Exosite-interactive Regions in the A1 and A2 Domains of Factor VIII Facilitate Thrombin-catalyzed Cleavage of Heavy Chain*

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Keiji Nogami‡, Qian Zhou‡, Timothy Myles‡, Lawrence L. K. Leung‡, Hironao Wakabayashi‡, and Philip J. Fay‡

From the ‡Departments of Biochemistry and Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642 and the §Division of Hematology, Stanford University School of Medicine, Stanford, California 94305

Thrombin catalyzes the proteolytic activation of factor VIII, cleaving two sites in the heavy chain and one site in the light chain of the procofactor. Evaluation of thrombin binding the reaction products from heavy chain cleavage by steady state fluorescence energy transfer using a fluorophore-labeled, active site-modified thrombin as well as by solid phase binding assays using a thrombin Ser205→Ala mutant indicated a high affinity site in the A1 subunit (Kd ~ 5 nM) that was dependent upon the Na+/H11545-dependent conversion of factor X to Xa (1). Factor VIII is synthesized as a multidomain, single chain molecule (A1-A2-B-A3-C1-C2) consisting of 2332 amino acid residues with a molecular mass of ~300 kDa (2, 3). Factor VIII is processed to a series of divervalent metal ion-linked heterodimers by cleavage at the B-A3 junction, generating a variably sized heavy chain (90–210 kDa) consisting of the A1, A2, and heterogeneous fragments of partially proteolized B domains together with a light chain (80 kDa) consisting of the A3, C1, and C2 domains (2–4).

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Facilitate Thrombin-catalyzed Cleavage of Heavy Chain*

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Interactions of Thrombin with Factor VIII Heavy Chain

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The fast form for the anticoagulant substrate protein C (18, 23). The fast form also shows high efficiency in the activation of factor V and factor VIII (17, 24).

Limited information is available on thrombin-interactive sites in the factor VIII substrate. An interactive site for thrombin that facilitates cleavage at Arg<sup>1869</sup>{sup>1}</sup> has been located within the C2 domain in the light chain of factor VIII (25). However, analogous site(s) for tethering interactions with factor VIII heavy chain remain unknown. In this report, we examine the mechanisms of interaction of thrombin with the factor VIII heavy chain by using a combination of functional and physical approaches employing synthetic peptides and recombinant factor VIII and thrombin mutants. Our results indicate that the Na<sup>+</sup>-bound form of thrombin interacts with the A1 domain with significantly greater affinity compared with the A2, whereas the latter interaction appears to be largely independent of the occupancy of the Na<sup>+</sup>-binding site in thrombin. Interactions with the A1 and A2 subunits were shown to be mediated primarily by exosites I and II, respectively. Furthermore, the acidic region comprising residues 389–394 in the A2 domain interacts with thrombin to facilitate the cleavage of the heavy chain at Arg<sup>40</sup>{sup>1}</sup>. This cleavage is likely required to yield the functional cofactor, as remnant fragments remaining at the C terminus of A2 subunit appear to markedly reduce cofactor activity.

MATERIALS AND METHODS

Reagents—Purified recombinant factor VIII preparations were generous gifts from Bayer Corp. (Berkeley, CA). The monoclonal antibodies 58.12 (26) and C5 (27) recognizing the N and C termini of the A1 subunit, respectively, were gifts from Drs. Lisa Regan and Zaverio Ruggeri. The monoclonal antibodies R8B12 (28) recognizing the A2 domain and 10104 recognizing the N-terminal end of factor VIII light chain (a3 segment) were obtained from Green Mountain Antibodies (Burlington, VT) and QED Bioscience Inc. (San Diego, CA), respectively. The reagents human α-thrombin, factor Xa, factor X, and factor Xa were from Enzyme Research Laboratories, South Bend, IN; chromogenic factor Xa substrate S-2765 (N-a-benzoylcarbonyl-n-arginylgly-cyl-3-arginyl-p-nitroanilide dihydrochloride) was from DiaPharm Group, Westchester, OH; thrombin substrate S-2393 (H-N-phenylala-nyl-l-piperyl-l-arginine-p-nitroaniline dihydrochloride) was from Chromobio, Italy; N-hydroxysuccinimido-biotin was from Pierce; recombinant nonsulfated hirudin-(1–65) and horseradish peroxidaseylated streptavidin were from Calbiochem; unfractionated and recombinant factor VIII and thrombin mutants. Our results indicate that the Na<sup>+</sup>-bound form of thrombin interacts with the A1 domain with significantly greater affinity compared with the A2, whereas the latter interaction appears to be largely independent of the occupancy of the Na<sup>+</sup>-binding site in thrombin. Interactions with the A1 and A2 subunits were shown to be mediated primarily by exosites I and II, respectively. Furthermore, the acidic region comprising residues 389–394 in the A2 domain interacts with thrombin to facilitate the cleavage of the heavy chain at Arg<sup>40</sup>{sup>1}</sup>. This cleavage is likely required to yield the functional cofactor, as remnant fragments remaining at the C terminus of A2 subunit appear to markedly reduce cofactor activity.

1 The abbreviations used are: ATA-FPR-CH<sub>2</sub>Cl, N-acetyl-l-phenylalamine-2-dimethylaminonaphthalene-CH<sub>2</sub>Cl; aspartic acid, Asp; DAPA-CH<sub>2</sub>Cl, 4-[2-aminoethylamino]-2-[(aminoiminomethyl)amino]-2-nitroaniline dihydrochloride; FPR-thrombin labeled with acrylodan; Fl-FPR-thrombin, FPR-thrombin labeled with fluorescein; PBS, phosphate-buffered saline.

The activity and antigen levels of factor VIII proteins were determined by a one-stage clotting assay and a sandwich enzyme-linked immunosorbent assay, respectively, and the latter used two anti-factor VIII monoclonal antibodies as described previously (33). Recombinant wild type thrombin and thrombin S205A, in which the active site Ser<sup>205</sup>{sup>2}</sup> (based on the numbering of human α-thrombin) was replaced by Ala, were constructed, expressed, and purified using methods described previously (32). The activity and antigen levels of factor VIII proteins were determined by a one-stage clotting assay and a sandwich enzyme-linked immunosorbent assay, respectively, and the latter used two anti-factor VIII monoclonal antibodies as described previously (33). Recombinant wild type thrombin and thrombin S205A, in which the active site Ser<sup>205</sup>{sup>2}</sup> (based on the numbering of human α-thrombin) was replaced by Ala, were constructed, expressed, and purified as described previously (34). A chromogenic assay using S-2238 revealed ~0.2% residual thrombin activity of thrombin S205A compared with wild type.

