Involvement of Thrombin Anion-binding Exosites 1 and 2 in the Activation of Factor V and Factor VIII*

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Thrombin is a multifunctional trypsin-like serine protease that recognizes macromolecular substrates and cofactors through anion-binding regions, called exosites, that are remote from its catalytic site. Exosite 1 binds fibrinogen, the fifth and sixth epidermal growth factor domains of thrombomodulin, the COOH-terminal domain of hirudin, and the hirudin-like region of the thrombin receptor, whereas exosite 2 binds heparin, the chondroitin sulfate moiety of thrombomodulin, fragment 2 of prothrombin, and possibly fibrin (1–3).

The role of anion-binding exosites of thrombin in the activation of factor V and factor VIII was studied using thrombin Arg93 → Ala, Arg97 → Ala, and Arg101 → Ala (thrombin RA), a recombinant exosite 2 defective mutant, and a synthetic N-acetylated dodecapeptide, Ac-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-O-SO4(−)-Leu (hirugen), which competitively inhibits binding of macromolecules to exosite 1. The catalytic efficiency of the activation of factor VIII of or of the first step of factor V activation by thrombin RA was approximately 10% that of wild-type thrombin. The overall rate of conversion to factor Va was not influenced by the mutation. In contrast to factor V, the slow activation of factor VIII by thrombin RA was associated with a decreased rate of cleavage at all three proteolytic sites (Arg372, Arg740, and Arg1689). Hirugen inhibited factor V and factor VIII activation. These results indicate that both anion-binding exosites of thrombin are involved in the recognition of factor V and factor VIII.

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† The abbreviations used are: thrombin RA, thrombin Arg93 → Ala, Arg97 → Ala, and Arg101 → Ala; factor VIIIa, activated factor VIII (in this study referring solely to thrombin-activated factor VIII); factor Vα, activated factor V (in this study referring solely to thrombin-activated factor V); vWF, von Willebrand factor; PAGE, polyacrylamide gel electrophoresis.
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Extinction Coefficients and Molecular Masses—The following extinction coefficients (mM⁻¹ cm⁻¹ at 280 nm and molecular masses were used: human factor VIII 1.2, 210 kDa (24); human thrombin, 1.74, 37 kDa (25); human factor IXαI 1.33, 46 kDa (26); porcine factor X 1.04, 57 kDa (21); bovine factor V 0.96, 330 kDa (27); and human vWF 0.75 (23), 270 kDa subunit (28).

Achromogenic substrate assay for activation of factor VIII by thrombin enables thrombin was employed in which limiting amounts of factor VIII, human factor IXαI and unilamellar phosphatidylcholine/phosphatidylserine vesicles were used to activate FX as follows. Factor VIII (100 nM) plus or minus vWF (250 nM subunits) was activated with thrombin (0.2 nM) or thrombin RA (0.2 or 2 nM as indicated in the figure legends) in 0.14 M NaCl, 5 mM Hepes, 0.2 mM CaCl₂, 0.01% Tween 80, pH 7.4. At various times, the activation mixture was diluted 125-fold into the same buffer containing 2 nM human factor IXαI and 20 μM unilamellar phosphatidylcholine/phosphatidylserine vesicles, followed immediately by the addition of FX to 300 nM. The initial velocity of FX activation, which is proportional to the concentration of activated factor VIII in the sample, was measured as described previously and expressed in units of nM/min (22).

Factor V Activation by Thrombin—Bovine factor V (0.5 mg/ml, 1.6 μM) in 0.1 M NaCl, 0.02 M Tris-HCl, 2 mM CaCl₂ was incubated with thrombin or thrombin RA (1 nM) for the times indicated. When lower thrombin concentrations were employed for kinetic analysis, factor V concentration and the buffer remained constant. Factor Va formation was monitored by the one-stage clotting assay using factor V-deficient plasma as described (8).

Fibrinogen Clotting Assay—Lyophilized human fibrinogen was dissolved in water, resulting in a buffer that was 70 mM NaCl, 40 mM sodium citrate, and 10 mg/ml fibrinogen. Clotting was performed by adding 0.1 ml of fibrinogen to a solution of thrombin diluted to 0.3 ml of 0.1 M NaCl, 0.02 M Tris-HCl, 1 mg/ml gelatin, pH 7.5 at 37°C. Clotting was detected visually. NIH standard thrombin was used as the reference, and a standard curve was prepared by plotting clotting time on the ordinate and the reciprocal of the thrombin concentration on the abscissa. Standard curves were also prepared for thrombin RA and wild-type thrombin, and the slopes were identical.

