Regions Remote from the Site of Cleavage Determine Macromolecular Substrate Recognition by the Prothrombinase Complex*

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Andreas Betz‡ and Srima Krishnaswamy§
From the Department of Medicine, Emory University, Atlanta, Georgia 30322

The proteolytic formation of thrombin is catalyzed by the prothrombinase complex of blood coagulation. The kinetics of prethrombin 2 cleavage was studied to delineate macromolecular substrate structures necessary for recognition at the exosite(s) of prothrombinase. The product, α-thrombin, was a linear competitive inhibitor of prethrombin 2 activation without significantly inhibiting peptidyl substrate cleavage by prothrombinase. Prethrombin 2 and α-thrombin compete for binding to the exosite without restricting access to the active site of factor Xa within prothrombinase. Inhibition by α-thrombin was not altered by saturating concentrations of low molecular weight heparin. Furthermore, proteolytic removal of the fibrinogen recognition site in α-thrombin only had a modest effect on its inhibitory properties. Both α-thrombin and prethrombin 2 were cleaved with chymotrypsin at Trp148 and separated into component domains. The C-terminal-derived 2 fragment retained the ability to selectively inhibit macromolecular substrate cleavage by prothrombinase, while the 1 fragment was without effect. As the 2 fragment lacks the fibrinogen recognition site, the P1-P3 residues or the intact cleavage site, specific recognition of the macromolecular substrate by the exosite in prothrombinase is achieved through substrate regions, distinct from the fibrinogen recognition or heparin-binding sites, and spatially removed from structures surrounding the scissile bond.

The conversion of prothrombin to the serine proteinase, α-thrombin, is pivotal for the maintenance of hemostasis. The specific proteolytic activation of prothrombin is catalyzed by the prothrombinase complex, which assembles through reversible interactions between the trypsin-like serine proteinase, factor Xa, and the cofactor protein, factor Va, on appropriate phospholipid surfaces in the presence of Ca2+ ions (6, 7). Although factor Xa itself can catalyze prothrombin cleavage, the macromolecular interactions which stabilize prothrombinase lead to a profound increase, by a factor of ~100,000, in the catalytic efficiency of prothrombin activation (6–8).

A major fraction of the increased catalytic efficiency observed upon assembly of factor Xa into the prothrombinase complex likely arises as a result of the influence of factor Va on the catalyst (6). However, the molecular basis for the ability of factor Va to enhance the catalytic efficiency of factor Xa within prothrombinase is poorly understood as is the basis for the narrow and distinctive macromolecular substrate specificity of factor Xa, despite its high degree of homology with trypsin (6, 9). These two aspects of enzymic function appear closely related since the increased rate of prothrombin activation that results from the incorporation of factor Xa into the prothrombinase complex is not accompanied by changes in the rate of cleavage of synthetic peptidyl substrates, in the reaction with active site-directed reagents or even in the rate constant for inhibition by macromolecular inhibitors such as antithrombin III (10–12).

Suggestions for the importance of specific macromolecular recognition, by prothrombinase, through interactions at extended recognition sites removed from the active site of factor Xa (exosites) were initially derived from studies with tick anticoagulant peptide (TAP)1 (13). Work with a mutant derivative of TAP suggested that the selective modulation of such exosite interactions following the assembly of factor Xa into prothrombinase could lead to large changes in affinity and kinetic mechanism in the interaction of the enzyme with macromolecules (14). However, the significance of these findings toward prothrombin activation by prothrombinase has required documentation by appropriate functional studies.

The interpretation of kinetic studies of prothrombin activation is complicated by the fact that the conversion of prothrombin to thrombin involves the cleavage of two peptide bonds and is the sum of two consecutive enzyme-catalyzed reactions (6, 15). However, kinetic interpretations are simplified by the use of prethrombin 2 as a substrate analog, which requires cleavage at a single site to yield thrombin (16). The kinetics of recognition and cleavage of this bond in prethrombin 2 are established to be indistinguishable from the cleavage of the same site in intact prothrombin (15).

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This work is dedicated to the memory of our late colleague, Dr. Stuart Stone.

‡Present address: COR Therapeutics Inc., 256 E. Grand Ave., South San Francisco, CA 94080.
§Supported by Grant HL-52883 from the National Institutes of Health. To whom all correspondence should be addressed: Joseph Stokes, Jr. Research Institute, Children’s Hospital of Philadelphia, 310 Abramson, 324 South 34th St., Philadelphia, PA 19104. Tel: 215-590-3346; Fax: 215-590-3660.

