Binding of Substrate in Two Conformations to Human Prothrombinase Drives Consecutive Cleavage at Two Sites in Prothrombin*

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Thrombin formation results from cleavage of prothrombin following Arg$^{271}$ and Arg$^{320}$. Both bonds are accessible for cleavage, yet the sequential action of prothrombinase on Arg$^{270}$ followed by Arg$^{271}$ is implied by the intermediate observed during prothrombin activation. We have studied the individual cleavage reactions catalyzed by prothrombinase by using a series of recombinant derivatives: wild type prothrombin (IIWT) contained both cleavage sites; IIQ$^{271}$ contained a single cleavable site at Arg$^{270}$; IIQ$^{320}$ and IIA$^{320}$ contained a single cleavable site at Arg$^{320}$; and IIQQ was resistant to cleavage. Cleavage at Arg$^{270}$ in IIWT could account for the initial cleavage reaction leading to the consumption of either plasma prothrombin or IIWT, whereas cleavage at Arg$^{271}$ in either IIQ$^{320}$ or IIA$^{320}$ was found to be ≈30-fold slower. Equivalent kinetic constants were obtained for three of the four possible half-reactions. Slow cleavage at Arg$^{271}$ in intact prothrombin resulted from an ≈30-fold reduction in $V_{\text{max}}$. Thus, the observed pathway of bond cleavage by prothrombinase can be explained by the kinetic constants for the four possible individual cleavage reactions. IIQ$^{320}$ was a competitive inhibitor of IIQ$^{271}$ cleavage, and IIQQ was a competitive inhibitor for each reaction with $K_{i} \approx K_{m}$. The data are inconsistent with previous proposals and suggest a model in which substrates for each of the four possible half-reactions bind in a mutually exclusive manner and with equal affinity to prothrombinase in a cleavage site-independent way. Despite equivalent exosite binding interactions between all four possible substrates and the enzyme, we propose that ordered bond cleavage results from the constraints associated with the binding of substrates in one of two conformations to a single form of prothrombinase.

The formation of thrombin, a key reaction of the blood coagulation cascade, arises from specific and limited proteolysis of prothrombin (1). Although the serine proteinase, factor Xa, can catalyze this reaction, the rate of thrombin formation is greatly increased following its assembly into prothrombinase through interactions with membranes and factor Va (1–3). Prothrombinase is considered the physiologically relevant catalyst for rapid thrombin formation following the initiation of coagulation (2, 4).

Thrombin formation results from cleavage of human prothrombin following Arg$^{271}$ and Arg$^{320}$ (5, 6). Initial cleavage at Arg$^{271}$ followed by cleavage at Arg$^{320}$ (Scheme I, Reactions 3 and 4) yields thrombin via the formation of prethrombin 2 and fragment 1.2 (P2 plus F1.2) as intermediates. This cleavage pathway is evident in the action of factor Xa on prothrombin (7, 8). Cleavage at Arg$^{320}$ followed by cleavage at Arg$^{271}$ (Scheme I, Reactions 1 and 2) results in thrombin formation via production of meizothrombin (mIIa) as an intermediate. Within detection limits, bond cleavage in this order appears to quantitatively account for thrombin formation catalyzed by prothrombinase (9–11). Prothrombinase cleaves the substrate in an apparently ordered fashion even though both Arg$^{270}$ and Arg$^{320}$ appear accessible to cleavage in prothrombin (9). The kinetic and molecular bases for these observations remain obscure.

Kinetic explanations for bond selectivity in prothrombin have been sought from studies using P2 plus F1.2 and mIIa as substrates (9–13). The individual bonds in both intermediates are cleaved by prothrombinase (Scheme I, Reactions 2 or 4) with approximately equal catalytic efficiency (9–12). Consequently, an explanation for ordered bond cleavage by prothrombinase requires that Arg$^{271}$ and Arg$^{320}$ in intact prothrombin are cleaved with different catalytic efficiencies. Therefore, formal consideration of the reactions of prothrombin activation requires a distinction to be made between cleavage at Arg$^{271}$ before and after Arg$^{320}$ cleavage (Arg$^{271}$ and Arg$^{271}$ as Scheme I) or at Arg$^{320}$ before and after Arg$^{271}$ cleavage (Arg$^{320}$ and Arg$^{271}$).

