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Novel Heparin Mimetics Reveal Cooperativity Between Exosite 2 and Sodium-Binding Site of Thrombin

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Abstract

Introduction—Thrombin is a primary target of most anticoagulants. Yet, thrombin's dual and opposing role in pro- as well as anti-coagulant processes imposes considerable challenges in discovering finely tuned regulators that maintain homeostasis, rather than disproportionately changing the equilibrium to one side. In this connection, we have been studying exosite 2-mediated allosteric modulation of thrombin activity using synthetic agents called low molecular weight lignins (LMWLs). Although the aromatic scaffold of LMWLs is completely different from the polysaccharidic scaffold of heparin, the presence of multiple negatively charged groups on both ligands induces binding to exosite 2 of thrombin. This work characterizes the nature of interactions between LMWLs and thrombin to understand the energetic cooperativity between exosite 2 and active site of thrombin.

Materials and Methods—The thermodynamics of thrombin–LMWL complexes was studied using spectrofluorimetric titrations as a function of ionic strength and temperature of the buffer. The contributions of enthalpy and entropy to binding was evaluated using classic thermodynamic equations. Label-free surface plasmon resonance was used to assess the role of sodium ion in LMWL binding to thrombin at a fixed ionic strength.

Results and conclusions—Exosite 2-induced conformational change in thrombin's active site is strongly dependent on the structure of the ligand, which has consequences with respect to regulation of thrombin. The ionic and non-ionic contributions to binding affinity and the thermodynamic signature were highly ligand specific. Interestingly, LMWLs display preference for the sodium-bound form of thrombin, which supports the existence of an energetic coupling between exosite 2 and sodium-binding site of thrombin.

Keywords

Allosteric inhibition; Exosites; Heparin; Heparin mimetics; Thrombin

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Introduction

Anticoagulants are the mainstay treatment for thrombotic disorders such as pulmonary embolism, deep vein thrombosis, myocardial infarction and unstable angina. Several anticoagulants target thrombin, a key serine protease that regulates the hemostasis. These agents include direct thrombin inhibitors such as hirudins, peptidomimetic inhibitors such as ximelagatran and melagatran, and indirect inhibitors such as unfractionated heparin (UFH). Each of these anticoagulants suffer from side effects, of which bleeding is most common [1,2].

While UFH and other heparins are indirect anticoagulants, they also bind directly to thrombin in a site called exosite 2, or alternatively, the heparin-binding site, which carries a number of positively charged residues including Arg93, Arg97, Arg101, Arg126, Arg165, Lys169, Arg173, Arg175, Arg233, Lys235, Lys236 and Lys240 [3–8]. This direct binding does not induce catalytic inhibition of thrombin. Yet, several molecules have now been discovered to inhibit thrombin by binding in exosite 2. Sulfated lignin-based oligomeric inhibitors, called low molecular weight lignins (LMWLs), are a unique group of inhibitors that belong to this new class (Figure 1). Interestingly, LMWLs display promising anticoagulant potency in inhibiting blood coagulation [3,4,9].

Despite the presence of anionic groups on both exosite 2 ligands, UFH and LMWLs are significantly different at a structural level. LMWLs have an aromatic backbone that enables hydrophobic interactions in contrast to polysaccharidic backbone of UFH. The presence of multiple sulfate and carboxylate groups on LMWLs afford ionic interactions, thereby generating a combination of molecular forces that engineer novel physicochemical properties [10,11]. Although hydrophobic amino acid residues present in exosite 2 of thrombin that interact with LMWLs remain to be identified, site directed mutagenesis studies have pinpointed that one of the LMWLs, called CDSO3, utilizes mainly Arg93 and Arg175 of exosite 2 for binding and inhibition of thrombin [5]. This has implied that the energy of LMWL binding to exosite 2 can be transmitted to the active site of thrombin resulting in inhibition, whereas UFH binding to the same region does not appear to transmit an inhibitory conformational change. The structure-dependent cooperativity between exosite 2 and active site of thrombin is interesting and raises possibility of tweaking it to induce regulation of thrombin's procoagulant function.

With regard to cooperativity, a large number of studies, of which many are contradictory, have studied ligand binding to thrombin's exosites 1 and 2, active site and sodium binding site [12–22]. LMWLs offer a novel opportunity to study thrombin allostery. How is the binding of LMWLs different from heparins? Do LMWLs affect sodium's allosteric effect on thrombin's catalytic function? Sodium is known to decrease the affinity of fragment 1.2 for exosite 2 [23]. Does sodium decrease affinity of LMWL for exosite 2? Does the drastic difference between the nature of scaffolds of LMWLs (aromatic) and UFH (polysaccharide) induce an altered thermodynamic signature upon binding to thrombin?

Elucidating these fundamental properties may help understand the complex allosteric network of thrombin so as to enable discovery of new modulators with potentially better thrombin regulating properties. In this work, we elucidate the role of electrostatics, temperature and sodium in the interaction of LMWLs with thrombin. We find that the structurally similar inhibitors, CDSO3 and FDSO3, possess different interaction profiles. While CDSO3 binds mainly through non-ionic interactions and favorable entropic contributions, FDSO3 utilizes more balanced forces with a greater dependence on enthalpic contribution. This is significantly different from the interaction of UFH with thrombin. Yet, both LMWLs displayed heat capacity changes similar to UFH. Finally, the interaction of LMWLs with thrombin appears to be thermodynamically less favored in the absence of sodium, which indicate a positive coupling between exosite 2 and sodium binding site, which is in contrast to that observed with fragment 1.2 [23].

