Designing Small, Nonsugar Activators of Antithrombin Using Hydropathic Interaction Analyses†

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Conformational activation of antithrombin is a critical mechanism for the inhibition of factor Xa, a proteinase of the blood coagulation cascade, and is typically achieved with heparin, a polyanionic polysaccharide clinically used for anticoagulation. Although numerous efforts have been directed toward the design of better activators, a fundamental tenet of these studies has been the assumed requirement of an oligo- or a polysaccharide backbone. We demonstrate here a concept that small nonsaccharidic nonpolymeric molecules may be rationally designed to interact with and activate antithrombin for enhanced inhibition of factor Xa. The rational design strategy is based on a study of complexes of natural and mutant antithrombins with heparin-based oligosaccharides using hydropathic interaction (HINT) technique, a quantitative computerized tool for analysis of molecular interactions. A linear correlation was observed between the free energy of binding for antithrombin−oligosaccharide complexes and the HINT score over a wide range of ∼13 kcal/mol, indicating strong predictive capability of the HINT technique. Using this approach, a small, nonsugar, aromatic molecule, (−)-epicatechin sulfate (ECS), was designed to mimic the nonreducing end trisaccharide unit DEF of the sequence specific heparin pentasaccharide DEFGH. HINT suggested a comparable antithrombin-binding geometry and interaction profile for ECS and trisaccharide DEF. Biochemical studies indicated that ECS binds antithrombin with equilibrium dissociation constants of 10.5 and 66 μM at pH 6.0, *I* 0.025, and pH 7.4, *I* 0.035, respectively, that compare favorably with 2 and 80 μM observed for the natural activator DEF. ECS accelerates the antithrombin inhibition of factor Xa nearly 8-fold demonstrating for the first time that conformational activation of antithrombin is feasible with appropriately designed small nonsugar organic molecules. The results present unique opportunities for de novo activator design based on this first-generation lead.

Introduction

Antithrombin, a plasma glycoprotein, is a member of the serpin, serine proteinase inhibitor, family of proteins that demonstrate similar three-dimensional fold and mechanism of inhibition.[1,2] It is a major inhibitor of several proteinases, especially factor IXa, factor Xa, and thrombin, which play pivotal roles in the coagulation phenomena. However, antithrombin by itself is a rather poor inhibitor of these enzymes under physiological conditions. This serpin requires the presence of heparin, a polysaccharide, that greatly (∼1000-fold) accelerates its reaction with coagulation enzymes.[3,4] This is the basis for the widespread clinical use of heparin as an anticoagulant. Nearly 500 million doses of heparin and its derivatives, low-molecular-weight heparins (LMW-Hs), are used annually in the U.S. alone to treat several cardiovascular conditions.[5]

Clinically used heparin, called unfractionated heparin (UFH), is exclusively obtained from animal sources including porcine intestine and bovine lung, while LMW-Hs are prepared from UFH through either chemical or enzymatic methods.[6,7] The animal origin of these pharmaceuticals raises significant concern regarding safety against prion-borne diseases. In addition, heparin (or LMW-H) therapy is beset with numerous complications, including enhanced risk of hemorrhage, thrombocytopenia, and inconsistent patient response.[8−11]

Heparin is a linear polymer of 1 → 4-linked uronic acid (glucuronic or iduronic) and glucosamine (*N*-acetylated or *N*-sulfated) residues[6,7] with differentially sulfated hydroxyls, resulting in a highly heterogeneous, polyanionic, polydisperse molecule. The LMW-Hs have reduced overall anionic character and polydispersity but retain many of the adverse effects of UFH. A specific five residue sequence in heparin, called the DEFGH (heparin pentasaccharide DEFGH) sequence (Figure 1), is involved in high-affinity binding to antithrombin.[12,13]

Mechanistically, the binding of pentasaccharide DEFGH (or polymeric heparin containing the DEFGH sequence) to antithrombin is a two step process in which the first step is the recognition of the heparin-binding domain in the serpin to form a low-affinity initial recognition complex (AT:H, Figure 2).[14,15] In the second step, the energy made available by initial binding is utilized to induce a major conformational change in the inhibitor, resulting in the formation of a noncovalent complex in which antithrombin exists in the activated state (AT*:H, Figure 2). Conformational change in antithrombin dramatically increases the binding affinity of DEFGH by nearly 3 orders of magnitude resulting in tight binding and induces multidomain movements

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† Abbreviations: HINT, hydropathic interaction analyses; ECS, (−)-epicatechin sulfate; LMW-H, low-molecular-weight heparins; UFH, unfractionated heparin; DEFGH, heparin pentasaccharide DEFGH; DEFGH′, heparin pentasaccharide DEFGH with an additional sulfate at the 3-position of residue H; DEF, trisaccharide DEF of DEFGH; TNS, 2-(*p*-toluidinyl)naphthalene-6-sulfonic acid.

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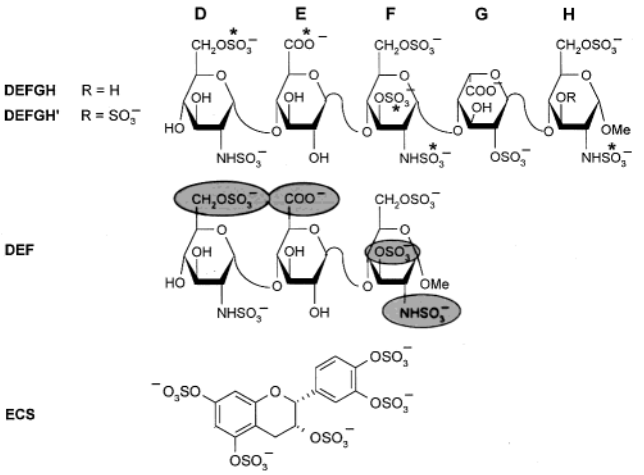


Figure 1. Structures of oligosaccharide activators of antithrombin and ECS. DEFGH represents the specific pentasaccharide sequence in heparin that is important for anticoagulant activity. Negatively charged groups in pentasaccharide DEFGH deemed critical for tight binding to antithrombin are marked with asterisks. DEFGH′ contains an additional 3-OSO_3^- group in residue H of the natural pentasaccharide DEFGH. Encircled groups in trisaccharide DEF constituted the pharmacophore for the design of ECS.

