Restricted Active Site Docking by Enzyme-bound Substrate Enforces the Ordered Cleavage of Prothrombin by Prothrombinase*

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The preferred pathway for prothrombin activation by prothrombinase involves initial cleavage at Arg320 to produce meizothrombin, which is then cleaved at Arg271 to liberate thrombin. Exosite binding drives substrate affinity and is independent of the bond being cleaved. The pathway for cleavage is determined by large differences in Vmax for cleavage at the two sites within intact prothrombin. By fluorescence binding studies in the absence of catalysis, we have assessed the ability of the individual cleavage sites to engage the active site of Xa within prothrombinase at equilibrium. Using a panel of recombinant cleavage site mutants, we show that in intact prothrombin, the Arg320 site effectively engages the active site in a 1:1 interaction between substrate and enzyme. In contrast, the Arg271 site binds to the active site poorly in an interaction that is ~600-fold weaker. Perceived substrate affinity is independent of active site engagement by either cleavage site. We further show that prior cleavage at the 320 site or the stabilization of the uncleaved zymogen in a proteinase-like state facilitates efficient docking of Arg271 at the active site of prothrombinase. Therefore, we establish direct relationships between docking of either cleavage site at the active site of the catalyst, the Vmax for cleavage at that site, substrate conformation, and the resulting pathway for prothrombin cleavage. Exosite tethering of the substrate in either the zymogen or proteinase conformation dictates which cleavage site can engage the active site of the catalyst and enforces the sequential cleavage of prothrombin by prothrombinase.

Thrombin, the key effector product of the coagulation cascade, is produced by specific proteolysis of the zymogen precursor, prothrombin (1). The physiologically relevant catalyst for this reaction is considered to be prothrombinase, a complex that assembles through reversible interactions between the serine proteinase, factor Xa, and the cofactor protein, factor Va on membranes containing acidic phospholipids (2, 3). The fundamental role of this reaction system in normal hemostasis is evidenced from profound bleeding associated with deleterious mutations in any one of its components (4, 5).

Prothrombinase acts specifically on prothrombin and is not known to catalyze cleavage of other coagulation zymogens at an appreciable rate. A series of studies have established a predominant role for interactions between extended surfaces on prothrombinase (exosites) and its protein substrate, independent of the active site of the catalyst, in determining function (6). Exosite binding drives substrate affinity and confers specificity by restricting the action of factor Xa within prothrombinase to protein substrate species that can engage the enzyme complex in this way (6). Docking of residues flanking the cleavage site in the substrate to the active site of factor Xa within prothrombinase occurs in a second, intramolecular binding step that contributes little to substrate affinity but has instead been proposed to affect the Vmax for bond cleavage (7).

Thrombin formation requires proteolytic cleavage of prothrombin following Arg271 and Arg320 (Scheme I, reaction 1).2 When catalyzed by prothrombinase, the reaction occurs through two sequential enzyme-catalyzed reactions that proceed largely via initial cleavage of prothrombin at Arg320 followed by subsequent cleavage at Arg271 (8). Meizothrombin (mlla),3 produced as the sole detectable intermediate, accumulates transiently at concentrations in vast excess over the concentration of enzyme and cleavage of the two bonds in the opposite order is below experimental limits of detection (9, 10). Although recent studies indicate that some intermediate produced via the alternate pathway can be detected (11), it is clear that both cleavage sites in prothrombin are accessible to externally added proteinase, yet prothrombinase preferentially rec

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2 Residue numbers in prothrombin represent those obtained by consecutive numbering of the 579 residues in the mature protein.

3 The abbreviations used are: mlla, meizothrombin; FPR-CH2Cl, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone; FPR-I1Q271, prothrombin with Arg271 replaced with Gln; I1Q320, prothrombin with Arg320 replaced with Gln; I1Q, prothrombin containing Gln replacing both Arg271 and Arg320; I1KY, recombinant wild type human prothrombin; Met-SC1–325His6, recombinant fragment containing an N-terminal methionine, residues 1–325 of staphylocoagulase followed by a His tag; pAB, 4-aminobenzamidine; PC, L-α-phosphatidylcholine; PS, L-α-phosphatidylserine; PCPSLUV, large unilamellar vesicles composed of 75% (w/w) PC and 25% (w/w) PS; PCPS, small unilamellar vesicles containing 75% (w/w) PC and 25% (w/w) PS; RVK6, factor Xa activating proteinase from Russell’s viper venom; Xa3195A, recombinant human factor Xa containing Ala in place of the catalytic Ser3195; HEK, human embryonic kidney.
Substrate Binding and Catalysis in Prothrombin Cleavage