1 Sequence numbers represent those obtained by consecutive numbering of the 579 residues in mature human prothrombin.
2 Prothrombin fragments are denoted by the following abbreviations: prothrombin, II; fragment 1.2, F1.2; prethrombin 2, P2; meizothrombin, mIIa; and thrombin, Ia. Ia is composed of the thrombin A chain, (Ia$_{A}$) in disulfide linkage with thrombin B chain, (Ia$_{B}$). mIIa contains fragment 1.2-thrombin A (F1.2-A) and Ia$_{B}$, linked by a disulfide bond.
3 The abbreviations used are: mIIa, meizothrombin; PC, L-α-phosphatidylcholine; ATA-FPR-CH$_{2}$Cl, acetothioacetyl FPR-CH$_{2}$Cl; DAPA, dapsyl-L-arginine N-[(3-ethyl1-L-pentanedisyl)amide; FPR-CH$_{2}$Cl, N-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone; IIQ$^{271}$, prothrombin purified from human plasma; IIQ$^{320}$, recombinant prothrombin with Arg at residue 271 replaced with Gln; IIQ$^{320}$, recombinant prothrombin with Arg at residue 320 replaced with Gln; IIQ$^{320}$, recombinant prothrombin with Arg at residue 320 replaced with Ala; IIA$^{320}$, recombinant prothrombin containing Gin at both 271 and 320; IIWT, recombinant wild type human prothrombin; mIIa, meizothrombin inactivated with ATA-FPR-CH$_{2}$Cl and modified with 6-(iodoacetamido)fluorescein following thioester hydrolysis; mIIa, meizothrombin inactivated with FPR-CH$_{2}$Cl; PS, L-α-phosphatidylserine; S2238, H$_{2}$N-phenylalanyl-L-pipeolicyl-L-arginine-p-nitroanilide; BioTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; MES, 4-morpholineethanesulfonic acid; SELDI/TOF/MS, time-of-flight mass spectrometry using a surface-enhanced laser desorption instrument.

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and Arg\textsuperscript{220}, Scheme I). As previous measurements have established that recognition and cleavage at Arg\textsuperscript{220} are independent of prior cleavage at Arg\textsuperscript{271} (11, 12), apparently ordered bond cleavage by prothrombinase can only result if cleavage at Arg\textsuperscript{271} is slow in comparison to cleavage at Arg\textsuperscript{271*} (11, 12).

Despite general agreement with this logical construct, comparable kinetic constants have been reported for the action of prothrombinase on each of the four individual cleavage reactions assessed using recombinant derivatives of prothrombin (12). Kinetic discrepancies have also led to the suggestion that a significant or large fraction of thrombin is produced by channeling without intermediate release (14–16). Studies with recombinant human prothrombin derivatives have yielded the novel suggestion that the two bonds in the substrate are recognized and cleaved by kinetically distinct and slowly interconverting conformers of prothrombinase (12). Yet binding studies indicate that all possible substrates and product bind competitively through exosite interactions to prothrombinase with affinities that are independent of the active site of the enzyme (17). These contradictory findings point to difficulties in providing a valid explanation for the action of prothrombinase on prothrombin by using kinetic models such as Scheme I. They are also inconsistent with models implicating a major role for exosite binding in substrate recognition (18–21).

We have studied the action of human prothrombinase on a series of recombinant derivatives of human prothrombin to investigate these contradictory ideas. We present a model that adequately explains the pathway for thrombin cleavage on the basis of the kinetic constants for the four possible enzymecatalyzed reactions illustrated in Scheme I. Our findings are inconsistent with the previous proposal (12), and instead suggest equivalent exosite binding interactions between all four possible substrate species and prothrombinase. We propose that ordered cleavage of the two bonds is driven by interactions between a single form of prothrombinase and two distinct conformations of substrate generated in the pathway for cleavage.

**EXPERIMENTAL PROCEDURES**

**Materials**—Hem egg \(\alpha\)-phosphatidylcholine (PC) and porcine brain \(\alpha\)-phosphatidylserine (PS) were from Avanti Polar Lipids (Alabaster, AL); 6-iodoacetamidofluorescein was from Molecular Probes (Eugene, OR); and 6-(iodoacetamido)fluorescein was from Molecular Probes (Eugene, OR). The proteinases inhibitors \(\alpha\)-phenylalanl-\(l\)-proplyl-\(l\)-arginine chloromethyl ketone (FPR-CH\(_2\)Cl, Calbiochem), p-aminophenylmethylene-sulfonyl fluoride (Sigma), and dansyl-\(l\)-arginine \(N\)-\((3\text{-ethyl}-1,5\text{-pentanediyl})\)hemithiocyanate (Sigma) were obtained from the sources indicated. The acetohydroxamate derivative of FPR-CH\(_2\)Cl (ATA-FPR-CH\(_2\)Cl) was prepared as described. The peptidyl substrate, \(H\)-\(l\)-phenylalanl-\(l\)-proplyl-\(l\)-arginine-p-nitroanilide (S2238) was from Chromogenix (West Chester, OH). Reagents for recombinant DNA manipulation were from Invitrogen as were cell culture media and most biochemicals. Human factors Xa and Va were prepared and characterized as described previously (5). Ecarin was purified and characterized by established procedures (5). Ecarin was treated with 5 mM benzamidine and stored at \(-20^\circ\text{C}\).