Experimental

Proteins, LMWLs, Chemicals and Reagents

Sulfated LMWLs CDSO3 and FDSO3 were synthesized in two steps from 4-hydroxycinnamic acid monomers, caffeic acid and ferulic acid respectively, as described earlier [3]. Porcine heparin (UFH) was purchased from Sigma (St. Louis, MO). Human α -thrombin and active site inhibited thrombin labeled with fluorescein (α FPR-Th) was purchased from Haematologic Technologies (Essex Junction, VT). Stock solution of thrombin for SPR experiments was prepared in 10 mM sodium acetate buffer (pH 5.2) while α FPR-Th was prepared in 20 mM Tris-HCl buffer (pH 7.4). Monothiol carboxyl SAM gold slides were purchased from Reichert Technologies (Buffalo, NY). The slides are mixed, self-assembled monolayer of alkanethiolates generated from the combination of polyethylene glycol-terminated alkanethiol (90%) and COOH-terminated alkanethiol (10%). All other chemicals were analytical reagent grade from either Sigma Chemicals (St. Louis, MO) or Fisher (Pittsburgh, PA) and used as such.

α FPR-Thrombin–LMWLs Interaction

α FPR-Th was freshly prepared from stock solution to get a final concentration of 50 nM in 20 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl, 2.5 mM CaCl_2 and 0.1 % polyethylene glycol (PEG) 8000 at 25 °C in a fluorescence quartz cuvette. Experiments were performed using a QM4 fluorometer (Photon Technology International, Birmingham, NJ) thermostated with a Lauda-Brinkmann circulating water bath. Equilibrium dissociation constants ($K_{D,app}$) for either CDSO3– or FDSO3–thrombin complex were determined by titrating the polymer into a solution of α FPR-Th and monitoring the decrease in the fluorescence ($\lambda_{EX} = 490$ nm, $\lambda_{EM} = 525$ nm). LMWLs were screened for interference at the emission wavelength of detection to rule out interfere with the readings (Supplementary Figure S1). The slit widths on the excitation and emission side were 1 and 2 mm, respectively. The decrease in fluorescence signal upon complex formation was fit to the standard Hill equation (equation 1) for cooperative ligand binding using Sigmaplot 12.0 (SPSS, Inc. Chicago, IL) by manually typing in the equation to obtain the apparent dissociation constant of interaction ($K_{D,app}$). In this equation, F is the change in fluorescence due to formation of the complex following each addition of LMWL from the

initial fluorescence F_0 , F_{max} represents the maximum change in fluorescence observed on saturation of α FPR-Th and Hill coefficient (n) is a measure for the cooperativity of binding.

$$\frac{\Delta F}{F_0} = \Delta F_{max} \times \frac{[LMWL]^n}{(K_{D,app})^n + [LMWL]^n} \quad (\text{Equation 1})$$

Salt Dependence of LMWL– α FPR-Thrombin Interaction

The contribution of ionic and non-ionic binding energies to thrombin–LMWL interaction was determined by measuring the $K_{D,app}$ of the binding in buffers with increasing salt concentration using fluorescence titrations. NaCl (100 – 300 mM) was added to 50 nM α FPR-Th in 20 mM Tris-HCl buffer, pH 7.4, containing 2.5 mM CaCl_2 and 0.1 % PEG 8000 to prepare buffers with varying ionic strengths. The plot of $\log K_{D,app}$ versus $\log [\text{Na}^+]$ affords ionic and non-ionic forces involved in binding by analyzing the data using linear equation 2. In this equation, $K_{D,NI}$ is the equilibrium dissociation constant for the non-ionic component of binding (at 1 M Na^+), Z is the number of ionic interactions formed between the protein and the polyelectrolyte during binding and ψ is the fraction of counter-ions released following interaction. The value of ψ has been experimentally determined to be ~0.8 for heparins [24] and this value was also used for LMWLs.

$$\log K_{D,obs} = \log K_{D,NI} + Z\psi \log [\text{Na}^+] \quad (\text{Equation 2})$$

Thermodynamics of Heparin and LMWLs Interaction with α FPR-Thrombin

The thermodynamic parameters of interaction were determined by measuring the $K_{D,app}$ of the binding at different temperatures (5 – 45 °C) in 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM CaCl_2 and 0.1 % polyethylene glycol (PEG) 8000 using fluorescence titrations for either CDSO3, FDSO3 or UFH binding to α FPR-Th. Integrated Van't Hoff equation 3 assumes a constant heat capacity within the narrow temperature range studied and was used to calculate the heat capacity change (C_p) for complex formation.

$$\ln K_A = \frac{\Delta C_p^0}{R} \left[\frac{T_H}{T} - \ln \left(\frac{T_S}{T} \right) - 1 \right] \quad (\text{Equation 3})$$

In this equation, T_H and T_S are specific temperature values, where the binding enthalpy and entropy vanish, respectively. T_H in particular is the absolute temperature where the equilibrium dissociation constant assumes its minimum value. This equation has been applied before to thrombin interactions with several of its natural substrates like hirudin and thrombomodulin, and in multiple other biological systems as well [25–27]. A plot of K_A versus $1/T$ yields a non-linear profile and data can be analyzed using equation 3 to determine C_p , T_H and T_S . Sigmaplot 12.0 (SPSS, Inc. Chicago, IL) was used to perform non-linear

curve fitting by manually typing in the equation in which C_p , T_H and T_S were allowed to float. Equation 3 was also rearranged to obtain H and S at each studied temperature, where

$$\Delta S^o = \Delta C_p^o \ln \left(\frac{T}{T_S} \right) \quad (\text{Equation 4})$$

