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# Substantial non-electrostatic forces are needed to induce allosteric disruption of thrombin's active site through exosite 2

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ABSTRACT

Sulfated β-O4 lignin (SbO4L), a non-saccharide glycosaminoglycan mimetic, was recently disclosed as a novel exosite 2-directed thrombin inhibitor with the capability of mimicking sulfated tyrosine sequences of glycoprotein Ibα resulting in dual anticoagulant and antiplatelet activities. SbO4L engages essentially the same residues of exosite 2 as heparin and yet induces allosteric inhibition. Fluorescence spectroscopic studies indicate that SbO4L reduces access of the active site to molecular probes and affinity studies at varying salt concentrations show nearly 6 ionic interactions, similar to heparin, but much higher non-ionic contribution. The results suggest that subtle increase in non-electrostatic forces arising from SbO4L's aromatic scaffold appear to be critical for inducing allosteric dysfunction of thrombin's active site.



## 1. Introduction

Hemostasis is a fine-tuned process that maintains a delicate balance between bleeding and clotting through a dynamic sequence of events involving soluble molecules, cells and sub-vasculature factors. A key aspect of this process is the blood coagulation cascade, which relies on highly selective recognition of macromolecular substrates by their proteases [1–3]. Selectivity for most enzyme–substrate pairs arises from complementarity of three-dimensional features around the active site, but that for coagulation proteases appears to rely on allosteric sites [2–4]. For example, thrombin can function either as a procoagulant or an anticoagulant depending on whether its macromolecular partner is fibrinogen or thrombomodulin [4,5].

Thrombin is arguably the most important coagulation protease interacting with many proteins including pro-cofactors and cell surface receptors to effect its function. In addition to its one-of-a-kind active site, it is endowed several exosites including the sodium binding site, and anion-binding exosites 1 and 2 [6–7]. The three exosites are located several angstroms away from the active site and modulate thrombin's catalytic activity. Recent studies indicate that thrombin is a mobile protease exhibiting multiple conformations that can be differentially stabilized by using appropriate exosite binding ligands [6,8]. Molecular forces that contribute to these processes are just beginning to be unraveled.

Understanding thrombin allosterism is important because of the possibility of developing anticoagulants for treating thrombotic disorders and procoagulants for treating hemophilic disorders. *A priori*, allosterism offers regulatory advantages that orthosterism cannot [4,9,10]. For example, allosteric inhibitors can be designed to maximally inhibit less than 100%, as shown earlier [11], which may be of special value when complete inhibition results in deleterious consequences. Considering that thrombin is a highly plastic enzyme, understanding forces contributing to allosteric networks may help develop novel inhibitors with potentially reduced bleeding complications.

Recently, we have developed sulfated β-O4 lignin (SbO4L), a sulfated non-saccharide glycosaminoglycan mimetic (NSGM), as a macromolecular allosteric inhibitor of thrombin [12]. SbO4L induced a reduction in the catalytic efficiency of thrombin by binding to several positively charged residues of exosite 2. This was in striking contrast to heparin, which also binds exosite 2 with similar set of residues, and yet does not induce catalytic dysfunction [13]. Further, the allosteric disruption of catalysis by SbO4L occurred only for thrombin suggesting a highly selective process. In contrast, heparin binds to several coagulation proteases and demonstrates essentially no selectivity [14]. Finally, SbO4L was found to compete with glycoprotein Ibα (GPIbα) of platelets in binding to exosite 2 of thrombin and thereby exert dual anticoagulant and antiplatelet activities. SbO4L is the first synthetic macromolecule to exhibit such a mechanism and offers an interesting platform to study the

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nature of allosterism and its dependence on the structure of effector ligand. This work discusses studies focused on understanding why SbO4L induces catalytic dysfunction and deciphering similarities and differences between thrombin interactions with SbO4L and heparin. The results provide new avenues to explore in developing sulfated NSGMs as allosteric regulators of thrombin.

## 2. Materials and methods

### 2.1. Materials

Active site labeled fluorescein-FPR-thrombin (*f*FPR-thrombin) was purchased from Haematologic Technologies (Essex Junction, VT). Fluorescence measurements were recorded using a QM4 spectrofluorometer (Photon Technology International, Birmingham, NJ). All other reagents were reagent grade and purchased from either Sigma Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

### 2.2. Fluorescence quenching studies

Quenching studies using a collisional quencher were performed in 20 mM Tris–HCl buffer containing 100 mM NaCl, 2.5 mM $CaCl_2$, and 0.1% PEG 8000 at pH 7.4. A mixture of *f*FPR-thrombin (170 nM) and 44 μg/mL SbO4L (or buffer) was titrated with increasing concentrations of acrylamide (10 M stock, 1 μL additions) and fluorescence intensity at 522 nm ($\lambda_{EX}$ = 490 nm) was monitored at 25 °C. Excitation and emission slit widths were 1 mm each. The ratio of the fluorescence without the quencher ($F_0$) to that in the presence of the quencher ($F$) was plotted against the quencher concentration ($Q$), which yielded a linear relationship that could be fitted using the Stern–Volmer Eq. (1) [15]. In this equation, $Q$ is the concentration of the quencher, $K_{SV}$ is the Stern–Volmer constant and is equal to $k_q\tau_0$ ($k_q$ = bimolecular quenching constant, $\tau_0$ = fluorophore lifetime in the absence of the quencher):

