Exosite Binding Tethers the Macromolecular Substrate to the Prothrombinase Complex and Directs Cleavage at Two Spatially Distinct Sites*

Danilo S. Boskovic and Sriram Krishnaswamy‡

From the Joseph Stokes Research Institute, Children's Hospital of Philadelphia and Department of Pediatrics, University of Pennsylvania, Philadelphia, Pennsylvania 19104

The prothrombinase complex, composed of the protease, factor Xa, bound to factor Va on membranes, catalyzes thrombin formation by the specific and ordered proteolysis of prothrombin at Arg^{225}Ile^{234}, followed by cleavage at Arg^{324}Thr^{325}. We have used a fluorescent derivative of meizothrombin des fragment 1 (mIIaAF1) as a substrate analog to assess the mechanism of substrate recognition in the second half-reaction of bovine prothrombin activation. Cleavage of mIIaAF1 exhibits pseudo-first order kinetics regardless of the substrate concentration relative to $K_m$. This phenomenon arises from competitive product inhibition by thrombin, which binds to prothrombinase with exactly the same affinity as mIIaAF1. As thrombin is known to bind to an exosite on prothrombinase, initial interactions at an exosite likely play a role in the enzyme-substrate interaction. Occupation of the active site of prothrombinase by a reversible inhibitor does not exclude the binding of mIIaAF1 to the enzyme. Specific recognition of mIIaAF1 is achieved through an initial bimolecular reaction with an enzymic exosite, followed by an active site docking step in an intramolecular reaction prior to bond cleavage. By alternate substrate studies, we have resolved the contributions of the individual binding steps to substrate affinity and catalysis. This pathway for substrate binding is identical to that previously determined with a substrate analog for the first half-reaction of prothrombin activation (2). Prothrombinase is an archetypal enzyme complex of blood coagulation (2). The enzyme complex assembles through well characterized, reversible, protein-protein and protein-membrane interactions between the serine protease, factor Xa, the cofactor, factor Va, and membranes in the presence of calcium ions (2–4). The resulting complex catalyzes the conversion of prothrombin to thrombin at a greatly enhanced rate, compared with the reaction rate catalyzed by factor Xa alone (2).

Prothrombin is activated by proteolytic cleavage at two sites, Arg^{274}Thr^{275} and Arg^{323}Ile^{324}, which yields the fragment 1.2 activation peptide and thrombin (5, 6). The reaction catalyzed by prothrombinase proceeds almost exclusively via the initial cleavage at Arg^{323}Ile^{324}, yielding meizothrombin as an intermediate, followed by cleavage at Arg^{374}Thr^{375} to yield the final products of the reaction (7, 8). Single turnover kinetic studies indicate that the overall process is likely the sum of two consecutive enzyme-catalyzed reactions (8). Consequently, steady state kinetic constants derived from measurements of the conversion of prothrombin to thrombin are difficult to interpret and are unlikely to provide valid mechanistic insights into this process. This problem can be circumvented by the use of proteolytic derivatives of prothrombin as analog substrates for the individual half-reactions of prothrombin activation (8–11).

Prethrombin 2, generated by preparative cleavage at Arg^{274}Thr^{275}, has been shown to be a valid substrate analog for kinetic studies of the cleavage at Arg^{231}Ile^{234}, which represents the first cleavage reaction in the activation of prothrombin by prothrombinase (8, 12). Prior cleavage at Arg^{274}Thr^{275} was shown to have no effect on the recognition and cleavage of the Arg^{323}Ile^{324} site by prothrombinase (8). Considerable advances in the understanding of substrate-prothrombinase interactions have been gained by studies with prethrombin 2 in the bovine system (12–14). The results support a model in which the substrate, prethrombin 2, binds to prothrombinase via a multistep reaction (13, 14). The affinity of the enzyme for the substrate is determined by a bimolecular reaction between prethrombin 2 and extended macromolecular recognition sites (exosites) on the enzyme complex. This step is followed by interactions between elements surrounding the scissile bond with the active site of the enzyme followed by bond cleavage. The resulting product, thrombin, remains bound to the exosite and requires dissociation for subsequent rounds of catalysis. Since active site interactions between substrate and enzyme were found to be unfavorable, it has been suggested that bind-

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‡ To whom all correspondence should be addressed: Joseph Stokes Research Inst., Children's Hospital of Philadelphia, 310 Abramson, 3516 Civic Center Blvd., Philadelphia, PA 19104. Tel.: 215-590-3346; Fax: 215-590-2320; E-mail: skrishna@mail.med.upenn.edu.

3 Residue numbers in prothrombin and derivatives are based on consecutive numbering of the 582 residues in the bovine zymogen. Thrombin consists of disulfide-linked A (residues 275–323) and B (residues 324–582) chains. Meizothrombin consists of disulfide-linked fragment 1.2-A (residues 1–323) and B (residues 324–582) chains with intact Arg^{274}Thr^{275} bond. Meizothrombin des fragment 1 consists of disulfide-linked fragment 2-A (residues 157–323) and B (residues 324–582) chains with intact Arg^{324}Thr^{375} bond. Prothrombin 2 consists of residues 275–582 with intact Arg^{225}Ile^{234} bond. Fragment 1.2 is residues 1–274. Fragment 2 is residues 157–274. Fragment 1 is residues 1–156.
ing specificity for cleavage at Arg<sup>223</sup>Ile<sup>224</sup> is largely determined by exosite interactions with the enzyme (13).

Comparative information on the second half-reaction, in which the Arg<sup>274</sup>-Thr<sup>275</sup> peptide bond is cleaved by prothrombinase, is lacking. Although prior cleavage at Arg<sup>274</sup>-Thr<sup>275</sup> does not influence the kinetics of cleavage at Arg<sup>223</sup>Ile<sup>224</sup>, the reverse is not true (8). Initial cleavage at Arg<sup>223</sup>Ile<sup>224</sup> has been suggested to enhance the rate of cleavage at Arg<sup>274</sup>-Thr<sup>275</sup> by a factor of 10 (11). The rate of cleavage at Arg<sup>274</sup>-Thr<sup>275</sup> which converts meizothrombin to thrombin, is only modestly stimulated by factor Va (9, 15).

Structural models based on x-ray diffraction studies indicate that the two cleavage sites in prothrombin are spatially distinct and separated by as much as 36 Å (16, 17). Finally, rapid kinetic studies support the possibility that the two cleavage reactions catalyzed by prothrombinase derive from two distinct types of substrate-enzyme interactions (8). Taken together, these observations suggest that there may be significant differences in the mechanisms underlying the recognition and cleavage of the two bonds in prothrombin by prothrombinase.

