 1983). PCPS were prepared as previously described (Higgins & Mann, 1983). Phospholipid concentrations were determined by a colorimetric phosphate assay following oxidation (Gomori, 1942), and concentrations are expressed in terms of total phospholipid. Characterization of the vesicles by quasielastic light scattering (Nicomp 370, NICOMP Instruments, Santa Barbara, CA) yielded a gaussian distribution centered at $d = 20.5 \pm 8.5$ nm. These dimensions yield an average of ~3000 phospholipids/vesicle on the basis of published values for the surface area occupied by head groups on outer and inner leaflets of small unilamellar vesicles (Mason & Huang, 1978).

Protein

Prothrombin and factor X was purified from bovine plasma as previously described (Krishnaswamy et al., 1986, 1990). Factor X was activated using the purified activator from Russell's viper venom and the resultant factor Xa was purified using benzamidine sepharose (Jesty & Nemerson, 1976; Krishnaswamy et al., 1987). Bovine factor Va was purified using established procedures (Krishnaswamy & Mann, 1988; Kalafatis et al., 1993). wt-TAP was expressed in Pichia Pastoris and purified as described (Laroche et al., 1994). The purified monoclonal IgG, αHII-5, directed against a peptidyl sequence present in the kringle 2 domain of prothrombin (Church et al., 1991), was a generous gift of Dr. William Church, University of Vermont. Prothrombin fragment 1.2, prethrombin 1, and thrombin were prepared as previously described (Mann et al., 1981; Lundblad et al., 1976). The isolation of prethrombin 2 and fragment 2 was according to modifications of existing methods (Mann et al., 1981; Carlisle et al., 1990). Prethrombin 1 (180 μM, 150 mg) in 0.85 M Na_3Citrate was treated with 220 nM factor Xa. Following cleavage for 30 min at room temperature, the reaction mixture was directly applied to benzamidine sepharose (1.5 × 8 cm) (Pharmacia-LKB, Piscataway, NJ) equilibrated in the same buffer. Development of the resin with 20 mM Hepes, 0.15 M NaCl, pH 7.4, resulted in the elution of a sharp peak containing prethrombin 2 and fragment 2. Thrombin and factor Xa bound to the resin could be eluted with the same buffer containing 4 mM benzamidine. Fractions containing prethrombin 2 and fragment 2 were pooled, applied to a column of trypsin inhibitor sepharose (1.6 × 5 cm) (Sigma) to remove trace amounts of factor Xa, dialyzed against 25 mM sodium phosphate, pH 6.5, and applied to a column (2.5 × 30 cm) of *S*-sepharose equilibrated in the same buffer. Fragment 2 was not retained by the resin. Bound prethrombin 2 was eluted with a linear gradient of increasing NaCl (0 to 1.0 M, 4 mL/min, 150 min) prepared in the same buffer. Fractions containing prethrombin 2 and fragment 2 were pooled separately and precipitated with ammonium sulfate (80% saturation). Precipitated protein was collected by centrifugation (53000*g*, 30 min) and dissolved in 50% glycerol. The individual protein preparations were then subject to gel-filtration chromatography using Sephadex G-75 (2.5 × 100 cm) equilibrated in 20 mM Hepes, 2.5 M NaCl, pH 7.4, to remove possible traces of cross-contaminating fragments (Carlisle et al., 1990). In each case, the pooled fractions were dialyzed against 20 mM Hepes, pH 7.4, concentrated by precipitation with ammonium sulfate and centrifugation and stored at −20 °C as concentrated solutions (>200 μM) in 50% glycerol. Typical yields were 40 mg of prethrombin 2 and 20 mg of fragment 2. Prethrombin 2, fragment 2, and fragment 1.2 were exchanged into 20 mM Hepes, 0.15 M NaCl, 2.0 mM $CaCl_2$, 0.1% (w/v) PEG, pH 7.4, either by dialysis or by desalting using centrifuge columns (Sephadex G-25, 5 mL) before use.

The purity of protein preparations was established by SDS−PAGE followed by staining with Coomassie Brilliant Blue (Laemmli, 1970). SDS−PAGE analysis revealed three closely spaced bands in all fragment 2 preparations. N-terminal sequencing yielded a single sequence expected for bovine fragment 2 (Mann et al., 1981). Laser desorption mass spectrometry of fragment 2 (peptide formula weight 12 791) yielded a major species at 12 825 with a minor peak at 13 456. The basis for this heterogeneity was not further investigated but may be related to partial glycosylation (Carlisle et al., 1990) or heterogeneity in some other modification (Owen et al., 1974). Titration of factor Xa or thrombin with *p*-nitrophenyl p′-guanidinobenzoate (Chase, & Shaw, 1967) yielded 1.12 and 0.96 mol active sites/mol protein, respectively. Protein concentrations were determined using the following molecular weights and extinction coefficients ($E^{0.1\%}_{280}$): factor Xa, 45 300, 1.24 (Jackson et al., 1968; Fujikawa et al., 1974); factor Va, 168 000, 1.74 (Laue et al., 1984; Krishnaswamy & Mann, 1988), prethrombin 1, 50 200, 1.64; prethrombin 2, 37 400, 1.95; fragment 1.2, 34 800, 1.12; fragment 2, 12 800, 1.25 (Mann et al., 1981); wt-TAP, 6 980, 2.54 (Krishnaswamy et al., 1994).

Progress Curves for Thrombin Formation

All kinetic measurements were performed in 20 mM Hepes, 0.15 M NaCl, 0.1% (w/v) PEG, 2 mM $CaCl_2$, pH 7.4. Final concentrations of 1.4 μM prethrombin 2, 1.4 μM prethrombin 2 *plus* 2.8 μM fragment 2, or 1.4 μM prethrombin 2 *plus* 2.8 μM fragment 1.2 were used for these measurements. Reaction mixtures (290 μL) were prepared containing 1.4 μM substrate with no addition, with 50 μM PCPS, 30 nM Va or 30 nM Va *plus* 50 μM PCPS and were incubated for 10 min at 25 °C. Thrombin formation was initiated by the addition of a factor Xa solution (10 μL) to achieve the indicated concentration and the reactions were maintained at 25 °C. Samples (10 μL) were withdrawn at various times and quenched by mixing with 90 μL of 20 mM Hepes, 0.15 M NaCl, 50 mM EDTA, 0.1% (w/v) PEG, 2 μM wt-TAP, pH 7.4. Quenched samples were then further diluted in the same buffer lacking wt-TAP and initial

[1] The abbreviations used are PCPS, small unilamellar vesicles composed of 75% (w/w) L-α-phosphatidylcholine and 25% (w/w) L-α-phosphatidylserine; PEG, polyethylene glycol; S2238, H-D-phenylalanylpipecoylarginine *p*-nitroaniline; wt-TAP, recombinant Tick Anticoagulant Peptide.

velocities of S2238 hydrolysis were determined in 96-well plates at ambient temperature in a Vmax kinetic plate reader (Molecular Devices, Menlo Park, CA) using 200 μM S2238 in 20 mM Hepes, 0.15 M NaCl, 50 mM EDTA, 0.1% (w/v) PEG, pH 7.4. Measured rates were related to the concentration of thrombin from the linear dependence of initial velocity on known concentrations of thrombin determined in each experiment. The addition of fragment 2 or fragment 1.2 at concentrations exceeding those carried over into the assay had no detectable effect on the rate of S2238 hydrolysis by thrombin. For certain substrate combinations, the apparent lack of quantitative conversion of substrate to thrombin in complete progress curves was established to arise from significant product inhibition by thrombin.

