HD1, a Thrombin-directed Aptamer, Binds Exosite 1 on Prothrombin with High Affinity and Inhibits Its Activation by Prothrombinase*

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Incorporation of prothrombin into the prothrombinase complex is essential for rapid thrombin generation at sites of vascular injury. Prothrombin binds directly to anionic phospholipid membrane surfaces where it interacts with the enzyme, factor Xa, and its cofactor, factor Va. We demonstrate that HD1, a thrombin-directed aptamer, binds prothrombin and thrombin with similar affinities ($K_d$ values of 86 and 34 nM, respectively) and attenuates prothrombin activation by prothrombinase by over 90% without altering the activation pathway. HD1-mediated inhibition of prothrombin activation by prothrombinase is factor Va-dependent because (a) the inhibitory activity of HD1 is lost if factor Va is omitted from the prothrombinase complex and (b) prothrombin binding to immobilized HD1 is reduced by factor Va. These data suggest that HD1 competes with factor Va for prothrombin binding. Kinetic analyses reveal that HD1 produces a 2-fold reduction in the $k_{cat}$ for prothrombin activation by prothrombinase and a 6-fold increase in the $K_m$, highlighting the contribution of the factor Va-prothrombin interaction to prothrombin activation. As a high affinity, prothrombin exosite 1-directed ligand, HD1 inhibits prothrombin activation more efficiently than Hir54–65(SO3$^-$$^-$) (f-Hir54–65(SO3$^-$$^-$)), the exosite 1-binding COOH terminus of hirudin, binds thrombin with an affinity 130-fold higher than that for prothrombin ($K_d$ values of 25 nM and 3.2 μM, respectively). Prothrombin activation intermediates display intermediate affinities for f-Hir54–65(SO3$^-$$^-$) that increase with the extent of activation (11, 12). Diminished affinity of other thrombin ligands for proexosite 1 on prothrombin also has been observed (13, 14).

In contrast to the progressive maturation of proexosite 1, exosite 2 displays more abrupt development. Exosite 2 is not accessible until fragment 2 (F2) is released from prothrombin. Thus, prethrombin 2 (pre2) and thrombin have similar affinities for heparin, whereas meizothrombin (mIIa) and meizothrombin des F1 [mIIa(-F1)], which retain the F2 domain, do not bind heparin (15).

Understanding the functional maturation of the exosites on thrombin has increased in importance with emerging evidence that the exosites serve not only as binding domains but also as allosteric regulators of thrombin activity (16–18). Numerous studies reveal that ligand binding to either exosite can modify the activity of thrombin. Thus, peptide (16, 19), glycosaminoglycan (20, 21), and nucleotide (22) ligands have all been shown to modulate thrombin. Thrombin-binding DNA aptamers represent a unique class of ligand. These engineered oligonucleotides, which fold into characteristic secondary structures, form binding pockets for specific ligands (23, 24). In the case of thrombin, aptamer HD1 binds selectively to exosite 1 (25), whereas aptamer HD22 binds to exosite 2 (26). Because of as heparin (3, 4) and platelet glycoprotein Ibα (5–7), serves to tether thrombin for subsequent interactions with substrates or inhibitors.

Prothrombin, the precursor of thrombin, lacks an active site and has immature or inaccessible exosites (8–10). Because exosite 1 on prothrombin exhibits reduced affinity for certain ligands, it has been designated proexosite 1 (8). This proexosite gains functional activity during prothrombin conversion to thrombin, as evidenced by fluorescent ligand binding studies (11). Thus, Anderson and Bock (11) reported that fluorescein-hirudin$^{54–65}$(SO3$^-$$^-$) (f-Hir$^{54–65}$(SO3$^-$$^-$)), the exosite 1-binding COOH terminus of hirudin, binds thrombin with an affinity 130-fold higher than that for prothrombin ($K_d$ values of 25 nM and 3.2 μM, respectively). Prothrombin activation intermediates display intermediate affinities for f-Hir$^{54–65}$(SO3$^-$$^-$) that increase with the extent of activation (11, 12). Diminished affinity of other thrombin ligands for proexosite 1 on prothrombin also has been observed (13, 14).

