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The Structural Integrity of Anion Binding Exosite I of Thrombin Is Required and Sufficient for Timely Cleavage and Activation of Factor V and Factor VIII*

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α -Thrombin has two separate electropositive binding exosites (anion binding exosite I, ABE-I and anion binding exosite II, ABE-II) that are involved in substrate tethering necessary for efficient catalysis. α -Thrombin catalyzes the activation of factor V and factor VIII following discrete proteolytic cleavages. Requirement for both anion binding exosites of the enzyme has been suggested for the activation of both procofactors by α -thrombin. We have used plasma-derived α -thrombin, β -thrombin (a thrombin molecule that has only ABE-II available), and a recombinant prothrombin molecule rMZ-II (R155A/R284A/R271A) that can only be cleaved at Arg³²⁰ (resulting in an enzymatically active molecule that has only ABE-I exposed, rMZ-IIa) to ascertain the role of each exosite for procofactor activation. We have also employed a synthetic sulfated pentapeptide (DY(SO₃⁻)DY(SO₃⁻)Q, designated D5Q1,2) as an exosite-directed inhibitor of thrombin. The clotting time obtained with β -thrombin was increased by ~8-fold, whereas rMZ-IIa was 4-fold less efficient in promoting clotting than α -thrombin under similar experimental conditions. α -Thrombin readily activated factor V following cleavages at Arg⁷⁰⁹, Arg¹⁰¹⁸, and Arg¹⁵⁴⁵ and factor VIII following proteolysis at Arg³⁷², Arg⁷⁴⁰, and Arg¹⁶⁸⁹. Cleavage of both procofactors by α -thrombin was significantly inhibited by D5Q1,2. In contrast, β -thrombin was unable to cleave factor V at Arg¹⁵⁴⁵ and factor VIII at both Arg³⁷² and Arg¹⁶⁸⁹. The former is required for light chain formation and expression of optimum factor Va cofactor activity, whereas the latter two cleavages are a prerequisite for expression of factor VIIIa cofactor activity. β -Thrombin was found to cleave factor V at Arg⁷⁰⁹ and factor VIII at Arg⁷⁴⁰, albeit less efficiently than α -thrombin. The sulfated pentapeptide inhibited moderately both cleavages by β -thrombin. Under similar experimental conditions, membrane-bound rMZ-IIa cleaved and activated both procofactor molecules. Activation of the two procofactors by membrane-bound rMZ-IIa was severely impaired by D5Q1,2. Overall the data demonstrate that ABE-I alone of α -thrombin can account for the interaction of both procofactors with α -thrombin resulting in their timely and efficient activation. Because formation of meizothrombin precedes that of α -thrombin, our findings also imply that meizothrombin may be the physiological activator of both procofactors *in vivo* in the presence of a procoagulant membrane surface during the early stages of coagulation.

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Blood clotting involves a multitude of proteins, which act in concert in response to vascular injury to produce the procoagulant enzyme thrombin, which in turn is responsible for the generation of the fibrin plug (1). The two procoagulant enzymatic complexes (*i.e.* intrinsic tenase and prothrombinase) that are critical for thrombin formation are similar in structure and composed of an enzyme, a cofactor, and the substrate associated on a cell surface in the presence of divalent metal ions. The intrinsic tenase complex is composed of the enzyme, factor IXa, and the cofactor, factor VIIIa, associated on the cell membrane in the presence of divalent metal ions. Similarly, the prothrombinase complex is composed of the enzyme, factor Xa, and the cofactor, factor Va, associated on a cell surface in the presence of divalent metal ions. Although both enzymes factor IXa and factor Xa alone, can activate factor X and prothrombin, respectively, their catalytic efficiencies are poor, and the overall reactions are incompatible with survival and the arrest of bleeding. Incorporation of the protein cofactor into both complexes results in a substantial increase in the catalytic efficiency of both enzymes, for cleavage and activation of their substrates insuring normal hemostasis (2–8). The increase in the overall enzymatic reaction is attributed to a large increase in the k_{cat} of the reactions, which in turn is solely attributed to the interaction of the cofactor molecules with both the membrane-bound enzyme and the membrane-bound substrate. Thus, the activities of the prothrombinase complex, as well as the intrinsic tenase complex, are limited by the presence of the two soluble, non-enzymatic cofactors, factor Va and factor VIIIa.

The procofactors, factor V and factor VIII, do not interact with the components of prothrombinase and intrinsic tenase, respectively. Proteolytic processing of factor V by α -thrombin at Arg⁷⁰⁹, Arg¹⁰¹⁸, and Arg¹⁵⁴⁵, results in the production of the active cofactor, factor Va, which consists of a heavy chain (M_r 105,000) and a light chain (M_r 74,000), and factor Va is required for the interaction of the cofactor with the members of prothrombinase (9–11) (Fig. 1). Similarly, factor VIII is activated following proteolysis by α -thrombin at Arg³⁷², Arg⁷⁴⁰, and Arg¹⁶⁸⁹ (12–15) (Fig. 1). Although cleavages at Arg³⁷² and Arg¹⁶⁸⁹ by α -thrombin are required for activation of factor VIII and expression of full procoagulant activity, requirement for cleavage at Arg⁷⁴⁰ for expression of activity has not yet been rigorously established (16–18). Both procofactors have in common the fact that all activating cleavage sites (with the exception of cleavage at Arg¹⁰¹⁸ in factor V) are preceded by a region rich in acidic amino acids that contain sulfated tyrosines (19) (Fig. 2). These regions (a1, a2, and a3 in factor VIII and b1 and b2 in factor V) have been reported to be required for appropriate interaction of both procofactors with α -thrombin. Although the exact location of the six sulfated tyrosines in factor VIII has been confirmed independently by several laboratories (20–22), confirmation of the exact positions of sulfated tyrosines in factor V has not been yet reported. However, earlier data have shown that sulfation is important for factor V activation by α -thrombin and cofactor function (23, 24). These residues in factor V and factor VIII

Cleavage at Arg³²⁰ in prothrombin resulting in meizothrombin generation is required and sufficient for the formation of the active site of α -thrombin and complete exposure of ABE-I of the molecule (45–51). However, meizothrombin does not have a fully exposed ABE-II (50–56). Optimal exposure of this exosite, which is partially covered by fragment 2 of prothrombin, requires cleavage at Arg²⁷¹ (51–57). Hence, most functions associated with ABE-II of α -thrombin are impaired in meizothrombin. For this reason, meizothrombin has reduced clotting activity (48).

Autolysis of native human α -thrombin, or controlled proteolysis of the enzyme by TPCK-trypsin at Arg⁶⁷ and Arg⁷⁷ results in the formation of β -thrombin (58–61). This conversion is responsible for a dramatic but differential loss of several of α -thrombin's enzymatic properties. For example, while β -thrombin conserves its amidolytic activity, it loses its fibrinogen clotting activity and the ability to activate protein C in the presence of thrombomodulin. The loss of these activities is attributed to the removal of eleven amino acids from the B chain of α -thrombin (amino acid residues 63–73) containing critical residues from ABE-I of α -thrombin and resulting in a spatial rearrangement of the remaining amino acids that make up ABE-I. It is quite surprising that the active site histidine, which is contained within the 62-amino acid peptide moiety attached to the rest of the β -thrombin molecule by non-covalent bonds, retains its precise role during catalysis. Whereas the active site of the enzyme appears to be only subtly changed, ABE-I-dependent functions of β -thrombin are dramatically impaired. Thus, the β -thrombin and meizothrombin molecules provide natural probes to examine the functions of the two ABEs independently. The present study utilizes these two thrombin derivatives to elucidate the importance of each ABE in the activation of factor V and factor VIII.