Meizothrombin (mIIa),<sup>2</sup> produced as the intermediate of prothrombin activation by prothrombinase following initial cleavage at Arg<sup>223</sup>Ile<sup>224</sup>, is the relevant substrate analog for kinetic studies of the action of prothrombinase on the Arg<sup>274</sup>-Thr<sup>275</sup> cleavage site (8, 9, 15). Single turnover kinetic studies have established that proteolytic removal of the membrane-binding fragment 1 domain from mIIa to yield meizothrombin des fragment 1 (mIIaF1) does not affect the kinetics of substrate recognition and cleavage by prothrombinase (8). Thus, although mIIa can bind membranes (18, 19), this interaction does not play an obvious enhancing role in its ability to be recognized and cleaved by prothrombinase. Therefore, mIIaΔF1 is a valid substrate analog for the second half-reaction of prothrombin activation that permits studies of the enzyme-substrate interaction in the absence of the obscuring effects of membrane-mediated substrate delivery steps (8, 20, 21). We have therefore pursued steady state kinetic studies of the cleavage of bovine mIIaΔF1 by bovine prothrombinase to further investigate the mechanisms underlying macromolecular substrate recognition by prothrombinase.

**EXPERIMENTAL PROCEDURES**

**Materials**—Hepes, L-α-phosphatidylcholine, L-α-phosphatidyl-L-serine, Coomassie Brilliant Blue R 250, Sephadex G-25, and Trizma base were obtained from Sigma. Crude lyophilized venom from *Echis carinatus pyramidum* was obtained from Latoxan (Valence, France). Dansyl-L-glutamyl-glycyl-L-arginine chloromethyl ketone (DEGR-CH<sub>2</sub>Cl), D-phenylalanyl-L-phenylalanyl-L-arginine chloromethyl ketone (FFR-CH<sub>2</sub>Cl), and D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (FPR-CH<sub>2</sub>Cl) were from Calbiochem-Novachem (La Jolla, CA). H-L-alanyl-L-pipeollyl-L-arginyl p-nitroanilide (S-2238) was from Chromogenex (West Chester, OH), and methoxycarbonyl-cyclohexylglycyl-L-arginyl p-nitroanilide (spectrozyme Xa) was obtained from American Diagnostica (Greenwich, CT). Substrate solutions were prepared in water, and concentrations were verified using ε<sub>280</sub> = 8270 M<sup>−1</sup> cm<sup>−1</sup> (22). Polyethylene glycol with average M<sub>r</sub> = 8000 was from J.T. Baker (Danvers, MA). Sucinimidyl (acetylthio)acetate and 6-(iodoacetamido)fluorescein were from Molecular Probes (Eugene, OR). The inhibitor 4-aminobenzamidine (PAB) was from Aldrich (Milwaukee, WI).

The concentration of Fab was determined using ε<sub>280</sub> = 14,400 M<sup>−1</sup> cm<sup>−1</sup> (23). Small unilamellar phospholipid vesicles composed of 75% (w/w) phosphatidylcholine and 25% (w/w) phosphatidylethanolamine: PAG, polyacrylamide gel electrophoresis.

**Proteins**—Ecarin was purified from crude *E. carinatus pyramidum* venom by modifications to published procedures (9, 15). Venom protein (10–19 mg/ml) dissolved in 50 mM Tris, pH 8.0, was clarified by centrifugation (10,000 × g, 45 min) and applied to a column of DEAE-cellulose (2.5 × 8.5 cm) equilibrated in the same buffer. Following washing with 100 mM Tris, pH 8.0, bound protein containing ecarin was eluted using 100 mM Tris, 100 mM NaCl, pH 8.0. Ecarin activity was detected by an assay that relied on the coupling of calcium-independent prothrombin activation to S-2238 hydrolysis (27). Fractions containing activity were pooled, treated with TFR-CH<sub>2</sub>Cl (5 μM) to inhibit trace contaminant activity, diluted with 19 volumes of 20 mM Hepes, pH 7.5, and subjected to high resolution cation exchange using a Poros HR5/5 BioTempt Membrane, Framingham, MA. Bound ecarin was eluted at 2 ml/min with a gradient of increasing NaCl (0.05–1.0 M, 50 ml) in 20 mM Hepes, pH 7.5. Fractions containing ecarin were pooled, precipitated with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, collected by centrifugation (100,000 × g, 45 min), dissolved in 50% (v/v) glycerol, and stored at −20 °C.

Bovine prothrombin, factor Xa, and factor Va were purified and characterized as described previously (8, 24, 28, 29). Thrombin, prethrombin 1, and fragment 2 were proteolytically prepared from prothrombin and purified as described (12, 14, 29, 30). Thrombin was chemically inactivated by treatment with FPR-CH<sub>2</sub>Cl to yield IIa<sub>i</sub>. Bovine thrombin (24 mg, 200 μM) in 20 mM Heps, 150 mM NaCl, pH 7.5, was inactivated by two sequential additions of 330 μM FPR-CH<sub>2</sub>Cl followed by incubation at 25 °C for 20 min. Excess FPR-CH<sub>2</sub>Cl was removed by gel filtration on Sephadex G-25 (1.5 × 49 cm) equilibrated in the same buffer.

Three types of catalytically inactivated derivatives of mIIaΔF1 were prepared by preparative cleavage of prethrombin 1 by ecarin in the presence of the appropriate peptidyl chloromethyl ketone. The nonfluorescent, inactivated derivative mIIaΔF1 was prepared by cleavage in the presence of FPR-CH<sub>2</sub>Cl. Dansyl-modified product, mIIaΔF1<sub>D</sub>, was obtained by cleavage in the presence of DEGR-CH<sub>2</sub>Cl as described previously (8). The fluorescein derivative, mIIaΔF1<sub>F</sub>, was prepared by cleavage in the presence of ATA-FPR-CH<sub>2</sub>Cl followed by thioester hydrolysis and modification with 6-(iodoacetamidofluorescein). Typically, mIIaΔF1 was prepared by cleavage of prethrombin 1 (12.7 μM, 35 mg) in 20 mM Hepes, 50 mM NaCl, pH 7.5, by ecarin (8 μg/ml) at 25 °C in the presence of 50 μM FPR-CH<sub>2</sub>Cl. After 25 min, the reaction mixture was quenched by the addition of 10 mM EDTA, and excess inhibitor was removed by gel filtration using a column (2.5 × 120 cm) of Sephadex G-25 equilibrated in 20 mM Heps, pH 7.5. Protein eluting in the void volume was pooled and applied directly to a Poros HS/M column (4.6 × 100 mm) by ecarin (8 μg/ml) at 25 °C rapidly absorb ecarin and side products. Unbound protein was pooled and subjected to high resolution anion exchange using a Poros HR5/5 BioTempt Membrane (PerSeptive Biosystems). Bound mIIaΔF1 derivatives were eluted at 6.4 ml/min with a gradient of increasing NaCl (0.05–0.2 M, 96 ml) in 20 mM Hepes, pH 7.5. Fractions containing mIIaΔF1 were pooled, precipitated with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, collected by centrifugation (100,000 × g, 45 min), dissolved in 50% (v/v) glycerol, and stored at −20 °C.