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2 The abbreviations used are: Hir$^{54–65}$(SO3$^-$$^-$), Tyr$^{65}$-sulfated COOH-terminal hirudin peptide amino acids 54–65; FITC, fluorescein isothiocyanate; PCP5S, N,N,N’,N’,N’-pentanediyl)amide; Chz-Th, chromozym thrombin.
their high affinity and selective binding, HD1 and HD22 serve as useful tools to probe the structure-function relationship of the exosites on thrombin. The crystal structure of the thrombin-HD1 complex has been defined (27), as has the structure of the thrombin-Hir\textsuperscript{54–65}(SO\textsubscript{3})\textsubscript{3} complex (28). These structures suggest that the two exosite 1-directed ligands bind to overlapping, but discrete, subdomains. Thus, Hir\textsuperscript{54–65}(SO\textsubscript{3})\textsubscript{3} largely interacts with the hydrophobic cleft of exosite 1, whereas HD1 binds to charged residues surrounding this cleft. Given their distinct binding sites on thrombin, it is possible that HD1 and Hir\textsuperscript{54–65}(SO\textsubscript{3})\textsubscript{3} do not exhibit identical interactions with prothrombin and its intermediates. To explore this possibility, we used HD1 and Hir\textsuperscript{54–65}(SO\textsubscript{3})\textsubscript{3} to examine the functional maturation of exosite 1 and HD22, the exosite 2-binding DNA aptamer, to report exosite 2 maturation.

**EXPERIMENTAL PROCEDURES**

**Materials**

Reagents—Human prothrombin, thrombin, and factor Xa were obtained from Enzyme Research Laboratories, Inc. (South Bend, IN). Factor Va and dansylarginine N-(3-ethyl-1,5-pentanediyl)amide (DAPA) were from Hematologic Technologies, Inc (Essex Junction, VT). d-Phe-Pro-Arg chloromethyl ketone and 1,5-dansyl-Glu-Gly-Arg chloromethyl ketone were obtained from Calbiochem. Fluorescein isothiocyanate (FITC) was from Sigma. HD1 (5'-GGTTGTTGTTGTTG-3'), HD22 (5'-AGTCCGTTGTTGAGCGGTTGGTGACT-3'), the exosite 1 and 2-directed DNA aptamers, respectively, and HD23 (5'-AGTCCGTTAAGCGGTTAAATGACT-3'), a scrambled oligonucleotide sequence of HD22, and their 3' FITC- or biotin-labeled counterparts, were synthesized by the Molecular Biology and Biotechnology Institute at McMaster University (Hamilton, Canada). Before use, all of the aptamers were subjected to renaturation by heating to 95 °C for 5 min followed by cooling on ice for 10 min (22). Ecarin, a snake venom protein derived from *Echis carcinus*, was from Pentapharm (Basel, Switzerland). DEAE-Sepharose, PD-10 Sephadex, Mono Q-Sepharose, and SP-C50 Sephadex were obtained from GE Healthcare (Dorval, Canada). Recombinant tick anticoagulant peptide, a factor Xa-directed inhibitor, was a generous gift from Dr. G. Vlasuk (Corvas International, Inc., San Diego, CA). Hirudin and its Tyr\textsupersulfated COOH-terminal peptide, Hir\textsuperscript{54–65}(SO\textsubscript{3})\textsubscript{3}, were from Bachem (King of Prussia, PA). Chromozym thrombin (Chz-Th) was from Roche Applied Science, whereas S2765 and S2238 were from Chromogenix (Milano, Italy).

L-α-Phosphatidyl-l-serine from bovine brain and L-α-phosphatidyl-choline type III-E from egg yolk were from Avanti Polar Lipids Inc. (Alabaster, AL) and Sigma, respectively. PCPS vesicles were synthesized using a modification of previously published methods (29, 30). A phosphate assay was used to determine the concentration of PCPS vesicles (31). The vesicles were stored at −80 °C in 10% sucrose.

**Labeled Proteins**—To label Hir\textsuperscript{54–65}(SO\textsubscript{3})\textsubscript{3} with FITC, 0.11 mg of peptide was dissolved in 250 μl of 0.2 m Na\textsubscript{2}HCO\textsubscript{3} buffer, pH 9.0, and 20 μl of FITC (25.7 μM in Me\textsubscript{2}SO) was added to a final concentration of 2 μM. After wrapping the mixture in aluminum foil and mixing the sample end-over-end for 90 min at 23 °C, 20 μl of 1 m NH\textsubscript{4}Cl was added to stop the reaction. The sample was then applied to a 10-ml G10 Sephadex column that was pretreated with 5 mg/ml ovalbumin and washed with 20 mM Tris-HCl, 0.15 mM NaCl, pH 7.4 (Tris-buffered saline). 0.5-ml fractions were collected, and the fluorescent fraction, which was identified by monitoring the effluent with a UV light, was recovered in a single tube. Absorbance of the fractions was determined at 492 nm, and protein concentrations were calculated based on ε = 6.8 × 10\textsuperscript{4} M\textsuperscript{-1} cm\textsuperscript{-1} (32), assuming 1:1 incorporation of FITC into Hir\textsuperscript{54–65}(SO\textsubscript{3})\textsubscript{3}.

