Exosites Determine Macromolecular Substrate Recognition by Prothrombinase†

Sriram Krishnaswamy* and Andreas Betz

Department of Medicine, Division of Hematology/Oncology, Emory University, Atlanta, Georgia 30322

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ABSTRACT: The prothrombinase complex, composed of factor Xa and factor Va assembled on a membrane surface, catalyzes the proteolytic formation of thrombin during blood coagulation. The molecular basis for the macromolecular substrate specificity of prothrombinase is poorly understood. By kinetic studies of prethrombin 2 cleavage by prothrombinase in the presence or absence of fragment 1.2, we show that occupation of the active site of the catalyst by inhibitors or alternate peptidyl substrates does not alter the affinity for prethrombin 2. Productive recognition of the macromolecular substrate therefore results from an initial interaction at enzymic sites (exosites) distinct from the active site, which largely determines substrate affinity. This interaction at exosites is evident even in the absence of activation peptide domains responsible for mediating the binding of the substrate to membranes or factor Va. Interactions at the active site with structures surrounding the scissile bond then precede bond cleavage and product release. The second binding step, which appears unfavorable, does not affect substrate affinity but contributes to the maximum catalytic rate. Therefore, binding specificity of prothrombinase for the macromolecular substrate is determined by exosites on the enzyme. We show that competitive inhibition of prethrombin 2 cleavage can be accomplished by interfering with the exosite binding step without obscuring the active site of the enzyme. These findings suggest limitations to the common approach of inferring the basis of factor Xa specificity with active site mutants or the targeting the active site of factor Xa with reversible inhibitors for therapeutic purposes. The achievement of distinctive macromolecular substrate specificities through exosite interactions and modulation of maximum catalytic rate through binding steps may also underlie the reactions catalyzed by the other coagulation complexes containing trypsin-like enzymes.

Several of the highly specific proteolytic activation steps of the clotting cascade are catalyzed by trypsin-like serine proteinases which assemble into a membrane- or surface-bound enzyme complex through interactions with a cofactor protein (Mann et al., 1988). This architecture is typified by the enzyme complex that catalyzes the conversion of prothrombin to thrombin. The specific recognition and cleavage of two peptide bonds in prothrombin is catalyzed by prothrombinase that assembles through reversible interactions between a serine protease, factor Xa and a cofactor protein, factor Va, in the presence of calcium ions and negatively charged membranes (Mann et al., 1988).

The incorporation of factor Xa into prothrombinase leads to a profound increase of ~100000-fold in the catalytic efficiency for prothrombin activation (Mann et al., 1988). Consequently, although factor Xa itself can cleave prothrombin, it is prothrombinase that is considered the relevant catalyst for thrombin formation. Alterations in factor Xa, which result from its interaction with factor Va on the membrane surface, probably play an important role in these changes. However, the increased catalytic efficiency for prothrombin activation is not paralleled by increases in the cleavage of synthetic peptidyl substrates, or in the reaction with serine proteinase inhibitors such as antithrombin III which are directed toward the active site of factor Xa (Walker & Krishnaswamy, 1993; Ellis et al., 1984). Thus, the accelerating effects of the cofactor appear related to the macromolecular substrate specificity of factor Xa. An understanding of the basis for this specificity of prothrombinase is likely to provide new insights into the mechanism(s) by which factor Va increases reaction rate and suggest appropriate strategies for the therapeutic targeting of this coagulation reaction.

Recently, interactions at extended macromolecular recognition sites (exosites) have been implicated in the highly specific recognition of a macromolecular inhibitor by factor Xa (Jordan et al., 1992). Studies with tick anticoagulant peptide have provided evidence for factor Va-induced changes at these recognition sites in factor Xa upon its incorporation into prothrombinase (Betz et al., 1997). The contribution of such effects at exosites, defined as enzymic sites removed from the catalytic residues or the traditional P1-P3 determinants[1] of protease specificity, to substrate recognition or the enhanced rate of prothrombin activation by prothrombinase is unknown. In the present study, we have used reversible inhibitors or alternate peptidyl substrates directed toward the active site of factor Xa to probe the role of interactions at exosites in the recognition and cleavage of the macromolecular substrate by prothrombinase.

EXPERIMENTAL PROCEDURES

Reagents. The peptidyl *p*-nitroanilide substrates were methoxycarbonyl-cyclohexyl-glycyl-glycyl -arginyl *p*-nitro-

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* Address correspondence to this author at Department of Medicine, Division of Hematology/Oncology, 1014 Woodruff Memorial Building, 1639 Pierce Drive, Emory University, Atlanta, GA 30322. Tel: (404) 727-3806. Fax: (404) 727-3404. E-mail: skris01@emory.edu.