$$\Delta H^o = \Delta C_p^o (T - T_H) \quad (\text{Equation 5})$$

FDSO3–Thrombin Interaction as a Function of Sodium Levels

The affinity of FDSO3 binding to thrombin was determined using label-free surface plasmon resonance (SPR) technique with 10 mM HEPES buffer (pH 7.4) containing 2.5 mM CaCl_2 , 0.1 % PEG 8000 as the running buffer with variable concentrations of NaCl. Thrombin was loaded at a flow rate of 10 $\mu\text{l}/\text{min}$ for 20 min to the sample channel of the sensor chip using a standard amine coupling technique [28]. The loading was monitored by the increase in response unit (~ 2000 μRU) that indicates thrombin attachment. After loading thrombin, the remaining activated surface on the sample as well as the reference channel was blocked twice with 1M ethanolamine (pH 8.5) to prevent non-specific binding. A serial dilution of FDSO3 (20 – 2400 nM) was injected at a flow rate of 50 $\mu\text{l}/\text{min}$ on the two channels. Different buffers with increasing NaCl concentrations (0 – 200 mM) were used to measure the affinity, where the ionic strength was kept constant at 0.2 M using compensating amount of choline chloride. The binding cycle consisted of 3 min for association, 6 min for dissociation, followed by regeneration of the slide surface using 20 mM NaOH for 15 sec. We used Reichert Two Channel SR7000DC instrument. The data (k_{ON} and k_{OFF}) were analyzed using Scrubber, which was a third-party module provided by the manufacturer. Scrubber implemented non-linear regression using 1:1 Langmuir binding isotherm.

Results

LMWLs bind thrombin with affinities higher than that of heparin

Earlier studies have shown that CDSO3 and FDSO3 bind in exosite 2 of thrombin, which is also the site of heparin binding [4,5]. To assess whether the electrostatic similarity (sulfates and carboxylates, see Figure 1) between LMWLs and heparin induces similar binding affinities of the complexes we utilized fluorescence spectroscopy. The interaction of CDSO3 or FDSO3 with $\alpha\text{FPR-Th}$ resulted in a $\sim 70\%$ decrease in fluorescence (Figure 2). This change in signal could be analyzed using the Hill equation to calculate the affinities of the two LMWLs, which were found to be 90 nM for CDSO3 and 350 nM for FDSO3 ($\Delta G = -9.65$ and -8.83 kcal/mol, respectively). These nanomolar affinities indicate a fairly strong interaction between LMWLs and alpha-thrombin in comparison to sulfated polysaccharide ligands, such as UFH, which have been reported to bind with affinities in the range of 0.5–10 μM [15,24,29,30]. The difference in affinities between LMWLs and UFH amount to

nearly 2–10-fold. Thus, although both groups of exosite 2 ligands carry multiple negative charges, the interaction affinities are significantly different. Most probably, this difference originates from the striking difference in the nature of the two backbones; aromatic *versus* polysaccharidic. It is also important to note that the fluorophore in *AFPR*-Th is localized approximately in the P4 position. This indicates that conformational changes in the active site are being felt by groups as far removed as the P4 position. Thus, LMWLs appear to be able to induce fairly widespread changes in thrombin structure in the vicinity of the active site.

CDSO3 and FDSO3 exhibit considerable differences in molecular forces of interaction

The difference in thrombin affinities of LMWLs and UFH suggest an underlying difference in the nature of molecular forces governing the interactions. To identify these differences, the dissociation constants for both LMWLs were studied under variable salt concentrations using spectrofluorometric titrations. As predicted by the protein–polyelectrolyte theory and utilized by a number of researchers, especially for polyanions binding to thrombin [24], the affinity of binding decreased as sodium concentration increased for both CDSO3 and FDSO3 (Table 1, Supplementary Figures S2 and S3). The protein–polyelectrolyte theory resolves the overall binding energy into a sum of non-ionic and ionic components, which can be obtained from the intercept and slope of the linear plot of $\log K_D$ *versus* $\log [Na^+]$, respectively (Figure 3). The intercept offers $K_{D,NI}$, the non-ionic affinity, while the slope offers Z , the number of ion-pair interactions involved in complexation.

The slope for CDSO3 was found to be 1.27 ± 0.15 corresponding to a Z value of 1.6 ± 0.2 . This indicates that CDSO3 forms only 1 to 2 ion-pair interactions with thrombin. In contrast, the Z value for FDSO3 was 4.2 ± 0.6 (slope = 3.34 ± 0.48), which indicates formation of 4 ionic interactions. Similar analysis performed earlier for UFH–thrombin system had led to a slope of 4.8 ± 0.2 , which is equivalent to 5 ionic interactions [24].

The intercept for CDSO3 was measured to be -6.02 ± 0.10 , which corresponds to $K_{D,NI}$ of $0.97 \pm 0.10 \mu M$ and G_{NI} of -8.24 ± 0.06 kcal/mol. On the other hand, FDSO3–thrombin system displayed an intercept of -3.54 ± 0.38 , which implies a $K_{D,NI}$ of $292 \pm 31 \mu M$ ($G_{NI} = -4.84 \pm 0.06$ kcal/mol). For thrombin – UFH pair, the $K_{D,NI}$ has been measured to be 200 ± 100 mM [24], which is approximately 1000–100,000-fold higher than that of LMWLs.