Fluorophore Labeling of Proteins—Acrylodan-labeled (Ac-) isolated A1 and A2 subunits were prepared as described previously (35). Approximately 0.9–1.1 mol of acrylodan was incorporated per mol of A1 or A2 subunit. Fluorescein-labeled FPR-thrombin was prepared as described previously (36) following minor modifications. Thrombin (15 μM) was incubated with a 2.5-fold molar excess of ATA-FPR-CH<sub>2</sub>Cl in 50 mM HEPES, pH 7.2, and 0.1 mM NaCl at 22 °C for 2 h. A chromogenic assay using S-2238 revealed less than 0.1% residual thrombin activity. After dialysis, ATA-FPR-thrombin was reacted with a 40-fold molar excess of fluorescein 5-maleimide in the dark at 22 °C for 4 h. The unbound fluorescein 5-maleimide was removed by extensively dialyzing the reaction mixture at 4 °C in the above buffer. Approximately 0.8–1.0 mol of fluorescein 5-maleimide incorporated per mol of thrombin was determined by a one-stage clotting assay using 5 μM CaCl<sub>2</sub>, 0.2% Tween 20, and 200 μg/ml BSA for 2 h. Results were expressed under three different conditions to assess slow thrombin (10 mM NaCl and 37 °C), fast thrombin (140 mM NaCl and 22 °C), and physiologic conditions (140 mM NaCl at 37 °C), which yield a mixture of slow and fast forms. Samples were excited at 395 nm, and emission spectra were monitored at 420–550 nm. Emission scans were run at 3 nm/s in quadruplicate, and the average value of the spectra was recorded. All spectra were corrected for background fluorescence resulting from the reaction buffer and (Fi)-FPR-thrombin. Percent donor quenching was calculated from integrated fluorescence intensities at λ = 465–480 nm as shown in Equation 1,

\[
\text{% donor quenching} = \frac{F_D - (F_{DA} - F_A)}{F_D} \times 100
\]

where \(F_D\) is the fluorescence intensity of donor plus unlabeled acceptor (Ac-A1 or Ac-A2/FPR-thrombin); \(F_{DA}\) is the fluorescence intensity of labeled donor plus labeled acceptor (Ac-A1 or Ac-A2/FI-FPR-thrombin), and \(F_A\) is the fluorescence intensity of unlabeled donor plus labeled acceptor (A1 or A2/FI-FPR-thrombin).

Factor VIII Binding Phase Assay—Microtiter wells were coated with 50 μl of thrombin S205A (100 nM) in PBS, pH 7.4, overnight at 4 °C. Prior to each subsequent addition of receptor, the wells were rapidly washed with PBS containing 0.02% Tween 20. Wells were blocked with PBS containing 5% BSA for 3 h at 22 °C (fast form) or 37 °C (slow or slow/fast form). This step was followed by addition of the indicated factor VIII subunit in 20 mM HEPES, pH 7.2, 5 mM CaCl<sub>2</sub>, and 0.02% Tween 20 containing 1% BSA for 2 h and containing 10 mM NaCl at 37 °C (slow form), 140 mM NaCl at 37 °C (slow/fast form), and 140 mM NaCl at 22 °C (fast form), respectively. Biotinylated factor VIII monoclonal antibody IgG (1 μg) was added to each well, and bound IgG was detected by addition of horseradish peroxidase-labeled streptavidin. Quantitation of the latter reagent was determined following addition of the substrate o-phenylenediamine dihydrochloride (1 mg/ml, Sigma). Reactions were stopped by the addition of 2 x H<sub>2</sub>SO<sub>4</sub>, and the absorbance was measured at 490 nm using a Vmax microtiter plate reader (Molecular Devices, Sunnyvale, CA). The amount of nonspecific binding of biotinylated IgG observed in the absence of factor VIII subunit was <5% of the total signal, and the amount of specific binding was obtained by subtracting the amount of nonspecific binding of biotinylated IgG. Factor VIII Generation Assay—The rate of conversion of factor X to factor Xa was monitored in a purified system (37). These reactions were run at 22 °C in a buffer containing 20 mM HEPES, pH 7.2, 0.1 mM NaCl, 5 mM CaCl<sub>2</sub>, and 0.01% Tween 20. Factor VIII (1 nM) was activated by the addition of thrombin (0.05 nM) in the presence of phospholipid vesicles (10 μM). Thrombin activity was inhibited after 5 min by the addition of hirudin (0.2 units/ml), and factor X generation reactions were initiated with the addition of factor Xa (20 nM) and factor X (300 units/ml). Reactions were initiated with the addition of factor Xa (20 nM) and factor X (300 units/ml).
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nm). Aliquots were removed at appropriate times to assess initial rates of product formation and added to tubes containing EDTA (50 mM final concentration) to stop the reaction. Rates of factor Xa generation were determined by the detection of the chromogenic substrate, S-2765 (0.46 mM final concentration). Reactions were run at 405 nm for 10 min using a V_{max} microtiter plate reader.

Cleavage of the Recombinant Factor VIII or Isolated Heavy Chain Subunit by Thrombin or Factor Xa—Recombinant factor VIII (100 nM) or the isolated heavy chain (50 nM) was reacted with the indicated concentrations of thrombin. Factor VIII (100 nM) was also reacted with factor Xa (10 nM) in the presence of phospholipid vesicles (10 μM). These reactions were run at 22 °C in a buffer containing 20 mM HEPEs, pH 7.2, 0.1 M NaCl, 5 mM CaCl₂, and 0.01% Tween 20. Samples were taken at the indicated times, and the reactions were immediately terminated and prepared for SDS-PAGE by adding the SDS-containing sample solution and boiling for 3 min.

Electrophoresis and Western Blotting—SDS-PAGE was performed on 8% gels using the procedure of Laemmli (38). Electrophoresis was carried out using a Bio-Rad mini gel apparatus at 150 V for 1 h. The protein was transferred to a polyvinylidene fluoride membrane, and then probed using the indicated anti-factor VIII monoclonal antibody followed by goat anti-mouse alkaline phosphatase-linked secondary antibody (Sigma). The signal was detected using the enhanced chemiluminescence system (Amersham Biosciences), and the blots were scanned at 570 nm using Storm 860 (Molecular Devices). Densitometric scans were quantitated using ImageQuant software (Molecular Devices).

Data Analyses—All experiments were performed at least three separate times, and the average values are shown. Nonlinear least squares regression analysis was performed by using Kaleidograph (Synergy, Reading, PA), and the parameters and their standard errors were obtained. Analyses of the interactions between factor VIII subunits and thrombin using fluorescence spectroscopy and solid phase binding assay were performed by using a single-site binding model and a two-site binding model (nonidentical and independent sites) using Equations 2 and 3, respectively,

\[
\% \text{ donor quenching or absorbance} = \frac{A_{\text{max}}[S]}{K_f + [S]} (\text{Eq. 2})
\]

and

\[
\% \text{ donor quenching or absorbance} = \frac{A_{\text{max}}[S]}{K_f + [S]} + \frac{A_{\text{max}}[S]}{K_d + [S]} (\text{Eq. 3})
\]

where [S] is the FI-FPR-thrombin or factor VIII subunit (A1, A2, or heavy chain) in the fluorescence spectroscopy or solid phase binding assay, respectively; K_f, K_d, and K_s are the dissociation constants; and A_{max}, A_{max1}, and A_{max2} represent maximum percent donor quenching or absorbance signals when the sites are saturated by FI-FPR-thrombin or factor VIII subunit. The model to best fit the data was determined by an F test comparing the sum of squares from each fitting. Degrees of freedom for each data set were obtained by subtracting the number of parameters from the number of data points. The percentage points (probability) for the F distribution were calculated using Microsoft Excel.

