Published in final edited form as:
Appl Microbiol Biotechnol. 2007 February ; 74(2): 263–272.

Anticoagulant Heparan Sulfate: Structural Specificity and Biosynthesis

Jian Liu[1,*] and **Lars C. Pedersen**[2]

1 *Division of Medicinal Chemistry and Natural Products, School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599, USA*

2 *Laboratory of Structural Biology, NIEHS, National Institutes of Health, Research Triangle Park, NC 27709, USA*

Summary

Heparan sulfate (HS) is present on the surface of endothelial and surrounding tissues in large quantities. It plays important roles in regulating numerous functions of the blood vessel wall, including blood coagulation, inflammation response and cell differentiation. HS is a highly sulfated polysaccharide containing glucosamine and glucuronic/iduronic acid repeating disaccharide units. The unique sulfated saccharide sequences of HS determine its specific functions. Heparin, an analogue of heparan sulfate, is the most commonly used anticoagulant drug. Because of its wide range of biological functions, HS has become an interesting molecule to biochemists, medicinal chemists and developmental biologists. Here, we summarize recent progress towards understanding the interaction between heparan sulfate and blood coagulating factors, the biosynthesis of anticoagulant heparan sulfate and the mechanism of action of heparan sulfate biosynthetic enzymes. Further, knowledge of the biosynthesis of HS facilitates the development of novel enzymatic approaches to synthesize HS from bacterial capsular polysaccharides and to produce polysaccharide end products with high specificity for the biological target. These advancements provide the foundation for the development of polysaccharide-based therapeutic agents.

Introduction

Heparin is a commonly used anticoagulant drug with annual sale close to $3 billion worldwide. Heparin is an analog of heparan sulfate (HS), a unique class of macromolecule that is widely expressed on the cell surface and in the extracellular matrix. HS is a highly sulfated polysaccharide consisting of the repeating disaccharide unit of 1→4-linked glucosamine and glucuronic/iduronic acid that may contain sulfo groups. A large body of evidence has demonstrated that this highly sulfated polysaccharide plays roles in regulating embryonic development, inflammatory responses, assisting viral and bacterial infections as well as blood coagulation (1–5). Under physiological pH, the sulfo groups exhibit considerable negative charges that influence binding properties to more than one hundred proteins. Thus, HS is a molecule with a high density of information and significant potential for therapeutic applications. Understanding the mechanism employed by HS to regulate a specific biological process could aid the development of polysaccharide-based therapeutic agents with anticancer and antiviral activities as well as improve the pharmacological drug properties of anticoagulant heparin.

* To whom correspondence should be addressed: Rm 309, Beard Hall, University of North Carolina, Chapel Hill, NC 27599. Tel: 919-843-6511; Fax: 919-843-5432; Email: jian_liu@unc.edu

It is believed that HS achieves its selectivity by interacting with unique protein effectors to modulate the activities of these effectors. Decoding the nature of the interactions between HS and associated proteins has attracted considerable interest to delineate the involvement of HS in these important biological functions at the molecular level(6,7). The prevailing hypothesis is that a HS polysaccharide with specific sulfation patterns determines the binding affinity and the extent of the activation of a target protein. Our laboratories focus on understanding the HS biosynthetic pathway via biochemical and structural biology methods. In this review, we summarize recent developments regarding aspects of the biosynthesis of anticoagulant HS, the interaction of anticoagulant HS with thrombin and antithrombin, the interaction of HS and its biosynthetic enzymes and enzyme-based synthesis of HS. The interaction between HS and growth factors represent another important arena of HS research: the regulation of embryonic development, angiogenesis and cancer growth. These topics have been reviewed by several groups, and they are beyond the scope of this article (8,9).

The interactions of HS and blood coagulating factors

The blood coagulation pathway is composed of a cascade of proteolytic reactions ultimately generating fibrin thrombi. The proanticoagulant activity of this cascade is balanced by several natural anticoagulant mechanisms. Binding of HS to antithrombin (AT) represents the most important of these mechanisms. HS achieves its anticoagulant activity by interacting with AT, which then undergoes a conformation change, with the generation of the active form of AT that inhibits blood coagulation factors Xa and thrombin (IIa). Factor Xa or thrombin binding to AT results in cleavage of the P1-P1′ scissle bond in the reactive center loop (RCL) loop trapping the coagulant protease in a covalently bound in-active intermediate with the AT molecule(10–12).

HS is found on the surface of endothelial cells. This suggests a physiologically relevant role in regulating levels of anticoagulant activity within the blood vessel. Pharmaceutical heparin is a product of mast cells that is isolated from porcine intestinal tissue with a stronger anticoagulant activity than endothelial HS. Heparin is considered a specialized form of HS with higher levels of sulfation per saccharide unit and iduronic acid content. For example, HS contains about 0.6 sulfo groups per disaccharide unit, while heparin contains 2.6 sulfo groups. Further, about 40% of uronic acid in HS is iduronic, while 90% uronic acid in heparin is iduronic. Heparin has remained the drug of choice for the treatment of arterial and venous thrombotic disorders for more than a decade (13). This is largely due to: heparin is the only drug that inhibits the activities of both factor Xa and thrombin; heparin has a rapid anticoagulant response; the excessive anticoagulant activity of heparin is reversible by protamine (14). A major side effect of purified heparin, however, is thrombocytopenia. This condition is due to the induction of antibodies against the complex of platelet factor 4 and heparin (15). The development of a new generation of heparin-based anticoagulant drugs with reduced side effects remains a high priority.

Structure and anticoagulant activity relationship

Only a fraction of HS binds to AT and exhibits anticoagulant activity. About 1–10% of the HS isolated from tissues binds to AT, while about 30% of pharmaceutical heparin binds to AT, suggesting that AT recognizes a unique structure of HS. Structural and functional studies have revealed that a short pentasaccharide domain of HS is critical for binding to AT (12,16). Based on the structure of the AT-binding pentasaccharide, a new synthetic pentasaccharide anticoagulant under the trade name of Arixtra has been approved by FDA(17). However, the pentasaccharide unit only inhibits the activity of factor Xa mediated by AT. A much larger oligosaccharide is required to exhibit complete anti-thrombin activity (18,19).

Selectivity for HS by protein binding partners stems from a number of different structural variables that affect the overall charge and conformation. The positions of sulfo groups and the length of the oligosaccharide are essential. In addition, the three dimensional positions of these sulfo groups can be affected by rotation around glycosidic linkage as well as through changes in ring conformation. Although GlcUA and GlcNAc are typically found in the $^1C_4$ " chair" conformation, IdoUA is capable of being in either the $^1C_4$ or $^2S_0$ "skew-boat" orientation (20,21). Multiple conformations of the IdoUA units can present the sulfates on the surface of the HS in different arrangements such that the HS can have a variety of different structural and electrostatic properties that help to optimize protein interactions with the sulfates as well as optimize van der Waals surface contacts with the protein for increased substrate specificity.

The structural requirement for HS binding to AT was proved by chemically synthesizing a series of pentasaccharides with various combinations of sulfo groups and carboxyl groups (17). Removal of certain sulfo or carboxyl groups significantly compromised the binding affinity to AT, suggesting that these groups are critical (Figure 1A) (22). One of the critical motifs involved in binding to AT is the sulfation at the 3-OH position of this saccharide. Indeed, a pentasaccharide (Figure 1A) that lacks the 3-*O*-sulfo group from unit C has a decreased binding affinity to AT by nearly 20,000-fold (22). Functional and structural studies investigating the interaction of HS with AT thus reveal a convincing example for demonstrating that a specific sulfated saccharide sequence controls the anticoagulant activity.