For the preparation of the fluorescein derivative, the activation reaction was conducted in the presence of 50 μM ATA-FPR-CH<sub>2</sub>Cl. Following quenching with EDTA, the reaction mixture was treated with 100 mM NH<sub>4</sub>OH (1.0 M stock solution in 0.1 M Hepes, 0.3 mM NaCl, 1 mM EDTA, pH 7.0) and 150 μM 6-(iodoacetamido)fluorescein (1.94 mM stock solution in 0.2 M Hepes, pH 7.0), followed by incubation at 25 °C for 25 min. In some instances, excess ATA-FPR-CH<sub>2</sub>Cl was removed by gel filtration prior to thioester hydrolysis and thiol modification with ecarin as described above. The remaining reaction was subjected to the gel filtration and two ion-exchange steps described above to yield mIIaΔF1<sub>F</sub>.

Protein concentrations were determined using the following molecular weights and extinction coefficients (ε<sub>280</sub>): ecarin, 71,000, 1.0 (31); factor Va, 168,000, 1.74 (32, 33); factor Xa, 45,300, 1.24 (34, 35); prethrombin 1, 50,200, 1.64 (36); fragment 2, 12,900, 1.25 (36); and thrombin 1, 50,200, 1.64 (36).
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bin, 37,500, 1.95 (30). The molecular weight and extinction coefficient for all mIIaΔF1 derivatives were assumed to correspond to the values determined for prothrombin 1.

Steady State Fluorescence Measurements—All fluorescence measurements were performed in stirred quartz cells (1 X 1 cm) in 20 mM Hepes, 0.15 M NaCl, 2 mM CaCl2, 0.1% (w/v) dimethylglyoxyme with Mf = 8000, pH 7.5 (assay buffer), maintained at 25 °C. The cleavage of mIIaΔF1 was studied using a LS50B fluorescence spectrophotometer (PerkinElmer Life Sciences) using λex = 494 nm and λem = 525 nm. Scattered light was minimized using a long pass filter (KV-500, Schott, Dureay, PA) in the emission beam and slits were adjusted to minimize photobleaching. The cleavage of mIIaΔF1 was measured in a modified SLM8000 fluorescence spectrophotometer (37), using λem = 280 nm and monitoring broadband fluorescence (λem ≥ 500 nm) isolated with a long pass filter (Schott KV-500) in the emission beam. Experiments with both substrates were also conducted using a customized rapid scanning fluorescence spectrophotometer (RSM-1000, On-Line Instrument Systems, Bogart, GA). Spectral-time data sets were obtained using the appropriate excitation wavelength for the probe studied and rapidly scanning the emission monochromator. Data collection times, time constants, and integration times were adjusted to permit the collection of 400 or 1000 spectra over 8–10 half-lives of the measured reaction.

For all kinetic studies, protein substrates and product inhibitors were exchanged into assay buffer either by dialysis or by column centrifugation using vacuum G-25 prior to use.

Progress Curve Analysis of mIIaΔF1 Cleavage—The reaction mixture contained 8.0 μM mIIaΔF1 (0.2 μM mIIaΔF1 plus 7.8 μM mIIaF1), 50 μM PCPS, and 30 nM factor Va in assay buffer. Cleavage was initiated by the addition of 1 nM Xa, and fluorescence was monitored continuously. At the indicated times, aliquots (65 μl) were withdrawn from a parallel reaction mixture and quenched by mixing with 35 μl of 156 mM Tris, 29 mM EDTA, 25% (v/v) glycerol, 5% (v/v) SDS, 0.02% (v/v) bromphenol blue, pH 6.8, heated, and analyzed by SDS-PAGE (38).

Following electrophoresis, fluorescent bands were detected and analyzed using a Storm 840 fluorescence scanner and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Protein bands were visualized by staining with Coomassie Brilliant Blue, imaged with transmitted light using a Kodak DC120 digital camera (Eastman Kodak Co.), and analyzed using ImageQuant. The exposure and aperture settings for the imaging step were selected to yield a linear densitometric response.

Steady State Kinetics of mIIaΔF1 Cleavage—The dependence of cleavage rate on the concentration of mIIaΔF1 was determined using reaction mixtures containing increasing concentrations of substrate (0.1 μM mIIaΔF1 plus 0–14.9 μM mIIaF1), 50 μM PCPS, and 30 nM factor Va in assay buffer. Following initiation by the addition of 1 nM Xa, reaction progress was monitored by continuous measurements of fluorescence intensity. Similar experiments with dansyl-modified substrate (mIIaΔF1) were conducted by monitoring the decrease in probe fluorescence intensity following initiation of reaction mixtures containing increasing concentrations of mIIaΔF1, (0–8 μM), 40 μM PCPS, and 25 nM factor Va in assay buffer with 0.5–30 nM Xa.

Inhibition Kinetics of mIIaΔF1 Cleavage—For inhibition studies with fragment 2, reaction mixtures contained 0.2 μM mIIaΔF1, 40 μM PCPS, 40 nM factor Va, and increasing concentrations of fragment 2 in assay buffer. Cleavage was initiated by the addition of 2.8 nM Xa followed by continuous measurements of probe fluorescence. Inhibition measurements with IIa, were performed using reaction mixtures containing 0.2 μM mIIaΔF1, 40 μM PCPS, 40 nM factor Va, and increasing concentrations of IIa, in assay buffer. Cleavage was initiated by the addition of 1 nM Xa.

Inhibition by PAB was determined using increasing concentrations of mIIaΔF1 (0.12 μM mIIaΔF1 plus 0.38–14.88 μM mIIaF1), 30 nM factor Va, 50 μM PCPS, and different fixed concentrations of PAB. Cleavage was initiated by the addition of factor Xa to a final concentration of 2, 3, or 6 nM, and reaction progress was inferred by continuously monitoring fluorescence intensity of the fluorescein probe.

Inhibition Kinetics of Peptidyl Substrate Cleavage—mIIaΔF1 used in alternate substrate studies was treated with an excess of amido phenylmethylsulfonyl fluoride to inactivate traces of active proteinase followed by dialysis into assay buffer prior to use. Reaction mixtures (170 μl) were prepared in wells of a 96-well plate, contained increasing concentrations of SpXa, 50 μM PCPS, 40 nM Va, and different fixed concentrations of mIIaΔF1 (0–40 μM) added shortly before initiation. The reactions were initiated by the addition of Xa (30 μl) to achieve a final concentration of 0.5 nM. Following mixing by brief vibration, peptidyl substrate hydrolysis was measured by continuously monitoring absorbance at 405 nm in a kinetic plate reader (Spectramax 250, Molecular Devices, Sunnyvale, CA). Control experiments without added Xa indicated that the velocity contribution due to active proteinase contamination in the mIIaΔF1 preparation was negligible (≤1%), at the highest concentrations of mIIaΔF1.