1. Overall reaction

2. Cleavage at R\textsubscript{320}

3. Cleavage at R\textsubscript{271}

4. Cleavage at R\textsubscript{271}

5. No Cleavage

**Scheme I. Cleavage of prothrombin and variants.** Prothrombinase converts prothrombin (II\textsubscript{WT}) to thrombin (I\textsubscript{a}) and the activation peptide F1.2 by catalyzing cleavage at Arg\textsubscript{271} and Arg\textsubscript{320} (reaction 1). The individual reactions have been studied using recombinant derivatives (IIQ\textsubscript{271}, IIQ\textsubscript{320}) or the proteolytic intermediate mII\textsubscript{a} with the indicated representative steady state kinetic constants (12). The overall reaction can be accounted for by the essentially ordered cleavage of prothrombin at Arg\textsubscript{271} to form mII\textsubscript{a} (reaction 2) followed by cleavage at Arg\textsubscript{320} (reaction 3). Cleavage of bonds in the opposite order is not readily detected because of the low V\textsubscript{max} for cleavage at Arg\textsubscript{320} in IIQ\textsubscript{320} to yield prethrombin 2 (P2) and F1.2 (reaction 4). Although IIQ\textsubscript{271} is not cleaved by prothrombinase (reaction 3), it acts as a competitive inhibitor for the cleavage of the other substrates.

Oligonucleotides and antibodies were purchased from the indicated suppliers. The concentration of pAB was determined using E\textsubscript{293} = 15,000 M\textsuperscript{-1} cm\textsuperscript{-1} (18). Crude lyophilized venoms from *Echis carinatus pyramidum* and *Daboia russelli russelli* (Russell’s viper) were obtained from Latoxan (Valence, France). Human plasma used for protein isolation was a generous gift of the Plasmapheresis Unit of the Hospital of the University of Pennsylvania. All fluorescence and activity measurements were performed in 20 mm Hepes, 150 mm NaCl, 5 mm CaCl\textsubscript{2}, 0.1% (w/v) polyethylene glycol 8000, pH 7.5 (Assay Buffer), at 25 °C.

**Experimental Procedures**

Reagents—Small unilamellar phospholipid vesicles (PCPS) composed of 75% (w/w) hen egg t-\alpha-phosphatidylycholine and 25% (w/w) porcine brain t-\alpha-phosphatidylserine (Avanti Polar Lipids) were prepared and characterized as described (9). Large unilamellar vesicles of the same composition (PCPS\textsubscript{LUV}) for control as previously detailed (7, 21). Recombinant wild type human prothrombin (II\textsubscript{WT}), human prothrombin 1-\alpha-antiplasmin (RVVXCP) and the factor X activator (RVVXCP) were expressed in HEK293 cells on a large scale.

**Materials**

- **Ecarin** and the factor X activator (RVVXCP) were purified from the appropriate crude venom using established procedures (14, 19). A fragment of staphylocoagulase containing an additional Met residue at the N terminus and a His\textsubscript{6} extension at the COOH terminus (Met-SC\textsubscript{1-325}-His\textsubscript{6}) was expressed in *Escherichia coli* and purified as described (13, 20).
- Procedures for the purification of factors V and X from human plasma have been described (7, 21). Factors Va and Xa were produced by preparative activation of factor V by thrombin or factor X by RVV\textsubscript{XCP} followed by re-purification and quality control as previously detailed (7, 21).
- Recombinant wild type human prothrombin (II\textsubscript{WT}), prothrombin containing Gln in place of Arg at position 320 (IIQ\textsubscript{320}), prothrombin containing Gln in place of Arg at position 271 (IIQ\textsubscript{271}), and prothrombin containing Gln in place of Arg at both positions 271 and 320 (IIQ\textsubscript{Q}) (Scheme I) were expressed in HEK293 cells on a large scale.

