Characterization of Proexosite I on Prothrombin*

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Activation of prothrombin by factor Xa is accompanied by expression of regulatory exosites I and II on the blood coagulation proteinase, thrombin. Quantitative affinity chromatography and equilibrium binding studies with a fluorescein-labeled derivative of the exosite I-specific peptide ligand, hirudin<sup>54–65</sup> ((5F)<sub>H</sub>ir<sup>54–65</sup>(SO<sub>3</sub>)<sub>−</sub>) were employed to identify and characterize this site on human and bovine prothrombin and its expression on thrombin. [5F]<sub>H</sub>ir<sup>54–65</sup>(SO<sub>3</sub>) was shown to have distinctive fluorescence excitation spectral differences in complexes with prothrombin and thrombin and bound to human prothrombin and thrombin with dissociation constants of 3.2 ± 0.3 μM and 25 ± 2 nM, respectively, demonstrating a 130-fold increase in affinity for the active proteinase. The bovine proteins similarly showed a 150-fold higher affinity of [5F]<sub>H</sub>ir<sup>54–65</sup>(SO<sub>3</sub>) for thrombin compared with prothrombin, despite a 2–5-fold lower affinity of the peptides for the bovine proteins. Unlabeled, Tyr<sup>64</sup>-sulfated and nonsulfated hirudin peptides bound competitively with [5F]<sub>H</sub>ir<sup>54–65</sup>(SO<sub>3</sub>) to human and bovine prothrombin and thrombin, exhibiting similar, 40–70-fold higher affinities for the proteinases, although nonsulfated Hir<sup>54–65</sup> bound with 7–17-fold lower affinity than the sulfated analog. These studies characterize proexosite I for the first time as a specific binding site for hirudin peptides on both human and bovine prothrombin that is present in a conformationally distinct, low affinity state and is activated with a ~100-fold increase in affinity when thrombin is formed.

Thrombin is generated in the penultimate step of the blood clotting cascade through activation of the zymogen, prothrombin, by the proteinase factor Xa in reactions regulated by phospholipid membrane surfaces, calcium, and the protein cofactor, factor Va (1). Cleavage of two peptide bonds in prothrombin by factor Xa activates the serine proteinase catalytic domain (prethrombin 2) and releases it as thrombin along with prothrombin activation fragments 1 and 2 (1). Activation of prothrombin is accompanied by the expression of regulatory exosites I and II on thrombin (2–4). Exosite I has been well characterized structurally and functionally on thrombin as an electropositive site that binds fibrinogen (5), thrombomodulin (6), the platelet thrombin receptor (7), factor V and Va (8–10), heparin cofactor II (11), and COOH-terminal hirudin peptides and their analogs specifically (12–14). This site plays a critical role in mediating the binding of these and other specific protein substrates, inhibitors, and macromolecular effectors to thrombin (2, 15, 16). The characteristics of this site on prothrombin and prothrombin activation intermediates, however, are not clearly established. Equilibrium binding studies with a fluorescein-labeled hirudin peptide, hirudin<sup>53–64</sup> (14), as an exosite I-specific probe showed that bovine prothrombin had no detectable affinity for the peptide (3). Expression of exosite I on thrombin was concluded to result from conformational changes that accompany either of the two factor Xa cleavages that give rise to the alternate prothrombin activation intermediates, prothrombin 2 and meizothrombin (3). Contrasting the results for hirugen, an anti-exosite I antibody and thrombomodulin showed no detectable affinity for the human prothrombin activation intermediates, whereas these ligands bind to exosite I of thrombin specifically (4). Binding to human prothrombin of a nonsulfated hirudin peptide analog, N-acetyl-Hir<sup>55–65</sup>, containing a Gly substitution at residue 65 was demonstrated by NMR, but the peptide bound to prothrombin with an estimated dissociation constant of ~500 μM, indicating a very low affinity of uncertain significance (17). Although there is good agreement that the affinity of exosite I for hirudin peptides and macromolecular ligands is increased on conversion of prothrombin to thrombin, this site has not been directly characterized on prothrombin, and the information available from previous binding studies favors the idea that the site is absent from the intactzymogen as a functionally significant site.