Electrophoresis—SDS slab gel polyacrylamide gel electrophoresis was used to identify factor V and factor VIII cleavage products was done by using the Laemmli buffer system (29) followed by staining with Coomassie Brilliant Blue R-250. The concentration of polyacrylamide used was 6% for factor V gels and 7% for factor VIII gels. Prior to electrophoresis of factor VIII, samples (0.22 ml, 5 μg of factor VIII) from the activation mixtures were concentrated by precipitation with 5 volumes of ice-cold acetone followed by centrifugation at 13,000 × g for 2 min, and solution of the pellet in SDS-PAGE sample buffer (30). The kinetics of cleavage of the factor VIII light chain was quantified by using an LKB Ultrascan XL laser densitometer. Peaks were integrated by fitting to an exponentially modified Gaussian distribution by using a commercially available program (PeakFit, Jandel Scientific).

RESULTS

Activation of Factor VIII by Thrombin and Thrombin RA—

The activation of factor VIII by recombinant, wild-type thrombin, or thrombin RA in the absence and the presence of vWF were compared using a plasma-free assay based on the ability of limiting amounts of activated factor VIII to support the activation of FX by factor IXαI. The vWF concentration used (250 nM subunits) was sufficient to completely bind factor VIII. Initial experiments revealed that recombinant and plasma-derived human thrombin-activated factor VIII similarly (not shown). Fig. 1 shows that an equivalent concentration of thrombin RA activates factor VIII considerably slower than wild-type thrombin. vWF did not have a significant effect on the activation of factor VIII by wild-type thrombin or thrombin RA. The activation profiles of wild-type thrombin and thrombin RA are similar when a 10-fold higher concentration of thrombin RA than wild-type thrombin is used, indicating that the catalytic efficiency of thrombin RA is approximately 10% that of wild-type thrombin.

The rates of production of proteolytic cleavages of factor VIII by 0.2 nM wild-type and 0.2 nM thrombin RA were compared by SDS-PAGE analysis of samples from the activation mixtures (Fig. 2). Cleavage by thrombin RA at all three proteolytic sites was impaired in either the absence or the presence of vWF. At 2 nM, thrombin RA produced a cleavage pattern similar to 0.2 nM wild-type thrombin, consistent with the functional activity shown in Fig. 1 (not shown). In the absence of vWF, cleavage at Arg1689 is necessary for measurement of full activity (11, 31), whereas significant activity can be generated in the absence of cleavage at Arg1220 (32, 33). Cleavage at Arg1689 is not necessary for activation (31). In the presence of vWF, cleavage at Arg1689 is necessary for factor VIII activation because this cleavage releases factor VIII from vWF (23, 32), which in turn is necessary for factor VIIIa binding to phospholipid (34, 35). Thus, in the absence of vWF, slow cleavage at Arg1689 accounts for the slow activation of factor VIII by thrombin RA, whereas in the presence of vWF, slow cleavages at Arg1689 and Arg1220 can contribute to the slow activation of factor VIII by thrombin RA.

Cleavages of 1 nM factor VIII catalyzed by 3.7 nM thrombin RA for 6 min were not affected by up to 10 units/ml unfractionated heparin (not shown), as expected because thrombin RA does not bind heparin (16). This concentration of heparin inhibits the rate of all cleavages of factor VIII by normal thrombin by greater than 95% (15). This indicates that the inhibition by heparin of factor VIII activation requires binding to thrombin. Combined with the slow activation of factor VIII by thrombin RA, these studies suggest that thrombin binding exosite 2 is directly involved in binding factor VIII. With heparin or the RA mutations, it is likely that the reduction in factor VIII activation is due to loss of specific side chain interactions with Arg93, Arg97, and Arg101 that are disrupted by these mutations or by charge neutralization of Arg93, Arg97, and/or Arg101 with heparin. Heparin inhibits factor VIII activation by thrombin somewhat more effectively than the RA mutation. Because heparin interacts with other basic residues in exosite 2 (36–38), it is possible that these residues may also contribute to factor VIII interaction or that heparin also interferes with steric hindrance.