EXPERIMENTAL PROCEDURES

Materials, Reagents, and Proteins—Diisopropyl fluorophosphate, Hepes, Trizma (Tris base), Coomassie Blue R-250, hirudin 55–65 (sulfated hirudin, Hir^{55–65} (SO₃⁻)), hirudin 54–65 (Hir^{54–65}, non-sulfated hirudin), and factor V-deficient plasma were purchased from Sigma. The secondary anti-mouse and anti-sheep IgG coupled to peroxidase were from Southern Biotechnology Associates Inc. (Birmingham, AL). L- α -Phosphatidylserine (PS) and L- α -phosphatidylcholine (PC) were from Avanti Polar Lipids (Alabaster, AL). The chemiluminescent reagent ECL⁺ and heparin-Sepharose were from Amersham Biosciences. Normal reference plasma, prothrombin-deficient plasma, and the chromogenic substrate Spectrozyme-TH were from American Diagnostica Inc. (Greenwich, CT). The thromboplastin reagent (Recombiplastin) for the clotting assays was purchased from Beckman (Fullerton, CA). The fluorescent thrombin inhibitor dansylarginine-N-(3-ethyl-1,5-pentanediylo)amide, human factor Xa, human thrombin, human prothrombin, the monoclonal antibody α hFV#1 coupled to Sepharose, and polyclonal monospecific antibody to prothrombin, were from Hematologic Technologies Inc. (Essex Junction, VT). Human β -thrombin was prepared as described (62) and was purchased from Hematologic Technologies Inc. The B chain of the β -thrombin molecule was cleaved at Arg⁶² and Arg⁷³ (residues numbered consecutively from the beginning of the B chain of thrombin (63)) or Arg⁶⁷ and Arg⁷⁷ in chymotrypsin numbering (64, 65) as determined by NH₂-terminal sequencing (not shown). The β -thrombin used in this study is composed of amino acids 1–62 attached to the rest of the molecule (amino acid residues 73–259) by non-covalent bonds as previously suggested (58, 62). The two monoclonal antibodies to human factor V (against the heavy and light chains of the cofactor, *i.e.* α HFV_{HC}#17 and α HFV_{LC}#9, respectively) were provided by Dr. Kenneth G. Mann (Dept. of Biochemistry, University of

Vermont, Burlington VT) and have been extensively characterized (66, 67). The pentapeptide DYDYQ (D5Q) that was previously shown to inhibit prothrombinase activity and delay factor V activation (68) as well as its sulfated version (D5Q1,2) were custom synthesized by New England Peptide Inc. (Gardner, MA) and by American Peptide Company (Sunnyvale, CA) respectively, purified by high-performance liquid chromatography, and characterized by mass-spectrometry/liquid chromatography. Initial experiments were performed with D5Q1,2 synthesized and characterized in the laboratory of Dr. Satya Yadav at the Cleveland Clinic Foundation. The pentadecapeptide P15H, containing sequence 337–351 of factor Va heavy chain, was also custom synthesized and purchased from New England Peptide Inc. Peptides were usually dissolved in water to a given concentration and used immediately. Although no problem was encountered when working with the sulfated peptide, it is noteworthy that D5Q was highly insoluble at concentrations of >5 mM. D5Q precipitated over time when incubated on ice. Thus, for all experiments D5Q was made fresh and kept at room temperature. Recombinant prothrombin rMZ-II that has only one cleavage site for factor Xa (prothrombin with the substitution Arg¹⁵⁵ → Ala, Arg²⁸⁴ → Ala, and Arg²⁷¹ → Ala) was prepared and purified as described (48, 69, 70). Recombinant purified human factor VIII was provided by Dr. Lisa Regan (Bayer Incorporation, Berkeley, CA). Human factor V was purified using methodologies previously described employing the monoclonal antibody α hFV#1 coupled to Sepharose (71). The cofactor activity of the factor Va preparations was measured by a clotting assay using factor V-deficient plasma and standardized to the percentage of control as described (71). Phospholipids vesicles composed of 75% PC and 25% PS (referred to as PCPS vesicles throughout the manuscript) were prepared as previously described (72). The concentration of phospholipids vesicles was determined by phosphorous assay as described earlier and is given as the concentration of inorganic phosphate (73).

Molecular Dynamics Simulation of the Peptides—The initial three-dimensional models of D5Q and D5Q1,2 were built using Pymol.³ The parameter set used was a modified version of GROMOS-87 (75), implemented in the GROMACS package (76–78) as “ffgmx.” A topology file for D5Q1,2 was generated by the PRODRG server (79). The “pdb2gmx” program from the GROMACS package was used to generate the topology file for D5Q. For the molecular dynamics simulations, the peptide D5Q or D5Q1,2, was placed in a water box by setting the distance between the box walls and the peptide to 10 Å. Initial velocities for atoms were taken from the Maxwellian distribution at 300 K. As solvent we used the single point charge water model (80). Na⁺ ions were added using the “genion” program from the GROMACS package (76–78) to keep the system neutral, by replacing water molecules with Na⁺ ions. Bonds were constrained using the LINCS algorithm (81).

Energy minimization was carried out with the steepest-descent method with the initial step set to 0.1 Å. For the neighbor search, the “grid” option was used, and the list update frequency was set to 10. Periodic boundary conditions were employed in all three dimensions. The cutoff distance for the short-range neighbor list was set to 9 Å. Long range electrostatic interactions were treated using the particle mesh Ewald summation method (82, 83). Ewald summation was performed in all three dimensions. Grid dimension for the fast Fourier transforms was set to 1.6 Å, and the interpolation order was set to 4. Bonds were not constrained during energy minimization. Position restraint molecular dynamics simulation was performed using the “md” integrator with all bonds constrained. The heavy atoms from the peptide were restrained to move around their initial position with a harmonic oscillator func-

³ W. L. DeLano (2002) DeLano Scientific, San Carlos, CA.

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tion. The integration step was set to 2 fs. Position restraint was run for 20 ps. Long range electrostatic interactions were calculated as described in the energy minimization step. The NPT ensemble (constant number of atoms, constant pressure, and constant temperature) was used in all simulations as well as a reference temperature of 300 K. Constant pressure was maintained, *i.e.* reference pressure of 1 bar, using Parrinello-Rahman isotropic pressure coupling with the compressibility set to $4.5 \times 10^{-5} \text{ bar}^{-1}$. All parameters for the molecular dynamics simulations were as described in the position restraint molecular dynamic simulations except that the heavy atoms were allowed to move freely. The peptide, solvent, and the Na^+ ions were separately coupled to a Berendsen thermostat (84) with a reference temperature of 300 K. The molecular dynamics simulation time was set to 50 ns. Distance analysis was performed using the "g_dist" analysis program from the GROMACS package.

Meizothrombin Activation—For experiments performed with rMZ-II, reaction mixtures composed of 30 μM PCPS, 10 nM FVa, 0.5 nM FXa, and 1.4 μM rMZ-II were incubated in buffer composed of 20 mM Tris, 0.15 M NaCl, pH 7.4 (Tris-buffered saline), 5 mM Ca^{2+} at ambient temperature. Activation of rMZ-II was monitored by removing aliquots of the reaction mixture at various times and diluting to 50 nM rMZ-II into a quench buffer in a 96-well plate. A 50 nM dilution of α -thrombin was also incubated into quench buffer for comparison. Both samples were incubated with Spectrozyme-TH, and the optical density was determined at 405 nm. Under these conditions, it was established that a 7.5-min incubation of rMZ-II with prothrombinase yielded optimally active meizothrombin (rMZ-IIa). In various experiments the level of activity of rMZ-IIa obtained with respect to cleavage of Spectrozyme-TH was 92–118% of the activity obtained with a similar concentration of α -thrombin. These findings are in agreement with previous data showing the potency of rMZ-IIa against a chromogenic substrate measuring thrombin activity (85). Thus, in all experiments described, rMZ-II was incubated with 0.5 nM prothrombinase for 7.5 min followed by the addition of a 2-fold excess of tick anticoagulant protein (1 nM) to stop the reaction. Prior to all experiments using rMZ-II, 50 nM rMZ-IIa and 50 nM thrombin were always compared for activity by chromogenic substrate to ensure that activation occurred, and rMZ-IIa was used immediately following activation.

Thrombin Time Assays—Peptides were screened for their capability to inhibit thrombin's ability to generate a fibrin clot in a thrombin assay using normal hemostasis reference plasma. Various concentrations of peptide were incubated with 20 nM thrombin for 5 min at ambient temperature in a final volume of 75 μl and then mixed with an equal volume of plasma. Final concentration of the enzyme was 10 nM in all assays. The reaction mixture was incubated in a water bath set to 37 °C and monitored by manually tilting the tube until a fibrin clot was observed. All reactions were performed in triplicate, and the various clotting times were plotted as time (seconds) *versus* final peptide concentration (micromolar).

Prothrombin Clotting Assays—To screen the ability of the peptides to inhibit prothrombin function in whole plasma a clotting assay using prothrombin-deficient plasma was employed. To determine the optimal concentration of prothrombin to be used in these assays, a standard curve was first generated using prothrombin concentrations in a 350 nM to 2.5 nM range. The clotting assay was performed as follows: 50 μl of prothrombin-deficient plasma was added to 100 μl of thromboplastin reagent, and the reaction was started by the addition of 50 μl of sample. The reaction was incubated at 37 °C in a water bath, and the time to clot generation was measured. Under these conditions, 25 nM prothrombin (final concentration in the assay) was found to produce an average clot-

ting time of 27 s, which was located on the linear portion of the standard curve. This concentration of prothrombin was used for all clotting assays. Peptides were incubated with prothrombin for 5 min at ambient temperature before being used in the clotting assay. All experiments were performed at least in triplicate.