Studies of the effects of hirudin peptides on the kinetics of human prothrombin 2 activation, however, demonstrate inhibition of the factor Va-stimulated rate, suggesting an unexpected function for a hirudin peptide binding site in prothrombin 2 activation that might extend to prothrombin as well (3). Kinetic studies of bovine prothrombin 2 activation by the factor Xa-factor Va-phospholipid complex showed inhibition by hirugen in an apparently competitive manner with the substrate (18), suggesting a role for a hirudin peptide binding site on productive prothrombin 2 interactions with the factor Xa-factor Va catalytic complex. Although these studies indicate that hirudin peptide binding sites are involved in prothrombin 2 activation, the mechanism has not been fully defined. Whether a similar mechanism exists for the natural substrate, prothrombin, has not been considered, largely because of the unknown status of exosite I on prothrombin.

Equilibrium binding studies employing a fluorescein-labeled derivative of the peptide, hirudin<sup>54–65</sup> ((5F)<sub>H</sub>ir<sup>54–65</sup>(SO<sub>3</sub>)) were undertaken to identify and characterize the properties of

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The abbreviations used are: Hir<sup>54–65</sup>, Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln; Hir<sup>54–65</sup>(SO<sub>3</sub>); Tyr<sup>64</sup>-sulfated Hir<sup>54–65</sup>; [5F]<sub>H</sub>ir<sup>54–65</sup>(SO<sub>3</sub>); Hir<sup>54–65</sup>(SO<sub>3</sub>) labeled at the amino terminus with 5-carboxyfluorescein; FPR-CH<sub>3</sub>Cl, d-Phe-Pro-Arg-CH<sub>3</sub>Cl.
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Prothrombin and bovine thrombin have different affinities for Hirudin peptides (14), and because proteins of both species have been studied previously in this context (3, 4, 17), bovine prothrombin and thrombin were compared here as analogs of the human proteins to delineate species-specific functional differences in the proexosite and exosite interactions. [5F]Hir54–65(SO3 2-) and the unlabeled peptide (Hir54–65(SO3 2-)) bound to human prothrombin with higher affinity than previously studied analogs, which allowed characterization of the proexosite for the first time. The results demonstrate that proexosite I is present on human and bovine prothrombin as a specific binding site for Hirudin peptides that exhibit an ~100-fold lower affinity compared with thrombin. Human and bovine proteins showed species-specific differences in affinities for the peptides, which were 2–5-fold lower for the bovine proteins. Comparison of the binding of Hirudin peptide analogs lacking the fluorescein probe or Try 63 sulfation was consistent with the peptides interacting with similar specificity through the same site on prothrombin and thrombin for both species. In the companion paper (19), evidence is presented for a role of proexosite I in the mechanism of specific recognition of prothrombin as the substrate of the factor Xa-factor Va catalytic complex.

Experimental Procedures

Protein and Peptide Purification and Characterization—Human prothrombin was purified from plasma or from a plasma fraction obtained from Hyland Laboratories (20). Bovine prothrombin and thrombin were obtained from Hematologic Technologies. Prothrombin was chromatographed on sulfopropyl-Sephadex (1 cm × 9 cm) in 50 mM Hepes, 0.125 M NaCl, 2 mM EDTA, 0.1 mM FPR-Cl2, 1 mg/ml polyethylene glycol 8000, pH 7.4, to eliminate traces of thrombin. Human α-thrombin was purified as described previously (20) or obtained from Dr. John Fenton (New York State Department of Health, Albany, NY). Thrombin concentrations were determined by active-site titration with p-nitrophenyl p'-guanidinobenzoate (20). Thrombin preparations were ≥90% active. Protein concentrations were determined by absorbance at 280 nm with the following absorption coefficients (mg/ml) -1 cm -1 and molecular weights (21, 22), respectively: human prothrombin, 1.47, 71,600; bovine prothrombin, 1.44, 72,100; human thrombin, 1.83 (0.1 M NaOH) or 1.74 (buffer), 36,600; and bovine thrombin, 1.95, 36,700.

Hir54–65(SO3 2-) and the nonsulfated peptide (Sigma or Bachem) were dissolved in water or buffer, and the concentration was determined by the purity and peptide content specified by the manufacturers. The concentration of nonsulfated peptide determined from the tyrosine absorbance at 293 nm in 0.1 M NaOH with an absorption coefficient of 2381 M -1 cm -1 (23) agreed to within 10% of the calculated concentration. An absorbance calibration curve confirmed the concentration and composition (≥20%) for both peptides. Hir54–65(SO3 2-) was labeled at the amino terminus with 5-carboxyfluorescein and characterized as described previously (24).