Initial Velocity Measurements

Initial, steady state velocities for thrombin formation were measured using discontinuous measurements of S2238 hydrolysis (above) in samples withdrawn at 0, 0.5, 1, 1.5, 2, and 3 min following initiation with factor Xa. The steady state rate of thrombin formation was extracted from the slope of the linear appearance of product as a function of time. When prethrombin 2 *plus* fragment 2 or prethrombin 2 *plus* fragment 1.2 was the varied substrate, the activation fragment was present at 1.5-fold molar excess to ensure saturation of prethrombin 2. Steady state kinetic constants were also determined by varying prethrombin 2 in the presence of a single fixed concentration (55 μM) of fragment 2.

Western Blot Analysis

Samples containing 5 μg of protein separated by SDS−PAGE were electrophoretically transferred to Immobilon PSQ membranes (Millipore, Bedford, MA) using a semidry blotting apparatus (Hoeffer Scientific, San Francisco, CA). Electrophoretic transfer was achieved in 10 mM CAPS, 10% (v/v) methanol, pH 11.0, at 0.04 mA cm^{-2} for 16 h. Following blocking of the membranes with 20 mM Hepes, 0.15 M NaCl, 0.1% (v/v) Tween-20, 0.1% (w/v) bovine serum albumin, pH 7.4, the membrane was incubated with a solution of 5 μg/mL αHII-5 antibody for 45 min in the same buffer lacking albumin. Following several washes with buffer, bound antibody was detected using biotinoylated antimouse IgG, avidin and biotinoylated horseradish peroxidase provided with the Vectastain Elite kit (Vector, Burlingame, CA) and following the instructions of the manufacturer. Immunoreactive bands were visualized using H_2O_2 and 4-chloronaphthol or at higher sensitivity with the Luminol reagent (Amersham, Arlington Heights, IL) followed by detection of chemiluminescence by exposure to photographic film.

Calorimetry

Isothermal titration calorimetry was carried out using a Microcal Omega 2 (Microcal, Northhampton, MA) at the Biocalorimetry Center, Johns Hopkins University, Baltimore, MD. The sample cell (1.362 mL) contained either 80 or 120 μM prethrombin 2 in 20 mM Hepes, 0.15 M NaCl, 0.1% (w/v) PEG, 2 mM $CaCl_2$, pH 7.4. Titrations were performed at 24.8 °C by successive injections (20 or 30 μL each at 4 min intervals) of 870 μM fragment 2 in the same buffer. Titrations were terminated when further additions of fragment 2 did not yield appreciable heat flow. Numerical integration and data analysis were performed using Origin software (Microcal) with appropriate script files provided by the manufacturer to extract ΔH, Kd, and stoichiometry.

Data Analysis

Steady State Kinetic Constants. The results of initial velocity measurements were analyzed according to the Henri−Michaelis−Menten equation by nonlinear least squares analysis using the Marquardt algorithm (Bevington, 1969). The fitted parameters are reported ±95% confidence limits. When substrate concentrations were well below Km, the kcat/Km term was calculated by linear regression analysis weighted according to the reciprocal of 1 standard deviation in the measured velocity (Bevington, 1969). When the kcat/Km term was calculated from fitted values of kcat and Km, the uncertainty in the calculated value was determined by propagating the errors of the fitted constants.

Equilibrium Constant for Prothrombinase Assembly. The initial velocity for prethrombin 2 activation increased with increasing concentrations of factor Va at a single fixed concentration of factor Xa and a saturating concentration of PCPS. The concentration of PCPS was chosen to bind most (>90%) of the factor Xa present yet ensuring that the concentration of vesicles was not vastly greater than the concentration of protease. Assuming that the increase in velocity results from the assembly of prothrombinase, the data were analyzed using the model and equations previously developed to yield the equilibrium dissociation constant for the interaction of factor Va with factor Xa on the membrane surface, the stoichiometry for the interaction, velocities in the absence of Va (v_o) and at infinite concentrations of factor Va (v_∞) (Krishnaswamy, 1990; Krishnaswamy et al., 1987). The assembly of prothrombinase is accompanied by a large decrease in the Km for prethrombin 2. As a result, prothrombinase assembly is favored in the presence of prethrombin 2. The dissociation constant for the Xa−Va interaction on membranes inferred from rates of prethrombin 2 cleavage therefore requires correction for the enhancing effect of the substrate on this interaction. The observed Kd from activity measurements was corrected for substrate effects assuming Km = Ks and the relationship

$$\mathrm{Kd} = \mathrm{Kd}_{\mathrm{obs}}\left(\frac{1 + \dfrac{S}{\mathrm{Ks}_{\mathrm{XaVaL}}}}{1 + \dfrac{S}{\mathrm{Ks}_{\mathrm{XaL}}}}\right) \qquad (1)$$

where *S* is the concentration of prethrombin 2, and the Km values for prothrombinase (Ks_{XaVaL} = 2.58 μM) and for the Xa−PCPS binary complex (Ks_{XaL} = 197 μM) were taken from Table 2. In addition to the rapid equilibrium assumption, this correction also requires that $kcat_{XaL}/Ks_{XaL} \ll kcat_{XaVaL}/Ks_{XaVaL}$ as a result of $Ks_{XaL} \gg Ks_{XaVaL}$ and/or $kcat_{XaL} \ll kcat_{XaVaL}$. This requirement is borne out by the data (Table 2). The correction was relatively small since the prethrombin 2 concentration was lower than both Km (Ks) terms.

Equilibrium Constant for the Interaction of Prethrombin 2 with Fragment 2. Initial velocity measurements of prethrombin 2 cleavage by prothrombinase at increasing concentrations of fragment 2 were analyzed assuming that fragment 2 could function as a nonessential activator by binding prethrombin 2 (Segel, 1975). The initial velocity

Table 1: Influence of Fragment 2 and Fragment 1.2 on the Ability of Factor Va to Accelerate Cleavage at $Arg^{323}-Ile^{324}$ [a]

		prethrombin 2		prethrombin 2/fragment 2		prethrombin 2/fragment 1.2	
enzyme components[b]	enzyme species[c]	initial velocity[d] (nM IIa/min/nM Xa)	rel rate[e]	initial velocity (nM IIa/min/nM Xa)	rel rate	initial velocity (nM IIa/min/nM Xa)	rel rate
Xa	Xa	0.016	1	0.026	1	0.45	1
Xa, PCPS	Xa−PCPS	0.036	2.3	0.029	1.1	0.99	2.2
Xa, Va	Xa + (Xa−Va)	0.09	5.9	0.97	37.3	98.16	219.1
Xa, Va, PCPS	Xa−Va−PCPS	28.80	1858	75.17	2891	1530	3415

[a] Initial velocities were determined from progress curves as illustrated in Figure 2. [b] With the exception of the Xa + Va data set, concentrations of PCPS and factor Va were selected to saturate factor Xa with PCPS or factor Va and PCPS. [c] Predicted distribution of enzyme species on the basis of published dissociation constants. Species in parentheses represent an unknown fraction of the total factor Xa present. [d] Initial velocities were normalized to the total concentration of factor Xa in the reaction mixture. [e] Relative rates were calculated for each substrate by assigning the initial velocity observed with factor Xa alone a value of 1.