The above results imply that nearly 85% and 55% of the total binding energy of the CDSO3 and FDSO3 complexes with thrombin arises from non-ionic forces. In contrast, the heparin–thrombin system displays non-ionic forces to contribute less than 20% of total binding energy [24]. This implies that although CDSO3 and FDSO3 carry multiple sulfates and carboxylates, in a manner similar to heparin, the nature of their interaction with thrombin is completely different from the polysulfated polysaccharide. Heparin–thrombin system is considered to be the prototypic non-specific glycosaminoglycan–protein system and the primary reason for its non-specificity is the low level of non-ionic binding energy [24]. Considering that both LMWLs display higher proportion of non-ionic binding energy, it is possible that certain chains of these molecules, especially CDSO3, may possess high level of specificity for exosite 2 of thrombin.

Another insight that these results bring about is the correlation between the proportion of non-ionic binding energy and inhibition potency. CDSO3 exhibits highest thrombin inhibition potency, FDSO3 is the next best, while heparin is non-inhibitory. Considering that the three molecules also display a similar change in non-ionic forces involved in binding to thrombin, it is likely that inhibition arises from interaction with non-ionic residues in exosite 2. Although our work with smaller, homogeneous molecules that bind in exosite 2 has shown early promise in discovery of allosteric modulators of thrombin [6–8], the residue-level cooperativity between exosite 2 and active site remains to be identified.

Exosite 2 Interactions Display a Characteristic Positive Change in Heat Capacity

To further parse scaffold-dependent differences in other thermodynamic parameters, e.g., heat capacity, we measured affinities of CDSO3, FDSO3 and UFH for thrombin at varying temperatures. The decrease in fluorescence due to the complex formation was used to obtain the $K_{D,app}$ ($= 1/K_A$) for CDSO3, FDSO3 or UFH binding to β PR-thrombin at the temperature studied (5–45 °C) (Table 2, Supplementary Figures S4 to S6). The plots of $\ln K_A$ versus $1/T$ for each of the system were found to display a characteristic parabolic profile (Figure 4). Using the integrated form of the van't Hoff equation, we calculated C_p , T_H and T_S for the complexes (Table 3). The change in heat capacity (C_p) of interaction was found to be positive for all three systems and ranged between 0.38 and 0.69 kcal/K mol. The positive heat capacity change indicates that complex formation involves burial of polar surface area on thrombin. Considering that all three molecules engage the same site of thrombin exosite 2, it is not unusual that C_p values are fairly similar for all three electronegative ligands.

The enthalpic (ΔH) and entropic ($-\Delta T \Delta S$) contributions to complex formation were resolved using equations 4 and 5, respectively. The ΔH contributions for CDSO3 and UFH were similar at each temperature studied in the range 278 to 318 K, whereas those for FDSO3 followed an identical trend but with considerable difference in magnitude (Table 4). Likewise, CDSO3 and UFH were more similar in entropic contributions. Quantitatively, entropic contributions were higher than enthalpic contributions at all temperatures studied for CDSO3 and UFH. This implies that the dominant force stabilizing thrombin–ligand complexes was entropy for CDSO3 and UFH. It is now known that heparin–protein interactions, e.g., UFH–antithrombin, are controlled by entropic changes, which typically arises from the release of Na^+ ions and/or H_2O molecules upon GAG–protein complex formation [31,32]. Yet, an interesting departure from this general observation was that FDSO3–thrombin complex was enthalpically stable at temperatures up to 303K because of more ionic interactions. Finally, for all three allosteric agents, enthalpy was unfavorable at physiologic temperature of 310K (~37°C, Table 4).

FDSO3 Reveals Cooperativity between Exosite 2 and Sodium Binding Site

Sodium is a well-known allosteric effector for thrombin. Thrombin activity increases upon binding to sodium [33–35], which has led to the classification of “slow” and “fast” forms of thrombin. Sodium binds in an allosteric site defined by residues Glu217, Asp222 and Tyr225, which are approximately 16–20 Å away from the catalytic triad, yet a mere 5 Å from D189 of S1 site. Several studies have shown that sodium binding alters the structure of

the enzyme. Sodium binding is also linked to changes in exosite 1 conformation [17,33–36] but its cooperativity with exosite 2 ligands has been variably interpreted. Whereas some researchers have not detected any cooperativity [17,37], others have noted negative cooperativity [23]. Considering that LMWLs are unique allosteric inhibitors of thrombin, a question arises whether LMWLs binding is energetically linked to sodium binding. More specifically, does sodium affect the affinity of LMWLs for thrombin?

To address this, we measured FDSO3 binding isotherms in buffers of constant ionic strength ($I = 0.2$ M) while varying the concentration of Na^+ using SPR. Human α -thrombin was immobilized onto gold surface using traditional amine coupling chemistry, as described earlier [28] and sensorgrams arising from the association and dissociation of FDSO3 followed as a function of time. Figure 5A shows representative association/dissociation profiles measured, which were then used to calculate the k_{ON} and k_{OFF} of the binding (Table S1). Figure 5B shows change in $K_D (=k_{\text{OFF}}/k_{\text{ON}})$ as a function of sodium concentrations, which displays a characteristic inverse hyperbola. The K_D of FDSO3–thrombin complex decreased from 49 nM to 1.3 nM, a change of some 30-fold, as Na^+ concentration increased from 0 to 200 mM. This implied that FDSO3 loses affinity for thrombin as sodium levels were reduced. Alternatively, FDSO3 prefers to bind thrombin in the presence of Na^+ and not in its absence. This is contrast to the observation with fragment 1.2, which displays negative cooperativity with saturation of sodium binding site [23]. A key reason for this difference is likely to be different amino acid residues engaged by the two ligands, despite binding in the same site. Our earlier work with a group of sulfated benzofuran oligomers also showed such microscopic differences [8], which probably arise when considerable structural differences exist between the two ligands (e.g., protein *versus* sulfated glycosaminoglycan mimetic).