Quantitative Affinity Chromatography—Binding of Hir54–65(SO3 2-) to prothrombin was investigated by quantitative affinity chromatography on Hir54–65(SO3 2-) agarose. The affinity matrices were prepared by coupling of 2 mg of Hir54–65(SO3 2-) to 2–3 ml of Affi-Gel 10 (Bio-Rad) by mixing in 50 mM Hepes, 0.1 M NaCl, 80 mM CaCl2, pH 7.5, at room temperature for 1 h. The immobilization reaction was stopped by blocking the gel with 0.1 M ethanolamine, pH 8.0, and mixing for 2 h. The 0.8-cm × 5.5-cm columns were washed with 100 ml of buffer, followed by buffer containing 2 M NaCl to remove any unreacted peptide. The capacity of the matrix for prothrombin was determined by analysis of batchwise titrations of small volumes of gel (50 µl) with prothrombin and measurement of binding from the decrease in solution protein absorbance. Chromatography experiments were performed by loading 300 µl of 4.7 µM prothrombin (0.1 mg) in 50 mM Hepes, 0.11 M NaCl, 5 mM CaCl2, 1 mg/ml polyethylene glycol 8000, pH 7.4, onto the 2.7-ml columns. The flow rate was regulated at 1.5 ml/h, and 0.28-ml fractions were collected. Prothrombin was preincubated with 1 µM FPR-CH2Cl and various concentrations of Hir54–65(SO3 2-) for 30 min at 25 °C before chromatography at room temperature, and the elution volume (Vₑ) was determined from the midpoint of the protein peak measured from the absorbance at 230 nm. A control matrix, prepared in the same manner described above but without any peptide, was used to determine the elution volume for prothrombin under conditions where it did not interact with the matrix (Vₑ). These results were also compared with the elution of prothrombin from Hir54–65(SO3 2-) agarose in buffer containing 2 mM NaCl. Recovery of the protein eluted from the column was quantitated by integration of the absorbance peaks.

On the basis of characterization of the matrix used for prothrombin affinity chromatography, concentration of prothrombin binding sites in the gel volume was ≥24 µM, at least 5-fold greater than the highest prothrombin concentrations in the experiments of ≤4.7 µM. Saturation of the condition that only a small fraction of matrix binding sites were occupied allowed the dependence of the fractional change in elution volume of prothrombin (Vₑ - Vₑo)/Vₑo) on the total concentration of competing Hir54–65(SO3 2-) to be approximated by the hyperbolic Equation 1 (25, 26).

\[
\frac{Vₑ - Vₑo}{Vₑo} = \frac{(Xo/Ko)}{(Ko + [Hir54–65(SO3 2-)])}
\]

(Eq. 1)

The fractional change in the elution volume of prothrombin was measured for fixed concentrations of Hir54–65(SO3 2-) and fit by Equation 1 to obtain the dissociation constant (Ko) for binding of the peptide to free prothrombin in solution and the ratio (Xo/Ko) of the maximum concentration of prothrombin binding sites in the gel volume (Xo) to the dissociation constant (Ko) of prothrombin for the matrix (25, 26).

Fluorescence Studies—Fluorescence was measured with an SLM 8100 fluorometer in the ratio mode, using acrylic cuvettes coated with polyethylene glycol 20,000. All experiments were performed in 50 mM Hepes, 0.11 M NaCl, 5 mM CaCl2, pH 7.4, and at 25 °C. Experiments with prothrombin contained 1 µM FPR-CH2Cl. Fluorescence excitation spectra of 0.2 µM [5F]Hir54–65(SO3 2-) with near-saturating concentrations of human (20 µM) or bovine (40 µM) prothrombin or thrombin (1 µM) were recorded at the emission maximum of 520 nm (2 nm excitation band pass and 4 nm emission band pass). Spectra were corrected for small variations in the initial fluorescence of [5F]Hir54–65(SO3 2-) between experiments by normalization of the fluorescence to the initial intensity measured with excitation at 491 nm. Corrections for dilution were ≤6%, with the exception of bovine prothrombin, where the lower affinity necessitated corrections of ≤20%. Corrections for background (≤1%) were made from parallel measurements on blanks lacking the labeled peptide.

Binding of [5F]Hir54–65(SO3 2-) to prothrombin or thrombin was measured by titrating the labeled peptide with each protein, monitored by the fluorescence changes at three excitation wavelengths selected from the difference spectrum. The changes in fluorescence of [5F]Hir54–65(SO3 2-) were expressed as (Fₑ - Fₒ)/Fₒ = ∆F/Fₒ, as a function of total prothrombin or thrombin concentration, were fit simultaneously with the fractional change in the elution volume of prothrombin (Vₑo) determined for each excitation wavelength (∆Vₑo/Fₒ), a single dissociation constant (Kₒ), and the stoichiometric factor (n).

