Extended Interactions with Prothrombinase Enforce Affinity and Specificity for Its Macromolecular Substrate*

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The specific action of serine proteinases on protein substrates is a hallmark of blood coagulation and numerous other physiological processes. Enzymic recognition of substrate sequences preceding the scissile bond is considered to contribute dominantly to specificity and function. We have investigated the contribution of active site docking by unique substrate residues preceding the scissile bond to the function of prothrombinase. Mutagenesis of the authentic P_1-P_3 sequence in prethrombin 2/fragment 1.2 yielded substrate variants that could be converted to thrombin by prothrombinase. Proteolytic activation was also observed with a substrate variant containing the P_1-P_3 sequence found in a coagulation zymogen not known to be activated by prothrombinase. Lower rates of activation of the variants derived from a decrease in maximum catalytic rate but not in substrate affinity. Replacement of the P_1 residue with Gln yielded an uncleavable derivative that retained the affinity of the wild type substrate for prothrombinase but did not engage the active site of the enzyme. Thus, active site docking of the substrate contributes to catalytic efficiency, but it is does not determine substrate affinity nor does it fully explain the specificity of prothrombinase. Therefore, extended interactions between prothrombinase and substrate regions removed from the cleavage site drive substrate affinity and enforce the substrate specificity of this enzyme complex.

Blood coagulation is dependent on a series of highly specific proteolytic activation steps that are catalyzed by membrane-assembled enzyme complexes (1, 2). The catalytic component in each of these reactions is a serine proteinase of the chymotrypsin family that can act on arginine-containing substrates (3). Yet, the coagulation enzymes act on their protein substrates with marked and distinctive specificity (1). Narrow specificity is also observed for proteinases of this family that function in diverse physiological processes (3). Based on the structural paradigms established for substrate recognition by the less selective digestive serine proteinases (4), the precise recognition of side chains flanking the cleavage site by the active site of the proteinase is considered a fundamental contributor to substrate affinity and specificity in these systems (5).

The proteolytic conversion of prothrombin to thrombin by the cleavage of two peptide bonds is a key step of coagulation. It is catalyzed by prothrombinase, an archetypal enzyme complex of coagulation, consisting of the serine protease factor Xa and its cofactor Va assembled through reversible interactions on membrane surfaces in a Ca^{2+}-dependent manner (1). The formation of thrombin results from two proteolytic cleavages in prothrombin each following an Asp/Glu-Gly-Arg sequence that is not found at the activation sites of other coagulation zymogens (6). Thus, the specific action of prothrombinase on prothrombin could be explained by the precise engagement of unique sequences preceding the scissile bond in the substrate with complementary sites at the active site of the enzyme. However, narrow specificity is not evident in the action of prothrombinase on peptidyl substrates (7) and the assembly of Xa into prothrombinase greatly enhances protein substrate cleavage without affecting the action of the enzyme on active site-directed ligands (8).

Studies using proteolytic derivatives of prothrombin containing a single cleavage site have resolved a multistep pathway for protein substrate recognition by prothrombinase (9, 10). The initial interaction occurs between extended surfaces on the enzyme complex removed from the active site (exosites) and surfaces on the substrate distinct from the site of cleavage (11). This is followed by active site engagement of residues surrounding the scissile bond and substrate cleavage (10). Exosite binding of the substrate is proposed to determine substrate affinity, whereas the intramolecular binding step that docks the substrate to the active site is paradoxically proposed to contribute to the maximum catalytic rate (9).

Enzymic specificity arising purely from the established paradigm of specific recognition by the active site of the enzyme implies that substrate affinity is dictated by interactions with sequences surrounding the scissile bond. In contrast, the proposed ideas for exosite-dependent substrate recognition indicate that altered active site interactions between the substrate and the enzyme will affect V_{max} without adversely affecting substrate affinity. Prethrombin 2 reconstituted with fragment 1.2, a substrate derivative with a single cleavage site, faithfully recapitulates the kinetics of the first half-reaction of prothrombin activation by prothrombinase (12). We have prepared a series of recombinant activation site derivatives of prethrombin 2 bearing different P_1-P_3[1] sequences to distinguish between these models and further investigate the basis of substrate specificity of prothrombinase.