The necessary size for an oligosaccharide to inhibit thrombin activity is much longer (14–20 saccharides) than the pentasaccharide that is required to bind AT and inhibit factor Xa(23). A synthetic hexadecasaccharide (SR123781) that displays excellent anticoagulant activity against thrombin *in vitro* and *in vivo* has been produced (20). This oligosaccharide consists of an AT-binding domain, a nonsulfated linker region and a thrombin-binding domain (Figure 1B). The structural requirement for binding of this oligosaccharide to thrombin, a cluster of sulfated saccharide units, appears to be less selective than for AT. Petitou and colleagues proved that the order of the three domains is essential for thrombin inhibition (19). Namely, the AT-binding domain is positioned at the reducing end of the nonsulfated linker, and the thrombin-binding domain at the nonreducing end of the linker. It should be noted that the synthesized hexadecasaccharide is not a HS, rather a HS mimetic carrying sulfo groups. Several structural differences between a native HS oligosaccharide and the synthetic hexadecasaccharide are noted (Figure 1B). This HS mimetic contains methylated hydroxyls and glucose instead of glucosamine at the AT-binding domain (S12–S16). These glucose units contain 2-*O*-sulfo groups instead of the *N*-sulfo groups as would be present on glucosamine units. In addition, highly sulfated glucose units (thrombin-binding domain, S1–S5) mimic the binding of -IdoUA2S-GlcNS6S- in heparin, provided that the binding of HS to thrombin requires only highly sulfated saccharide units and thus is non-specific. Despite structural differences, the binding of AT and thrombin to this hexadecasaccharide appears to mimic the interaction of AT, thrombin and HS (12). Chemically synthesized HS hexadecasaccharides have not been reported.

Crystal structure of ternary complex AT/Thrombin/hexadecasaccharide

Details of the molecular interactions have been ascertained from X-ray crystal structures of the binary complex of AT/HS as well as from ternary complexes of AT/thrombin and SR123781 (10,12,16). These structures have revealed interactions between AT and heparin and heparin and thrombin that account for the increased level of inhibition of thrombin by AT in the presence of heparin (Figure 2).

Numerous contacts between the sulfo and carboxyl groups of the oligosaccharide with the proteins were observed in the ternary complex, in particular in the region of AT and the AT-

binding domain of the hexadecasaccharide (Figure 2). All of the saccharide units in the AT-binding domain are in the chair conformation with the exception of the iduronic acid (S15) at the reducing end, which is present in the "skew-boat" conformation. There is a kink in the domain between this iduronic acid and the neighboring glucose (S14) that contains the specific 3-*O*-sulfo group. Indeed, the skew boat conformation of this iduronic acid unit is essential for binding to AT as demonstrated by Das and colleagues (24). The interactions between AT and HS involve the sulfo groups on the glucosamine equivalent glucose molecules at the 2,3 and 6 position as well as with carboxylate groups from the uronic acid saccharides. These interactions confirm the conclusions from the biochemical studies (Figure 1A) (reference ternary structure papers). The functional groups that form interactions with AT are the 2-*O*-sulfo of S16, the carboxylate from S15, the 2-*O*-sulfo and 3-*O*-sulfo groups from S14, the carboxylate from S13, and the 6-*O*-sulfo group from S12. These specific interactions involve both side chain and backbone interactions with AT. The critical 3-*O*-sulfo group on S14 for AT binding is within hydrogen bonding distance to two lysines and one asparagine side chain. Given the numerous interactions between the pentasaccharide and AT, it is unclear why the 3-*O*-sulfo group from S14 is critical to binding. One possibility may be that locking this position down leads to the kink in the pentasaccharide with subsequent optimization of van der Waals contacts and ionic interactions with AT.

Biosynthesis of anticoagulant HS

Biosynthetic pathway

Cellular HS is present in the form of HS proteoglycan, which consists of a core protein and polysaccharide side chains. HS polysaccharide is attached to the core protein through a serine residue. The core protein functions as a carrier of the HS chains on the cell surface and in the extracellular matrix. Although the binding of HS polysaccharide side chains with protein effectors is believed to result in specific biological functions, the expression of core proteins plays a critical role in temporal and spatial regulation of cellular HS (25). The biosynthesis of HS is performed by a complex pathway involving chain elongation and multiple modification steps (Figure 3). Synthesis is initiated with addition of a linkage region domain (LRD) to a specific Ser residues on the protein followed by elongation steps to generate the unsulfated and unepimerized polysaccharide backbone consisting of GlcUA-GlcNAc disaccharide repeats (26,27).

After chain elongation, the polymer is modified by a series of sulfotransferases and a C5 epimerase (Figure 3). The first modification is *N*-sulfonation to form the *N*-sulfo glucosamine unit by *N*-deacetylase/*N*-sulfotransferase (NDST). NDST is a dual function enzyme that catalyzes the removal of the acetyl group from a GlcNAc residue and the transfer of a sulfo group to the amino group of the "deacetylated" unit. The C5-epimerase converts the neighboring GlcUA on the nonreducing side to an IdoUA unit. The chain modification proceeds with 2-*O*-sulfonation at the iduronic acid (or to a lesser extent at a GlcUA), 6-*O*-sulfonation at the glucosamine and 3-*O*-sulfonation at the glucosamine. These modifications are catalyzed by 2-*O*-sulfotransferase (2-OST), 6-*O*-sulfotransferase (6-OST), and 3-*O*-sulfotransferase (3-OST), respectively (Figure 3).

Biosynthesis of a polysaccharide with defined sequences

The mechanism for regulating synthesis of polysaccharides with defined saccharide sequences is essentially unknown because the synthesis is not a template-driven process. Nevertheless, it appears that structures of HS from a single cell line are consistent between generations, suggesting that the synthesis of HSs with unique saccharide sequences are genetically controlled (28). In addition, synthesis of specific saccharide sequences can be regulated by the

expression levels of specialized biosynthetic enzymes and the accessibility of the unique substrates for those specialized biosynthetic enzymes. These observations have led to a widely accepted hypothesis: that the overall sulfation pattern of HS is dictated by the substrate specificities of HS biosynthetic enzymes. However, the detailed regulation of the sulfation pattern on a polysaccharide are not well understood.

The biosynthetic enzymes exhibit the substrate specificities at two different levels: the regio-selectivity of the residue to be modified (the monosaccharide level) and the saccharide structures around the modified residue (at the oligosaccharide level). The regio-selectivity for a given HS sulfotransferase is nearly exclusive (29–32). For example, 2-OST recognizes an iduronic acid (or a glucuronic acid) residue, and specifically transfers the sulfo group to the 2-OH position to form IdoUA2S (or GlcUA2S).

The best evidence of substrate specificities at the oligosaccharide levels is demonstrated by the fact that different isoforms recognize unique sulfation patterns around the modification site (33). HS biosynthetic enzymes are present in multiple isoforms with the exception of 2-OST and C_5 epimerase. NDST is present in four different isoforms, 6-OST is present in three different isoforms and 3-OST in seven. The isoforms have slightly different substrate specificities and unique tissue specific expression patterns (34–37). It has been hypothesized that cells regulate expression levels of different isoforms of the biosynthetic enzyme in order to synthesize HS with defined saccharide sequences to achieve unique biological functions (38). Indeed, 3-OST isoforms are essential for the synthesis of heparin/HS with a variety of saccharide sequences that exhibit different substrate specificities, and the products display distinct biological functions as described in the section below. 6-OST isoforms are important for generating HS responsible for fibroblast growth factors and fibroblast growth factor receptor complex formation, although the substrate specificities among 6-OSTs are not experimentally distinguishable (39,40). The substrate specificities of NDST isoforms are more difficult to determine with current techniques, and thus, are largely unknown.