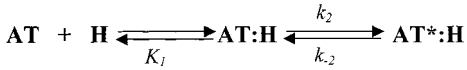


Figure 2. Two step induced fit mechanism of heparin activation of antithrombin. In the first step, the pentasaccharide recognizes the heparin-binding domain on antithrombin to form a low-affinity initial recognition complex (AT:H) governed by the dissociation constant for rapid equilibrium K_1. In the second step, a conformational change occurs in antithrombin that alters the conformation of the heparin-binding domain to give a high-affinity complex (AT*:H) in which antithrombin is activated. k_2 and k_{-2} represent the rate constants for the forward and reverse conformational change, respectively. Activated antithrombin reacts with procoagulant enzymes at a greatly accelerated rate that forms the basis of heparin's clinical use as an anticoagulant.

in the inhibitor, including an increased exposure of a 15 residue proteinase recognition sequence containing the scissile bond.[16,17] This conformational change results in a ~300-fold enhancement in the rate of factor Xa inhibition.[15,17] Whereas heparin-induced antithrombin conformational change is a major factor in the accelerated inhibition of factor Xa, it is not as critical for thrombin inhibition. Simultaneous bridging of the inhibitor and the proteinase on a heparin chain is more important for the enhancement in the rate of thrombin inhibition.[15,18,19] Recently, a similar bridging mechanism that enhances the inhibition of factor Xa, in addition to the conformational activation mechanism, has been demonstrated.[20,21]

Biochemical and crystal structure studies indicate that an asymmetric positively charged region, comprised of helix A, helix D, and the polypeptide N terminus, forms the pentasaccharide-binding site in antithrombin.[22−27] Positively charged residues Lys11, Arg13, Arg46, Arg47, Lys114, Lys125, and Arg129 interact with the carboxylate and sulfate groups of the pentasaccharide.[22] Studies with the truncated variants of the pentasaccharide DEFGH indicate that the trisaccharide unit DEF from the nonreducing end of the pentasaccharide is critical for both initial recognition and conformational activation processes.[28−30] More importantly, synthetic trisaccharide DEF (Figure 1) was found to activate antithrombin nearly 300-fold.[28] This is equivalent to the acceleration achieved with pentasaccharide DEFGH, suggesting that residues G and H are not as critical in the conformational activation process. It appears that these residues are more important for increasing the binding affinity to effect a high-affinity interaction under physiological conditions.

Numerous efforts have been directed toward the design of synthetic antithrombin activators. These include modifications in the functional groups in polymeric heparin and related polysaccharides, including curdlan sulfate, fucoidan, galactomannan, and chitosan derivatives.[31−37] Additionally, several modifications in the native heparin pentasaccharide DEFGH structure have also been explored.[38−45] However, a fundamental tenet of these studies has been the assumed requirement of (i) a saccharide skeleton and (ii) a minimum size corresponding to the five residue sequence for a high-affinity interaction with antithrombin. These were apparently supported by the dramatic loss in acceleration following a saccharide ring disruption in the pentasaccharide framework.[45] A major concern with the polysaccharide-based approach is the difficulty of synthesis that leads to much-reduced cost effectiveness. We reasoned that it should be feasible to activate antithrombin for accelerated factor Xa inhibition by a suitable small molecule that is not saccharide-based. This first-generation molecule may be equivalent in size to trisaccharide DEF, rather than pentasaccharide DEFGH.

In this paper, we demonstrate this concept by rationally designing the first organic nonsugar activator of antithrombin. First, we demonstrate the applicability of hydropathic interaction (HINT) technique to quantify and predict the interactions of antithrombin at a molecular level, followed by its usage in designing a small nonsugar skeleton, (−)-epicatechin sulfate (ECS, Figure 1). ECS binds antithrombin with an affinity comparable to DEF and accelerates the inhibition of factor Xa nearly 8-fold. The success of this endeavor highlights the unique opportunities of designing small organic activators of antithrombin.

Materials and Methods

Proteins and Chemicals. Human antithrombin and human factor Xa were a generous gift from Professor Steven T. Olson of the University of Illinois−Chicago. Molar concentrations of the inhibitor were calculated from absorbance measurements at 280 nm using a ϵ_{max} of 37 700 M^{-1} cm^{-1}. (−)-Epicatechin and 2-(*p*-toluidinyl)naphthalene-6-sulfonic acid (TNS) were purchased from Aldrich Chemical (Milwaukee, WI) and used without further purification. Triethylamine−sulfur trioxide complex was prepared using the literature procedure.[46]

Nuclear Magnetic Resonance (NMR) Spectroscopy. ^1H and ^{13}C NMR spectra of (−)-epicatechin and ECS were recorded using a Varian 300 MHz Gemini instrument at room temperature. The samples were dissolved in ^2H_2O and lyophilized twice before the final dissolution in 500 μL to give ~10−25 mM solution.

Experimental Conditions. Antithrombin interaction and activation studies were performed at 25 °C and in 20 mM sodium phosphate buffer, containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1%(w/v) poly(ethylene glycol) (PEG) 8000, adjusted to either pH 6.0 or pH 7.4. The ionic strength of buffer in the absence of any added salt is either

0.025 (pH 6.0) or 0.035 (pH 7.4). Because of the reported decrease in stability of the inhibitor activity at lower pH,[47] antithrombin solutions in pH 6.0 buffer were prepared by fresh dilution from a stock solution at pH 7.4 (>100-fold). No significant losses in inhibitor activity were noted over the time frame of the experiments performed in this study.

Modeling Antithrombins. Sybyl 6.5 software (Tripos Associates, St. Louis, MO) was used for molecular modeling experiments. Structures of antithrombin alone (accession number, "2ant"[48]) and pentasaccharide-complexed ("1azx"[22]) were acquired from Research Collaboratory for Structural Bioinformatics (http://www.rcsb.org/pdb/). In both of these crystal structures, the inhibitor exists as a dimer of an inhibitory and a latent molecule. Chain I corresponding to the inhibitory monomer was extracted from each of these dimers and used as a model of the native (2ant) and activated (1azx) conformations. Residues 34−42 (2ant) and 1, 26−38, and 432 (1azx) are absent in the reported crystal structures. Previous biochemical studies do not implicate these absent amino acids in heparin binding; hence, the residues were not reengineered into the polypeptide chains. More importantly, the residues known to be important were present in both the native and the activated inhibitory polypeptide chains. Individual atoms were assigned Gasteiger−Hückel charges, and the polypeptide chain was minimized using Tripos force field until a terminating gradient of 0.5 kcal/mol Å^2 was reached. Mutations, including Arg129His, Lys125Met, and Lys114Gln, were introduced in the inhibitor in the activated form (1azx) using the mutation protocol in Sybyl. The mutant polypeptide chains were prepared for further experiments by minimization with the Tripos force field, in a manner similar to the parent chains.

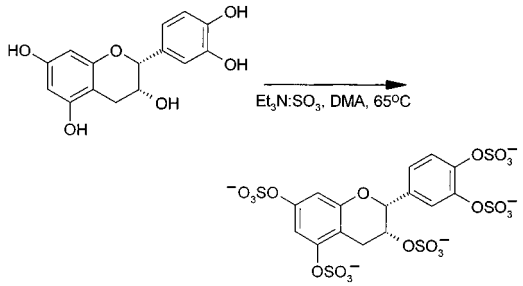
Modeling DEFGH, DEFGH′, DEF, and ECS. The structure of the pentasaccharide, retrieved from the antithrombin−pentasaccharide complex (1azx), formed the starting structure for modeling the oligosaccharide ligands. This structure, called DEFGH′ (Figure 1), has a sulfate group ($-OSO_3^-$) at the 3-position of residue H, while the natural pentasaccharide DEFGH has an unmodified 3-OH group instead. In addition, pentasaccharide DEFGH′ contains OSO_3^- groups at the 2-positions in residues D, F, and H rather than $NHSO_3^-$ groups present in natural pentasaccharide DEFGH. These structural changes were introduced in the DEFGH′ structure to obtain the model of native natural pentasaccharide DEFGH. Trisaccharide DEF (Figure 1) was built by extracting the three residues from the nonreducing end of natural pentasaccharide DEFGH. The DEFGH, DEFGH′, and DEF structures so prepared were expected to most closely represent the conformations of these molecules in the native state and hence were not further energy-minimized.