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

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anilide (Spectrozyme Xa,[2] American Diagnostica), H-D-phenylalanyl-L-pipecolyl-L-arginyl *p*-nitroanilide (S2238, Pharmacia-Hepar), and benzoyl isoleucyl-glutamyl-glycinyl-arginyl *p*-nitroanilide (S2222, Pharmacia-Hepar). The inhibitors were 4-aminobenzamidine (PAB, Aldrich) and Nα tosylglycyl-3-DL-amidinophenylalanyl methyl ester (TAPA, Celsus). Published extinction coefficients were used to determine the concentrations of PAB ($\epsilon_{293} = 15\,000$ M^{-1} cm^{-1}) (Evans et al., 1982) and peptidyl *p*-nitroanilide substrates ($\epsilon_{342} = 8270$ M^{-1} cm^{-1}) (Lottenberg & Jackson, 1983). L-α-phosphatidylcholine (hen egg) and L-α-phosphatidylserine (bovine brain) were from Sigma (St. Louis). Small unilamellar phospholipid vesicles composed of 75% phosphatidylcholine and 25% phosphatidylserine (PCPS) were prepared and characterized as previously described (Krishnaswamy et al., 1993; Krishnaswamy & Walker, 1997).

Proteins. Bovine factors Xa and Va were purified and characterized by established procedures (Krishnaswamy et al., 1993; Walker & Krishnaswamy, 1994). The bovine prothrombin derivatives, prethrombin 1, prethrombin 2 fragment 1.2, and thrombin were prepared as described (Krishnaswamy & Walker, 1997; Lundblad et al., 1976). Titration of factor Xa and thrombin with *p*-nitrophenyl *p*′-guanidinobenzoate (Chase & Shaw, 1967), yielded 1.16 and 0.96 mol of active site/mol of protein, respectively. Thrombin (22 mg, 150 μM in 20 mM Hepes, 0.15 M NaCl, pH 7.4) was inactivated with three sequential additions of 460 μM *p*-amidino phenylmethanesulfonyl fluoride (APMSF, Calbiochem). Following dialysis against the same buffer, residual active thrombin was further depleted by chromatography on a 1.5 × 3 cm column of benzamidine sepharose (Pharmacia-LKB). The resulting preparation of inactivated thrombin (IIa_i) possessed less than 0.001% activity toward S2238. Protein concentrations were determined using the following molecular weights and extinction coefficients ($E_{280}^{0.1\%}$): factor Xa, 1.24, 45 300 (Jackson et al., 1968; Fujikawa et al., 1974); factor Va, 1.74, 168 000 (Krishnaswamy & Mann, 1988; Laue et al., 1984); prethrombin 2, 1.95, 37 400; fragment 1.2, 1.12, 34 800; thrombin, 1.95, 37 400 (Mann et al., 1981).

Activity Measurements. All prothrombinase activity measurements were performed in 20 mM Hepes, 0.15 M NaCl, 0.1% PEG-8000, 2 mM $CaCl_2$, pH 7.4 (assay buffer). The concentrations of factor Va and PCPS were chosen to saturably incorporate factor Xa into the prothrombinase complex based on known equilibrium constants for the individual discrete interactions (Krishnaswamy, 1990). Measured initial rates were therefore normalized per unit concentration of prothrombinase by dividing by the concentration of factor Xa. The linear dependence of rate on the concentration of factor Xa at saturating concentrations of factor Va and PCPS was established in separate experiments.

Kinetics of Peptidyl Substrate Cleavage. The kinetics of Spectrozyme Xa hydrolysis by prothrombinase was measured using increasing concentrations of substrate at different fixed concentrations of inhibitor. The initial steady state rate was determined by continuously monitoring the change in absorbance at 405 nm at room temperature in a V_{max} kinetic plate reader (Molecular Devices). For inhibition studies with IIa_i, control experiments in the absence of prothrombinase were used to determine and subtract the velocity contribution of traces of active thrombin toward Spectrozyme Xa hydrolysis. This correction was minor (<7%) even at the highest concentrations of IIa_i used.

Discontinuous Measurements of Prethrombin 2 Cleavage. The effect of inhibitors on thrombin formation catalyzed by prothrombinase was measured using increasing concentrations of prethrombin 2 or prethrombin 2 *plus* fragment 1.2 in the presence of different fixed concentrations of inhibitor. The initial, steady state rate of thrombin formation at 25 °C was determined using six serially quenched samples exactly as previously described (Krishnaswamy & Walker, 1997). Control experiments established that the quenched samples were sufficiently diluted so that the inhibitors had a negligible direct effect on thrombin activity.

Inhibition of Prethrombin 2 Cleavage by Alternate Peptidyl Substrates. Alternate tripeptidyl substrates for prothrombinase (S2238 and S2222) are also readily cleaved by thrombin. Therefore, initial velocity measurements of prethrombin 2 cleavage in the presence of these alternate tripeptidyl substrates were determined continuously in a coupled assay. Prethrombin 2 used for these experiments was pretreated with APMSF followed by extensive dialysis to inactivate traces of thrombin and eliminate background hydrolytic activity. Reaction mixtures containing preassembled prothrombinase in assay buffer (100 μL, 0.1 nM Xa, 52 nM Va, and 130 μM PCPS) were prepared in wells of a microtitre plate and initiated with an equal volume of a mixture of prethrombin 2 and the tripeptidyl substrate prepared in the same buffer immediately prior to use. The substrate mixture contained varying concentrations of prethrombin 2 (18 values, 0−22 μM) in the presence of different fixed concentrations of S2222 (300, 400, and 1200 μM) or S2238 (200 and 300 μM). The final concentrations were therefore one-half those present in the initial enzyme or substrate solutions. Following mixing by brief vibration, absorbance at 405 nm was monitored continuously at room temperature. The velocity contribution of prothrombinase toward the cleavage of these peptidyl substrates was established to be negligible relative to that of the thrombin produced in the reaction. The resulting parabolic curves therefore continuously describe prethrombin 2 activation inferred from the enzymatic activity of the product, thrombin, in the presence of alternate peptidyl substrates for prothrombinase. Initial velocities of thrombin formation in the absence of alternate substrates for prothrombinase were determined in parallel using the discontinuous assay method described above.