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1 The abbreviations used are: TAP, recombinant wild type tick anticoagulant peptide; APMSF, p-amidinophenylmethanesulfonyl fluoride; ATA-EGR-CH2Cl, acetothioacetyl derivative of EGR-CH2Cl; ATA-EGR-Xa, factor Xa inactivated with ATA-EGR-CH2Cl; EGR-CH2Cl, glutamyl-glycyl-glycyl-arginyl chloromethylketone; IIa, thrombin inactivated with APMSF; LMW heparin, low molecular weight heparin (average Mr = 3,000); [OG488]-EGR-Xa, adduct of ATA-EGR-Xa and Oregon Green488 iodoacetamide; PEG, polyethylene glycol; PC, 1,α-phosphatidylcholine; PS, 1,α-phosphatidylserine; PCL, small unilamellar vesicles composed of 75% (w/w) PC and 25% (w/w) PS; S2238, H-n-phenylalanyl-l-pipecolyl-l-arginyl p-nitroanilide; SpXa, methoxyacyrbonyl-cyclohexyl-glycyl-glycyl-arginyl p-nitroanilide; Xa, factor Xa inactivated with APMSF; dansyl, 5-dimethylaminonaphthalene-1-sulfon; TPCK, tosyl-l-1-phenylalanine chloromethyl ketone; MES, 4-morpholineethanesulfonic acid; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis.
Studies with reversible inhibitors targeting the active site of factor Xa have provided evidence for a significant contribution from exosite interactions within prothrombinase in the recognition of prethrombin 2 (17). Active site-directed reversible inhibitors as well as oligopeptidyl alternate substrates are classical noncompetitive inhibitors of macromolecular substrate cleavage by prothrombinase despite their established ability to compete for substrate binding to the active site (17). In contrast, thrombin is a competitive product inhibitor of prethrombin 2 activation but does not interfere with oligopeptidyl substrate cleavage by prothrombinase (17). These findings indicate that the affinity and binding specificity for prethrombin 2 is determined by interactions at exosites rather than by interactions between elements surrounding the scissile bond and the active site of the protease (17). Thus, competitive inhibition of macromolecular substrate cleavage by thrombin is achieved by competition for the initial exosite interaction between the substrate and prothrombinase.

It therefore follows that the structural features of prethrombin 2 that determine substrate affinity, through interactions with the exosite, are spatially distinct from residues surrounding the scissile bond. Following cleavage, the polypeptide sequence N-terminal to the scissile bond is not released, but retained in the two chain product, thrombin, through a disulfide bond (18). Since thrombin competes for prethrombin 2 binding without obscuring access to the active site of prothrombinase, it also follows that the interaction between thrombin and prothrombinase is achieved by product domains spatially distinct from the P1-P3 residues found in the A-chain of thrombin. We have used proteolytic derivatives of thrombin and prethrombin 2 as well as ligands established to bind to specific sites in the substrate and product, to test these predictions and delineate the regions of the substrate that contribute to binding specificity through interactions with the prothrombinase exosite.

**EXPERIMENTAL PROCEDURES**

**Materials**

Hepes, Trisin (Tris base), L-α-phosphatidylserine (PS), L-α-phosphatidylcholine (PC), p-amidophenylmethylsulfonyl fluoride (APMSF), bovine fibrinogen (Fraction I, Type IV), and soybean trypsin inhibitor immobilized on Sepharose were from Sigma. Glu-Gly-Arg chloromethyl ketone (EGR-CH2Cl) was from Calbiochem (La Jolla, CA). Succinimidyl acetylthioacetate and Oregon Green488 iodoacetamide was performed in 20 mM Hepes, 0.15 M NaCl, 2 mM CaCl2, 0.1% (w/v) PEG, (Greenwich, CT), respectively. Stock solutions (cyclohexyl-Gly-Gly-Arg acetyl-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-SO3-Leu) (22), N-terminally (YM10, Amicon, Danvers, MA) to a concentration of 182 μM was obtained from J. T. Baker (Davens, MA). All kinetic studies were performed in 20 mM Hepes, 0.15 M NaCl, 2 mM CaCl2, 0.1% (w/v) MPEG, pH 7.4 (assay buffer) at 25 °C. Phospholipid vesicles (PCPS) composed of 75% (w/w) PC and 25% (w/w) PS were prepared as described previously (20). The concentration of phospholipid was determined after oxidation by a colorimetric phosphate assay and is stated as the concentration of inorganic phosphate (21). The concentration of unfragmented heparin (ESI, Cherry Hill, NJ) was calculated assuming an average molecular weight of 20,000. Low molecular weight heparin (LMW heparin) was obtained from Celsus Laboratories (Cincinnati, OH). The average molecular weight (3,000) of this preparation is consistent with the predominant species being composed of 5 disaccharides. Hirugen2, a synthetic peptide comprising the C-terminal region of hirudin, acetyl-Glu-Aap-Phe-Glu-Ile-Pro-Glu-Thr-SO3-Leu (22), originally manufactured by Biogen (Cambridge, MA) was a gift from Drs. S. R. Hanson and L. A. Harker (Emory University).

Proteins

Prethrombin and factor X were purified from bovine plasma as described previously (23). Bovine factor Xa was converted to factor Xa by using the purified activator from Russell’s viper venom and further purified by chromatography using benzamidine Sepharose (24, 25). Kinetic titration of factor Xa with p-nitrophosphoryl-p’-guanidino benzoate (26), typically yielded 1.12–1.15 mol of active sites/mmol of factor Xa. Bovine factor Va was purified using an established procedure (27) and recombinant TAP was produced and purified as described (28). Prethrombin 2 and α-thrombin were prepared as described previously (16, 18). Gel-filtration chromatography of prethrombin 2 on Sephadex G-50 in 20 mM Hepes, 2.5 mM sodium chloride, pH 7.4 (16), resolved prethrombin 2 from a polymeric form eluting at the void volume. The aggregated species was discarded as it was found to be a potent inhibitor of prethrombin 2 cleavage by prothrombinase. Hirugen-Sepharose 4B was prepared as described and extensively precycled prior to use (29). Buffer components of the hirugen preparation were removed by preparative reverse phase HPLC (Aquapore C-18, ABI, San Jose, CA) and elution with a gradient of increasing CH3CN in 0.1%(v/v) trifluoroacetic acid. Multiple peaks were identified by monitoring absorbance at 215 nm. Heterogeneity in the sample was confirmed by microbore reversed phase HPLC. However, amino acid analysis was consistent with the primary structure of hirugen and the basis for sample heterogeneity could not be determined by further analysis using either laser desorption (MALDI) or electron spray mass spectrometry. As the individual species identified by HPLC could not be adequately resolved on a preparative scale, a single pool was prepared, lyophilized, and dissolved in assay buffer. Peptide content was established by amino acid analysis.