EXPERIMENTAL PROCEDURES

Materials—Peptidyl substrates methoxycarbonyl-D-cyclohexylglycyl-glycyl-L-arginine-*p*-nitroanilide (SpXa)[2] and H-D-phenylalanyl-L-pipe-

[1] Nomenclature of Schechter and Berger (13).
[2] The abbreviations used are: SpXa, methoxycarbonyl-D-cyclohexyl-

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colyl-L-arginine-*p*-nitroanilide (S2238) were from American Diagnostica (Greenwich, CT) and Chromogenix (West Chester, OH), respectively. Stock solutions (~4 mM) were prepared in water, and concentrations were determined using $E_{342} = 8270$ M^{-1}.cm^{-1} (14). Small unilamellar vesicles (PCPS) composed of 75% (w/w) L-α-phosphatidylcholine and 25% (w/w) L-α-phosphatidylserine (Avanti Polar Lipids, Alabaster, AL) were prepared and characterized as described previously (12).

Proteins—Human prothrombin, factor X, and factor V were isolated from plasma obtained as a gift from the Plasmapheresis Unit of the Hospital of the University of Pennsylvania as described previously (15). The derivatives, prethrombin 2 (p-Pre2), fragment 1.2 (F1.2), and thrombin were prepared by proteolysis of human prothrombin and purified by established procedures (6). Human factors Xa and Va were prepared, purified, and characterized as described (16). Protein concentrations were calculated using the following molecular weights and extinction coefficients (E_{280} mg^{-1}.cm^2): human Xa, 45,300, 1.16 (17); human Va, 168,000, 1.74, (16, 18); F1.2, 34,800, 1.12 (6); and p-Pre2 and all its recombinant variants, 37,500, 1.95 (6).

Recombinant Prethrombin 2 Variants—A cassette encoding the tissue plasminogen activator leader sequence followed by cDNA encoding human prethrombin 2 (Thr^{271}-Glu^{583} of mature prothrombin), and a stop codon was constructed by the technique of splicing by overlap extension (19) using pDEX (20) and the cDNA for human prothrombin (21) as templates. The cassette was ligated into the pNUT vector (22) for expression and for the preparation of recombinant mutants with the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). Recombinant forms of prethrombin 2 (r-Pre2) were designed to yield the following amino acids preceding the scissile bond: Asp-Gly-Arg (r-Pre2$_{WT}$), Asp-Gly-Lys (r-Pre2$_{DGK}$), Leu-Thr-Arg (r-Pre2$_{LTR}$), Leu-Thr-Lys (r-Pre2$_{LTK}$), and Asp-Gly-Gln (r-Pre2$_{DGQ}$). The integrity of all constructs was established by DNA sequence analysis. Baby hamster kidney cells were transfected with DNA using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) and were selected for growth in the presence of 440 μM methotrexate. Stable cell lines were generated by expanding resistant colonies on the basis of protein production. Stable cells were grown to confluence in Triple Flasks (Nalge-Nunc, Naperville, IL) and maintained in Opti-MEM (Invitrogen) supplemented with 40 μM ZnCl$_2$ and 1% (v/v) UltroSerG (BioSepra SA, Cergy Ste. Christopher, France). Conditioned media, harvested twice daily, was treated with 1 mM benzamidine and stored at −20 °C.

Pooled conditioned media (8–10 liters) was adjusted to 20 mM MES, pH 6.7, and applied to a column (4.8 × 6 cm) of S-Sepharose (Amersham Biosciences, Piscataway, NJ) equilibrated with 20 mM MES, 1 mM benzamidine, pH 6.5. The column was washed with 20 mM MES, 0.15 M NaCl, pH 6.5, and bound protein was eluted with a gradient of increasing NaCl (0.15 to 1.0 M, 10 ml/min, 30 min). Fractions containing protein were pooled, dialyzed into 20 mM MES, 0.11 M NaCl, pH 6.5, and applied (5 ml/min) to a 0.46 × 10 cm POROS HS/M column (Applied Biosystems, Framingham, MA) equilibrated in 20 mM MES, pH 6.5. Bound protein was eluted with a gradient of increasing NaCl (0 to 1.0 M, 5 ml/min, 9 min) prepared in the same buffer. Fractions containing prethrombin 2 were pooled, precipitated with (NH$_4$)$_2$SO$_4$ (80% saturation), collected by centrifugation (56,000 × *g*, 30 min), and dissolved in 20 mM Hepes, 0.15 M NaCl, pH 7.5. Gel filtration chromatography using Sephacryl S-300 (2.6 × 100 cm) (Amersham Biosciences) in the same buffer removed traces of high molecular weight contaminants. Fractions containing prethrombin 2 were pooled, precipitated with (NH$_4$)$_2$SO$_4$ (80% saturation), collected by centrifugation (56,000 × *g*, 30 min), dissolved in 50% (v/v) glycerol, and stored at −20 °C.