The coupled assay approach for the analysis of zymogen activation is well described in other systems (Christensen & Mullertz, 1977) and has also been previously used to study prothrombin activation (Kosow & Orthner, 1979). Linear regression analysis of the first derivative of each progress curve was used to establish the data range over which the inferred concentration of thrombin increased linearly as a function of time. Parabolic progress curves of absorbance *versus* time truncated at the 200−600 s mark, based on this

[2] Abbreviations: APMSF, *p*-amidino phenylmethanesulfonyl fluoride; IIa_i, thrombin inactivated with APMSF; PCPS, small unilamellar phospholipid vesicles composed of 75% phosphatidylcholine and 25% phosphatidylserine; PAB, 4-aminobenzamidine; S2222, benzoyl isoleucyl-glutamyl-glycinyl-arginyl *p*-nitroanilide; S2238, H-D-phenylalanyl-L-pipecolyl-L-arginyl *p*-nitroanilide; Spectrozyme Xa, methoxycarbonyl-cyclohexyl-glycyl glycyl-arginyl *p*-nitroanilide; TAPA, Nα tosylglycyl-3-DL-amidinophenylalanyl methyl ester.

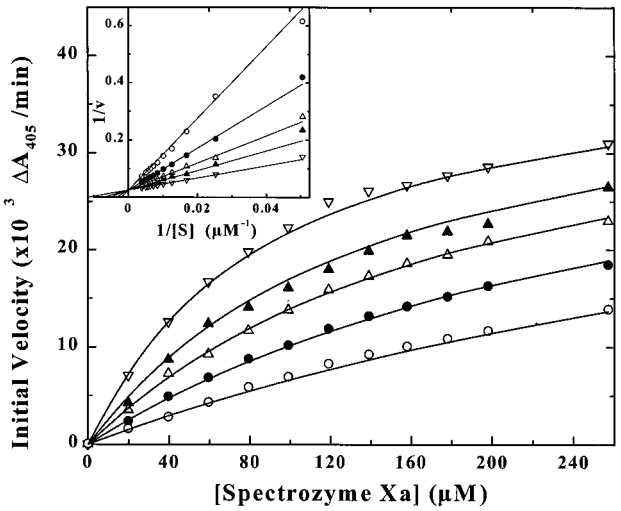


FIGURE 1: Inhibition kinetics of peptidyl substrate hydrolysis by prothrombinase. Initial velocities were measured using 0.5 nM prothrombinase (0.5 nM Xa, 20 nM Va, 50 μM PCPS), increasing concentrations of Spectrozyme Xa with 0 (▽), 15 μM (▲), 30 μM (△), 60 μM (●) and 120 μM (○) PAB. The lines are drawn according to linear competitive inhibition, with the fitted constants listed in Table 1. (Inset) Double reciprocal plot showing effect of PAB on K_m but not on the V_{max} for the reaction.

criterion, were then analyzed by nonlinear least-squares regression analysis according to eq 25 of Christensen and Mullertz (1977). The linear dependence of the rate in change of absorbance as a function of increasing known concentrations of thrombin performed as a control at each concentration of S2222 or S2238 was then used to determine the initial rate of thrombin formation in molar terms. Separate experiments over the complete range of prethrombin 2 and peptidyl substrate concentrations used established that inferred initial rate was linearly proportional to the concentration of prothrombinase.

Data Analysis. Initial velocity data were analyzed by nonlinear least-squares regression analysis using established steady state rate expressions for linear competitive or classical noncompetitive inhibition (Segel, 1975). Alternative inhibition mechanisms were excluded on the basis of significantly poorer fits as judged by the root mean squared deviation and errors of the fitted parameters. In each case, data are presented in conventional double reciprocal form solely to provide a convenient visual corroboration of the conclusions derived from the numerical analyses. The fitted constants are listed ±95% confidence limits and representative data from one of at least two similar experiments are presented in each case.

RESULTS

Factor Xa and other trypsin-like serine proteinases are inhibited by 4-aminobenzamidine (PAB) which binds reversibly to the S1 or primary specificity pocket in the protease (Evans et al., 1982; Bode & Schwager, 1975). This is consistent with the kinetic behavior of PAB as a linear competitive inhibitor of Spectrozyme Xa hydrolysis by the prothrombinase complex (Figure 1). Linear competitive inhibition implies that PAB binding to the primary specificity pocket of factor Xa within prothrombinase precludes synthetic peptidyl substrate binding to the active site. Apparent affinity for the peptidyl substrate is systematically decreased to undetectable levels by increasing concentrations of inhibitor. Inhibition at any inhibitor concentration can be completely overcome by very high concentrations of substrate. This is evident as an increased K_m in the presence of inhibitor while the V_{max} remains unchanged (Figure 1).