The atom types for the oxygen atom in the SO_3^- and COO^- moieties were modified to ensure that the bond angles and bond lengths of the simulated sulfate and carboxylate groups were equivalent to that observed in the crystal structure of the complex. The partial charge for each atom in the pentasaccharide was computed using the Gasteiger−Hückel method that is in-built in Sybyl. The protocol assigns a charge of −0.521 and −0.46 to the oxygens of the SO_3^- and COO^- groups, respectively.

The structure of ECS (Figure 1) was built using the small molecule builder module in Sybyl 6.5. The global minimum energy conformation of ECS was searched by systematic variation of bond angles in all its rotatable bonds.

Docking. The flexidock module in Sybyl was used to dock oligosaccharides (DEF, DEFGH, or DEFGH′) and ECS onto the heparin-binding domain in antithrombin and its mutants. To ensure similar starting geometries of each ligand in the binding site of native and activated forms of each inhibitor, the crystal structure of the activated form (1azx) was used as a reference. The binding site was defined as all residues within 4 Å distance from Arg46, Arg47, Lys114, Lys125, Arg129, Arg132, and Lys133.[22] All basic amino acid residues lining the binding site were positively charged. Rotatable bonds of these residues, primarily the side chain single bonds, were allowed conformational flexibility in the docking process, while the backbone and remaining bonds were held rigid. Water was excluded from the simulation, although a distance-dependent dielectric constant was used to simulate the presence of water. Docking was performed with a rigid oligosaccharide model (no ligand flexibility) without the introduction of any additional constraints such as explicit formation of hydrogen bond between selected donor−acceptor sites. In general, Flexidock provides nearly 20 solutions for each docking experiment. Each of these structures was minimized as described earlier to eliminate bad electronic and/or steric contacts, and the structure with lowest energy was used for HINT analysis.

Scheme 1



Scoring. HINT (EduSoft LC, Ashland, VA) was used to evaluate the binding of oligosaccharides and ECS to antithrombin. In the HINT, model specific interactions between a small molecule and a macromolecule are described as a double sum over the atoms within each component[49,50]

$$B = \sum_{i=1}^{\text{atoms}} \sum_{j=1}^{\text{atoms}} b_{ij} = \sum \sum (S_i a_i S_j a_j R_{ij} T_{ij} + r_{ij}) \qquad \text{(I)}$$

where S is the solvent accessible surface area, a is the hydrophobic atom constant, T is a descriptor function (vide infra), and R and r are functions of the distance between atoms i and j. From this equation, a binding score is calculated where b_{ij} describes specific interaction between atoms i and j and B describes the total interaction score between the two species. A detailed description of the HINT interaction analysis can be found in refs 49−51.

Antithrombin was assigned HINT parameters from a dictionary of previously determined values. Only polar hydrogens were explicitly used in the partitioning of the protein and the ligand, following which an interaction score using eq I was calculated for each final docked structure. Because HINT parameters for the sulfur atoms in higher oxidation states including sulfates are not well-established, sulfur interaction scores were eliminated. Three-dimensional maps that pictorially represent the noncovalent interactions deduced by HINT were calculated on a 1 Å grid.

Synthesis and Characterization of ECS. ECS was synthesized from (−)-epicatechin by sulfation with triethylamine−sulfur trioxide complex (Scheme 1) at 65 °C in dimethylacetamide.[52] The mixture was poured into acetone under basic conditions and left for 24 h at 4 °C. The crude oil formed at the bottom was washed with acetone and suspended in 30% sodium acetate. The suspension was added to ethanol to precipitate the sodium salt of ECS. The concentration of ECS was estimated from its weight and confirmed by its extinction coefficient at 277 nm (ϵ_{277} = 4200 M^{-1} cm^{-1}). The IR spectrum indicated a strong band at 1250 cm^{-1}, characteristic of $-SO_3^-$ stretch vibrations. A comparison of the 300 MHz ^{1}H NMR spectra of (−)-epicatechin and ECS shows downfield shift of 0.5−0.9 ppm for all protons suggesting sulfation of all five hydroxyl groups. The electronanospray mass spectrum in acetic acid matrix showed a molecular ion peak at 734 m/z corresponding to $[M + 3H + 2Na]^+$ ion. The detection of Na atoms in the molecular ion peaks for sulfated molecules has been noted earlier.[53] Elemental analysis of repeatedly purified sample indicated the presence of $6H_2O$ and $0.5Na_2SO_4$ mol-

ecules for every ECS molecule. IR (KBr) cm^{-1}: 1250 (S═O in $-OSO_3^-$ stretch). ^1H NMR (^2H_2O): δ 3.10 (dd, 1H, ^2J = 18 Hz, ^3J = 4.2 Hz, Ar−CH_2), 3.45 (dd, 1H, ^2J = 18 Hz, ^3J = 6 Hz, Ar−CH_2), 5.00 (m, 1H, CH−OSO_3^-), 5.10 (d, 1H, ^3J = 8.1 Hz, O−CH−Ar), 6.80−7.70 (m, 5H, Ar). MS (electronanospray, AcOH medium): 734 m/z $(M + 3H + 2Na)^+$, Elemental analysis: calcd (%) for $C_{15}H_9O_{21}S_5Na_5 + 6H_2O + 0.5Na_2SO_4$: C, 17.9; H, 2.1; S, 17.8. Found (%): C, 17.29; H, 2.21; S, 18.00.

Fluorescence Spectroscopy and Equilibrium Binding Studies. Fluorescence experiments were performed with a PCI Spectrofluorometer (ISS Instruments, Champaign, IL) at room temperature. Emission spectra were obtained in the pH 6.0 buffer with 1 μM antithrombin and 100 μM ECS in ratio mode at 1 nm wavelength resolution by excitation at 280 nm. Corrections for Raman bands and any background signal from the buffer were made by subtracting the buffer spectra.