**Plasma Proteins**—Proteins—The purification of factor X, factor V, and prothrombin from plasmapheresis plasma have been described (21, 24). Human factors Xa and Va were prepared and characterized as described previously (9, 25, 26). Kinetic titration of Xa preparations with \(p\)-nitrophenol-p'\-guanidinobenzonate (27) yielded 0.96–1.22 mol of active sites/mol of factor Xa. Further quality control of factor Va preparations was performed by fluorescence binding measurements assessing its ability to assemble into prothrombinase as described previously (26). Typical results from this approach yielded \(K_{d} = 1.74 \pm 0.45\) mmol/mol for the complex of thrombin with FPR-CH\(_2\)Cl and \(K_{d} = 1.37 \pm 0.02\) mol of Va bound/mmol of Xa at saturation. Proteolytic derivatives of human prothrombin (IIa\(_{1}\)), fragment II (FL2), prothrombin 2 (P2), and thrombin were purified and characterized by established procedures (5). Ecarin was purified from the venom of Echis carinatus pyramidum (Latoxan, Valence, France) (19). Modifications to procedures developed bovine prothrombin were employed to prepare and purify human mIa\(_{1}\) covalently activated with FPR-CH\(_{2}\)Cl and labeled with 6-iodoacetamidofluorescein (mIa\(_{2}\)) (17, 19). Following purification, both mIa\(_{1}\) and mIa\(_{2}\) were dialyzed into 20 mm Hepes, 0.15 m NaCl, \(pH\) 7.5, and concentrated by ultrafiltration, and stored at \(-20^\circ\text{C}\). Protein concentrations were determined using the

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following extinction coefficients ($E_{280}$, $^m$ cm$^{-1}$) and molecular weights: human Xa, 1.16, 45,300 (28); human Va, 1.75, 173,000 (29), F1, 1.12, 34,900; P2, 1.94, 37,500 (5); IIa, 1.94, 37,500 (30); mIIa derivatives, 1.42, 72,000. All prothrombin derivatives were exchanged into Assay Buffer either by dialysis or by centrifugal gel filtration before use.

Characterization of Prothrombin Variants—N-terminal sequence analysis of prothrombin species was performed by automated Edman degradation at the Emory University Microchemical Facility. Prothrombin species and their cleavage products were characterized by sequencing bands excised following SDS-PAGE and electrophototyping as described (24). Analysis of 4-carboxyglutamyl acid content was performed by base hydrolysis and quantitative determination of Gla and Aax separated by high performance liquid chromatography and detected following post-column derivatization (31, 32). Molecular weights were determined by mass spectrometry using SELDI/TOF/MS (Ciphergen, Fremont, CA).

Prolonged Digestion of Prothrombin Variants—Reaction mixtures in Assay Buffer contained 2.8 μM of each prothrombin variant with no additions (−) or 50 μM PCPS, 95 μM DAPA, 70 mM Va, and 3 mM Na+(•). Samples (20 μl), withdrawn following a 30-min incubation at 25 °C, were mixed with 15 μl of SDS quenching buffer to achieve final concentrations of 62.5 mM Tris, 25 mM EDTA, 62 mM diithiothreitol, 1% (v/v) SDS, 10% (v/v) glycerol, 0.02% (w/v) bromphenol blue, pH 6.8, and heated at 90 °C for 2.5 min. SDS-PAGE of quenched samples (25 μl, −2.8 μg of protein) was performed using a 12% NOVEX Bis/Tris gradient gels run with MES buffer (Invitrogen). Bands were visualized following staining with 0.25% (w/v) Coomassie Brilliant Blue R-250 in 45% (v/v) MeOH, 10% (v/v) AcOH, 0.1% (v/v) glycerol and processed with a consistent staining and destaining protocol as described (24). Therefore, the concentration of prothrombinase (E) was considered equal to the limiting concentration of factor Xa present in the reaction mixture (36). As a result of this experimental design, only a vanishingly small fraction (−0.2%) of all small unilamellar PCPS vesicles would be expected to be populated with prothrombinase at the low concentrations of factor Xa used for this purpose. To achieve reliable initial velocity measurements, rate-limiting mass transfer of membrane-binding reactants between nonproductive and productive vesicles in this system is known to lead to the slow assembly of factor Xa into prothrombinase and dominate the rate of substrate delivery (37–40). Kinetic complexities arising from these physical limitations were avoided by decreasing the vesicle concentration and maximally unfolding the enzyme as applied to the initial velocity studies. Calculations based on measured vesicle size, a head group surface area of 74 Å$^2$ (41), and a 50:50 distribution of lipids between the two leaflets indicated that >54% of the PCPS$_{UV}$ vesicles would be expected to be populated with enzyme at the lowest concentration of prothrombinase used.