**Methods**

**Preparation of Prothrombin Activation Intermediates**—All of the prothrombin activation intermediates were prepared using modifications of published methods (33, 34). The progress of each reaction was monitored using SDS-PAGE analysis (35) on 4–15% acrylamide Ready Gels (Bio-Rad) under reducing and nonreducing conditions.

Pre1 was prepared by incubating prothrombin (1 mg/ml) with 200 mM thrombin in 17 mM imidazole-HCl, 144 mM NaCl, pH 7.4, for 2 h at 37 °C. The reaction was terminated by the addition of 500 mM FPR-ck and, the absence of residual thrombin activity was confirmed using Chz-Th. The sample was then subjected to chromatography on a 5-ml DEAE-Sepharose column.

Pre2 was prepared by digesting pre1 with factor Xa. Briefly, 1 mg/ml pre1 in 25% trisodium citrate buffer, pH 8.8, was incubated with 50 mM factor Xa for 45 min at 37 °C. The reaction was terminated by the addition of 1 mM FPR-ck and 1 mM dansyl-Glu-Gly-Arg chloromethyl ketone, and inhibition of the residual thrombin or factor Xa activity was confirmed with Chz-Th or S2765, thrombin- and factor Xa-directed substrates, respectively. The mixture was then subjected to chromatography on a SP-C50 Sephadex column.

mlla and mlla(-F1) were generated by treating prothrombin or pre1, respectively, with Ecarin. The reactions were conducted in the presence of FPR-ck to prevent autocatalytic cleavage. Prothrombin or pre1 (1 mg/ml in Tris-buffered saline) was incubated with 250 μg/ml Ecarin diluted in 10 mM HEPES, 150 mM NaCl, pH 7.0, containing 25 mM CaCl\textsubscript{2} in the presence of 20 μM FPR-ck for 3 h at 37 °C. The samples were then subjected to anion exchange chromatography on a Q-Sepharose column.

Protein concentrations were determined at 280 nm using the following extinction coefficients: ε = 1.64, ε = 1.95, ε = 1.44, and ε = 1.64 mM\textsuperscript{-1} cm\textsuperscript{-1} for pre1, pre2, mlla, and mlla(-F1), respectively (36). All of the proteins were concentrated using an Amicon Centriprep YM-10 (Beverly, MA). The integrity of each of the prothrombin intermediates was assessed by SDS-PAGE, and aliquots were stored at −80 °C.

**Binding Studies**—Functional assessment of exosites 1 and 2 was performed using fluorescein-labeled HD1 (f-HD1) and HD22 (f-HD22), respectively. f-HD1 or f-HD22 (30 nl), diluted in Tris-buffered saline containing 2 mM CaCl\textsubscript{2}, 5 mM KCl, 1 mM MgCl\textsubscript{2}, and 0.1% polyethylene glycol (aptamer buffer), was added to a 10 × 4-mm quartz cuvette maintained at 23 °C with a circulating water bath and stirred using a micro stir bar. Fluorescence was monitored at an emission wavelength of 535 nm.
HD1 Binds Prothrombin Exosite 1 and Inhibits Prothrombinase

A discontinuous assay was designed to measure the effect of HD1 on the kinetics of prothrombin activation by prothrombinase using a modification of a previously published method (37). A 10× stock solution of prothrombinase, consisting of 60 μM PCPS, 2.5 nm factor Xa, and 6 nm factor Va, diluted in aptamer buffer was preincubated for 10 min at 23 °C. 1 μM prothrombin was incubated with HD1 or Hir54–65(SO3−)2, in concentrations ranging from 0 to 2.5 μM, for 5 min in a series of wells in a 96-well plate (90-μl volume). To start the reactions, 10 μl of the prothrombinase stock solution was added to each prothrombin-containing well. At intervals up to 10 min, individual reactions were terminated by addition of 4.5 μl of a solution containing 5.5 μM tick anticoagulant peptide and 200 mM EDTA. Generated thrombin was quantified by measuring hydrolysis of 600 μM Chz-Th at 405 nm for 10 min using a SpectraMax 340 plate reader ( Molecular Devices, Sunnyvale, CA). Rates of substrate cleavage (mOD/min), as determined by instrument software, were used to calculate thrombin concentration based on the specific activity of thrombin cleavage of Chz-Th, as determined in a separate experiment. By plotting thrombin concentration versus time, rates of prothrombin activation were determined. In some experiments, the effect of HD1 on prothrombin activation by factor Xa was measured in the absence of factor Va or PCPS. For these studies, 1 μM prothrombin was activated either with 50 nm factor Xa and 6 μM PCPS, 0.25 nm factor Xa and 0.3 nm factor Va, or with 2 nm factor Xa and 20 nm factor Va. As a control, the effect of HD1 on Ecarin-mediated prothrombin activation also was examined. For these studies, 1 μM prothrombin was activated with 100 μg/ml Ecarin in the absence of presence of 10 μM HD1, and thrombin generation was measured as described above.