Equilibrium dissociation constants (K_D) for antithrombin−ECS or antithrombin−DEF complexes were determined by titrating the activators into a solution of antithrombin−TNS complex or antithrombin and monitoring the decrease in the fluorescence at 432 nm (λ_{ex} = 330 nm) or the increase in fluorescence at 330 nm (λ_{ex} = 280 nm), respectively. The decrease or the increase in fluorescence signal with ECS or DEF concentration, respectively, was fit to the quadratic equilibrium binding eq II to obtain the K_D of interaction, wherein ΔF represents the change in fluorescence at each addition of the activator ($[ACT]_O$) from the initial fluorescence F_O, and ΔF_{max} represents the maximal change in fluorescence observed on saturation of antithrombin ($[AT]_O$).

$$\frac{\Delta F}{F_O} = \frac{\Delta F_{max}}{F_O} \times \frac{([AT]_O + [ACT]_O + K_D) - \{([AT]_O + [ACT]_O + K_D)^2 - 4*[AT]_O[ACT]_O\}^{1/2}}{2*[AT]_O} \quad \text{(II)}$$

Factor Xa Inhibition Studies. The accelerating effect of ECS on the kinetics of the antithrombin inhibition of factor Xa was measured under pseudo-first-order conditions. A fixed 10 nM concentration of factor Xa was incubated with antithrombin (1 μM) and ECS (0−100 μM) in pH 6.0 buffer at 25 °C. After incubation for 60 min, the reactions were quenched with 900 μL of 100 μM Spectrozyme FXa (American Diagnostics, Greenwich, CT) in 20 mM sodium phosphate buffer, containing 100 mM sodium chloride, 0.1 mM EDTA, 0.1% (w/v) PEG 8000, 50 μM Polybrene, at pH 7.4. The residual factor Xa activity was then measured spectrophotometrically from the initial rate of substrate hydrolysis at 405 nm. The second-order rate constant of factor Xa inhibition (k_{ECS}) was determined using eq III (This equation was deduced from pseudo-first-ordered reaction equation ($T = T_O \exp(-k_{OBS} \times t)$) for single turnover kinetics in the presence (T_2, $k_{OBS} = k_{UNCAT}[AT]_O + k_{ECS}[AT:ECS]_O$) and absence ($T_1$, $k_{OBS} = k_{UNCAT}[AT]_O$) of ECS where T_O is the initial activity of the enzyme.), wherein T_1 and T_2 are the residual factor Xa activities in the absence and presence of ECS, respectively, at time t for a fixed antithrombin concentration. K_D is the equilibrium dissociation constant of antithrombin−ECS interaction at pH 6.0.

$$\frac{\ln(T_1/T_2)}{t} = k_{ECS} \times \frac{([AT]_O + [ECS]_O + K_D) - \{([AT]_O + [ECS]_O + K_D)^2 - 4[AT]_O[ECS]_O\}^{1/2}}{2[AT]_O} \quad \text{(III)}$$

The accelerating effect of trisaccharide DEF on the kinetics of antithrombin inhibition of factor Xa was measured by following the reaction as a function of time to determine the observed rate constant (k_{OBS}) from the exponential decrease of residual active factor Xa concentration in the presence of a fixed concentration of the activator. The observed rate constant was determined at three concentrations of DEF. The second-order rate constant of antithrombin inhibition of factor Xa in the presence of DEF (k_{DEF}) was then determined from the slope of the linear plot (eq IV) of k_{OBS} against $[AT:DEF]_O$ (obtained from the quadratic equilibrium binding equation with the K_D value obtained for the interaction at pH 7.4, I 0.135, 25 °C). The uncatalyzed second-order rate constant for antithrombin inhibition of factor Xa (k_{UNCAT}) was obtained from the intercept of this plot.[22,23,41]

$$k_{OBS} = k_{UNCAT}[AT]_O + k_{DEF}[AT:DEF]_O \quad \text{(IV)}$$

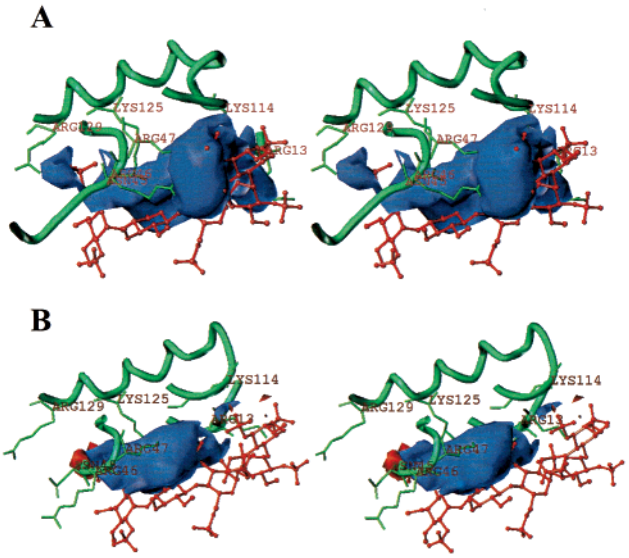


Figure 3. HINT map of activated (A) and native (B) antithrombin interacting with heparin pentasaccharide DEFGH. The pentasaccharide (red ball-and-sticks) was docked onto the heparin-binding site in antithrombin (turquoise ribbon) using Flexidock followed by HINT analysis. Side chains of important amino acid residues are shown in green. The blue "cloud" represents the contours of the atomic level interactions. Contours at +1.0 and −1.0 levels, representing either favorable (blue, positive) or unfavorable (red, negative; almost nonexistent at the level of sampling) interactions, were mapped for better visualization. Helix A, helix D, and the polypeptide N terminus form the heparin-binding domain in antithrombin. See text for details.

Results

HINT Analyses of Antithrombin Interactions. To test the applicability of HINT for analyzing heparin−antithrombin interactions, we studied complexes of the inhibitor with DEFGH and DEFGH′ in the native and activated states. In each case, docking resulted in a single family of structures that differed from each other in small torsional angle variations, giving rise to slightly different energies (not shown). Comparison of the docked structure of activated antithrombin−DEFGH complex (Figure 3A) with the crystal structure complex[22] showed nearly identical interactions. Likewise, the orientation of the pentasaccharide (DEFGH or DEFGH′) in the heparin-binding site of activated antithrombin or its mutants was similar (not shown). However, the position of pentasaccharide DEFGH in the binding site of native antithrombin (Figure 3B) was different as compared to that in the activated form. The pentasaccharide was found to move closer to Arg129, the C-terminal end of helix D, in the activated form relative to that in the native form, thus suggesting the possibility of a lateral movement of the pentasaccharide in the binding process.

Table 1. Comparison of the Overall HINT Score and the Experimentally Determined Free Energy of Binding for Complexes of Antithrombin and Its Mutants with Oligosaccharide Ligands

			HINT score		
complex[a]	$K_{D,OBS}$[b] (M)	ΔG_{OBS}^{O} (kcal/mol)	nonpolar[c]	polar[d]	total
AT:DEFGH	2×10^{-5}	6.4	−2425	5733	3308
AT*:DEF	2×10^{-6}	7.8	−1816	7964	6148
K114Q*:DEFGH	1.5×10^{-7}	9.4	−2337	10 128	7791
K125M*:DEFGH	7×10^{-8}	12.5	−1211	9338	8127
R129H*:DEFGH	1×10^{-8}	13.7	−1851	9489	7638
AT*:DEFGH	1×10^{-12}	16.0	−1862	10 396	8534
AT*:DEFGH′	1×10^{-14}	19.0	−2563	14 035	11 472

[a] AT = native antithrombin; asterisk (*) indicates the activated form of either antithrombin or its mutant. [b] Equilibrium dissociation constant experimentally measured under nearly identical set of conditions.[25−29,53,54] [c] Includes a composite total of all base−base, base−hydrophobic, and hydrophobic−hydrophobic interactions deduced by HINT analysis. [d] Includes a composite total of all hydrogen-bonding and acid−base interactions deduced by HINT analysis.