Data Analysis—Reactant concentrations were chosen to ensure that the concentrations of PCPS and Va were saturating relative to the concentration of factor Xa. Based on measured equilibrium and rate constants (39, 40), greater than 95% of the factor Xa, under these conditions, is expected to be incorporated into the prothrombin complex rapidly (t½ > 30 ms) following initiation of the reaction with factor Xa. The concentration of enzyme (prothrombinase) was therefore considered equivalent to the limiting concentration of factor Xa present in the reaction mixture. This expectation was empirically verified by documenting a linear dependence of reaction rate on the concentration of factor Xa, provided its concentration was limiting.

Data were analyzed according to the indicated equations using nonlinear least squares analysis (41). The quality of the fit was assessed by the criteria described (42). Fitted parameters are reported ± 95% confidence limits.

The initial, steady state rate of product formation was derived from the initial slope of the fluorescence traces and converted to concentration terms using the limits of the progress curve to signify 0% and 100% conversion of substrate to product. This approach is justified by the results of densitometry analysis following SDS-PAGE (below).

Images of fluorescent or Coomassie Blue-stained bands were analyzed by volume integration (pixel intensity integrated over band area) and normalized using the signal obtained by integration over the entire lane. The resultant normalized densitometry results were converted to concentrations of substrate and product at each time point using the considerations described in detail (8).

Time-resolved emission spectra, obtained by rapid scanning fluorescence measurements, were analyzed by singular value decomposition (43), to determine the number of kinetically and optically resolved species, followed by analysis of the eigenvectors to yield rate constant information. Global analysis was approached to a single exponential, to yield the component spectra of the starting and limiting species, a normalized wavelength-independent kinetic trace for the spectral transition, and kobs for the transition.

Fixed wavelength kinetic traces were analyzed according to a single exponential.

\[
F_t = F_0 + (F_\infty - F_0) \left(1 - e^{-k_{\text{obs}} t}\right)
\]  
(Eq. 1)

The signal at t (Ft) is determined by the limits of the signal at zero (F0) and infinite (F\infty) time and the observed pseudo-first order rate constant (k_{\text{obs}}) for the process. Non-linear least squares analysis yielded fitted values for F0, F\infty, and k_{\text{obs}}.

The dependence of k_{\text{obs}} on the initial concentrations of substrate (S) and product (P) was analyzed according to the rate expression developed by Mihalyi (44), for the limiting case where substrate and product bind competitively to the enzyme with identical affinities.

\[
k_{\text{obs}} = \frac{V_{\text{max}}E_P}{K_m + (S + P)}
\]  
(Eq. 2)

E_P, V_{\text{max}}, and K_m have the usual meaning with the constraint \(K_m = K_p\). K_m refers to the equilibrium dissociation constant for the binding of P to E.

Initial velocity data for the cleavage of mIIaΔF1 (S) obtained at increasing concentrations of substrate in the presence of different fixed concentrations of PAB (I) were analyzed according to the rate expression for Scheme I previously developed using the rapid equilibrium assumption (13).

\[
S \cdot E_P \cdot k_{\text{obs}} \left(1 + K_m^{-1} + \frac{1}{K_p^{-1}}\right)^{-1}
\]  
(Eq. 3)

K_m, K_p, and K_{\text{obs}} represent the equilibrium dissociation constants for the steps denoted in Scheme 1 (below), and k_{\text{obs}} is the rate constant for catalysis. In the absence of I, Equation 3 is of the same form as the Henri-Michaelis-Menten equation where the observed kinetic constants
are related to the constants illustrated in Scheme 1 by Equations 4 and 5.

$$K_{n\text{ (obs)}} = K_n \cdot \left( \frac{K_n^*}{1 + K_n^*} \right)$$

(Eq. 4)

$$\frac{V_{\text{max}}}{E_p} = \frac{k_{\text{cat}}}{(1 + K_n^*)}$$

(Eq. 5)

Alternate substrate effects of the protein substrate (S) on the cleavage of the peptidyl substrate (SpXa) were analyzed according to Scheme I, assuming that the binding of SpXa to the enzyme occurs in steps equivalent to those illustrated for the binding of active site-directed ligand, I, followed by cleavage to yield the chromophoric product measured by monitoring absorbance at 405 nm.

$$\frac{dA_{\text{obs}}}{dt} = \frac{\text{SpXa} \cdot E_p \cdot k_{\text{cat,obs}}}{\text{SpXa} + \frac{1}{K_{n\text{ (obs)}}} + \frac{S}{K_n^* \cdot K_n^* + K_n^* + S}}$$

SpXa refers to the concentration of the peptidyl substrate, $k_{\text{cat,obs}}$ and $K_{n\text{ (obs)}}$ are the kinetic constants for the cleavage of SpXa by prothrombinase, S is the concentration of the protein substrate, and the constants $K_n^*$ and $K_n^*$ refer to the equilibrium dissociation constants illustrated in Scheme I. Initial velocity measurements of the rate of SpXa cleavage at increasing concentrations of SpXa in the presence of different fixed concentrations of S, were analyzed according to Equation 6 to yield fitted values of $k_{\text{cat,obs}}$, $K_{n\text{ (obs)}}$, $K_n^*$, and $K_n^*$.

RESULTS

Experimental Design—Because of autocatalytic degradative reactions, mIIa and mIIαΔF1 can only be produced in stable form in the presence of proteinase inhibitors (9, 45). Stable derivatives of mIIa and mIIαΔF1 have previously been produced by inactivation with DEGR-CH₂Cl, wherein the dansyl moiety serves as a reporter group for subsequent cleavage by prothrombinase (10). Measurements with mIIαΔF1 have relied on energy transfer from aromatic side chains to the dansyl moiety to yield a fluorescence intensity change associated with cleavage at Arg²⁷⁴-Thr²⁷⁵ (8). In this approach, the useful substrate concentration range is limited by probe sensitivity and the inner filter effect. Some of these limitations could be overcome by the preparation of a fluorescent derivative, mIIαΔF1Δ, containing 6-(idoacetamido)fluorescein incorporated into the active site with a peptidyl chloromethylketone linker.