To examine the effect of HD1 on the kinetics of prothrombin activation by prothrombinase, prothrombin (in concentrations ranging from 0 to 8 μM) was incubated with prothrombinase (0.25 nm factor Xa, 0.6 nm factor Va, and 5 μM PCPS) in the absence or presence of 25 μM HD1 for varying intervals up to 60 s. Initial rates of thrombin production (nmol/s) were plotted versus prothrombin concentration (nm) and fit by nonlinear regression to the Michaelis-Menten equation,

$$ V = \frac{(V_{\text{max}}) \times (S)}{(K_m + S)} $$

where $V_{\text{max}}$ is the Michaelis-Menten constant, and $V_{\text{max}}$ is the maximum rate of prothrombin activation (nmol/s). $k_{\text{cat}}$ was calculated by dividing $V_{\text{max}}$ by the factor Xa concentration.

**SDS-PAGE Analysis of Prothrombin Activation**—To examine the effect of HD1 on the prothrombin activation pathway, activation intermediates generated in the absence or presence of HD1 were assessed by SDS-PAGE. Prothrombin (14 μM) was incubated with 70 μM DAPA in the absence or presence of 50 μM HD1. The reactions were initiated by the addition of the preassembled prothrombinase complex, consisting of 200 nm factor Xa, 20 nm factor Va, and 6 μM PCPS vesicles (final concentrations) diluted in aptamer buffer. At intervals, 5- μl aliquots were removed into sample buffer, boiled for 2 min, and subjected to SDS-PAGE analysis under reducing and nonreducing conditions.

**Statistical Methods**—Unless otherwise indicated, the experiments were performed at least three times. The results are presented as the means ± S.E.
**RESULTS**

**Competitive Binding of Exosite 1 Ligands to Thrombin and Prothrombin**—Inspection of the crystal structures of thrombin in complex with Hir$^{54-65}$($\text{SO}_3^-$) or with HD1 suggests that these exosite 1-directed ligands interact with distinct but partially overlapping domains on thrombin (27, 28). Whether these ligands interact with the same domains on prothrombin as they do on thrombin is currently unknown. To begin to address this, we first examined the capacity of Hir$^{54-65}$($\text{SO}_3^-$) to displace f-HD1 from prothrombin or thrombin. As illustrated in Fig. 1A, the fluorescence intensity increases by 17 ± 1.1% when 110 nM thrombin is added to a cuvette containing 30 nM f-HD1. This increase in fluorescence intensity is negated when unlabeled HD1 is added (data not shown), consistent with reversible binding. The fluorescence intensity value also returns to base line when the f-HD1-thrombin complex is titrated with Hir$^{54-65}$($\text{SO}_3^-$), suggesting that the Hir$^{54-65}$($\text{SO}_3^-$)-binding site on thrombin overlaps with that of HD1 (Fig. 1A). In the reciprocal experiments, the addition of thrombin to f-Hir$^{54-65}$($\text{SO}_3^-$) results in a 10 ± 2.8% reduction in fluorescence intensity (Fig. 1B). When the f-Hir$^{54-65}$($\text{SO}_3^-$)-thrombin complex is titrated with HD1, the fluorescence intensity increases but does not return to base line. These findings suggest that the HD1-binding site on thrombin overlaps with the Hir$^{54-65}$($\text{SO}_3^-$)-binding site.

