

Chemistry and Biology of Heparin and Heparan Sulfate  
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## Chapter 17

### Antithrombin Activation and Designing Novel Heparin Mimics

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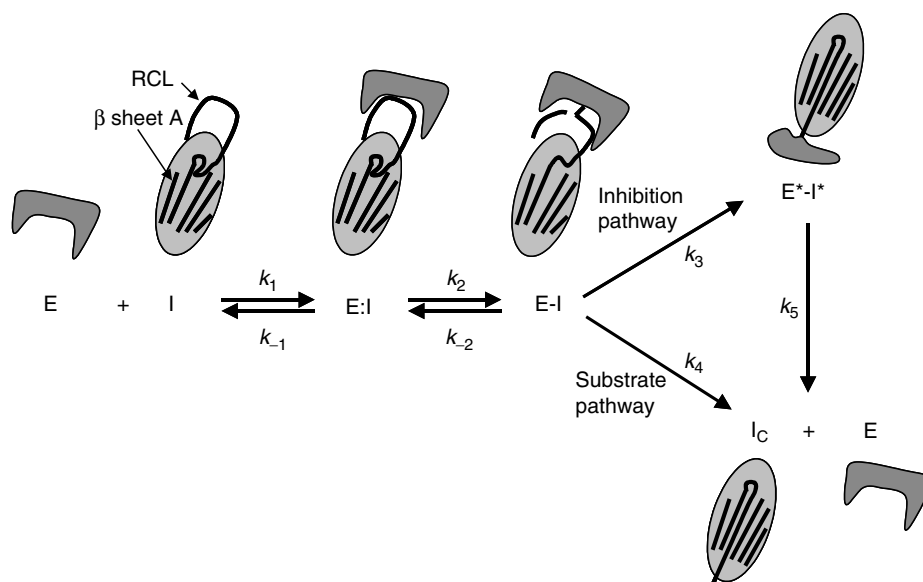
#### I. Introduction

Antithrombin (AT) is a key molecule regulating the clotting cascade in humans. The importance of the antithrombin can be assessed from numerous cases of deficiencies, congenital or acquired, which lead to enhanced risk for thrombosis in the affected individuals (1–4). More importantly, targeted disruption of the antithrombin gene in mice resulted in death of all  $AT^{-/-}$  embryos due to consumptive coagulopathy and/or liver dysfunction supporting its central role in regulation of blood coagulation in the myocardium and liver (5,6). Antithrombin is a plasma glycoprotein that inhibits several enzymes of the coagulation cascade, thereby functioning as a principal anticoagulant agent. Yet, current knowledge indicates that the physiological targets of antithrombin are factor IXa (fIXa), factor Xa (fXa), and thrombin (fIIa).

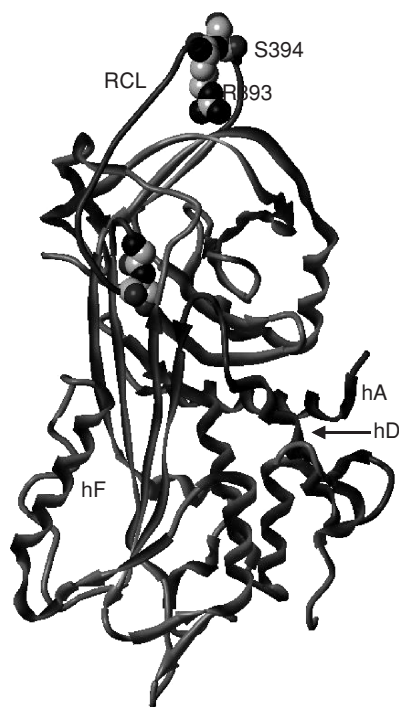
The circulating form of human antithrombin has 432 residues with a molecular weight of 58,200 Da.  $\alpha$ -Antithrombin, the major form circulating in plasma, has four Asn residues glycosylated, while the minor form ( $\beta$ -antithrombin) has an unglycosylated Asn135. The inhibitor is present in human plasma in fairly high concentration (2–3  $\mu$ M) and for a long time served as a principal source of material for study. Nowadays, recombinant antithrombin is routinely expressed in a number of cell systems including baculovirus infected insect cells and mammalian cells, with properties similar to the plasma protein. In addition, the milk of transgenic dairy

goats has been exploited to provide a homogeneous, well defined, and abundant supply of this inhibitor for commercial purposes (7).

Antithrombin belongs to the *serpin* superfamily of proteins, in which most members inhibit serine proteinases, although several are known to inhibit cysteine proteinases, while some are non-inhibitory (8,9). Antithrombin shows structural and functional similarity with prototypic serpin members including  $\alpha_1$ -proteinase inhibitor, heparin cofactor II, and plasminogen activator inhibitor I. Like these inhibitory serpins, antithrombin inhibits fIXa, fXa, and fIIa through the formation of stable complex. The serpin inhibition mechanism, studied extensively in the past decade, can be referred to as the “mousetrap” mechanism. In this mechanism, the inhibitor (**I**) acts as bait to trap the target enzyme (**E**) in an equimolar, covalent, inactive complex (**E<sup>\*</sup>-I<sup>\*</sup>**) (Fig. 1). The reactive center loop (RCL), an exposed sequence of residues containing the enzyme recognition site, first interacts with the active site of the proteinase as in a normal substrate reaction to form a Michaelis complex (**E:I**). This is rapidly followed by cleavage of the scissile bond P1-P1 “in the RCL to form an acyl-enzyme intermediate (**E-I**), which undergoes a major rearrangement to disrupt the enzyme” catalytic triad (10,11) resulting in inhibition (**E<sup>\*</sup>-I<sup>\*</sup>**) (inhibition pathway, Fig. 1). A competing process, called the substrate pathway (Fig. 1), may operate



**Figure 1** A model of the antithrombin inhibition mechanism. E, target enzyme (fIXa, fXa, or thrombin); AT, antithrombin; E:AT, Michaelis-Menten complex; E-AT, acyl-enzyme intermediate; E<sup>\*</sup>-AT<sup>\*</sup>, antithrombin-enzyme complex; AT<sub>C</sub>, cleaved antithrombin; RCL, reactive center loop; “k”, microscopic rate constants. See text for details. Reprinted with permission from Desai (2004) *Med. Res. Rev.* 24:151–181. Wiley Periodicals, Inc.



**Figure 2** Structure of native antithrombin showing partially inserted reactive center loop and the main body of the inhibitor. The structure of inhibitory form of plasma antithrombin was obtained from RCSB (file name “1E04” – chain I). Arg393 (P1)–Ser394 (P1') bond and partially inserted P14–P15 residues (Ser380–Gly379) of the RCL are shown in ball and stick representation. hD, helix D; hA, helix A; hF, helix F; sA,  $\beta$ -sheet A; see text for details.

in parallel and diminish the efficacy of inhibition. In the substrate pathway, structural perturbations in antithrombin (e.g., mutational changes) may facilitate rapid hydrolysis of the acyl enzyme intermediate **E–I** to yield an active enzyme (**E**) and a cleaved inhibitor (**I<sub>C</sub>**). *In vitro*, inhibition of fIXa, fXa, and thrombin by plasma antithrombin has no contribution from the substrate pathway, thus the reaction has a stoichiometry of inhibition of 1. However, alterations in the structure of the inhibitor (e.g., introduction of certain mutations) may enhance the contribution of the substrate pathway, thereby increasing the inhibition stoichiometry significantly. Such antithrombins may not function well as anticoagulants.

The central role of antithrombin has resulted in some 18 crystal structures, in which the inhibitor is either present alone or in complex with other molecules. Interestingly, each reported crystal structure is a dimer containing antithrombin in either the inhibitory, cleaved or latent form. To date, monomeric antithrombin has not been crystallized, thus the exact structure of the circulating form remains unclear. The structure of intact, uncleaved free antithrombin shows 9  $\alpha$ -helices

surrounding 3  $\beta$ -sheets (12–14). Of these secondary structures, two features are striking – a dominant five-stranded  $\beta$ -sheet Approximately in the center of the inhibitor and an exposed 15-residue sequence containing the reactive bond Arg393–Ser394, the so-called RCL at the “op” of the molecule (Fig. 2). These features are common to all serpins. In addition, antithrombin shows an extremely interesting feature known to be present in only one other serpin to-date, heparin cofactor II (15). Two residues, P15-P14 (Gly379–Ser380) at the N-terminal end of the RCL, are inserted as a short  $\beta$ -strand in-between strands 3 and 4 of  $\beta$ -sheet A in the inhibitor (Fig. 2). This feature is called the partial insertion of RCL and the reason(s) for such a preferential fold in antithrombin is not clear.