In the porcine system, vWF is a cofactor for cleavage of factor VIII at Arg1689 (39). This cleavage dissociates factor VIII from vWF, which is necessary for membrane-dependent intrinsic pathway factor X activation. Fig. 2 (A and C) in the present study suggest that human vWF accelerates cleavage of Arg1689 in human factor VIII by wild-type thrombin. The vWF cofactor effect is also observed using thrombin RA (cf. Fig. 2, B and D).
In contrast, Pittman et al. (40) did not observe an effect of human vWF on cleavage of recombinant human factor VIII by human thrombin at Arg1689. To resolve this discrepancy, we examined the kinetics of light chain cleavage of human factor VIII by SDS-PAGE under conditions identical to those reported by Pittman et al., which employed 0.5 μg/ml human thrombin (13 nM), 10 μg/ml human factor VIII (∼40 nM) in the presence or the absence of 30 μg/ml human vWF (110 nM) in 0.15 M NaCl, 50 mM Tris-Cl, 2.5 mM CaCl₂, 5% glycerol, pH 7.5. Fig. 3 shows that the reaction was 60–70% completed by the time the first sample was taken, which was slightly faster than reported by Pittman et al. There is no apparent acceleration due to vWF. When the reaction was slowed by decreasing the thrombin concentration 65-fold to 0.2 nM, vWF produces a 2-fold increase in product formation at the earliest time point (Fig. 3). Increasing the vWF concentration 5-fold to 550 nM did not further increase the rate of light chain cleavage, indicating that vWF was saturating at 100 nM (not shown). When 0.2 nM porcine thrombin, 40 nM porcine factor VIII, and 110 nM porcine vWF were substituted for the human proteins under these conditions, porcine vWF produced a 3.5-fold increase in product formation at 2 min (not shown).

This increase is indistinguishable from previously reported experiments in the porcine system at this concentration of factor VIII (39). In that study, the increase in catalytic efficiency (kcat/Km) of light chain cleavage produced by vWF was calculated to be 7-fold and the Km in the presence of vWF was calculated to be 27 nM. At 40 nM, the concentration of factor VIII is above the Km for thrombin in the presence of vWF and approximately forty times greater than the physiological concentration of factor VIII. Thus, results of this study demonstrate a significant cofactor effect of human vWF on the cleavage of the human factor VIII light chain. Based on the present studies, the controversy over whether vWF can accelerate factor VIII activation appears to be largely explained by differences in the factor VIII concentrations employed and the time points selected in the two studies.
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**DISCUSSION**

In this study, we have examined the role of anion binding exosites 1 and 2 in thrombin in the recognition of factor V and factor VIII. Our results with the exosite 2 mutant, thrombin RA, and the exosite 1 ligand, hirugen, indicate that the activation of factor VIII by thrombin involves both anion binding exosites 1 and 2. Hirugen also inhibits factor V activation. This role is anticipated, because thrombomodulin, which binds exosite 1, blocks factor V activation (43). The role of exosite 2 in factor V recognition appears more complex. Exosite 2 contributes to the rapid cleavage at Arg^{713} that releases the heavy chain of factor V but does not influence the rate of the slower cleavage at Arg^{1536} that releases the light chain. The surprising feature, however, was that heparin failed to inhibit either

**Involvement of Anion Binding Exosites 1 and 2 in Factor V Activation—**To determine whether factor V and factor VIII utilize anion binding exosite 2 to the same extent, the kinetics of factor V activation by thrombin and thrombin RA was studied in the presence and the absence of hirugen in furosemide, and the presence and the absence of hepatic. The time courses of factor V processing by thrombin and the RA mutant were somewhat different (Fig. 6). The first, very rapid cleavage at Arg^{713} to release the factor Va heavy chain was slower with the RA mutant than with wild-type thrombin. This is clearly seen in the time course because all of the factor V has been cleaved within 30 s by wild-type thrombin, but detectable factor V is present at 2 min with thrombin RA. In contrast, the slow cleavage at Arg^{1536} to generate the factor Va light chain and the large B domain followed approximately the same time course. Four gel time courses were done at thrombin concentrations ranging from 1.1 to 11 nM. Using the lower thrombin levels, it took thrombin RA 3–8-fold longer than wild-type thrombin to completely cleave the intact factor V. The overall patterns were independent of thrombin concentration, except that as observed previously (8), at the low thrombin concentrations the reactions tended to stop at the B-A3-C1-C2 intermediate (data not shown). A 7-fold increase in factor V activity was obtained with both enzymes. These data indicate that the first factor V cleavage may involve these Arg residues in exosite 2, but the second cleavage does not. We also determined whether heparin inhibits the rate of cleavage of any of the bonds in factor V significantly. Fig. 6 (C and D) indicate that despite the implication that exosite 2 is involved in factor V activation, thrombin RA studies, heparin does not affect the rate of cleavages at either Arg^{1536} or Arg^{1536}. The basis for this is unclear if exosite 2 is involved. It could be that factor V interacts with one of the three Arg residues that are mutated in thrombin RA and that this residue is not in contact with heparin or that occupancy of exosite 1 displaces the heparin from exosite 2. SDS-PAGE analysis was done in the presence and the absence of hirugen under the same conditions. At 5 μM, hirugen slowed the cleavages at both activation sites 4–8-fold (not shown).
cleavage in factor V. Possibly, exosite 2 plays less of a role in factor V activation than factor VIII activation.