**Preparation of Thrombin Derivatives**

Inactivation of α-thrombin with APMSF to yield α-IIa, was performed as described previously (17). For the preparation of bovine γ-thrombin, α-thrombin in assay buffer (22 μM, 45 μM) was incubated with 0.7 μM trypsin for 3 h at room temperature. The reaction was quenched with 10 μM soybean trypsin inhibitor, dialyzed against 20 mM Tris-PO4, 0.1% (w/v) PEG, pH 5.8, 40 mM NaCl, 0.1% (w/v) PEG for 4 h at 4 °C and applied to a column (1.5 × 12.5 cm) of S-Sepharose equilibrated in the same buffer. Bound protein was eluted (4 ml/min, 120 min) with a linear gradient of increasing NaCl (40–700 mM) in 20 mM Hepes, 0.1% (v/v) PEG, pH 7.4. Pooled material was concentrated by ultrafiltration in a stirred cell. The concentration of phospholipid was determined after precipitation of S2238. Fractions from the leading peak, containing γ-thrombin with an estimated contamination of 5% undigested material determined by SDS-PAGE were pooled, dialyzed against 20 mM Hepes, 40 mM NaCl, 0.1% (w/v) PEG, pH 7.4, and subject to affinity chromatography using a 4.5 × 17-cm column of fibrin-Sepharose equilibrated in the same buffer. Bound protein was eluted with a linear gradient of increasing NaCl (40–600 mM) in 20 mM Hepes, 0.1% (w/v) PEG, pH 7.4. The γ-thrombin containing fractions were pooled and reapplied to a second fibrin-Sepharose column with isocratic elution to remove traces of remaining α-thrombin. The flow-through fractions were characterized by the same specific activity as α-thrombin toward S2238 with ~2.1 mol active site/mol peptide in a fibrin clotting assay (18). Pooled material was concentrated by ultrafiltration in a stirred cell (YM10, Amicon, Danvers, MA) to a concentration of ~100 μM, inactivated by the addition of 1 mM APMSF followed by brief incubation at room temperature and dialyzed against assay buffer. The resulting preparation of inactivated γ-thrombin (γ-thrombin, possessed <0.01% catalytic activity when compared with α-thrombin. Protein sequencing of the fibrin-Sepharose resolved by SDS-PAGE yielded the exact sequence for bovine γ-thrombin (3–5, 31). In addition, the mass of γ-thrombin was determined by MALDI mass spectrometry and found to be consistent with removal of the undescapeceteptide (Ile68-Arg73)3.

Bovine γ-thrombin was prepared by treatment of α-thrombin (27 μM, 2) The residues of α-thrombin have been numbered after the corresponding amino acids in chymotrypsin using the convention of Bode et al. (2). Cleavage of human α-thrombin by trypsin at Arg30, Arg30, and Lys406 leads to the loss of the Ile68-Arg73 peptide and yields γ-thrombin.

*2 Nomenclature of Schechter and Berger (1).*
15 ml) with a linear gradient of increasing NaCl (0–350 mM) in 20 mM MES, pH 6.5, with 1 nM chymotrypsin for 3 h at room temperature. The reaction was quenched with 50 mM Tris, 30% (v/v) CH3CN, pH 9.0, for 4 h at room temperature. Following clarification by centrifugation (50,000 × g, 20 min), aliquots (3 ml) were fractionated by cation exchange HPLC (Aquapore Cation 7 μm, 0.46 × 22.2 cm, ABI). Elution (1 ml/min, 30 min) with a linear gradient of increasing NaCl (0–350 mM) in 20 mM Tris, 30% (v/v) CH3CN, pH 9.0, resolved two peaks corresponding to the two thrombin fragments. Material from each of the peaks accumulated from successive runs was pooled, dialyzed against 0.1% (v/v) trifluoroacetic acid, concentrated, and applied to a column (22.2 cm, ABI). Bound protein was eluted (1 ml/min) with a biphasic gradient of increasing Buffer B (20 mM NEt3-HCl, pH 2.5) and fractionated in ~1-ng aliquots using an Aquapore Phenyl column (0.46 × 22.2 cm, ABI). Protein sequencing and mass spectrometry. Pools were prepared for each of the resolved peaks of cleaved material. The purity of all protein preparations was judged by SDS-PAGE (33).