Regulation of the biosynthesis of anticoagulant HS

The key control of anticoagulant HS synthesis *in vivo* by cells is regulation of the expression level of HS 3-*O*-sulfotransferase isoform 1 and isoform 5 (3-OST-1 and 3-OST-5) (32,34). 3-OST's transfers the sulfo group to the 3-OH position of a glucosamine unit to form a 3-*O*-sulfated glucosamine. The detailed substrate specificities of 3-OST isoforms are described below. Overexpression of 3-OST-1 and 3-OST-5 in cells significantly elevates the level of anticoagulant HS (41,42). The existence of multiple isoforms may explain why 3-OST-1 knockout mice failed to produce thrombotic phenotypes (41).

Substrate specificities and mechanism of action of 3-OST isoforms

Substrate specificities of 3-OST isoforms

HS 3-OST is present in seven isoforms, including 3-OST-1, -2, -3A, 3B, -4, -5, and 6 (33,34, 43). These enzymes catalyze the transfer of a sulfo group from the sulfo donor, 3′-phosphoadenosine-5′-phosphosulfate (PAPS), to the 3-OH position of a glucosamine unit. The amino acid sequences of the different isoforms have greater than 60% homology in the sulfotransferase domains. The isoforms transfer the sulfo group to the glucosamine residues that are linked to different saccharide units at the nonreducing end. The modifications predominately fall into three types (Figure 4). First, the sulfo group is transferred to the glucosamine unit that is adjacent to an unsulfated glucuronic acid (GlcUA). This type of modification is carried out by 3-OST-1. Second, the sulfo group is transferred to the glucosamine unit that is adjacent to a 2-*O*-sulfo iduronic acid (IdoUA2S). This type of

modification is performed by 3-OST-2, -3A, -3B, -4, and -6. Among these, 3-OST-3A and 3-OST-3B have nearly identical amino acid sequence in the sulfotransferase domain, and thus, 3-OST-3 represents both 3-OST-3A and 3-OST-3B. Third, the sulfo group is transferred to the glucosamine unit that is adjacent to GlcUA, IdoUA and IdoUA2S. This type of modification is carried out by 3-OST-5 (44).

The unique substrate specificities of different 3-OST isoforms endow HS with distinct biological functions. As described above, 3-OST-1 and 3-OST-5 carry out critical 3-*O*-sulfation that permits the resultant HS binds to AT and exert anticoagulant activity. On the other hand, other 3-OST isoforms, including 3-OST-2, -3, -4, -5, and -6, carry out the critical 3-*O*-sulfation that generates HS with activity that promotes the infection of herpes simplex virus type 1 (HSV-1) (43,45). HS modified by these isoforms interacts with herpes virus envelope glycoprotein gD and serves as an entry receptor for the virus.

Crystal structures of 3-OST-1 and 3-OST-3

Because 3-OSTs play a critical role in controlling the biosynthesis of anticoagulant HS and unique substrate specificities among the isoforms, we investigated the mechanism of 3-OSTs using a structural biology approach coupled with site-directed mutagenesis(46,47). From these studies, several key amino acid residues have been identified that contribute to the interactions with the sulfo donor PAPS, as well as with polysaccharide substrates.

The crystal structure of 3-OST-1 in complex with PAP was solved at 2.5 Å. In addition, a ternary complex of 3-OST-3/PAP/tetrasaccharide substrate was solved at 1.9Å (46,47). We have found that 3-OST-1 and 3-OST-3 have very similar overall structure. Here, we focus our attention on describing the structure of 3-OST-3/PAP/tetrasaccharide (Figure 5A and 5B). The tetrasaccharide used in this study binds in a positively charged cleft. As seen in heparin binding to AT, a kink is induced by the protein between the two central residues I2 and G3 of the tetrasaccharide. The G1 residue forms interactions with the protein via the *N*-sulfo group and a bound sodium, as well as with the 3-hydroxyl. The I2 residue is found in the “skew-boat” conformation and forms interactions with its carboxylate group and 3-hydroxyl. The substrate residue G3 is oriented in a catalytically relevant position with the 3 hydroxyl acceptor positioned 5 Å from the 5′-phosphate of the leaving group PAP and within hydrogen bonding distance of the proposed general base E184. In addition, the *N*-sulfo group is hydrogen bonded to a backbone amide. At the nonreducing end of the tetrasaccharide is the terminal uronate residue (U4) with an unsaturated 4,5 carbon bond which results from the polysaccharide cleavage by a heparin lyase. This residue dictates substrate specificity between 3-OST-1 and 3-OST-3 and forms ionic interactions with both the 2-*O*-sulfo group and the carboxylate group as well as a possible interaction with the 3′ hydroxyl An unsulfated glucuronic acid at this position (as is the case for the 3-OST-1 substrate) would likely have a different ring conformation and thus likely would not form the same interactions. Mutations have been made in these regions of 3-OST-3 and 3-OST-1 to support the conjecture that these residues determine substrate specificity. By understanding these subtle differences, we may be able to alter the substrate specificity of these enzymes to create more specific enzymes or novel sulfation patterns on heparin to improve the requirements for use of HS as therapeutic agents. For example, structure-activity studies suggest that an extra 3-*O*-sulfo group at unit E (Figure 1A) improves the binding affinity to AT nearly 100-fold (18). A pentasaccharide consisting of two 3-*O*-sulfated glucosamine units has not yet been isolated from natural sources. The addition of a 3-*O*-sulfate group using genetically modified 3-OST enzymes could increase the specificity of the synthesized HS for AT.

Chemoenzymatic synthesis of HS with biological activities

Synthesis of HS oligosaccharides has proved to be successful for the development of synthetic versions of HS-based anticoagulant drugs (17). This endeavor is largely based on the fact the side effects of heparin drugs stem from the structural heterogeneity of the polysaccharide (17).

Although chemical synthesis has been the major route to obtain structurally defined heparin oligosaccharides, total chemical synthesis is time consuming and costly. Further, chemical synthesis of non-analog heparin and HS oligosaccharides, larger than hexasaccharides, is extremely difficult, if not impossible, based on currently available methods for carbohydrate synthesis. While a number of groups continue to pursue the synthesis of heparin (48–50), it is likely that chemical synthesis alone will be incapable of generating most larger oligosaccharide structures. The application of HS biosynthetic enzymes for generating large heparin and HS oligosaccharides with desired biological activities offers a promising alternative approach.