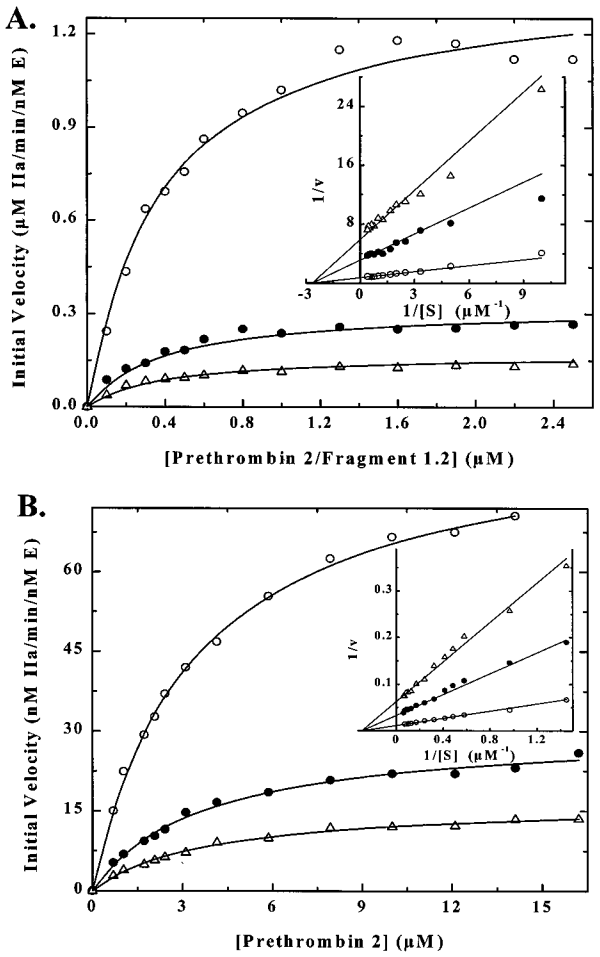


FIGURE 2: Inhibition kinetics of macromolecular substrate cleavage by prothrombinase. The initial velocity for thrombin formation (rate normalized/nanomolar prothrombinase) was determined at increasing concentrations of prethrombin 2 *plus* fragment 1.2 with 0.25 nM prothrombinase (0.25 nM factor Xa, 54 μM PCPS, and 24 nM Va) and 0 (○), 189 μM (●) or 409 μM PAB (△) (Panel A) or increasing concentrations of prethrombin 2 with 5 nM prothrombinase (5 nM Xa, 54 μM PCPS, and 24 nM Va) and 0 (○), 60 μM (●) and 160 μM PAB (△) (panel B). The lines are drawn following analysis according to classical noncompetitive inhibition, with the constants $K_{m_{obs}} = 0.38 \pm 0.02$ μM, $V_{max_{obs}}/E_T = 23 \pm 4$ s^{-1}, and $K_i = 57.3 \pm 4.7$ μM (panel A) or $K_{m_{obs}} = 3.39 \pm 0.1$ μM, $V_{max_{obs}}/E_T = 1.46 \pm 0.02$ s^{-1}, and $K_i = 31.8 \pm 0.64$ μM (panel B). Insets illustrate that PAB changes V_{max} but not K_m.

The conversion of prothrombin to thrombin results from cleavage at Arg^{323}-Ile^{324} followed by Arg^{274}-Thr^{275} by prothrombinase (Mann et al., 1988). The first cleavage reaction is the one most significantly affected by factor Va or membranes (Nesheim & Mann, 1983). Kinetic analyses are greatly simplified by using the substrate derivative prethrombin 2 *plus* fragment 1.2 that requires cleavage at a single site (Arg^{323}-Ile^{324}) in the prethrombin 2 domain by prothrombinase to form thrombin. The kinetics of recognition and cleavage at this site in prethrombin 2 is indistinguishable from the cleavage at the same site in intact prothrombin (Walker & Krishnaswamy, 1994).

Initial velocity studies of prethrombin 2 *plus* fragment 1.2 cleavage by prothrombinase indicate that PAB acts as a classical noncompetitive inhibitor of this reaction (Figure

Table 1: Inhibition Kinetics of Synthetic Peptidyl or Macromolecular Substrate Cleavage by Prothrombinase

substrate	inhibitor[a]	inhibition type[b]	K_m (μM) ± SD	V_{max}/E_T[c] (s^{-1}) ± SD	K_i (μM) ± SD
Spectrozyme Xa	PAB	competitive	85 ± 5	234 ± 4	24.5 ± 1.2
	TAPA	competitive	82.7 ± 3.5	233 ± 3	1.87± 0.3
	S2238[d]	competitive	114 ± 3.2	238 ± 3	51.3 ± 1.3
Prethrombin 2	PAB	noncompetitive	3.39 ± 0.1	1.46 ± 0.02	31.8 ± 0.64
	TAPA	noncompetitive	2.97 ± 0.14	1.49 ± 0.02	4.37 ± 0.15
	S2238[e]	noncompetitive	2.35 ± 0.1	1.37 ± 0.02	28.9 ± 1.6
	S2222[e]	noncompetitive	2.51 ± 0.22	1.02 ± 0.03	361.8 ± 22.3

[a] Reversible active site directed inhibitors or alternate peptidyl substrates for factor Xa. [b] Linear competitive or classical noncompetitive inhibition mechanisms were established as described and steady state kinetic constants determined from data as illustrated in Figures 1 and 2 and are reported ±95% confidence limits. [c] Maximum velocity divided by total enzyme concentration. [d] S2238 cleavage by prothrombinase is characterized by a greatly reduced k_{cat} relative to Spectrozyme Xa (Lottenberg et al., 1986). Inhibition by S2238 acting as an alternate substrate is therefore adequately described by linear competitive inhibition even though both substrates yield a common product. [e] Initial velocity measurements of prethrombin 2 cleavage in the presence of alternate tripeptidyl substrates were determined continuously in a coupled assay.