The unexpected and untested relationship between substrate binding at the active site and the perceived rate constant for catalysis is a key feature of the current model proposed for the ordered action of prothrombinase on prothrombin. The Circe Effect provides a formal consideration of the strategies by which enzymes may utilize the intrinsic binding energy of the substrate to affect the rate constant for catalysis (16). However, this relationship is typically inferred through negative findings and has proved difficult to establish experimentally with an unambiguous mechanistic basis (16, 17). Furthermore, kinetic approaches, such as those that have been used to frame our ideas, are notorious for yielding interpretations that can be greatly affected by the model and assumptions chosen for analysis. We now directly examine the previously proposed relationship between binding and catalysis. Our approach employs equilibrium binding measurements in the absence of catalysis to provide a model-independent assessment of the ability of the individual cleavage site prothrombin derivatives to dock at the active site of factor Xa within prothrombinase.
Substrate Binding and Catalysis in Prothrombin Cleavage

scale, purified, and characterized as described (12). Recombinant factor X containing Ala in place of Ser at position 195 was prepared by site-directed mutagenesis of the cDNA encoding wild type factor X, expressed in HEK293 cells, and purified as described for other recombinant factor X mutants (22, 23). This factor X derivative was activated with RVVSCP and the resulting XaS195A was purified by affinity chromatography using soybean trypsin inhibitor Sepharose (Sigma) (24). N-terminal sequencing and quantitative chemical analysis of 4-carboxyglutamic acid content established that XaS195A and all recombinant derivatives of prothrombin possessed a correctly processed N terminus with 4-carboxyglutamic acid content that was indistinguishable from that of their counterparts purified from plasma. IIQ271 was converted to mII and catalytically inactivated by preparative cleavage using ecarin in the presence of FPR-CH2Cl followed by purification as described (12). IIQ270 was conformationally activated using Met-SC1–325-His6 and stabilized in a proteinase-like state by covalent inactivation with FPR-CH2Cl followed by dissociation of Met-SC1–325-His6 and purification of the conformationally stabilized FPR-IIQ270 adduct as described (13). All prothrombin derivatives and XaS195A were exchanged into Assay Buffer by centrifugal gel filtration before use. Protein concentrations were determined using the following molecular weights and extinction coefficients (27).

\[ \text{FPR-CH}_{2}\text{Cl} \]

- IIQQ, 1.47 (26);
- IIQ320, 1.78 (26);
- all recombinant II variants and mIIa, 72,000,
- 1.2 (25);
- Va, 200 μM PCPS;
- 10 μM FPR-CH2Cl;
- B (no enzyme), 1.2 μM Va,
- 10 μM FPR-CH2Cl;
- 10 μM FPR-CH2Cl,
- and 25 μM pAB;
- C (experimental), 1 μM XaS195A,
- 1.2 μM Va, 200 μM PCPS;
- 10 μM FPR-CH2Cl,
- and 25 μM pAB;
- Emission spectra were collected before and after the addition of the indicated concentrations of the prothrombin variant. For titrations, steady state intensity was measured 5 and 10 min after incremental additions of the indicated prothrombin variant. All emission spectra were corrected for dilution (max 6%) and spectra from samples B and C were corrected for scatter using reaction A. The resulting spectra from B and C were normalized by setting the peak of spectrum B (free probe) to 1. In intensity titrations, fluorescence intensity was corrected for scatter and expressed in terms of the fluorescence of free pAB using:

\[ F_{\text{OBS}}/F_{\text{P,Free}} = (F_{\text{C}} - F_{\text{A}})/(F_{\text{B}} - F_{\text{A}}) \]  

(1)

where the subscripts indicate the fluorescence intensities measured for reactions A, B, and C. To accommodate minor variations in \( F_{\text{OBS}}/F_{\text{P,Free}} \) from systematic error in experiments conducted over several months, all titration curves were normalized to a reference data set with \( F_{\text{OBS}}/F_{\text{P,Free}} = 1.371 \) in the absence of added prothrombin. Control experiments documented that the inclusion of 10 μM FPR-CH2Cl was without effect on the binding of pAB to XaS195A but was sufficient to abrogate any detectable cleavage of the prothrombin variants by trace contaminating active proteinase over the extended period of the experiment. Titrations were discontinued if the scattering signal was unexpectedly high or if the samples turned visibly turbid. Problems associated with turbidity and high scattering were minimized by the use of PCPS within 2 days of preparation.