Competitive binding of [5F]Hir54–65(SO3 2-) and unlabeled peptides was quantitated in titrations measuring the reversal of the fluorescence change of mixtures of [5F]Hir54–65(SO3 2-) and prothrombin or thrombin as a function of competitor concentration. The direct titrations of [5F]Hir54–65(SO3 2-) with excitation at 491 nm and the competitive binding curves collected at one or more excitation wavelengths were fit simultaneously by the cubic equation for competitive binding to determine ∆Fₑo/Fₒ, the dissociation constant, and stoichiometric factor for peptide binding to prothrombin or thrombin (24, 27, 28). In these experiments, measurements were additionally corrected for a small (≤5%) linear increase in fluorescence of [5F]Hir54–65(SO3 2-) when titrated with the unlabeled peptide at high concentrations. Parameters for [5F]Hir54–65(SO3 2-) binding were allowed to vary in nonlinear least squares analysis of the competitive binding experiments to optimize the fits. The resulting best fit parameters were within the experimental error of the independently determined values. Data were analyzed by least squares fitting with Scientist software (MicroMath). Reported errors in the parameters are ± 2 S.D.

Results

Quantitative Affinity Chromatography of Prothrombin on Hir54–65(SO3 2-) agarose—Hir54–65(SO3 2-) interactions with prothrombin were investigated first by small-zone quantitative affinity chromatography. Human prothrombin eluted from an immobilized Hir54–65(SO3 2-) peptide matrix as a broad peak at an elution volume well beyond the void volume in 0.15 M, 5 mM CaCl₂, pH 7.4 buffer. Prothrombin eluted near the void volume
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Fig. 1. Quantitative affinity chromatography of human and bovine prothrombin on Hir54–65(SO3)2-agarose. A, the 230-nm absorbance elution profiles are shown for chromatography of 4.7 \( \mu \text{M} \) human prothrombin in 0.15 \( M \) buffer on Hir54–65(SO3)2-agarose ( ), on a control column lacking the peptide ( ), and on Hir4–65(SO3)2-agarose ( ) in buffer containing 2 \( M \) NaCl ( ). Results are also shown for chromatography of prothrombin in the presence of Hir54–65(SO3)2 at 1 \( \mu \text{M} \) ( ), 2 \( \mu \text{M} \) ( ), and 4 \( \mu \text{M} \) ( ). Inset, the fractional change in elution volume (\( V_e / V_0 \)) as a function of total Hir54–65(SO3)2 concentration (\( [\text{Hir54–65(SO3)}_2]_t \)) was fit by Equation 1 with the parameters given in the text. B, elution profiles determined as in A are shown for chromatography of 4.6 \( \mu \text{M} \) bovine prothrombin in 0.15 \( M \) buffer on Hir54–65(SO3)2-agarose ( ), on a control column prepared without peptide ( ), and on Hir4–65(SO3)2-agarose in buffer containing 2 \( M \) NaCl ( ). Chromatography was performed and analyzed as described under “Experimental Procedures.”

- Spectroscopic Characterization of [5F]Hir54–65(SO3)2 binding to prothrombin and thrombin. Fluorescence excitation difference spectra of [5F]Hir54–65(SO3)2 binding to prothrombin and thrombin. Fluorescence excitation difference spectra (\( \Delta F \)) are shown for 0.2 \( \mu \text{M} \) [5F]Hir54–65(SO3)2 and 20 \( \mu \text{M} \) human (→) or 40 \( \mu \text{M} \) bovine (→→) prothrombin, representing 86% and 73% saturation, respectively. Results are also shown for 1 \( \mu \text{M} \) human (→) or bovine (→→) thrombin, which represented 91–97% saturation. Spectra were collected and analyzed as described under “Experimental Procedures.”

- Direct binding of [5F]Hir54–65(SO3)2 to human and bovine prothrombin and thrombin. A, the fractional changes in fluorescence (\( \Delta F / F_0 \)) of 50 \( \mu \text{M} \) [5F]Hir54–65(SO3)2 monitored at the excitation wavelengths of 491 \( \text{nm} \) ( ), 504 \( \text{nm} \) ( ), and 513 \( \text{nm} \) ( ) plotted as a function of total human prothrombin concentration ([Pro]o). B, fluorescence changes of [5F]Hir54–65(SO3)2 with bovine prothrombin, measured as described in A at excitation wavelengths of 491 \( \text{nm} \) ( ), 508 \( \text{nm} \) ( ), and 513 \( \text{nm} \) ( ). C, fluorescence changes of [5F]Hir54–65(SO3)2 with bovine thrombin measured as described in C. The lines represent the nonlinear least squares fit of the data by the quadratic binding equation for each of the excitation wavelengths, with the parameters given in Table I. Titrations were performed and analyzed as described under “Experimental Procedures.”