Gel Electrophoresis and Western Blotting—SDS-PAGE analyses were performed using 4–12% gradient gels (for factor V analyses) or 5–15% (for factor VIII analyses) according to the method of Laemmli (86). In several experiments, proteins were transferred to polyvinylidene difluoride membranes according to the method described by Towbin *et al.* (87). After transfer to nitrocellulose, factor V heavy and light chain(s) were detected using the appropriate monoclonal and polyclonal antibodies. Immunoreactive fragments were visualized with chemiluminescence.

Inhibition of Factor V and Factor VIII Cleavage by D5Q and D5Q1,2—The ability of the peptides to inhibit α -thrombin, β -thrombin, and rMZ-IIa to cleave and activate the cofactors, was assessed over time by SDS-PAGE. Peptides or an equivalent amount of Tris-buffered saline with Ca^{2+} were incubated with the enzymes for 5 min at ambient temperature. Reaction mixtures containing 500 nM cofactor (plasma-derived human factor V or recombinant human factor VIII) were diluted in Tris-buffered saline plus Ca^{2+} . An aliquot was removed at zero time for analysis by SDS-PAGE. In experiments with rMZ-IIa, 20 μM PCPS was also added into the reaction mixtures. The final concentrations of peptides and various enzymes in the mixtures were 100 μM and 2 nM, respectively, or as indicated. Time courses were started by the addition of the enzyme species and peptide mixtures, and samples were removed at time points described in the legend to the figures. In the case of all the factor VIII activation experiments, 10 μg of protein was removed per time point, and gels were stained by Coomassie Brilliant Blue. In the case of all factor V activation experiments, 1 μg of protein was removed per time point for gel electrophoresis. Gels were transferred to polyvinylidene difluoride membranes followed by immunoblotting with specific monoclonal antibodies. In all experiments, reactions were performed simultaneously in the presence and in absence of peptides.

Immunoblotting of Whole Plasma Samples—The modified clotting assay used in our studies to compare the efficiency of prothrombin and meizothrombin for clot formation was described in details elsewhere (66). Using the same methodology and prothrombin-deficient plasma, the capability of rMZ-II to induce clotting was also assayed. In this latter case, plasma was diluted 10-fold in a CaCl_2 -containing buffer, and 140 nM rMZ-II was added prior to the initiation of clotting by the addition of PCPS vesicles. At selected time intervals before and after clot formation given in the legend to the figures, samples were withdrawn and analyzed by SDS-PAGE following by immunoblotting with monoclonal antibodies to factor V or with a monospecific polyclonal antibody to prothrombin. Immunoreactive fragments were visualized with chemiluminescence. The films were scanned with a Lexmark printer/scanner; the final images were captured as TIFF files and analyzed by densitometry using the software UN-SCAN-IT gel (Silk Scientific, Orem, UT).

RESULTS

Thrombin Derivatives and Thrombin Time—Meizothrombin is an intermediate formed during prothrombin activation that has enzymatic activity. Its properties are difficult to study because of autocatalytic events resulting in its instability. Recombinant meizothrombin that has been mutated to contain only one cleavage site for factor Xa (*i.e.* at Arg³²⁰, rMZ-II) and is stable for several weeks at 4 °C (69) was activated to produce rMZ-IIa. Thrombin time of normal human plasma in the

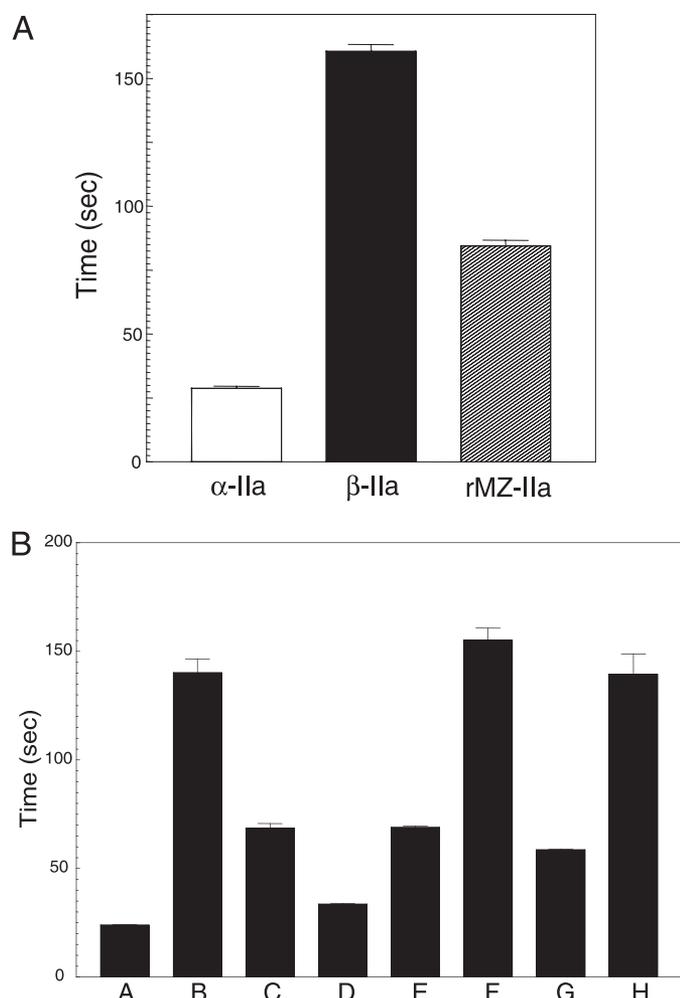


FIGURE 3. *A*, comparison of the three forms of thrombin. The average clotting times obtained in six different measurements in the presence of 10 nM enzyme are shown as follows: white, α -thrombin; black, β -thrombin; and hatched, rMZ-IIa. *B*, comparison of the activity of the peptides. The average clotting time found in three different measurements in prothrombin-deficient plasma are shown as follows: *A*, 25 nM prothrombin; *B*, 25 nM rMZ-II; *C*, 25 nM prothrombin that was preincubated with 250 μ M D5Q1,2; *D*, 25 nM prothrombin that was preincubated with 250 μ M P15H; *E*, 100 nM rMZ-II; *F*, 100 nM rMZ-II that was preincubated with 250 μ M D5Q1,2; *G*, 25 nM prothrombin that was preincubated with 50 μ M Hir⁵⁴⁻⁶⁵; *H*, 25 nM prothrombin that was preincubated with 50 μ M Hir⁵⁵⁻⁶⁵(SO₃⁻).

presence of 10 nM α -thrombin was 26 s, while under similar experimental conditions, thrombin time with β -thrombin was 160 s (Fig. 3A). A similar thrombin time was obtained in a control experiment when buffer was substituted for protein (170 ± 12 s); thus, β -thrombin has negligible clotting activity. An analogous experiment with rMZ-IIa resulted in a thrombin time that was ~ 4 -fold increased (97 s) corresponding to $\sim 7\%$ of the clotting activity of α -thrombin (Fig. 3A). These data demonstrate that both ABEs of α -thrombin are required for timely clot formation in whole plasma. However, ABE-I appears to be more important for α -thrombin functions in whole plasma, because rMZ-IIa promotes fibrinogen clotting faster than β -thrombin albeit less efficiently than α -thrombin.

Effect of Synthetic Peptides on Prothrombin Activity during Clotting—We have previously shown that a pentapeptide from the COOH terminus of factor Va heavy chain inhibits thrombin and prothrombin functions, because it represents a thrombin/prothrombin binding exosite for factor V and factor Va, respectively (DYDYQ, D5Q) (68). The pentapeptide contains two tyrosine residues that have the potential to be sulfated. To ascertain the effect of tyrosine sulfation on the inhibitory potential of

the pentapeptide, we obtained the double sulfated version of the pentapeptide, and we tested its ability to inhibit clotting in prothrombin-deficient plasma. In Fig. 3B, bars *A*, *B*, and *E* show control clotting times obtained when 25 nM prothrombin, 25 nM rMZ-II, and 100 nM rMZ-II, respectively, were added to prothrombin-deficient plasma. In the presence of 250 μ M D5Q1,2 the clotting time of prothrombin and rMZ-II was increased 3-fold (bars *C* and *F*, respectively). This effect of D5Q1,2 was similar to effect observed with non-sulfated hirudin (Hir⁵⁴⁻⁶⁵, bar *G*) but was 2-fold lower than the effect produced by sulfated hirudin (Hir⁵⁵⁻⁶⁵(SO₃⁻), bar *H*). Overall the data demonstrate that D5Q1,2 is a potent inhibitor of α -thrombin and prothrombin functions in whole plasma.