1-thrombin and 2-thrombin. Thrombin (30 mg) treated with chymotrypsin (as above) was dialyzed against 20 mM Tris, 30% (v/v) CH3CN, pH 9.0, for 4 h at room temperature. Following clarification by centrifugation (50,000 × g, 20 min), aliquots (3 ml) were fractionated by cation exchange HPLC (Aquapore Cation 7 μm, 0.46 × 22.2 cm, ABI). Elution (1 ml/min, 30 min) with a linear gradient of increasing NaCl (0–350 mM) in 20 mM Tris, 30% (v/v) CH3CN, pH 9.0, resolved two peaks corresponding to the two thrombin fragments. Material from each of the peaks accumulated from successive runs was pooled, dialyzed against 0.1% (v/v) trifluoroacetic acid, concentrated, and further purified by reversed phase HPLC. Each of the pools was dialyzed against Buffer A (20 mM NEt3-HCl, pH 2.5) and fractionated in ~1-ng aliquots using an Aquapore Phenyl column (0.46 × 22.2 cm, ABI). Bound protein was eluted (1 ml/min) with a biphasic gradient of increasing Buffer B (20 mM NEt3-HCl, 80% (v/v) CH3CN, pH 2.5) of 0–24% Buffer B in 25 min followed by 30–37% Buffer B in 80 min. Analysis by SDS-PAGE confirmed quantitative separation of the two fragments which were identified as 1-thrombin and 2-thrombin based on N-terminal sequence analysis and mass spectrometry. Pools were prepared for each of the resolved peaks from each of the fractions. Lyophilized, dialyzed against assay buffer, and clarified by centrifugation (50,000 × g, 20 min). The purity of all protein preparations was judged by SDS-PAGE (33). Protein concentrations were determined using the following molecular weights and extinction coefficients (37, 86): bovine factor Xa, 45,300, 1.24 (34, 35); bovine factor Va, 168,000, 1.74 (27, 36); bovine prethrombin 2, 57,400, 1.95 (31); bovine a-thrombin, 37,400, 1.95 (18); trypsin, 23,800, 1.58 (37); chymotrypsin, 24,200, 1.88 (37). The concentrations of the thrombin and prethrombin 2 derivatives were determined using molecular weights determined from the primary structure and extinction coefficients calculated by the method of Gill and von Hippel (38): γ1-thrombin, 34,100, 1.89; γ2-thrombin, 37,400, 1.85; 1-thrombin or 1-prethrombin 2, 23,000, 1.83; 2-thrombin (or 2-prethrombin 2, 12,450, 1.90.

Preparation of Prethrombin 2 Derivatives

The two chymotryptic fragments of prethrombin 2 were prepared by treating prethrombin 2 (27 μl, 5 mg) in 0.25 mM sodium phosphate, pH 6.5, with 1 nM chymotrypsin for 3 h at room temperature. The reaction mixture (300 μl) was applied to a column (1.5 × 120 cm) of S-Sepharose equilibrated in 50 mM sodium phosphate, pH 2.5, with isotropic elution. The resulting ATA-EGR-CH2Cl was identified, concentrated, and characterized as described (39). Factor Xa in 0.5 mM Tris, 0.1% (v/v) PEG, pH 7.5, was inactivated by sequential additions of ATA-EGR-CH2Cl and the resulting modified factor Xa (ATA-EGR-Xa) towards, 37,400, 1.95 (31); bovine...
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EGR-Xa. When necessary, the reaction at each concentration of titrant was calculated by subtracting the anisotropy observed in the absence of factor Va. Displacement experiments were performed by preforming prothrombinase in assay buffer using 28.8 nm [OG488]-EGR-Xa, 50 μM PCPS and either 20 or 60 nM factor Va. Anisotropy was measured following titration with increasing concentrations of factor Va. The initial steady state velocity of thrombin formation or of SpXa hydrolysis was measured following initiation with 5 nM (●) or 0.25 nM (○) factor Xa. For illustrative purposes, each case, the velocities were normalized to the rate observed in the absence of α-IIa. The lines are drawn following analysis by linear regression (Eq. 1) or according to the rate expression for linear competitive inhibition (Eq. 2) with $K_i = 2.02 \pm 0.11 \mu M$ and $K_m = 2.45 \pm 0.07 \mu M$.

RESULTS

Kinetics of Inhibition of Prothrombinase by α-Thrombin—Previous studies have shown that α-thrombin acts as an exosite-directed product inhibitor of macromolecular substrate cleavage by prothrombinase (17). Thrombin, activated with APMSF (α-IIa) was tested for its ability to inhibit the cleavage of either a tripeptidyl substrate (SpXa) or the macromolecular substrate analog, prethrombin 2, by prothrombinase (Fig. 1). Even though either substrate was present at approximately the same multiple of $K_m$ (0.7 $\times$ $K_m$), increasing concentrations of α-IIa yielded significant inhibition of prethrombin 2 activation ($K_i = 2.02 \pm 0.11 \mu M$) with a minor effect ($K_i \approx 80 \mu M$) on the initial rate of SpXa hydrolysis (Fig. 1). Initial velocity studies using increasing concentrations of prethrombin 2 at different fixed concentrations of α-IIa (not shown) yielded linear competitive inhibition with $K_i = 2.15 \pm 0.3 \mu M$ (Table 1). Thus, α-IIa and prethrombin 2 bind in a mutually exclusive fashion to prothrombinase. Because α-IIa has a minor effect on pep tidyl substrate hydrolysis by prothrombinase, competitive inhibition of prethrombin 2 cleavage is achieved without restricting access to the active site of factor Xa within the prothrombinase complex. Such observations, in part, form the basis for the previous suggestion that the affinity of the enzyme complex for macromolecular substrates such as prothrombin 2 is determined by binding interactions at exosites and not the active site of factor Xa within prothrombinase (17).