Kinetic Studies—All kinetic measurements were performed in 20 mM Hepes, 0.15 M NaCl, 5 mM CaCl$_2$, and 0.1% (w/v) polyethylene glycol (M_r 8000), pH 7.5 (Assay Buffer), at 25 °C. Reaction mixtures containing the indicated concentrations of prethrombin 2 *plus* F1.2, PCPS, and factor Va in assay buffer were initiated with factor Xa. Thrombin formation was measured discontinuously exactly as previously described (16). Initial, steady-state rates of thrombin formation were determined from

glycyl-glycyl-L-arginine-*p*-nitroanilide; S2238, H-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide; F1.2, fragment 1.2 comprising residues 1–270 of mature human prothrombin; MES, 4-morpholineethanesulfonic acid; PCPS, small unilamellar phospholipid vesicles composed of 75% (w/w) L-α-phosphatidylcholine and 25% (w/w) L-α-phosphatidylserine; p-Pre2, prethrombin 2 prepared by preparative proteolysis of plasma-derived prothrombin; r-Pre2$_{WT}$, recombinant wild type human prethrombin 2 comprising residues 271–583 of mature human prothrombin. For the other recombinant activation site variants, the subscripted letters denote the three residues preceding the scissile bond in single-letter code.
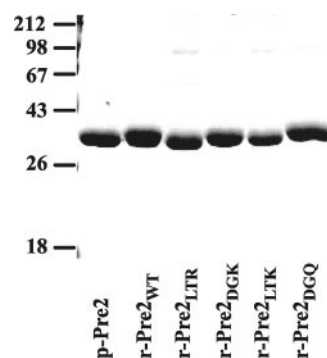

FIG. 1. **SDS-PAGE analysis of prethrombin 2 variants.** Approximately 5 μg of each variant was analyzed following disulfide bond reduction and visualized by staining with Coomassie Brilliant Blue R-250. The *numbers* in the *left margin* denote relative molecular weights (×10^3) of the standards.

the linear appearance of product with time measured in six serially quenched samples (16). For measurements with prothrombinase, concentrations of factor Va and PCPS were selected to be saturating with respect to the established equilibrium dissociation constants and stoichiometries for binding Xa (16). Thus, the concentration of enzyme was considered equal to the limiting concentration of factor Xa. Kinetic studies with each variant established that the concentration of F1.2 was saturating with respect to the concentration of prethrombin 2. Initial rates were fit to the Henri-Michaelis-Menten equation using non-linear least squares regression analysis to yield fitted values of K_m and V_{max}/E_t. Inhibition studies with r-Pre2$_{DGQ}$ were analyzed according to the rate expression for complete competitive inhibition (23) to yield fitted values for K_m, K_i, and V_{max}/E_t.

RESULTS

The various recombinant forms of Pre2 were purified with typical yields of 0.5–2 mg/liter conditioned media. All recombinant Pre2 variants comigrated with p-Pre2 upon analysis by SDS-PAGE (Fig. 1). Mass spectrometry revealed microheterogeneity in the molecular weight for r-Pre2$_{WT}$, likely reflecting heterogeneous glycosylation. However, the major species displayed a molecular weight that was slightly greater (1690–1800 atomic mass units) than the molecular weight of p-Pre2. Hydrodynamic molecular weights determined by sedimentation velocity were consistent with this finding. N-terminal sequencing indicated that p-Pre2 was truncated by 13 residues from the expected site of cleavage, most likely due to the action of thrombin during isolation (24). In contrast the N terminus of r-Pre2$_{WT}$ was extended by three residues due to uniform and incomplete processing of the signal peptide (20). These findings provide an adequate explanation for the slightly larger molecular weight of the recombinant Pre2 species.