Data from studies assessing the A2 synthetic peptide, hirudin, and heparin-dependent inhibition of thrombin interaction with isolated factor VIIIa subunits were fitted by nonlinear least squares regression by using Equation 4,

\[
\% \text{ binding} = \frac{B_{\text{max}}([A1 \ or \ A2 \ subunit])}{K_f + [L]} + C (\text{Eq. 4})
\]

where L represents the concentration of ligand (hirudin or heparin) or peptide; B_{max} represents maximum binding; K_f is the dissociation constant for the interaction between A1 or A2 subunit and thrombin S205A; K_s is the (apparent) inhibition constant for L; and C is a constant for binding of factor VIII subunit and thrombin that was unaffected by L.

RESULTS

Energy Transfer between FI-FPR-thrombin and Ac-A1 or Ac-A2 Subunit—Whereas a thrombin-interactive site that contributes to enzyme docking and facilitates catalysis of cleavage of factor VIII light chain at Arg1689 has been localized within the C2 domain (25), analogous sites within factor VIII heavy chain have not been identified. To address this question, an initial series of experiments was performed to assess thrombin binding to the isolated A1 and A2 subunits of factor VIIIa. The rationale for this approach of employing the products of thrombin cleavage of heavy chain was based upon the hypothesis that an interactive site for thrombin may be localized within rather than span the contiguous A domains. Affinity between the A1 or A2 subunit in the heavy chain and thrombin was initially examined using steady state fluorescence resonance energy transfer. The A1 and A2 subunits each possess a free, reactive Cys residue at Cys310 and Cys892, respectively (39), which was utilized for incorporation of acrylodan (fluorescence donor). Thrombin was modified at its active site using ATA-FPR-CH₂Cl and was subsequently labeled at the reactive ATA moiety using fluorescein 5-maleimide (fluorescence acceptor). The use of this fluorophore pairing was based upon an earlier study examining inter-subunit affinity following reconstitution of factor VIIIa using fluorophore-labeled subunits (35). Ac-A1 and Ac-A2 subunits retained >80% specific activity compared with unlabeled subunits based upon reconstitution of factor VIIIa (data not shown).

Fluorescence experiments were conducted using 60 nM Ac-A1 or Ac-A2 subunit and varying levels of FI-FPR-thrombin as described under "Materials and Methods," and the results are shown in Fig. 1 and Table I. Reactions were performed using low (10 nm) and physiological (140 nm) Na⁺ concentrations to evaluate slow and fast forms of thrombin, respectively. Saturable (or near saturable) donor quenching was observed for interactions of slow and fast thrombin with both the A1 and A2 subunits. At 140 mM NaCl and 22 °C, conditions yielding >95% fast thrombin (21), we observed high affinity and low affinity sites for interaction with the A1 subunit. Similar affinity sites were observed at this Na⁺ concentration and 37 °C, which yields a rapid equilibrium between fast and slow thrombin forms (19). Use of the two-site thrombin-binding model for the fast form and slow/fast mixture was supported by an F-test showing appropriate statistical significance parameters (Table I). However, there was no significant difference between a single-site and two-site binding model for interaction of the slow form of thrombin with the A1 subunit, which yielded a single affinity site ~60-fold weaker than the high affinity site identified for fast thrombin. Evaluation of thrombin interactions with the A2 subunit yielded a single moderate affinity site (K_d ~80–140 nm) that appeared independent of occupancy of the Na⁺ site in thrombin. Comparison of thrombin interactions with the heavy chain-derived subunits indicated that the slow form of thrombin exhibits ~4-fold higher affinity for A2 compared with the A1 subunit, whereas interactions of the slow/fast and fast forms show ~20-fold higher affinity for A1 than the A2 subunit. These results suggest different mechanisms for thrombin interaction with the A1 and A2 subunits.

Examination of donor quenching data values for fast thrombin binding to the moderate affinity site in the A2 subunit yielded significantly less quenching than slow thrombin interaction with that subunit. By assuming the binding sites are identical, these data suggest the change in conformation from fast to slow forms results in a closer inter-fluorophore spatial separation between the thrombin active site and the acrylodan fluorophore in A2. Evaluation of quenching values for the thrombin-A1 subunit interaction is more complex based upon the two-site model. We observed a very low quenching value for fast thrombin binding to the high affinity site, and a value for the low affinity site similar to that observed for interaction with the A2 subunit. Binding of slow thrombin to A1 yielded a
Ser205 was replaced by Ala (thrombin S205A) was used to assess thrombin binding to the reaction products of heavy proach, independent of protein modification, was employed to drance by virtue of the fluorescein probe, an alternative ap/sites on the enzyme as well as producing potential steric hin-

levels of Fl-FPR-thrombin were reacted in a buffer of 20 mM HEPES, pH or Ac-A2 subunit.

form, (open circles) or a two-site ( dashed line) binding model as indicated that affinity of the heavy chain substrate for thrombin was from the continuity of the two domains. However, the observa-

can not rule out the existence of cooperative effects for thrombin binding between the two domains. The reason for the reduced absorbance signal for heavy chain compared with signals observed for the A1 and A2 subunits is not known but may reflect fewer moles of intact heavy chain bound compared with the isolated subunits as a result of blocking accessible binding sites because of its larger size.