Influence of Thrombin on the Assembly of the Prothrombinase Complex—Factor Xa assembled into the ternary prothrombinase complex with saturating concentrations of factor Va and phospholipid membranes catalyzes prethrombin 2 cleavage with greatly increased catalytic efficiency compared with factor Xa in solution or saturated with membranes (16). Since the three enzyme species only exhibit minor differences in the kinetics of hydrolysis of SpXa and other tripeptidyl substrates (11), inhibition of the assembly of prothrombinase by α-IIa could provide a trivial explanation for the selective inhibition of macromolecular substrate cleavage.

Equilibrium and kinetic measurements of the assembly of prothrombinase have previously relied on the use of a dansyl reporter group covalently tethered to the active site of factor Xa.
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Table I

| Inhibitor<sup>a</sup> | $K_i$ ± S.E.<sup>b</sup> | $K_m$ ± S.E. | $V_{max}/E$ ± S.E.<sup>c</sup> |
|----------------------|----------------------------|---------------|-------------------------------|
| None                | 3.9 ± 0.7                  | 0.84 ± 0.2    |                              |
| $\alpha$-IIa<sup>2</sup> | 2.15 ± 0.3                | 4.0 ± 0.5     | 0.95 ± 0.05                   |
| $\gamma$-IIa<sup>2</sup> | 7.0 ± 1.0                  | 3.2 ± 0.5     | 0.87 ± 0.04                   |
| $\zeta$-IIa        | 17.0 ± 2.6                 | 3.9 ± 0.9     | 0.98 ± 0.04                   |
| $\alpha$-IIa + LMW heparin | 1.4 ± 0.1                | 3.4 ± 0.3     | 1.27 ± 0.04                   |
| $\alpha$-IIa       | 2.6 ± 0.3                  | 7.6 ± 1.2     | 7.5 ± 0.46                    |

<sup>a</sup> The inhibitors were catalytically inactivated derivatives of thrombin identified in Fig. 3.
<sup>b</sup> Initial steady state velocities were determined using increasing concentrations of prothrombin 2 (8–14 values) at different fixed concentrations of the indicated inhibitor (3–5 values). Kinetic constants were determined by analysis according to the rate expression for linear concentration of prethrombin 2 (8–14 values) at different fixed concentrations of prothrombinase.
<sup>c</sup> $V_{max}$ divided by the total concentration of prothrombinase. The resulting value is not equivalent to the first order rate constant for catalysis (17).

Fluorescence titrations using a single fixed concentration of $[OG_{as8}]-EGR-Xa$, saturating PCPS, and increasing concentrations of factor Va were compared in the presence or absence of 12 µM $\alpha$-IIa, (Fig. 2B). The data were normalized using the anisotropy in the absence of factor Va because the presence of $\alpha$-IIa led to a small but reproducible increase (0.004) in the anisotropy of $[OG_{as8}]-EGR-Xa$ either in the presence or absence of PCPS (not shown). The two titration curves were indistinguishable from each other and could be adequately described by equilibrium parameters comparable to those established above. Thus, $\alpha$-IIa has no detectable effect on the assembly of prothrombinase at concentrations which yield substantial inhibition of prothrombin 2 cleavage. These findings exclude a potentially trivial explanation for the ability of $\alpha$-IIa to selectively inhibit macromolecular substrate cleavage by prothrombinase.

**Dissection of Interaction Sites of Thrombin and Prothrombin 2 with Prothrombinase**—A schematic representation of prothrombin 2, $\alpha$-thrombin, and known proteolytic derivatives is provided in Fig. 3A. The corresponding products isolated on a preparative scale were analyzed by SDS-PAGE (Fig. 3B). Activation of the single chain zymogen, prothrombin 2, converts it to the two chain $\alpha$-thrombin, which retains the P1-P3 residues (Fig. 3A). Functional and structural studies have established the presence of sites on $\alpha$-thrombin, removed from the P1-P3 residues, that play a role in the diverse macromolecular interactions of the protease (50). The fibrinogen recognition site and the heparin-binding site in $\alpha$-thrombin (Fig. 3A), are at least partially expressed in prothrombin 2 as well (51).

Limited proteolysis of $\alpha$-thrombin by trypsin in the fibrinogen recognition site releases a peptide comprising residues Ile<sup>68</sup>-Arg<sup>77A</sup> (3–5). The resulting $\gamma$-thrombin (Fig. 3A) is unable to cleave fibrinogen, shows dramatically reduced affinity for hirudin but retains full catalytic activity toward oligopeptidyl substrates (3, 5). $\gamma$-Thrombin, with these expected properties was isolated (Fig. 3B) and inactivated with APMSF to yield $\gamma$-IIa. Product inhibition studies of prothrombin 2 activation by prothrombinase using $\gamma$-IIa, yielded linear competitive inhibition (Table I). In contrast to the large reduction in fibrinogen cleavage following proteolytic removal of the fibrinogen recognition site, the $K_i$ for the inhibition of prothrombinase by $\gamma$-IIa, was only modestly increased in comparison to $\alpha$-IIa (Table I). However, some variability was noted in the inhibitory properties of different preparations of $\gamma$-IIa, illustrated by the representative $K_i$ values in Table I. These effects are likely related to difficulties associated with the reproducibility of partial protease digests, stability of the proteolysed product, and variable trace contamination (<5%) with undigested material. These points lead to the qualified conclusion that tryptic removal of the fibrinogen recognition site may have a small effect on but does not eliminate the ability of thrombin to act as an exosite-directed product inhibitor of macromolecular substrate cleavage by prothrombinase.