Effect of D5Q1,2 on Procofactor Cleavage by α -Thrombin—Based on several previous observations suggesting that activation of factor V and factor VIII requires the integrity of both ABEs of thrombin (32, 33), we initiated a series of studies on the cleavage and activation of factor V and factor VIII by α -thrombin and β -thrombin to ascertain the contribution of each of the two ABEs separately to procofactor cleavage and activation. We have also evaluated the inhibitory effect of D5Q1,2 on all enzymes studied. Fig. 4A, lanes 2–9, show that, under the conditions employed, factor V activation by α -thrombin is complete by 15 min. Preincubation of α -thrombin with 100 μ M D5Q1,2 resulted in considerable decrease of the rate of cleavage at Arg⁷⁰⁹ while cleavage at Arg¹⁵⁴⁵ was almost completely abrogated (Fig. 4B). Similar results were found with the non-sulfated version of the peptide however, the non-sulfated peptide was less effective in inhibiting factor V activation under similar experimental conditions (not shown). Analogous experiments conducted with β -thrombin in the absence of D5Q1,2 demonstrated slow cleavage at Arg⁷⁰⁹ while cleavage at Arg¹⁵⁴⁵ did not occur during the time interval studied (Fig. 5A, lanes 2–9). Following incubation of β -thrombin with D5Q1,2, slight inhibition of cleavage at Arg⁷⁰⁹ was observed (Fig. 5B, lanes 2–9). Overall our findings provide evidence for the requirement of ABE-I of α -thrombin for timely cleavage at Arg¹⁵⁴⁵ of factor V. This cleavage that results in the formation of the light chain of factor Va, is rate-limiting during activation of factor V and is required for expression of optimal cofactor activity. Thus, α -thrombin interaction with factor V through ABE-I is required for cleavage of the procofactor at Arg¹⁵⁴⁵ and expression of optimum cofactor activity.

We next assessed the effect of α - and β -thrombin on factor VIII activation (Fig. 6). Under the conditions employed, factor VIII was cleaved by α -thrombin at all three activating cleavage sites to produce the expected fragments (Fig. 6A). Although β -thrombin cleaved factor VIII slowly at Arg⁷⁴⁰ to produce the M_r 90,000 heavy chain (Fig. 6B, lanes 2–8), the enzyme was unable to cleave factor VIII at Arg³⁷² and Arg¹⁶⁸⁹ (Fig. 6B, lanes 2–8). These two latter cleavages are required for procofactor activation (16–18). Cleavage at Arg³⁷² and Arg¹⁶⁸⁹ occurred readily following incubation of the β -thrombin-cleaved procofactor with α -thrombin (Fig. 6B, lane 9). These data demonstrate requirement of ABE-I for factor VIII activation. Factor VIII activation by α -thrombin was severely inhibited by D5Q1,2 because of impaired cleavage at all three sites. However, cleavages at Arg³⁷² and Arg¹⁶⁸⁹ by α -thrombin were more susceptible to inhibition by the sulfated pentapeptide than cleavage at Arg⁷⁴⁰ (Fig. 6C). The non-sulfated peptide inhibited α -thrombin activation of factor VIII albeit less efficiently (not shown). Fig. 6D shows that cleavage of factor VIII by β -thrombin at Arg⁷⁴⁰ was also inhibited by D5Q1,2. Overall, the data demonstrate that, like factor V activation, the two required factor VIII-activating cleavages necessitate the integrity of ABE-I. Altogether, our findings suggest that exposure of ABE-I alone is required and sufficient for efficient cleavage and activation of both procofactor molecules.

Role of ABE-I for Factors V and VIII Activation

FIGURE 4. Effect of D5Q1,2 on factor V activation by α -thrombin. *A*, activation of factor V (500 nM) by α -thrombin alone (2 nM). Factor V was incubated with α -thrombin as described under "Experimental Procedures." *B*, α -thrombin was preincubated with D5Q1,2, and the mixture was added to factor V. At selected time intervals, aliquots of the mixtures were removed, mixed with 2% SDS, heated for 5 min at 90 °C, and analyzed on a 4–12% SDS-PAGE followed by immunoblotting. Fragments were identified following staining with a mixture of monoclonal antibodies α HFV#17 that recognizes an epitope between amino acid residues 307–506 of factor V and α HFV#9 that recognizes an epitope on the light chain of the active cofactor. Immunoreactive bands were visualized with chemiluminescence. *Lane 1* in both panels depicts aliquots of the mixture withdrawn from the reaction before the addition of thrombin or α -thrombin/peptide mixture, whereas *lanes 2–9* show aliquots of the reaction mixture withdrawn at 30 s, 1 min, 5 min, 10 min, 15 min, 30 min, 1 h, and 2 h following the addition of α -thrombin alone or of α -thrombin/peptide mixture. The positions of the light and heavy chain of factor V as well as of single chain factor V are indicated at the *right* (for more details refer to Fig. 1).

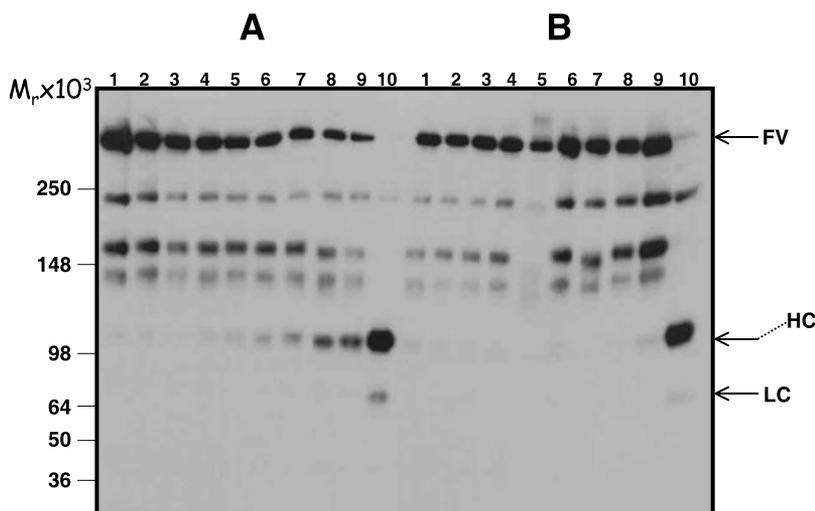
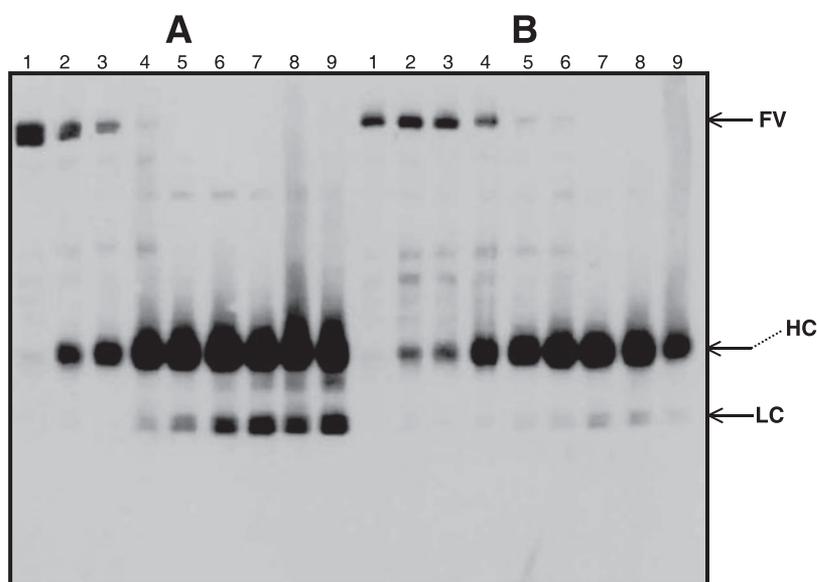


FIGURE 5. Effect of D5Q1,2 on factor V activation by β -thrombin. *A*, activation of factor V (500 nM) by β -thrombin alone (2 nM). Factor V was incubated with β -thrombin as described under "Experimental Procedures." *B*, β -thrombin was preincubated with D5Q1,2, and the mixture was added to factor V. At selected time intervals, aliquots of the mixtures were removed and treated as described in the legend to Fig. 4. *Lane 1* in both panels depicts aliquots of the mixture withdrawn from the reaction before the addition of thrombin or thrombin/peptide mixture, whereas *lanes 2–9* show aliquots of the reaction mixture withdrawn at 30 s, 1 min, 5 min, 10 min, 15 min, 30 min, 1 h, and 2 h following the addition of thrombin alone or of β -thrombin/peptide mixture. *Lane 10* in both panels represents an aliquot of the mixture shown in *lane 9* treated with α -thrombin for an additional 10 min. The positions of the light and heavy chains of factor V as well as of single chain factor V are indicated at the *right* (for more details refer to Fig. 1).