Evaluation of Exosite Ligands on the Interaction of Thrombin with the A1 and A2 Subunits—Significant literature indicates that thrombin interacts with substrates and cofactors through electrostatic interactions involving anion-binding exosites I and/or II. A previous report demonstrates that both exosites were involved in the recognition of factor VIII (16). To clarify the role(s) of thrombin exosites in the binding of A1 and A2 subunit, the effects of two exosite-specific competitors, hirudin and heparin, which bind exosite I and exosite II, respectively, were examined in the solid phase assay. In this experiment, a fixed concentration of the A1 (160 nM) or A2 (60 nM) subunit was incubated with immobilized thrombin S205A in the pres-

**FIG. 1.** Energy transfer between Fl-FPR-thrombin and Ac-A1 or Ac-A2 subunit. Ac-A1 (A) or Ac-A2 (B) subunit (60 nM) and varying levels of Fl-FPR-thrombin were reacted in a buffer of 20 mM HEPES, pH 7.2, 5 mM CaCl$_2$, 0.01% Tween 20, and 200 µg/ml BSA containing 10 mM NaCl at 37 °C (slow form, closed circles), 140 mM NaCl at 37 °C (slow/fast form, open circles), or 140 mM NaCl at 22 °C (fast form, open squares), respectively, for 2 h as described under “Materials and Methods.” The emission intensity of Ac-A1 or Ac-A2 was measured at $\lambda = 465$–480 nm and recorded in the absence and presence of the indicated levels of F1-FPR-thrombin. Percent donor quenching refers to the fluorescence intensity of Ac-A1 or Ac-A2 in the presence of Fl-FPR-thrombin relative to that of Ac-A1 or Ac-A2 alone. These values were plotted as a function of the concentration of F1-FPR-thrombin, and data were fitted according to a single-site (solid line) or a two-site (dashed line) binding model as described by Equations 2 and 3, respectively under "Materials and Methods.”

significantly lower quenching value than that observed for binding to A2 subunit.

**Binding of Factor VIII Subunits to Thrombin S205A Mutant in a Solid Phase Assay**—Because F1-labeled ATA-FPR probe binds to the active site of thrombin, occluding the $S_1$-$S_3$$_2$ subsites on the enzyme as well as producing potential steric hindrance by virtue of the fluorescein probe, an alternative approach, independent of protein modification, was employed to assess thrombin binding to the reaction products of heavy chain cleavage. A missense mutation in which the active site Ser$^{205}$ was replaced by Ala (thrombin S205A) was used to evaluate binding by using a solid phase assay. For these experiments, varying amounts of the factor VIIIa subunit were reacted with 100 nM thrombin S205A, which had been immobilized onto microtiter wells as described under “Materials and Methods.” Bound subunits were detected using an appropriate biotinylated anti-factor VIII monoclonal antibody. Results from this study are shown in Fig. 2 and Table II. Evaluation of both A1 (Fig. 2A) and A2 (Fig. 2B) subunits yielded apparent saturable binding curves. Because this method is not a true equilibrium binding assay, the $K_d$ values represent an apparent $K_d$ for interaction of the subunits with thrombin S205A.

Binding of fast thrombin to A1 subunit was best described by a two-site model, whereas slow thrombin interaction with this subunit was defined by a single binding site. The affinity values for the slow/fast thrombin mixture and fast thrombin forms for the A1 subunit were $\sim$10 and $\sim$50-fold higher, respectively, than that of the slow form for the A1 subunit. Binding of thrombin to the A2 subunit suggested a single, moderate affinity site independent upon the reaction conditions employed. Overall, affinity values from this study compared favorably with those obtained in the fluorescence energy transfer analysis. Thus the results from the two binding assays are mutually supportive. Taken together, these results suggest that thrombin binding to the A1 subunit is dependent upon reaction conditions, with the fast form binding this subunit with high affinity, whereas thrombin binding the A2 subunit occurs at a moderate affinity site independent of occupancy of the Na$^+$ site in thrombin. Furthermore, the latter experiment validates the fluorescence data and suggests that potential perturbations resulting from fluorophore incorporation at the active site were benign with respect to interaction of thrombin with the factor VIIIa subunits.

The intact heavy chain also bound to immobilized thrombin S205A with lower apparent $K_d$ values for all forms of thrombin compared with those observed for the A1 or A2 subunit (Fig. 2C and Table II). This somewhat higher affinity value may derive from a synergistic effect of the two binding domains and/or a conformational change in a single interactive site resulting from the continuity of the two domains. However, the observation that affinity of the heavy chain substrate for thrombin was not markedly greater than affinities determined for the individual reaction products supports the original hypothesis that a significant interactive site for thrombin is not shared between the two $\alpha$ domains. This observation also suggests that cleavage at Arg$^{272}$ did not likely introduce changes in the protein-fold that affected the thrombin-binding site(s). However, we can not rule out the existence of cooperative effects for thrombin binding between the two domains. The reason for the reduced absorbance signal for heavy chain compared with signals observed for the A1 and A2 subunits is not known but may reflect fewer moles of intact heavy chain bound compared with the isolated subunits as a result of blocking accessible binding sites because of its larger size.

2 Nomenclature from Schechter and Berger (64).
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Reactions were performed as described under “Materials and Methods.” Parameter values were calculated by nonlinear least squares regression of the data shown in Fig. 1 using Equations 2 and 3 shown under “Materials and Methods.” The F test was performed to compare and identify the better curve fit using a two-site binding model.

### Table I

| Factor VIII subunit | Thrombin form | $K_d$ (% donor quenching) | $K_{d2}$ (% donor quenching) | F value | p value |
|--------------------|---------------|---------------------------|-----------------------------|--------|--------|
| Ac-A1              | Slow          | 307 ± 38                  | 301 ± 20                    | 0.40   | >0.05  |
|                    | Slow/fast     | 4.6 ± 1.7a                | 1182 ± 943a                 | 7.08   | <0.001 |
|                    | Fast          | 5.4 ± 4.3a                | 643 ± 471a                  | 4.42   | <0.005 |
| Ac-A2              | Slow          | 76.6 ± 3.3                | 52.3 ± 0.8                  | 1.67   | >0.05  |
|                    | Slow/fast     | 138 ± 30                  | 16.3 ± 1.4                  | 3.57   | >0.05  |
|                    | Fast          | 129 ± 20                  | 12.3 ± 0.8                  | 1.28   | >0.05  |

*a The F test was performed to compare and identify the better curve fit using a two-site binding model.

The $F$ test was performed to compare and identify the better curve fit using a two-site binding model.

Of varying concentrations of hirudin or heparin. Reaction conditions (Na\(^+\) and temperature) were varied to evaluate both slow and fast (and/or slow/fast) thrombin forms, and the results are shown in Fig. 3 and Table III. We observed that hirudin blocked the interaction of A1 subunit with thrombin in a dose-dependent manner by >50% by using physiologic reaction conditions in assessing the slow/fast mixture (Fig. 3A, left panel). By using low Na\(^+\) conditions, the apparent $K_d$ value for hirudin-dependent inhibition of slow thrombin binding to A1 subunit was increased by ~15-fold, and this observation was consistent with an earlier report showing preferential binding of the inhibitor to fast thrombin (18). Binding of fast thrombin to A1 was not determined due to very low signal in the binding assay. The observation that the hirudin-dependent inhibition of binding to A1 was incomplete suggested that thrombin may also interact with this subunit through an exosite I-independent mechanism, consistent with the two-site binding model suggested above for this interaction. On the other hand, the interaction of A2 subunit with thrombin appeared to be independent of exosite I because little, if any, inhibition was observed even at the highest levels of hirudin tested, independent of the form of thrombin (Fig. 3A, right panel).