## II. Antithrombin Inhibition of Procoagulant Proteinases

Antithrombin has been reported to inhibit most enzymes of the clotting cascade including kallikrein, fXIIa, fXIa, fIXa, fXa, thrombin, and factor VIIa. Yet, the rates of antithrombin inhibition of these procoagulant proteinases under physiological conditions are small (Table 1). Typical uncatalyzed inhibition rate constants ( $k_{\text{UNCAT}}$ ) lie in the range of  $10$ – $10,000 \text{ M}^{-1}\text{s}^{-1}$ , thus suggesting that antithrombin alone is not very effective in inhibiting these enzymes. The reason for the slow inhibition likely resides in the structure of antithrombin. Previously, it was believed that partial insertion of the RCL introduces a structural constraint in antithrombin that is not conducive for rapid reaction with procoagulant proteinases (16,17), yet recent evidence suggests proteinase recognition by native antithrombin is encoded primarily in the P1Arg residue and that the conformation of RCL plays a minimal role (18–20).

The slow rates of antithrombin inhibition of these enzymes are greatly enhanced (Table 1) in the presence of heparin, a natural linear sulfated polysaccharide. Whereas the inactivation of thrombin, fXa, and fIXa reached nearly diffusion-controlled second-order rate constants of  $10^6$ – $10^7 \text{ M}^{-1}\text{s}^{-1}$  in the presence of full-length heparin, the inhibition of other proteinases was some 20-fold slower

**Table 1** Rates of Antithrombin Inhibition of Pro-Coagulant Proteinases Under Physiological Conditions

	fIXa <sup>a</sup> ( $\text{M}^{-1}\text{s}^{-1}$ )	fXa <sup>b</sup> ( $\text{M}^{-1}\text{s}^{-1}$ )	fIIa <sup>c</sup> ( $\text{M}^{-1}\text{s}^{-1}$ )
AT	$5.8 \times 10^1$	$2.6 \times 10^3$	$8.7 \times 10^3$
AT:H <sub>5</sub> <sup>d</sup>	$3.1 \times 10^4$	$81.2 \times 10^5$	$14.6 \times 10^3$
AT:H <sup>e</sup>	$\sim 2 \times 10^7$	$140 \times 10^6$	$37 \times 10^6$

<sup>a</sup>Data taken from Bedsted et al. (2003) *Biochemistry* 42:8143.

<sup>b</sup>Data taken from Rezaie and Olson (2000) *Biochemistry* 39:12083.

<sup>c</sup>Data taken from Olson et al. (1992) *J. Biol. Chem.* 267:12528.

<sup>d</sup>Antithrombin–heparin pentasaccharide complex.

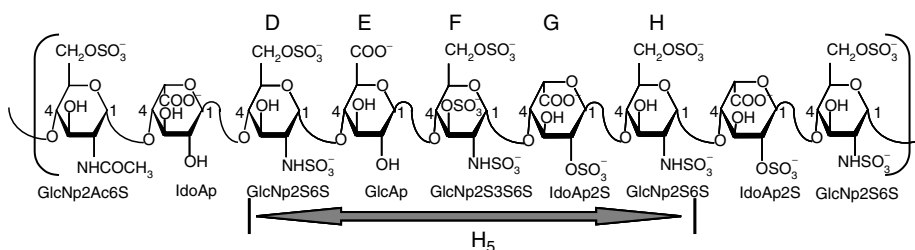
<sup>e</sup>Antithrombin–high-affinity-heparin complex.

(11,21–24). These increases in rate constants represent an acceleration of  $\sim 600$ -fold for fXa,  $\sim 2000$ -fold for thrombin, and  $\sim 10^6$ -fold for fIXa. These dramatic enhancements in inhibition of three key enzymes form the basis for heparin's clinical use as an anticoagulant since 1940s. Likewise, the manifold activity of polysaccharide heparin on other proteinases of the coagulation and fibrinolysis systems may also be responsible for the bleeding complications observed with heparin therapy.

### III. Structure of Heparin and Heparin Pentasaccharide

Heparin is a  $1 \rightarrow 4$ -linked linear copolymer of glucosamine (2-amino-2-deoxyglucopyranose) and uronic acid (pyranosyluronic acid) residues that are variously sulfated (in this chapter, the  $\text{NHSO}_3^-$  group is called *N*-sulfate and the  $\text{OSO}_3^-$  group is called sulfate) (Fig. 3). This structure, called glycosaminoglycan (GAG), is obtained by chemical processing of proteoglycan heparin present in porcine or bovine intestinal mucosa and lung (25). Glycosaminoglycan heparin is a complex mixture of polymeric chains. The structural complexity arises primarily from the apparently incomplete biosynthesis of proteoglycan heparin, in which multiple enzyme systems are involved (26,27), and secondarily from its preparative processing with alkali, enzymes and/or bleaching agents.

Glycosaminoglycan heparin, referred to as unfractionated heparin (UFH), consists of polysaccharide chains having molecular weights in the range of 5000–40,000 Da with an average molecular weight of  $\sim 14,000$  Da. Several substitutions are possible in the glucosamine and uronic acid residues. The  $\beta$ -D-glucosamines may be *N*-sulfated or acetylated, and may also contain sulfates at 2-, 3-, and 6-positions, while the uronic acid residues may be either  $\beta$ -D-glucuronic acid (GlcAp)



**Figure 3** Plausible structure of a high-affinity heparin chain containing heparin pentasaccharide sequence  $\text{H}_5$  (also called DEFHG). GlcNp,  $\beta$ -D-2-amino-2-deoxy-glucopyranose; GlcAp,  $\beta$ -D-glucopyranosyluronic acid; IdoAp,  $\alpha$ -L-idopyranosyluronic acid; S, sulfate group; Ac, acetate group. The glucosamines may have sulfate groups at 3- or 6-positions, while the uronic acids may have sulfate at the 2-position. The glucosamines may be *N*-acetylated or *N*-sulfated. Heparin chain is  $1 \rightarrow 4$ -linked.  $\text{H}_5$  sequence, marked as a double-headed arrow, consists of  $\rightarrow 4$ )-GlcNp2S6S-( $1 \rightarrow 4$ )-GlcAp-( $1 \rightarrow 4$ )-GlcNp2S3S6S-( $1 \rightarrow 4$ )-IdoAp2S-( $1 \rightarrow 4$ )-GlcNp2S6S-( $1 \rightarrow$

or  $\alpha$ -L-iduronic acid (IdoAp), and may be sulfated at 2-position (Fig. 3). Typically, there are more *N*-sulfated glucosamines than *N*-acetylated, and more IdoAp residues than GlcAp. Heparin is the strongest acid in our body ( $pK_a$  of sulfate groups  $\approx 0.2$ ) and consequently is highly anionic at physiological pH. The molecular weight dispersity, structural variability, and polyanionic character introduce some novel and challenging properties in heparin.

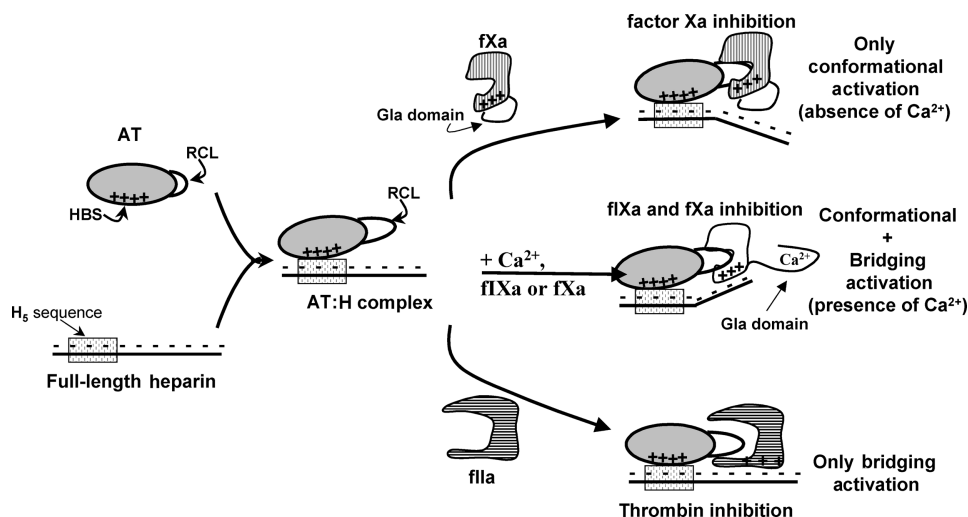
Heparin is an anticoagulant because it recognizes antithrombin with high affinity and activates the inhibitor, as discussed above. The high-affinity interaction arises from a specific sequence **H<sub>5</sub>** in a polymeric chain of heparin – called the high-affinity heparin (HAH) chain (Fig. 3). **H<sub>5</sub>** is composed of three 2-*N*- and 6-sulfated glucosamines ( $\beta$ -D-GlcNp2S, 6S) interspersed with a 2-sulfated iduronic acid ( $\alpha$ -L-IdoAp2S) and a glucuronic acid ( $\beta$ -D-GlcAp), in which the central glucosamine residue has a unique 3-sulfate group. This sequence is labeled as **DEFGH** (28–31), which reflects its history of identification. High-affinity heparin binds to plasma antithrombin, mostly  $\alpha$ -antithrombin, with an affinity of  $\sim 10$  nM at physiological pH. This high affinity originates from a combination of multiple ionic and nonionic interactions between the sulfate and carboxylate groups of DEFGH and positively charged residues of antithrombin (22).