Densitometry analysis of digital camera images was performed using the program ImageQuant TL (Amersham Biosciences). Staining intensity of each band was determined by volume integration (pixel intensity integrated over area of band image), corrected for background staining and normalized to the total staining intensity in each lane. For each experiment, images acquired at different shutter speeds were used to test for saturation-related artifacts. Reaction profiles for the action of prothrombinase on IIa, IIb, or IIb were analyzed using a strategy previously established in the bovine system (19). Measurements were performed in a SpectraMaxGesture (Molecular Devices, Sunnyvale, CA) kinetic plate reader using black polystyrene 96-well plates that had been pretreated with 0.1% (v/v) Tween 20 in Assay Buffer and air-dried before use. Reaction mixtures (200 μl) containing 70 mM mIIa, increasing concentrations of mIIa, 30 μM PCPS$_{UV}$, and 35 mM Va in Assay Buffer were initiated with 30 pm Xa. Following brief mixing by vibration, fluorescence intensity was continuously monitored at 25 °C using λex = 470 nm and λem = 530 nm and monitored at 20-min intervals. Progress of substrate depletion was followed by the change in fluorescence using a strategy previously established in the bovine system (19). Inhibition studies were performed using a reaction mixture containing 10 μg of DAPA, 3 μM PCPS$_{UV}$, and 50 mM Va, 28–30 μM prothrombinase, 20 μM PCPS, 20 μM DAPA, and 50 mM Va in Assay Buffer and maintained at 25 °C were initiated by the addition of 1 mM Xa. Samples (15 μl) withdrawn at the indicated times were quenched by mixing with an equal volume of 125 mM Tris, 20% (v/v) glycerol, 0.8 M NaCl, pH 7.5. The unbound fraction was then applied to a column (0.25 ml) of Q-Sepharose (Amersham Biosciences) equilibrated in the same buffer to adsorb the mIIai variants. Bound protein was eluted with 20 mM Hepes, 0.8 M NaCl, pH 7.5 (2.0 ml), and desalted by concentration of P2, and the concentration of substrate was considered to be equal to the concentration of P2 (33). The product produced by the action of prothrombinase on IIQ271 was assumed to behave equivalently to thrombin in measurements of S2238 hydrolysis.

Initial Velocity Measurements of Cleavage of mIIa by prothrombinase—Cleavage of mIIa by prothrombinase was accompanied by ~25% enhancement in fluorescence intensity of the fluorescein moiety. The kinetics of cleavage at Arg$^{774}$ in mIIa was inferred from continuous measurements of this fluorescence change using a strategy previously established in the bovine system (19). The initial, steady state, rate of fluorescence change was converted to concentration terms using the total concentration of mIIa and the limits of the fluorescence signal signifying 0 and ~100% conversion of substrate to product (19). Inhibition studies were performed using the same approach but in the presence of the indicated mIIa concentration at saturating phospholipids by the use of PCPS$_{UV}$.
Characterization of Recombinant Prothrombin Variants—The N-terminal sequence for all prothrombin variants (Table I) was consistent with appropriate processing of the leader and propeptide during secretion by the stable HEK293 cell lines. Molecular weights obtained by SELDI/TOF/MS were in agreement with the molecular weight of IIPL (Table I). The slightly lower molecular weights observed for all recombinant prothrombin species might reflect differences in carbohydrate content with IIPL. Gla content was generally consistent with the isolation of fully carboxylated recombinant prothrombin variants (Table I). However, IIWT and IIQ271 consistently yielded a Gla content greater than the expected value of 10 (Table I). SDS-PAGE implicated trace contamination with co-purified fragment 1 in this phenomenon, and calculations revealed that minor contamination with fragment 1 (<5 mol %) could skew the measured Gla/Asx ratio sufficiently to account for the findings. This suggestion is consistent with the expected Gla content observed for fragment 1 purified following quantitative proteolysis of IIWT (Table I). We assert that the purified recombinant prothrombin preparations contain the appropriate complement of key post-translational modifications known to be necessary for function.

Equivalence between IIPL and IIWT was further documented by comparing full progress curves for product formation obtained by discontinuous measurements of S2238 cleavage following the addition of prothrombinase (not shown). IIPL and IIWT yielded product at the same rate and to a comparable extent. Accordingly, SDS-PAGE analysis following prolonged digestion of IIPL or IIWT with prothrombinase yielded bands indicating the production of thrombin and F1.2 arising from cleavage at both Arg320 and Arg271 (Fig. 1 and Scheme I). The action of prothrombinase on IIQ271 yielded bands consistent with the limiting formation of mIIa following cleavage only at Arg320, whereas cleavage of IIQ320 produced bands consistent with the formation of P2 and F1.2 (Fig. 1 and Scheme I). IIQ4 was resistant to digestion by prothrombinase (Fig. 1). The identities of bands produced from the recombinant species were also unambiguously established by N-terminal sequence analysis. In agreement with previous studies (12), the data establish the utility of the recombinant prothrombin derivatives as reagents for measurements of the individual cleavage reactions in intact prothrombin.

Kinetics of Bond Cleavage Catalyzed by Prothrombinase—Analyses by SDS-PAGE indicated that the two bonds in IIPL and IIWT were cleaved in an equivalent manner by prothrombinase (Fig. 2, A and B). In each case, the disappearance of prothrombin was accompanied by the transient appearance of a band corresponding to F1.2-A. Bands corresponding to F1.2 and IIaB predominated at longer reaction times (Fig. 2, A and B). The results agree with published findings in the bovine and human systems, implicating the sequential action of prothrombinase at Arg320 and at Arg271 (9–11, 42).