Studies were then repeated using prothrombin in place of thrombin. As illustrated in Fig. 1C, the addition of prothrombin to f-HD1 results in a 16 ± 0.5% increase in fluorescence intensity. Upon titration with Hir$^{54-65}$($\text{SO}_3^-$), the fluorescence intensity decreases but does not reach base-line levels. In the reverse experiment, fluorescence intensity decreases by 5 ± 0.4% when prothrombin is added to f-Hir$^{54-65}$($\text{SO}_3^-$). There is no change in fluorescence intensity when the f-Hir$^{54-65}$($\text{SO}_3^-$)-prothrombin complex is titrated with HD1, suggesting that the HD1-binding site on prothrombin does not overlap with the f-Hir$^{54-65}$($\text{SO}_3^-$)-binding site. The addition of unlabeled Hir$^{54-65}$($\text{SO}_3^-$) to the cuvette resulted in fluorescence returning to base line, confirming reversibility (data not shown). These data suggest that prothrombin binds both exosite 1 ligands and that the Hir$^{54-65}$($\text{SO}_3^-$)-binding site on prothrombin only partially overlaps with the HD1-binding site.

**Binding of Aptamers to Prothrombin Derivatives**—Because the change in fluorescence intensity that occurs when f-HD1 complexes with prothrombin is similar in magnitude to that which occurs when it binds thrombin, we measured the affinity of f-HD1 for prothrombin, prothrombin intermediates, and thrombin. The fluorescence intensity of 30 nM f-HD1 was monitored before and after titration with prothrombin, prothrombin intermediates, or thrombin, and the relative changes in intensity signal were plotted versus protein concentration. The addition of prothrombin to f-HD1 results in a maximal 33 ± 1.7% increase in fluorescence intensity and yields a saturable curve with a $K_d$ value of 86 ± 8.4 nM (Fig. 2). Thus, the affinity of f-HD1 for prothrombin is much higher than that of Hir$^{54-65}$($\text{SO}_3^-$) (below) and is comparable with the $K_d$ value of 40 nM reported for domain 2 of staphylococcal aureolysin (38). Binding experiments were subsequently performed to measure the affinity of f-HD1 for prothrombin derivatives. As outlined in Table 1, f-HD1 binds mIIa, mIIa(-F1), pre1, and pre2 with $K_d$ values of 35 ± 5.5, 45 ± 0.3, 86 ± 1.0, and 66 ± 16 nM, respectively. f-HD1 binds thrombin with a $K_d$ value of 34 ± 4.8 nM. Thus, f-HD1 binds prothrombin and all its activation intermediates with an affinity similar to that for thrombin. These findings suggest that the HD1-binding site on prothrombin undergoes little structural change during prothrombin conversion to thrombin.

Studies were then done using f-Hir$^{54-65}$($\text{SO}_3^-$) in place of HD1. Consistent with previous reports (11, 12), Hir$^{54-65}$($\text{SO}_3^-$) binds prothrombin with an affinity 44-fold lower than that for thrombin ($K_d$ values of 3000 ± 1400 and 68 ± 5.2 nM, respectively; Table 1). These data suggest that, unlike the HD1-binding site on prothrombin, the f-Hir$^{54-65}$($\text{SO}_3^-$)-binding site on prothrombin does not overlap with the HD1-binding site.
HD1 Binds Prothrombin Exosite 1 and Inhibits Prothrombinase

To begin to define the mechanism by which HD1 inhibits this reaction, prothrombin was activated by factor Xa in the presence or absence of individual components of the prothrombinase complex (Fig. 3). When PCPS vesicles were omitted from the prothrombinase complex, HD1 inhibited prothrombin activation by over 85% with factor Va concentrations of either 0.3 or 20 nM, yielding IC50 values of 505 ± 97 and 472 ± 366 nM, respectively. In contrast, HD1 had no effect on prothrombin activation when factor Va was omitted from the prothrombinase complex. These results suggest that the inhibitory effect of HD1 on prothrombin activation is factor Va-dependent, consistent with other known exosite 1 ligands (42–44). In support of this concept, HD1 had no effect on prothrombin activation by Ecarin (data not shown), a factor Va-independent activator of prothrombin.

Previous structural (39) and functional (40, 41) data indicate that F2 binds exosite 2. Consequently, exosite 2 should only be accessible on thrombin and pre2, derivatives lacking F2. To explore this concept, we measured the affinity of f-HD22, an exosite 2-directed DNA aptamer, for prothrombin, prothrombin intermediates, and thrombin. As expected, f-HD22 binds only to pre2 and thrombin, with $K_d$ values of 42 ± 6.8 and 29 ± 3.1 nM, respectively (Table 1). Thus, these data confirm the concept that exosite 2 is only accessible on prothrombin derivatives lacking F2.