expression for this case is given by

$$V = \frac{E_T\left(\mathrm{kcat}_{P2}\frac{P2}{Ks_{P2}} + \mathrm{kcat}_{P2F2}\frac{P2F2}{Ks_{P2F2}}\right)}{1 + \frac{P2}{Ks_{P2}} + \frac{P2F2}{Ks_{P2F2}}} \quad (2)$$

where E_T is the total concentration of prothrombinase, P2 is the free concentration of prethrombin 2, P2F2 is the concentration of prethrombin 2 bound to fragment 2, and the subscripted kcat and Ks terms are the kinetic constants for the cleavage of the two substrates. The data were analyzed assuming Km = Ks using mean values of Ks_{P2} = 2.58 μM and Ks_{P2F2} = 14.18 μM (Table 2). The free and bound concentrations of prethrombin 2 are related to total concentrations by the expression

$$P2F2 = \frac{(P2_T + nF2_T + Kd_{P2,F2}) - \sqrt{(P2_T + nF2_T + Kd_{P2,F2})^2 - 4P2_T nF2_T}}{2} \quad (3)$$

where $P2_T$ and $F2_T$ represent total concentrations of prethrombin 2 and fragment 2, *n* mol of F2 combine per mol of P2, and $Kd_{P2,F2}$ is the equilibrium dissociation constant for that interaction. Analysis of the data according to eqs 2 and 3 yielded fitted values of $Kd_{P2,F2}$, $n_{P2,F2}$, $kcat_{P2}$, and $kcat_{P2F2}$. The kcat terms were also fitted to accommodate the uncertainty in the ability of averaged kinetic constants to accurately predict velocities at any one given substrate concentration.

RESULTS

Effects of Prothrombinase Components on the Rate of Cleavage at $Arg^{323}-Ile^{324}$

Progress curves illustrating the effects of prothrombinase components on the conversion of prethrombin 2 to thrombin are provided in Figure 2A. Factor Xa was a poor catalyst for the activation of prethrombin 2. Minor differences in the rate of thrombin formation were evident when either PCPS (50 μM) or factor Va (30 nM) were individually added to the reaction mixture (Figure 2A).

Factor Xa binds reversibly to PCPS with moderate affinity (Nelsestuen & Broderius, 1977; Krishnaswamy et al., 1988). On the basis of published equilibrium constants for this interaction, greater than 90% of the factor Xa would be expected to be membrane bound under these conditions (Krishnaswamy et al., 1988; Krishnaswamy, 1990). Thus,

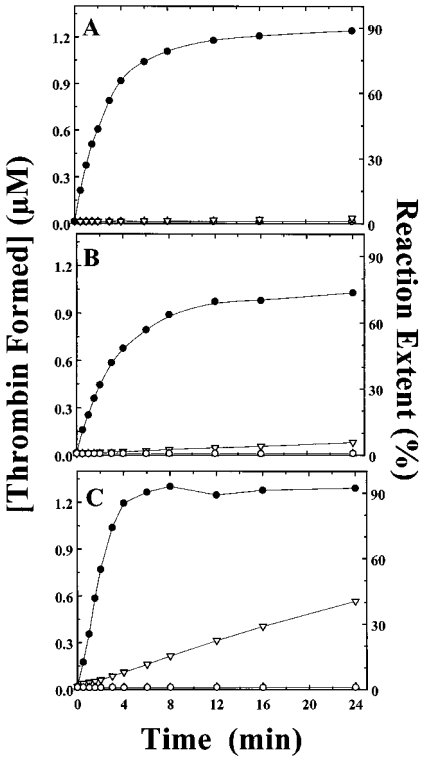

FIGURE 2: Effect of factor Va on the rate of cleavage of prothrombin derivatives at $Arg^{323}-Ile^{324}$. Progress curves are illustrated for thrombin formation from 1.4 μM prethrombin 2 (panel A), 1.4 μM prethrombin 2 plus 2.8 μM fragment 2 (panel B), and 1.4 μM prethrombin 2 plus 2.8 μM fragment 1.2 (panel C) using factor Xa concentrations of 10 nM (panel A), 3 nM (panel B), and 0.25 nM (panel C). In each panel, the reaction mixtures contained no addition (○), 55 μM PCPS (△), 30 nM factor Va (▽), or 55 μM PCPS and 30 nM factor Va (●). The lines were arbitrarily drawn.

membrane binding by factor Xa alone is not sufficient to elicit large changes in the rate of cleavage at $Arg^{323}-Ile^{324}$ in prethrombin 2. Factor Va interacts weakly with factor Xa in solution with an equilibrium dissociation constant of 10^{-6} M (Boskovic et al., 1990; Pryzdial & Mann, 1991) and only a small fraction (~1.5%) of factor Xa present would be expected to be complexed with factor Va at the concentrations used in the absence of membranes. This expectation is consistent with the small differences in the rate of thrombin formation from prethrombin 2 by factor Xa alone in comparison to a mixture of factors Xa and Va.

The rate of thrombin formation catalyzed by factor Xa was dramatically enhanced when both factor Va and PCPS were present in the reaction mixture (Figure 2A). On the basis of equilibrium constants determined in physical studies of prothrombinase assembly (Krishnaswamy et al., 1988;

Krishnaswamy, 1990), the concentrations of Va and PCPS were sufficient to incorporate more than 95% of the factor Xa present into the prothrombinase complex. A comparison of the initial rate of thrombin formation by the Xa−PCPS binary complex to the rate observed with prothrombinase (Table 1), indicates that the presence of the cofactor in the prothrombinase complex increases the rate of $Arg^{323}-Ile^{324}$ cleavage by a factor of ~1000. Since prethrombin 2 lacks the fragment 2 domain, which is considered to mediate the substrate−cofactor interaction, it follows that factor Va can significantly accelerate substrate cleavage by factor Xa within the membrane-assembled prothrombinase complex by 1000-fold even in the absence of substrate−cofactor interactions mediated by fragment 2.

Previous studies have established a minor accelerating effect of factor Va on prethrombin 2 cleavage relative to the effect of the cofactor on the cleavage of prethrombin 2 *plus* fragment 2 (Esmon & Jackson, 1974). Therefore, equivalent experiments were conducted in the presence of a molar excess of fragment 2 (Figure 2B) or fragment 1.2 (Figure 2C) to assess the effects of the fragment 2 domain on the ability of factor Va to accelerate cleavage at the $Arg^{323}-Ile^{324}$ peptide bond in prethrombin 2. The rate of thrombin formation was significantly enhanced by the presence of fragment 2 and more so by fragment 1.2, requiring the use of lower concentrations of catalyst. However, the relationship between initial rates of thrombin formation catalyzed by factor Xa, the Xa−PCPS binary complex or by factor Xa saturably incorporated into the Xa−Va−PCPS ternary complex was largely maintained (Figure 2). For each substrate, the rate of product formation was increased by a factor of $\sim 10^3$ following the addition of saturating concentrations of factor Va to the Xa−PCPS binary complex.