2A). Classical noncompetitive inhibition, evident as an effect of the inhibitor on the apparent V_{max} with no change in K_m, implies that PAB bound to the active site of the enzyme does not alter the affinity for prethrombin 2 *plus* fragment 1.2 but appears to change the rate constant for catalysis.

The fragment 1.2 activation peptide associates tightly but reversibly with prethrombin 2 and imparts membrane binding and factor Va binding properties to the substrate (Myrmel et al., 1976; Esmon & Jackson, 1974; Gitel et al., 1973). These interactions could dominate substrate binding and are unlikely to be influenced by active site directed inhibitors. Classical noncompetitive inhibition by PAB was also observed for prethrombin 2 cleavage by prothrombinase in the absence of fragment 1.2 (Figure 2B). Occupation of the primary specificity pocket of factor Xa within the prothrombinase complex by PAB therefore has no obvious effect on the K_m for macromolecular substrate cleavage even in the absence of activation peptide domains responsible for membrane and factor Va binding by the substrate.

This discrepancy between the kinetics of cleavage of tripeptidyl substrates and prethrombin 2 by prothrombinase was sustained with other inhibitors and alternate peptidyl substrates (Table 1). In each case, reagents directed toward the active site of factor Xa within the prothrombinase complex acted as linear competitive inhibitors of Spectrozyme Xa cleavage but yielded classical noncompetitive inhibition of prethrombin 2 cleavage. Surprisingly, peptidyl substrates that are cleaved by prothrombinase were not competitive inhibitors of macromolecular substrate cleavage. Coupled assays performed in the presence of different concentrations of S2238 yielded classical noncompetitive inhibition by this alternate substrate (Figure 3). This observation confirms the inhibitory effects of S2238 in coupled assays of prothrombin activation noted previously (Mann et al., 1981). Noncompetitive inhibition of prethrombin 2 hydrolysis by alternate peptidyl substrates of prothrombinase indicates that the K_m for prethrombin 2 is not changed even when a peptidyl substrate is productively bound and cleaved at the active site. The results with S2222 (Table 1) are particularly significant because the peptidyl sequence of this substrate is identical to the P4-P1 sequence preceding the scissile bond in prethrombin 2. It therefore appears that the primary determinants of macromolecular substrate affinity for prothrombinase reside on exosites in the enzyme complex that are distinct from those involved in the binding of active site directed ligands or oligopeptidyl substrate analogs.

Since cleavage of prethrombin 2 by factor Xa within the prothrombinase complex must involve binding interactions

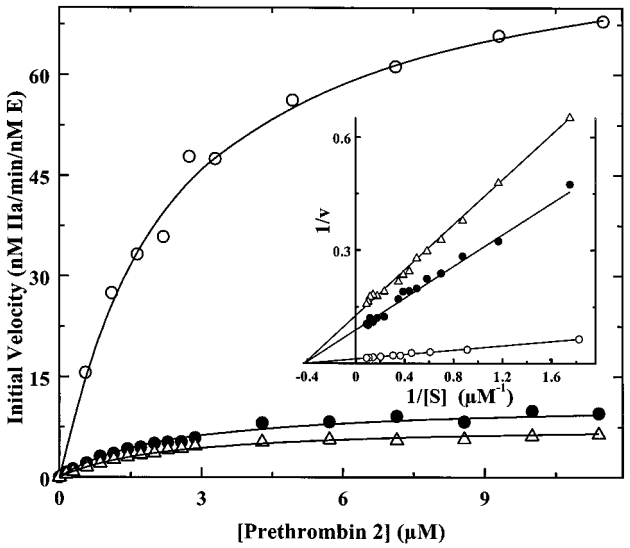
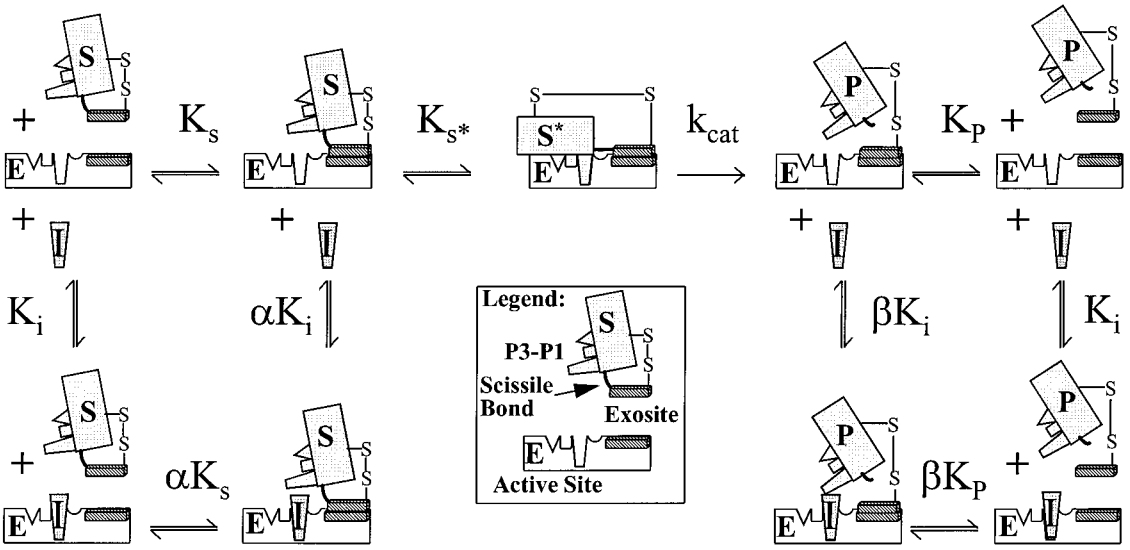