Activation of Factor V and Factor VIII by Meizothrombin—To ascertain if exposure of ABE-I alone is sufficient for efficient procofactor activation we used rMZ-IIa. Meizothrombin has been reported to cleave and activate factor V only in the presence of a membrane surface (47, 49). The data obtained herein demonstrate that factor V is readily cleaved and activated by membrane-bound rMZ-IIa (Fig. 7A). Factor V activation by membrane-bound rMZ-IIa was completely abrogated by D5Q1,2 because of impaired cleavages at Arg⁷⁰⁹, Arg¹⁰¹⁸, and Arg¹⁵⁴⁵ (Fig. 7B). Direct comparison of the rates of cleavage of membrane-bound factor V by α -thrombin and meizothrombin revealed that, under similar experimental conditions, meizothrombin cleaves membrane-bound factor V with a rate that is 5-fold faster than the rate of cleavage of the membrane-bound procofactor by α -thrombin (not shown). Overall the data demonstrate that amino acids from ABE-I interact with factor V in order for the molecule to be efficiently cleaved at all three sites by membrane-bound rMZ-IIa. Thus, exposure of ABE-II is not required and does not appear to play any significant role in factor V activation.

We next assessed the capability of membrane-bound rMZ-IIa to cleave and activate recombinant human factor VIII. Activation of the procofactor by membrane-bound rMZ-IIa occurred readily during a 20-min incubation period (Fig. 8A, *lanes 1–8*). Cleavage and activation

of factor VIII by membrane-bound rMZ-IIa was completely inhibited by 100 μ M D5Q1,2 (Fig. 8B, *lanes 1–8*). Even cleavage at Arg⁷⁴⁰ by membrane-bound rMZ-IIa was susceptible to inhibition by the sulfated pentapeptide. This cleavage was partially resistant to inhibition when using α -thrombin (see Fig. 6C). These data demonstrate that membrane-bound rMZ-IIa is more sensitive to D5Q1,2 inhibition than α -thrombin.

Activation of factor V during Clot Formation—Our data suggest that meizothrombin is a potent activator of both procofactors. However, while extensive data in the literature provides evidence that meizothrombin is impaired in its fibrinogen-clotting activity, no data have been yet reported assessing the clotting activity of meizothrombin in prothrombin-deficient plasma.

To assess the activity of meizothrombin in whole plasma, a previously described modified clotting assay was employed (66). When PCPS vesicles and CaCl₂ were added to normal plasma, clotting was observed in 35 min. Under similar experimental conditions, where prothrombin-deficient plasma was complemented with rMZ-II, clotting also occurred following ~35-min incubation (Fig. 9, *vertical arrows*). Fig. 9A depicts products from factor V formed during the experiment prior and after clot formation. Clot formation in both cases correlates with appearance of both the heavy and light chains of factor Va. Prolonged incubation of

FIGURE 6. Effect of D5Q1,2 on factor VIII activation by α - and β -thrombin. *A*, activation of factor VIII (500 nM) by α -thrombin alone (2 nM). *B*, activation of factor VIII (500 nM) by β -thrombin alone (2 nM). *C*, α -thrombin was preincubated with D5Q1,2, and the mixture was added to factor VIII. *D*, β -thrombin was preincubated with D5Q1,2, and the mixture was added to factor VIII. At selected time intervals, aliquots of the mixtures were removed mixed with 2% SDS, 2% β -mercaptoethanol, heated for 5 min at 90 °C, and analyzed on a 5–15% SDS-PAGE followed by staining with Coomassie Blue. *Lane 1* in all panels depicts aliquots of the mixture withdrawn from the reaction before the addition of thrombin or thrombin/peptide mixture, whereas *lanes 2–8* show aliquots of the reaction mixture withdrawn at 30 s, 1 min, 3 min, 5 min, 10 min, 15 min, and 20 min following the addition of thrombin alone or of thrombin/peptide mixture. *Lane 9* in *B* and *D* is an aliquot of the mixture at 20 min treated with α -thrombin for an additional 10 min. The positions of all factor VIII fragments are indicated at the *right* (for more details refer to Fig. 1).

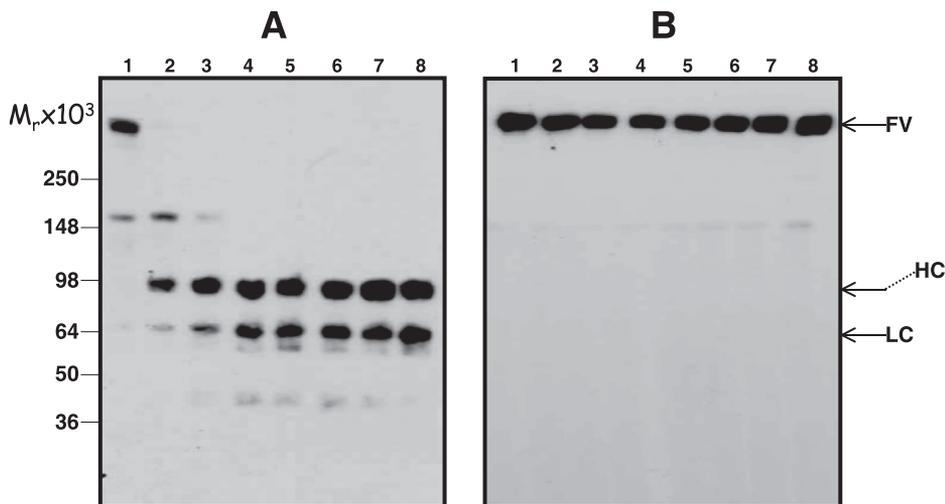
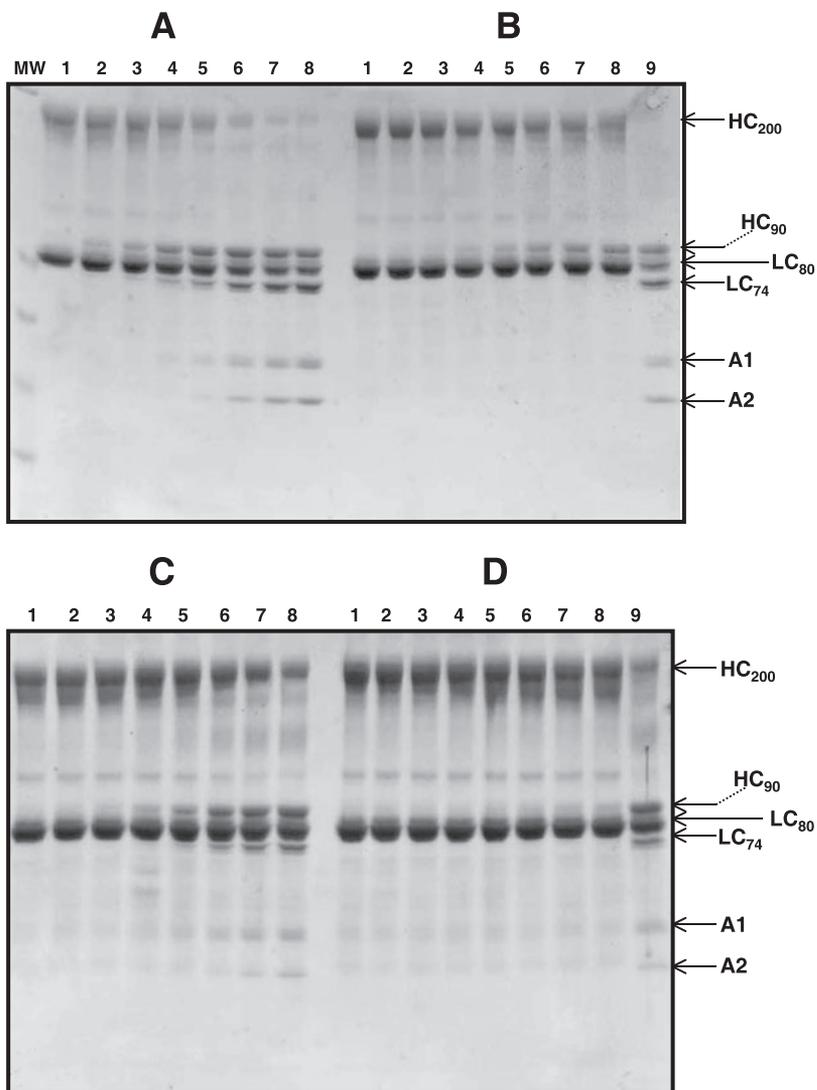