#### IV. Mechanism of Heparin Activation of Antithrombin

Nature has engineered two distinct mechanisms for heparin acceleration of antithrombin inhibition of fIXa, fXa, and thrombin. These two mechanisms are (i) allosteric activation of antithrombin by heparin pentasaccharide DEFGH; and (ii) activation through bridging of the inhibitor and the enzyme (Fig. 4).

The allosteric activation of antithrombin, also called the conformational activation mechanism, is an important mechanism that enhances the rate of inhibition of fXa and fIXa. The binding of heparin pentasaccharide DEFGH to antithrombin increases the second-order rate constant nearly 300-fold for fXa (22) and  $\sim 300$ –500-fold for fIXa (23). Up until a couple of years ago, the conformational activation mechanism was thought to occur through the expulsion of the partially inserted RCL residues. In this mechanism, first proposed by van Boeckel et al. (32) the binding of DEFGH in the heparin-binding site of antithrombin results in elongation of helix D by 1–2 turns introduces some pressure in sheet A, thereby causing it to close with concomitant exposure of the RCL. This expulsion of the partially inserted residues was expected to significantly change the conformation of RCL residues, especially the P1–P1' reactive center, making it more favorable for recognition and cleavage by the proteinase (33,34) (Fig. 4).

Several discrete steps of the hypothesis have found support. For example, mutagenesis of C-terminal end residues of helix D to prevent elongation resulted in impaired heparin activation (35,36), while mutation of the critical hinge region residue P14Ser to Glu (additional charge), Trp (steric bulk), or Cys-fluorescein (bulk) resulted in activation without the need for heparin (37–39). Likewise, comparison of the X-ray crystal structures of native and pentasaccharide-complexed



**Figure 4** Bridging and conformational activation mechanism of antithrombin inhibition of factor Xa, factor IXa, and thrombin in the presence of heparin. AT:H, antithrombin–heparin complex; H<sub>5</sub>, high-affinity pentasaccharide sequence in heparin; RCL, reactive center loop; fXa, factor Xa; fIXa, factor IXa; Gla, Gla domain on fIXa/fXa; “+ + +”, exosite on enzyme; HBS, heparin-binding site. See text for details.

antithrombin (14,33) shows a clear difference in conformation of the hinge region residues, although the P1Arg was still directed inward and bound to Glu255. Despite this apparent lack of structural change in the critical P1 residue in the crystalline state, heparin-dependent modification of P1Arg residue in solution supported the idea that this critical proteinase recognition site becomes available in the activated form (16). In addition, earlier it had been demonstrated that NBD fluorophore at the P1Cys position underwent a dramatic increase in fluorescence on DEFGH binding, thereby supporting significant changes in the conformation of RCL hypothesis (40).

Although these experiments supported the idea of heparin-induced RCL conformational change, another body of experiments suggested that the changes introduced are not enough to explain the 300–500-fold accelerations achieved. Specifically, when Olson and coworkers mutated the antithrombin RCL sequence to a more thrombin specific sequence, a maximal acceleration achieved was only 2–9-fold due to heparin binding (18,19,41). Interestingly, these RCL mutations did not decrease pentasaccharide acceleration of fXa inhibition, thus supporting the idea that RCL sequence, except for P1Arg, or conformation had little to do with heparin activation of antithrombin for fXa (20). Thus, it was hypothesized that there must be exosites outside of RCL in antithrombin that recognize the target proteinases and that these exosites become available only in the activated state of the inhibitor. This exosite has now been localized using chimeric antithrombins to strand 3 of  $\beta$ -sheet C, which when changed to  $\alpha$ 1-proteinase inhibitor sequence

shows no enhancement in the rate of fXa/fIXa inhibition (42). The strand 3C sequence contains three conserved residues, Tyr253, Glu255, and Lys257, which appear to be good candidates as exosites. An alternative proposal, based on modeling antithrombin–fXa Michaelis complex, suggests strands 1B, 3B, and 4C as the likely proteinase recognition domains (43).

Complementing the antithrombin exosite hypothesis are studies on identifying the exosite(s) on fXa that is involved in the acceleration phenomenon. Through a series of mutations in the surface loops on fXa, including the 39, 60, sodium-binding, and the autolysis loop, Rezaie and coworkers (44,45) have identified Arg150 of the autolysis loop as a primary determinant of the recognition of the activation conformation of antithrombin. Interestingly, when the autolysis loop of fXa was introduced into corresponding positions in thrombin and activated protein C, the acceleration achieved with heparin pentasaccharide was 37- and 375-fold, respectively, thus suggesting that the autolysis loop of fXa plays a major role (46).

Taking these studies on the importance of RCL structure and exosites in antithrombin and the role of exosites in fXa/fIXa into consideration, Olson and Chuang (20) hypothesize that antithrombin RCL sequence evolved to *not* optimally recognize proteinases for a specific reason. In addition to inhibiting procoagulant proteinases, antithrombin can also possibly inhibit activated protein C because of its P1Arg specificity. Activated protein C is a natural anticoagulant and thus to avoid being simultaneously conflicting, anticoagulant when inhibiting fIXa, fXa or thrombin and procoagulant when inhibiting activated protein C, antithrombin evolved to not recognize its potential targets properly! In fact, antithrombin is an extremely poor inhibitor of activated protein C even in the presence of heparin (47). The use of exosites as a means to introduce specificity and reactivity is nature's ingenuity in regulation.

In contrast to fXa inhibition, thrombin inhibition is accelerated only 1.7-fold through allosteric activation of antithrombin by heparin pentasaccharide (22). The predominant effect of heparin in accelerating thrombin inhibition arises from a bridging mechanism. Tight binding of antithrombin to the **H<sub>5</sub>** sequence in full-length heparin is followed by the binding of thrombin to the same chain to form an antithrombin–heparin–thrombin ternary complex (Fig. 4). The binding of heparin to thrombin at the so-called exosite II is electrostatically driven resulting in poor heparin structure specificity (21,48). Thrombin then diffuses along the polyanionic chain to encounter the inhibitor resulting in a ~2000-fold acceleration in inhibition under physiological conditions. The lack of specificity with respect to heparin structure is probably necessary for thrombin to glide along the sulfated polysaccharide chain. A chain length of ~18 residues is needed to simultaneously hold thrombin and antithrombin for the accelerated inhibition (49,50). Thus, while sequence-specific **H<sub>5</sub>** is necessary for tight binding of heparin chains, **H<sub>5</sub>** alone cannot potentiate antithrombin inhibition of thrombin. The bridging mechanism exemplifies the fine use of specificity and nonspecificity to enhance recognition and reactivity.