Cleavage of thrombin in the autolysis loop at Trp<sup>148</sup> has also been shown to modulate the interaction of thrombin with macromolecules such as fibrinogen and antithrombin III (5). Thrombin was digested with chymotrypsin under conditions comparable to those previously established (52) to yield $\zeta$-thrombin (Fig. 3A). Purification of the derivative yielded two fragments evident by SDS-PAGE of the expected size (Fig. 3B).
and cleavage at Trp\textsuperscript{148} was confirmed by N-terminal sequencing and mass spectrometry. The resulting \( \xi \)-thrombin, inactivated with APMSF to yield \( \xi \)-IIa, retained the ability to act as a competitive inhibitor of prethrombin 2 hydrolysis with a \( K_d \) that was slightly lower than that of \( \alpha \)-IIa (Table I). Therefore, the binding of macromolecular product to the exosite on prothrombinase is unaffected by prior cleavage in the autolysis loop.

Potential contributions from the heparin-binding site were evaluated by examining the effects of saturating concentrations of LMW heparin on the ability of \( \alpha \)-IIa to selectively inhibit prethrombin 2 activation by prothrombinase. Since both thrombin and prethrombin 2 are known to bind heparin with high affinity, LMW heparin concentrations were chosen to be high relative to the published \( K_d \) for binding thrombin as well as to provide sufficient binding sites for both prethrombin 2 and thrombin at the highest concentrations used (53). In preliminary experiments, LMW heparin was found to substantially enhance prethrombin 2 activation by increasing both the \( K_m \) and \( V_{\text{max}} \) (Table I). While the basis for these effects was not extensively investigated, they appear related to the ability of prethrombin 2 to bind heparin since LMW heparin had no effect on the kinetics of SpXa cleavage by prothrombinase (not shown). However, the presence of saturating concentrations of LMW heparin had no detectable effect on the kinetics of inhibition by \( \alpha \)-IIa (Table I). Comparable experiments with unfractinated heparin yielded equivalent results except that initial velocity measurements at high substrate concentrations were compromised by protein precipitation possibly due to interactions between thrombin and long chain heparin at multiple sites (53). Since occupation of the heparin-binding site(s) in \( \alpha \)-IIa does not affect its ability to act as a product inhibitor, it is unlikely that this macromolecular interaction site in the product is either directly or indirectly involved in mediating recognition by the exosite in prothrombinase.

**Kinetics of Exosite-dependent Product Inhibition in the Presence of Hirugen—**Hirugen binds to the fibrinogen recognition site and thereby competitively inhibits interactions between macromolecules and \( \alpha \)-thrombin at this site (22). Kinetic studies with hirugen were used to further test the conclusions derived from inhibition studies with \( \gamma \)-IIa and \( \xi \)-IIa.

In initial experiments, hirugen was found to directly inhibit

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**FIG. 2. Fluorescence measurements of prothrombinase assembly.** Panel A, prothrombinase assembly in the absence of \( \alpha \)-IIa. Reaction mixtures containing 14.4 nM (○) [OG\textsubscript{end}]-EGR-Xa and 30 µM PCPS in assay buffer were titrated with increasing concentrations of factor Va at 25°C. Fluorescence anisotropy was measured as described under "Experimental Procedures." The lines were drawn according to Equation 2 with the fitted constants: \( K_a = 0.89 \pm 0.08 \text{ nM} \), \( n = 0.94 \pm 0.01 \text{ mol of Va/mol [OG\textsubscript{end}]-EGR-Xa} \) at saturation, \( \Delta r = 0.176 \pm 0.001 \) and \( \Delta r = 0.060 \pm 0.001 \). Residuals to the fitted lines are illustrated in the upper panel. Titration in assay buffer containing 2 mM EDTA instead of Ca\textsuperscript{2+} (▲). Inset, reaction mixtures containing 28.8 nM [OG\textsubscript{end}]-EGR-Xa, 30 µM PCPS, and 20 (○) or 60 (▼) µM factor Va in assay buffer were titrated with increasing concentrations of Xa and 30 nM [OG\textsubscript{end}]-EGR-Xa at saturation, \( K_{a\text{comp}} = 1.74 \pm 0.52 \text{ nM} \), and \( \Delta r = 0.060 \pm 0.001 \) assuming both n and \( \alpha \)-IIa (Table I). Therefore, the binding of macromolecular product to the exosite on prothrombinase is unaffected by prior cleavage in the autolysis loop.