Initial rates normalized to the concentration of factor Xa as well as rates relative to those observed with solution phase factor Xa are presented in Table 1. Interpretation of these data requires explicit knowledge of catalyst concentration, which can be reliably predicted at saturating concentrations of PCPS or Va and PCPS on the basis of direct equilibrium binding measurements (Krishnaswamy et al., 1988; Krishnaswamy, 1990). In the case of each substrate, the rate of the reaction observed with Xa−PCPS as the catalyst was increased by a factor of 800 to 2500 upon saturable incorporation into the membrane assembled prothrombinase complex (Xa−Va−PCPS). Thus, the bulk of the factor Va-dependent increase in reaction rate under these conditions appears largely independent of the presence of the fragment 2 domain.

In contrast, results of initial velocity measurements comparing rates observed with factor Xa alone with those observed in the presence of nanomolar concentrations of factor Va in the absence of membranes imply a significant effect of the activation fragments on the factor Va-dependent rate enhancement (Figure 2, Table 1). These findings, illustrated for the purposes of comparison, are in good agreement with previous observations (Esmon & Jackson, 1974). However, since the fractional saturation of factor Xa by factor Va is small, rate comparisons normalized to the total concentration of factor Xa are invalid. These conditions are also particularly sensitive to even small effects of different substrates or activation fragments on the apparent affinity for the solution-phase Xa−Va interaction. Significant effects of this type are implied by an apparent Kd of 25 nM measured from initial velocity measurements of prothrombin activation (Skogen et al., 1984), relative to a directly measured equilibrium dissociation constant of 1−2 μM for the Xa−Va interaction in solution (Boskovic et al., 1990; Pryzdial & Mann, 1991; Krishnaswamy et al., 1993).

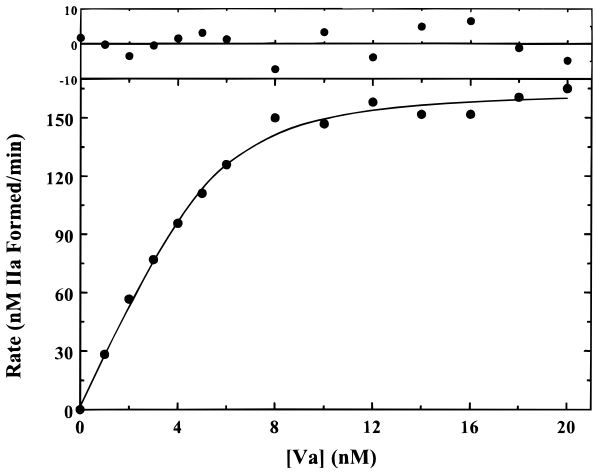

FIGURE 3: Dependence on the rate of cleavage of prethrombin 2 on prothrombinase assembly. Reaction mixtures containing 1.4 μM prethrombin 2, 58 μM PCPS, and the indicated concentrations of factor Va in 20 mM Hepes, 0.15 M NaCl, 2 mM $CaCl_2$, 0.1% (w/v) PEG, pH 7.5, at 25 °C, were initiated with 5 nM factor Xa. Initial velocities determined as a function of factor Va were analyzed using the assumptions and equations described under Data Analysis. The line was drawn using Kd = 0.71 ± 0.25 nM, n = 0.89 ± 0.09 mol of Va/mol of Xa at saturation and v_∞ = 164 ± 6.6 nM IIa/min. The residuals to the fitted line are shown in the upper panel.

Detailed kinetic information that is necessary for the adequate interpretation of initial velocities obtained with factor Xa partially saturated with factor Va in solution is presently unavailable. Thus, only reaction rates observed with factor Xa alone, Xa saturated with PCPS, or Xa saturated with Va and PCPS can be interpreted in a meaningful way. The initial velocity measurements (Table 1) with these combinations of catalyst components permit the preliminary conclusion that while there may be minor differences in the extent of rate enhancement by the cofactor in the presence of fragment 2 or fragment 1.2, the predominant accelerating effect of factor Va on the rate of cleavage at $Arg^{323}-Ile^{324}$ by the prothrombinase complex does not require the fragment 2 domain.

Effect of Prothrombinase Assembly on the Rate of Cleavage of Prethrombin 2

The initial velocity measurements (Table 1) at saturating concentrations of factor Va imply that the accelerated cleavage of prethrombin 2 in the absence of added prothrombin fragments is the consequence of the effect of factor Va within the membrane assembled prothrombinase complex. This interpretation requires that acceleration of prethrombin 2 cleavage should correspond directly to the known requirements for the assembly of the prothrombinase complex.

Measurements of the conversion of prethrombin 2 to thrombin were conducted using increasing concentrations of factor Va at a single, fixed concentration of factor Xa and PCPS (Figure 3). The initial velocity was found to increase saturably with increasing concentrations of the cofactor,

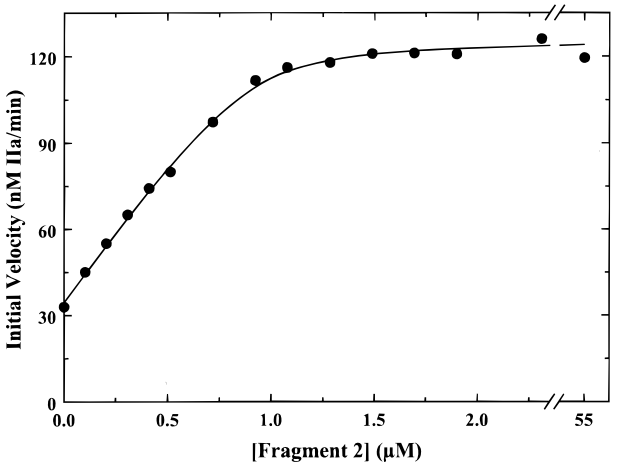

FIGURE 4: Effect of the fragment 2 domain on the rate of cleavage at $Arg^{323}-Ile^{324}$ by prothrombinase. Reaction mixtures containing 1.0 μM prethrombin 2, the indicated concentrations of fragment 2, 55 μM PCPS, and 26 nM factor Va in 20 mM Hepes, 0.15 M NaCl, 2 mM $CaCl_2$, 0.1% (w/v) PEG, pH 7.5, were initiated with 2 nM factor Xa. Initial rates were determined at 25 °C. The line was drawn following analysis according to eqs 2 and 3 using the terms $Kd_{P2,F2} = 33 \pm 13$ nM, $n_{P2,F2} = 1.1 \pm 0.05$ mol of F2/mol of P2, $kcat_{P2} = 1.0 \pm 0.03$ s^{-1}, and $kcat_{P2F2} = 17.1 \pm 0.95$ s^{-1}.

reaching a maximum value that was approximately 1000-fold greater than the rate observed in its absence. As the concentration of PCPS was saturating relative to the individual equilibrium dissociation constants for the binding of Xa or Va to these membranes (Krishnaswamy, 1990), the data were analyzed as described under Experimental Procedures to extract the equilibrium parameters describing the interaction between factors Xa and Va on the membrane surface. The inferred dissociation constant ($Kd = 0.71 \pm 0.25$ nM) and stoichiometry ($n = 0.89 \pm 0.09$ mol of Va/mol of Xa) are comparable to those previously determined from direct physical or activity measurements using similar assumptions under a wide range of conditions (Krishnaswamy, 1990). Thus, the increase in the rate of cleavage of prethrombin 2 observed following the addition of saturating concentrations of factor Va to Xa−PCPS (Table 1) results from a change which approximates the saturable incorporation of the protease into the membrane-assembled prothrombinase complex.