Considering that K_D refers to 50% saturation of a binding site, the change in K_D of FDSO3–thrombin complex as a function of Na^+ could be used to estimate the K_D of sodium binding to thrombin using the hyperbolic binding isotherm. This non-linear regression analysis, presented in Figure 5B, resulted in a K_D of thrombin– Na^+ complex of 1.9 ± 0.4 mM, which is in good agreement with the value of ~ 4 mM reported in the literature under similar conditions [23]. Thus, FDSO3 prefers to bind to the “fast” form of thrombin, which is the clinically relevant form.

Discussion

LMWLs are structurally and mechanistically distinct from any thrombin inhibitor known to date. Although they were initially designed as UFH mimetics, they were found to inhibit thrombin directly in an antithrombin-independent manner [3,4]. LMWLs have been shown to allosterically inhibit thrombin by binding to exosite 2. This work shows that the thrombin affinity of these agents is high and comparable to sulfated aromatic ligands interacting with coagulation enzymes and serpins [38].

This work shows that although the two LMWLs studied here are chemically related, some of their physicochemical properties are similar (C_p), while others are dissimilar ($K_{D,NI}$). Interestingly, major differences were found in the number of ion-pair interactions formed by

the two LMWLs with thrombin. Whereas CDSO3 utilizes 1 – 2 ionic interactions, FDSO3 relies on 4 – 5. More importantly, CDSO3 appears to exhibit elements of thrombin recognition specificity, which could be further exploited in the design of specific allosteric modulators.

Macromolecular interactions that are dominated by electrostatic interactions are accompanied by burial of polar surfaces of the protein. These interactions usually display a positive ΔG , e.g., xanthine oxidase – superoxide dismutase [39], phosphofructokinase tetramerization [40], and translation initiation factor eIF4E – mRNA [26,27]. GAGs also display similar thermodynamic signature, e.g., heparin/heparan sulfate binding to cationic cell penetrating peptides, or brain natriuretic peptide, or HIV-1 *trans*-acting activator of transcription [41–43]. Therefore, the current results imply that LMWLs are good mimetics of UFH as far as ΔG is concerned.

A major conclusion of this work is the energetic coupling between sodium binding site and exosite 2 as far as LMWLs are concerned. The cooperative network between sodium binding site, active site, and exosite 1 has been discussed in numerous papers. Subtle ligand-specific cooperativity has been noted in several studies. Yet, the interplay of exosite 2 and sodium binding site has remained unclear. Our results with FDSO3 shows positive cooperativity, in contrast to either neutral [17,37] or negativity cooperativity [23], between exosite 2 and sodium binding site. This positive cooperativity implies that FDSO3 functions as a better inhibitor of thrombin in the sodium-bound form, which theoretically is the target of a clinically relevant anticoagulant. This implies that the structurally unique characteristics of LMWLs should enable discovery of true allosteric regulators of thrombin.

Overall, this work presents fundamental insights into ligand recognition of exosite 2. The results show that LMWLs are novel and potent allosteric ligands of thrombin, which increased binding in the presence of sodium. A summative directive of this work is that each thrombin–ligand pair appears to exhibit distinctive features, which are important to elucidate to advance the design of allosteric regulators of thrombin that truly balance its pro- and anti-coagulation roles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

LMWH	low molecular weight heparin
LMWL	low molecular weight lignin
PEG	polyethylene glycol
UFH	unfractionated heparin