In buffer containing 2 \( M \) NaCl and, similarly, from a control column lacking immobilized peptide, indicating that the peptide bound specifically to prothrombin at physiological ionic strength (Fig. 1B). Prothrombin was eluted in progressively smaller volumes from the peptide matrix when it was equilibrated with increasing concentrations of Hir54–65(SO3)2 (Fig. 1). Analysis of the decrease in elution volume of prothrombin as a function of increasing concentration of Hir54–65(SO3)2 gave a dissociation constant of 1.3 ± 0.2 \( \mu \text{M} \) for binding of Hir54–65(SO3)2 to prothrombin in solution and an intercept of 2.3 ± 0.1 for \([X_l]/K_X\) (Fig. 1A, inset). The latter value was in good agreement with the value of 2.6 obtained independently from titration of the matrix with prothrombin (see “Experimental Procedures”), supporting the consistency of the analysis. The recovery of prothrombin eluted from the column was 77–102%, indicating that binding was not due to a minor species in prothrombin preparations with a higher affinity for the peptide but, instead, represented a homogeneous property of prothrombin. Bovine prothrombin was not as tightly bound to Hir54–65(SO3)2-agarose as the human protein, eluting slightly but reproducibly shifted to higher elution volume (Fig. 1B) by an amount that reflected too low an affinity to be determined. By contrast, thrombin was quantitatively bound by the peptide column over much larger elution volumes (results not shown), indicating much higher affinity binding.

Spectroscopic Characterization of [5F]Hir54–65(SO3)2 Binding to Human and Bovine Prothrombin and Thrombin—Interactions of [5F]Hir54–65(SO3)2 with thrombin and prothrombin were characterized in fluorescence excitation and emission spectral studies. As shown in Fig. 2, the excitation difference spectra of [5F]Hir54–65(SO3)2 binding to prothrombin and thrombin revealed differences that allowed binding of the two proteins to be distinguished experimentally by choice of excitation wavelengths. The S-shaped difference spectrum for human prothrombin and [5F]Hir54–65(SO3)2 had a minimum at 491 ± 2 \( \text{nm} \), an apparent isosbestic point at 504 ± 2 \( \text{nm} \), and a fluorescence enhancement maximum at 513 ± 2 \( \text{nm} \) (Fig. 2). By contrast, the shape and amplitude of the fluorescence changes were quite different for binding of the peptide to thrombin (Fig. 2). Thrombin thus decreased the fluorescence at all three wavelengths by 29 ± 1% at 491 \( \text{nm} \), 19 ± 1% at 504 \( \text{nm} \), and 10 ± 1% at 513 \( \text{nm} \). Additional spectra collected as a function of prothrombin concentration confirmed that the crossover point
in the difference spectrum was an isosbestic wavelength (results not shown). This indicated that the fluorescence changes at different excitation wavelengths were well described by no more than two states, consistent with a single binding event. The fluorescence emission spectrum of [5F]Hir54–65(SO3)2 showed a maximum at 520 nm and exhibited only small spectral shifts (±2 nm) on binding to bovine or human prothrombin and thrombin. The excitation difference spectra for bovine prothrombin and thrombin were qualitatively similar to those of the corresponding human proteins but quantitatively different in the amplitudes of the fluorescence changes and showed a small but significant difference in the position of the isosbestic point from 504 (human) to 508 nm (bovine) (Fig. 2). These results demonstrated distinctly different environments of the probe when bound to proexosite I on prothrombin compared with exosite I on thrombin, which was maintained by proteins of both human and bovine sources.