The HINT map shows a favorable interaction interface between most amino acid residues of helix A, helix D, and the polypeptide N terminus with the sulfate and carboxylate groups of the pentasaccharide (Figure 3A). This interface spans the entire length of the pentasaccharide, supporting previous results that all five residues contribute significant binding energy.[54] In contrast, the HINT map of the native antithrombin−DEFGH complex indicates a much reduced interaction interface that is localized primarily around residues D, E, and F of the pentasaccharide (Figure 3B). Rapid kinetic studies with tri- and tetrasaccharide variants of pentasaccharide DEFGH indicate that residues D, E, and F, but not G and H, are involved in the initial recognition of the activator.[28,29] We predict that our simulated native antithrombin−DEFGH complex is equivalent to the initial recognition complex (AT:H, Figure 2) that is detected in rapid kinetic experiments as a complex in rapid equilibrium with native antithrombin. This simulation aided by HINT analysis affords an inside look at the interaction of antithrombin in the ground (native) state (see below) and is expected to be especially useful for ligands that show weak interaction in the activated state.

Overall HINT Score and Free Energy of Binding. Besides the interaction of pentasaccharide DEFGH with native (AT:DEFGH) and activated (AT*:DEFGH) forms of antithrombin, we simulated its interaction with single-point antithrombin mutants, Arg129His, Lys125Met, and Lys114Gln. Extensive biochemical studies indicate that mutation of Arg129, Lys125, and Lys114 results in loss of binding affinity of 2.3, 3.5, and 6.6 kcal/mol, respectively.[25,27,55,56] In addition, we simulated the binding of trisaccharide DEF and pentasaccharide DEFGH′ (Figure 1) to plasma antithrombin. Our earlier biochemical study indicated that free energies of DEF and DEFGH′ binding to plasma antithrombin were 7.8 and 19 kcal/mol, respectively.[28] Together, the ΔG^{O} for the seven complexes studied span a range of 12.6 kcal/mol and an equilibrium dissociation constant range of ∼9 log units.

Table 1 lists the overall HINT scores for the seven simulated complexes. The HINT scores are positive and range from ∼3000 to ∼11 000, a substantial ∼4-fold change. A plot of the total HINT score against the observed ΔG^{O} for these complexes (Figure 4) suggests that the two are linearly correlated with a slope of 491 ± 105 HINT score (kcal mol^{-1}) and an intercept of 1622 ± 1346 HINT score. Considering that the flexibility of amino acid side chains, so ubiquitous and critical in the binding process, is difficult to simulate, the linear correlation observed is particularly good.

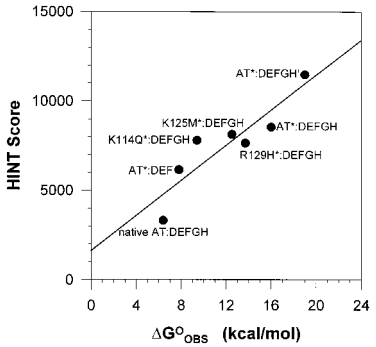


Figure 4. Correlation between the observed free energy of binding (ΔG_{OBS}^{O}) complexes and the total HINT scores for the complexes of antithrombin. Individual complexes are labeled. Asterisk (*) represents antithrombin or its mutant in the activated state. The solid line is the regression obtained by fitting the data to a linear equation with a slope of 491 ± 105 HINT score/kcal mol^{-1} and an intercept of 1622 ± 1346 HINT score. See text for details.

Residue Level HINT Score and Free Energy of Binding. To understand whether HINT predicts the interactions of antithrombin at a residue level, the scores for individual atoms belonging to each amino acid residue were combined to obtain a residue level HINT score. Table 2 lists the polar (hydrogen bonding + acid−base) and nonpolar (base−base + hydrophobic) HINT scores for interactions of the native and activated forms of antithrombin. Positive HINT scores indicate favorable interactions while negative scores indicate otherwise.

HINT analysis suggests that numerous residues, in addition to those deduced in the biochemical studies, interact with the pentasaccharide. These include Cys8, Thr9, Ala10, Lys11, Pro12, Arg13, Asp14, Ala43, Thr44, Asn45, Arg46, Arg47, Val48, Glu50, Ser112, Glu113, Lys114, Thr115, Gln118, Phe121, Phe122, Lys125, Arg129, Arg132, and Leu417. Of these, residues of the polypeptide N terminus (Cys8→Asp14) primarily interact unfavorably, while positively charged residues dominate the interaction through the formation of either hydrogen bonds or salt bridges. The binding energy contribution of residues Arg129, Lys125, Lys114, Arg47, and Arg46, calculated from the linear correlation deduced above, is 1, 3.7, 4.3, 5.0, and 0.6 kcal/mol, respectively (Table 2). A comparison with the binding energy defects observed on mutation of these residues suggests that the ΔG^{O} values of Arg129 and Lys114 are

Table 2. HINT Analyses of Interactions of Native and Activated Forms of Plasma Antithrombin and Its Mutants with Pentasaccharides DEFGH and DEFGH

	plasma antithrombin						antithrombin mutant					
	AT:DEFGH[a]		AT*:DEFGH[a]		AT*:DEFGH′[a]		K114Q*:DEFGH[a]		K125M*:DEFGH[a]		R129H*:DEFGH[a]	
residue	nonpolar[c]	polar[d]	nonpolar	polar	nonpolar	polar	nonpolar[c]	polar[d]	nonpolar	polar	nonpolar	polar
Lys11	0	0	−83	1304	−96	1481	−53	1414	−69	1696	−84	118
Arg13	−207	109	−6	168	−100	862	−20	294	−10	209	−31	91
Thr44	−178	635	−314	29	−301	27	−258	15	−199	16	−185	0
Asn45	−10	0	−297	1323	−420	1367	−246	1248	−298	1389	−355	1281
Arg46	0	0	34	308	−17	1698	−12	324	0	236	0	131
Arg47	−132	2525	134	2448	−39	3542	−25	2908	0	3066	145	2929
Lys114	−52	0	−28	2110	−271	2206	−888	1384	12	2279	26	2170
Lys125	−36	2156	−69	1832	−79	1863	−98	1986	−229	0	−21	2396
Arg129	0	0	−46	489	−38	523	−14	384	−11	272	−48	58
other[b]	−1810	308	−1187	385	−1202	462	−723	171	−407	175	−1304	315

[a] AT = native antithrombin; AT* = activated antithrombin. [b] Includes interactions of Thr9, Ala10, Pro12, Asp14, Ala43, Val48, Glu50, Ser112, Glu113, Gln118, Phe121,Phe122, Arg132, and Leu417 residues. [c] Includes a composite total of all base−base, base−hydrophobic, and hydrophobic−hydrophobic interactions deduced by HINT analysis. [d] Includes a composite total of all hydrogen-bonding and acid−base interactions deduced by HINT analysis.