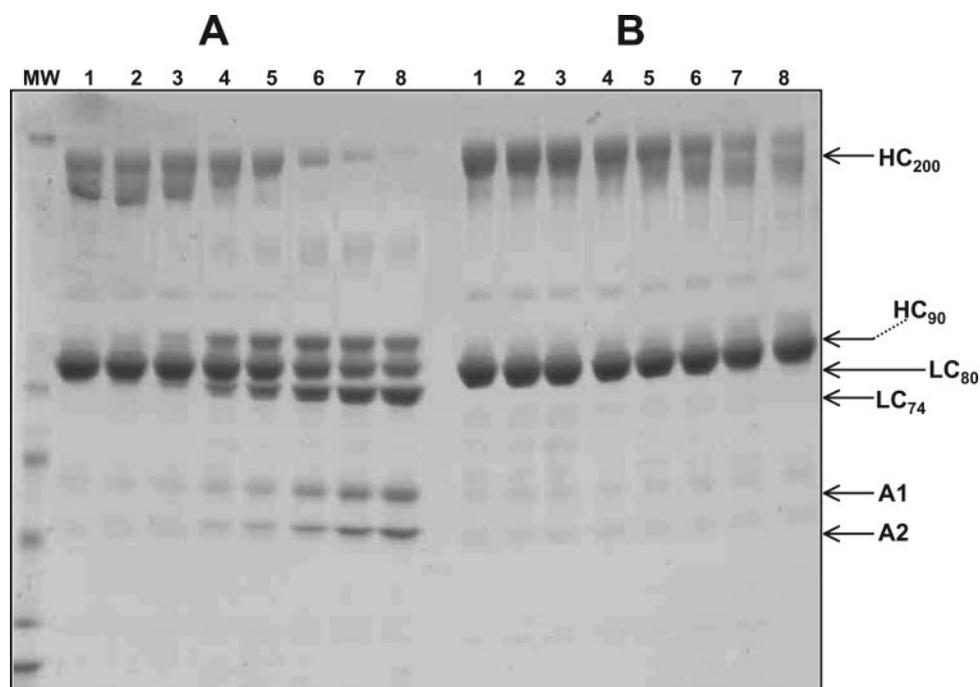
FIGURE 7. Effect of D5Q1,2 on factor V activation by meizothrombin. *A*, activation of factor V (500 nM) by membrane-bound rMZ-IIa (2 nM). *B*, membrane-bound meizothrombin was preincubated with D5Q1,2, and the mixture was added to factor V. At selected time intervals, aliquots of the mixtures were removed and treated as described in the legend to Fig. 4. *Lane 1* in both panels depicts aliquots of the mixture withdrawn from the reaction before the addition of rMZIIa or rMZIIa/peptide mixture; whereas *lanes 2–8* show aliquots of the reaction mixture withdrawn at 30 s, 1 min, 3 min, 5 min, 10 min, 15 min, and 20 min following the addition of rMZ-IIa alone or of rMZ-IIa/peptide mixture. The positions of the light and heavy chains of factor V as well as of single chain factor V are indicated at the *right* (for more details refer to Fig. 1).

the samples at 37 °C results in the appearance of fragments of M_r 75,000, 54,000, and 30,000 characteristic of the degradation of factor Va heavy chain by intrinsically formed activated protein C (APC) (2, 66, 67). Surprisingly, when prothrombin-deficient plasma is supplemented with physiological concentrations of rMZ-II the heavy chain of factor Va appears to be significantly more degraded than the factor Va heavy

chain obtained in normal plasma (compare in Fig. 9A, lanes 14 and 7, respectively). Scanning densitometry demonstrated that when prothrombin-deficient plasma is incubated with plasma prothrombin, 85% of the heavy chain remains following 60-min incubation (Fig. 9A, lane 7). In contrast, when prothrombin-deficient plasma is supplemented with rMZ-II, only 35% of the heavy chain remains following 60-min

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FIGURE 8. Effect of D5Q1,2 on factor VIII activation by meizothrombin. *A*, activation of factor VIII (500 nM) by membrane-bound rMZ-IIa (2 nM). *B*, membrane-bound meizothrombin was preincubated with D5Q1,2, and the mixture was added to factor VIII. At selected time intervals, aliquots of the mixtures were removed and treated as described in the legend to Fig. 6. Lane 1 in both panels depicts aliquots of the mixture withdrawn from the reaction before the addition of rMZIIa or rMZIIa/peptide mixture, whereas lanes 2–8 show aliquots of the reaction mixture withdrawn at 30 s, 1 min, 3 min, 5 min, 10 min, 15 min, and 20 min following the addition of rMZ-IIa alone or of rMZ-IIa/peptide mixture. The positions of the light and heavy chains of factor VIII are indicated at the right (for more details refer to Fig. 1).



incubation. A logical explanation for this significant difference in the degradation of factor Va heavy chain, can be provided by the fact that under the experimental conditions employed more APC is generated following clot formation by meizothrombin than by α -thrombin. These data are in total agreement with previous kinetic studies (48) and demonstrate for the first time that, in whole plasma, meizothrombin is a better activator of protein C in the presence of PCPS than α -thrombin. Data shown in Fig. 9B demonstrate that clotting coincides with the formation of thrombin (arrows). Overall, our findings establish meizothrombin as a potent procoagulant and verify the fact that, in the presence of a procoagulant membrane surface, and in the absence of thrombomodulin (TM), meizothrombin is a more efficient anticoagulant than α -thrombin with respect to protein C activation and factor Va heavy chain cleavage.

Molecular Dynamics Simulations of the Inhibitory Peptides—In addition to all the data thus far presented, we have identified D5Q1,2 as a potent inhibitor of α -thrombin. The peptide appears to interact with ABE-I of the enzyme impairing several ABE-I-related functions. Sulfation of D5Q on both tyrosines significantly increases its potency with respect to α -thrombin inhibition when compared with the non-sulfated peptide (68). To ascertain the differences in peptide conformation following sulfation of D5Q, a 50-ns molecular dynamics simulation of the peptides in aqueous solution was performed, and snapshots of each molecule are shown in Fig. 10. Although the distance between the α carbon of Asp1 and the α carbon of Gln5 was always greater than 1 nm, D5Q was found to interchange conformations periodically between a linear and a packed conformation. In $\sim 40\%$ of the simulation time, D5Q adopted a packed conformation with Tyr² and Tyr⁴ facing each other (see Fig. 10A). Although the distance between the hydroxyl groups of Tyr² and Tyr⁴ varies between 3.5 and 5 Å, which is approximately twice the minimal distance allowed for a typical hydrogen bond to occur (~ 1.8 Å), the packed conformation appeared to be the preferred conformation for D5Q approximately half the time in solution. Thus, the fact that the two phenyl groups in D5Q face each other periodically, may explain the insolubility problems encountered when working with high concentrations of D5Q. In contrast, D5Q1,2 preferred a linear rather than a packed conformation (Fig. 10B). D5Q1,2 was found in the latter