**Direct Binding of [5F]Hir54–65(SO3)2 to Human and Bovine Prothrombin and Thrombin**—The spectral changes accompanying binding of [5F]Hir54–65(SO3)2 were used to quantitate the interactions and to distinguish between binding of the peptide to prothrombin and to thrombin. The amplitudes of the fluorescence changes in titrations of [5F]Hir54–65(SO3)2 paralleled the fluorescence changes seen in the difference spectra for prothrombin and thrombin. Analysis of titrations of [5F]Hir54–65(SO3)2 with human prothrombin monitored at excitation wavelengths of 491, 504, and 513 nm gave a dissociation constant of 3.2 ± 0.3 μM (Fig. 3A, Table I). The maximum fluorescence change with excitation at 504 nm of −0.9 ± 0.3% indicated no trace contamination of prothrombin preparations with thrombin, which would have been detected by a decrease of the fluorescence at this isosbestic wavelength. Fluorescence titrations of [5F]Hir54–65(SO3)2 with human thrombin revealed binding of 0.84 ± 0.06 mol of peptide/mol of thrombin with a 130-fold tighter dissociation constant of 25 ± 2 nm compared with prothrombin (Fig. 3B). The dissociation constant for thrombin was in agreement with the previously reported values in the presence of EDTA (24), indicating no effect of calcium on peptide binding. Titrations of [5F]Hir54–65(SO3)2 with bovine prothrombin and thrombin resulted in a 15 ± 2 μM dissociation constant for prothrombin (Fig. 3C, Table I) and a 99 ± 9 nm dissociation constant for thrombin (Fig. 3D), indicating a similar, 150-fold enhanced affinity of the peptide for thrombin. The bovine proteins bound the fluorescein-labeled peptide with 4–5-fold lower affinity when compared with human prothrombin and thrombin.

**Competitive Binding of Unlabeled Hir54–65(SO3)2 to Human and Bovine Prothrombin**—To examine the influence of the presence of the fluorescein probe on interactions of the labeled peptide, binding of unlabeled Hir54–65(SO3)2 to human prothrombin was quantitated from its competitive effect on binding of [5F]Hir54–65(SO3)2. Titrations of fixed concentrations of [5F]Hir54–65(SO3)2 and various fixed concentrations of human prothrombin with Hir54–65(SO3)2 as the competitor resulted in a return of the fluorescence intensity of the probe at each of three excitation wavelengths toward their original values (Fig. 4). Simultaneous fitting of the data with a competitive binding model yielded a stoichiometry of 0.74 ± 0.09 mol Hir54–65(SO3)2/mol of prothrombin and a dissociation constant of 2.6 ± 0.6 μM (Table I). The maximum change in fluorescence observed at 504 nm was a negligible, −1.3 ± 0.5%, supporting further the conclusion that a single interaction with only prothrombin was responsible for the spectral changes.

**Competitive Binding of Unlabeled Hir54–65(SO3)2 to Human and Bovine Thrombin**—Binding of Hir54–65(SO3)2 to human and bovine thrombin were similarly compared in competitive binding experiments (Fig. 6). Fitting of the titrations gave

### Table I

Parameters determined for binding of hirudin peptides to human and bovine prothrombin and thrombin

| Interaction Method | Human Parameters | Bovine Parameters |
|-------------------|------------------|------------------|
|                   | KD (μM) | ΔFmax/Fo (%) | KD (μM) | ΔFmax/Fo (%) |
| Prothrombin [5F]Hir54–65(SO3)2 | 3.2 ± 0.3 | −17.6 ± 0.5 | 15 ± 2 | −22.5 ± 1.0 |
|                   | 491 nm   | −0.9 ± 0.3 | 13.0 ± 0.5 | 8.5 ± 0.6 |
|                   | 504/508 nm | −13.0 ± 0.3 | 12.8 ± 0.4 |
|                   | 513 nm   | −18.7 ± 0.4 | 8 ± 1 | −25.6 ± 1.5 |
|                   | 504/508 nm | 0.74 ± 0.09 | 79 ± 11 |
|                   | 513 nm   | 0.74 ± 0.09 |
| Hir54–65(SO3)2 | 2.6 ± 0.6 | 8 ± 1 | −24.0 ± 1.3 |
|                   | 491 nm   | −18.4 ± 0.5 | 1.3 ± 0.1 |
|                   | 504/508 nm | 0.909 ± 0.009 |
|                   | 513 nm   | 0.190 ± 0.027 |
|                   | 504/508 nm | 0.099 ± 0.009 |
|                   | 513 nm   | −29.4 ± 0.5 |
| Hir54–65(SO3)2 | 0.038 ± 0.015 | 0.73 ± 0.38 |
|                   | 491 nm   | −214 ± 0.8 |
|                   | 504/508 nm | −11.5 ± 0.7 |
|                   | 513 nm   | −29.4 ± 0.5 |
| Hir54–65 | 0.65 ± 0.08 | 1.3 ± 0.1 | −24.1 ± 0.3 |

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![Image](http://www.jbc.org/Downloaded from http://www.jbc.org/July 22, 2018)
Effect of Tyr63 Sulfation on the Affinity of Hir54–65 for Prothrombin and Thrombin—To examine the dependence of proexosite I affinity on structural changes in Hir54–65(SO3)