Despite these differences, progress curves obtained in the presence of saturating F1.2 illustrated that p-Pre2 and r-Pre2$_{WT}$ were activated at the same rate and to the same extent by prothrombinase (Fig. 2*A*). Surprisingly, several of the other variants possessing mutations at the P$_1$-P$_3$ residues were also activated at an appreciable rate and to a comparable extent by prothrombinase (*e.g.* r-Pre2$_{DGK}$, Fig. 2*B*). In each case, omission of factor Va greatly reduced the rate of thrombin formation (Fig. 2). Initial rate measurements indicated that all zymogen variants with a basic P$_1$ side chain were activated at detectable rates by prothrombinase (Table I). Because formation of an active serine proteinase product requires the generation of a new N terminus beginning at residue 16 (25),[3] it follows that prothrombinase is capable of cleaving the correct peptide bond in these zymogen variants. A product was not detected with Pre2$_{DGQ}$, nor was cleavage of this zymogen spe-

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[3] Proteinase domain residues are numbered according to the topologically equivalent residues in chymotrypsinogen (26).

FIG. 2. **Activation of prethrombin 2.** *A*, progress curves of the activation of r-Pre2$_{WT}$ *plus* 2.2 μM F1.2 (○, ●) or p-Pre2 *plus* 2.8 μM F1.2 (△, ▲) using 0.125 nM Xa and 50 μM PCPS. *B*, progress curves for activation of r-Pre2$_{DGK}$ *plus* 2.2 μM F1.2 (▽, ▼) with 2 nM Xa and 50 μM PCPS. Activation was monitored either in the presence (*closed symbols*) or absence (*open symbols*) of 30 nM Va using 1.4 μM Pre2 variant.
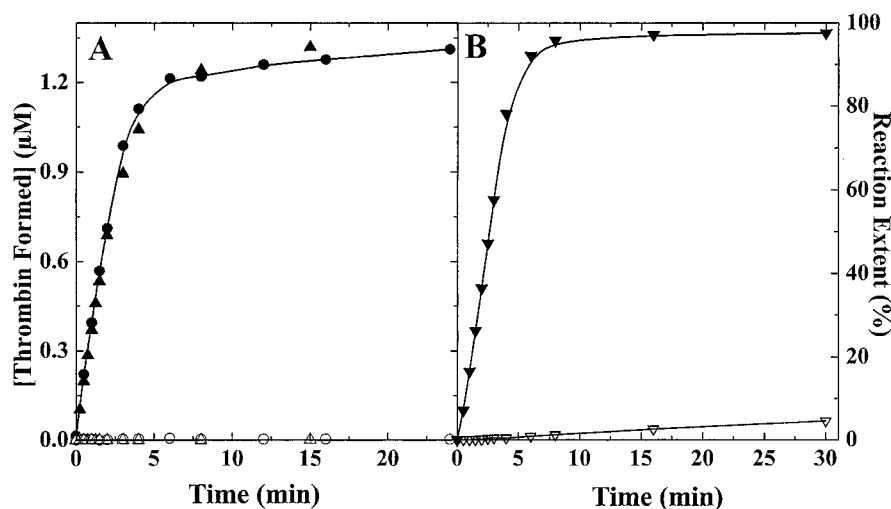
TABLE I

Activation of prethrombin 2 variants in the presence of saturating fragment 1.2

Substrate[a]	Rate$_{Xa-L}$[b]	Rate$_{Xa-Va-L}$[c]	Rate$_{+Va}$/Rate$_{-Va}$
	nM IIa/min/nM E		
p-Pre2	0.27	1467	5433
r-Pre2$_{WT}$	0.30	1456	4853
r-Pre2$_{DGK}$	0.04	150	3750
r-Pre2$_{LTR}$	0.017	32	1882
r-Pre2$_{LTK}$	0.001	1.33	1330
r-Pre2$_{DGQ}$	ND[d]	ND	