Cleavage of mIIαΔF1, monitored using the fluorescein probe in mIIαΔF1Δ, yielded a useful change in fluorescence intensity. Following initiation of cleavage with prothrombinase, fluorescence intensity increased with time to yield a limiting enhancement of ~9% (Fig. 1). SDS-PAGE analysis and imaging of the fluorescent bands indicated that the increase in fluorescence intensity correlated with the appearance of the expected products following cleavage of the Arg²⁷⁴-Thr²⁷⁵ bond in mIIαΔF1Δ (Fig. 1, inset). Densitometry analysis of product formation either from the fluorescence gel image or following staining for total protein with Coomassie Brilliant Blue yielded progress curves that were essentially indistinguishable from each other and from the continuously measured fluorescence trace (Fig. 1). The substrate solution (0.2 μM mIIαΔF1Δ plus 7.8 μM mIIαΔF1) contained an excess of the inactivated but nonfluorescent derivative (mIIαΔF1Δ), relative to the concentration of mIIαΔF1Δ. The densitometry results (Fig. 1) indicated that mIIαΔF1Δ and mIIαΔF1 are cleaved at identical rates. Within experimental error, the change in fluorescence intensity of mIIαΔF1Δ is coincident with the kinetics of cleavage of the Arg²⁷⁴-Thr²⁷⁵ bond in the substrate. Thus, mIIαΔF1Δ can be utilized as a valid tracer to report the cleavage of the Arg²⁷⁴-Thr²⁷⁵ site in the bulk substrate. This was further validated by showing that the rate of product formation, inferred from the fluorescence change, was independent of the ratio of the fluorescent to nonfluorescent substrate species at a fixed total concentration of mIIαΔF1 (data not shown). Therefore, the total substrate concentration (mIIαΔF1Δ) was considered equal to the sum of the concentrations of mIIαΔF1Δ and mIIαΔF1 and initial velocities of mIIαΔF1 cleavage were calculated using the limits of the fluorescence trace to signify 0 and 100% conversion of bulk substrate to product.

The amplitude of the fluorescence change, observed upon substrate cleavage, was influenced by the total concentration of mIIαΔF1, even when the concentration of mIIαΔF1Δ was held constant. The limiting enhancement varied from 20% at 0.1 μM mIIαΔF1Δ to 6% at 0.1 μM mIIαΔF1Δ plus 14.9 μM mIIαΔF1, despite the fact that quantitative cleavage of the substrate could be established by SDS-PAGE. Further studies indicated that equivalent fluorescence quenching of the products could be achieved with increasing concentrations of fragment 2 (data not shown). This observation is consistent with the reported finding that fragment 2 binds with modest affinity to thrombin ($K_d = 5 \mu M$) and quenches the fluorescence of 6-(idoacetamido)fluorescein incorporated into the active site using an ATAPFR-CH₂Cl tether (46). Thus, at least part of the fluorescence change upon cleavage of mIIαΔF1Δ by prothrombinase likely arises from the dissociation of fragment 2 from the fluorescent thrombin species following cleavage at Arg²⁷⁴-Thr²⁷⁵. The equivalence between kinetics of bond cleavage and the fluorescence change (Fig. 1) implies that such steps which may contribute to the increase in fluorescence are not rate-limiting and do not compromise the use of the fluorescence signal to infer rates of bond cleavage.

Rapid Scanning Measurements of mIIαΔF1 Cleavage—The possibility that the change in intensity upon cleavage of mIIαΔF1Δ arises from discrete steps was further investigated by rapid scanning fluorescence measurements. Spectral-time data sets, obtained upon cleavage of 0.3 μM mIIαΔF1Δ by prothrombinase, were globally analyzed by singular value decomposition (Fig. 2). The wavelength-independent kinetic trace could be adequately described by a single exponential transition from a less fluorescent species to one with ~20% greater fluorescence intensity as denoted by the deconvoluted component spectra (Fig. 2, inset). Thus, the change in probe fluorescence is coincident with cleavage at Arg²⁷⁴-Thr²⁷⁵ in the substrate with no evidence to suggest the presence of additional
complicating steps represented by spectrally or kinetically resolved intermediates on the steady state timescale.

Steady State Kinetics of mIIaF1 Cleavage—Steady state kinetic constants for the cleavage of mIIaF1 by prothrombinase were determined from initial velocity measurements using mixtures of mIIaF1p and mIIaF1p. Initial velocities determined at increasing concentrations of total substrate (mIIaF1) could be adequately described by the Henri-Michaelis-Menten equation (Fig. 3) yielding $K_m = 4.2 \pm 0.3 \mu M$ and $V_{max}/E_T = 91 \pm 3 \, s^{-1}$. Similar experiments with mIIaF1D (data not shown), where the total substrate concentration was equal to the fluorophore-labeled species yielded $K_m = 2.0 \pm 0.3 \mu M$ and $V_{max}/E_T = 105 \pm 6 \, s^{-1}$. Thus, kinetic constants in tolerable agreement are obtained, regardless of the fluorophore or peptidyl tether incorporated into the active site of mIIaF1. However, the finding that bond cleavage proceeds with pseudo-first order kinetics at a substrate concentration well above the determined $K_m$ (Fig. 1) questions the physical significance of these constants and implies a violation of assumptions underlying the derivation of the Henri-Michaelis-Menten equation (47).

Further analysis revealed that pseudo-first order kinetics were observed for mIIaF1 cleavage by prothrombinase, regardless of the substrate concentration relative to $K_m$. This unusual observation was sustained in measurements with mIIaF1p and mIIaF1p, as well as by direct measurements of bond cleavage by SDS-PAGE (e.g. Fig. 1). Similar observations were also made when the reversible fluorescent inhibitor, dapsylarginine-$N$-(3-ethyl-1,5-pentanediyl)amide, was used to infer reaction progress (9). This is illustrated by the adequate fit of progress curves to a single exponential rise at total substrate concentrations corresponding to $\sim 0.07 \times K_m$ or $\sim 3 \times K_m$ (Fig. 4). The observed rate constant was found to decrease with increasing substrate concentration.

As described by Mihalyi (44), these circumstances are expected when there is competitive product inhibition, with the constraint that substrate and product bind to the enzyme with exactly equal affinity i.e. $K_m = K_p$. The rate expression developed by Mihalyi (44) for these limiting conditions (Equation 2), predicts a linear dependence of the pseudo-first order rate constant ($k_{obs}$) on the concentration of enzyme and a hyperbolic decrease in $k_{obs}$ with increasing initial substrate concentrations. These criteria were met for the cleavage of mIIaF1 (Fig. 5). The data were adequately described by Equation 2, to yield $K_m = K_p = 2.6 \pm 0.2 \mu M$ and $V_{max}/E_T = 70.2 \pm 3.5 \, s^{-1}$. Similar experiments with mIIaF1p yielded comparable fits with the constants $K_m = K_p = 2.0 \pm 0.1 \mu M$ and $V_{max}/E_T = 107 \pm 5 \, s^{-1}$. The linear dependence of $k_{obs}$ on enzyme concentration (Fig. 5, inset), further ensures that the peculiarity of the observed kinetics does not arise from a rate-limiting unimolecular process that follows the cleavage of mIIaF1p.