References

1. Henry, BL., Desai, UR. Burger's Medicinal Chemistry, Drug Discovery and Development. John Wiley & Sons, Inc; 2010. Anticoagulants.
2. Weitz JI, Harenberg J. New developments in anticoagulants: Past, present and future. *Thromb Haemost.* 2017; 117(7):1283–1288. [PubMed: 28594426]
3. Monien BH, Henry BL, Raghuraman A, Hindle M, Desai UR. Novel chemo-enzymatic oligomers of cinnamic acids as direct and indirect inhibitors of coagulation proteinases. *Bioorg Med Chem.* 2006; 14:7988–7998. [PubMed: 16914317]
4. Henry BL, Monien BH, Bock PE, Desai UR. A novel allosteric pathway of thrombin inhibition: Exosite II mediated potent inhibition of thrombin by chemo-enzymatic, sulfated dehydropolymers of 4-hydroxycinnamic acids. *J Biol Chem.* 2007; 282:31891–31899. [PubMed: 17804413]
5. Abdel Aziz MH, Mosier PD, Desai UR. Identification of the site of binding of sulfated, low molecular weight lignins on thrombin. *Biochem Biophys Res Commun.* 2011; 413:348–352. [PubMed: 21893043]
6. Sidhu PS, Liang A, Mehta AY, Abdel Aziz MH, Zhou Q, Desai UR. Rational design of potent, small, synthetic allosteric inhibitors of thrombin. *J Med Chem.* 2011; 54:5522–5531. [PubMed: 21714536]
7. Abdel Aziz MH, Sidhu PS, Liang A, Kim JY, Mosier PD, Zhou Q, Farrell DH, Desai UR. Designing allosteric regulators of thrombin. Monosulfated benzofuran dimers selectively interact with Arg173 of exosite 2 to induce inhibition. *J Med Chem.* 2012; 55:6888–6897. [PubMed: 22788964]
8. Sidhu PS, Abdel Aziz MH, Sarkar A, Mehta AY, Zhou Q, Desai UR. Designing allosteric regulators of thrombin. Exosite 2 features multiple subsites that can be targeted by sulfated small molecules for inducing inhibition. *J Med Chem.* 2013; 56:5059–5070. [PubMed: 23718540]
9. Henry BL, Thakkar JN, Martin EJ, Brophy DF, Desai UR. Characterization of the plasma and blood anticoagulant potential of structurally and mechanistically novel oligomers of 4-hydroxycinnamic acids. *Blood Coag Fibrinol.* 2009; 20:27–34.
10. Liang A, Thakkar JN, Desai UR. Study of physico-chemical properties of novel highly sulfated, aromatic, mimetics of heparin and heparan sulfate. *J Pharm Sci.* 2010; 99:1207–1216. [PubMed: 19711446]
11. Mehta AY, Desai UR. Substantial non-electrostatic forces are needed to induce allosteric disruption of thrombin's active site through exosite 2. *Biochem Biophys Res Commun.* 2014; 452:813–816. [PubMed: 25201728]
12. Fredenburgh JC, Stafford AR, Weitz JI. Evidence for allosteric linkage between exosites 1 and 2 of thrombin. *J Biol Chem.* 1997; 272:25493–25499. [PubMed: 9325262]
13. Verhamme IM, Olson ST, Tollefsen DM, Bock PE. Binding of exosite ligands to human thrombin. Re-evaluation of allosteric linkage between thrombin exosites I and II. *J Biol Chem.* 2002; 277:6788–6798. [PubMed: 11724802]
14. Sabo TM, Farrell DH, Maurer MC. Conformational analysis of gamma' peptide (410–427) interactions with thrombin anion binding exosite II. *Biochemistry.* 2006; 45:7434–7445. [PubMed: 16768439]
15. Petrera NS, Stafford AR, Leslie BA, Kretz CA, Fredenburgh JC, Weitz JI. Long range communication between exosites 1 and 2 modulates thrombin function. *J Biol Chem.* 2009; 284:25620–25629. [PubMed: 19589779]
16. Sabo TM, Maurer MC. Biophysical investigation of GpIb alpha binding to thrombin anion binding exosite II. *Biochemistry.* 2009; 48:7110–7122. [PubMed: 19591434]
17. Qureshi SH, Yang L, Manithody C, Iakhiaev AV, Rezaie AR. Mutagenesis studies toward understanding allostery in thrombin. *Biochemistry.* 2009; 48:8261–8270. [PubMed: 19640005]
18. Li W, Johnson DJ, Adams TE, Pozzi N, De Filippis V, Huntington JA. Thrombin inhibition by serpins disrupts exosite II. *J Biol Chem.* 2010; 285:38621–38629. [PubMed: 20889971]
19. Malovichko MV, Sabo TM, Maurer MC. Ligand binding to anion-binding exosites regulates conformational properties of thrombin. *J Biol Chem.* 2013; 288:8667–8678. [PubMed: 23378535]

20. Yeh CH, Stafford AR, Leslie BA, Fredenburgh JC, Weitz JI. Dabigatran and argatroban diametrically modulate thrombin exosite function. *PLoS One*. 2016; 11:e0157471. [PubMed: 27305147]
21. Chen K, Stafford AR, Wu C, Yeh CH, Kim PY, Fredenburgh JC, Weitz JI. Exosite 2-directed ligands attenuate protein C activation by the thrombin-thrombomodulin complex. *Biochemistry*. 2017; 56:3119–3128. [PubMed: 28549218]
22. Xiao J, Melvin RL, Salsbury FR. Mechanistic insights into thrombin's switch between "slow" and "fast" forms. *Phys Chem Chem Phys*. 2017; 19:24522–24533. [PubMed: 28849814]
23. Kamath P, Huntington JA, Krishnaswamy S. Ligand binding shuttles thrombin along a continuum of zymogen- and proteinase-like states. *J Biol Chem*. 2010; 285:28651–28658. [PubMed: 20639195]
24. Olson ST, Halvorson HR, Bjork I. Quantitative characterization of the thrombin-heparin interaction. Discrimination between specific and nonspecific binding models. *J Biol Chem*. 1991; 266:6342–6352. [PubMed: 2007587]
25. Vindigni A, White CE, Komives EA, Di Cera E. Energetics of thrombin-thrombomodulin interaction. *Biochemistry*. 1997; 36:6674–6681. [PubMed: 9184147]
26. Niedzwiecka A, Stepinski J, Darzynkiewicz E, Sonenberg N, Stolarski R. Positive heat capacity change upon specific binding of translation initiation factor eIF4E to mRNA 5' cap. *Biochemistry*. 2002; 41:12140–12148. [PubMed: 12356315]
27. Kiraga-Motoszko K, Niedzwiecka A, Modrak-Wojcik A, Stepinski J, Darzynkiewicz E, Stolarski R. Thermodynamics of molecular recognition of mRNA 5' cap by yeast eukaryotic initiation factor 4E. *J Phys Chem B*. 2011; 115:8746–8754. [PubMed: 21650456]
28. Fischer MJ. Amine coupling through EDC/NHS: a practical approach. *Methods Mol Biol*. 2010; 627:55–73. [PubMed: 20217613]
29. Carter WJ, Cama E, Huntington JA. Crystal structure of thrombin bound to heparin. *J Biol Chem*. 2005; 280:2745–2749. [PubMed: 15548541]
30. Hogg PJ, Jackson CM, Labanowski JK, Bock PE. Binding of fibrin monomer and heparin to thrombin in a ternary complex alters the environment of the thrombin catalytic site, reduces affinity for hirudin, and inhibits cleavage of fibrinogen. *J Biol Chem*. 1996; 271:26088–26095. [PubMed: 8824251]
31. Sarkar A, Yu W, Desai UR, MacKerell AD, Mosier PD. Estimating glycosaminoglycan-protein interaction affinity: water dominates the specific antithrombin-heparin interaction. *Glycobiology*. 2016; 26:1041–1047. [PubMed: 27496757]
32. Sarkar A, Desai UR. A simple method for discovering druggable, specific glycosaminoglycan-protein systems. Elucidation of key principles from heparin/heparan sulfate-binding proteins. *PLoS One*. 2015; 10:e0141127. [PubMed: 26488293]
33. Di Cera E. Thrombin as procoagulant and anticoagulant. *J Thromb Haemost*. 2007; 5:196–202. [PubMed: 17635727]
34. Di Cera E, Page MJ, Bah A, Bush-Pelc LA, Garvey LC. Thrombin allostery. *Phys Chem Chem Phys*. 2007; 9:1291–1306. [PubMed: 17347701]
35. Di Cera E. Thrombin. *Mol Aspects Med*. 2008; 29:203–254. [PubMed: 18329094]
36. Zavyalova E, Tagiltsev G, Reshetnikov R, Arutyunyan A, Kopylov A. Cation coordination alters the conformation of a thrombin-binding G-quadruplex DNA aptamer that affects inhibition of thrombin. *Nucleic Acid Ther*. 2016; 26:299–308. [PubMed: 27159247]
37. Mutch NJ, Myles T, Leung LL, Morrissey JH. Polyphosphate binds with high affinity to exosite II of thrombin. *J Thromb Haemost*. 2010; 8:548–555. [PubMed: 20002544]
38. Henry BL, Connell J, Liang A, Krishnasamy C, Desai UR. Interaction of antithrombin with sulfated, low molecular weight lignins: opportunities for potent, selective modulation of antithrombin function. *J Biol Chem*. 2009; 284:20897–20908. [PubMed: 19497853]
39. Zhou Y, Liao J, Du F, Liang Y. Thermodynamics of the interaction of xanthine oxidase with superoxide dismutase studied by isothermal titration calorimetry and fluorescence spectroscopy. *Thermochimica Acta*. 2005; 426:173–178.