Contribution of Fragment 2 to Prethrombin 2 Cleavage Catalysed by Prothrombinase

Evidence for a tight interaction between fragment 2 and thrombin ($Kd = 0.8$ nM) or prethrombin 2 ($Kd = 0.13$ nM) derives from measurements performed at low ionic strength (Myrmel et al., 1976). An ionic strength dependence for this interaction is implied by the much larger equilibrium dissociation constant determined for the binding of fragment 2 to thrombin ($Kd = 5$ μM) at $I = 0.15$ M (Bock, 1992). Therefore, the relatively small effects of fragment 2 on the rate of prethrombin 2 cleavage by prothrombinase (Table 1) could reflect incomplete saturation by fragment 2 at physiological ionic strength.

This possibility was investigated by initial velocity measurements of prethrombin 2 cleavage by prothrombinase in the presence of increasing concentrations of fragment 2 (Figure 4). Initial rate was found to increase saturably with increasing concentrations of fragment 2 and approached a limiting value at concentrations just slightly greater than the fixed concentration of prethrombin 2. The initial velocity observed at saturating concentrations of fragment 2 was ~3.5-fold greater than that observed with prethrombin 2 alone. Analysis assuming that the observed increase in velocity results from the interaction between prethrombin 2 and fragment 2 (Data Analysis) yielded an inferred equilibrium dissociation constant of 33 ± 13 nM for the binding of fragment 2 to prethrombin 2 with a stoichiometry of approximately 1.

No obvious evidence for inhibition by fragment 2 was observed at concentrations as high as 55 μM (Figure 4). This observation implies that putative interactions between fragment 2 and factor Va contribute weakly, if at all, to productive cleavage at $Arg^{323}-Ile^{324}$ in prethrombin 2 by prothrombinase. Therefore, nonessential activation schemes more complex than that accounted for by eqs 2 and 3 need not be invoked to account for the data (Segel, 1975).

The interaction between fragment 2 and prethrombin 2 in the absence of catalyst was further investigated by isothermal titration calorimetry (data not shown). This approach yielded $\Delta H = -8.8 \pm 0.06$ kcal mol^{-1}, $n = 1.03$ mol of fragment 2/mol of prethrombin 2 and an upper limit estimate of $Kd_{P2,F2} < 0.2$ μM. Collectively, the data indicate that the interaction between prethrombin 2 and fragment 2 is tight relative to the concentrations used in this study and that saturating concentrations of fragment 2 lead to a modest increase in the rate of cleavage at $Arg^{323}-Ile^{324}$ in prethrombin 2 by prothrombinase.

Assessment of the Extent of Contamination of Prethrombin 2 by Fragment 2 Containing Derivatives

The observation that substoichiometric concentrations of fragment 2 or fragment 1.2 are sufficient to increase the rate of cleavage of most of the prethrombin 2 present has led to the suggestion that these activation peptides may function catalytically in thrombin formation (Esmon et al., 1974b). This type of phenomenon and contamination of prethrombin 2 by small amounts of these polypeptides could explain the large enhancement in prethrombin 2 cleavage by the assembly of the prothrombinase complex observed in the present study.

SDS−PAGE analysis of several prethrombin 2 preparations showed no obvious evidence of contamination by other prothrombin derivatives including fragment 2 or fragment 1.2. Staining of heavily overloaded gels indicated that, if these fragments were present, the level of contamination was probably less than 10 mol % of the prethrombin 2. This was further investigated using a monoclonal antibody directed against a polypeptide sequence present in the kringle 2 domain of human prothrombin (Church et al., 1991). Western blotting indicated that this antibody could detect bovine prothrombin and all possible derivatives containing fragment 2 (prethrombin 1, meizothrombin, meizothrombin des fragment 1, fragment 1.2, fragment 2) with moderate sensitivity (data not shown).

A representative Western Blot of a gel containing prethrombin 2, fragment 2 and fragment 1.2 is illustrated in Figure 5B along with a gel analyzed by protein staining (Figure 5A). Prethrombin 2 and fragment 1.2 yielded single bands, but three closely spaced bands were observed with fragment 2 (Figure 5A). The antibody used could readily

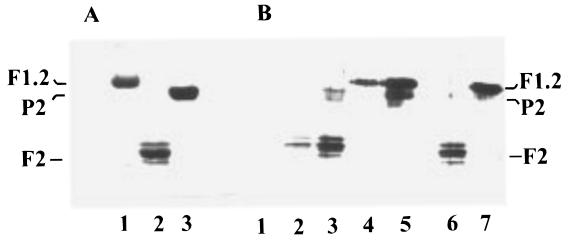

FIGURE 5: SDS−PAGE and Western Blot analysis of prethrombin 2. Panel A. Representative preparations of fragment 1.2 (lane 1), fragment 2 (lane 2), and prethrombin 2 (lane 3) were subject to SDS−PAGE following disulphide bond reduction, and protein bands were visualized by staining with Coomassie Brilliant Blue. Each lane contained 12 μg of protein. Panel B. Proteins initially separated by SDS−PAGE following disulphide bond reduction were electrophoretically transferred to PVDF membranes, and bands immunoreactive with αHII-5 antibody were visualized as dercribed under Experimental Procedures. The lanes contained (1) 5 μg of prethrombin 2; (2) 5 μg of prethrombin 2 + 34 ng of fragment 2; (3) 5 μg of prethrombin 2 + 85 ng of fragment 2; (4) 5 μg of prethrombin 2 + 90 ng of fragment 1.2; (5) 5 μg of prethrombin 2 + 230 ng of fragment 1.2; (6) 85 ng of fragment 2 and (7) 230 ng of fragment 1.2. The migration of fragment 1.2 (F1.2), prethrombin 2 (P2), and fragment 2 (F2) is indicated in the margins.

detect small amounts of purified fragment 2 or fragment 1.2 (Figure 5B). However, no antibody reactivity was detected in the lane containing purified prethrombin 2, indicating the absence of prothrombin or derivatives containing the fragment 2 domain. Fragment 2 or fragment 1.2 intentionally added to prethrombin 2 could be detected at levels corresponding to 2 mol % contamination. These data indicate that possible contamination of prethrombin 2 by these activation fragments is less than 2 mol %.

An indistinct band migrating in the region of prethrombin 2 was reproducibly revealed by the antibody when higher amounts of fragment 1.2 or fragment 2 were added to prethrombin 2 independent of protein preparation or the presence of protease inhibitors (lanes 5 and 3, Figure 5B). Although the data suggest some interaction between these proteins during SDS−PAGE even after disulphide reduction, this possibility seems unlikely. The basis for this observation was not further investigated.