FIGURE 3: Effect of alternate peptidyl substrates on prethrombin 2 cleavage by prothrombinase. The initial velocity for thrombin formation catalyzed by prothrombinase was determined using increasing concentrations of prethrombin 2 in the absence of S2238 (○) or by a coupled assay in the presence of 100 μM S2238 (●) or 150 μM S2238 (△). The lines are drawn following analysis according to classical noncompetitive inhibition, with the constants $K_m = 2.35 \pm 0.1$ μM, $V_{max}/E_T = 1.37 \pm 0.02$ s^{-1}, and $K_i = 28.9 \pm 1.6$ μM. (Inset) Double reciprocal plot showing that S2238 alters V_{max} but has no effect on K_m.

between the substrate and the active site of factor Xa, at least two binding steps precede cleavage of the scissile bond (Scheme 1). The first step involves interactions between prethrombin 2 and exosites in prothrombinase. This bimolecular step is not influenced by binding of small active site-directed inhibitors or synthetic peptidyl substrates to the enzyme. Equivalently, formation of the ES complex has no detectable effect on the binding of small molecules to the active site of factor Xa within prothrombinase. The formation of the initial complex is then followed by interactions between structures surrounding the scissile bond in the protein substrate and the active site of factor Xa, in a unimolecular step before catalysis, requiring prior dissociation of alternate substrates or inhibitors from the active site of the enzyme. Inhibition of macromolecular substrate cleavage by these reagents is not achieved by interfering with the bimolecular combination of enzyme and substrate but rather by influencing the formation of ES*. Since the unimolecular transformation of ES to ES* precedes catalysis, it is expected to contribute to the maximum catalytic rate. As a result, inhibitors that interfere with the interactions

Scheme 1: Kinetic Scheme Illustrating Cleavage of Prethrombin 2 (S) to the Two Chain Product, Thrombin (P) by Prothrombinase (E) in the Presence of Active Site-Directed Reversible Inhibitor (I)[a]



[a] K_s, K_i, and K_p refer to the equilibrium dissociation constants for the binding of S, I, and P to E respectively. K_{s*} is the equilibrium dissociation constant for substrate binding to the active site. Initial velocity measurements indicate that the multipliers α and β are indistinguishable from 1. The rate constant for the catalytic step is denoted by k_{cat}.

between the macromolecular substrate and the active site of the enzyme will be expected to reduce ES* formation and decrease the V_{max} for the reaction. This interpretation is consistent with the kinetics of prethrombin 2 inhibition by active site directed reagents.

Additional insights into the significance of the multistep mechanism for macromolecular substrate binding and cleavage are provided by the composite nature of the observed kinetic constants. This is most readily illustrated using the rapid equilibrium assumption:

$$K_{m_{obs}} = K_s \frac{K_{s*}\left(1 + \frac{I}{K_i}\right)}{\left[1 + K_{s*}\left(1 + \frac{I}{K_i}\right)\right]} \quad (1)$$

$$V_{max_{obs}} = \frac{E_T k_{cat}}{\left[1 + K_{s*}\left(1 + \frac{I}{K_i}\right)\right]} \quad (2)$$

The equilibrium constant (K_{s*}) for the interaction between the macromolecular substrate and the active site of factor Xa influences both the observed K_m and V_{max}. Therefore, the measured V_{max} is significantly influenced by a binding step. In spite of the fact that I and K_i terms modify both composite kinetic constants, the presence of inhibitor, I, leads to a decrease in $V_{max_{obs}}$ without affecting $K_{m_{obs}}$. Such findings are only expected when K_{s*} is much greater than 1, implying that the ES to ES* transition is unfavorable. These two major conclusions can also be drawn from the relevant steady state rate expressions (not shown).

It follows that reagents that compete with prethrombin 2 for its initial interaction with the enzyme should behave as linear competitive inhibitors. Additionally, if inhibition of exosite binding is indeed achieved at site(s) distinct from the active site, this type of inhibitor should not restrict access to the active site and therefore not affect synthetic tripeptidyl substrate cleavage by prothrombinase.

