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Novel heparin mimetics reveal cooperativity between exosite 2 and sodium-binding site of thrombin

May H. Abdel Aziz[a,1], Umesh R. Desai[a,b,*]

[a] Department of Medicinal Chemistry, Virginia Commonwealth University, Richmond, VA 23219, United States
[b] Institute for Structural Biology, Drug Discovery and Development, Virginia Commonwealth University, Richmond, VA 23219, United States

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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 were 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.

1. 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 (Fig. 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 affords 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

Abbreviations: LMWH, low molecular weight heparin; LMWL, low molecular weight lignin; PEG, polyethylene glycol; UFH, unfractionated heparin

* Corresponding author at: Department of Medicinal Chemistry, Virginia Commonwealth University, 800 East Leigh Street, Suite 212, Richmond, VA 23219, United States.
E-mail address: urdesai@vcu.edu (U.R. Desai).
[1] Current address: Ben and Maytee Fisch College of Pharmacy, The University of Texas, 3900 University Blvd., Tyler, TX.

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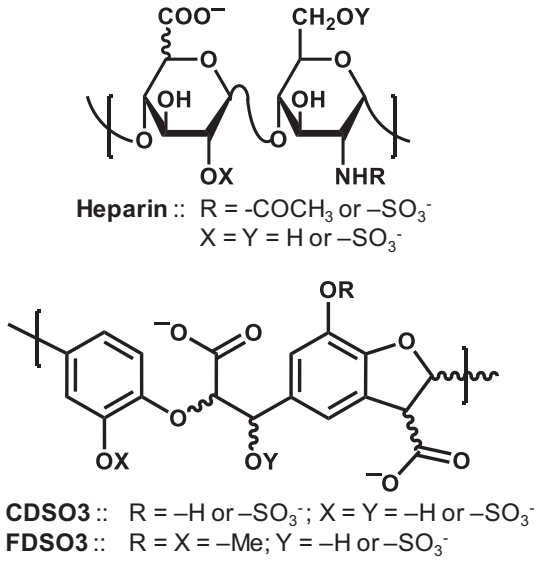

Fig. 1. Structures of animal-derived unfractionated heparin (UFH or heparin) and chemoenzymatically prepared two low molecular weight lignins (LMWLs), CDSO3 and FDSO3. Of these, the latter two possess average molecular weights of 3000–4000 Da (5–13-mers) in comparison to ~15,000 Da of the former (30–50-mers). Whereas UFH has a polysaccharide scaffold, LMWLs are based on an aromatic scaffold called lignin. All three agents contain sulfate (—OSO_3^-) and carboxylate groups (—COO^-), which is the reason for their high negative charge at physiologic pH and high water solubility. Chiral centers are identified by curvy bonds.

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].

2. Experimental

2.1. 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 (*f*FPR-Th) were 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 *f*FPR-Th was prepared in 20 mM Tris-HCl buffer (pH 7.4). Monothiol carboxyl SAM gold slides were purchase 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.

2.2. *f*FPR-thrombin–LMWLs interaction

*f*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 *f*FPR-Th and monitoring the decrease in the fluorescence (λ_{EX} = 490 nm, λ_{EM} = 525 nm). LMWLs were screened for interference at the emission wavelength of detection to rule out interfere with the readings (Supplementary Fig. 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 (Eq. (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, ΔF is the change in fluorescence due to formation of the complex following each addition of LMWL from the initial fluorescence F_0, ΔF_{max} represents the maximum change in fluorescence observed on saturation of *f*FPR-Th and Hill coefficient (n) is a measure for the cooperativity of binding.

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

2.3. Salt dependence of LMWL–*f*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 *f*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 $[Na^+]$ affords ionic and non-ionic forces involved in binding by analyzing the data using linear Eq. (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.

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