Other lines of evidence also indicate that the observed acceleration of prethrombin 2 cleavage by prothrombinase assembly is probably unrelated to trace contamination by fragment 2 containing derivatives of prothrombin: (1) The initial velocity measurements (Table 1) could be reproduced with eight different preparations of prethrombin 2. (2) Passage of prethrombin 2 over a column of immobilized αHII-5 antibody did not alter cleavage kinetics or behavior on western blots. Control experiments established that the antibody column could deplete fragment 2 from preparations of prethrombin 2 intentionally mixed with the activation peptide. (3) The dependence of reaction rate or kinetic constants (Table 2, below) on the concentration of fragment 2 is inconsistent with trace contamination of prethrombin 2 with fragment 2.

Steady State Kinetics of the Cleavage at Arg^{323}−Ile^{324}

The basis for the effects of prothrombinase assembly on the rate of cleavage at Arg^{323}−Ile^{324} was investigated by steady state kinetic studies of the conversion of prethrombin 2, prethrombin 2 *plus* fragment 2, and prethrombin 2 *plus* fragment 1.2. Kinetic constants for the cleavage of each of these substrates were obtained using factor Xa saturated with PCPS or factor Xa saturated with PCPS and with factor Va. This approach was used to permit an assessment of both the kinetic contribution of factor Va within the prothrombinase complex to the rate of cleavage at Arg^{323}−Ile^{324} as well as the contribution of the substrate−cofactor interaction mediated by the fragment 2 domain to the ability of saturating concentrations of factor Va to accelerate bond cleavage.

Representative plots showing the dependence of initial velocity on increasing substrate concentrations are presented in Figure 6 and the derived steady state kinetic constants from these and other experiments are listed in Table 2. For each substrate, addition of saturating concentrations of factor Va to the Xa−PCPS binary complex resulted in an equivalent increase in the catalytic efficiency (kcat/Km). Comparable constants were observed for several different preparations of prethrombin 2, fragment 2, and fragment 1.2, indicating that unforeseen trace contamination of one or more of these protein preparations is not a likely explanation for the observed results. The factor Va-dependent increase in catalytic efficiency ranged from 1000-fold for prethrombin 2 to 10 000-fold for prethrombin 2 *plus* fragment 2 or 2200-fold for prethrombin 2 *plus* fragment 1.2. A principal source of uncertainty in some of these comparisons derives from the low rates in the absence of the cofactor coupled with the need for high substrate concentrations for these measurements. Thus, while there may be some contribution of the fragment 2 domain to the ability of factor Va to increase the catalytic efficiency for cleavage at Arg^{323}−Ile^{324}, the data in Table 2 indicate that the majority of the effects of the cofactor on the catalytic efficiency of the prothrombinase complex are independent of the fragment 2 domain and the resulting interactions between substrate and factor Va that this domain may mediate.

For all three substrates, obvious evidence for saturation was obtained when the catalyst was composed of the prothrombinase complex. Addition of fragment 2 or fragment 1.2 to prethrombin 2 resulted in a large change (10−20-fold) in the kcat for product formation catalyzed by prothrombinase. These results are consistent with significant effects of fragment 2 or fragment 1.2 on prethrombin 2 cleavage previously established in a number of studies. The presence of saturating concentrations of fragment 2 was found to increase both Km and kcat for prethrombin 2 cleavage by prothrombinase by a factor of ∼10 leading to a 2-fold increase in catalytic efficiency. As these changes appear unrelated to effects mediated by factor Va, the data imply that the interaction between fragment 2 and prethrombin 2 in some way alters substrate structures important for productive recognition of the Arg^{323}−Ile^{324} bond and its cleavage by the prothrombinase complex.

The reversible interaction of fragment 1.2 with prethrombin 2 confers the substrate with the potential macromolecular recognition elements of the fragment 2 domain as well as with membrane binding properties mediated by the fragment 1 region (Figure 1). If the effects of these domains are indeed independent, the listed kinetic constants for prothrombinase indicate that a reduction in the Km term is a dominant consequence of membrane binding by the substrate. This conclusion is consistent with prior kinetic models for prothrombin activation (Rosing et al., 1980; Nesheim et al., 1979, 1984; van Rijn et al., 1984) as well as more recent studies demonstrating a large effect of the substrate−

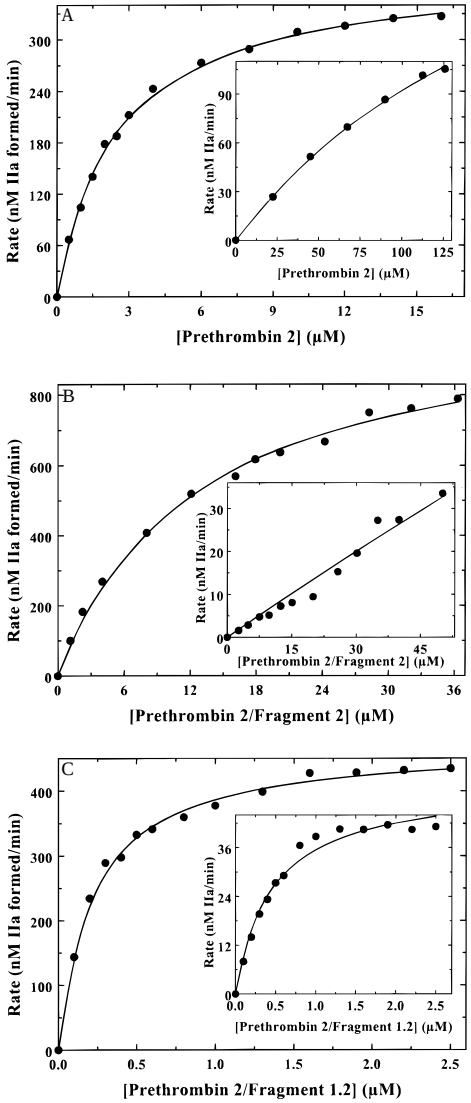

FIGURE 6: Effect of Factor Va and the Fragment 2 Domain on the Kinetic Constants for Cleavage at Arg^{323}−Ile^{324}. Representative v versus S plots are illustrated for the activation of prethrombin 2 (panel A), prethrombin 2 plus fragment 2 (panel B) and prethrombin 2 plus fragment 1.2 (panel C) either by factor Xa assembled in the prothrombinase complex or by the Xa−PCPS binary complex (insets). When prethrombin 2 was the varied substrate, reactant concentrations were 55 μM PCPS, 25 nM factor Va, and 5 nM factor Xa (panel A) or 200 μM PCPS and 48 nM factor Xa (panel A, inset). When prethrombin 2 plus fragment 2 was varied, the concentration of fragment 2 was 1.5-fold greater than the concentration of prethrombin 2. Other reactant concentrations were 55 μM PCPS, 25 nM factor Va, and 1 nM factor Xa (panel B) or 200 μM PCPS and 98 nM factor Xa (panel B, inset). The total concentration of prethrombin 2 plus fragment 1.2 was varied at a constant ratio of 1:1.5, using 55 μM PCPS, 25 nM factor Va, and 0.25 nM factor Xa (panel C) or 200 μM PCPS and 50 nM factor Xa (panel C, inset). The lines are drawn following analysis as described under Experimental Procedures using the fitted steady state kinetic constants illustrated in Table 2.

membrane interaction on the rate of cleavage at Arg^{323}−Ile^{324} by prothrombinase (Walker & Krishnaswamy, 1994).