[a] Initial, steady-state rates were determined in assay buffer using the indicated substrate variant (1 μM) *plus* 1.5 μM F1.2.
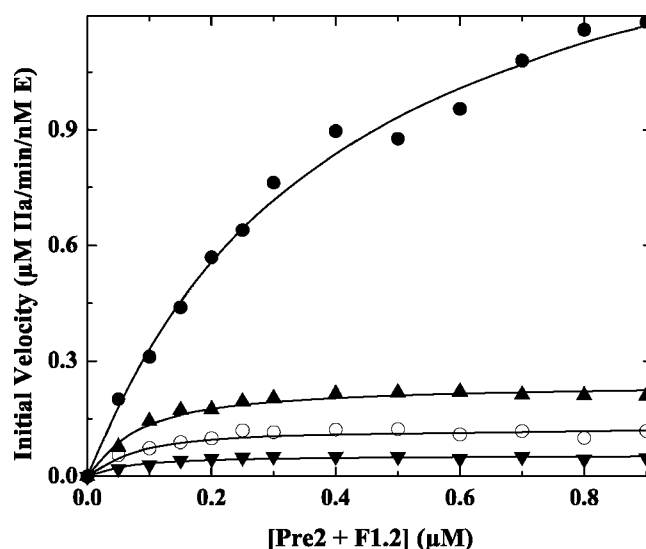
[b] Rates were determined at different concentrations of factor Xa and saturating (50 μM) PCPS. Enzyme concentration was considered equal to the limiting concentration of factor Xa.

[c] Rates were determined at different concentrations of factor Xa, 50 μM PCPS, and 60 nM Va. Enzyme concentration was considered equal to the limiting concentration of factor Xa.

[d] ND, not detected.

FIG. 3. **Initial velocity studies of the activation of prethrombin 2 variants.** Initial velocities for thrombin formation were determined using increasing concentrations of r-Pre2$_{WT}$ (●), r-Pre2$_{DGK}$ (▲), r-Pre2$_{LTR}$ (▼), and r-Pre2$_{LTK}$ (○) in the presence of 1.5 equivalents of F1.2, 50 μM PCPS, 30 nM Va, and different concentrations of factor Xa. Initial rate was normalized to the concentration of Xa. Velocities obtained with r-Pre2$_{LTK}$ were multiplied by 100 to facilitate presentation on the same scale.

cies evident by SDS-PAGE analysis following prolonged incubation with high concentrations of prothrombinase (not shown). For the derivatives that could be activated, the reaction rate was affected by the nature of the amino acid substitutions but was greatly enhanced by the addition of saturating concentrations of factor Va (Table I). Variability in the extent of rate enhancement by factor Va partly arises from imprecision in the very low rates observed in the absence of factor Va. Although these data indicate that the P$_1$-P$_3$ residues in the substrate indeed affect the rate at which the zymogen is activated, they also yield the conclusion that neither cleavage specificity nor rate enhancement by factor Va is absolutely dependent on the Asp-Gly-Arg sequence that precedes the scissile bond in the wild type zymogen.

Zymogen variants, with the exception of Pre2$_{DGQ}$, were further characterized by initial velocity studies of their activation by prothrombinase in the presence of saturating concentrations of F1.2 (Fig. 3). In each case, the dependence of reaction rate on substrate concentration could be adequately described by the Henri-Michaelis-Menten equation (Fig. 3). The activation site mutants yielded thrombin at lower rates than those observed for r-Pre2$_{WT}$, at all substrate concentrations (Fig. 3). The resulting steady-state kinetic constants (Table II) indicated that p-Pre2 and r-Pre2$_{WT}$ were indistinguishable substrates for prothrombinase in the presence of F1.2. The overall second order rate constant ($V_{max}/E_t/K_m$) was significantly lower for r-Pre2$_{DGK}$, r-Pre2$_{LTR}$, and r-Pre2$_{LTK}$. This decrease arose from a large reduction in maximum catalytic rate (V_{max}/E_t) with no

obvious increase in K_m (Table II). A noticeable decrease in K_m was observed with some of the variants (Table II), which could reflect a contribution from non-productive binding, linkage between exosite binding and active site docking, or failure of the rapid equilibrium assumption. Interpretation of such effects according to classic theory is confounded by the complex contributions of the equilibrium constants for the two substrate binding steps to the observed steady-state kinetic constants (9, 10). However, the data illustrate that, although alterations in the P$_1$-P$_3$ residues in the zymogen are expected to affect substrate docking at the active site of factor Xa within prothrombinase, these mutations do not significantly decrease the perceived affinity of the enzyme for the protein substrate. Instead, impaired docking interactions between elements surrounding the scissile bond and the active site of the enzyme are primarily realized as a V_{max} defect.