**Kinetic Studies of IIQ271 Cleavage**—Initial velocity studies of IIQ271 cleavage were conducted as previously detailed (12). Reaction mixtures in assay buffer and maintained at 25 °C, containing 0.3 μM IIQ271, 27.5 μM PCPS, 31 nm Va, and increasing concentrations of either IIQQ or IIQ320 were initiated with 0.02 nM Xa. Samples were quenched at 0, 0.5, 1, 1.5, 2, and 3 min and product formation was assessed discontinuously as described (15), to yield the initial, steady state rate of IIQ271 cleavage.

**Data Analysis**—Representative results from two or more experiments, typically done with different protein preparations, are presented throughout. Data were fitted by the indi-
cated equations using the Levenberg-Marquardt algorithm (30). Fitted constants are presented ±95% confidence limits. Analysis according to Scheme II was performed by combining the numerical solution of multiple equilibria with non-linear error minimization using Dynafit (31), obtained as a generous gift from Petr Kuzmic (BioKin, Pullman, WA). In this case, errors in the parameters reflect linear approximations of 95% confidence limits.

In all experiments with prothrombinase, the concentrations of Va and PCPS were chosen to saturably incorporate all Xa (or Xₐₛ₁₉₅ₐ) into prothrombinase based on measured equilibrium constants and stoichiometries (32). Therefore, the concentration of prothrombinase was considered equal to the limiting concentration of factor Xa. The rationale for using PCPS(LUV) in activity measurements at subnanomolar concentrations of enzyme has been described (12). This approach permits the use of saturating concentrations of phospholipid to minimize kinetic complexity associated with non-productive binding of substrate to vesicles lacking enzyme (12).

Fluorescence titrations to assess pAB binding assumed a stoichiometry of 1 mol of pAB bound per mol of enzyme at saturation. Analysis as previously described (29) yielded fitted values for _Kₛ* and the maximal fluorescence change at saturation (∆Fₘₐₓ/ΔF_free), which reflects the-fold increase in fluorescence that accompanies the binding of pAB to enzyme.

Analysis according to Scheme II assumed _Kₑₛ = _Kₘ (or _Kₖ₊), with _Kₑₛ fixed at 260 nm, which represents an average of the affinity terms listed in Scheme I. _Kₑₚ was fixed at the measured value of 50 μM, 1 mol of P was assumed to be bound per mol of E at saturation and α was assumed to be equal to 1 based on previous kinetic studies (15). In addition, the fluorescence yields of P bound to either E or ES were assumed to be equal. Fitting according to Scheme II with these constraints provided estimates for _Kₛ* and the fluorescence signals for free and enzyme-bound P normalized on a molar basis. Division of these two fluorescence yields along with the propagation of the individual errors provided a fitted value for ∆Fₘₐₓ/ΔF_free, equivalent to the term derived from the pAB binding studies. In titrations with a saturable decrease in normalized fluorescence to 1, uncertainty in the stoichiometry for the binding of S to E was accommodated by also fitting the concentration of E. This approach is valid because the fixed concentration of E was substantially greater than _Kₑₛ. The stoichiometry determined in this way was fixed in titrations that produced only a small change in fluorescence. Although _Kₛ* was robustly determined within these constraints, the fitted value of _Kₛ* was found to be highly correlated with the assumption that _Eₖ and _Eₚ exhibit identical fluorescence properties. For this reason, we have deliberately qualified fitted values of _Kₛ* as upper or lower limit estimates without assigning significance to fitted confidence limits in this parameter.

RESULTS

Experimental Strategy—We pursued studies with Xₐₛ₁₉₅ₐ and pAB to develop a strategy assessing the ability of the substrate to engage the active site but in the absence of catalysis. This approach is based on the extensive literature in the serine proteinase field establishing that mutation of the catalytic serine (Ser¹⁹⁵) or its chemical conversion to dehydroalanine abolishes catalysis without impacting, in a major way, binding interactions at the active site (33–36). The use of pAB as a reversible fluorescent probe for the S₁ pocket⁵ of arginine-specific serine proteinases, including Xa, has been established in numerous studies (28). Because pAB and other ligands cannot simultaneously be bound at the S₁ site, probe binding and dissociation, inferred from fluorescence, has been used extensively to characterize reaction pathways between inhibitors and proteinases (28).