Thrombin, inactivated with APMSF (IIa_i), was found to be an effective product inhibitor of prethrombin 2 cleavage of prothrombinase. Initial velocity studies established that IIa_i was a linear competitive inhibitor of prethrombin 2 activation with $K_p = 2.2\ \mu M$ (Figure 4A, Scheme 1). The same concentrations of IIa_i had a small effect, if at all, on the rate of Spectrozyme Xa hydrolysis by prothrombinase (Figure 4B). These properties are predicted for an inhibitor that competes with the macromolecular substrate for interactions at the exosite of prothrombinase. Thus, the product release steps following cleavage of the Arg^{323}-Ile^{324} peptide bond in prethrombin 2 also involve interactions between newly formed thrombin and some exosite on prothrombinase that precludes prethrombin 2 binding but does not obscure access of small ligands to the active site of factor Xa (Scheme 1).

DISCUSSION

There is considerable interest in the therapeutic modulation of coagulation by specific factor Xa inhibitors. The recent availability of X-ray structures of factor Xa will likely direct and refine structure aided design of specific active site-directed inhibitors of factor Xa (Brandstetter et al., 1996; Padmanabhan et al., 1993). While these inhibitors may display appropriate properties when assessed with synthetic peptidyl substrates for factor Xa, our empirical observations suggest that targeting the active site will fail to yield competitive inhibitors of macromolecular substrate cleavage by the prothrombinase complex.

The observations can be adequately explained by the fact that binding specificity for the macromolecular substrate is determined by two resolvable steps (Scheme 1), involving an interaction at an exosite followed by an interaction at the active site. The fact that active site-directed reagents yield classical noncompetitive inhibition while the exosite-directed product yields linear competitive inhibition with no detectable affect on the active site of prothrombinase suggests an order to the two-step binding process, as illustrated. While binding interactions in the alternate order cannot be completely

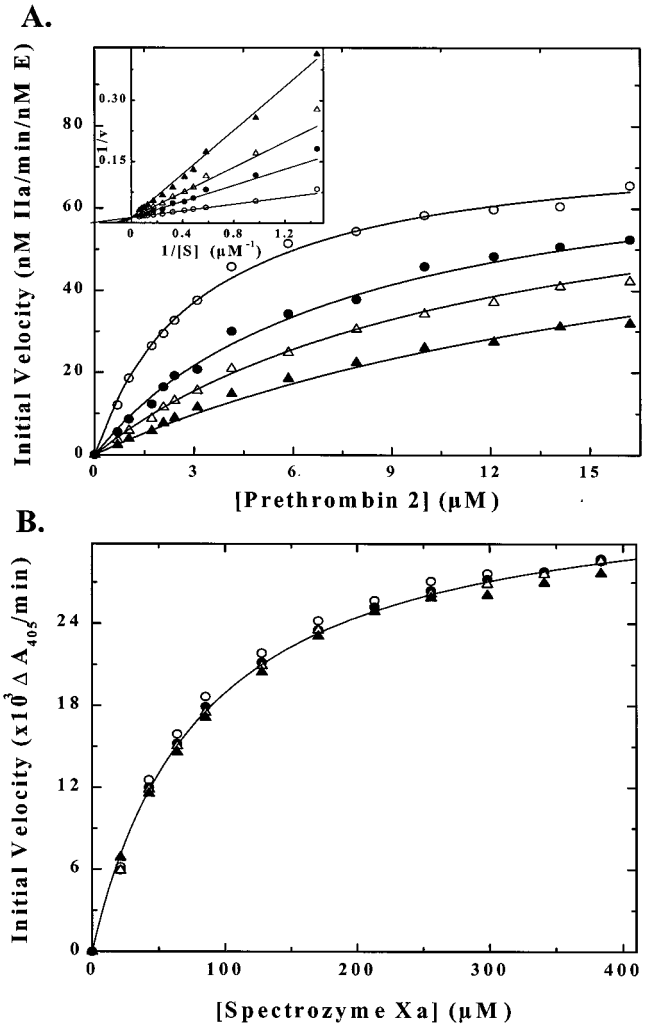


FIGURE 4: Effect of inactivated thrombin on substrate hydrolysis by prothrombinase. Initial rates of prethrombin 2 cleavage (panel A) or Spectrozyme Xa cleavage (panel B) by prothrombinase were determined in the presence of 0 μM (○), 3 μM (●), 6 μM (△), or 12 μM (▲) thrombin inactivated with APMSF (IIa_i). (Panel A) Initial rates of thrombin formation from prethrombin 2 measured with 5 nM prothrombinase (5 nM Xa, 25 nM factor Va, 44 μM PCPS). The lines are drawn following analysis according to linear competitive inhibition with the constants: $K_{m_{obs}} = 3.17 \pm 0.13$ μM, $V_{max_{obs}}/E_T = 1.28 \pm 0.02$ s^{-1} and $K_p = 2.2 \pm 0.08$ μM. (Panel B) Initial rates of Spectrozyme Xa hydrolysis measured with 0.5 nM prothrombinase (0.5 nM Xa, 25 nM factor Va, 44 μM PCPS). Fitting of all data to the Michaelis−Menten equation yielded $K_m = 82.3 \pm 2$ μM and $k_{cat} = 235 \pm 7$ s^{-1}.

excluded, the data suggest that velocity contributions from such an alternate pathway are so small so as to be within experimental error over the wide range of substrate and inhibitor concentrations used in the present work.