The bridging mechanism also called the template or approximation mechanism, also plays an important role in accelerated inhibition of fIXa and fXa

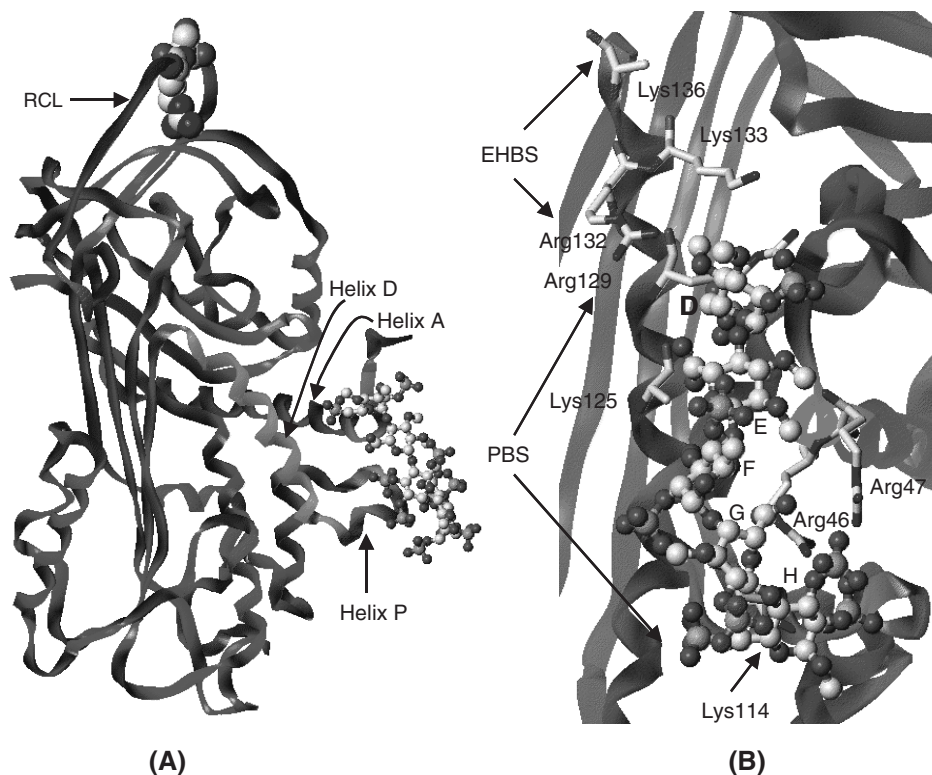


(23,24,51–53). Under physiological concentrations of  $\text{Ca}^{2+}$  ions, additional enhancement in the inhibition rate is observed. For fXa, polysaccharide heparin accelerated antithrombin inhibition more than 100-fold in the presence of 2.5 mM  $\text{Ca}^{2+}$  over and above the acceleration achieved in the presence of DEFGH (51,52), while for fIXa this acceleration reaches 130–1000-fold (23). The increased acceleration in the presence of  $\text{Ca}^{2+}$  is mediated by a bridging of the proteinase and the inhibitor on polysaccharide heparin chains. In the absence of  $\text{Ca}^{2+}$  ions, the N-terminal Gla domain containing several  $\gamma$ -carboxy glutamic acid residues, which are negatively charged, folds over an electropositive exosite that recognizes heparin. Binding of calcium ions to the Gla domain of both fIXa and fXa results in the release of the positively charged exosite on the proteinase, thus facilitating ternary complex formation with full-length heparin (Fig. 4) (54,55). For fXa, the maximal effect due to  $\text{Ca}^{2+}$  was observed with HAH containing  $\sim 35$  (or higher) saccharide residues, while for fIXa nearly half as long a polysaccharide appears to be necessary (53).

## V. The Heparin Binding Site in Antithrombin

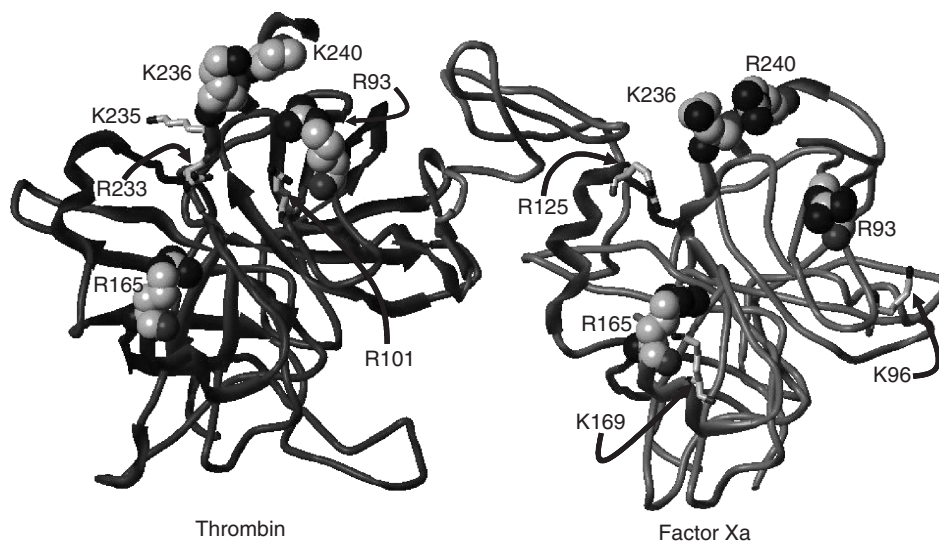
The heparin-binding site in antithrombin is an engineering masterpiece (Fig. 5). The binding site is located some 20 Å away from the RCL and hence the mechanism of conformational activation is called the allosteric activation mechanism. This binding site specifically recognizes **H<sub>5</sub>** with high affinity, yet is capable of binding to numerous structurally different molecules that bear resemblance to the pentasaccharide. The heparin-binding domain in antithrombin is formed by positively charged residues of helices A and D, and the polypeptide N-terminus. The crystal structure of antithrombin–pentasaccharide co-complex shows that residues Lys114, Lys125, and Arg129 in this region interact strongly with **H<sub>5</sub>** (33). Biochemical studies with antithrombin mutants suggest that the three residues contribute  $\sim 50$ ,  $\sim 25$ –33, and  $\sim 28$ –35% of the total binding energy, respectively (56–58). Interestingly, Lys114 and Arg129 cannot be interchanged, while Lys125  $\rightarrow$  Arg interchange was essentially silent, indicating a high level of structural specificity (59). The domain formed by these three residues is called the pentasaccharide-binding site (PBS, Fig. 6). The PBS also contains other residues that play a smaller role in heparin binding. These residues include Lys11, Arg13, Arg24, Arg46, Arg47, and Trp49 (60–62). Each of these residues contributes 5–15% of total binding energy, except for Trp49, which contributes nearly 20% binding energy (62). It is to be expected that the many charged interaction points in PBS, each capable of hydrogen bonding in addition, raise the level of specificity as well as enhance affinity for a molecule with perfect fit (e.g., the heparin pentasaccharide DEFGH).

In addition to interacting with PBS, full-length heparin binds to an extended region formed by residues Arg132, Lys133, and Lys136 at the C-terminal end of helix D. This extended region is designated as the extended heparin-binding site (EHBS, Fig. 5) (63). This interaction is supported by biochemical studies that show that mutation of Arg132 and Lys133 selectively impairs the binding of polysaccharide



**Figure 5** Structure of heparin-binding site of antithrombin: (A) shows plasma antithrombin complexed to natural pentasaccharide DEFGH, while (B) shows a close up view of the heparin-binding site. The structure of co-complex was obtained from RCSB (filename "1E03"). In both figures, green ribbon shows antithrombin, red represents RCL, and magenta represents the heparin-binding site. Pentasaccharide DEFGH is shown in ball-and-stick representation and individual residues are marked in (B). Helices D, P and A (C-terminal end) form the heparin-binding site. Helix D extends by 1.5 turns in the complex as compared to free antithrombin (Fig. 2). In addition, helix P is not present in uncomplexed antithrombin. Arg46, Arg47, Lys114 [hidden in (B)], Lys125 and Arg129 form the pentasaccharide binding site (PBS), while Arg132, Lys133, and Lys136 form the extended heparin-binding site (EHBS). See text for details. Reprinted with permission from Desai (2004) *Med. Res. Rev.* 24:151–181. Wiley Periodicals, Inc.

heparin, but not of DEFGH (64). In addition, salt dependence of binding affinity shows that whereas DEFGH forms 4 ion-pair interactions, full-length heparin forms 5 (22). Finally, crystallographic studies on antithrombin–heparin–thrombin ternary complexes also suggest that these positively charged residues could form ion-pairs with sulfate groups on heparin (65,66), although the heparin used in these studies had a neutral linker.



**Figure 6** Comparison of heparin-binding exosite in factor Xa and thrombin. The X-ray crystal structures of Gla-domainless fXa (RCSB file “*1hcg*”) and thrombin (from RCSB file “*1tb6*”) were used for comparison. The enzymes are placed in approximately similar orientation. Key heparin-binding residues common in both proteinases are shown in space-filling model, while other residues implicated in heparin binding are shown in capped stick representation. See text for details.

It is important to recognize that the allosteric activation phenomenon involves the conformational changes at both ends – at the RCL as well as at the heparin-binding site. At a molecular level, following heparin binding at least three changes occur in the HBS. One, a short  $3_{10}$  P helix, containing the critical Lys114 residue, is formed at the N-terminal end of helix D. Two, a small kink present in helix D before heparin binding is straightened out and three, helix D is extended by 1.5 turns, the elongation discussed above. These changes in the heparin-binding site are coupled to changes in the RCL and the exosite in  $\beta$ -strand 3C. Whereas the molecular details at the two ends of the allosteric activation mechanism are fairly well understood, much less is understood regarding the molecular mechanism of transmission of heparin binding energy.