Product Inhibition of mIIaF1 Cleavage—Cleavage of the Arg$^{74}$Thr$^{275}$ bond in mIIaF1 by prothrombinase yields fragment 2 and thrombin (45). The suggestion of a significant role for product inhibition in the cleavage of mIIaF1 was further pursued by inhibition studies with the individual products. Increasing concentrations of fragment 2 did not detectably decrease $k_{obs}$ for mIIaF1p cleavage at concentrations as high as 12 $\mu M$ (Fig. 6, upper panel). In contrast, increasing concentrations of IIa yielded a hyperbolic decrease in $k_{obs}$ (Fig. 6, lower panel). Progress curve analysis of bond cleavage by SDS-PAGE verified these findings (data not shown). The data indicate that it is thrombin and not fragment 2 that is responsible
rate constants were determined with 1 nM Xa. The fractional change in
mixture containing 0.2 mIIa was initiated with Xa. The fractional change in
presence of the product (obs,P) to the rate constant observed in the
Equation 2 to yield 

Further verification of the initial conclusions was obtained by the analysis of data from all experiments conducted in the presence or absence of IIa as a product inhibitor. These data, combined from separate experiments at different concentrations of mIIaF1 and IIai, could be adequately described by Equation 2 to yield . The residuals to the fitted line are shown in

for competitive product inhibition, with equal affinity as the substrate, during mIIaF1 cleavage by prothrombinase. This finding is somewhat surprising since the COOH terminus of the fragment 2 domain retains the residues preceding the cleaved scissile bond that are expected to interact with the active site of factor Xa within the prothrombinase complex.

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Prior work has established that IIa binds to prothrombinase through an enzymic exosite, without restricting access of small molecules to the catalytic site of factor Xa with the enzyme complex. The observation that IIa and mIIaF1 bind to the enzyme in a mutually exclusive fashion, with apparently identical affinity, raises the possibility that the initial interaction between mIIaF1 (S) and prothrombinase (E) to form ES, may also occur through exosite interactions with the enzyme. Initial interactions at an enzymic exosite must be followed by docking interactions between substrate structures surrounding the scissile bond and the active site of the enzyme prior to catalysis. This sequence of substrate binding steps, previously delineated for the recognition and cleavage of the Arg235-IIa bond in prothrombin 2 by prothrombinase (13), is illustrated in Scheme I as one possible explanation for the steps underlying the productive interaction between mIIaF1 and the enzyme complex.

Exosite-dependent Recognition of mIIaF1 by Prothrombinase—Previous studies have established a facile approach using active site-directed reversible inhibitors to infer the relative contributions of active site versus exosite interactions toward protein substrate recognition by coagulation enzymes (13, 48). A hallmark of ordered, exosite-driven, protein substrate recognition is that active site-directed reversible inhibitors that exclude peptidyl substrate binding at the active site of the enzyme fail to act as competitive inhibitors of protein substrate cleavage (13, 48).

The speculated sequence of substrate binding events (Scheme I) was further investigated by steady state inhibition studies with PAB. PAB is expected to bind reversibly to the S1 specificity site of arginine-specific serine proteinases (49) and is established to act as a well behaved, classical competitive inhibitor of peptidyl substrate cleavage by prothrombinase (13).
Inhibition of mⅠaΔF1 cleavage by PAB obviously deviated from classical competitive inhibition (Fig. 7). This observation indicates that PAB and mⅠaΔF1 do not bind in a mutually exclusive way to the enzyme. Analysis according to the rate expression for classical noncompetitive inhibition yielded a reasonable fit, suggesting that PAB binding to the active site of factor Xa within prothrombinase minimally alters mⅠaΔF1 binding and vice versa. This observation provides empirical support for the hypothesis that the initial binding of S to E (Scheme I), likely involves interactions at sites removed from the active site of the enzyme.

Small but systematic deviations were observed between the data and the lines fitted according to classical noncompetitive inhibition (Fig. 7). The $K_s$ for PAB, determined by this analysis, was approximately 2-fold greater than the measured $K_s$ for PAB binding to prothrombinase (50), further implying a systematic error in the description of the data by this rate expression. Classical noncompetitive inhibition is expected for the mechanism depicted in Scheme I for the limiting case when the formation of ES* is unfavorable ($K_s^* \gg 1$) (13, 48). However, deviation from classical noncompetitive inhibition is expected when this criterion is not satisfied. The complete initial velocity expression for Scheme I (Equation 3) provided a superior fit to the data (Fig. 7), and yielded fitted values for $K_s$ in agreement with the measured $K_s$ for the binding of PAB to prothrombinase (50). Provided Scheme I adequately accounts for mⅠaΔF1 binding to prothrombinase, the fitted estimates $K_s = 17.4 \pm 4.4 \, \mu M$ and $K_{s}^{*} = 0.37 \pm 0.14$ yield the tentative conclusion that interactions between the substrate and the active site of the enzyme, depicted by the equilibrium distribution between ES and ES*, are favorable.

These preliminary conclusions were further pursued by initial velocity studies of the ability of mⅠaΔF1 to inhibit oligopeptidyl substrate cleavage by prothrombinase. This approach was feasible because the products of mⅠaΔF1 cleavage do not exhibit catalytic activity. The experimental strategy relies on the previous observation that protein substrate or product interactions at the exosite of prothrombinase have no detectable effect on the ability of the enzyme to bind and cleave peptidyl substrates (13, 14). Thus, in initial velocity studies of peptidyl substrate cleavage in the presence of the protein substrate, $E$ and $E$S (Scheme I) are expected to exhibit equivalent kinetic constants while $E$S* is not expected to bind and cleave the peptidyl substrate. The resulting rate expression (Equation 6) describes the dependence of the initial velocity of peptidyl substrate hydrolysis on the concentration of peptidyl substrate (SpXa) in the presence of increasing concentrations of the protein substrate (S).

The initial velocity for SpXa cleavage by prothrombinase was decreased in the presence of mⅠaΔF1 (Fig. 8). However, mⅠaΔF1 was a partial inhibitor of SpXa hydrolysis, as illustrated by the residual activity of ~50% at saturating concentrations of mⅠaΔF1 and $-K_{m}$ concentrations of SpXa (Fig. 8, inset). The family of curves obtained at different fixed concentrations of mⅠaΔF1 could be adequately described by Equation 6 to yield fitted values of $K_s = 11.7 \pm 1.4 \, \mu M$ and $K_{s}^{*} = 0.26 \pm 0.02$ (Fig. 8).

Collectively, the inhibition studies yield results consistent with the substrate binding steps illustrated in Scheme I. We therefore propose that the reaction between mⅠaΔF1 and prothrombinase results from interactions at an enzymic exosite governed by $K_s$ (Scheme I). The formation of ES is followed by an intramolecular binding step, governed by $K_s^*$ (Scheme I), which permits structures about the scissile to interact with the active site of the enzyme in a modestly favorable step, prior to catalysis.