Attempts to synthesize HS using biosynthetic enzymes have been reported. Although it is unclear whether the enzymatic approach synthesizes structurally defined polysaccharides, this method generates products with desired anticoagulant activity. Kuberan and Rosenberg utilized this approach to synthesize HS containing AT binding sites, with anticoagulant activity (51–53). While this approach demonstrated for the first time the feasibility of enzymatic synthesis of HS, only microgram amounts of product were generated, making extensive biological studies impossible. Recently, Lindahl and colleagues reported an alternative chemoenzymatic approach for the synthesis of anticoagulant heparin from heparosan, an *E. coli* K5 capsular polysaccharide (54). This method utilized the C_5 epimerase to convert D-glucuronic acid (GlcUA) to L-iduronic acid (IdoUA), followed by the chemical persulfonation and finally selective desulfonation. While this approach affords gram quantities of a heparin-like polysaccharide with anticoagulant activity, unnatural saccharide units, such as 3-*O*-sulfo-D-glucuronic acid, are present in the product, suggesting a limitation in the selectivity of chemical sulfonation/desulfonation in HS synthesis. To this point, an effective approach for the synthesis of structurally defined large oligosaccharides is not yet available.

Following the same strategy we have developed an efficient approach to synthesize the HS with desired biological activities in multi-milligram scales. To achieve enzymatic synthesis of HS at a practical pharmaceutical level on a large scale, two obstacles need to be overcome; the availability of large amount of HS sulfotransferases and the inhibition effect of 5′-phosphoadenosine 3′-phosphate (PAP), the desulfated product of PAPS. A proof of principle study has been recently published using completely desulfated and *N*-sulfated heparin (1) as a starting material (Figure 6) to conduct the *O*-sulfonation reactions (55). First, highly active HS *O*-sulfotransferases were expressed in *E. coli*, in order to provide sufficient quantities of sulfotransferases. Second, the sulfotransfer reactions were coupled with the PAPS regeneration system that was developed by the Wong group(56). This strategy eliminates the inhibition effect of PAP. The PAPS regeneration system utilizes arylsulfotransferase IV, which can converts PAP to PAPS as illustrated in Figure 6B. In addition, the PAPS regeneration system utilizes the *p*-nitrophenol sulfate PNPS as the sulfo donor and requires only catalytic amounts of PAP, thereby reducing the cost of synthesis by more than 1000-fold (56). Immobilized enzymes are used in this approach for reuse and improved thermal stability of the HS sulfotransferases. Thus the protocol can be efficiently scaled up. This method is capable of generating milligram quantities of specifically sulfated polysaccharide for desired biological activities: these include the anticoagulant activity as measured by the anti-Xa and anti-thrombin activities (compounds **5** and **8**), the activity in triggering FGF/FGF receptor signaling (compounds **4a** and **4b**) and binding to herpes simplex virus glycoprotein D (compound **6**).

Conclusions

In addition to the anticoagulant activity of HS, many novel biological functions have emerged in recent years. These functions include roles of HS in regulating cancer growth, assisting viral and bacterial infections and inflammatory responses. Indeed, a low molecular weight heparin mimetic, PI88, is under phase II clinical trial to treat herpes simplex viral infections (57). In addition, PI88 also potentially serves as an anticancer drug, inhibiting the activity of heparanase in facilitating tumor metastasis (58). Knowledge of HS/heparin interactions with blood coagulating factors and how the biosynthesis of anticoagulant HS is performed will provide a model system for investigating the roles of HS in other related biological processes. It is clear that a chemoenzymatic approach is a viable method for generating the polysaccharides with different sulfation patterns and biological functions. The next challenge for HS/heparin research is the development of a technique to prepare “recombinant polysaccharides” with more precise structures so as to achieve high functional selectivity. The improved understanding of the regulation of HS biosynthesis and/or simplified synthetic techniques will help to achieve this goal. In the coming years of the glycomics era, continuing efforts on in HS/heparin research will likely lead to novel therapeutic agents.

Acknowledgements

The authors are grateful to A.F. Moon and Dr. L.G. Pedersen for critical reading of the manuscript. The Liu lab is supported by a grant from the National Institutes of Health (AI050050). This research was supported in part by the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences.

References

1. Hacker U, Nybakken K, Perrimon N. Heparan sulfate proteoglycans:The sweet side of development. Nat Rev Mol Cell Biol 2005;6:530–541. [PubMed: 16072037]
2. Esko JD, Selleck SB. Order out of chaos: Assembly of ligand binding sites in heparan sulfate. Ann Rev Biochem 2002;71:435–471. [PubMed: 12045103]
3. Taylor KR, Gallo RL. Glycosaminoglycans and their proteoglycans: host-associated molecular patterns for initiation and modulation of inflammation. FASB J 2006;20:9–22.
4. Liu J, Thorp SC. Heparan sulfate and the roles in assisting viral infections. Med Res Rev 2002;22:1–25. [PubMed: 11746174]
5. Rosenberg RD, Showrak NW, Liu J, Schwartz JJ, Zhang L. Heparan sulfate proteoglycans of the cardiovascular system: specific structures emerge but how is synthesis regulated? J Clin Invest 1997;99:2062–2070. [PubMed: 9151776]
6. Raman R, Sasisekharan V, Sasisekharan R. Structural insights into biological roles of protein-glycosaminoglycan interactions. Chemistry & Biology 2005;12:267–277. [PubMed: 15797210]
7. Coombe DR, Kett WC. Heparan sulfate-protein interactions: therapeutic potential through structure-function insights. Cell Mol Life Sci 2005;62:410–424. [PubMed: 15719168]
8. Sasisekharan R, Shriver Z, Venkataraman G, Narayanasami U. Roles of heparin-sulphate glycosaminoglycans in cancer. Nat Rev Cancer 2002;2:521–528. [PubMed: 12094238]
9. Sanderson RD, Yang Y, Suva LJ, Kelly T. Heparan sulfate proteglycans and heparanase-partners in osteolytic tumor growth and metastasis. Matrix Biol 2004;23:341–352. [PubMed: 15533755]
10. Dementiev A, Petitou M, Herbert J-M, Gettins PG. The ternary complex of antithrombin-anhydrothrombin-heparin reveals the basis of inhibitor specificity. Nat Struct Mol Biol 2004;11:867–863.
11. Gettins PG. Serpin structure, mechanism, and function. Chem Rev 2002;102:4751–4803. [PubMed: 12475206]
12. Li W, Johnson DJ, Esmon CT, Huntington JA. Structure of the antithrombin-thrombin-heparin ternary complex reveals the antithrombotic mechanism of heparin. Nat Struct Mol Biol 2004;11:857–862. [PubMed: 15311269]