The binding of pAB to the active site of Xa in solution or to the active site of factor Xa within prothrombinase was accomplished by a large and saturable increase in probe fluorescence intensity (Fig. 1). Addition of 100 μM FPR-CH₂Cl to covalently modify the active site led to a rapid decay in fluorescence denoting the displacement of pAB from the active site of the catalyst (not shown). The assembly of factor Xa into prothrombinase produced a further enhancement in probe fluorescence, sug-

⁵ Nomenclature of Schechter and Berger (44).
gesting some detectable perturbation in the active site of Xa when it assembles into prothrombinase. The equilibrium dissociation constant inferred for the binding of pAB to Xa (Table 1), was in excellent agreement with published results (28). The assembly of factor Xa into prothrombinase was found to produce a minor but detectable enhancement in pAB binding (Table 1). Equivalent results were obtained with XaS195A either in solution or upon its assembly into prothrombinase with saturating concentrations of Va and PCPS although the enhancement in probe fluorescence was considerably lower (Table 1). These results were unrelated to the inefficient incorporation of XaS195A into prothrombinase as binding studies established that XaS195A was indistinguishable from Xa in its ability to assemble into prothrombinase (not shown). Lower probe fluorescence observed with XaS195A could reflect the contribution of hydrogen bonding between the amino group of the probe and Ser195 absent in this Xa variant (37, 38). These data establish the ability of pAB to bind reversibly to the S1 site of XaS195A within prothrombinase, albeit with lower fluorescence intensity. Substrates or ligands that engage the active site are expected to displace the probe and upon saturation, reduce fluorescence intensity to that of pAB in solution.

**Probe Displacement by Prothrombin Variants**—Because prothrombin is cleaved by prothrombinase, at least one of the two cleavage sites in the zymogen must engage the active site of the catalyst. The ability of prothrombin to displace pAB from XaS195A within prothrombinase was assessed using fluorescence emission spectra. Addition of XaS195A to a reaction mixture containing a non-saturating concentration of pAB (0.5 × \( K_d \)) and saturating concentrations of Va and PCPS yielded the expected increase in fluorescence intensity and a small blue shift in the emission spectrum (Fig. 2A). Subsequent addition of 1.4 \( \mu M \) IIWT decreased emission intensity to that observed in the absence of XaS195A, implying that IIWT could engage the active site of the catalyst and completely displace pAB (Fig. 2A). The addition of higher concentrations of IIWT did not further decrease probe fluorescence (below). In parallel experiments, addition of the same concentration of IIQQ, which is not cleaved by prothrombinase, produced no obvious evidence for displacement for pAB from the active site of prothrombinase (Fig. 2B). Because substitution of the two P1 Arg residues with Gln in IIQQ is expected to abrogate docking at the active site, these findings lend support to the initial conclusion that the decrease in pAB fluorescence to the value expected for free probe following addition of IIWT (Fig. 2A) reflects the displacement of pAB resulting from the engagement of at least one of the two possible cleavage sites in IIWT with the active site of XaS195A within prothrombinase.

This idea was further investigated using prothrombin variants (IIQ271 and IIQ320) in which the two cleavage sites were singly rendered uncleavable (12). Emission spectra obtained following the addition of IIQ320, which retains a cleavable site at Arg271, paralleled those obtained with IIWT (Fig. 2C). In contrast, spectra obtained following the addition of IIQ320 with a cleavable site at Arg271, mirrored those obtained with IIQQ (Fig. 2D). It follows from studies with these variants that in intact prothrombin, the Arg271 site can engage the active site of the catalyst, whereas the Arg271 site cannot do so as readily. It also follows that

### Table 1

**Binding of 4-aminobenzamide to Xa and prothrombinase**

| Enzyme species | \( K_d \) S.D. | \( \Delta F_{	ext{sat}}/F_{	ext{free}} \) S.D. |
|----------------|--------------|------------------|
| Xa            | 85 ± 7.5     | 114 ± 4          |
| Xa, Va, PCPS  | 31 ± 0.8     | 189 ± 1          |
| XaS195A       | 117 ± 14     | 23 ± 1           |
| XaS195A, Va, PCPS | 50 ± 5.5 | 30 ± 1           |

* Fluorescence titrations were performed using 1.0 \( \mu M \) Xa or XaS195A, with no other additions or in the presence of saturating concentrations of Xa (1.2 \( \mu M \)) and PCPS (200 \( \mu M \)).