Conversely, heparin blocked the thrombin interaction with the A2 subunit in a dose-dependent manner by >60% and with similar apparent $K_d$ values as observed using both slow and slow/fast forms (Fig. 3B, right panel). However, heparin markedly blocked the A2-fast thrombin interaction by >90% with an ~100-fold lower $K_d$ value. This value (~32 nM) was similar to an earlier report of the binding affinity for the thrombin-heparin interaction (30–130 nM (40)) and was consistent with the observed heparin-dependent inhibition of thrombin-catalyzed factor VIII cleavage (1IC\(_{50}\) ~40 nM (41)). The observation that heparin resulted in near complete inhibition of this binding suggested that the majority of the thermodynamic stability for this interaction was dependent upon exosite II of thrombin. Little if any contribution to this inhibition was from direct binding of heparin to the putative heparin-binding site in A2 subunit, residues 558–565 (42), because a peptide (residues 555–566) encompassing this sequence did not affect the observed heparin-dependent inhibition of the thrombin-A2 interaction (see Fig. 7). A more marginal effect of heparin on the A1 and thrombin interaction was observed with ~20% inhibition of the A1 subunit binding at the highest heparin concentration employed (Fig. 3B, left panel). However, this high heparin concentration may impair anion-binding exosite I interactions. Taken together, these data indicate that thrombin predominantly interacts with the A2 subunit via exosite II, whereas interaction with the A1 subunit is affected by exosite I with the possible contribution of exosite II.

Effects of A2 N- and C-terminal, Acidic-rich Peptides on Factor VIII Activation and Cleavage by Thrombin—Based upon the observations that the affinity of thrombin for A2 subunit appears independent upon Na\(^+\) occupancy of the enzyme, likely reflects binding to a single site, and relies heavily on the anion-binding exosite II for this interaction, we focused a series of studies on the interaction of thrombin with this subunit. Examination of the A2 domain sequence revealed two regions of clustered acidic residues, 398–394 (Glu-Glu-Glu-Asp-Trp-Asp) and 720–725 (Glu-Asp-Ser-Tyr-Glu-Asp), that are localized ~20 residues distal from the P\(_1\) residues Arg\(^{372}\) and Arg\(^{410}\), respectively, that are cleaved by thrombin during activation of factor VIII (see Fig. 4). Most interestingly, these two acidic regions in the A2 domain of factor VIII are highly conserved in other species. Thus, we hypothesized that one or both of these acidic regions could represent (an) interactive region(s) for exosite II of thrombin. To test this hypothesis, two synthetic peptides derived from sequences 373–395 and 719–740, which encompass the clustered acidic regions, were prepared, and the inhibitory effects of these peptides on factor VIII activation by thrombin were determined in a factor Xa generation assay.

Mixtures containing factor VIII (1 nM) and increasing concentrations of each peptide were reacted with thrombin (0.05 nM) for 5 min, after which hirudin was added to quench thrombin activity. Reactions were run under physiologic ionic strength (including 100 mM Na\(^+\)) and at 22 °C to yield the fast form of thrombin. The extent of cofactor activation was assessed following addition of factor Xa (20 nM) and factor X (300 nM) in the presence of phospholipid vesicles (10 μM) as described under “Materials and Methods.” Furthermore, in a separate experiment, factor VIII was activated by thrombin in the absence of peptide, which was then added to the reaction mixture prior to addition of the other reactants to assess factor Xa generation. This control reaction served to determine the effect of peptide on the rate of factor Xa generation independent of the cofactor activation step. Results shown in Fig. 5 indicated that both peptides yielded complete inhibition of the thrombin-catalyzed activation of the procofactor while minimally affecting subsequent steps in the generation of factor Xa. IC\(_{50}\) values determined for the 373–395 and 719–740 peptides were 7.9 ± 0.7 and 3.2 ± 0.2 μM, respectively (Fig. 5, A and B, respectively), indicating a high affinity of the peptides for thrombin. In addition, a control peptide composed of residues 373–385 that lacked the acidic cluster region of the 373–395 peptide showed no effect on the activation of factor VIII by thrombin.
A–C, respectively) subunits were reacted with thrombin S205A (100 mM HEPES, pH 7.2, 5 mM CaCl₂, 0.01% Tween 20, and 1% BSA at 37 °C (slow/fast form, open squares), or 140 mM NaCl at 22 °C (fast form, open circles), or 140 mM NaCl at 22 °C (fast form, open circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 37 °C (slow form, closed squares), respectively, for 2 h as described under “Materials and Methods.” Bound factor VIII subunits were detected using biotinylated anti-A1 (58.12) and/or anti-A2 (R8B12) IgG, respectively. Absorbance values were plotted as a function of the concentration of factor VIII subunit, and data were fitted according to a single-site (solid line) or a two-site (dashed line) binding model as described by Equations 2 and 3, respectively under “Materials and Methods.”

**FIG. 2.** Binding of factor VIII subunits to thrombin S205A in a solid phase assay. Varying amounts of A1, A2, and intact heavy chain (A–C, respectively) subunits were reacted with thrombin S205A (100 mM HEPES, pH 7.2, 5 mM CaCl₂, 0.01% Tween 20, and 1% BSA containing 10 mM NaCl at 37 °C (slow form, open circles), 140 mM NaCl at 37 °C (slow form, closed circles), or 140 mM NaCl at 22 °C (fast form, open squares), respectively, for 2 h as described under “Materials and Methods.” Bound factor VIII subunits were detected using biotinylated anti-A1 (58.12) and/or anti-A2 (R8B12) IgG, respectively. Absorbance values were plotted as a function of the concentration of factor VIII subunit, and data were fitted according to a single-site (solid line) or a two-site (dashed line) binding model as described by Equations 2 and 3, respectively under “Materials and Methods.”

These data suggest that the acidic clusters contained within regions 373–395 and 719–740 are thrombin-interactive and may contribute to the activation of factor VIII.

SDS-PAGE was employed to visualize the effects of the peptides on thrombin-catalyzed cleavage of the factor VIII heavy chain. For this analysis, isolated factor VIII heavy chain was used as substrate in order to eliminate any contribution of light chain to the interaction with thrombin. Heavy chain (50 nM), free of detectable light chain (data not shown), was reacted for 5 min with thrombin (1 nM) in the presence of increasing concentrations of either the 373–395 or the 719–740 peptide. Fig. 6 shows the results from Western blotting of the cleavage reactions using an anti-A2 monoclonal antibody. Both peptides were observed to block cleavages at the A1-A2 (Arg³⁷²) and A2-B (Arg⁷⁴⁰) domain junctions in a dose-dependent manner. At the highest peptide concentrations employed (256 μM), both cleavages were inhibited by >90%, as judged by the generation of the A2 subunit as well as the residual levels of the intact (A1-A2-B) heavy chain. Estimation of the IC5₀ values from scanning densitometry data suggested similar values of 40 ± 10 and 31 ± 7 μM for cleavage at Arg⁷⁷² by the 373–395 and 719–740 peptides, respectively. On the other hand, cleavage at the A2-B junction (Arg⁷⁴⁰) yielded IC₅₀ values of 120 ± 20 and 30 ± 9 μM for the 373–395 and 719–740 peptides, respectively, suggesting a possible bond-specific selectivity for the inhibition observed for the former peptide. Furthermore, the 373–385 peptide lacking the acidic region as well as an acidic rich peptide to residues 337–372 (separating the A1 and A2 domains) showed no effect on the cleavage of isolated heavy chain by thrombin (data not shown). These data are consistent with the results observed in the thrombin-catalyzed activation of the procofactor described above and add further support to the potential contributions of these acidic regions in the A2 domain to a specific thrombin-interactive site that facilitates cleavages in the heavy chain.