## VI. The Heparin Binding Site in Factor Xa and Thrombin

Both fXa and thrombin can bind heparin polysaccharide, under appropriate conditions, as can be concluded from the above discussion. Heparin affinity of fXa and thrombin has been mostly inferred from indirect experiments involving formation of ternary complexes at high concentrations of full-length heparin, although direct titrations have been performed for thrombin to ascertain the apparent affinities so

measured (67,68). High- and low-affinity heparin (for antithrombin) bind to thrombin at physiological pH 7.4 and ionic strength I 0.15, but in absence of  $\text{Ca}^{2+}$ , with an apparent  $K_D$  of 0.7–2  $\mu\text{M}$ . This indistinguishable apparent affinity for the two forms of heparin polysaccharide indicates less stringent structural specificity. Moreover, affinity may be different in the presence of calcium ions. In contrast, the wild-type fXa–high affinity heparin complex, in which the proteinase lacks the N-terminal Gla domain, has an apparent dissociation constant of  $\sim 0.4 \mu\text{M}$  in the presence of 2.5 mM  $\text{Ca}^{2+}$ . Whether the affinity of fXa for heparin is dependent on the sequence of the polysaccharide remains to be tested, although one can expect minimal structural specificity. It is interesting that there is a small  $\sim 2$ –5-fold difference in affinity for heparin between the two proteinases, although the  $\text{Ca}^{2+}$  concentrations were different.

Both fXa and thrombin contain electropositive domains, called exosites that bind heparin. A number of mutagenesis experiments have identified the amino acid residues that interact with sulfate groups on heparin. The residues identified common in both enzymes include Arg93, Arg165, Lys236, and Lys/Arg240 [residues are numbered following the chymotrypsin numbering system (69)] (68,70–72, Fig. 6). In addition, Arg101, Arg233, and Lys235 of thrombin have been implicated (70–72), while for fXa the additional residues include Lys96, Arg125, and Lys169 (68). According to the defect introduced in heparin binding, the rank order of importance of these residues in thrombin is Arg93 > Lys236 > Lys240 > Arg101 > Arg233, for fXa the order is Arg240 > Lys236 > Lys96, Lys169 > Arg165 > Arg93.

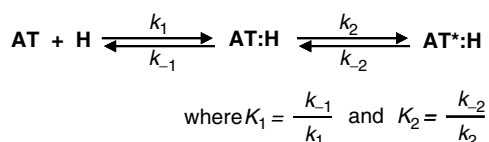
Whereas the crystal structure of fXa–heparin complex remains unknown, that for thrombin has recently been solved (73). In addition, the crystal structure of antithrombin–heparin–thrombin ternary complex, discussed above (65), also provides an indepth view of the structure of heparin binding exosite II in thrombin. Compiling this structural information with the crystal structure of  $\gamma$ -Gla, domainless fXa shows striking similarity between the exosites (Fig. 6). The residues common to both (R93, R165, K236 and K/R240) are in identical positions on exposed surfaces scattered across helices and loops. Yet, other residues are positioned differently, especially Arg101 of fIIa and, Lys96 and Lys169 of fXa. Thus, differences do exist that may result in small changes in affinity and specificity. It is likely that the electropositive density of thrombin exosite is more than that of fXa. Finally, the reason for the difference in the rank order of importance of residues is not obvious. A simple calculation suggests that both exosites are nearly 15–20 Å long capable of binding 3–4 saccharide residues.

## VII. Thermodynamics and Kinetics of Heparin Binding to Antithrombin

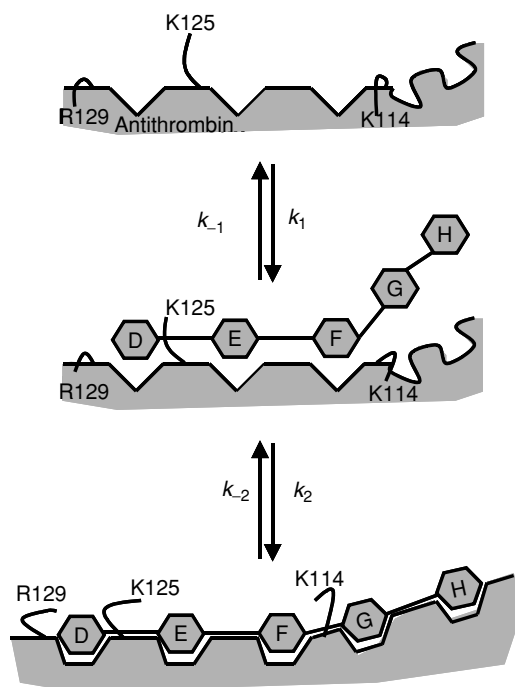
The high-affinity interaction between heparin and antithrombin is mediated through heparin pentasaccharide DEFGH that contains a unique 3-*O*-sulfate substituent on glucosamine F (see Fig. 3, 28–31). DEFGH accounts for  $\sim 95\%$  of the free energy of binding of the HAH species to antithrombin (22), which arises

primarily due to sulfates and carboxylates of DEFGH interacting with positively charged residues, identified above, of the PBS. Ionic binding energy contributes  $\sim 40\%$  of the total standard free energy of binding under physiological conditions (22) arising from nearly four ion-pair interactions (22,74,75). In addition, our molecular modeling study using hydropathic interaction (HINT) analysis, based on the crystal structure of pentasaccharide–antithrombin complex, indicates that the remaining  $60\%$   $\Delta G^\circ$  also arises from the hydrogen bond-type interactions between these positively and negatively charged groups (76). This implies that according to HINT analysis the saccharide backbone makes minimal contribution to the binding affinity. It is likely that the pentasaccharide backbone is important to primarily position the critical  $\text{OSO}_3^-$  and  $\text{COO}^-$  groups for optimal interaction with antithrombin, a concept deemed extremely important for designing new antithrombin activators.

The binding of DEFGH, or HAH, to antithrombin is a two-step process involving the formation of an initial low-affinity complex (AT:H) in rapid equilibrium. This process may be called the “recognition” process. This is followed by a major conformational change to give a high-affinity  $\text{AT}^*\text{:H}$  complex in which antithrombin has been conformationally activated (Scheme I, Fig. 7) (22,77). This process may be thought of as the “ctivation” process. This conformational change has been linked to the expulsion of the RCL for accelerated inhibition of fXa (40). Analysis of the two-step induced fit binding process for antithrombin mutants and heparin pentasaccharide derivatives has yielded some important insight into how activation occurs at a molecular level on the heparin side. Our investigations with the truncated forms of DEFGH indicate that full conformational activation of antithrombin ( $\sim 300$ -fold acceleration) can also be achieved with trisaccharide DEF under saturating conditions suggesting that recognition and activation is built-in in the trisaccharide (74). Thus, DEF residues play a major role in both the first and second step of the induced-fit pathway interacting with all three critical residues, Arg129, Lys125, and Lys114, of the PBS (56–59). Of these, only Lys125 is known to play a major role in the recognition of DEF, although it is likely that other point charges are involved to lesser extent (57). Yet, the affinity of DEF for antithrombin is much weaker ( $K_D \sim 66 \mu\text{M}$ ) under physiological conditions. This affinity is dramatically enhanced by the presence of disaccharide unit GH that interacts with Lys114. Thus, residues GH play an important role in the conformational activation step to greatly stabilize the activated state of antithrombin (58,74) (Fig. 7). Overall, in addition to the three critical residues, many other positively



**Scheme 1**



**Figure 7** A model of conformational changes occurring in the pentasaccharide-binding site of antithrombin during the two-step binding process. The PBS in native antithrombin can recognize only the DEF part of the pentasaccharide (step 1). In addition, it is not fully complementary to the trisaccharide unit result in weaker affinity. In this first step, only K125 residue appears to play a major role. The small amount of binding energy made available due to this initial recognition results in conformational changes in the PBS that generates binding sites for residues G and H, as well as introduces greater complementarity for residues D, E, and F. In this second step, Arg129 and Lys114 play a major role. It is important to note that many other positively charged residues of the PBS take part in this process. See text for details.

charged residues play a secondary role, primarily in the second step. Activation of antithrombin aligns these residues to make optimal contacts with the heparin pentasaccharide, thus enhancing the stability of the activated form (see Fig. 9B).