DISCUSSION

Evidence for a major role of exosite interactions in the productive pathway for protein substrate recognition by prothrombinase has been previously developed in kinetic studies using prethrombin 2 as a substrate analog for cleavage at the Arg272-Ile274 peptide bond in prothrombin (13, 14). Data obtained in the present work using mⅠaΔF1 suggest that equivalent exosite interactions are relevant for protein substrate recognition and cleavage at the Arg272-Thr275 site as well.

Evidence to support this conclusion derives from the observation that the product, thrombin (Ⅰa), acts as a competitive inhibitor of the cleavage of either prethrombin 2 (Arg272-Ile274 cleavage) or mⅠaΔF1 (Arg272-Thr275 cleavage) by prothrombinase. In contrast, the binding of Ⅰa to prothrombinase has no obvious effect on the access of small ligands and peptidyl substrates to the active site of Xa within prothrombinase (13, 14). Competitive inhibition of prethrombin 2 activation by Ⅰa, oc-

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**FIG. 7.** Kinetics of inhibition of mⅠaΔF1 cleavage by amino-benzamidine. Initial velocities were determined using reaction mixtures containing 0.12 μM mⅠaΔF1, plus 0.38–14.88 μM mⅠaΔF1, 50 μM PCPS, 30 nM factor Va and 0 (○), 110 (●), or 330 μM (▲) PAB. Reactions were initiated with 2, 3, or 6 nM Xa, respectively. The data were analyzed using the rate expression for classical noncompetitive inhibition (dotted lines) or by analysis according to Equation 5 using the fitted parameters: $k_{cat} = 206 \pm 29 \, s^{-1}$, $K_s = 17.3 \pm 4.4 \, \mu M$, $K_{s}^{*} = 0.37 \pm 0.14$, and $K_s = 31.8 \pm 3.6 \, \mu M$.

**FIG. 8.** Inhibition of peptidyl substrate hydrolysis by prothrombinase. Reaction mixtures in assay buffer contained increasing concentrations of SpXa, 30 nM Va, 50 μM PCPS, and 0 (○), 2 (●), 6 (▲), 10 (△), 20 (▼), or 40 μM (▲) mⅠaΔF1. The reaction was initiated with 0.5 nM Xa, and initial velocities of peptidyl substrate hydrolysis were determined by continuously monitoring absorbance at 405 nm. The lines are drawn following analysis according to Equation 6 using the fitted parameters: $k_{cat}/K_{m}^{SpXa} = 158 \pm 3 \, s^{-1}$, $K_{m}^{SpXa} = 82 \pm 5 \, \mu M$, $K_s = 11.66 \pm 1.4 \, \mu M$ and $K_{s}^{*} = 0.27 \pm 0.02$. Inset, plot of percentage of activity observed at 166 μM SpXa and increasing concentrations of mⅠaΔF1.
curs with a $K_s$ comparable to the affinity of prothrombinase for the substrate (13). In the case of mIIaΔF1 cleavage, the $K_i$ for IIa is exactly equal to the apparent affinity of the enzyme for this substrate. Thrombin and prethrombin 2 share a series of structural features (17), while thrombin represents the COOH-terminal domain of mIIaΔF1 (16). These points justify the reasonable conclusion that equivalent interactions with an enzymic exosite underlie the recognition and cleavage of both analog substrates for the two half-reactions of prothrombin activation.

Alternate substrate studies with mIIaΔF1 have permitted a resolution of the thermodynamic contributions of the presumed exosite ($K_s$, Scheme I) and active site interactions ($K_i$, Scheme I) to substrate affinity. Although the overall affinity of prothrombinase for mIIaΔF1 is equal to the affinity for IIa (i.e. $K_m = K_p$), this equivalence does not apply to the inferred thermodynamics of the exosite-binding step. The inferred equilibrium dissociation constant for the binding of mIIaΔF1 to the exosite ($K_s \approx 12 \mu M$) is approximately 4-fold greater than the values determined for exosite binding by prethrombin 2 or IIa (13, 14). However, mIIaΔF1 contains the fragment 2 domain that is established to modulate interactions between the protease domain of the substrate and other macromolecular ligands (51–53). Thus, the data obtained in studies of mIIaΔF1 cleavage require comparison with the kinetics of activation of prothrombin 2 saturated with fragment 2. The value for $K_s$ inferred for mIIaΔF1 in the present work is strikingly similar to the $K_m$ previously determined for prothrombin 2 plus fragment 2 (12). For prethrombin 2 activation, the data are consistent with an unfavorable active site docking step, implying $K_i^{\ast} \gg 1$ (estimated by simulations at $K_i^{\ast} \approx 8$) and $K_m = K_i$ in substrate binding steps equivalent to those illustrated in Scheme I (13).

The present results, and the application of Equations 4 and 5 to the steady state kinetic constants for prethrombin 2 plus fragment 2 (12), allow for a more appropriate comparison of the stepwise binding interactions that lead to the recognition of the two bonds in the substrate by prothrombinase followed by catalysis (Scheme II). Although prethrombin 2 plus fragment 2 and mIIaΔF1 are individually cleaved by prothrombinase with different steady state kinetic constants, the dissociation constant for the exosite binding step ($K_s$) and the inferred rate constant for catalysis ($k_{cat}$) for these two substrates are equivalent (Scheme II). The major difference appears to lie in the equilibrium dissociation constant for the active site docking step ($K_i^{\ast}$) that precedes bond cleavage. Active site interactions that precede the cleavage of the Arg274-Thr275 site in mIIaΔF1 appear to be modestly favorable, whereas the comparable binding step that precedes cleavage of the Arg323-Ile324 site in prethrombin 2 plus fragment 2 is an unfavorable step (Scheme II).

Comparable values for $K_i$ inferred for both protein substrate analogs (Scheme II) supports the contention that the initial interaction between both substrates and prothrombinase involves equivalent exosite binding steps. Since prethrombin 2 and thrombin appear to bind this enzymic exosite with greater affinity ($K_{i^{\ast}} \approx 3 \mu M$) (13, 14), it follows that the relatively high affinity interaction between fragment 2 and prethrombin 2 (12) modestly decreases the affinity of the resultant substrate for the enzymic exosite by a factor of 4. The binding of fragment 2 to prethrombin 2 has also been established to increase the $V_{max}$ for substrate cleavage by prothrombinase by approximately the same factor (12). Thus, while the rate-enhancing effects of fragment 2 on the cleavage of prethrombin 2 by prothrombinase are well established in the literature (12, 54, 55), the data are most consistent with the interpretation that the binding of fragment 2 to prethrombin 2 somehow alters the structure of the substrate, leading to a modest perturbation in the kinetic constants (12). Based on previous studies with proteolytic fragments of prethrombin 2 and thrombin (14), it seems probable that the reduced affinity of the fragment 2-prethrombin 2 complex for exosite binding to prothrombinase arises from linkage between distinct sites in the proteinase domain of the substrate that mediate fragment 2 binding and interactions with the enzymic exosite.