Previous studies have concluded that factor Va functions in the prothrombinase complex to increase the kcat for prothrombin activation (Rosing et al., 1980; Nesheim et al., 1979, 1984; van Rijn et al., 1984). These conclusions are based on a comparison of steady state kinetic constants for prothrombin activation catalyzed by reaction mixtures containing Xa and PCPS relative to those containing Xa, Va, and PCPS. A similar comparison can be made for the kinetics of cleavage at Arg^{323}−Ile^{324} using the membrane-binding substrate, prethrombin 2 *plus* fragment 1.2 (Table 2). In agreement with the observations made with intact prothrombin, the kinetic constants obtained with this substrate suggest that factor Va alters the kcat for bond cleavage without significantly changing the Km term. However, the physical significance of kinetic constants obtained for such a membrane-binding substrate is questionable (Gentry et al., 1995; Lu & Nelsestuen, 1996a). These apparent kinetic constants have been shown to vary with the concentration of PCPS, membrane composition and vesicle size (Rosing et al., 1980; Gerads et al., 1990; Pei et al., 1993; Giesen et al., 1991; Lu & Nelsestuen, 1996a). These findings are consistent with the demonstration that the rate of the substrate−membrane interaction can limit catalysis by membrane-assembled prothrombinase (Giesen et al., 1991). If membrane binding by the substrate indeed precedes catalysis as is implied by rapid kinetic measurements (Walker & Krishnaswamy, 1994), both steady state kinetic constants are likely to be influenced in an unpredictable, mechanism-specific way by the rate constants for membrane binding and dissociation, which are estimated to be slow relative to catalysis (Nelsestuen & Lim, 1977; Kung et al., 1994; Lu & Nelsestuen, 1996b). It therefore seems unlikely that a superficial interpretation of the observed kcat change for prethrombin 2 *plus* fragment 1.2 cleavage following the addition of factor Va accurately reflects the effects of the cofactor on the function of the prothrombinase complex.

Complexities arising from the substrate−membrane interaction are mimized in the case of prethrombin 2 or prethrombin 2 *plus* fragment 2. For example, a 4-fold increase in the concentration of PCPS had no obvious effect on the kinetic constants for cleavage of either substrate by prothrombinase (data not shown). Comparisons with these substrates indicate that, in the absence of the fragment 1 domain, addition of factor Va to the Xa−PCPS binary complex results in a large change in the Km for the reaction. This is suggested by the linear increase in initial velocity of thrombin formation in the absence of factor Va over a wide range of substrate concentrations. In the case of prethrombin 2 where a rough estimate for the Km for cleavage by Xa−PCPS could in fact be obtained, the data indicate that the ∼1000-fold increase in kcat/Km is achieved through a large decrease in Km (∼80-fold) and a smaller increase in kcat (∼12-fold). Since these effects were observed in the absence of the fragment 2 domain, the data suggest that an important functional consequence of the interaction between factors Xa and Va on the membrane surface is an enhanced affinity of the protease component for its macromolecular substrate.

DISCUSSION

The enhanced activity of prothrombinase toward its biological substrate, prothrombin, is a consequence of macromolecular interactions within this enzyme complex mediated by the membrane surface as well as the cofactor (Mann et al., 1988, 1990). In the present study, we have applied the accumulated information on the thermodynamics of the various binary and ternary interactions within the enzyme complex to examine the potential contribution of interactions between prothrombin and factor Va to cofactor function.

Table 2: Kinetic Constants for the Cleavage at $Arg^{323}-Ile^{324}$ in the Absence and Presence of Factor Va[a]

substrate[b]	enzyme species[c]	kcat[d] ($s^{-1} \pm$ SE)	Km (μM $\pm$ SE)	kcat/Km ($M^{-1}\ s^{-1} \pm$ SE)
Prethrombin 2	Xa−PCPS	ND[e]	ND	576 ± 19
		0.1 (est)	197 (est)	482 ± 56
	Xa−Va−PCPS	1.27 ± 0.01	2.44 ± 0.1	$(5.2 \pm 0.2) \times 10^5$
		1.3 ± 0.02	2.58 ± 0.15	$(5.04 \pm 0.3) \times 10^5$
		1.75 ± 0.03	2.73 ± 0.15	$(6.41 \pm 0.4) \times 10^5$
Prethrombin 2 plus Fragment 2	Xa−PCPS	>0.06 (est)	>500 (est)	114 ± 3.2
	Xa−Va−PCPS	15.8 ± 1.1	16.06 ± 1.5	$(9.8 \pm 0.8) \times 10^5$
		17.5 ± 0.5	12.3 ± 0.9	$(1.42 \pm 0.1) \times 10^6$
		15.0 ± 0.6[f]	11.95 ± 1.15[f]	$(1.26 \pm 0.1) \times 10^6$
Prethrombin 2 plus Fragment 1.2	Xa−PCPS	0.017 ± 0.001	0.45 ± 0.05	$(3.8 \pm 0.5) \times 10^4$
	Xa−Va−PCPS	26.3 ± 0.3	0.22 ± 0.01	$(1.2 \pm 0.07) \times 10^8$
		21.5 ± 0.6	0.32 ± 0.03	$(6.83 \pm 0.75) \times 10^7$
		23.1 ± 0.7	0.38 ± 0.04	$(5.99 \pm 0.63) \times 10^7$

[a] Kinetic constants were determined as illustrated in Figure 6. The results of multiple experiments are presented. [b] Except where noted, when kinetic constants were determined in the presence of fragment 2 or fragment 1.2, the prethrombin 2 concentration was varied at a fixed ratio of 1:1.5 molar equiv of each fragment. [c] Factor Xa was either saturated with PCPS or with factor Va and PCPS. [d] kcat was calculated by dividing Vmax by the total concentration of factor Xa. [e] ND = not determined. (est) = estimated. [f] Constants determined using 14 different concentrations of prethrombin 2 (0−36 μM) in the presence of 55.0 μM fragment 2.

Steady state kinetic measurements were conducted using prethrombin 2 derivatives which have been established to be appropriate analog substrates for the first half reaction of prothrombin activation catalyzed by the prothrombinase complex (Walker & Krishnaswamy, 1994). This approach was used to examine the effects of factor Va on the kinetics of cleavage of the $Arg^{323}-Ile^{324}$ peptide bond in the absence or presence of prothrombin activation fragments, which are known to bind tightly to prethrombin 2 and contain the cofactor binding domain or both the cofactor binding domain as well as the membrane binding domain (Figure 1). Initial rate and catalytic efficiency of cleavage at $Arg^{323}-Ile^{324}$ in prethrombin 2 or prethrombin 2 *plus* fragment 2 was increased to an equivalent extent by the addition of saturating concentrations of factor Va to the Xa−PCPS binary complex. This observation indicates that the cofactor has a large accelerating effect on prothrombinase function even when the substrate is unable to interact with factor Va. The presence of saturating concentrations of fragment 2 further contributed in a relatively small way to the ability of factor Va to increase reaction rate. It therefore follows that interactions between substrate and factor Va mediated by the fragment 2 domain are neither requisite nor a major influence on the large increase in activity that accompanies the assembly of the prothrombinase complex.