40. Luther MA, Gilbert HF, Lee JC. Self-association of rabbit muscle phosphofructokinase: role of subunit interaction in regulation of enzymatic activity. *Biochemistry*. 1983; 22:5494–5500. [PubMed: 6228252]
41. Ziegler A, Seelig J. Binding and clustering of glycosaminoglycans: a common property of mono- and multivalent cell-penetrating compounds. *Biophys J*. 2008; 94:2142–2149. [PubMed: 18065465]
42. Hileman RE, Jennings RN, Linhardt RJ. Thermodynamic analysis of the heparin interaction with a basic cyclic peptide using isothermal titration calorimetry. *Biochemistry*. 1998; 37:15231–15237. [PubMed: 9790687]
43. Ziegler A, Seelig J. Interaction of the protein transduction domain of HIV-1 TAT with heparan sulfate: binding mechanism and thermodynamic parameters. *Biophys J*. 2004; 86:254–263. [PubMed: 14695267]

Highlights

- Thrombin's sodium binding site shows positive cooperativity with certain exosite 2 ligands.
- Non-saccharide glycosaminoglycan mimetics display better thrombin inhibition in the presence of sodium
- Different allosteric inhibitors may engage different set of residues although binding in the same site of binding

Heparin :: $R = -\text{COCH}_3 \text{ or } -\text{SO}_3^-$
 $X = Y = \text{H or } -\text{SO}_3^-$

CDSO3 :: $R = -\text{H or } -\text{SO}_3^-$; $X = Y = -\text{H or } -\text{SO}_3^-$
FDSO3 :: $R = X = -\text{Me}$; $Y = -\text{H or } -\text{SO}_3^-$

Figure 1.

Structures of animal-derived unfractionated heparin (UFH or heparin) and chemoenzymatically prepared two low molecular weight lignins (LMWLs), CDSO3 and FDSO3. Of these, the latter two possess average molecular weights of 3000 – 4000 Da (5–13-mers) in comparison to ~15,000 Da of the former (30–50-mers). Whereas UFH has a polysaccharide scaffold, LMWLs are based on an aromatic scaffold called lignin. All three agents contain sulfate ($-\text{OSO}_3^-$) and carboxylate groups ($-\text{COO}^-$), which is the reason for their high negative charge at physiologic pH and high water solubility. Chiral centers are identified by curvy bonds.

Figure 2.

Representative fluorescence titrations showing binding of FPR-Th to LMWLs, CDSO3 and FDSO3. The fluorescence of 50 nM fluorescein-labeled thrombin was monitored in 20 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl, 2.5 mM CaCl₂ and 0.1 % PEG 8000 as a function of LMWL concentration at 25 °C in a quartz cuvette. Solid lines represent fits using equation 3 to obtain the apparent K_D , as described in 'Experimental'.

Figure 3.

A plot showing the variation in the observed binding affinity ($\log K_{D,app}$) of FDSO3 as a function of the ionic strength ($\log [Na^+]$) of the buffer at pH 7.4. The binding affinities were measured using spectrofluorimetric titrations at ionic strengths in the range of 50 – 500 mM NaCl. Solid line represents linear fits of the data using equation 2 to obtain the binding affinity arising from non-ionic forces ($K_{D,NI}$) and the number of ion-pair interactions formed between the enzyme and ligand (Z), as described in ‘Experimental’.

Figure 4.