The interaction of fragment 2 with thrombin is also likely to reduce the affinity of the product for exosite binding. If the
affinity changes observed with prethrombin 2 directly apply to thrombin, it follows that the equilibrium dissociation constant for the interaction between thrombin and the enzymic exosite is approximately 4-fold lower than that of the fragment 2-thrombin complex i.e. \( K_d = 3 \mu \text{M} \), \( \beta K_{D,\text{IIa}} = 12 \mu \text{M} \), \( \beta = 4 \) (Scheme I). Consequently, the previously measured equilibrium dissociation constant for the binding of fragment 2 to thrombin \( (K_d = 5 \mu \text{M}) \) (46) is also expected to be increased by a factor of 4 in the presence of prothrombinase i.e. \( K_{D,\text{IIa}} = 20 \mu \text{M} \) (Scheme I). These points provide a reasonable quantitative accounting for the inhibition of mIIaAF1 cleavage by IIa, as well as the modest increase in reaction rate observed in the presence of increasing concentrations of fragment 2 (Fig. 6).

Evidence for a high affinity interaction between fragment 2 and thrombin \( (K_d = 0.8 \text{M}) \) has previously been obtained at an ionic strength much lower than those used in the present work (56). A strong ionic strength dependence of this interaction is implied by the substantially larger equilibrium dissociation constant measured by Bock at \( I = 0.15 \) M (46), which seems to represent the most appropriate value for considerations of product inhibition in the present studies. However, a value of \( K_{D,\text{IIa}} \) substantially lower than 5 \( \mu \text{M} \) would provide more compelling support for the conclusion that fragment 2 binding does not enhance the ability of IIa to bind prothrombinase and would imply a far stronger destabilizing effect of fragment 2 on this interaction.

Significant differences in the rate constant for catalysis, inferred by division of \( V_{\text{max}} \) by \( K_M \), have been previously noted for the two cleavage reactions in the protein substrate catalyzed by prothrombinase (9, 10). An obvious explanation for this finding has not been forthcoming since identical \( P_1-P_4 \) residues precede both cleavage sites in prothrombin (36). Assuming that Scheme II provides an adequate description of the binding steps in substrate recognition, the present results indicate that the rate constant for catalysis is essentially the same for the two cleavage reactions and is comparable to values observed for the cleavage of peptidyl substrates bearing the same \( P_1-P_4 \) sequence encountered in the protein substrate (57).

Structural models for mIIaAF1 and prethrombin 2 from x-ray diffraction data indicate that the residues preceding the Arg232-Ile234 bond are either disorderd or require significant rearrangement to be successfully docked into the active site of factor Xa (17). Such features are not observed for the identical residues preceding the Arg274-Thr275 bond in mIIaAF1 (16). These observations may provide a structural explanation for the large differences in the equilibrium constant for the active site docking step \( (K_d^*, \text{Scheme II}) \) inferred for the two protein substrates. Previous work has established that, although cleavage at Arg232-Ile234 in prethrombin 2 is greatly accelerated by factor Va, the cofactor has a much smaller effect on the cleavage at the Arg274-Thr275 bond in mIIaAF1 (9, 16). If it is indeed true that exosite binding by the protein substrate is only significant following assembly of the prothrombinase complex (13, 50, 58), then this initial interaction, which serves to tether the substrate to the enzyme complex, is likely to disproportionately enhance cleavage at the disordered Arg232-Ile234 site governed by an unfavorable active site docking step in contrast to cleavage at the Arg274-Thr275 site, which results from a favorable interaction at the active site. This hypothesis implies that the rate-enhancing effects of factor Va at least partly arise from indirect or direct contributions toward exosite binding by the protein substrate. This initial tethering reaction could overcome inefficient catalysis at suboptimally configured cleavage sites in the protein substrate.

Structural studies of prothrombin derivatives indicate that the two scissile bonds in the substrate are separated by as much as 36 A (16), yet the present data indicate that both cleavages derive from equivalent exosite interactions that initially tether the substrate to prothrombinase. Therefore, there must be a considerable rearrangement of the protein substrate to permit the structures surrounding spatially distinct scissile bonds to interact with the active site before cleavage (Scheme II). Prior work has established that prothrombin activation by prothrombinase proceeds by cleavage at Arg232-Ile234 followed by cleavage at Arg274-Thr275 (7, 8). Within experimental error, cleavage in the opposite order has been undetectable despite the fact that both bonds appear accessible in the protein substrate (8). An explanation for these observations may lie in the geometric constraints imposed by exosite binding on the accessibility of the individual bonds to the active site of the enzyme. Such an explanation would suggest that geometric constraints on the substrate bound to prothrombinase by exosite interactions somehow restrict access of the Arg274-Thr275 site to the active site of the enzyme until the Arg232-Ile234 bond is cleaved. Rapid kinetic measurements of prothrombin cleavage suggest such potential substrate rearrangements that could follow cleavage at Arg232-Ile234 and permit active site access to the Arg274-Thr275 site may be related to the resultantzymogen-proteinase transition and may be rate-limiting (8).

Extended interactions between enzyme and substrate are likely to be relevant to a variety of enzymic systems that act on macromolecular substrates. In such cases, the productive interaction between substrate and enzyme is likely to result from two or more binding steps that contribute differentially to the perceived affinity of the enzyme for the substrate and the inferred rate constant for catalysis. The kinetic concepts and approaches developed in this work and in prior studies with prethrombin 2 (13, 14) may prove generally useful in studies with such systems. Several other trypsin-like enzymes of coagulation act specifically to cleave their protein substrates at more than one site (2). Exosite-dependent substrate tethering may play a role in determining specificity and cleavage order in some of these reactions as well. In addition to specific proteolytic reactions such considerations may also be relevant to other reaction systems such as protein carboxylation (59), phosphorylation (60), or acetylation (61), for example, where enzymic catalysts act with defined specificity but at multiple sites on macromolecular substrates.

In summary, the results of the present study of the cleavage of mIIaAF1 by prothrombinase suggest that exosite binding by the substrate plays a central role in both cleavage reactions of prothrombin activation. Enzymic specificity is apparently achieved by stepwise interactions of the protein substrate with an exosite followed by an active site docking step prior to bond cleavage. The two cleavage reactions are characterized by equivalent exosite interactions and rate constants for catalysis but differ significantly in the inferred thermodynamics of the active site docking step. Thus, binding to the enzymic exosite tethers the protein substrate to prothrombinase and directs cleavage at two spatially distinct sites. These findings provide novel insights into the basis for protein substrate specificity, the reaction pathway for the conversion of prothrombin to thrombin, and the function of the prothrombinase complex.

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Exosite Binding Tethers the Macromolecular Substrate to the Prothrombinase Complex and Directs Cleavage at Two Spatially Distinct Sites
Danilo S. Boskovic and Sriram Krishnaswamy

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