Several independent lines of evidence support the ability of prothrombin derivatives containing the fragment 2 domain to interact reversibly with factor Va (Esmon et al., 1973; Luckow et al., 1989; Guinto & Esmon, 1984; Boskovic et al., 1990). However, the relevance of this interaction toward the function of factor Va within the prothrombinase complex is essentially entirely based on initial velocity measurements with prethrombin 2 in the absence of membranes using concentrations of factor Va ($\sim 10^{-8}$ M) well below those required to saturate the Xa−Va interaction in solution (Esmon & Jackson, 1974). These measurements have formed the basis for the prevailing idea that the ability of the cofactor to accelerate thrombin formation is specifically dependent upon the interaction between the fragment 2 region and factor Va (Esmon & Jackson, 1974). Initial velocity measurements under comparable conditions illustrate that the perceived effects of the cofactor at nonsaturating concentrations of factor Va and substrate appear to be influenced by the presence of fragment 2 and more so by fragment 1.2 (Table 1). However, this finding is not borne out either by initial velocity measurements or by steady state kinetic constants measured in the presence of membranes and saturating concentrations of factor Va. Since factor Va accelerates cleavage at $Arg^{323}-Ile^{324}$ in prethrombin 2 greatly and to a comparable extent both in the presence or absence of fragment 2, the data suggest that putative interactions between cofactor and the fragment 2 domain of the substrate are not required for this aspect of Va function within the prothrombinase complex.

Detailed kinetic information required for adequate interpretation of initial velocity measurements with solution-phase factors Xa and Va is not presently available. The available information is, however, consistent with the preliminary conclusion that disproportionate changes observed in the presence of fragment 2 or fragment 1.2 may be related to their effects on the apparent affinity for the interaction between factors Xa and Va in solution. This could readily arise from effects of the activation fragments on the large difference in the Ks for substrate binding to the two forms of enzyme (eq 1). Alternatively, the interaction between cofactor and the fragment 2 region of the substrate (Kd $\approx$ 10 μM) could have a significant enhancing effect on the weak interaction between Xa and Va in solution that is not evident in the presence of membranes. This type of consideration may also help explain the recent observation that prothrombin enhances prothrombinase assembly on membranes with suboptimal PS content, conditions which might be expected to engender weak interactions between factors Xa and Va (Billy et al., 1995).

The function of the fragment 2 domain in prothrombin activation has previously been assessed using a recombinant deletion mutant lacking the kringle 2 region (Kotkow et al., 1995). Deletion of this domain led to the demonstrable loss of factor Va binding, yet only yielded a marginal change in Km and a 7-fold decrease in kcat for the reaction catalyzed by prothrombinase (Kotkow et al., 1995). These findings also suggest that the bulk of the rate enhancement observed in the presence of factor Va does not derive from an interaction between the cofactor and the substrate.

Although the addition of factor Va resulted in an equivalent increase in activity for all three substrates, cleavage at

$Arg^{323}-Ile^{324}$ in prethrombin 2 by prothrombinase was itself enhanced by the presence of fragment 2 or fragment 1.2. A comparison of the kinetic constants (kcat/Km) for the cleavage of $Arg^{323}-Ile^{324}$ in prethrombin 2 or prethrombin 2 *plus* fragment 2 by prothrombinase raises the possibility that a major consequence of the interaction between fragment 2 and prethrombin 2 is to alter substrate structure and increase the probability of productive recognition and cleavage of the scissile bond rather than to lead to catalytic improvements by simply providing added binding interactions with the cofactor within the enzyme complex. A similar suggestion has been made on the basis of studies with a recombinant derivative of factor Xa (Rezaie & Esmon, 1995).

In contrast to the small effect of fragment 2 on the catalytic efficiency of the cleavage of prethrombin 2 by prothrombinase, fragment 1.2 caused a large change in the kcat/Km term for this reaction (Table 2). Assuming that the fragment 1 and fragment 2 domains contribute independently to the kinetic effects, these results indicate an increase in catalytic efficiency by a factor of ~70 which can be attributed to the effects of membrane binding by the substrate. This conclusion is consistent with previous rapid kinetic studies indicating a 60-fold decrease in the rate constant for cleavage at $Arg^{323}-Ile^{324}$ following proteolytic removal of the fragment 1 domain (Walker & Krishnaswamy, 1994).

The contribution of factor Va to prothrombinase function has been previously inferred from comparisons of the steady state kinetic constants for prothrombin activation by factor Xa in the presence of membranes relative to those obtained in the presence of membranes as well as factor Va (Rosing et al., 1980; Nesheim et al., 1979, 1984; van Rijn et al., 1984). Such comparisons are complicated by the fact that two cleavage reactions are required to convert prothrombin to the final product and because factor Va substantially changes the relative contributions of the two possible pathways for thrombin formation (Krishnaswamy et al., 1986, 1987; Rosing et al., 1986; Esmon et al., 1974a). These particular complexities are eliminated in the interpretation of the kinetic constants for the cleavage at $Arg^{323}-Ile^{324}$ provided in the present work. However, the potential problems in the interpretation of kinetic constants under conditions where membrane binding by the substrate can precede and limit the rate of catalysis have not been circumvented. Consequently, we have restricted our comparisons to the steady state kinetic constants obtained with prethrombin 2 or prethrombin 2 *plus* fragment 2 with the caveat that the changes observed with these substrates may actually pertain to the effects observed on the intrinsic constants for prethrombin 2 *plus* fragment 1.2 cleavage if the obscuring effects of the substrate−membrane interaction could eventually be accounted for. For these substrates, it appears that the effect of factor Va on the catalytic efficiency of factor Xa derives from a significant decrease in the Km as well as an increase in the kcat. In the case of prethrombin 2 where Km and kcat terms could be roughly estimated in the absence of factor Va, the most significant effect of factor Va appears to be on the Km term. These data are consistent with the conclusion that an important effect of factor Va on prothrombinase function may be to alter factor Xa so as to result in enhanced binding interactions between the protease and the substrate and yield a large improvement in catalytic efficiency for cleavage at $Arg^{323}-Ile^{324}$.

In summary, the present results indicate that substrate−cofactor interactions mediated by the fragment 2 domain are not a critical feature of the mechanism(s) underlying the accelerating effects of factor Va on the function of factor Xa within the membrane-assembled prothrombinase complex. This conclusion, based entirely on kinetic studies of cleavage at $Arg^{323}-Ile^{324}$ in the biological substrate, seems justified since the second cleavage reaction (cleavage at $Arg^{274}-Thr^{275}$) is only modestly influenced by factor Va. Clearly, we are unable to exclude the possibilities that interactions between fragment 2 and factor Va have a significant effect on some other aspect of factor Va or prothrombinase function or in some way influence reaction steps in the kinetic pathway that are not rate-limiting for catalysis by membrane-assembled prothrombinase. It is also possible that previously undetected interactions between the substrate and factor Va mediated by regions outside fragment 2 become significant upon assembly of the prothrombinase complex. Finally, it is also conceivable that the interaction between fragment 2 and factor Va has significant functional consequences only in the presence of the fragment 1 domain which imparts membrane binding properties to the substrate. This possibility cannot be addressed without a more comprehensive knowledge of the kinetic pathway for the cleavage of the membrane binding substrate. Bearing these qualifications in mind, the data are consistent with the interpretation that the dominant mechanism by which factor Va increases the catalytic efficiency of factor Xa within the prothrombinase complex is by altering the properties of the protease rather than by binding the substrate through the fragment 2 domain and altering the structure about the scissile bond(s).

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