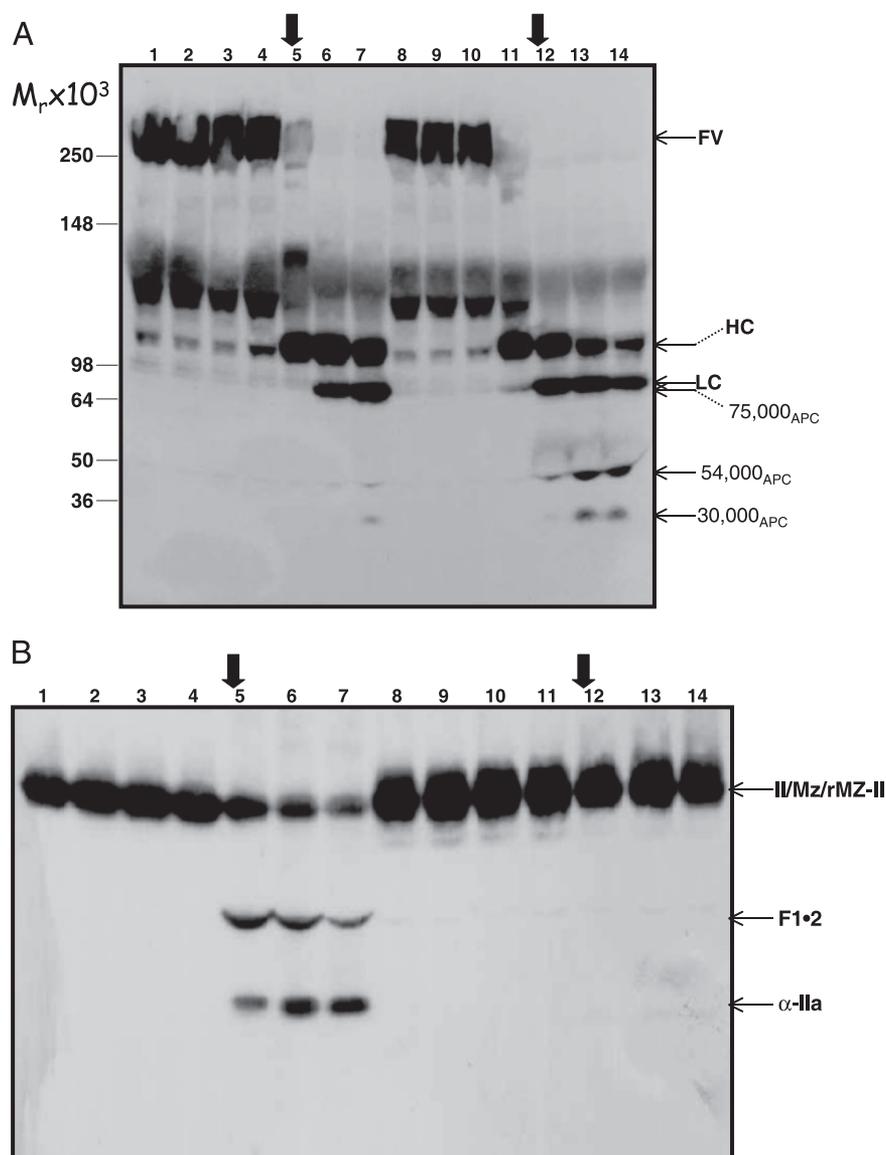
conformation only 1% of the total simulation time. In addition, no solubility problems were encountered when working with D5Q1,2. The data suggest that the two sulfate moieties in D5Q1,2 are repulsive leading to an open conformation most of the time, which in turn, must favor interaction of D5Q1,2 with α -thrombin compared with D5Q. Moreover, D5Q1,2 has two additional negative charges that can interact with basic amino acids from ABE-I of α -thrombin and meizothrombin. In sum, D5Q1,2 has an advantage over D5Q, because it possesses overall more negative charges available to interact with more positive charges from ABE-I of α -thrombin. All these facts may explain the increased potency for inhibition of α -thrombin function by D5Q1,2 when compared with inhibition of the enzyme by D5Q.

DISCUSSION

The data presented herein using natural and minimal modified recombinant proteins demonstrate for the first time that: 1) exposure of ABE-I of α -thrombin alone is required and sufficient for timely and efficient activation of factor V and factor VIII; 2) a thrombin molecule that has only ABE-II available is unable to efficiently cleave and activate both procofactors; 3) membrane-bound meizothrombin can efficiently cleave and activate factor VIII; 4) meizothrombin is a more efficient activator of factor V than α -thrombin in the presence of a procoagulant membrane surface; 5) membrane-bound meizothrombin, at the place of vascular injury, can efficiently account for both clot initiation as well as protein C activation (in the absence of TM); and 6) a sulfated pentapeptide mimicking an α -thrombin interactive site on factor V (D5Q1,2) is a new ABE-I ligand that impedes α -thrombin function in whole plasma and impairs activation of both procofactors. Collectively, the data presented in this report strongly support the notion that membrane-bound meizothrombin can sustain initiation and termination of blood clotting during normal hemostasis.

Human thrombin's function is allosterically regulated through the binding of Na⁺ (88–91). The important amino acids for the expression of thrombin's sodium-induced allosteric regulation were identified using 78 alanine mutants of thrombin (92). The fast form of thrombin (bound to Na⁺) is procoagulant, because it cleaves fibrinogen more efficiently than the slow form of the enzyme (Na⁺ free), which in turn is

FIGURE 9. Proteolysis of factor V and prothrombin during clot formation. Normal plasma (lanes 1–7) was diluted 10-fold in a CaCl_2 -containing buffer following by the addition of PCPS vesicles. Prothrombin-deficient plasma (lanes 8–14) was also diluted 10-fold in a CaCl_2 -containing buffer, supplemented with rMz-II (140 nM) and clotting was initiated with PCPS vesicles. Clotting in both samples occurred at ~ 32 min (arrowheads on top). Lanes 1 and 8 represent control samples prior to the addition of PCPS vesicles. Lanes 2–7 and 9–14 depict aliquots of the reaction mixture at 5, 15, 25, 35, 45, and 60 min following the addition of PCPS vesicles. All samples were analyzed by SDS-PAGE (4–12% linear gradient) under non-reducing conditions followed by transfer to polyvinylidene difluoride membranes. **A**, results of an immunoblot probed with a mixture of monoclonal antibodies to human factor Va (i.e. $\alpha\text{HFV-17}$ that recognizes an epitope on the heavy chain of the cofactor and $\alpha\text{HFV-9}$ that recognizes an epitope on the light chain of factor V (66)). The arrows at right depict the position of single chain factor V (FV), the M_r 105,000 heavy chain of factor Va (HC), the M_r 74,000 light chain of the cofactor (LC), the M_r 75,000 fragment deriving from cleavage of the heavy chain at Arg^{306} (amino acids 1–506, $75,000_{\text{APC}}$), the M_r 30,000 fragment obtained from cleavage of factor Va at $\text{Arg}^{506}/\text{Arg}^{306}$ (amino acids 307–506, $30,000_{\text{APC}}$), and the M_r 54,000 fragment deriving from initial cleavage of the heavy chain by APC at Arg^{306} amino acids (306–679, $54,000_{\text{APC}}$). The positions of the molecular weight markers are indicated at the left. **B**, result of an immunoblot probed with a polyclonal monospecific antibody to human prothrombin made in sheep. The arrows at the right represent the position of fragment 1·2 (amino acids 1–271) and α -thrombin.



considered to be the anticoagulant version of thrombin, because it activates protein C more efficiently (91). The fast form of human thrombin specifically recognizes substrates such as factor V and factor VIII (33, 93). Recent data have shown that murine thrombin does not respond to sodium regulation, because the enzyme is locked in a conformation that favors the fast form (94).

It has been established that two cleavages in factor VIII (i.e. Arg^{372} and Arg^{1689}) and one cleavage in factor V (i.e. Arg^{1545}) are required for expression of optimum cofactor activity. Both anion binding exosites of thrombin were reported to be implicated in the cleavage and activation of both procofactors (32, 33, 93, 95). Previous data using site-directed mutagenesis suggested that, while the integrity of anion binding exosite I represents a requirement for efficient proteolytic cleavage of factor VIII at Arg^{372} and Arg^{1689} , anion binding exosite II appears to have a small contribution for the cleavage at Arg^{372} . Alanine scanning mutagenesis also assigned important roles for ABE-I, ABE-II, and the Na^+ binding site of thrombin for the activation of factor V. However, the minimum structural determinants from thrombin, necessary for the activation of both procofactors have not yet been determined.

Earlier data demonstrated that purified bovine β -thrombin (a natural derivative of α -thrombin) can slowly cleave bovine factor V at Arg^{1006}

(equivalent to Arg^{1018} in the human molecule) (9). Further, cleavages at Arg^{713} and Arg^{1536} (equivalent to Arg^{709} and Arg^{1545} , respectively, in human factor V) and formation of the heavy and light chains were severely impaired when using β -thrombin, with cleavage at Arg^{1536} being almost completely resistant to β -thrombin (no light chain formation following a 20-min incubation period). Moreover, cleavage at Arg^{713} and Arg^{1536} occurred readily following incubation of the β -thrombin-cleaved bovine procofactor with bovine α -thrombin (9). Our data show that β -thrombin that has poor clotting activity and only ABE-II available, is unable to cleave plasma-derived human factor V at Arg^{1545} and recombinant factor VIII at Arg^{372} and Arg^{1689} but can promote cleavage at Arg^{709} in factor V and at Arg^{740} in factor VIII, albeit less efficiently than α -thrombin. In contrast, meizothrombin, which unlike β -thrombin is impaired in all of ABE-II-related functions of α -thrombin, can efficiently cleave and activate both procofactors. Altogether, the data suggest that, while both ABEs have been reported to be involved in factor V and factor VIII activation to various degrees, amino acids from ABE-I appear to be solely responsible and sufficient for the primary interaction between the enzyme and the procofactors, thereby controlling efficient activation. In addition, because purified human β -thrombin is a natural derivative of α -thrombin, all concerns relating