* Fitted parameters are listed ±95% confidence limits.

* The fitted amplitude of the fluorescence change at saturation reflects the fold increase in fluorescence of free pAB upon binding the enzyme assuming 1 mol of pAB bound per mol of enzyme.

### Figure 2

**Displacement of pAB from prothrombinase.** Technical fluorescence emission spectra (\( \lambda_{	ext{ex}} = 320 \) nm) were obtained using reaction mixtures containing 25 \( \mu M \) pAB, 200 \( \mu M \) PCPS, 1.2 \( \mu M \) Va (P), the reaction mixture in P but also containing 1 \( \mu M \) XaS195A (P + E), and the reaction mixture in P + E also containing 1.4 \( \mu M \) IIWT (panel A, P + E + IIWT), 1.4 \( \mu M \) IIQQ (panel B, P + E + IIQQ), 1.4 \( \mu M \) IIQ271 (panel C, P + E + IIQ271), or 1.4 \( \mu M \) IIQ320 (panel D, P + E + IIQ320). Each spectrum, collected by averaging three scans, was corrected for scattering and normalized by setting the peak fluorescence of reaction P to 1.
probe displacement by II\textsubscript{WT} can be accounted for by active site docking by the Arg\textsuperscript{320} site. These conclusions are in agreement with the ability of cleavage at Arg\textsuperscript{320} and not at Arg\textsuperscript{271}, to quantitatively explain the initial action of prothrombinase on pro-thrombin (Scheme I, reaction 2 versus 4) (12).

**Contribution of Active Site Docking to Substrate Affinity**—The inability of II\textsubscript{QQ} and II\textsubscript{QQ} to displace pAB from the active site of X\textsubscript{A\textsubscript{S195A}} within prothrombinase could arise from a compromised affinity for the enzyme. This possibility was assessed by initial velocity studies of II\textsubscript{QQ} cleavage by prothrombinase in the presence of II\textsubscript{QQ} as an inhibitor or II\textsubscript{Q271} as an alternate substrate (Fig. 3). Near equivalent curves were obtained with either prothrombin variant. The data could be adequately described by the rate expression for complete competitive inhibition to yield approximately equal affinities for II\textsubscript{Q271}; II\textsubscript{QQ} and II\textsubscript{QQ} (Fig. 3). The findings indicate that the affinity of these variants for functional prothrombinase is independent of their ability to engage the active site of prothrombinase as inferred from the displacement of pAB.

In classical competitive inhibition, the initial rate for the indicator reaction tends to zero at infinite concentrations of inhibitor or alternate substrate (39). Adequate description of the data by the rate expression for this form of inhibition over a relatively wide range of concentrations of II\textsubscript{Q271} and II\textsubscript{QQ} (as high as $\sim 22 \times K_i$ with $S = 1.5 \times K_m$) obviates the need to invoke partial inhibition by alternate substrates in this system as has previously been suggested (10).

**Analysis of Active Site Docking by the Individual Sites in Intact Prothrombin**—The displacement of pAB from the active site of X\textsubscript{A\textsubscript{S195A}} within prothrombinase was studied further with more comprehensive fluorescence titrations (Fig. 4). Increasing concentrations of II\textsubscript{WT} and II\textsubscript{Q271} yielded sharp breaking displacement curves that were indistinguishable from each other (Fig. 4). In either case, fluorescence intensity saturated at the signal for free pAB at approximately 1 eq of added substrate (Fig. 4). The data imply that active site docking by either II\textsubscript{WT} or II\textsubscript{Q271} and the associated displacement of pAB, results from a tight binding interaction, wherein, 1 mol of either prothrombin variant is bound per mol of prothrombinase.