**Binding of the 373–395 and 719–740 Peptides to Thrombin**—In order to confirm that the inhibitory activity observed for the peptides resulted from their binding to thrombin, we performed a series of competition experiments to examine the effects of the 373–395 and 719–740 peptides on the binding of the A2 subunit to thrombin S205A in a solid phase assay. The A2 subunit (60 nM) was reacted with immobilized thrombin S205A under conditions that yield the fast form in the presence of increasing concentration of peptides as described under “Materials and Methods,” and the results are shown in Fig. 7. Both the 373–395 and 719–740 peptides blocked the A2 subunit binding to thrombin S205A by ~90%, and these effects were dose-dependent. The apparent Kᵢ values obtained from curve fitting for the 373–395 and 719–740 peptides were similar (16.0 ± 3.3 and 20.4 ± 5.0 μM, respectively). Furthermore, an equimolar mixture of both peptides yielded enhanced inhibitory activity with an indicated apparent Kᵢ value of 9.0 ± 0.8 μM. This value was ~2-fold reduced compared with the values obtained for the individual peptides and suggests an additive effect of the peptides. A control experiment using the 373–385 peptide showed weak inhibition of the A2 subunit with thrombin with >80% residual binding observed at the highest concentration (~300 μM) of peptide employed. We also assessed the effects of the A2 peptides on blocking the interaction of A2 with slow and slow/fast thrombin forms. Both peptides exhibited inhibitory activity yielding similar Kᵢ values to those...
above, independent of the thrombin form (data not shown). Overall, these observations suggest the two acidic cluster peptides represent effective probes for blocking the thrombin exosite-A2 domain interactions as judged by inhibition of activation and heavy chain cleavage, as well as direct binding to A2 subunit. Together these results strongly suggest that the 373–395 and/or 719–740 acidic regions in A2 domain represent a thrombin-interactive site(s).

**Table II**

| Factor VIIIa subunit | Thrombin form | $K_d (B_{max})$ | $K_{fit} (B_{max})$ | $F$ value | $p$ value |
|----------------------|---------------|-----------------|---------------------|------------|-----------|
| A1                   | Slow          | 166 $\pm$ 13 (0.41 $\pm$ 0.13) | 1211 $\pm$ 854$^a$ (0.41 $\pm$ 0.20) | 2.29 | >0.05 |
|                      | Slow/fast     | 12.4 $\pm$ 6.0$^a$ (0.04 $\pm$ 0.01) | 1030 $\pm$ 193$^a$ (0.15 $\pm$ 0.02) | 31.6 | <0.001 |
| A2                   | Slow          | 42.6 $\pm$ 2.3 (0.91 $\pm$ 0.01) | 1.01 | >0.05 |
|                      | Slow/fast     | 64.8 $\pm$ 6.8 (0.62 $\pm$ 0.21) | 2.86 | >0.05 |
|                      | Fast          | 71.5 $\pm$ 9.6 (0.31 $\pm$ 0.01) | 3.95 | >0.05 |
| Heavy chain          | Slow          | 22.8 $\pm$ 2.0 (0.22 $\pm$ 0.01) | 1.76 | >0.05 |
|                      | Slow/fast     | 1.3 $\pm$ 0.6$^a$ (0.02 $\pm$ 0.004) | 114 $\pm$ 27$^a$ (0.11 $\pm$ 0.005) | 8.57 | <0.05 |
|                      | Fast          | 0.62 $\pm$ 0.35$^a$ (0.007 $\pm$ 0.001) | 1030 $\pm$ 193$^a$ (0.08 $\pm$ 0.006) | 4.88 | <0.05 |

$^a$ The $F$ test was performed to compare and identify the better curve fit using a two-site binding model.

**Fig. 3.** Effects of hirudin and heparin on the binding of A1 or A2 subunit to thrombin S205A. A1 (left panel) or A2 (right panel) subunit (160 or 60 nM, respectively) in the presence of the indicated levels of hirudin (A) or heparin (B) was reacted with thrombin S205A (100 nM) immobilized onto microtiter wells under conditions for slow thrombin (open circles), slow/fast thrombin forms (closed circles), or fast thrombin (open squares) as described under “Materials and Methods.” Bound factor VIII subunits were detected using biotinylated anti-A1 (58.12) or anti-A2 (R8B12) IgG, respectively. The absorbance values for the A1 or A2 binding to thrombin S205A in the absence of each competitor represented the 100% level. The percentage of A1 or A2 binding was plotted as a function of hirudin or heparin concentration, and the plotted data were fitted by nonlinear least squares regression to Equation 4 under “Materials and Methods.”

**Thrombin Interaction with a Factor VIII D392A/D394A Double Mutant**—Additional support for a functional role for the acidic cluster residues within the 373–395 cluster was obtained following examination of a recombinant, B-domainless factor VIII molecule in which Ala was substituted for Asp$^{392}$ and Asp$^{394}$. Attempts to stably express other acidic cluster mutations within this segment as well as the A2 C-terminal acidic cluster have been unsuccessful for reasons that are not clear.
Reactions were performed as described under “Materials and Methods.” Parameter values were calculated by nonlinear least-squares regression of the data shown in Fig. 3 using Equation 4 as shown under “Materials and Methods.”

| Thrombin (form) | Hirudin | Heparin |
|-----------------|---------|---------|
|                 | A1 binding | A2 binding | A1 binding | A2 binding |
| Slow            | 169 ± 71  | a       | 10.7 ± 3.4 (3.4) |
| Slow/fast       | 9.9 ± 4.2 | a       | 7.5 ± 1.9 (2.1) |
| Fast            | ND       | a       | 0.11 ± 0.03 (0.022) |

| Species       | Amino acid sequence             |
|---------------|---------------------------------|
| Human (N-terminus) | SVAKKHPKTVHYIAPDEEDNY |
| Porcine       | EREEDNY                        |
| Marine        | EREEDNY                        |
| Canine        | EREEDNY                        |
| Human (C-terminus) | YEYSDYESIAYLLSKNAEIHF-CONH2 |
| Porcine       | YDNYTED                        |
| Marine        | YEEYTED                        |
| Canine        | YEEYTED                        |

Fig. 4. N- and C-terminal sequences of human A2 domain and the conservation of acidic residue-rich segments. Residues at the N-terminus (373–385) and the C-terminus (719–740) of human factor VIII (A2 domain) are indicated by the single-letter designation. Conserved acidic residues in regions 389–395 and 720–725 are underlined.