Effect of Tyr63 Sulfation on the Affinity of Hir54–65 for Prothrombin and Thrombin—To examine the dependence of proexosite I affinity on structural changes in Hir54–65(SO3), the role of sulfation of Tyr63 in binding affinity was investigated by characterizing the binding of the unlabeled, nonsulfated peptide (Fig. 7). The simultaneous fit of the competitive binding data collected for human prothrombin resulted in a dissociation constant of 45 ± 7 μM for binding of Hir54–65 (Fig. 7A, Table I), representing a 17-fold loss of affinity compared with Hir54–65(SO3) due to lack of sulfation of Tyr63. Similar competitive binding studies with bovine prothrombin gave a dissociation constant of 79 ± 11 μM (Fig. 7C, Table I), showing a similar, 10-fold lower affinity compared with the sulfated peptide. Titration of human or bovine thrombin with Hir54–65 resulted in dissociation constants of 0.65 ± 0.08 μM and 1.3 ± 0.1 μM (Fig. 7, B and D, Table I), respectively, which represented a 60–70-fold enhanced affinity compared with prothrombin. The results indicated that both prothrombin and thrombin bound the sulfated peptide with 10–17-fold higher affinity compared with prothrombin and thrombin bound the sulfated peptide with 10–17-fold higher affinity compared with prothrombin and thrombin bound the sulfated peptide with 10–17-fold higher affinity compared with prothrombin and thrombin bound the sulfated peptide with 10–17-fold higher affinity.

Dissociation constants of 0.038 ± 0.015 μM and 0.19 ± 0.027 μM for human and bovine thrombin, respectively (Table I). These results demonstrated competitive binding of the peptides to exosite I and binding of the unlabeled and fluorescein-labeled peptides with similar affinity. Comparison of the results for prothrombin and thrombin with Hir54–65(SO3) showed that proexosite I displayed a 70-fold (human) and 40-fold (bovine) increase in affinity on conversion of prothrombin to thrombin, somewhat smaller than the 130–150-fold change in affinity seen for [5F]Hir54–65(SO3) (Table I).

DISCUSSION

The results of these studies demonstrate that exosite I is present on both human and bovine prothrombin as a specific binding site and forms a complex with hirudin peptides that is conformationally distinct from the thrombin exosite I-peptide complex. The proexosite undergoes a ~100-fold increase in affinity for hirudin peptides upon conversion to thrombin. Several lines of evidence support the conclusion that binding of the peptides is a homogenous property of prothrombin and represents a specific site rather than binding to minor protein spe-
cies with different properties or nonspecific interactions of the probe or peptide. This evidence includes (a) the single binding site stoichiometry determined for binding of Hir₁₅₄–₁₆₅(SO₃)₂ to prothrombin, (b) the quantitative affinity chromatography results demonstrating binding of essentially all of prothrombin to the immobilized and free peptides, and (c) characterization of the excitation spectral changes, allowing the possible complicating contribution of minor species with high affinity for the peptides, notably thrombin, to be ruled out.

The favorable spectral properties and high affinity of [₅F]Hir₁₅₄–₁₆₅(SO₃)₂ for prothrombin allowed proexosite I of thezymogen to be directly characterized for the first time. In previous studies, the affinity of exosite I for specific ligands was undetectable or weak on bovine (3) or human (4, 17) prothrombin. The main differences between these studies were the different structures of the hirudin peptides studied, as well as the use of human and bovine proteins, which have different properties. Given the −4-fold lower affinity for thrombin of the hirudin₁₅₃–₁₆₄ peptide used in previous studies (3), and assuming a similar, 150-fold decrease in binding affinity, as that of seen for bovine prothrombin with [₅F]Hir₁₅₄–₁₆₅(SO₃)₂, the affinity of the labeled peptide for prothrombin may have diminished to an undetectable level under the conditions of the previous studies. The amplitude of the fluorescence change may also have been too small to detect binding of this particular peptide derivative to prothrombin.