Panel B, prothrombinase assembly in the presence of \( \alpha \)-IIa. Reaction mixtures containing 25 nM [OG\textsubscript{end}]-EGR-Xa, 60 µM PCPS, and 0 (●) or 12 µM \( \alpha \)-IIa (○) were titrated with increasing concentrations of factor Va at 25°C. Delta r was calculated as described and the line was drawn according to Equation 2, using the fitted constants: \( K_a = 0.82 \pm 0.2 \text{ nM} \) and \( \Delta r = 0.057 \pm 0.001 \), assuming \( n = 1 \) mol of [OG\textsubscript{end}]-EGR-Xa/mol of Va at saturation.
prethrombin 2 cleavage by prothrombinase. Increasing concentrations of hirugen were found to increase the $K_v$ for prethrombin 2 activation without affecting the $V_{\text{max}}$ (Fig. 4). Prethrombin 2 has been documented to bind hirugen (54). Thus, inhibition could arise from an interaction between hirugen and prethrombin 2 which precludes substrate binding to prothrombinase. The data could be adequately described by the rate expression for this type of inhibition (Equation 1, Fig. 4). However, the data could also be described equally well by rate expressions for alternate mechanisms, including partial inhibition resulting from a 5–7-fold decrease in substrate affinity following hirugen binding to prethrombin 2 or even by competitive inhibition resulting from an interaction between hirugen and prothrombinase.

Initial velocity studies illustrating the inhibition of prethrombin 2 activation by 30 $\mu M$ $\alpha$-IIa, in the absence or presence of 241 $\mu M$ hirugen are provided in Fig. 5. The presence of $\alpha$-IIa, at $-10 \times K_v$, yielded the expected and substantial inhibition of prethrombin 2 activation in the absence of hirugen. Inhibition by $\alpha$-IIa was alleviated in the presence of saturating concentrations of hirugen. These data suggest that the binding of hirugen to $\alpha$-IIa, attenuates or eliminates the ability of the product to bind to prothrombinase and inhibit prethrombin 2 activation. However, the direct inhibitory effect of hirugen on prethrombin 2 activation by prothrombinase suggests a complex mechanism of action and precludes definitive interpretation of these observations.

Localization of a Domain That Imparts Binding Specificity for Prethrombin 2—Cleavage of thrombin by chymotrypsin at Trp$^{148}$ (4-thrombin, Fig. 3A) yields two peptides that remain noncovalently associated (32, 52). The cleavage site separates thrombin into approximate hemispheres, each bearing elements of the catalytic triad, that can be reassociated following dissociation and separation to reconstitute enzymatic activity (52). The N-terminal domain (1-thrombin) bears the P1-P3 fibrinogen recognition site as well as some residues involved in heparin binding. The C-terminal domain (2-thrombin) bears elements of the heparin-binding site (Fig. 3A). By analogy, cleavage at the same site in
prethrombin 2 yields similar species denoted as ζ1- and ζ2-prethrombin 2 (Fig. 3A). While the ζ2 fragments from prethrombin 2 and thrombin are chemically identical, ζ1-prethrombin 2 retains the intact scissile bond acted upon by prothrombinase.

The resulting fragments from ζ-thrombin and ζ-prethrombin 2 were dissociated, preparatively purified by HPLC (Fig. 3B), and identified by mass spectrometry and N-terminal sequencing. As described previously (52), thrombin activity could be near-quantitatively recovered by mixing stoichiometric concentrations of the ζ1-thrombin and ζ1-prethrombin 2 on prethrombin 2 activation by prothrombinase.

Purified ζ1 and ζ2 fragments prepared from thrombin and prethrombin 2 were separately tested for their ability to inhibit SpXa cleavage and prethrombin 2 activation by prothrombinase (Fig. 6). Results from several experiments using different fragment preparations indicated that only the ζ2 fragment, from either thrombin or prethrombin 2, retained the ability to inhibit prethrombin 2 activation by prothrombinase. The $K_m$ for the inhibition of prethrombin 2 activation by the ζ2 fragment was equivalent to that observed with undissociated ζ-IIa (Fig. 6, Table I). In parallel experiments, the ζ2 fragment had a relatively small effect on the rate of hydrolysis of SpXa by prothrombinase (Fig. 6). In contrast, the ζ1 fragment derived from either prethrombin 2 or thrombin had no detectable effect ($K_m > 23 \mu M$) on prethrombin 2 activation by prothrombinase (Fig. 6, inset) or on the rate of SpXa cleavage by the enzyme complex (not shown).

The data indicate that the inhibitory properties of α-IIa, or ζ-IIa, can be completely repeated with ζ2-thrombin or with ζ2-prethrombin 2. Since the fibrinogen recognition site is not present in the ζ2 fragment (Fig. 3A) and heparin has no effect on the ability of α-IIa, to inhibit prethrombin 2 activation (Table I), the data suggest that binding to the exosite in prothrombinase is mediated by substrate/product regions that are distinct from the fibrinogen recognition site or the heparin-binding site. The lack of significant inhibition by ζ1-thrombin and ζ1-prethrombin 2 despite the presence of an intact cleavage site in the latter fragment supports the conclusion that the domain of the substrate and product that mediates recognition at the exosite of prothrombinase and determines binding specificity is spatially removed from sites immediately surrounding the scissile bond in the substrate.