### VIII. Rationale for Designing Functional Mimics of Heparin

Despite the phenomenal success of heparin as an anticoagulant drug, heparin therapy is beset with a number of drawbacks. Natural product heparin is a highly heterogeneous, polydisperse polymer that forms indiscriminate, ionic interactions with numerous soluble proteins and cell surface receptors (11,78). This introduces several problems: (i) appreciable patient-to-patient variability in response, thus

requiring continuous monitoring and hospital stay; (ii) enhanced risk for bleeding; (iii) possibility of heparin-induced-thrombocytopenia (HIT) and osteoporosis; (iv) poor bioavailability; and (v) short half-life.

This nonspecific binding, and the problems thereof, can be greatly reduced by decreasing the size and the highly anionic character of heparins. Low-molecular weight heparins (LMWHs), developed in the past decade, have much better defined chemical and biological properties. These agents are prepared using chemical or enzymatic depolymerization of heparin and possess distinct pharmacologic profile that is largely determined by their composition. Depolymerization reduces the average molecular weight to ~3000–6000 Da. The multitude of preparative methods makes each product a distinct entity (79). Moreover, several reports show that the critical antithrombin binding sequence, DEFGH, may be lost in some of the preparative methods (80,81). Yet, LMWHs possess several advantages. These include better bioavailability, fewer HIT incidences, and lesser risk of bleeding complications (79). With the use of these advantages, LMWHs have significantly changed outpatient anticoagulant management. Thus, the introduction of LMWHs represents a major advance in improving the use of heparin and to a large extent the LMWHs have replaced unfractionated heparin in most subcutaneous indications.

In 2001, a heparin pentasaccharide fondaparinux, based on sequence DEFGH, was introduced as a selective fXa inactivating agent. Fondaparinux, originally synthesized in 1983 by Choay et al. (82), represents a major advance in anticoagulation therapy, in terms of its applicability and its unprecedented massive preparation. It broke apart two commonly held beliefs surrounding heparins. One, that fXa inhibition only cannot be effective, and two, that the half-life of molecules smaller than full-length heparin will be much shorter. Current evidence indicates that fondaparinux is much better than both UFH and LMWHs. It has greater efficacy over LMWHs, is useful in both arterial and venous thrombosis, has a longer half-life (17 h), has no HIT adverse effects, and has limited patient-to-patient variability (83–86). The bleeding risk seems to not have been completely eliminated.

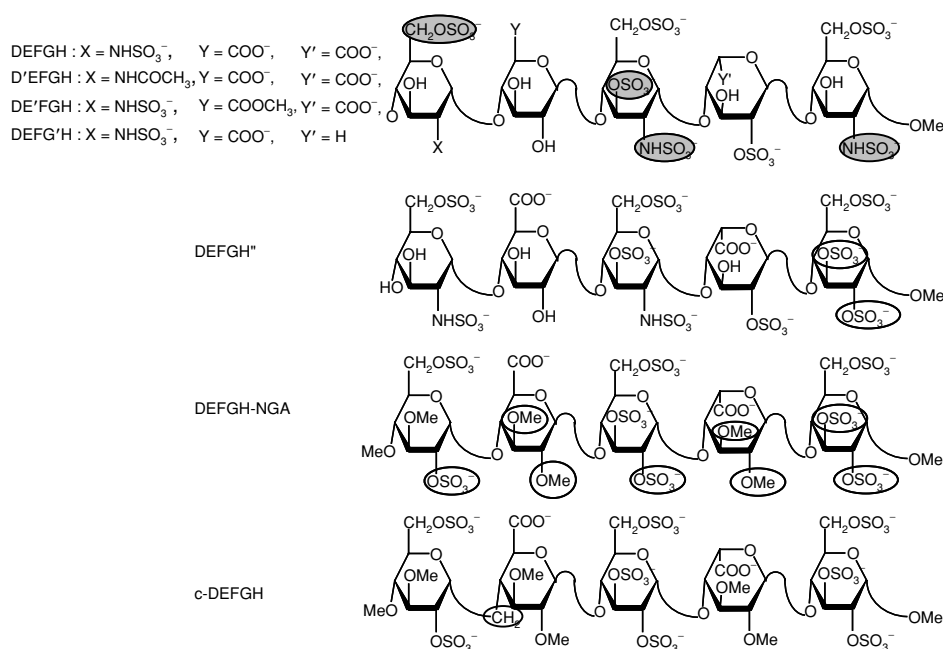
Although anticoagulation therapy has advanced dramatically in the last few years, the risk for bleeding seems to persist. The synthetic challenge with oligosaccharides introduces high costs (87). Additionally, we are way away from orally active heparins.

## **IX. Heparin Mimics**

### **A. Heparin Mimics that Selectively Utilize Conformational Activation Pathway**

Enormous effort has been directed toward discovering and/or designing new molecules with heparin-like anticoagulant activity. These new molecules can be classified into natural polysaccharides, synthetic modified heparins, synthetic oligosaccharides, and synthetic nonsugar heparin mimics (88). Recent efforts in designing novel heparin pentasaccharide derivatives have also been reviewed (89).

Petitou and coworkers (89,90) have made major inroads into pentasaccharide derivatives that are better than the native molecule. The natural pentasaccharide sequence has two variations of unequal activity arising from the nonreducing end glucosamine residue D being either *N*-sulfated (DEFGH) or *N*-acetylated (D'EFGH) (Fig. 8). The *N*-sulfated pentasaccharide (700 units/mg anti-fXa) is about twice as active as the *N*-acetylated variant. Structure–activity relationships were derived using synthetic variants of DEFGH. It was established that 4-sulfate groups, at the 6-position of residue D, 3- and 2-positions of residue F, and 2-position of residue H, are critical for high-affinity binding to antithrombin (Fig. 8). Of these, the 3-sulfate group on residue F was found to be most important for antithrombin



**Figure 8** Structure of natural pentasaccharide DEFGH and its derivatives. Molecules with *primes* have a residue whose structure differs slightly from natural pentasaccharide DEFGH. Sulfate groups in DEFGH highlighted as filled ovals (●) are critical for high-affinity interaction with antithrombin. Pentasaccharide “DEFGH” has residue H-containing sulfates at 2- and 3-positions (highlighted as empty ovals). DEFGH-NGA, the “nonglycosamino” derivative of the natural pentasaccharide, has no *N*-sulfates and contains uronic acids that have 2-OMe groups. Note the number of changes introduced in DEFGH-NGA to ease the synthesis and enhance the anti-fXa activity. C-DEFGH contains a methylene (–CH<sub>2</sub>) group replacing the interglycosidic oxygen atom between residues D and E. Reprinted with permission from Desai (2004) *Med. Res. Rev.* 24:151–181. Wiley Periodicals, Inc.



activation. Its antithrombin interaction counterpart is Lys114 (33), which has also been implicated as the most important residue (58,59). The carboxylate groups of GlcAp and IdoAp2S are also important as demonstrated by pentasaccharides DE'FGH and DEFG'H (Fig. 8) that exhibit less than 5% of the activity of the reference DEFGH.

Studies with DEFGH' and DEFGH-NGA (Fig. 8) led Petitou and coworkers to two major advances. Replacement of  $\text{NHSO}_3^-$  group in all three glucosamine residues with  $\text{OSO}_3^-$  and introduction of alkyl ethers at the available free hydroxyl groups not only retained anti-fXa activity, but enhanced it  $\sim 200\%$  (90). The new "on-glycosamino" pentasaccharide DEFGH-NGA contains glucose residues, instead of glucosamines, thus easing their synthesis significantly. The "on-glycosamino" pentasaccharide preserves the distribution of critical sulfate and carboxylate groups of the natural pentasaccharide sequence. Further, it is likely that the new pentasaccharide retains the conformational preference and flexibility of the parent molecule. Further, a C-pentasaccharide, consisting of a carbon ( $\text{CH}_2$ )-based interglycosidic bond between residues D and E (Fig. 8), was shown to hardly affect the biological properties of the natural pentasaccharide (91). This C-pentasaccharide shows an anti-fXa activity  $\sim 34\%$  better than DEFGH and represents the first member of new anti-fXa pentasaccharides.

Recently, major advances on chemo-enzymatic front have been made by Rosenberg and coworkers (92–94) to synthesize the high-affinity saccharides. This approach utilizes the tremendous advances made in cloning and expressing heparan sulfate sulfotransferases (*N*-deacetylase *N*-sulfotransferase, 2-*O*-sulfotransferase, 3-*O*-sulfotransferase and 6-*O*-sulfotransferase). The availability of these enzymes for *in vitro* use coupled with the availability of a polysaccharide from *Escherichia coli* strain K5 has resulted in a two-step enzymatic synthesis of HAH (93) and a six-step synthesis of heparin pentasaccharide (92) with reasonably good yields and in short time. In addition, the HAH prepared in this approach was found to be 3–4-fold better than commercial heparin because of the higher proportion of the **H**<sub>5</sub> sequence per full-length chain. Another chemo-enzymatic approach, which relied more on chemical sulfation/de-sulfation steps, was used by Lindahl et al. (95) to prepare HAH from bacterial K5 capsular polysaccharide. These chemo-enzymatic approaches are likely to be extremely useful in generating heparin (heparan sulfate) structures tailored to specific sulfation pattern (94).