The uncleavable nature of r-Pre2$_{DGQ}$ likely arises from severely impaired interactions between the P$_1$ Gln side chain and the primary specificity pocket at the active site of factor Xa within prothrombinase. Despite its inability to be activated by

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prothrombinase, r-Pre2$_{DGQ}$ was found to inhibit the activation of r-Pre2$_{WT}$. Initial velocity studies were conducted in the presence of saturating concentrations of F1.2 using a fixed concentration of r-Pre2$_{WT}$ and increasing concentrations of r-Pre2$_{DGQ}$ (not shown) or increasing concentrations of r-Pre2$_{WT}$ in the presence of different fixed concentrations of r-Pre2$_{DGQ}$ (Fig. 4A). Inhibition by r-Pre2$_{DGQ}$ could be adequately described by the rate expression for complete competitive inhibition with a K_i that was comparable to the K_m for r-Pre2$_{WT}$. Thus, the uncleavable nature of r-Pre2$_{DGQ}$ does not arise from an impaired affinity of the zymogen for prothrombinase even though it is expected to interact in a limited way with the active site of the enzyme. Despite the ability of r-Pre2$_{DGQ}$ to inhibit the activation of r-Pre2$_{WT}$, concentrations of the uncleavable zymogen *plus* F1.2 as high as ~10× K_i did not affect active site function of factor Xa within prothrombinase as assessed by the kinetics of cleavage of an active site-directed tripeptidyl substrate (Fig. 4B). These data imply that r-Pre2$_{WT}$ and r-Pre2$_{DGQ}$ bind to prothrombinase in a mutually exclusive manner with equivalent affinities despite the fact that r-Pre2$_{DGQ}$ does not detectably engage the active site of factor Xa within the enzyme complex. These data provide additional support for the conclusion that the affinity of prothrombinase for its protein substrate is predominantly determined by interactions at sites removed from the active site of the proteinase within the enzyme complex.

DISCUSSION

We have used recombinant activation site mutants of pre-thrombin 2 *plus* F1.2 as tools to distinguish between the contributions of exosite-dependent tethering *versus* active site engagement to the binding specificity of prothrombinase toward its macromolecular substrate. Mutagenesis of the P$_1$-P$_3$ residues preceding the single scissile bond in the substrate altered the maximum catalytic rate with which the substrate derivative was cleaved without detectably decreasing the perceived affinity for the enzyme. These findings are contrary to the expectations of a model wherein substrate affinity and binding specificity is solely driven by the precise engagement of unique residues preceding the scissile bond in the substrate with the active site of Xa within prothrombinase.

Our findings corroborate the predictions derived from the multistep pathway delineated for protein substrate recognition by prothrombinase (9). Mutations at substrate residues immediately surrounding the scissile bond are expected to perturb active site docking but not exosite binding to prothrombinase. The initial exosite binding step is proposed to determine the perceived affinity of prothrombinase for its protein substrate in a dominant way, whereas the subsequent active site docking interaction is proposed to primarily contribute to maximum catalytic rate (9). These ideas satisfactorily explain the decreased V_{max} for the cleavage of the activation site variants of prethrombin 2 by prothrombinase.

This conclusion is empirically illustrated by the ability of r-Pre2$_{DGQ}$ to bind prothrombinase with the same affinity as r-Pre2$_{WT}$ and act as a competitive inhibitor of protein substrate cleavage without occluding the active site of Xa within prothrombinase. Thus, r-Pre2$_{DGQ}$ is rendered uncleavable solely because of a defective V_{max} despite the fact that it is not expected to bind to the primary specificity pocket of Xa within prothrombinase.

TABLE II
Kinetic constants for the activation of variant substrate derivatives by prothrombinase

Steady-state kinetic constants were determined as illustrated in Fig. 3 using Xa saturated with PCPS and factor Va. The values are listed ±95% confidence limits. Two sets of constants are listed for each recombinant substrate derivative to illustrate the range of values observed in multiple determinations.

Substrate[a]	V_{max}/E_t ± S.D.	K_m ± S.D.	$V_{max}/E_t/K_m$ ± S.D.[b]
	nM IIa/min/nM E	*μM*	*μM^{-1}·s^{-1}*
p-Pre2	2535 ± 106	0.59 ± 0.05	71.2 ± 6.4
r-Pre2$_{WT}$	2280 ± 212	0.73 ± 0.1	51.9 ± 9.7
	2270 ± 128	0.69 ± 0.07	55.1 ± 6.4
r-Pre2$_{DGK}$	253 ± 21	0.20 ± 0.04	21.2 ± 4.1
	265 ± 12	0.10 ± 0.02	44.6 ± 7.5
r-Pre2$_{LTR}$	59.3 ± 5.7	0.07 ± 0.02	15.2 ± 4.2
	63.4 ± 5.7	0.09 ± 0.01	12.0 ± 1.1
r-Pre2$_{LTK}$	3.01 ± 0.6	0.21 ± 0.01	0.24 ± 0.05
	1.84 ± 0.13	0.07 ± 0.01	0.46 ± 0.08