13. Becker RC. Optimizing heparin compounds: A working construct for furture antithrombotic drug development. J Thromb Thrombolysis 2004;18:55–58. [PubMed: 15744556]
14. Hirsh J, O'Donnell M, Weitz JI. New anticoagulants. Blood 2005;105:453–463. [PubMed: 15191946]
15. Kelton JG. Heparin-induced thrombocytopenia: an overview. Blood Rev 2002;16:77–80. [PubMed: 11914002]
16. Jin L, Abrahams P, Skinner R, Petitou M, Pike RN, Carrell RW. The anticoagulant activation of antithrombin by heparin. Proc Natl Acad Sci 1997;94:14683–14688. [PubMed: 9405673]
17. Petitou M, van Boeckel CAA. A synthetic antithrombin III binding pentasaccharide is now a drug! What comes next? Angew Chem Int Ed 2004;43:3118–3133.
18. Petitou M, van Boeckel CAA. Chemical synthesis of heparin fragments and analogues. Fortschr Chem Org Naturst 1992;60:143–210. [PubMed: 1483612]
19. Petitou M, Herault L-P, Bernat A, Driguez P-A, Duchaussoy P, Lormeau J-C, Herbert J-M. Synthesis of thrombin-inhibiting heparin mimetics without side effects. Nature 1999;398:417–422. [PubMed: 10201371]
20. Mikhailov D, Mayo KH, Pervin A, Linhardt RJ. ^{13}C-NMR relaxation study of heparin-disaccharide interactions with tripeptide GRG and GKG. Biochem J 1996;315:447–454. [PubMed: 8615813]
21. Mulloy B, Forster M. Conformation and dynamics of heparin and heparan sulfate. Glycobiology 2000;10:1147–1156. [PubMed: 11087707]
22. Atha DH, Lormeau J-C, Petitou M, Rosenberg RD, Choay J. Contribution of monosaccharide residues in heparin binding to antithrombin III. Biochemistry 1985;24:6723–6729. [PubMed: 4084555]
23. OOsta GM, Gardner WT, Beeler DL, Rosenberg R. Multiple functional domains of the heparin molecule. Proc Natl Acad Sci 1981;78:829–833. [PubMed: 6940150]
24. Das S, Mallet J, J E, Driguez P, Duchaussoy P, Sizun P, Herault J, Herbert J, M P, P S. Synthesis of conformationally locked L-iduronic acid derivatives: direct evidence for a critical role of the skew-boat 2S0 conformer in the activation of antithrombin by heparin. Chemistry 2001;7:4821–4834. [PubMed: 11763451]
25. Bernfield M, Gotte M, Park PW, Reizes O, Fitzgerald ML, Lincecum J, Zako M. Functions of cell surface heparan sulfate proteoglycans. Ann Rev Biochem 1999;68:729–777. [PubMed: 10872465]
26. McCormick C, Leduc Y, Martindale D, Mattison K, Esford LE, Dyer AP, Tufaro F. The putitive tumour suppressor EXT1 alters the expression of cell-surface heparan sulfate. Nat Genet 1998;19:158–161. [PubMed: 9620772]
27. Lind T, Tufaro F, McCormick C, Lindahl U, Lidholt K. The putative tumor suppressors EXT1 and EXT2 are glycosyltransferases required for the biosynthesis of heparan sulfate. J Biol Chem 1998;273:26265–26268. [PubMed: 9756849]
28. Lindahl U, Kusche-Gullberg M, Kjellen L. Regulated diversity of heparan sulfate. J Biol Chem 1998;273:24979–24982. [PubMed: 9737951]
29. Habuchi H, Habuchi O, Kimata K. Purification and characterization of heparan sulfate 6-sulfotransferase from the culture medium of Chinese hamster ovary cells. J Biol Chem 1995;270:4172. [PubMed: 7876170]
30. Kobayashi M, Habuchi H, Habuchi O, Saito M, Kimata K. Purification and characterization of heparan sulfate 2-sulfotransferase from cultured Chinese hamster ovary cells. J Biol Chem 1996;271:7645–7653. [PubMed: 8631801]
31. Ishihara M, Guo Y, Wei Z, Yang Z, Swiedler SJ, Orellana A, Hirschberg CB. Regulation of biosynthesis of the basic fibroblast growth factor binding domains of heparan sulfate by heparan sulfate-*N*-deacetylase/*N*-sulfotransferase expression. J Biol Chem 1993;268:20091–20095. [PubMed: 8376367]
32. Liu J, Shworak NW, Fritze LMS, Edelberg JM, Rosenberg RD. Purification of heparan sulfate D-glucosaminyl 3-*O*-sulfotransferase. J Biol Chem 1996;271:27072–27082. [PubMed: 8900198]
33. Liu, J.; Rosenberg, RD. Heparan sulfate D-glucosaminyl 3-*O*-sulfotransferase. In: Taniguchi, N.; Fukuda, M., editors. Handbook of glycosyltransferases and their related genes. Tokyo: Springer-Verlag; 2002. p. 475.-483.

34. Xia G, Chen J, Tiwari V, Ju W, Li J-P, Malmström A, Shukla D, Liu J. Heparan sulfate 3-O-sulfotransferase isoform 5 generates both an antithrombin-binding site and an entry receptor for herpes simplex virus, type 1. J Biol Chem 2002;277:37912–37919. [PubMed: 12138164]
35. Aikawa, J-i; Grobe, K.; Tsujimoto, M.; Esko, JD. Multiple isozymes of heparan sulfates/heparin GlcNAc N-deacetylase/GlcN N-sulfotransferase: Structure and activity of the fourth member, NDST4. J Biol Chem 2001;276:5876–5882. [PubMed: 11087757]
36. Habuchi H, Tanaka M, Habuchi O, Yoshida K, Suzuki H, Ban K, Kimata K. The occurance of three isoforms of heparan sulfate 6-O-sulfotransferase having different specificities for hexuronic acid adjacent to the targeted N-sulfoglucosamine. J Biol Chem 2000;275:2859–2868. [PubMed: 10644753]
37. Shworak NW, Liu J, Petros LM, Zhang L, Kobayashi M, Copeland NG, Jenkins NA, Rosenberg RD. Diversity of the extensive heparan sulfate D-glucosaminyl 3-*O*-sulfotransferase (3-OST) multigene family. J Biol Chem 1999;274:5170–5184. [PubMed: 9988767]
38. Liu J, Shworak NW, Sinaÿ P, Schwartz JJ, Zhang L, Fritze LMS, Rosenberg RD. Expression of heparan sulfate D-glucosaminyl 3-*O*-sulfotransferase isoforms reveals novel substrate specificities. J Biol Chem 1999;274:5185–5192. [PubMed: 9988768]
39. Ashikari-Hada S, Habuchi H, Kariya Y, Itoh N, Reddi AH, Kimata K. Characterization of Growth Factor-binding Structures in Heparin/Heparan Sulfate Using an Octasaccharide Library. J Biol Chem 2004;279:12346–12354. [PubMed: 14707131]
40. Smeds E, Habuchi H, Do A-T, Hjertson E, Grundberg H, Kimata K, Lindahl U, Kusche-Gullberg M. Substrate specificities of mouse heparan sulphate glucosaminyl 6-O-sulfotransferases. Biochem J 2003;372 (pt2):371–380. [PubMed: 12611590]
41. Shworak NW, Liu J, Fritze LMS, Schwartz JJ, Zhang L, Logeart D, Rosenberg RD. Molecular cloning and expression of mouse and human cDNAs encoding heparan sulfate D-glucosaminyl 3-*O*-sulfotransferase. J Biol Chem 1997;272:28008–28019. [PubMed: 9346953]
42. Duncan MB, Chen J, Krise JP, Liu J. The biosynthesis of anticoagulant heparan sulphate by the heparan sulphate 3-O-sulphotransferase isoform 5. Biochim Biophys Acta 2004;1671:34–43. [PubMed: 15026143]
43. Xu D, Tiwari V, Xia G, Clement C, Shukla D, Liu J. Characterization of Heparan Sulfate 3-O-Sulfotransferase Isoform 6 and Its Role in Assisting the Entry of Herpes Simplex Virus, Type 1. Biochem J 2005;385:451–459. [PubMed: 15303968]
44. Chen J, Duncan MB, Carrick K, Pope M, Liu J. Biosynthesis of 3-O-sulfated heparan sulfate: unique substrate specificity of heparan sulfate 3-O-sulfotransferase isoform 5. Glycobiology 2003;13:785–794. [PubMed: 12907690]
45. Shukla D, Liu J, Blaiklock P, Shworak NW, Bai X, Esko JD, Cohen GH, Eisenberg RJ, Rosenberg RD, Spear PG. A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry. Cell 1999;99:13–22. [PubMed: 10520990]
46. Edavettal SC, Lee KA, Negishi M, Linhardt RJ, Liu J, Pedersen LC. Crystal structure and mutational analysis of heparan sulfate 3-O-sulfotransferase isoform 1. J Biol Chem 2004;279:25789–25797. [PubMed: 15060080]
47. Moon A, Edavettal SC, Krahn JX, Munoz EM, Negishi M, Linhardt RJ, Liu J, Pedersen LC. Structural analysis of the sulfotransferase (3-OST-3) involved in the biosynthesis of an entry receptor of herpes simplex virus 1. J Biol Chem 2004;279:45185–45193. [PubMed: 15304505]
48. Avci FY, Karst NA, Linhardt RJ. Curr. Pharmaceut Des 2003;9:2323–2335.
49. de Paz JL, Noti C, Seeberger PH. Microarrays of synthetic heparin oligosaccharides. J Am Chem Soc 2006;128:2766–2767. [PubMed: 16506732]
50. Lee JC, Lu XA, Kulkarni SS, Wen YS, Hung SC. Synthesis of heparin oligosaccharides. J Am Chem Soc 2004;126:476–477. [PubMed: 14719939]
51. Balagurunathan K, Beeler DL, Lech M, Wu ZL, Rosenberg RD. Chemoenzymatic Synthesis of Classical and Non-classical Anticoagulant Heparan Sulfate Polysaccharides. J Biol Chem 2003;278:52613 – 52621. [PubMed: 14519763]