In parallel experiments, cleavage of IIQ271 by prothrombinase at Arg320 also proceeded rapidly (Fig. 2C). Although residual substrate was obvious following prolonged digestion, the disappearance of thezymogen band at the early time points approximated the disappearance of IIPL or IIWT (see below). In contrast, IIQ320 was cleaved slowly at Arg271 (Fig. 2D), and product bands were only evident at longer time points. Comparable results were obtained with IIQ320 as a substrate (Fig. 2E). Thus, despite the presence of a single cleavable site at Arg271, both IIQ320 and IIQ4 are cleaved slowly by prothrombinase. The observations suggest that Arg271 is poorly accessible to cleavage by prothrombinase in the otherwise intactzymogen. This effect is independent whether Arg320 is rendered uncleavable by substitution with either Gln or Ala.

**RESULTS**

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**Analysis of Prothrombin Cleavage**—Quantitative densitometry yielded equivalent profiles for the cleavage of IIPL or IIWT (Fig. 3). In either case, product profiles could be accounted for by the consideration of substrates and products relevant to Reactions 1 and 2 in Scheme I. Prothrombin disappearance was accompanied by the transient accumulation of mIIa and the
delayed appearance of thrombin, with features typical of an A → B → C reaction series (Fig. 3) (43). For both IIPL and IIWT, progress curves for the appearance of F1.2 were indistinguishable from those describing the appearance of thrombin (not shown). This finding indicates that flux toward thrombin formation via the formation of P2 plus F1.2 (Scheme I, Reactions 3 and 4) is below the limit of detection. In agreement with this conclusion, the initial rate of disappearance of prothrombin was matched by the initial rate of appearance of mIIa, and thrombin accumulated with an obvious lag phase (Fig. 3). Thus, within experimental error, cleavage of prothrombin at Arg320 to yield mIIa (Scheme I, Reaction 1) is sufficient to account for the action of prothrombinase on the intact zymogen. Thrombin formation can seemingly be explained by the stepwise action of prothrombinase on Arg320 followed by Arg271* in successive enzyme catalyzed reactions (Scheme I, Reactions 1 and 2).

Quantitative densitometry of prothrombin consumption was used to compare the action of prothrombinase on prothrombin derivatives containing both cleavage sites (IIPL and IIWT) with its action on the individual sites in IIQ271 and IIQ320 (Fig. 4). IIQ271 was consumed, as a result of cleavage at Arg320, with an initial rate that was indistinguishable from the rate of consumption of either IIPL or IIWT (Fig. 4). Residual amounts of IIQ271 (~15%) remaining after even 30 min probably reflect the results of product inhibition. In contrast, both IIQ320 and IIQ320 were consumed at a rate that was ~30-fold lower than that observed with either IIWT or IIQ271 (Fig. 4).

The action of prothrombinase at the Arg320 site in IIQ271 can quantitatively account for the consumption of prothrombin even when both Arg271 and Arg320 (in IIWT or IIPL) are available for cleavage. In contrast, the Arg271 site in the intact zymogen is acted upon by prothrombinase with a greatly reduced rate. Consequently, the bulk of thrombin produced would be predicted to result from initial cleavage at Arg320 in prothrombin. These data provide an adequate explanation for the observed pathway for the action of prothrombinase on IIPL or IIWT (Fig. 3). However, more kinetic information is necessary to establish whether such rate differences can explain selectivity of bond cleavage at other reactant concentrations.

Kinetics of the Individual Cleavage Reactions—Initial velocity studies were pursued with large unilamellar PCPS vesicles (PCPSLUV) to eliminate interpretation problems arising from rate-limiting mass transfer of reactants at saturating concentrations of phospholipid and picomolar concentrations of prothrombinase (see “Data Analysis”). To aid in comparisons with the results of SDS-PAGE, principal kinetic findings were confirmed with small unilamellar vesicles as well (not shown). However, at picomolar enzyme and saturating phospholipid, reactions conducted with PCPSLUV were characterized by a 2–3-fold lower $K_m$ and an 3-fold higher $V_{max}$. Substrate inhibition, a pronounced problem with small unilamellar vesicles (44), was not obvious with PCPSLUV, and the initial rate was proportional to $[E]$ from the picomolar to the nanomolar range (not shown). On these bases, we have chosen to present findings obtained with PCPSLUV by assuming that they more amenable to meaningful kinetic interpretation.