[a] The indicated substrate variants were varied either using 1.5 equivalents of F1.2 or with F1.2 fixed at a single saturating concentration.
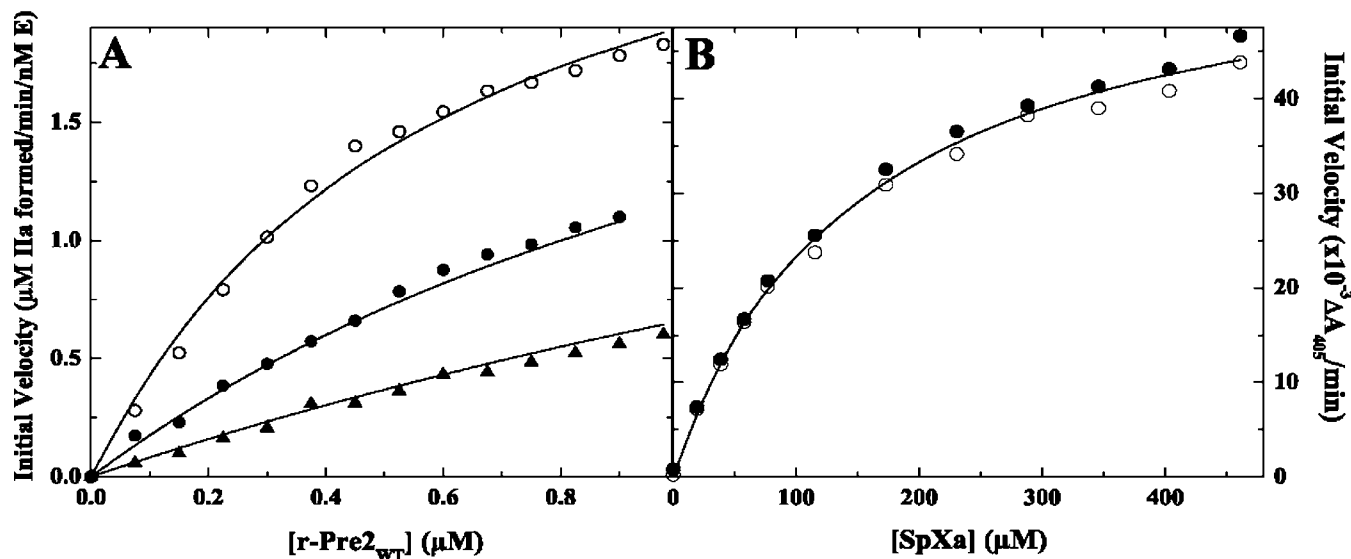[b] Uncertainty was determined by error propagation.

FIG. 4. **Inhibition of Prothrombinase by r-Pre2$_{DGQ}$.** A, initial velocities for thrombin formation were determined using increasing concentrations of r-Pre2$_{WT}$, 6 μM F1.2, 125 pM Xa, 50 μM PCPS, 30 nM Va with 0 μM (○), 1.4 μM (●), or 4 μM (▲) r-Pre2$_{DGQ}$. The *lines* are drawn according to complete competitive inhibition with the fitted constants: K_m = 0.69 ± 0.05 μM, K_i = 0.73 ± 0.05 μM, and V_{max}/E_t = 53.9 ± 3 s^{-1}. B, initial velocities were determined using increasing concentrations of SpXa, 1 nM Xa, 30 nM Va, 50 μM PCPS in either the absence (●) or presence (○) of 7 μM r-Pre2$_{DGQ}$ *plus* 10.5 μM F1.2. The *line* is drawn according to the Henri-Michaelis-Menten equation using the fitted constants K_m = 149 ± 6.8 μM and kcat = 170.5 ± 3 s^{-1}.

Factor Xa cleaves synthetic oligopeptidyl substrates containing Lys at P_1 with a greatly increased K_m compared with those containing Arg (7). However, P_1 Lys-containing prethrombin 2 variants were activated at significantly lower rates than those with Arg at P_1 because of a reduced V_{max}. This further documents the inadequacy of studies with oligopeptidyl probes targeting the active site of factor Xa in explaining the action of prothrombinase on its protein substrate.