52. Balagurunathan K, Lech MZ, Beeler DL, Wu ZL, Rosenberg RD. Enzymatic synthesis of antithrombin III-binding heparan sulfate pentasaccharide. Nat Biotechnol 2003;21:1343–1346. [PubMed: 14528313]

53. Kuberan B, Beeler DL, Lawrence R, Lech M, Rosenberg R. Rapid two-step synthesis of mitrin from heparosan: a replacement for heparin. J Am Chem Soc 2003;125:12424–12425. [PubMed: 14531682]

54. Lindahl U, Li J, Kusche-Gullberg M, Salmivirta M, Alaranta S, Veromaa T, Emies J, Roberts I, Taylor C, Oreste P, et al. Generation of "neoheparin" from *E. Coli* K5 capsular polysaccharide. J Med Chem 2005;48:349–352. [PubMed: 15658847]

55. Chen J, Avci FY, Muñoz EM, McDowell LM, Chen M, Pedersen LC, Zhang L, Linhardt RJ, Liu J. Enzymatically redesigning of biologically active heparan sulfate. J Biol Chem 2005;280:42817–42825. [PubMed: 16260789]

56. Burkart MD, Izumi M, Chapman E, Lin C, Wong C. Regeneration of PAPS for the enzymatic synthesis of sulfated oligosaccharides. J Org Chem 2000;65:5565–5574. [PubMed: 10970295]

57. Nyberg K, Ekblad M, Bergstrom T, Freeman C, CR P, Ferro V, Trybala E. The low molecular weight heparan sulfate-mimetic, PI-88, inhibits cell-to-cell spread of herpes simplex virus. Antiviral Res 2004;63:15–24. [PubMed: 15196816]

58. Ferro V, Don R. The development of the novel angiogenesis inhibitor PI-88 as an anticancer drug. Austr Biotechnol 2003;13:38–39.

59. Kraulis PJ. MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. J Appl Crystallogr 1991;24:946–950.

60. Merritt EA, Bacon DJ. Raster3D: Photorealistic molecular graphics. Meth Enz 1997;277:505–524.

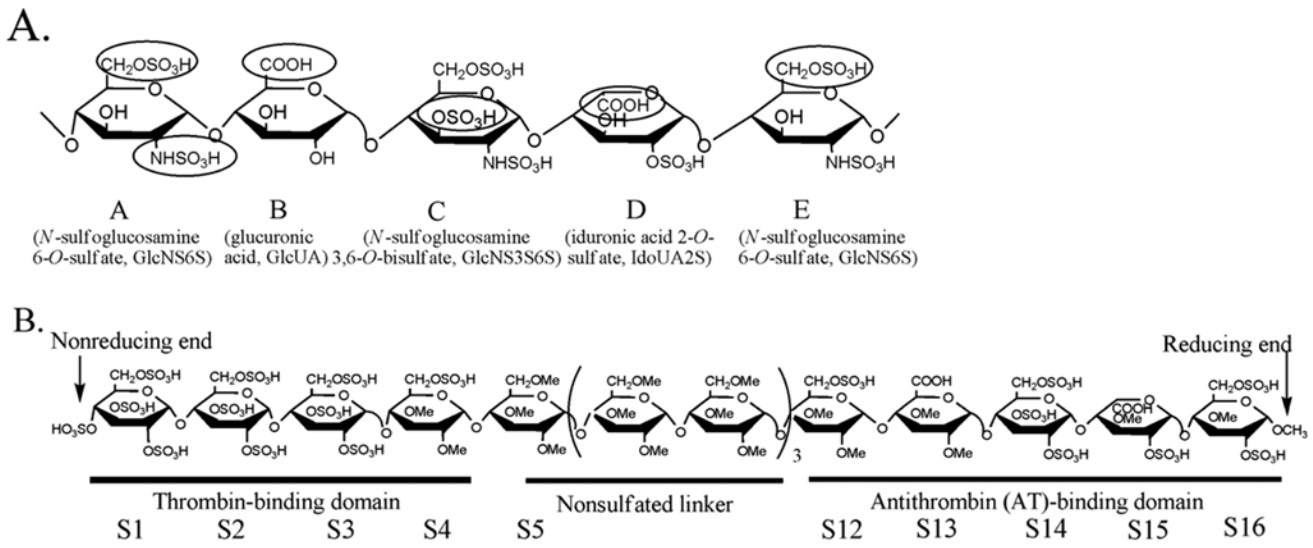

Figure 1. Structures of AT-binding domain and chemically synthesized hexadecasaccharide with anti-IIa activity

Panel A shows the structure of AT-binding pentasaccharide. The critical sulfo and carboxylate groups for the binding of AT in the pentasaccharide are circled. The name of individual saccharide unit is below each unit. Panel B shows the structure of the synthetic hexadecasaccharide (SR-123781). The AT-binding domain, nonsulfated linker and the thrombin-binding domain are indicated. Reducing and nonreducing ends are also indicated. S1, S2, …, S16 define the position of the saccharide units.