The temperature dependence of association constant (K_A) for CDSO3, FDSO3 and UFH. The K_A was calculated from the observed binding affinity ($K_{D,app} = 1/K_A$) using spectrofluorimetric titration of $\mathcal{F}$ PR-Th in 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM CaCl_2 , and 0.1% PEG 8000. The range of temperatures studied was 278–318 K (see ‘Experimental’). Solid lines represent regressional fit of the data using equation 3 where C_p , T_H and T_S were allowed to float.

Figure 5.

(A) Binding isotherms for binding of FDSO3 to the immobilized thrombin using surface plasmon resonance (grey lines). Increasing concentrations of FDSO3 (40, 80, 160, 240, 320, 400, 600 nM) were studied for binding to thrombin at 25 °C. Varying levels of NaCl and choline chloride were used such that the total ionic strength remained constant ($I = 0.2$). The association sensorgram was followed for 3 min to measure association rate constant (k_{ON}), while the dissociation sensorgram was followed for 6 min to obtain (k_{OFF}). The manufacturer's software was used to calculate the binding affinity ($= k_{OFF}/k_{ON}$) assuming a 1:1 binding model (black lines). (B) Variation in the apparent K_D of FDSO3–thrombin complex measured by SPR as a function of the concentration of sodium ion. Solid line represents the regressional fit of the data to equation 6 in which K_D , B_o and B_{max} were allowed to float.

Table 1

Salt dependence of dissociation constant of CDSO3 and FDSO3 binding to human alpha-thrombin.

[Na ⁺] (M)	K _{D,app} (nM) ^a	
	FDSO3	CDSO3
0.100	165 ± 16 ^b	ND ^c
0.150	353 ± 39	90 ± 4
0.200	1437 ± 217	119 ± 7
0.250	3260 ± 507	156 ± 6
0.300	ND	223 ± 10

^a K_{D,app} was measured in 20 mM Tris-HCl buffer, pH 7.4, containing 100 – 300 mM NaCl, 2.5 mM CaCl₂, and 0.1% polyethylene glycol (PEG) 8000 at 25 °C (see ‘Experimental’). Experiments were performed at least in duplicate and averaged for curve fitting

^b errors represent ±1 SD

^c Not determined

Table 2

Temperature dependence of dissociation constant of CDSO3, FDSO3 and UFH binding to human alpha-thrombin.

Temp (K)	$K_{D,app}$ (nM) ^a		
	CDSO3	FDSO3	UFH
278	53 ± 2^b	21 ± 3	163 ± 14
283	ND ^c	33 ± 5	187 ± 13
288	66 ± 3	54 ± 3	ND
293	65 ± 3	62 ± 4	198 ± 20
298	53 ± 2	105 ± 11	181 ± 13
303	48 ± 3	85 ± 6	149 ± 11
308	ND	76 ± 6	ND
313	40 ± 2	ND	113 ± 6
318	25 ± 3	68 ± 4	ND

^a $K_{D,app}$ was measured in 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM CaCl₂, and 0.1% polyethylene glycol (PEG) 8000 at variable temperatures (278 – 318 K) (see 'Experimental'). Experiments were performed at least in duplicate and averaged for curve fitting.

^bErrors represent ± 1 SD

^cNot determined

Table 3

Thermodynamic properties of CDSO3, FDSO3 and UFH in relation to binding to human alpha-thrombin.

	CDSO3	FDSO3	UFH
K_D (nM) ^a	53.3 ± 2.2^c	104.8 ± 11.4	181 ± 13.0
K_A (M ⁻¹)	$(1.88 \pm 0.08) \times 10^7$	$(0.95 \pm 0.10) \times 10^7$	$(0.55 \pm 0.04) \times 10^7$
G (kcal/mol)	-9.91 ± 0.02	-9.51 ± 0.06	-9.19 ± 0.04
C_p (kcal/K mol) ^b	0.38 ± 0.08	0.69 ± 0.11	0.38 ± 0.05
T_H (K) ^b	289 ± 2.2	304 ± 1.6	289 ± 1.0
T_S (K) ^b	265 ± 6.5	290 ± 1.5	267 ± 3.8

^a K_D was measured in 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM CaCl₂, and 0.1% polyethylene glycol (PEG) 8000 at variable temperatures (278 – 318 K) (see ‘Experimental’). Experiments were performed at least in duplicate and averaged for curve fitting.

^b C_p , T_H and T_S were obtained following analysis of K_A *versus* 1/T using equation 3 (see “Experimental”). Experiments were performed at least in duplicate and averaged for curve fitting.

^c Errors represent ± 1 SD

Table 4
Temperature dependence of thermodynamic parameters for CDSO3, FDSO3 and UFH binding to human alpha-thrombin.^a

Temp. (K)	CDSO3			FDSO3			UFH		
	H (kcal/mol)	-T S (kcal/mol)	H (kcal/mol)	-T S (kcal/mol)	H (kcal/mol)	-T S (kcal/mol)	H (kcal/mol)	-T S (kcal/mol)	
278	-4.20	-5.11	-18.12		8.28		-4.28	-4.40	
283	-2.29	-7.13	-14.69		4.97		-2.41	-6.37	
288	-0.37	-9.18	-11.26		1.60		-0.53	-8.37	
293	1.54	-11.27	-7.83		-1.83		1.34	-10.41	
298	3.45	-13.39	-4.40		-5.32		3.22	-12.48	
303	5.36	-15.54	-0.97		-8.87		5.09	-14.58	
310	8.03	-18.60	2.46		-12.47		6.97	-16.71	
313	9.18	-19.93	4.43		-16.13		8.84	-18.87	
318	11.09	-22.18	9.32		-19.85		10.72	-21.06	

^a H and S were obtained following measurements of Cp, TH and TS using equations 4 and 5 (see 'Experimental').