Role of ABE-I for Factors V and VIII Activation

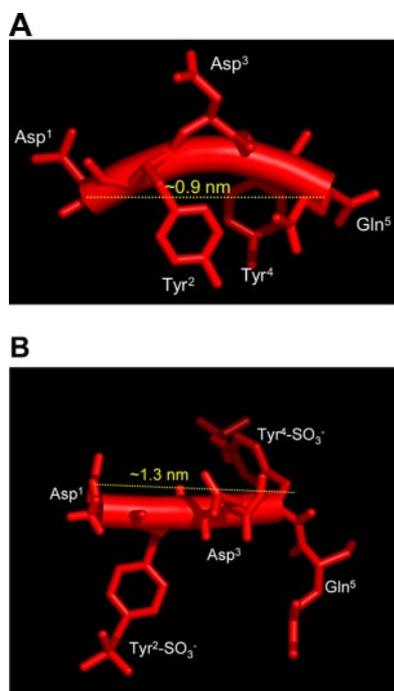


FIGURE 10. **Solution conformation of D5Q and D5Q1,2.** Molecular dynamic simulations of the peptides were performed as described under "Experimental Procedures." The two panels show a representation with schematics and sticks for D5Q (A) and D5Q1,2 (B). Snapshots for both peptides were taken at 10 ns. The dotted yellow line in both panels measures the length of the peptides between the C^α of Asp¹ and the C^α of Gln⁵.

to conformational changes resulting in functional alterations that are valid for recombinant proteins are not applicable to the β -thrombin molecule used in this study. Overall, our findings assign a novel function to ABE-I: interaction with amino acids from the $\alpha 1$ and $\alpha 3$ domains of factor VIII and the $\beta 2$ domain of factor V, which in turn are required for cleavage and activation of both procofactors. Further, because ABE-I of α -thrombin is readily available in meizothrombin, our data are in agreement with previous and recently published work, suggesting that meizothrombin may be responsible for procofactor activation *in vivo* during the critical initial steps of coagulation (46, 47, 49).

Complete knock-out of the TM gene causes embryonic lethality before the assembly of a vascular system (96, 97). In addition, a mouse model in which TM could not serve as a cofactor for thrombin for protein C activation (TM^{Pro/Pro}) generated mice with increased intravascular fibrin deposition in some tissues, however, the mutant mice remained free of thrombosis and exhibited normal life span (98, 99). Further, the mutant mice failed to show any fibrin deposition in the brain or kidney suggesting that anticoagulation could be regulated differently depending on the tissue (98). On the other hand, *in vitro* experiments have demonstrated that, in the presence of TM, thrombin and meizothrombin have similar catalytic efficiencies with respect to APC formation (48, 100, 101). However, in the presence of PCPS vesicles or a cell surface, the catalytic efficiency of meizothrombin for the activation of protein C is 6-fold higher than the activity of thrombin for the same reaction (48, 101), and this increased efficiency is specifically due to the binding of meizothrombin to the membrane surface, because meizothrombin desF1 has the same activity as thrombin for the activation of protein C in the presence of TM and in the presence or absence of a membrane surface (48). Our data show that, in whole plasma, in the absence of TM but in the presence of PCPS vesicles, meizothrombin generates APC much faster than thrombin as shown by the increase in appearance of APC-degradation products from factor Va heavy chain. Our findings thus provide a logical explanation for the fact that require-

ment for TM may be bypassed during normal anticoagulation, locally at the place of vascular injury by meizothrombin. Moreover, because meizothrombin remains bound to the injured surface through its fragment 1 component, it is most likely that it will come in contact with membrane-bound protein C as compared with thrombin that is free in solution. Collectively, our findings strongly support the notion that membrane-bound meizothrombin can initiate clotting and subsequent protein C activation in the presence of a procoagulant membrane surface exposed following injury of the vessel wall.

The importance of initial cleavage of prothrombin at Arg³²⁰, resulting in meizothrombin formation and total ABE-I exposure (55, 57, 69), compared with that of cleavage at Arg²⁷¹ alone, *in vivo*, is underscored by the severity of symptoms of individuals heterozygous for prothrombin San Antonio, which are in sharp contrast with the milder symptoms of homozygous and heterozygous individuals for prothrombin Dhahran, prothrombin Padua I, and prothrombin Barcelona (102–107). Prothrombin San Antonio has histidine instead of arginine at position 320 (102), prothrombin Dhahran and Padua I have histidine instead of arginine at position 271 (103–105), and prothrombin Barcelona has cysteine at position 271 (106, 107). Heterozygous individuals for prothrombin San Antonio have severe bleeding disorders following surgery or trauma, while homozygous patients for prothrombin Barcelona and Dhahran have mild to moderate bleeding episodes following challenge (102, 103, 107). Thus, while the *in vitro* data suggest that ABE-I, which is the primary binding site for fibrinogen, is fully exposed upon cleavage at Arg³²⁰, all individuals having a circulating prothrombin molecule that is unable to be cleaved at Arg²⁷¹ have mild/moderate tendency to bleed upon challenge (103–108). Nevertheless, the fact that all patients with hypothyrombinemia or dysprothrombinemia reported have some detectable levels of thrombin-like activity in their plasma, suggest that meizothrombin can assume the physiological functions of thrombin albeit less efficiently, hence the mild to moderate bleeding tendency in individuals homozygous for an amino acid substitution at Arg²⁷¹ (108). Data presented in this report verify this hypothesis and suggest that membrane-bound meizothrombin can catalyze most reactions resulting in clot formation and/or clotting arrest and usually ascribed to α -thrombin, locally at the place of vascular injury, when an appropriate membrane surface is provided. At this point it is important to emphasize the fact that considerable amounts of meizothrombin desF1 were identified in human arterial and venous thrombi as well as in clotted whole blood (109, 110). For this reason, although cleavage of prothrombin Dhahran, Barcelona, and/or Padua I at Arg³²⁰, which results in full exposure of ABE-I and produces an active enzyme with reduced (but not nil) fibrinogen clotting activity (because of exposure of ABE-I) (55, 57, 69); only heterozygous patients with prothrombin San Antonio were reported, and we can realistically speculate that homozygosity for prothrombin San Antonio would be equivalent to complete prothrombin deficiency and thus incompatible with survival. Bearing all these qualifications in mind, we must conclude that the scarcity of patient data identifying mutations at Arg³²⁰ and Arg²⁷¹ in prothrombin may reflect the fact that homozygosity for the former is incompatible with survival while heterozygosity for the latter may go undetectable. Thus, the mild/moderate severity of the symptoms of individuals with a circulating prothrombin molecule that is unable to be cleaved at Arg²⁷¹, coupled to the abundance of prothrombin in plasma, will result in an insignificant decrease of both the overall functional levels of prothrombin (*i.e.* thrombin and meizothrombin), and the bleeding phenotypes, which in turn are the hallmark for patient detection (108).

Hirudin inhibits factor V activation and prothrombinase function (29–32, 111). Hirudin^{54–65} (or hirugen), a 12-amino acid peptide from

the COOH-terminal portion of hirudin, also inhibits factor V activation and cofactor function. Sulfation of hirugen on its unique tyrosine residue (Tyr⁶³, Hir⁵⁴⁻⁶⁵(SO₃⁻)) increased its potency with respect to thrombin/prothrombin interaction. However, Hir⁵⁴⁻⁶⁵(SO₃⁻) has ~100-fold increased affinity for thrombin as compared with prothrombin (30, 56). Further, the sulfated tyrosine was found to interact with ABE-I of thrombin (112, 113). More recently, a mutant factor V molecule with the sequence ⁶⁹⁵DYDY⁶⁹⁸ mutated to ⁶⁹⁵KFKF⁶⁹⁸ was found to be partially resistant to α -thrombin activation and a peptide with the sequence DYDYQ impaired activation of single chain factor V by α -thrombin (68). Data presented herein demonstrate that the double sulfated version of the peptide is a better inhibitor of α -thrombin function in plasma when compared with the non-sulfated pentapeptide. This enhanced potency is most likely the result of a more linear conformation of the sulfated pentapeptide compared with the non-sulfated form, allowing more contact points with ABE-I of α -thrombin. The accumulated data also demonstrate that the amino acid motif E/DD adjacent to two critical activation cleavage sites in factor V and factor VIII are crucial for the interaction of the molecules with ABE-I of meizothrombin/ α -thrombin required for efficient procofactor activation. Inhibition of these points of interaction may result in the inhibition of meizothrombin/ α -thrombin function in whole plasma and may thus represent a new anticoagulant molecule.

In conclusion, the physiological consequences in individuals with natural prothrombin mutations together with data on record, and the findings presented herein, establish meizothrombin and ABE-I of thrombin as major players during the critical initial steps of the initiation/termination of blood coagulation. Our data also show that a sulfated pentapeptide inhibits several procoagulant ABE-I-related functions of α -thrombin and provide a target as well as the scaffold for the synthesis of an exosite-directed anticoagulant molecule that could inhibit and/or attenuate thrombin function in individuals with thrombotic tendencies.

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