Indeed, the D392A/D394A double mutant exhibited a markedly reduced specific activity (−0.1% the wild type level) consistent with a severe hemophilia A phenotype.

The D392A/D394A mutant (100 nM) was evaluated for cleavage by thrombin (2.5 nM) in time course reactions using SDS-PAGE analysis. Fig. 8 shows the results from Western blotting of the cleavage reactions. Expression of both wild type and mutant factor VIII indicated the presence of single chain material (contiguous heavy and light chains) as well as heterodimeric factor VIII. Fig. 8A shows a schematic of the domain structures for these forms along with sites of cleavage. Products were determined by using an anti-A2 domain monoclonal antibody (recognizing residues 563–740 (28), Fig. 8B), anti-A1 domain monoclonal antibody (recognizing residues 351–365 (27), Fig. 8C), and anti-light chain monoclonal antibody (recognizing residues 1649–1689, Fig. 8D). Thrombin-catalyzed cleavage of wild type factor VIII resulted in rapid loss of the A2 subunit (Fig. 8B) and A1 or the heavy chain did not appear impaired as judged by all three panels. Cleavage of the wild type factor VIII resulted in rapid loss of the A2 subunit (Fig. 8B). Thrombin demonstrated a moderate affinity for the Na⁺-free slow form of thrombin bound the A1 subunit with 50-fold reduced affinity compared with the fast form. On the other hand, thrombin demonstrated a moderate affinity for the A2 subunit that was essentially independent of occupancy of the Na⁺ site but showed high dependence on anion-binding exosite II.

**Discussion**

By using two complementary approaches, we demonstrated that thrombin binds both the isolated A1 and A2 subunits derived from cleavage of factor VIII heavy chain. Steady state fluorescence resonance energy transfer employed acrylodan-labeled factor VIIIa subunits and a fluorescent, active site-labeled thrombin, whereas a microtiter well-based solid phase binding assay yielded affinity values following interaction of the subunits with a thrombin molecule possessing a Ser205→Ala point mutation. Although each method has its limitations, e.g., the former requires chemical modification of the reactants that may alter binding specificity (43, 44), whereas the latter is a non-equilibrium method, the similarity in results supports the authenticity of the data. Our results indicated a high affinity interaction of the Na⁺-bound, fast form of thrombin for the A1 subunit that was 20-fold greater than its affinity for the A2 subunit. This high affinity interaction appeared partially dependent upon anion-binding exosite I. Furthermore, the Na⁺-free slow form of thrombin bound the A1 subunit with 50-fold reduced affinity compared with the fast form. On the other hand, thrombin demonstrated a moderate affinity for the A2 subunit that was essentially independent of occupancy of the Na⁺ site but showed high dependence on anion-binding exosite II.
The results in this study differed from earlier observations employing surface plasmon resonance to assess factor VIIIa subunits binding to immobilized anhydrothrombin (25) in which the active site serine was chemically altered to dehydroalanine by a $\beta$-elimination reaction (45, 46). In that report, a relatively weak affinity interaction was observed for the A2 subunit ($K_d \approx 500$ nM), whereas no binding to thrombin was detected for the A1 subunit. The reasons for the markedly weaker affinities observed in the earlier study are not clear but may relate to perturbations in the thrombin structure during its chemical modification.

Use of thrombin-derived products from the heavy chain cleavage reaction to study the thrombin-heavy chain interaction is justified because substrate recognition is primarily upon enzymic binding via exosites rather than active site docking at the P1 residue (47). The assumption that an exosite-interactive region did not span the A domains but rather that these regions were localized within a given domain appeared justified based upon similar affinity values of thrombin S205A for the A1 and A2 subunits compared with the intact factor VIII heavy chain as determined with the fast and slow forms of the enzyme. Earlier studies using the exosite site-specific inhibitors hirudin (16, 48) and heparin (41) suggested that both exosites I and II in thrombin participated in the activation of factor VIII. More recently, alanine-scanning mutagenesis identified several basic residues in each exosite as contributing to the interaction with substrate factor VIII (17). Specifically, exosite I appeared essential for factor VIII activation through cleavage of Arg$^{372}$ and Arg$^{1689}$, and exosite II contributed to cleavage of Arg$^{372}$ (17). By using the same inhibitors, our results indicate that exosite I appears to contribute to a high
affinity interaction with the A1 subunit, whereas a moderate affinity interaction between thrombin and the A2 subunit is mediated by exosite II. Whereas correlation of specific exosite interactions within the heavy chain to cleavage at Arg372 remains to be determined, the relative domain specificities of the exosites are not surprising. The A1 subunit terminates in a highly acidic, hirudin-like region, residues 337–372. Furthermore, sulfation of Tyr346 contributes to procofactor activation and cleavage at Arg372 by thrombin (49), paralleling the importance of sulfation at Tyr63 in hirudin in contributing to its high affinity interaction with thrombin (50). Of interest was the observation that the 337–372 peptide failed to appreciably inhibit thrombin-catalyzed cleavage of the factor VIII heavy chain, suggesting that the lack of sulfation at Tyr346 in the peptide may attenuate potential inhibitory capacity. Alternatively, the A2 domain does not possess an extended sequence rich in acidic residues, and sulfated Tyr residues contained within this domain at Tyr718, Tyr719, and Tyr723 do not affect the rates of activation and cleavage by thrombin (49).

The A2 subunit possesses two acidic residue-rich regions, residues 389–394 (Glu-Glu-Glu-Asp-Trp-Asp) and residues 720–725 (Glu-Asp-Ser-Tyr-Glu-Asp), localized close to the N and C termini, respectively. We propose that one or both of these regions in the folded protein interacts with exosite II of thrombin based upon our results showing that peptides encompassing the clustered acidic regions (i) blocked activation of factor VIII by thrombin, (ii) blocked the cleavages at Arg372 in the A1-A2 junction and Arg740 in the A2-B junction by thrombin, and (iii) blocked direct binding of A2 subunit to thrombin. Although little information exists on the contribution of the N-terminal acidic region in A2 to interaction with thrombin, several lines of evidence suggest the C-terminal acidic region does make a contribution. A monoclonal antibody directed against this region inhibited thrombin activation of factor VIII (51, 52), and recombinant B-domainless factor VIII molecules in which this region was partially deleted were observed to require higher thrombin concentrations for efficient activation of the procofactor (52, 53). Furthermore, in experiments using a factor VIII chimera where A2 residues 712–736 were replaced with a high affinity thrombin site from the serpin, heparin cofactor II showed increased rates of both cleavages at Arg372 by thrombin and overall factor VIII activation (54), suggesting that the C-terminal region of A2 influences cleavages at distal sites in the factor VIII molecule. Taken together, these earlier results coupled with the results from the current study suggest that the acidic regions localized to the N and/or C termini of the A2 domain appear indispensable for the exosite-dependent tethering of thrombin contributing to efficient cleavage of the heavy chain.