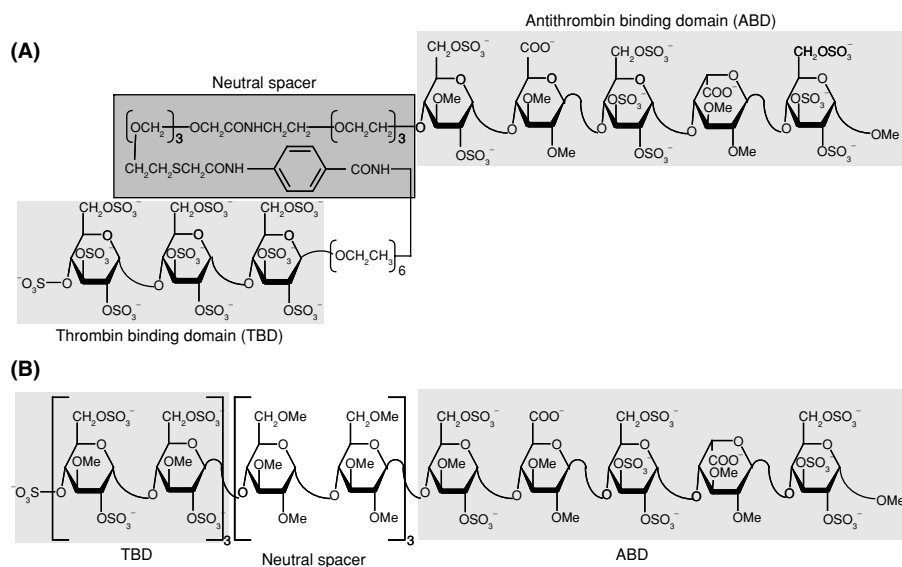
## B. Heparin Mimics that Use the Bridging Mechanism of Inhibition

As discussed above the dominant mechanism in heparin action appears to be the bridging pathway. Thus, it was reasoned that a heparin mimic could be easily designed based on a three-domain structure – an antithrombin-binding domain (ABD), a thrombin binding domain (TBD) and a linker joining the two. Essentially, heparin is a three-domain structure. Whereas the structure of ABD (i.e. DEFGH)

could not be changed greatly, the structure of TBD in heparin could be altered readily because thrombin–heparin interaction is relatively nonspecific (21,48). The TBD was predicted to compose of  $\sim 5$ –6 negative charges, recently confirmed by the crystal structure of thrombin–heparin complex (73), distributed on either 2 or 3 saccharides, most probably representing the dominant disaccharide sequence,  $\rightarrow 4$ -IdoAp2S–(1  $\rightarrow$  4)-GlcNp2S,6S–(1  $\rightarrow$ , of heparin. Previous molecular modeling studies (96), later confirmed by a crystallography study (33), had suggested that TBD was located on the nonreducing end of the DEFGH sequence. Thus, the relative orientation of the two domains could be fixed. However, questions remained on the constitution and length of spacer, as well as the fine structure of TBD.

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Chemical synthesis of oligosaccharides, containing the heparin pentasaccharide at the reducing end and heparin disaccharide at the nonreducing end of the chain, established that for accelerated thrombin inhibition a minimum chain length of either 15 or 16 residues is needed (97,98). Several different spacer structures were utilized (Fig. 9). A flexible polyethylene glycol type spacer was less active as anti-factor IIa inactivation agent than a rigid polyglucose type spacer (99). Finally,



**Figure 9** Structure of novel molecules designed to inhibit both factor Xa and factor IIa using the bridging mechanism: (A) shows antithrombin-binding domain (ABD) linked to a thrombin-binding domain (TBD) using a flexible polyethylene glycol type neutral linker to arrive at an appropriate length so as to facilitate ternary complex formation. The ABD is extended at its nonreducing terminus. (B) shows a similar concept, however with a relatively more rigid neutral spacer consisting of glucose units. The anti-fIIa activity of molecule (A) is much lower than that of molecule (B).

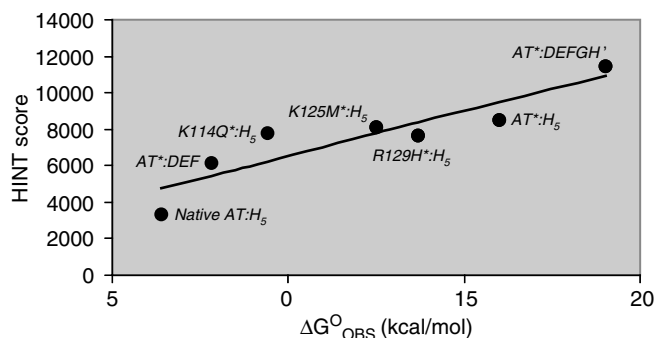
a heparin mimic was engineered that has the ABD connected to TBD through an uncharged hexasaccharide spacer. To ease the synthesis, the TBD was constructed of a disulfated glucose-based hexasaccharide (Fig. 9) (100,101). This heptadecasaccharide is an extremely potent anti-fXa and anti-factor IIa molecule and cannot be neutralized by PF4. It is likely that this molecule has negligible HIT adverse effect. Recently, a heparin mimic containing TBD, which is perphosphorylated rather than persulfated, has been found to exhibit an increase in antithrombin activity (102). Phosphate groups appear to be more potent at interacting with TBD than sulfate group. These full-length heparin mimics show tremendous promise of moving into clinical trials.

### C. Heparin Mimics with Nonheparin Scaffold

As may be apparent from the above discussion, all functional mimics of heparin started with a heparin scaffold. We questioned this fundamental tenet of the assumed requirement of saccharide skeleton for high-affinity interaction with antithrombin. A major concern with the saccharide-based approach is the difficulty of oligosaccharide synthesis that reduces the cost effectiveness. We have recently designed synthetic nonsugar molecules that exhibit antithrombin activation for accelerated inhibition of fXa (103,104). These molecules represent the first in the class of small, nonsugar activators.

To rationally design nonsugar activators a robust modeling tool was required that accurately simulates the interactions of antithrombin at a molecular level. First, hydrophathic interaction (HINT) analysis was used to quantitate the interactions of antithrombin (103). Hydrophathic interaction is molecular modeling tool that provides quantitative information regarding intermolecular interactions at an atomic level. Hydrophathic interaction accurately identifies and quantitates three common interactions, charge–charge interactions, hydrogen bonding interactions, van der Waals interactions, between proteins and its ligands. For antithrombin–pentasaccharide interaction, HINT map showed favorable interactions between positively charged residues of helix A, helix D, and the polypeptide N terminus and the sulfate and carboxylate groups of the pentasaccharide (105). Further, HINT predicted reasonably well the interaction of DEFGH with heparin-binding mutants of antithrombin (103) (Fig. 10). Using this as a basis, the interaction profile of designed *de novo* ligands was studied.

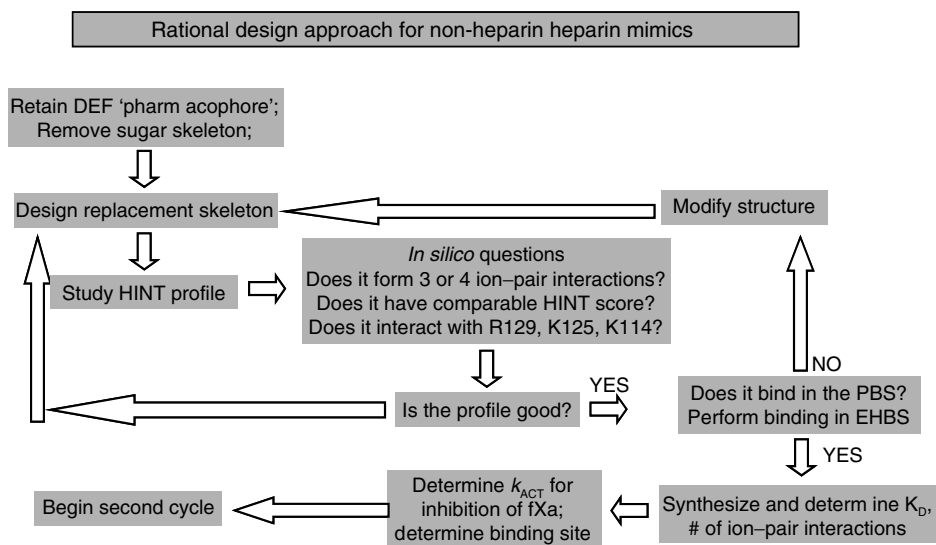
We reasoned that mimicking trisaccharide DEF would be a good starting point for designing heparin mimics for several reasons. DEF is nearly equivalent to pentasaccharide DEFGH in inducing acceleration in antithrombin inhibition of fXa, it is that part of the heparin pentasaccharide structure that exhibits considerable rigidity, and it is small enough for rapid synthesis of mimics. To design an antithrombin activator, a “harmacophore” 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 Fig. 8), groups that are thought to be important for antithrombin activation. Numerous molecular frameworks were