**Effect of HD1 on Prothrombin Activation by Prothrombinase**—Exosite 1 on prothrombin contributes to prothrombin activation by prothrombinase by mediating, at least in part, the interaction of prothrombin with factor Va (42). Because HD1 binds exosite 1 of prothrombin with high affinity, we set out to determine whether HD1 attenuates prothrombin activation by competing with factor Va for prothrombin binding. The rate of prothrombin activation by complete prothrombinase was measured in the absence or presence of HD1. HD1 attenuates prothrombin activation by prothrombinase in a dose-dependent and saturable fashion (Fig. 3), inhibiting the reaction by 93 ± 1.0% with an IC50 value of 134 ± 60 nM, a value comparable with the $K_d$ of f-HD1 for prothrombin.

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**Effect of HD1 on Prothrombin Activation by Prothrombinase**—Exosite 1 on prothrombin contributes to prothrombin activation by prothrombinase by mediating, at least in part, the interaction of prothrombin with factor Va (42). Because HD1 binds exosite 1 of prothrombin with high affinity, we set out to determine whether HD1 attenuates prothrombin activation by competing with factor Va for prothrombin binding. The rate of prothrombin activation by complete prothrombinase was measured in the absence or presence of HD1. HD1 attenuates prothrombin activation by prothrombinase in a dose-dependent and saturable fashion (Fig. 3), inhibiting the reaction by 93 ± 1.0% with an IC50 value of 134 ± 60 nM, a value comparable with the $K_d$ of f-HD1 for prothrombin.

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Exosite 2-directed DNA aptamer, for prothrombin, prothrombin intermediates, and thrombin. As expected, f-HD22 binds only to pre2 and thrombin, with $K_d$ values of 42 ± 6.8 and 29 ± 3.1 nM, respectively (Table 1). Thus, these data confirm the concept that exosite 2 is only accessible on prothrombin derivatives lacking F2.
mIIa is the predominant intermediate formed during prothrombin activation (45, 46). mIIa generation is PCPS- and factor Va-dependent, whereas pre2 is the predominant intermediate that is generated in the absence of cofactors. To determine whether HD1 alters the extent of mIIa generation, 14 μM prothrombin was activated by prothrombinase in the absence or presence of 50 μM HD1, and aliquots of the reaction mixture were subjected to SDS-PAGE analysis under reducing (Fig. 4) or nonreducing (data not shown) conditions to distinguish mIIa from prothrombin. In the absence of HD1, mIIa is the major intermediate, and prothrombin activation is complete by 30 min (Fig. 4). When HD1 is added, prothrombin activation also proceeds through mIIa, but prothrombin activation remains incomplete at 120 min. Delayed conversion of the F1.2.A fragment to F1.2 in the presence of HD1 reflects the inhibitory effect of HD1 on thrombin. Thus, HD1 attenuates prothrombin activation by prothrombinase but appears to have minimal effects on the prothrombin activation pathway.

In terms of the kinetic mechanism of prothrombin activation, current thinking is that factor Va increases the $k_{cat}$ for factor Xa cleavage of prothrombin, whereas PCPS lowers the $K_m$ of the reaction (47, 48). Because HD1 attenuates prothrombin activation by prothrombinase in a factor Va-dependent fashion, we examined the effect of HD1 on the kinetic parameters of activation. Prothrombin, in concentrations ranging from 0 to 8 μM, was activated with prothrombinase in the absence or presence of 25 μM HD1, and the rate of thrombin generation was quantified (Fig. 5). In the absence of HD1, the $K_m$ and $k_{cat}$ values are 255 ± 97 nM and 12.2 ± 1.4 s$^{-1}$, respectively, values comparable with the previously reported $K_m$ of 120 nM and $k_{cat}$ of 32.4 s$^{-1}$ (49). With 25 μM HD1, the reaction proceeds at a slower rate, yielding a $K_m$ of 1480 ± 223 nM and a $k_{cat}$ of 6.2 ± 0.7 s$^{-1}$. Thus, the catalytic efficiency is 12-fold lower in the presence of HD1 than it is in its absence, reflecting a 6-fold increase in $K_m$ and 2-fold reduction in $k_{cat}$. These findings explain why HD1 produces over 90% inhibition of prothrombin activation by prothrombinase (Fig. 3).

**Effect of HD1 on the Prothrombin-Factor Va Interaction**—To determine whether HD1 competes with factor Va for prothrombin binding, a competition experiment was conducted using surface plasmon resonance. In this experiment, prothrombin binding to immobilized b-HD1 was examined in the absence or presence of 1 μM factor Va. When factor Va is present, prothrombin binding to immobilized b-HD1 is reduced by 56 ± 2.2% (data not shown). These findings support the concept that HD1 disrupts the factor Va-prothrombin interaction.