Attempts to prepare and express mutations within the two A2 acidic regions were not highly successful for reasons that are not well understood. One mutant protein, representing a double mutation of D392A/D394A, was expressed in reasonable yield, and analysis of this reagent did offer insights into interactions with thrombin leading to proteolysis and cofactor activity. SDS-PAGE analysis revealed the mutant was defective in cleavage at Arg740, whereas cleavages at the other heavy chain site, Arg372, and the site in the light chain were essen-
tially unaffected. This observation suggests that tethering thrombin at the 389–394 acidic cluster affects the cleavage rate at the A2-B domain junction. Whether tethering of the enzyme at the C-terminal acidic cluster of A2 contributes to catalysis of bond cleavage at either site in the heavy chain remains to be determined. Structural information on the juxtaposition of these putative tethering sites with the scissile bonds is limited inasmuch as the C-terminal region of the A2 domain (following residue 719) is not accounted for in the ceruloplasmin-based A domain homology model (55).

However, by taking the above observations together, our results are consistent with a model where the primary interaction of thrombin with factor VIII heavy chain is a high affinity one involving the A1 domain with anion-binding exosite I and dependent upon occupancy of the Na⁺ site in thrombin. Although initial docking proceeds via exosite I, we speculate the subsequent association of thrombin exosite II with A2 domain residues, possibly 389–394, serves to initially orient the active site facilitating cleavage at the A2-B domain junction (Arg⁴⁷⁰). Cleavage at this site to remove the B domain or its fragments occurs rapidly in the activation mechanism and precedes appreciable cleavage at Arg⁴⁷² (4, 56). In the absence of exosite II interactions, cleavage at Arg⁴⁷⁰ is retarded as judged by results obtained with the D392A/D394A mutant, whereas attack at the Arg⁴⁷² site is not significantly affected. Thus cleavage at the A2-B junction is not a requirement for cleavage at the A1-A2 site. This model is consistent with observations showing that mutations in exosite II make marginal contributions to cleavage at the Arg⁴⁷² site, whereas mutations in either exosite I or the Na⁺-binding site show more dramatic effects in this reaction (17). Additional evidence in support of this hypothesis is the observation that an exosite II mutant shows ~60% reduced cleavage at Arg⁷⁰₉ at the factor V A2-B domain junction (24).

Most interestingly, the D392A/D394A mutation possessed a severe hemophilic phenotype consistent with a functional role for the N-terminal acidic residue domain. The basis for the lack of activity is not completely known. However, one may speculate that failure to cleave factor VIII at the A2-B junction contributes to this defect. This would suggest an A2 subunit possessing a C-terminal extension has limited activity relative to the authentic A2 subunit. Results in Fig. 8B clearly show significant accumulation of an A2-a3 intermediate that persists throughout the time course. However, modest levels of what appears to be authentic A2 subunits are also observed in the blot, suggesting a limited extent of cleavage at the Arg⁷⁰₉ site. Thus the basis for the ~0.1% cofactor activity observed for the mutant is not clear. One possibility is that cleavage at Arg⁷⁰₉ is indeed completely blocked in the mutant, and the latter A2 band possesses the small 14-residue extension of B domain that links the factor VIII heavy and light chains (see Fig. 8A). Lack of cofactor activity due to the C-terminal extension may result from interference of the interaction of this subunit with factor IXa in the intrinsic factor Xase complex because A2 contributes a significant, extended interface critical for modulating factor IXa activity (for review see Ref. 6). This alternative would suggest that cleavage at the A2-B junction also represents an essential step in the process for procofactor activation.

Factor VIII and factor V share homologous structures, and functions (57) also appear to share similarities in interactive regions for activating enzymes. Although factor V shows no acidic-rich segment separating the A1 and A2 domains, a functionally important acidic region was recently localized to the N-terminal region of the A2 domain of factor V (residues 323–331) (58). This region of the factor Va heavy chain was suggested to comprise a factor Xa-binding site required for the expression of cofactor activity (59). The C-terminal region of the A2 domain of factor V also contains two acidic sequences within residues 659–698 (60). A recent study demonstrated that a pentapeptide to an acidic segment comprising factor V residues 695–699 blocked its activation by thrombin (61). That study also demonstrated that mutations within this sequence yielded a factor V molecule that was partially resistant to thrombin- but not factor Xa-catalyzed activation. However, a previous report demonstrated that the factor Va heavy chain binds to thrombin via exosite I but not exosite II of the protease (62). This interaction, based upon a competitive binding assay using 2-anilinonaphthalene-6-sulfonic acid-labeled FPR-thrombin and hirudin-(54–65), was of significantly weaker affinity (Kᵣ ~ 600 nM) compared with the value we determined for factor VIII heavy chain using the solid phase assay (Kᵣ ~ 1 nM). Therefore, the mechanisms for thrombin binding to the heavy chain of factor Va and factor VIII may be somewhat distinct. This distinction is likely reflected in the requirement for cleavage at both the A1-A2 and A2-B domain junctions for factor VIII activation.

Factor Xa has been shown to attack factor VIII at sites identical to those by thrombin (5). Mechanistic studies of factor Xa-catalyzed proteolysis of factor VIII heavy chain indicated that the proteinase interacts with acidic residues localized within the 337–372 region separating the A1 and A2 domains to facilitate cleavages at both Arg⁷⁰₂ and Arg⁴⁷⁰ (32). Furthermore, this binding utilizes the heparin-binding exosite of factor Xa (63). Thus, whereas similar exosite interactions facilitate formation of the enzyme-substrate complexes, the sites for tethering appear to differ significantly. This contention is further supported by our observation that the 373–395 peptide blocked thrombin-catalyzed cleavage of isolated heavy chain, whereas this peptide showed no effect on factor Xa-catalyzed proteolysis (32). Furthermore, we observed that thrombin and factor Xa demonstrated markedly different reactivities toward cleaving the Arg⁴⁷₀–Ser⁴⁷₁ scissile bond in the factor VIII D392A/D394A mutant. These results emphasize the unique modes of substrate recognition relative to an identical point of attack exhibited by the two enzymes.

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Exosite-interactive Regions in the A1 and A2 Domains of Factor VIII Facilitate Thrombin-catalyzed Cleavage of Heavy Chain

Keiji Nogami, Qian Zhou, Timothy Myles, Lawrence L. K. Leung, Hironao Wakabayashi and Philip J. Fay

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