Initial velocity studies with IIQ271, established kinetic constants for the action of prothrombinase at the Arg320 site in intact prothrombin (Table II). The use of IIQ320 (cleavable at Arg271) as an alternative substrate yielded data that could adequately be described by the rate expression for classical competitive inhibition (Fig. 5A). The $K_i$ value for inhibition by IIQ320 was comparable to the $K_m$ value for IIQ271 (Table II). IIQ271 and IIQ320, each with a different cleavable site, bind to prothrombinase in a mutually exclusive manner with approximately equal affinity. The perceived affinity of prothrombinase for the substrate is therefore the same regardless of
whether the enzyme acts on Arg\(^{320}\) or Arg\(^{271}\) in the intact zymogen. Most surprisingly, it follows that the 30-fold lower rate of cleavage at Arg\(^{271}\) in \(\text{II}_{Q320}\) (Fig. 4) arises from a decreased \(V_{\text{max}}\) and not a compromised \(K_m\).

\(\Pi_{Q320}\) was also a classical competitive inhibitor of \(\Pi_{Q271}\) cleavage by prothrombincase with \(K_i \sim K_m\) (Fig. 5B and Table II). As both sites are uncleavable in \(\Pi_{Q271}\), the data confirm the conclusion that substrate affinity is independent of the cleavage site in the intact zymogen that is acted upon by prothrombincase. Competitive inhibition indicates mutually exclusive interactions between \(\Pi_{Q271}\) or \(\Pi_{Q271}\) and prothrombincase, completely independent of the availability of cleavage sites in the substrate. Thus, increasing concentrations of \(\Pi_{Q271}\) are expected to decrease the rate of cleavage of \(\Pi_{Q271}\) to zero at any concentration of \(\Pi_{Q271}\). Taken together with the fact that these findings were made in initial velocities determined following initiation with factor Xa, our findings obviate the need to invoke the differential recognition of the cleavage sites in the substrate by two slowly equilibrating but kinetically distinct enzyme conformers (12).

Kinetic constants for cleavage at the Arg\(^{320}\) site, assessed using P2 plus F1.2 as a substrate, were indistinguishable from those determined for cleavage at Arg\(^{271}\) using \(\Pi_{Q271}\) (Table II). Both \(\Pi_{Q320}\) and \(\Pi_{Q271}\) acted as classical competitive inhibitors of P2 plus F1.2 cleavage with \(K_i \sim K_m\) (Table II). In agreement with previous suggestions (11, 12), these findings document the kinetic equivalence of the action of prothrombincase on Arg\(^{320}\) and on Arg\(^{271}\).

Such equivalence was not evident for cleavage at Arg\(^{271}\) versus Arg\(^{271}\) in mIIa. Kinetic constants for the action of prothrombincase on Arg\(^{271}\) in mIIa were comparable with those observed for cleavage at Arg\(^{320}\) and Arg\(^{271}\) (Table II). These findings imply that Arg\(^{271}\) in mIIa is cleaved with a significantly greater \(V_{\text{max}}\) than is Arg\(^{271}\) in \(\Pi_{Q320}\). Thus, the \(V_{\text{max}}\) for cleav-
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TABLE II
Steady state kinetic constants for the individual half-reactions of prothrombin activation

| Substrate | Measured step | Alternative substrate | Cleavable site in alternative substrate | Inhibition type | $K_m \pm S.D.$ | $V/E \pm S.D.$ |
|-----------|---------------|------------------------|----------------------------------------|---------------|----------------|---------------|
| IIQ271-300 | Arg$^{320}$ | None | NA | Competitive | $176 \pm 14$ | $94 \pm 2$ |
| IIQ320 | Arg$^{321}$ | None | NA | Competitive | $198 \pm 8$ | $76 \pm 1$ |
| IIQ271-300 | None | Arg$^{321}$ | Competitive | $195 \pm 11$ | $80 \pm 1$ |
| IIQ320 | None | Arg$^{321}$ | Competitive | $176 \pm 11$ | $61 \pm 1$ |
| IIQ271-300 | None | Arg$^{321}$ | Competitive | $142 \pm 9$ | $80 \pm 1$ |
| IIQ320 | None | Arg$^{321}$ | Competitive | $162 \pm 12$ | $82 \pm 1$ |
|ockIIQ | Cleavage at Arg$^{3210}$ | None | NA | Competitive | $284 \pm 22$ | $114 \pm 3$ |
| IIQ271 | None | NA | NA | Competitive | $308 \pm 10$ | $108 \pm 2$ |

* Fitted steady state kinetic constants are reported ±95% confidence limits.

** $V/E = V_{max}[prothrombinase]$. 

** NA, not applicable.

Influence of Prior Cleavage at Arg$^{320}$ on Recognition and Cleavage at Arg$^{321}$—Implicit in Scheme II is the idea that prior cleavage at Arg$^{320}$ greatly enhances cleavage at Arg$^{321}$ by affecting the conversion of Arg$^{321}$ to Arg$^{321*}$ and the conversion of Arg$^{321*}$ to Arg$^{321*}$ (Fig. 2D) would be rectified if IIQ320 could be converted to mIIa. Ecarin cleaved all recombinant prothrombin variants to mIIa-like species regardless of the identity of the side chain at residue 320 (Fig. 7, inset). N-terminal sequence analysis con-
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FIG. 6. Inhibition of the cleavage of Arg<sup>271</sup> in mIIa. Initial velocities for cleavage at Arg<sup>271</sup> in mIIa were determined by continuous fluorescence measurements as described under "Experimental Procedures" using reaction mixtures containing 70 nM mIIa, increasing concentrations of mIIa (mIIa = mIIa<sub>E</sub> + mIIa<sub>F</sub>), and 30 pm prothrombinase (0.03 nM Xa, 35.4 nM Va, and 30 μM PCPS). The lines are drawn according to the fitted rate expression for classical competitive inhibition using the fitted constants listed in Table II.