DISCUSSION

Productive recognition of prethrombin 2 by prothrombinase proceeds through interactions at exosites on the enzyme complex followed by binding of substrate structures surrounding the scissile bond to the active site of factor Xa prior to cleavage and product release as illustrated in Scheme I (17). Substrate affinity is determined by the bimolecular interaction between the substrate and enzymic exosite(s) rather than by binding interactions at the active site which are unfavorable and instead contribute to maximum catalytic rate (17). One hallmark for this type of mechanism is that thrombin acts as a linear competitive inhibitor of macromolecular substrate cleavage without obscuring access of small molecule inhibitors and oligopeptidyl substrates to the active site of factor Xa within prothrombinase (17). The present results bear out predictions that necessarily follow from these conclusions and indicate that binding to the exosite(s) in prothrombinase is mediated by a domain of prethrombin 2 and thrombin distinct from the residues immediately surrounding the cleavage site. As illustrated in Scheme I, binding specificity is conferred by structures present in the C-terminal region of prethrombin 2 or thrombin, physically separable from the domain containing the P1-P3 sites or the intact scissile bond and distinct from the fibrinogen recognition or heparin-binding sites.

The results obtained with γ1-IIa, ζ-IIa, and the purified ζ2 fragment support the conclusion that the fibrinogen recognition site is not directly involved in the interaction of prethrombin 2 or thrombin with the prothrombinase exosite. The strong-
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est evidence to suggest this derives from the ability of the C2 fragment, which lacks the fibrinogen recognition site, to reproduce the inhibitory properties of α-IIa. While γ-IIa, does display reduced affinity for prothrombinase, the changes in $K_a$ are modest when compared with the large reduction in fibrinogen cleavage or hirudin binding which accompanies proteolysis in the fibrinogen recognition site (3–5). However, a role for the fibrinogen recognition site in exosite binding is suggested by the ability of hirugen to directly inhibit prothrombin 2 cleavage by increasing the $K_a$ and by saturating concentrations of hirugen to reduce the ability of α-IIa, to act as a product inhibitor. Given the observations with the thrombin derivatives, it seems unlikely that the fibrinogen recognition site is directly involved in the binding of the macromolecular substrate/product to the exosite(s) in prothrombinase. Instead, the data are more consistent with the possibility that hirugen binding to thrombin or prothrombin 2 elicits changes at sites distinct from the fibrinogen recognition site which in turn modulate binding to prothrombinase. Extensive allosteric linkage has been documented between the fibrinogen recognition site and the binding of ligands such as heparin, antithrombin III, fragment 2, and Na+ to other sites in thrombin (55–57). Recent binding measurements imply that hirugen and a fragment 2 peptide behave as competitive ligands even though they are known to bind to distinct sites on thrombin (56). It is also possible that the effects observed at high concentrations of hirugen in the present study are related to secondary interactions of the highly charged peptide. We are unable to distinguish between these possibilities.

Structure-function studies of substrate specificity, of the type described, are susceptible to interpretation problems arising from unanticipated effects of protein fragments on the stability of the prothrombinase complex or from inhibition derived from the ability of protein fragments containing Arg-X residues to act as alternate substrates. In the present study, direct equilibrium binding measurements of prothrombinase assembly, at reactant concentrations approaching those used in the kinetic measurements, were used to exclude inhibitory effects arising from the destabilization of the enzyme complex. In addition, all inhibitory proteolytic derivatives acted as selective inhibitors of prothrombin 2 activation with a minor effect on synthetic peptidyl substrate hydrolysis by prothrombinase. This fact rules out significant alternate substrate effects as a trivial explanation for the present findings.

The ability of thrombin to act as a linear competitive inhibitor of prothrombin 2 cleavage catalyzed by prothrombinase implies that prothrombin 2 and thrombin bind to the enzyme complex in a mutually exclusive fashion. The similarities in the known structures of prothrombin 2 and thrombin (51) and the substrate-product relationship between the two suggest that they are likely to compete for interactions at the same site in the enzyme complex as illustrated in Scheme I. While, alternate, more complicated explanations cannot be adequately excluded at present, this interpretation is supported by the fact that the C2 fragment from either species exhibits equivalent inhibitory properties toward prothrombin 2 activation.

The fragment 1 domain mediates the binding of prothrombin to membranes (58) and the fragment 2 region has been shown to be responsible for the interaction between the substrate and factor Va (59). Since prothrombin binding to factor Va and membranes does not involve the active site of factor Xa within prothrombinase, they represent potential exosite interactions. However, these domains are absent in purified prothrombin 2 and thrombin. Thus, it is possible that the exosite interactions inferred in this and previous work (17) involves specific recognition of the macromolecular substrate by sites on factor Xa itself that are removed from the active site. This possibility is supported by prior work documenting the inhibition of macro-molecular but not oligopeptidyl substrate cleavage by prothrombinase with a monoclonal antibody specific for factor Xa (60). However, it remains possible that previously unidentified interactions between prethrombin 2 or thrombin and other sites in the prothrombinase complex also contribute to substrate/product binding.

Explanations for the narrow and distinctive substrate specificity of factor Xa or prothrombinase have, thus far, been sought from the active site geometry based on the x-ray structure of factor Xa, by mutagenesis studies of residues surrounding the active site and by studies with synthetic peptidyl substrates (9, 61, 62). The results of this and previous studies (17) suggest that while such approaches may describe the properties of factor Xa in solution, they are inadequate for assessing the basis for the substrate specificity of the prothrombinase complex. The affinity of prothrombinase for the macromolecular substrate is not determined by binding of the substrate to the active site of factor Xa but rather by interactions between extended macromolecular recognition sites, distinct from the active site, and substrate regions spatially distinct from structures immediately surrounding the scissile bond.

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