The fluorescence spectral changes accompanying [₅F] Hir₁₅₄–₁₆₅(SO₃)₂ binding provided a comparison of the relative environments of the probe and, thereby, the exosite in the complexes with prothrombin and thrombin. Conversion of prothrombin to thrombin is accompanied by the conformational change in the prothrombin 2 domain that activates the thrombin catalytic site and dissociation of the prothrombin fragment 1 and 2 activation domains. Interactions of the fragment 2 domain with thrombin have been reported to affect exosite I affinity for hirudin peptide analogs (29, 30), and therefore, this domain-domain interaction within prothrombin could contribute to the differences observed between the prothrombin- and thrombin-peptide complexes. Crystallographic studies of thrombin and its complexes show that exosite I is initially disordered in free thrombin and prothrombin 2 and that it assumes a well defined conformation when the hirudin peptides, which are also disordered in solution, bind (31–33). It is not known if the proexosite on prothrombin is also initially disordered. Binding is thought to be driven first by favorable electrostatic interactions of the anionic peptide with the electropositive field created by basic residues in exosite I (34–37). Comparison of the structures of different thrombin complexes with hirudin peptide analogs show that many contacts are subsequently made between the peptide and binding site residues, but these studies are also consistent with flexibility in the sets of particular interactions of the peptides with exosite I residues that contribute to stability of the complexes (38, 39). The distinctive spectral changes of the fluorescence probe in the proexosite I and exosite I complexes were consistent with this flexibility and indicated that the environments of the exosite on prothrombin and thrombin were significantly different in the vicinity of the fluorescein probe at the amino terminus of hirudin₅₄–₆₅. This is not incompatible with the previous conclusion from NMR studies (17) that a hirudin peptide analog bound in the same conformation to prothrombin and thrombin because these studies employed peptides different from those used here. While the conformation of the bound peptides may be the same in some respects, the probe reports a change in the microenvironment of proexosite I near the amino terminus of the peptide when prothrombin is converted to thrombin.

Human thrombin showed a large increase of 130-fold in the affinity for [₅F]Hir₁₅₄–₁₆₅(SO₃)₂ when compared with prothrombin, which is concluded to represent activation of exosite I. The results of the competitive binding experiments for [₅F]Hir₁₅₄–₁₆₅(SO₃)₂, Hir₁₅₄–₁₆₅(SO₃)₂, and Hir₁₅₄–₁₆₅ indicated that the changes in fluorescence were due to specific interactions of the peptides with the same site on prothrombin and thrombin. [₅F]Hir₁₅₄–₁₆₅(SO₃)₂ showed the largest differential affinity, with a 130–150-fold difference between prothrombin and thrombin, whereas Hir₁₅₄–₁₆₅(SO₃)₂ and Hir₁₅₄–₁₆₅ showed slightly smaller, 40–70-fold and 60–70-fold enhancements (Table I). These differences provide some evidence for differences in the specificity of prothrombin and thrombin for the peptide analogs. Relatedly small differences in the interactions of the fluorescein probe presumably account for this effect.

The absence of Tyr²¹ sulfation of Hir₅₄–₆₅ resulted in a 7–10-fold (bovine) and 17-fold (human) lower affinity for both prothrombin and thrombin, confirming that the sulfate group of the peptide stabilizes the interaction with exosite I on thrombin (14, 36, 40, 41) and demonstrating that it has a similar effect in proexosite I on prothrombin. The magnitude of the change in affinity for thrombin due to sulfation is in agreement
with previous studies (14, 41). The similar effect of sulfation on the affinity of \( \text{Hir}^{54-66}(\text{SO}_3^-) \) for thrombin and prothrombin indicates that the hydrogen bonding network that stabilizes the interaction of exosite I residues with the sulfate group (40) is apparently unchanged in the prothrombin- and thrombin-peptide complexes. This aspect of the bound peptide conformation thus appears to be the same in the zymogen and enzyme complexes and does not contribute to activation of the exosite.

Comparison of human and bovine prothrombin and thrombin demonstrated quantitative differences in proexosite I and exosite I by a 2–5-fold lower affinity of the bovine proteins for the hirudin peptides and similar but quantitatively different spectral changes on binding \([5F]\text{Hir}^{54-66}(\text{SO}_3^-)\). Human and bovine thrombin B-chains differ by 34 amino acid substitutions, including substitution of Asn78 by Lys and Ile79 with Val near exosite I, and 7 less conservative differences in the autolysis loop (42). The two residues near the exosite are not involved in contacts of thrombin with the hirudin peptide and, therefore, cannot account for the lower affinity of the peptides for the two species (40, 42). However, Lys148c in the autolysis loop of human thrombin is thought to promote peptide binding by contributing to the positive electrostatic field of exosite I, a process that cannot occur in bovine thrombin because this residue is Glu (38). Human and bovine thrombin complexes with hirugen peptides and similar but quantitatively different species-specific differences in the prothrombin complexes and does not contribute to activation of the exosite.

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