**SCHEME 2.** Kinetics of the individual half-reactions of prothrombin activation.

- F1.2/P2.2 + E
  - Km = 176 nM
  - (V/E)<sub>R271</sub> = 94 s<sup>-1</sup>
- F1.2/P2 + E
  - Km = 284 nM
  - (V/E)<sub>R320</sub> = 144 s<sup>-1</sup>
- mIIa + E
  - Km = 284 nM
- IIa + F1.2 + E
- (V/E)<sub>R271</sub> = 94 s<sup>-1</sup>

**DISCUSSION**

Since the initial demonstration of the apparently ordered action of prothrombinase on the two scissile bonds in prothrombin (9, 10, 42), major gaps and contradictory ideas have prevailed, affecting the understanding of the kinetic and molecular bases for bond selectivity in this process. Our results are consistent with a model that accounts for prothrombin activation by prothrombinase based on the measured kinetic properties of each of the four possible cleavage reactions with mutually exclusive binding interactions between each of the four possible substrate species and the enzyme (Scheme II). Our findings also provide new insights into the mechanisms underlying the action of prothrombinase on the two scissile bonds in prothrombin.

Three of the four possible cleavage reactions are characterized by equivalent steady state kinetic constants (Scheme II). The reduced V<sub>max</sub> for cleavage at Arg<sup>271</sup> in intact prothrombin lies at the heart of providing an explanation for the cleavage pathway observed with prothrombinase. As a result of the reduced V<sub>max</sub> for this step, >96% of the thrombin formed at any substrate concentration will occur via cleavage at Arg<sup>320</sup> followed by cleavage at Arg<sup>271</sup>. Thus, whereas cleavage of the two bonds in prothrombin can indeed occur in either order, by far the predominant pathway for thrombin production is via the formation of mIIa. Flux via P2 plus F1.2 is expected to play a minor role in thrombin formation that is below the limits of detection. These conclusions explain observations in a series of studies of prothrombin activation catalyzed by prothrombinase using saturating concentrations of membranes and factor Va (9–12). Factor Va and the substrate-membrane interaction are established to differentially affect the individual cleavage reactions illustrated in Scheme II (9–13). Thus, changes in the contributions of the two possible pathways for prothrombin cleavage are expected in model systems in which factor Va and membranes are not saturating, cofactor function is altered, or membrane binding by the substrate is affected.

Slow cleavage at Arg<sup>271</sup> relative to Arg<sup>320</sup> in intact prothrom-
bin has been considered the most likely logical explanation for
the seemingly ordered action of prothrombinase on prothrom-
bin considered within the constraints of Scheme I (11, 12, 19).
However, experimental verification of this key prediction has
awaited the application of recombinant prothrombin deriva-
tives suited for an assessment of the individual cleavage reac-
tions in the otherwise intact zymogen. In contrast to our find-
ings with such an approach, recent studies have reported
approximately equal catalytic efficiencies for the action of pro-
thrombinase on Arg271 and Arg320 in intact prothrombin (12).
The inability of these observations to explain the action of prothrombinase on prothrombin was attributed to an inappro-
priate property of the recombinant mutant containing Ala in
place or Arg at 320, rather than a failure of simplified reaction
schemes (e.g. Scheme I) to adequately account for prothrombin
activation or unforeseen effects of two other substitutions in
that prothrombin derivative (12). We have documented equiva-
ently slow cleavage at Arg271 regardless of whether the 320
site is rendered uncleavable by substitution with Gln or Ala.

Full rescue of slow cleavage at Arg271 by prior cleavage at the
320 site further reduces the likelihood of deleterious effects on
cleavage at Arg271 by substitutions at 320. Thus, although we
presently cannot provide an explanation, unexpected effects
arising from replacing Arg at 320 with Ala cannot account for
the discrepancy between this and the previous study (12).

Thrombin formation arising from cleavage at Arg271 followed
by cleavage at Arg320* (Scheme I, Reactions 3 and 4) is pre-
dicted to contribute in an experimentally insignificant way to
prothrombin activation by prothrombinase. The intermediate,
mIIa, accumulates at concentrations in vast excess of the con-
centration of prothrombinase, and the initial rate of prothrom-
bin disappearance agrees with the rate of mIIa production.
These are the principal arguments against an obvious contri-
bution from channeling to thrombin formation (12, 14–16).
Although the overall process can adequately be described to
result from two consecutive enzyme-catalyzed reactions separ-
ated by a product release step, some fraction of mIIa must be
cleaved to thrombin without dissociating from the enzyme. The
data indicate that any contribution from such a pathway is
small and within experimental error.