**Figure 10** Predictability of affinity of heparin ligands for binding to antithrombin using HINT technique. Total HINT scores were obtained for heparin oligosaccharides binding to native antithrombin and its mutants *in silico* for which the affinities had been previously obtained. A linear correlation with correlation coefficient of 0.81 was obtained for standard free energy of binding ( $\Delta G^{\circ}_{\text{OBS}}$ ) range of 4–20 kcal/mol suggesting high predictability. Asterisk (\*) represents activated state. The solid line is a linear regression obtained by fitting data with slope of  $491 \pm 105$  HINT score/kcal/mol and an intercept of  $1622 \pm 1346$  HINT score. Adapted from Gunnarsson and Desai (2002) *J. Med. Chem.* 45:1233–1243.

screened based on their ability to satisfy the three-dimensional organization of the pharmacophore (Fig. 11). 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. The essence of our rational approach to arrive at the first nonsugar heparin-mimic scaffold was repetitive docking and scoring analysis to answer questions, such as: (i) does the new mimic form ion-pair interactions of the type formed by heparin pentasaccharide? (ii) does the mimic have HINT score comparable to DEF or DEFGH? (iii) does the mimic show interactions with R129, K125 and/or K114 (Fig. 11)? After a series of structural screens, a small nonsugar skeleton, (–)-epicatechin sulfate (ECS, Fig. 12A), was designed. Epicatechin sulfate was found to interact with antithrombin with an affinity comparable to DEF. More interestingly, it accelerated the inhibition of fXa nearly 8-fold, the first small nonsugar molecule known to activate antithrombin (103).

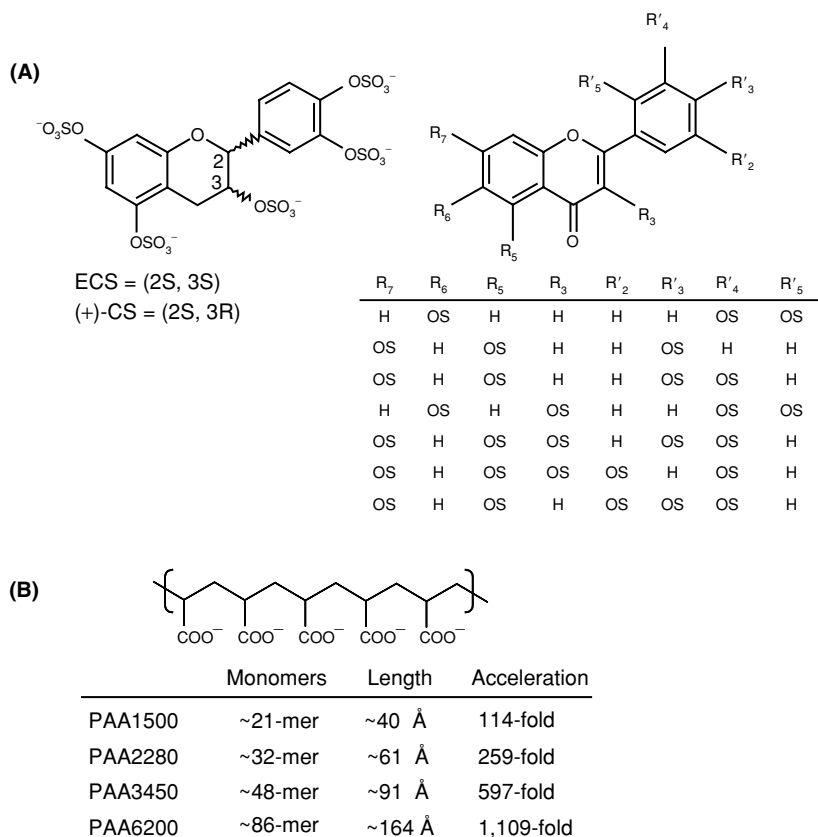
The design of the first nonsugar heparin mimic, albeit with a weak acceleration in inhibition of fXa, indicated a strong possibility of discovering more active molecules. Hence we screened several related molecules (Fig. 12A). However, the activation of antithrombin did not increase beyond ~20-fold in comparison to that known for DEF (~300-fold) (104,106,107). To examine the reason for this poor activation, we performed detailed competitive binding and molecular modeling studies. These experiments indicate although designed to bind to the PBS in antithrombin, our designed small molecules prefer to bind in an electropositive domain adjacent to the pentasaccharide binding site and formed by residues Arg132, Lys133, and Lys136, a site called the extended heparin-binding site (EHBS) (104). This site is known to play a secondary role in antithrombin activation



**Figure 11** Strategy for rational design of non-saccharide mimics of heparin. We started with DEF pharmacophore, rather than DEFGH, and used HINT and biochemical studies extensively to derive the first organic nonsugar mimics of heparin. See text for details.

and thus, the weaker activation potential of flavanoids and flavonoids could be rationalized. This exercise has given rise to a modified search algorithm that goes beyond the initial screening of new scaffolds. The new search algorithm requires an answer to the question whether the heparin-mimic is likely to prefer to bind to the PBS or the EHBS? Answer to this question may sometimes come only from biochemical studies using competitive inhibition experiments (Fig. 11).

Simultaneously we have exploited the bridging mechanism to arrive at non-heparin skeletons for antithrombin activation. A major advantage of the bridging mechanism is that it obviates conformational change in antithrombin. This is important because inducing conformational change in antithrombin is likely to be difficult with non-heparin mimics, although not impossible. Thus, we reasoned that activation should be possible with non-sulfated activators, based only on carboxylic acid groups. As a proof of the principle, linear poly(acrylic acid)s (PAAs) (Fig. 12B) have been found to bind to antithrombin and accelerate inhibition of fXa and thrombin (108). The acceleration in thrombin inhibition achieved with linear PAAs was in the range of 100–1100-fold depending on the length of the polymer, which is extremely encouraging. The affinity of these PAAs for antithrombin under physiological conditions is poor ( $\sim 100 \mu\text{M}$ ) and hence impossible to succeed as a drug. Yet, our work demonstrates that molecules completely devoid of sulfate groups can activate antithrombin effectively. These initial studies highlight the possibility that rational design of non-heparinoid molecules that recognize the PBS in antithrombin and that effectively bridge proteinases is possible.



**Figure 12** Structures of novel nonsugar activators of antithrombin. (A) Structure of flavanoids and flavonoids shown to interact with plasma antithrombin with  $\mu\text{M}$  affinity and inducing a 8–20-fold acceleration in inhibition of fXa. ECS [(–)-epicatechin sulfate] was the first rationally designed molecule to mimic trisaccharide DEF. “OS” in the substitution grid represents the sulfate group ( $\text{OSO}_3^-$ ). (B) Structure of poly(acrylic acid)s used to test the hypothesis that antithrombin can be activated in the absence of sulfate groups and in the presence of carboxylate groups only. Acceleration ( $k_{\text{CAT}}/k_{\text{UNCAT}}$  ratio) in thrombin inhibition shown here was determined in pH 7.4, I 0.05 buffer, wherein the affinity of these molecules for antithrombin is very poor. See text for details.

## X. Conclusions

Detailed knowledge on the mechanism of antithrombin activation, the structure of heparin-binding site in antithrombin, and the structure–function relationships in the heparin pentasaccharide sequence has made possible the rational design of new heparin mimics. Several interesting pentasaccharide derivatives have been made for selective inhibition of fXa. Longer chain polysaccharides have also been developed for effective simultaneous inhibition of fXa and thrombin. Novel small, rationally

designed, nonsugar molecules have shown early promise of antithrombin activation. Finally, good acceleration in antithrombin inhibition of thrombin has been achieved with non-sulfated polymers. These advances bode well for rational-design of high-affinity, high-activity indirect anticoagulants with minimal adverse effects.

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#### Author Queries

[q1] OK as edited?

[q2] OK as edited?