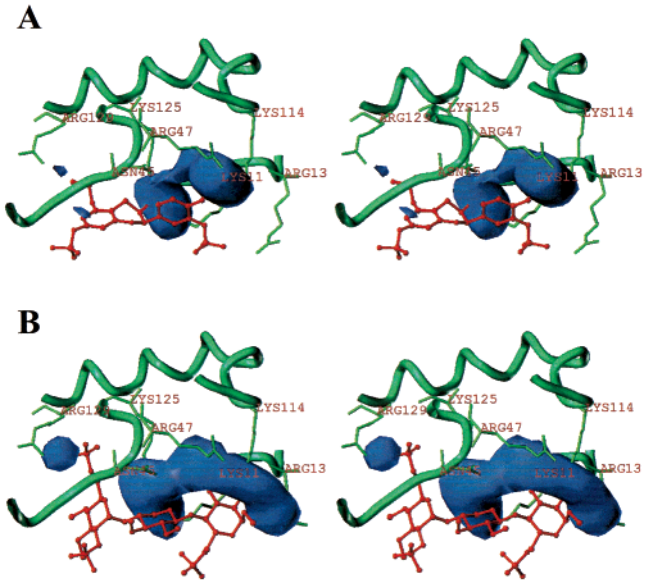


Figure 5. HINT map of ECS (A) and trisaccharide DEF (B) interacting with the activated form of antithrombin. DEF and ECS were docked onto the heparin-binding site in antithrombin (shown as turquoise ribbon) and the simulated cocomplexes analyzed by HINT analyses. All display parameters are similar to those in Figure 3 except for the red sticks representing either ECS (A) or DEF (B). See text for details.

underestimated by 1.3 and 2.3 kcal/mol, respectively, while that for Arg47 is higher by ∼4.0 kcal/mol.[25,27,55,56]

Simulation of Interaction of ECS and DEF with Antithrombin. Comparison of several solutions obtained after docking ECS and DEF onto the heparin-binding domain in antithrombin indicates a single family of binding geometries for both of the activators (not shown). The simulated DEF interaction was similar to that observed for the trisaccharide unit at the nonreducing end of DEFGH in the antithrombin−pentasaccharide cocrystal structure.[22] Analysis of the simulated ECS−antithrombin complex suggested that nearly all positively charged residues in the heparin-binding domain that bind to DEF interact with ECS (Figure 5).

Table 3 shows the HINT scores for the simulated binary complexes. Whereas the overall HINT score for the DEF interaction was 6024, that for ECS was 3687 suggesting comparable high-affinity interaction. The

Table 3. HINT Scores for Trisaccharide DEF and ECS Interacting with Plasma Antithrombin

residue	DEF	ECS	residue	DEF	ECS
Lys11	1,50	861	Lys114	1317	822
Ala43	−235	−250	Lys125	1818	946
Thr44	−330	−113	Arg129	603	306
Asn45	975	328	other[a]	245	−32
Arg47	73	715	total	6024	3687

[a] Includes the small contributions from Ala10, Asp14, Asp48, Glu50, Gln118, Phe121, Phe122, and Arg132 residues of the heparin-binding site.

scores indicate ∼12.3 and ∼7.5 kcal/mol free energy of binding for DEF and ECS, respectively, based on a value of 491 HINT score per kcal/mol deduced earlier. Analysis of HINT scores at the residue level suggests that although both ECS and DEF interact favorably with most positively charged residues of the heparin-binding site in antithrombin, the strength of interactions is not equivalent. Thus, whereas Asn45, Lys114, Lys125, and Arg129 show ∼1.5−3.0-fold better interaction with DEF, Arg47 interacts ∼10-fold more with ECS than with DEF.

Equilibrium Dissociation Constant of ECS−Plasma Antithrombin Interaction. The intrinsic protein fluorescence of antithrombin increases ∼30% when activated by pentasaccharide DEFGH or trisaccharide DEF affording a convenient probe for the determination of the K_D of the interaction.[28,29] The equilibrium dissociation constants for DEF−antithrombin interaction at pH 6.0 and pH 7.4 (Figure 6B) were observed to be 2.0 ± 0.6 and 66 ± 4 μM.[28,29] These correspond to a free energy of DEF binding to antithrombin of 7.8 and 5.7 kcal/mol, respectively (Table 4).

The fluorescence emission spectrum of ECS indicates significant fluorescence in the region 310−340 nm (not shown) that is amplified in the presence of antithrombin suggesting that intrinsic protein fluorescence may not be appropriate to monitor ECS binding. Hence, a nonspecific probe, TNS, was used to determine the equilibrium dissociation constant of interaction, as previously used for heparin− and pentasaccharide−antithrombin interactions.[57] The fluorescence of the antithrombin−TNS complex decreased as a function of ECS concentration reaching a limiting value of ∼62% (Figure 6A). An equilibrium dissociation constant (K_D)

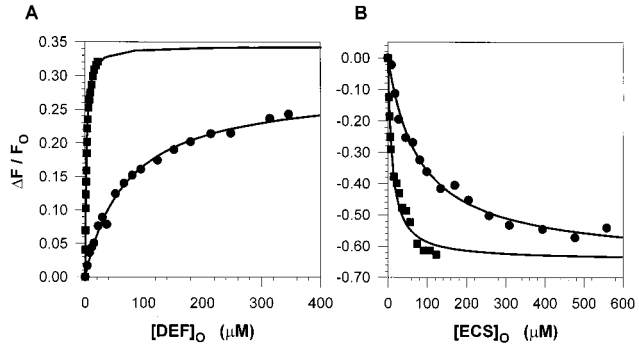


Figure 6. Interaction of ECS (A) and DEF (B) with plasma antithrombin. The K_D values for ECS interacting with plasma antithrombin were determined from the decrease in fluorescence of the bound TNS, a nonspecific fluorescent probe (A), while those for DEF were determined from the increase in the intrinsic protein fluorescence (B). Representative profile at pH 6.0, *I* 0.025 (l), and at pH 7.4, *I* 0.035 (*n*), are shown. The solid lines represent nonlinear regression fits of the data to obtain the K_D of interaction (see Table 2).

of 10.5 ± 1.2 μM (A K_D value of 7 μM was obtained from eq III (see Materials and Methods) when both K_D and k_{ECS} parameters were allowed to float supporting the assumed 1:1 binding stoichiometry for ECS interaction with antithrombin.) at pH 6.0, *I* 0.025, could be obtained by fitting the data to the quadratic binding eq II assuming a 1:1 binding stoichiometry (Table 4). This corresponds to a free energy of binding of 6.8 ± 0.1 kcal/mol. Equivalent decrease in TNS fluorescence was observed at pH 7.4, *I* 0.035 (~65%), corresponding to a K_D of 80 ± 4 μM (ΔG^O = 5.6 ± 0.1 kcal/mol).