**DISCUSSION**

Efficient prothrombin activation by prothrombinase is essential for generating a burst of thrombin at sites of vascular injury (50). For efficient activation to occur, prothrombin must incorporate into the prothrombinase complex. Assembly is accomplished not only by prothrombin γ-carboxylated glutamic acid domain-dependent interaction with negatively charged phospholipids (45), but also by prothrombin binding to factor Va (46). Current thinking is that exosite 1 on prothrombin mediates, at least in part, its interaction with negatively charged phospholipids (45), but also by prothrombin binding to factor Va (46). Current thinking is that exosite 1 on prothrombin based on the observation that Hir54–65(SO32−) binds prothrombin with an affinity much lower than that for thrombin and weakly attenuates prothrombin activation (8, 44). Our data with HD1 refine the concept of proexosite 1 on prothrombin and provide new information on proexosite maturation during prothrombin conversion to thrombin.
bin. We demonstrate that, unlike Hir54–65(SO3−), HD1 (a) binds prothrombin and thrombin with similar high affinity, (b) potently inhibits prothrombin activation by prothrombinase in a factor Va-dependent fashion, and (c) competes with factor Va for prothrombin binding. Therefore, HD1 provides unique insight into the structure and function of exosite 1 on prothrombin. The different inhibitory profiles of HD1 and Hir54–65(SO3−) can partly be explained by disparity in their binding interactions with prothrombin and thrombin.

**Comparison of Hir54–65(SO3−) and HD1 Binding to Prothrombin and Thrombin**—Examination of the crystal structures of the Hir54–65(SO3−) and HD1-thrombin complexes suggests that the two exosite 1-directed ligands bind to distinct but contiguous regions of exosite 1 (27, 28). Competition experiments support this concept because Hir54–65(SO3−) competes with F-HD1 for binding to both prothrombin and thrombin. These findings can be explained in two ways. First, the capacity of Hir54–65(SO3−) and HD1 to compete for binding could reflect extended conformational changes associated with ligand binding. Thus, exosite 1 residues are disordered in their native state. Once Hir54–65(SO3−) binds, however, these residues become highly ordered (28, 51), and this conformational rearrangement may displace HD1 from its nearby binding site. A second potential explanation is that Hir54–65(SO3−) directly competes for the charged residues that bind HD1. Consistent with a sterical mechanism, HD1 incompletely displaces Hir54–65(SO3−) from prothrombin or thrombin. Thus, HD1 displays a small thrombin-binding footprint with five residues identified as important for binding (52). In contrast, Hir54–65(SO3−), which makes extended contacts with thrombin (28), has a considerably larger footprint. The fact that HD1 only partially competes with Hir54–65(SO3−) suggests that the footprints of the two ligands overlap, but the residues mediating the interaction of HD1 with thrombin or prothrombin represent only a portion of those that mediate Hir54–65(SO3−) binding. Thus, our competition studies highlight differences in the structure of exosite 1 of prothrombin. The distinct interactions of HD1 and Hir54–65(SO3−) with prothrombin prompted investigation of the functional maturation of exosite 1 in prothrombin.

**Exosite 1 on Prothrombin**—In contrast to Hir54–65(SO3−), HD1 binds prothrombin and thrombin intermediates with affinities similar to that for thrombin. These findings suggest that the charged residues that constitute the HD1-binding site in exosite 1 undergo minimal conformational rearrangement during prothrombin conversion to thrombin. In contrast, the Hir54–65(SO3−)-binding site undergoes significant conformational maturation during prothrombin activation. Cleavage of the Arg328–Ile329 bond likely contributes to maturation of the exosite 1 hydrophobic cleft because the affinity of Hir54–65(SO3−) for the meizo-derivatives of prothrombin is much higher than those for prothrombin, pre1, and pre2 (11, 12).

Our data suggest that the concept of a proexosite on prothrombin needs to be refined. Although it may be a proexosite in terms of its affinity for thrombin substrates, such as fibrinogen, it does not serve as a proexosite for HD1. It also should not be considered a proexosite in the context of factor Va, whose primary role is to bind prothrombin rather than thrombin. In fact, our data, as well as those of others (8, 42, 44, 53), indicate that exosite 1 is an important contributor to the prothrombin/factor Va interaction.