The recent proposal that the cleavage sites in prothrombin
are differentially recognized and cleaved by two slowly inter-
converting forms of prothrombinase requires that the binding
of each of the four possible substrate species to the enzyme is
cleavage site-dependent and that the enzyme-substrate inter-
actions that govern cleavage at Arg271 versus Arg320 are not
mutually exclusive (12). This idea cannot readily co-exist with
the alternative suggestion of equivalent, exosite-dependent in-
teractions between bovine prothrombinase and the substrate
regardless of the site that is cleaved (19). The latter suggestion
is further supported by equilibrium binding studies showing
that all possible substrate derivatives and product bind to
prothrombinase in a mutually exclusive manner, independent
of the covalent occlusion of the active site of the enzyme (17).

Classical competitive inhibition of the cleavage at Arg320 in
IIQ271 by IIQ320 or by IIQQ indicates mutually exclusive binding
of these substrate derivatives to prothrombinase regardless of
the site available for cleavage. Further support for this idea is
provided by the ability of IIQQ to act as a classical competitive
inhibitor of three of the possible four half-reactions that could
be investigated (Table II). These data now discount the need to
incorporate two kinetically distinct enzyme isoforms in the
kinetic scheme accounting for the two cleavage reactions (12).
Instead, they are more consistent with the idea that each
cleavage reaction arises from mutually exclusive interactions
between the four possible substrate species and kinetically
identical forms of the enzyme (Scheme II).

Competitive inhibition of the cleavage reactions by IIQQ,
with \( K_i \approx K_m \), agrees with the idea that perceived affinity of
prothrombinase for the various substrate derivatives is domi-
nated by contributions from binding to an exosite on the en-
zyme (19, 20). Docking of structural elements surrounding the
scissile bond of the substrate with the active site of the enzyme
occurs in a following step leading to catalysis (19, 20). Whereas
exosite binding is a primary determinant of \( K_m \), the unimolecu-
lar active site docking step contributes to the \( V_{\text{max}} \) (19, 20).
Along this line of reasoning, mutually exclusive binding inter-
actions between IIQ320 and IIQQ271, with approximately equal
affinity indicate that both substrate analogs, each with a dif-
f erent cleavable site, bind to prothrombinase through equiva-
 lent exosite interactions. Structural data places the Arg320 and
Arg271 sites on opposite faces of the protease domain, separ-
ated by 36 Å (45, 46). These geometric constraints imply that
only one of the two cleavage sites will be appropriately posi-
tioned to engage effectively the active site of the enzyme. Op-
timized active site docking of structures surrounding the
Arg320 site and the reduced accessibility of those surrounding
Arg271 could explain the ~30-fold lower \( V_{\text{max}} \) for the cleavage
at Arg271 in IIQ320 in comparison to cleavage at Arg320 in IIQ271.
Flexibility in the conformation of prothrombin tethered
through exosite binding to prothrombinase is implied by the
fact that the \( V_{\text{max}} \) for cleavage at Arg271 in IIQ320 is not zero.

The increased \( V_{\text{max}} \) for cleavage at Arg271 in comparison to
Arg271 could arise from a change in the substrate following
cleavage at Arg320 that now facilitates enhanced docking of
structures surrounding this scissile bond with the active site of
the enzyme. Cleavage at Arg320 leads to the conversion of the
zymogen to proteinase and is associated with major conforma-
tional changes in the activation domains (45–49). Conforma-
tional changes associated with the transition of zymogen to
proteinase represent candidate explanations for changes in the
substrate that follow cleavage at Arg320 and now permit the
efficient docking of structures flanking Arg271* with the active
site. Geometric constraints associated with the exosite-depend-
ent tethering of the substrate in either the zymogen or the
proteinase forms could determine accessibility and drive pres-
tentation of the individual cleavage sites to the active site of
the enzyme. Such ideas provide a potential and experimentally
testable explanation for the equivalent kinetic constants ob-
served for cleavage at Arg320 and Arg271*, large differences in
the \( V_{\text{max}} \) for cleavage at Arg271 and Arg271*, and the observed
order of bond cleavage catalyzed by prothrombinase.

In contrast to the previous kinetic model requiring two forms
of prothrombinase to account for the two cleavage reactions
in prothrombinase (12), we suggest instead that a more compre-
hensive kinetic explanation can be provided by a simpler model
comprising the four individual cleavage reactions (Scheme II)
and a conformational change in the substrate following initial
cleavage at Arg320. We propose that the ordered action of
prothrombinase on the two sites in prothrombin arises from the
constraining effects of substrate bound in two distinct confor-
mations through equivalent exosite binding interactions to a
single form of prothrombinase.

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