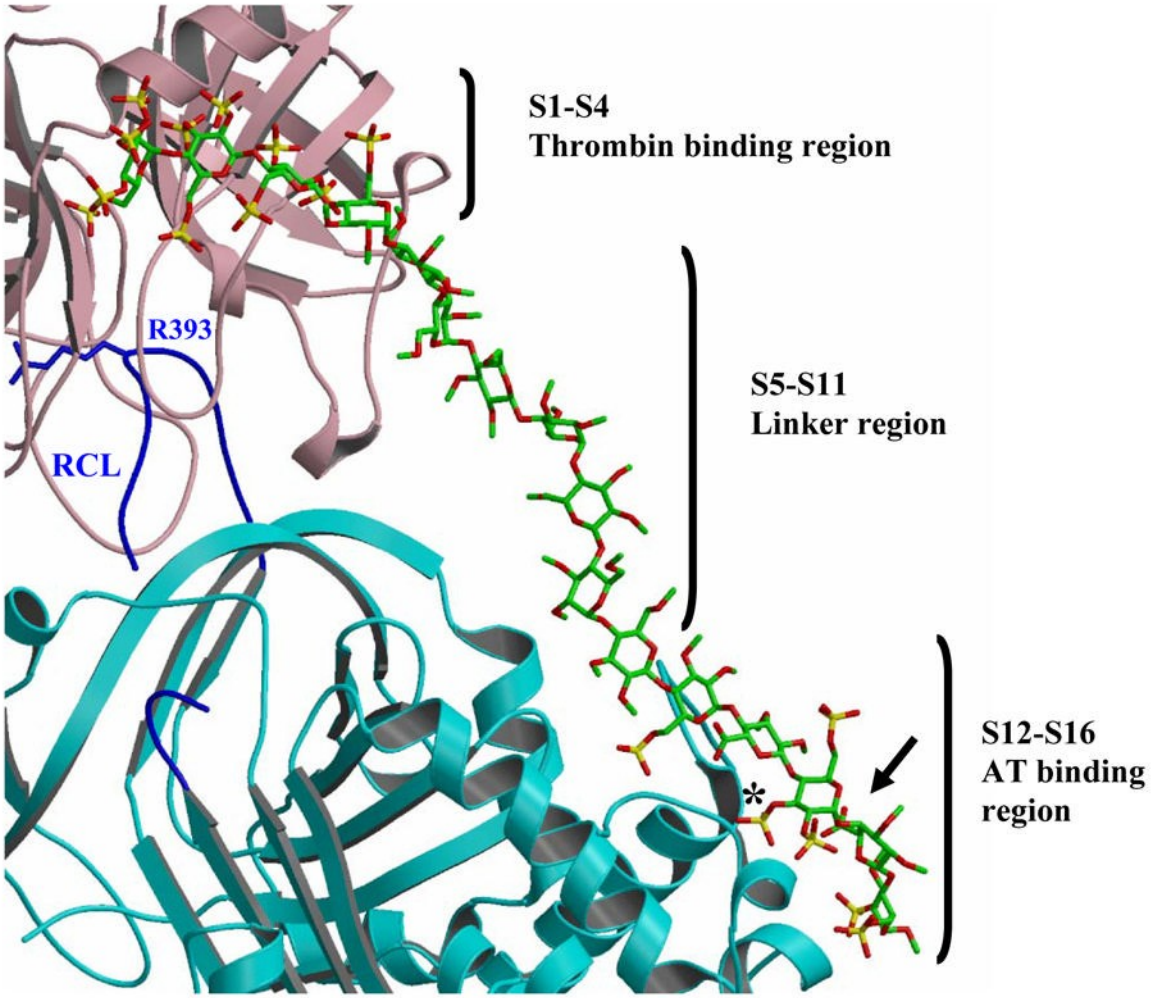

Figure 2. Heparin analog binding in the AT/thrombin complex

Crystal structure of the ternary complex between AT(cyan), thrombin(pink), and a synthetic hexadecasaccharide SR-123781(green). The critical 3-*O*-sulfo group for effective AT binding is labeled with an asterisks and an arrow locates the kink formed in the heparin analog by AT binding. The reactive center loop (RCL) of AT is shown in blue along with residue R393 of AT bound to the active site of thrombin (This figure was created using the PDB coordinates 1TB6 with the programs Molscript and Raster3D (59,60).).

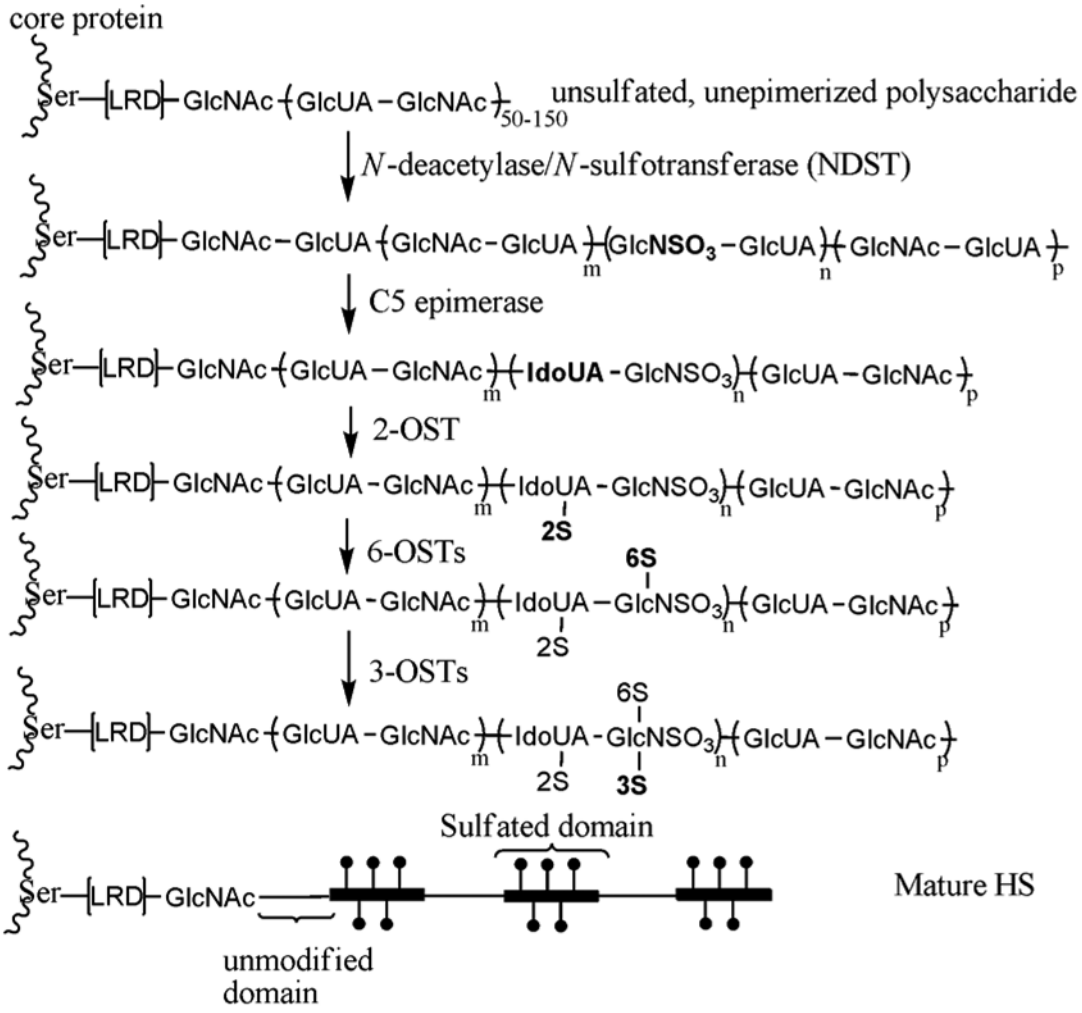

Figure 3. HS biosynthetic pathway

This scheme shows the sequential enzymatic modifications that occur after chain elongation. LRD represents linkage region domain.