Even though II\textsubscript{Q320} and II\textsubscript{QQ} bind to prothrombinase with the same affinity as II\textsubscript{Q271} (above), increasing concentrations of II\textsubscript{Q320} or II\textsubscript{QQ} produced only a minor change in fluorescence (Fig. 4). These findings again suggest the inability of these variants to effectively engage the active site of the catalyst (Fig. 4). Whereas II\textsubscript{QQ} is not cleaved by prothrombinase and is unlikely to engage the S1 pocket in the enzyme, the Arg\textsuperscript{271} site in II\textsubscript{Q320} is indeed cleaved by prothrombinase (Scheme I, reaction 4) and therefore must, however poorly, engage the active site of the enzyme. Comparable findings with II\textsubscript{Q320} and II\textsubscript{QQ} suggest that any possible active site docking by Arg\textsuperscript{271} in II\textsubscript{Q320} occurs so weakly so as to be within the precision of the measurement and cannot be distinguished from a completely negative result.

A series of previous studies have established that pAB acts as a classical noncompetitive inhibitor of protein substrate cleavage by prothrombinase. This is because it has no effect on exosite-dependent substrate binding but interferes with the subsequent docking of the substrate with the active site of the enzyme (15). These ideas, illustrated in Scheme II, provide a formal framework for further consideration of the behavior of the prothrombin variants in the pAB displacement studies. Analysis according to Scheme II, using the constraints and assumptions outlined under “Data Analysis,” provided an adequate description of the data (Fig. 4). Fitting of displacement curves obtained with increasing concentrations of either II\textsubscript{WT} or II\textsubscript{Q271} yielded a stoichiometry of near 1 mol of S bound/mol of E at saturation, a ratio of free and bound fluorescence yields that was consistent with the directly measured value and a fitted upper limit estimate of $K_s^* = 0.02$ (Fig. 4). For data obtained with II\textsubscript{Q320} and
II_Q320 analysis yielded a lower limit estimate for $K_s^*$ = 12 (Fig. 4). These estimates for $K_s^*$ provide the quantitative basis for the suggestion that Arg$^{271}$ in intact prothrombin readily engages the active site of prothrombinase, whereas Arg$^{271}$ does so poorly, with an estimated 600-fold larger equilibrium constant. 

Rescue of the Defective Ability of II_Q320 to Engage the Active Site of Prothrombinase—Prior cleavage of prothrombin at Arg$^{320}$ enhances the action of prothrombinase on the Arg$^{271}$ site (Scheme I) (12). The action of prothrombinase on Arg$^{271}$ can also be enhanced without prior substrate cleavage but by stabilizing the uncleaved zymogen in a proteinase-like state (13). Both approaches were applied to II_Q320 so as to rescue its defective ability to engage the active site of prothrombinase. Ecarin can cleave the 320 site in II_Q320 to form mIIa despite the absence of Arg at P1 (12). II_Q320 was converted to mIIa with ecarin, covalently inactivated with FPR-CH₂Cl, and purified (Fig. 5, inset). II_Q320 was also conformationally activated using Met-SC1–325-His₆, covalently modified with FPR-CH₂Cl, and purified (13). This quantitative analysis establishes that approximately 1 mol of substrate is bound per mol of prothrombinase at saturation (Scheme I). It also provides a physical explanation for the largely ordered action of prothrombinase on prothrombin.

The variants II_Q320, II_Q271, and II_Q320 all bind in a mutually exclusive manner to prothrombinase with equal affinity despite their differential abilities to engage the active site of the enzyme. Thus, the energetics of active site docking contributes undetectably to substrate affinity. This conclusion verifies a key, yet previously untested, prediction that arises from the multistep pathway for substrate recognition by prothrombinase initially proposed from kinetic studies (6). Accordingly, active site engagement inferred from pAB displacement can adequately be accounted for by the reactions illustrated in Scheme II assuming directly measured values of $K_{E,S}$ and $K_{E,P}$. This quantitative analysis establishes that approximately 1 mol of substrate is bound per mol of prothrombinase at saturation and provides measured estimates for $K_s^*$, the unimolecular equilibrium constant for active site engagement by the individual cleavage sites for the substrate in either the zymogen or proteinase configuration.