Factor VIII contains sulfated tyrosines at Tyr346, Tyr718, Tyr719, Tyr723, Tyr1664, and Tyr1680 (44). Site-directed mutagenesis studies indicate that tyrosine sulfation is necessary for efficient cleavage of factor VIII by thrombin at Arg172 and Arg1689 (45). Factor V also contains sulfated tyrosines whose exact location has not been established (46). These tyrosines are necessary for efficient activation by thrombin (47). These sulfation sites are likely targets for recognition by thrombin's exosites, although it is not possible to predict which exosite interacts with these sites. Tyr-O-SO4 63 in hirudin binds within anion binding exosite 1 (48, 49). The fact that anion binding exosite 2 is involved in interactions with sulfated carbohydrate polymers like heparin supports the concept that this exosite might be able to interact with sulfated tyrosines.

Thrombin docking to factor V and factor VIII apparently is considerably different than its docking to other substrates and inhibitors. Exosites 1 and 2 are located on nearly opposite sides of thrombin. Most other substrates and inhibitors interact with the active site and anion binding exosite 1 or only with the active site. For most substrates, specific interactions with Arg residues 93, 97, and/or 101 in exosite 2 have little influence on catalysis as evidenced by the fact that fibrinogen clotting, protein C activation, and inhibition by antithrombin are essentially normal with thrombin RA (16). Fibrinogen (50), the thrombin receptor (51), and heparin cofactor II (52) interact with both anion binding exosite 1 and the active site. Protein C and antithrombin are two examples of proteins that interact primarily with the active site (52, 53). Exosite 2 has been considered primarily as a docking site for glycosaminoglycans. This conclusion is based on both mutagenesis studies (16, 54, 55) and on the fact that the fragment 2 domain in meizothrombin des fragment 1, which binds to exosite 2 on thrombin, blocks heparin acceleration of glycosaminoglycan binding (55). Fibrin may also bind to exosite 2 because fibrin decreases the ability of heparin to accelerate inhibition by antithrombin (56). Direct interaction of fibrin with exosite 2 remains to be demonstrated, however, because thrombin can interact simultaneously with heparin and fibrin to form a ternary complex (57). Furthermore, thrombin RA retains the ability to bind fibrin. Anion binding exosite 2 can, however, interact with proteins. The clearest example is prothrombin activation fragment 2, which binds reversibly to thrombin, an interaction that involves salt bridges between fragment 2 and Arg93, Arg97, and Arg101 of thrombin (58). When viewed in the standard orientation (1), these thrombin residues are located above and to the left of a unique insertion loop, the Trp60D loop, that forms the upper lid of the S2 pocket (2) and are located in a position to make them candidates for docking to residues to the P2 side of the cleavage sites.

The diverse nature of binding interactions of thrombin with its substrates and inhibitors is quite remarkable. This diversity is illustrated by the fact that the two chains of fibrinogen appear to follow different binding interactions as they exit the active center of the enzyme and enter anion binding exosite 1 (1). When fibrinogen docks to thrombin, the P residues extend just under anion binding exosite 2 through the molecule and the P residues extend into the other side of thrombin, anion binding exosite 1. It seems likely that factor VIII and possibly factor V share these sites of interaction but extend the specific interactions on the P side into the anion binding exosite 2 region. The very extensive enzyme-substrate complementarity may account for the fact that thrombin is an extremely effective activator of factors V and VIII.

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2 J. I. Weitz and C. T. Esmon, unpublished observations.

3 The nomenclature of Schecter and Berger (59) is used to designate amino acid residues with respect to the scissile bond.