Accelerating Effect of ECS on Factor Xa Inhibition by Antithrombin. The second-order rate constant for uncatalyzed inhibition of factor Xa by antithrombin (k_{UNCAT}) was determined at pH 6.0, *I* 0.025, as previously described (Figure 7A).[28,29] A k_{UNCAT} value of 120 M^{-1} s^{-1} was obtained (Table 4), in agreement with previous results,[28,29] ~15-fold lower than that observed at pH 7.4, consistent with the reduced catalytic activity expected of factor Xa under these conditions. The second-order rate constant for the accelerated inhibition of factor Xa by antithrombin−ECS complex (k_{ECS}) was evaluated from the residual factor Xa activity of reactions with increasing concentrations of ECS reaching greater than 90% saturation of the inhibitor. The concentration dependence profile of factor Xa inhibition at pH 6.0, *I* 0.025, is shown in Figure 7B. Analysis of the data using eq III, with a K_D of 10.5 μM, gives a second-order rate constant (k_{ECS}) of 909 ± 40 M^{-1} s^{-1}. Thus, ECS increased the rate of antithrombin inhibition of factor Xa ~8-fold (Table 4).

Discussion

We reasoned that designing a new antithrombin activator, with structural features different from the natural activator, heparin (or pentasaccharide), would become easier if a robust modeling tool is available that accurately simulates the interactions of antithrombin. The wealth of biochemical information already available on the heparin−antithrombin interaction could serve to validate the modeling tool, which may then be used to predict de novo ligand behavior.

Although molecular modeling tools have been used to investigate the heparin-binding site on antithrombin[58] and also to study a ternary complex of heparin with antithrombin and thrombin,[59] these semiquantitative tools are not particularly adaptable for new activator design. HINT analysis is a scoring technique for studying interactions of individual atoms using a predefined set of parameters.[49,50] These parameters are obtained from partition coefficient measurements, and hence, are related to thermodynamic equilibrium constant measurements. A practical advantage of this technique is that it identifies and quantifies individual interactions at the atomic level. These include hydrophobic, hydrogen-bonding, and acid−base interactions that are classified into nonpolar or polar HINT scores. This atomic level description is particularly suitable for molecular design purposes because it rapidly identifies features that require changes.

The observation of a linear correlation over a wide range of binding affinity for oligosaccharide complexes with natural and mutant antithrombins is exciting and, if found to be true for small organic ligands, is expected to be particularly helpful in the design process. The slope deduced in this study (491 HINT score/kcal mol^{-1}) is similar to that determined for hemoglobin−small molecule ligand interactions (515 HINT score (kcal $mol)^{-1}$)[51] (Figure 4) and at the present time appears to be coincidental. The implication of a nonzero intercept (1622 HINT score) is unclear; however, it may reflect the HINT score to be expected of nonspecific interactions of antithrombin.

Although the linear relationship appears to effectively predict the antithrombin binding affinity of an oligosaccharide ligand, it arises despite the absence of large-scale movement of amino acid side chains in the docking process. It is likely that the interactions of Arg129 and Lys114, residues that play a major role only in the second step of the binding process, are not completed in docking, while simultaneously retaining the interaction with the most exposed residues including Arg47 (see below). Thus, the binding energy contributions of Arg129 and Lys114 are estimated lower, while that for Arg47 is higher. It is fortuitous that the loss in predicted contributions of Arg129 and Lys114 equals the gain in simulated binding energy for Arg47, resulting in linear correlation between the overall HINT score and the observed ΔG^O. Thus, we predict that our simulated complex with activated antithrombin (AT*:H, Figure 2)-resembles, but is not the same as, the final complex that is detected biochemically.[15,28,29]

Functional Roles of Ionic Heparin-Binding Residues. The simulation of the initial recognition (AT:H) and final activated (AT*:H) complexes affords an interesting opportunity of confirming and predicting the functional roles of key ionic amino acid residues. The high HINT scores of 2525 and 2156 for Arg47 and Lys125, respectively (Table 2), in the native antithrombin−pentasaccharide (AT:DEFGH) complex indicates that these residues are important for initial binding. Site-directed mutagenesis of Arg47 and Lys125 indicate that these residues play a major role in the initial recognition process supporting our modeling analysis.[26,55] Table 2 also indicates that Arg46, Lys114, and Arg129 give high HINT scores only for the activated state (AT*:DEFGH) indicating that these ionic residues are involved in the conformational activation step of the

Table 4. Equilibrium Dissociation Constants, Maximal Fluorescence Changes, and Second-Order Rate Constant of Inhibition for ECS and Trisaccharide DEF Interacting with Plasma Antithrombin[a]

	ECS		DEF	
	pH 6.0	pH 7.4	pH 6.0	pH 7.4
ΔF_{max} (%)	62 ± 2[b]	65 ± 2[b]	34 ± 3[c]	28 ± 2[c]
K_D (μM)	10.5 ± 1.2	80 ± 4	2.0 ± 0.6	66 ± 4
ΔG^O (kcal/mol)	6.8 ± 0.1	5.6 ± 0.1	7.8 ± 0.3	5.7 ± 0.1
$k_{activator}$[d] (M^{-1} s^{-1})	909 ± 40[f]		$45\,000 \pm 1000$[g]	
acceleration of inhibition[e]	7.6 ± 1.0		320 ± 20	

[a] See Materials and Methods; errors represent 1 SE. [b] Determined from the decrease in TNS fluorescence on ECS binding (see Figure 6b). [c] Determined by the increase in intrinsic protein fluorescence on DEF binding (See Figure 6a).[28,29] [d] k_{ECS} or k_{DEF}. [e] An uncatalyzed rate constant (k_{UNCAT}) of antithrombin factor Xa reaction of 120 ± 10 M^{-1} s^{-1} was independently measured. [f] Determined from the plot as in Figure 7b. [g] Determined from the plot as in Figure 7a.[28,29]

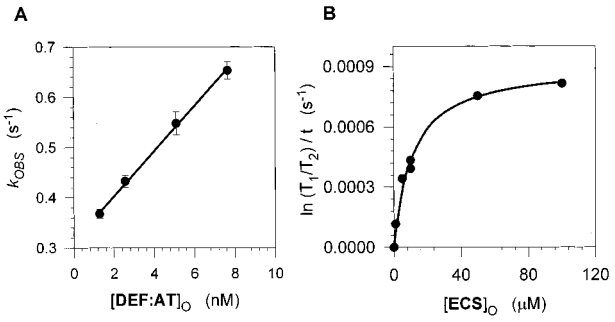


Figure 7. Activation of antithrombin with DEF (A) and ECS (B) for accelerated inhibition of factor Xa. The inhibition of factor Xa in the presence of increasing concentrations of DEF or ECS was monitored from the decrease in the concentration of residual active enzyme under pseudo-first-order conditions at pH 6.0, I 0.025, 25 °C. Independent observed rate constants were derived for DEF by monitoring the inhibition of the enzyme at each concentration to determine k_{DEF} (slope) from the above linear plot. A composite analysis in the absence (T_1) and presence (T_2) of ECS at a fixed time point (t) was used to determine the second-order rate constant of inhibition k_{ECS} (eq II). See Materials and Methods.

interaction (step 2, Figure 2). All three residues have been shown to play a major role in the second step of the binding process,[25,27,55] further confirming the predictive capabilities of our modeling study. These observations have important implications for design purposes.