**Prothrombin Interaction with Factor Va**—We show that HD1 inhibits prothrombin activation by prothrombinase in a factor Va-dependent fashion and that factor Va competes with prothrombin for HD1 binding. Taken together, these data highlight the importance of exosite 1 in the prothrombin/factor Va interaction, a concept supported by previous work with other exosite 1-directed ligands, such as Hir54–65(SO3−) (42, 44) or Bothrojaracin, a prothrombin-binding protein isolated from the venom of Bothrops jararaca (43). Although HD1 attenuates prothrombin activation, it does not totally block it because exosite 1 is not the sole binding site for factor Va. Thus, residues 473–487 of the B chain (54), F2 (55), and the γ-carboxylated glutamic acid domain (56) and kringle 1 of F1 (57) also contribute to the prothrombin/factor Va interaction. Despite the extensive contacts that prothrombin makes with factor Va, however, the potenti inhibitory effect of HD1 on prothrombin activation by prothrombinase suggests that exosite 1 on prothrombin plays an important part in the prothrombin/factor Va interaction.

The relative contribution of factor Va and L-α-phosphatidyl-L-serine membranes to direct factor Xa activity toward prothrombin is uncertain. Initial studies suggested that factor Va determines which prothrombin intermediate is generated by factor Xa-mediated cleavage (46, 58). More recent work challenges this concept, suggesting instead that L-α-phosphatidyl-L-serine dictates the activation pathway, whereas factor Va enhances the rate of thrombin production regardless of the pathway (45, 46, 59, 60). Our observation that HD1 reduces the rate of prothrombin activation by prothrombinase, without affecting the activation pathway through which prothrombin is converted to thrombin, suggests that exosite 1 on prothrombin is a major contact for its entry into the prothrombinase complex but does not contribute to directing factor Xa cleavage of prothrombin.

**Role of Exosite 1 on Prothrombin in Its Activation by Prothrombinase**—HD1 is one of several exosite 1-directed ligands that has been shown to inhibit prothrombin activation by prothrombinase. Hir54–65(SO3−) and Bothrojaracin inhibit prothrombin activation by the factor Xa-factor Va complex, but not by complete prothrombinase (43, 61). Similarly, DYDYQ, a pentapeptide analog of a portion of factor Va, binds prothrombin with a $K_d$ value of 850 nM and inhibits prothrombinase activity with an $IC_{50}$ value of 1.6 µM (62). Although all of these ligands bind exosite 1 on prothrombin, none inhibits prothrombin activation by prothrombinase as potently as HD1.

At saturating concentrations, HD1 inhibits prothrombin activation by prothrombinase by over 90%. Kinetic analysis indicates that HD1 affects the $K_m$ of the reaction more than the $k_{cat}$. This observation is consistent with the concept that exosite 1 on prothrombin is a major docking site for factor Va and is important for prothrombin incorporation into the prothrombinase complex. The continued enhancement of prothrombin activation observed in the presence of HD1 may reflect factor Va interactions with factor Xa and/or prothrombin that are independent of exosite 1 (55). Because of its high affinity for prothrombin, HD1 provides a unique tool to examine the con-
turbation of the prothrombin/factor Va interaction to efficient prothrombin activation by prothrombinase.

**Relevance**—Our data suggest that the notion that prothrombin possesses a proexosite 1 needs to be refined. Whereas Hir54–65(SO3)2 and fibrinogen bind prothrombin more weakly than thrombin, this is not the case with HD1. Likewise thrombomodulin may also bind prothrombin more tightly than Hir54–65(SO3)2 or fibrinogen because thrombomodulin binds mIIa and mIIa(-F1) with high affinity (40) and is a more potent inhibitor of prothrombin activation by prothrombinase than Hir54–65(SO3)2 (44). Therefore, exosite 1 on prothrombin is fully capacitated for binding ligands, such as factor Va, that have a direct prothrombin-dependent function. In contrast, this domain serves as a proexosite for thrombin-directed ligands or substrates.

HD1 was initially developed as an anticoagulant because of its capacity to directly inhibit thrombin clotting activity (22, 52). Our data suggest that HD1 has dual anticoagulant activity. Thus, in addition to blocking thrombin activity, HD1 also attenuates thrombin generation by inhibiting prothrombin activation by prothrombinase. Positioned at the junction between the intrinsic and extrinsic pathways of coagulation, prothrombinase is an attractive target for new anticoagulants. By attenuating thrombin generation, the intent is to preserve sufficient thrombin activity to permit hemostasis. Such an approach may produce less bleeding than one that targets thrombin directly. Our data suggest that an aptamer that preferentially binds prothrombin over thrombin would specifically inhibit thrombin directly. Our data suggest that an aptamer that preferentially binds prothrombin over thrombin would specifically inhibit thrombin directly. Such an approach may produce less bleeding than one that targets prothrombinase is an attractive target for new anticoagulants.

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