$$\frac{F_0}{F} = 1 + K_{SV}[Q] \tag{1}$$

### 2.3. Binding affinity studies

The affinity of SbO4L for *f*FPR-thrombin was measured in 20 mM Tris–HCl, pH 7.4, containing varying concentrations of NaCl (100–200 mM), 2.5 mM $CaCl_2$ and 0.1% PEG 8000. A concentration of 170 nM *f*FPR-thrombin was sufficient to give good fluorescence emission signal at 522 nm ($\lambda_{EX}$ = 490 nm) at 25 °C. Excitation and emission slit widths were 1 mm each. Titrations were performed by adding small aliquots of concentrated stock of SbO4L so as to not increase the total volume of the sample by more than 5% and monitoring the change in fluorescence at 522 nm. This change in fluorescence signal was fitted using the classic quadratic binding equation 2 to calculate the dissociation constant $K_D$ at various concentrations of salt. IN this equation, $\Delta F$ represents the change in fluorescence of *f*FPR-thrombin at each addition of SbO4L from the initial fluorescence $F_0$ and $\Delta F_{MAX}$ represents the maximal change in fluorescence observed when the enzyme is saturated with the inhibitor. The binding stoichiometry was assumed to be 1:1 for SbO4L–thrombin complex:

$$\frac{\Delta F}{F_0} = \frac{\Delta F_{MAX}}{F_0}\left\{\frac{[E]_0 + [\mathrm{SbO4L}]_0 + K_D - \sqrt{([E]_0 + [\mathrm{SbO4L}]_0 + K_D)^2 - 4[E]_0[\mathrm{SbO4L}]_0}}{2[E]_0}\right\} \tag{2}$$

To resolve contribution of the ionic and non-ionic forces to the SbO4L–thrombin interaction, Eq. (3) was used. The slope ($Z \times \Psi$) of the line correlates the sensitivity of the binding affinity to salt concentration, while the intercept corresponds to binding affinity when all ionic interactions are completely screened ([$Na^+$] = 1 M).

$$\log K_{D,OBS} = \log K_{D,NI} + Z \times \Psi \log [Na^+] \tag{3}$$

## 3. Results

### 3.1. Exosite 2 binding by SbO4L reduces steric access to thrombin's active site

To assess the steric accessibility of thrombin's active site upon SbO4L binding, quenching studies were carried out on *f*FPR-thrombin using a collisional quencher. We reasoned that if SbO4L binding to exosite 2 induces significant changes in the conformational state of the active site, then a collisional quencher would affect fluorescence of the active site probe at a rate different from that for the native enzyme. Acrylamide and iodide are two collisional quenchers typically used in the literature for this purpose with good success [15]. Both molecules quench protein's fluorescence through direct molecular contact, which implies that the method senses steric and electrostatic environment in the vicinity of the fluorophore. Fig. 1A shows the acrylamide-induced quenching of fluorescein fluorescence of *f*FPR-thrombin in free and SbO4L bound forms. At low levels of acrylamide (0.04–0.12 M), a rapid decrease in fluorescence was observed, which gradually reached a plateau at acrylamide concentrations of 0.56 M, for enzyme alone. However, in the presence of saturating concentrations of SbO4L, fluorophore quenching was significantly retarded such that at 0.56 M acrylamide, the fluorescence of the complex was significantly higher than the thrombin alone. Further, a bolus of SbO4L (44 μM final concentration) added thrombin pre-quenched with 0.56 M acrylamide resulted in a nearly complete recovery of fluorescence to levels observed in the titration of SbO4L–thrombin complex (not shown).

Collisional quenching can be quantitatively analyzed in terms of one or more species of fluorophores present on the protein using various forms of Stern–Volmer relationships [15]. Fig. 1B shows Stern–Volmer plots that can be explained well using a linear relationship (Eq. (1)) suggesting the presence of only one species of fluorophore for both free thrombin and thrombin–SbO4L complex. The slope of the linear regression corresponds to the quenching constant ($K_{SV}$), which was calculated to be 0.351 for free thrombin and 0.073 for the complex, a difference of almost 4.8-fold. This suggests that the allosteric conformational change induced by SbO4L in thrombin reduces the significant accessibility of the fluorophore to acrylamide. Considering that the fluorescein fluorophore is located almost at the P4 site ($^{P4}$fFPR$^{P1}$), these results indicate that the allosteric change induced by SbO4L is extensive and is not just limited to the catalytic triad.

### 3.2. SbO4L–thrombin interaction is primarily electrostatic, but with significant non-ionic component

To determine the nature of interactions made by SbO4L with thrombin, the dissociation constant of complex ($K_{D,OBS}$) was measured as a function of NaCl concentration. Fluorescence titrations were performed by monitoring the change in fluorescence of *f*FPR-thrombin with increasing concentrations of SbO4L (Fig. 2A), which could be fitted by quadratic binding Eq. (2) to calculate the affinity of the complex. A significant loss in binding affinity was observed for a relatively small change in the concentration of NaCl (100–200 mM) suggesting that the interaction was highly sensitive to ionic strength of the buffer. To resolve the forces contributing to the interaction, a plot of $\log K_{D,OBS}$ versus $\log[Na^+]$ was prepared following literature reports on the application of the protein–polyelectrolyte interaction theory [16,17]. According to