Limitations arising from the small fluorescence change exhibited upon the binding of pAB to XaS₁₉₅Δ and the need for large amounts of recombinant protein precluded more comprehensive studies to establish $K_s^*$ reliably. Nevertheless, the present estimates differ considerably from those provided by initial velocity studies with bovine prothrombinase using proteolytic derivatives of prothrombin lacking the membrane binding domain of the substrate (14, 15). Protein-membrane and possibly protein-protein interactions mediated by the fragment 1 domain of the substrate, by definition, provide addi-
Of the enzymology literature is replete with examples in which knowledge-based structural alterations in either the enzyme or the substrate expected to perturb substrate binding, yield disproportionately large changes in the rate constant for catalysis relative to substrate affinity. Such observations usually implicate the Circe Effect, proposed by Jencks (16), which encompasses a series of mechanisms by which intrinsic substrate binding energy may be coupled to drive ground state destabilization, decrease $\Delta G^\ddagger$, and improve catalysis. However, experimental approaches to establish the quantitative contributions of such mechanisms remain a challenge and computational approaches have yielded controversial solutions (40, 41).

When taken together, the kinetic and binding approaches with prothrombinase establish a direct relationship between substrate binding at the active site and the perceived rate constant for catalysis. The kinetic explanation for this relationship lies in the ordered multistep pathway for protein substrate recognition that results from exosite binding followed by active site engagement, which occurs in a unimolecular step prior to catalysis (6). This result is generalizable to any reaction in which substrate binds to the enzyme through stepwise interactions although the precise contribution of any unimolecular binding step to rate at saturating substrate would depend on the magnitude of the rate and equilibrium dissociation constants for the individual steps. Such a mechanism regulating substrate binding energies to catalysis could be considered potentially trivial because it does not arise from ground state destabilization and instead from interpretation of kinetics with incomplete consideration of the full mechanism for substrate binding. It may also be expected to be most relevant to reaction systems in which the substrate is a macromolecule and can engage the enzyme in an extended way. Nevertheless, because substrate affinity is typically determined by multiple bonds between the substrate and enzyme even in small substrates, it is unlikely that all such contacts occur simultaneously or that their stepwise formation is accounted for in most interpretations. Thus, this relationship between substrate binding and the perceived rate constant for catalysis may apply widely to interpretations of structure-function relationships in enzyme-catalyzed reactions.

For enzymes that act on macromolecular substrates, the relationship between binding and the perceived rate constant for catalysis is likely to greatly impact interpretations when the likelihood of substrate engagement through extended interactions is not fully accounted for. An illustration of such problems is provided by the fact that the Arg171 site in intact prothrombin is cleaved poorly because the substrate is technically non-productively bound to the enzyme. Yet, comparison of the steady state kinetic constants for cleavage at the individual sites provides no indication of this fact based on the definition of non-productive binding developed to explain the behavior of simpler enzyme systems (42).

Numerous studies have established the predominant role for exosite binding in determining the affinity of prothrombinase for its protein substrate (6). This strategy plays a major role in restricting the action of prothrombinase to prothrombin or its derivatives despite the fact that the active site of factor Xa, within the enzyme complex, can accommodate a wide variety of peptidyl sequences including those found at the activation sites of the other coagulationzymogens (7). By equilibrium binding studies, we now directly show that active site docking by the substrate that follows exosite binding enforces an additional level of specificity evident in the ability of prothrombinase to discriminate between the two cleavage sites in the substrate. Consequently, the largely ordered action of prothrombinase on prothrombin arises from differences in $V_{max}$ and not $K_m$ for the individual cleavage reactions related to the differential ability of the individual cleavage sites to engage the active site of the catalyst depending on whether the substrate is a zymogen or a proteinase. This guides the activation pathway via the formation of mIIa, a proteinase with a different spectrum of biological activities than thrombin (5). Geometric constraints imposed by exosite-dependent substrate tethering likely play a significant role in affecting active site docking by the two cleavage sites that are expected to be ~36 Å apart in the zymogen and that probably reposition following initial cleavage at the Arg271 site. This idea highlights a testable physical correlate of the findings in this and in our previous work (13).
Substrate Binding and Catalysis in Prothrombin Cleavage

Exosite-dependent substrate recognition is increasingly evident in the action of the other enzymes of coagulation on their protein substrates. Furthermore, many reactions of coagulation require multiple cleavage events in the substrate that frequently occur in a seemingly ordered fashion. Thus, the two step “dock and lock” strategy for substrate recognition and associated consequences delineated for prothrombin cleavage by prothrombinase may also apply to explain biological specificity and function in the other coagulation reactions.

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