Interactions at an exosite predominate the perceived affinity of enzyme for substrate. Active site-directed inhibitors of factor Xa therefore interfere with binding steps that follow the bimolecular combination of substrate with prothrombinase. Inhibitors directed to the exosite may help overcome the difficulties inherently associated with the inhibition of intramolecular binding steps (Jencks, 1987), as well as the preparation of reversible inhibitors that can specifically react with one in a family of trypsin-like coagulation proteases with homologous active sites (Neurath, 1984). Exosite-directed inhibitors may also offer the added advantage of permitting the further regulation of the already inhibited enzyme by circulating serine proteinase inhibitors that is otherwise abrogated upon binding of reversible inhibitors to the active site of factor Xa.

The known structure of prethrombin 2 indicates that structures surrounding the Arg^{323}-Ile^{324} scissile bond in the zymogen require significant rearrangement before they can be docked with the active site of factor Xa (Vijayalakshmi et al., 1994). This provides a structural correlate for the kinetic conclusion that the active site interaction is governed by an unfavorable step. It is therefore possible that substrate binding specificity for cleavage at Arg^{323}-Ile^{324} is largely determined by the exosite rather than active site interactions. This may limit conclusions regarding the molecular basis for the specificity of factor Xa inferred using synthetic peptidyl substrates and active site mutants of the protease.

The established ability of prothrombin to bind to factor Va through the fragment 2 domain, by definition, involves interactions at sites peripheral to the active site of factor Xa within prothrombinase (Esmon et al., 1973; Esmon & Jackson, 1974; Luckow et al., 1989; Guinto & Esmon, 1984; Boskovic et al., 1990). Previous studies of the reaction catalyzed by Xa partially saturated with factor Va in solution have implicated the substrate−cofactor interaction as an important contributor to the affinity of the enzyme for prothrombin (Boskovic et al., 1990). However, this is apparently not the case for substrate recognition by membrane assembled prothrombinase (Krishnaswamy & Walker, 1997). Recent kinetic studies in this case have indicated a very weak contribution, if at all, by the fragment 2 domain to substrate affinity or to mediating an interaction with the cofactor in the productive pathway for substrate recognition (Krishnaswamy & Walker, 1997). The present work provides evidence for a predominant contribution of exosite interactions to macromolecular substrate recognition even in the absence of the fragment 1.2 activation peptide established to be responsible for the binding of substrate to factor Va and to membranes (Gitel et al., 1973; Esmon et al., 1973; Luckow et al., 1989; Guinto & Esmon, 1984). Thus, the presently identified exosite interactions are unrelated to known interactions between the substrate and factor Va or the substrate and membranes. Consequently, while it seems reasonable to consider that substrate recognition results from interactions with extended surfaces on factor Xa assembled within prothrombinase, it is equally possible that this exosite binding step arises from previously unidentified interactions between prethrombin 2 and factor Va or from sites generated by structural contributions from both the protease and the cofactor within the prothrombinase complex.

Previous studies of prothrombin cleavage by factor Xa in the absence of factor Va have indicated that S2222 is a competitive inhibitor of prothrombin activation (Tans et al., 1980). This indicates that exosite-mediated substrate binding is not evident in the absence of the cofactor. However, the converse experiment by the same authors and others (Tans et al., 1980; Boskovic et al., 1990) indicates that prothrombin is unable to inhibit the cleavage of S2222 by factor Xa. While these observations form the basis for the suggestion of open and closed forms of prothrombin bound to factor Xa (Tans et al., 1980), it is not precisely clear whether these observations relate to the present findings or derive from kinetic complexities associated with the extremely poor affinity of prothrombin for factor Xa in the absence of factor Va.

The importance of exosite interactions in determining macromolecular substrate or inhibitor specificity is well-

established in the case of thrombin. Recent studies have implicated similar interactions in the recognition of plasminogen by tPA and of factor X by the VIIa−tissue factor complex (Ruf et al., 1992; Ke et al., 1997). It is therefore possible that optimization of substrate−enzyme interactions through exosite interactions is a prevalent strategy through which high and distinctive macromolecular substrate specificity is achieved by homologous, trypsin-like serine proteinases of coagulation and fibrinolysis.

Because of the multistep pathway for productive binding of prethrombin 2 to prothrombinase, binding steps that follow the initial bimolecular combination of substrate with enzyme can significantly contribute to the observed V_{max} for the reaction (eqs 1 and 2, Scheme 1). In the case of a membrane binding substrate such as prothrombin, it is likely that binding of substrate to membranes precedes substrate binding to the membrane-assembled enzyme (Walker & Krishnaswamy, 1994). In this case, the observed V_{max} will probably contain modifiers related to additional binding steps. The modulation of the binding of substrate to enzyme as a result of prothrombinase assembly can therefore lead to large changes in the observed V_{max} even though the rate constant for catalysis remains unchanged. Factor Va and the other cofactor proteins of coagulation accelerate reaction rate primarily by increasing the V_{max} for product formation. Historically, this has been interpreted to reflect an effect of the cofactor on the catalytic step. Instead, it is possible that V_{max} changes resulting from enhanced substrate binding steps in a multistep pathway provides a partial explanation for the function of factor Va and other cofactor proteins in the coagulation cascade.

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