Predictions on the Roles of Nonionic Residues. Although several nonionic residues are present in the heparin-binding domain of antithrombin, the contribution of these residues to the binding phenomenon is unclear. Our results suggest that Pro12, Ala43, Thr44, and Val48 give HINT scores of −360, −219, −314, and −208 (AT*:DEFGH) indicating that these residues adversely affect the interaction (Table 2). However, overall, the contribution of these residues appears to be less than 1−2 kcal/mol. A site-directed mutagenesis study suggests significant binding energy contribution of Trp49 residue.[60] This observation is not confirmed in the current modeling study. In fact, the HINT score for Trp49 was consistently zero for all complexes studied herein. This suggests that that Trp49 is most likely not a direct heparin-binding residue but rather exerts its effect through indirect means. Helix D in the heparin-binding site contains a unique phenylalanine trimer (Phe121−Phe122−Phe123) sequence, the functional role of which is unclear. More interestingly, the side chain aromatic ring of the Phe121 residue is more than 85% exposed to solvent. The HINT scores for the Phe trimer are small (100−200) implying that these residues do not directly interact with the pentasaccharide and may primarily contribute to the structural integrity of the heparin-binding domain.

Design of a Nonsugar Activator. To design an antithrombin activator that is small and not sugar-based, a "pharmacophore" was deduced from the DEF portion of the natural pentasaccharide, DEFGH. This assumed pharmacophore was built from four negatively charged residues, at the 6-position of residues D and E and at the 3- and 2-positions of residue F (see Figure 1), groups that are thought to be important for antithrombin activation.[45,61−63] Numerous molecular frameworks were screened based on their ability to satisfy the three-dimensional organization of the pharmacophore. These frameworks are constituted of saturated or unsaturated 6- or 10-membered rings joined by a linker that is one, two, or three bonds long. ECS was selected for investigation on the basis of HINT analyses (Table 3, Figure 5) that indicated strong positive correspondence between antithrombin interactions with trisaccharide DEF. This predicted similarity in overall interactions was confirmed with nearly equivalent antithrombin-binding affinities for DEF and ECS under two sets of conditions (Figure 6, Table 4).

It is important to compare how well ECS binds to antithrombin given that nonspecific interactions may play a significant role. However, ECS is also the first molecule that is both small and nonsaccharidic; hence, an appropriate reference molecule is not available at the present time. The free energy of ECS binding to antithrombin, 5.6 kcal/mol at pH 7.4, I 0.035, is comparable to that for low-affinity heparin and dermatan sulfate (5.0−6.0 kcal/mol), two polyanionic molecules that also activate antithrombin for enhanced inhibition of factor Xa.[19] However, the binding of these multidentate polysaccharides to antithrombin involves numerous nonspecific electrostatic interactions. The many negative charges available over the length of the polymeric chain of low-affinity heparin and dermatan sulfate may facilitate the interaction of positively charged residues that reside outside the heparin-binding domain resulting in an unusually high binding affinity of the polymers for the inhibitor.[24,64] The free energy of binding of a repeating unit in these polymers that could compare with ECS, e.g., a disaccharide, is therefore expected to be significantly lower than ∼6 kcal/mol, the observed binding energy.[19] Thus, although the interaction of ECS with antithrombin is much weaker than that for the natural pentasaccharide DEFGH, the affinity is greater than that expected on the basis of solely nonspecific interactions.

The free energy of binding for ECS−antithrombin interaction is nearly 1 kcal/mol lower than that for DEF−antithrombin interaction at pH 6.0, while the ΔG^{O} values are equivalent at pH 7.4 (Table 4). Salt dependence of the K_D of DEFGH−antithrombin interaction at the two pH values suggests that an additional charge−charge interaction is formed at pH 6.0, most probably originating from interactions of residue D with the C-terminal end of helix D including Lys125 and Arg129.[28] Thus, it is likely that ECS does not interact well with these residues at pH 6.0, although the comparable affinities of ECS and DEF at pH 7.4 and HINT analyses (Table 3) suggest significant similarity in the interactions of the two activators with antithrombin.

It is extremely encouraging that the rationally designed de novo molecule ECS activates antithrombin for enhanced inhibition of factor Xa. While ECS is found to activate antithrombin ∼8-fold, the acceleration is much smaller than that achieved with DEF (∼320-fold).[28,29] Accelerated inhibition of factor Xa by antithrombin is a function of the activating conformational change induced in the inhibitor by an activator, such as trisaccharide DEF, and acceleration can be thought of as a displacement of the equilibrium between the native and the activated forms in antithrombin toward the conformationally activated state. Assuming that this equilibrium is displaced 100% by trisaccharide DEF, ECS displaces only about 3%. Thus, although ECS conformationally activates antithrombin, it is much less effective in comparison to DEF despite their comparable high-affinity interactions. One reason for this discrepancy may be that the overall comparable binding affinities of ECS and DEF do not manifest from identical interactions at individual residue level (Figure 5, Table 3). For example, the interactions with Arg129 and Lys125, two critical residues that operate cooperatively,[25,26] are weaker for ECS than for DEF. This is also supported by the observation that ECS probably does not interact as well as DEF with Arg129 and Lys125 residues at pH 6.0. Second, it is possible that several modes of interactions exist for ECS resulting in some nonproductive binding. The HINT map (Figure 5) indicates a more extensive network of interactions for DEF that span the entire length of the activator than for ECS. The conformational activation process induced by activator binding is a series of concerted movements that encompass nearly all the domains of the serpin culminating in the expulsion of the 15 residue factor Xa recognition sequence.[65] Thus, it is possible that ECS does not interact as effectively with both ends of the heparin-binding domain as DEF resulting in weaker transmission of the available binding energy for conformational activation. Studies with modified ECS skeletons and/or antithrombin mutants will help elaborate this possibility.

Conclusions

The work demonstrates a concept that rationally designed small organic molecules can bind and conformationally activate antithrombin, a process central to the design of new antithrombin-based antithrombotics. The overall rational design process was initiated with computerized molecular modeling involving HINT analysis that predicts the antithrombin−oligosaccharide interactions with good accuracy. ECS, a small nonsugar molecule designed using HINT, was found to bind and activate antithrombin for enhanced inhibition of factor Xa. This is the first and only report of a high-affinity organic molecule that is both small and nonsaccharidic. ECS presents us unique opportunities of designing better activators based on this first-generation lead.

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