Human factor X, which contains a Leu-Thr-Arg sequence preceding its activation site, is not known to be activated by Xa or by prothrombinase. In contrast, r-Pre2$_{LTR}$ was activated at a detectable rate by Xa, and its cleavage was greatly accelerated by the assembly of prothrombinase. Thus, the ability of prothrombinase to discriminate in a near absolute way between prothrombin and other coagulation zymogens must also derive from exosite interactions specific for the cognate substrate.

Evidence implicates a major role for exosite interactions in determining substrate affinity in the activation of factor X by the VIIa/tissue factor complex (27). Exosite binding plays a role in the action of thrombin on its diverse protein substrates with different P_1-P_3 sequences preceding the scissile bond (28). Recent results with activation site mutants of thrombin-activated fibrinolysis inhibitor (TAFI) suggest that it is exosite binding rather than specific recognition of sequences surrounding the scissile bond that likely explains the ability of thrombomodulin to specifically enhance TAFI and protein C activation by thrombin (29). Thus, exosite-dependent substrate recognition may represent a prevalent mechanism underlying the function of the trypsin-like enzymes of coagulation.

The data also point to an important role for active site docking by the substrate in determining substrate specificity for prothrombinase, although these effects are realized through a modulation of the maximum catalytic rate. Therefore, overall protein substrate specificity for prothrombinase arises from the combined contributions of both exosite binding as well as active site docking steps. Factor Xa is apparently not markedly fastidious in the peptidyl sequences that can be accommodated at the active site and cleaved. Therefore, active site docking interactions are apparently insufficient to impart the requisite level of specificity to the enzyme. The initial interaction between the substrate and extended surfaces on the enzyme complex serves to further restrict the action of prothrombinase to only those protein substrates that can be appropriately tethered to the enzyme complex thereby permitting structural elements surrounding the scissile bond to engage the active site of the enzyme.

These ideas have not previously been investigated by directed mutagenesis of the P_1-P_3 residues preceding the activation site found in prethrombin 2. However, one naturally occurring mutant (prothrombin[San Antonio]) has been described in a patient with the P_1 arginine replaced with histidine (30). The proband was heterozygous for this mutation and found to have normal antigen levels and $\sim50\%$ prothrombin activity, yet suffered a history of bleeding associated with both trauma and surgery. However, the limited biochemical characterization reported in this study and the other active diseases in the proband preclude a satisfactory interpretation of the molecular basis for the bleeding phenotype from this patient study. Patients with bleeding phenotypes are typically identified only when there are very significant decreases in the antigenic and/or functional levels of prothrombin (31). We speculate that the paucity of patient data identifying mutations at the P_2 and P_3 positions at this cleavage site may reflect our conclusion that the P_1-P_3 sequence is not an absolute determinant of cleavage specificity by prothrombinase. Other sequences preceding the scissile bond could still yield substrate derivatives that can be acted upon by prothrombinase to yield adequate levels of thrombin to achieve hemostasis.

Insights into the basis of the specificity of Xa or prothrombinase have thus far been sought from considerations of active site geometry and/or mutagenesis of residues surrounding the active site. Substrate recognition at the S_1-S_4 enzymic sub-sites has been considered to dominate evolution and functional diversity of the serine proteinases (32). Instead, we propose that the distinctive substrate specificity of prothrombinase also derives from a major contribution of exosite binding to protein substrate recognition by prothrombinase. Extended interactions with the substrate, favored directly or indirectly by factor Va within prothrombinase (16, 33), serve to direct cleavage at the activation site that otherwise interacts weakly or unfavorably with the active site of Xa (9). The action of prothrombinase is thereby restricted to the cleavage of prothrombin or its derivatives that can bind appropriately to extended surfaces on the enzyme complex even though the active site of factor Xa within the enzyme complex is fully capable of catalyzing the cleavage of activation sites in other zymogens such as factor X. Thus, extended interactions between the protein substrate and prothrombinase provide a potential explanation for the enhanced action of prothrombinase on prothrombin as well as the inability of the enzyme complex to activate the other coagulation zymogens. These ideas may have general implications for the narrow and distinctive substrate specificities of the other enzyme complexes of coagulation and of other enzyme systems that act on macromolecular substrates.

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