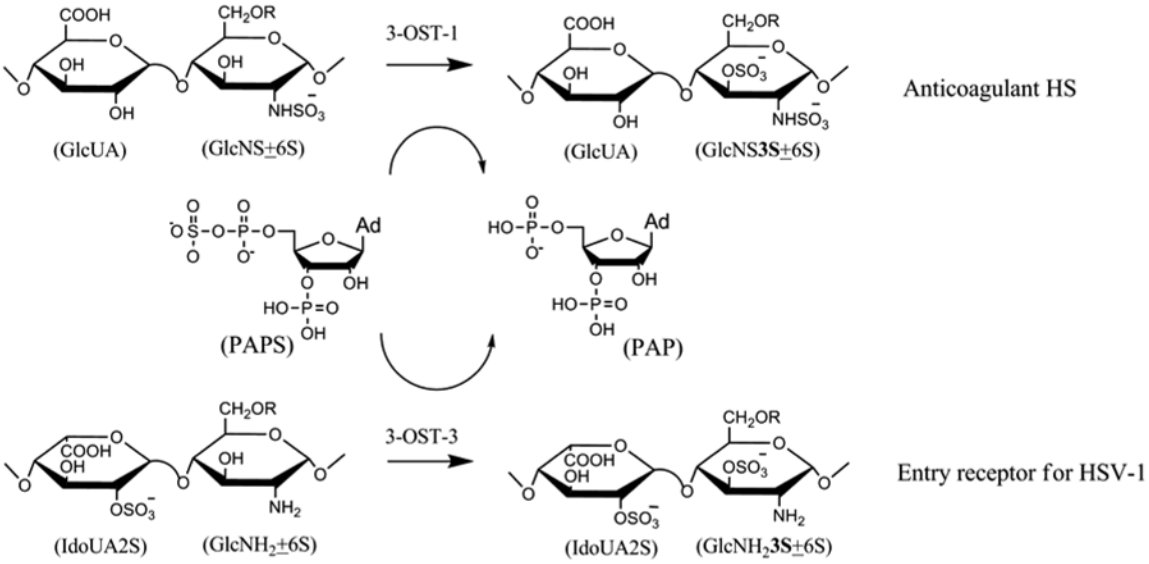

Figure 4. Substrate specificity of 3-OST isoforms

The reactions catalyzed by 3-OST-1 and 3-OST-3 are shown. 3-OST-1 transfers the sulfo group from PAPS to the 3-OH position of a glucosamine unit (GlcNS±6S) that is linked to a glucuronic acid unit (GlcUA), thereby forming the 3-*O*-sulfated HS containing GlcUA-GlcNS3S±6S (an anticoagulant HS). 3-OST-3 transfers the sulfo group to the 3-OH position of a glucosamine unit ($GlcNH_2$±6S) that is linked to a 2-*O*-sulfo iduronic acid unit (IdoUA2S), thereby forming the 3-*O*-sulfated HS containing IdoUA2S-$GlcNH_2$3S±6S (an entry receptor for herpes simplex virus type 1 (HSV-1). R represents a proton (−H) or a sulfo group ($-SO_3^-$).

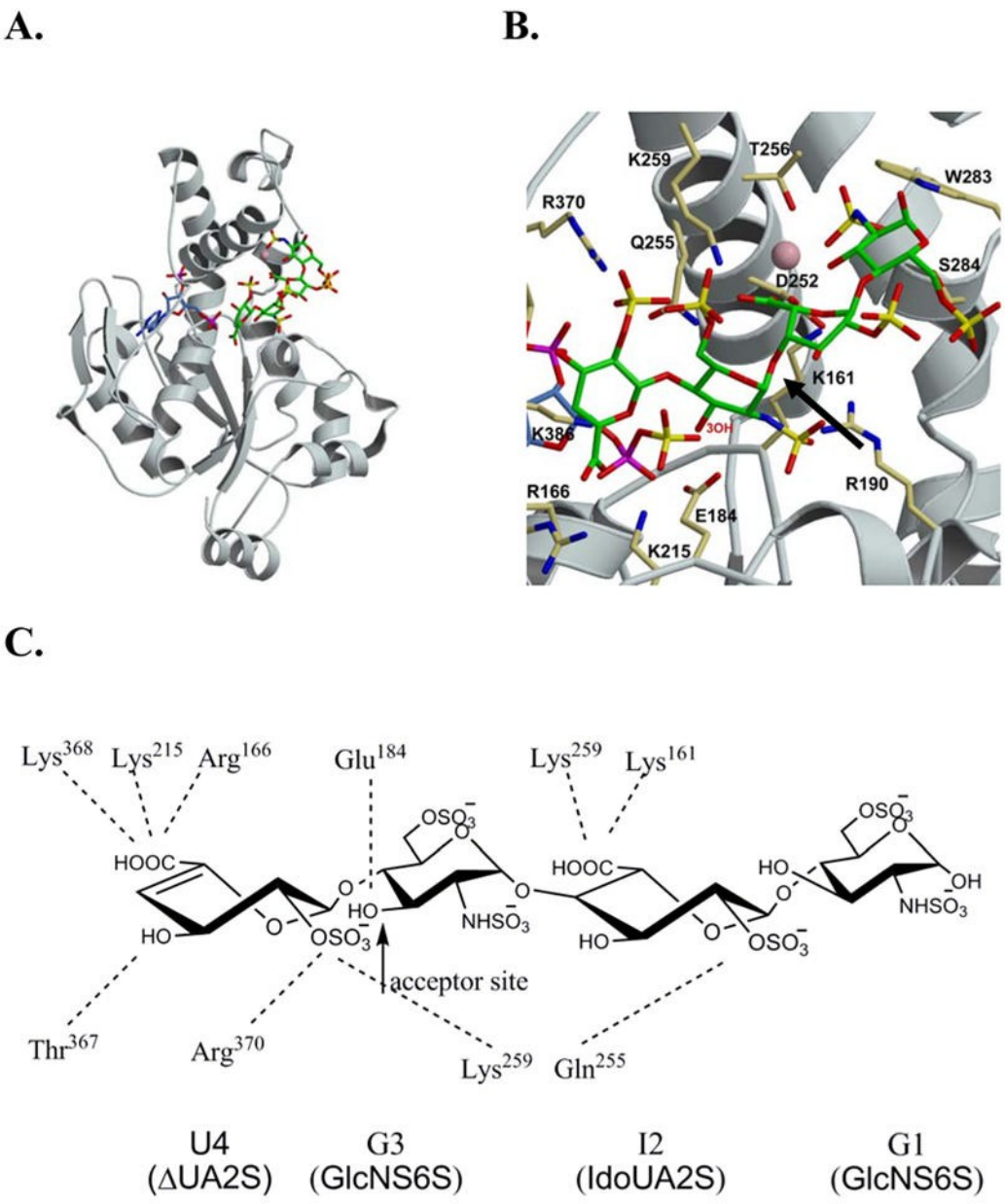

Figure 5. 3-OST-3a/PAP/tetrasaccharide complex

A) crystal structure of 3OST-3A in complex with PAP(blue) and a tetrasaccharide HS molecule (green). B) Superposition of PAPS onto PAP in the active site of the 3OST-3a crystal structure. This figure displays the relative orientation of the acceptor 3-hydroxyl to the sufo group being transferred from PAPS. Side chains that are involved in binding the tetrasaccharide are shown. A sodium ion involved in binding is pictured in pink. The location of the kink, in the polysaccharide, is indicated by an arrow. C) Figure displaying side chain with specific functional groups of the bound tetrasaccharide. Hydrogen bonds are indicated by dashed lines.

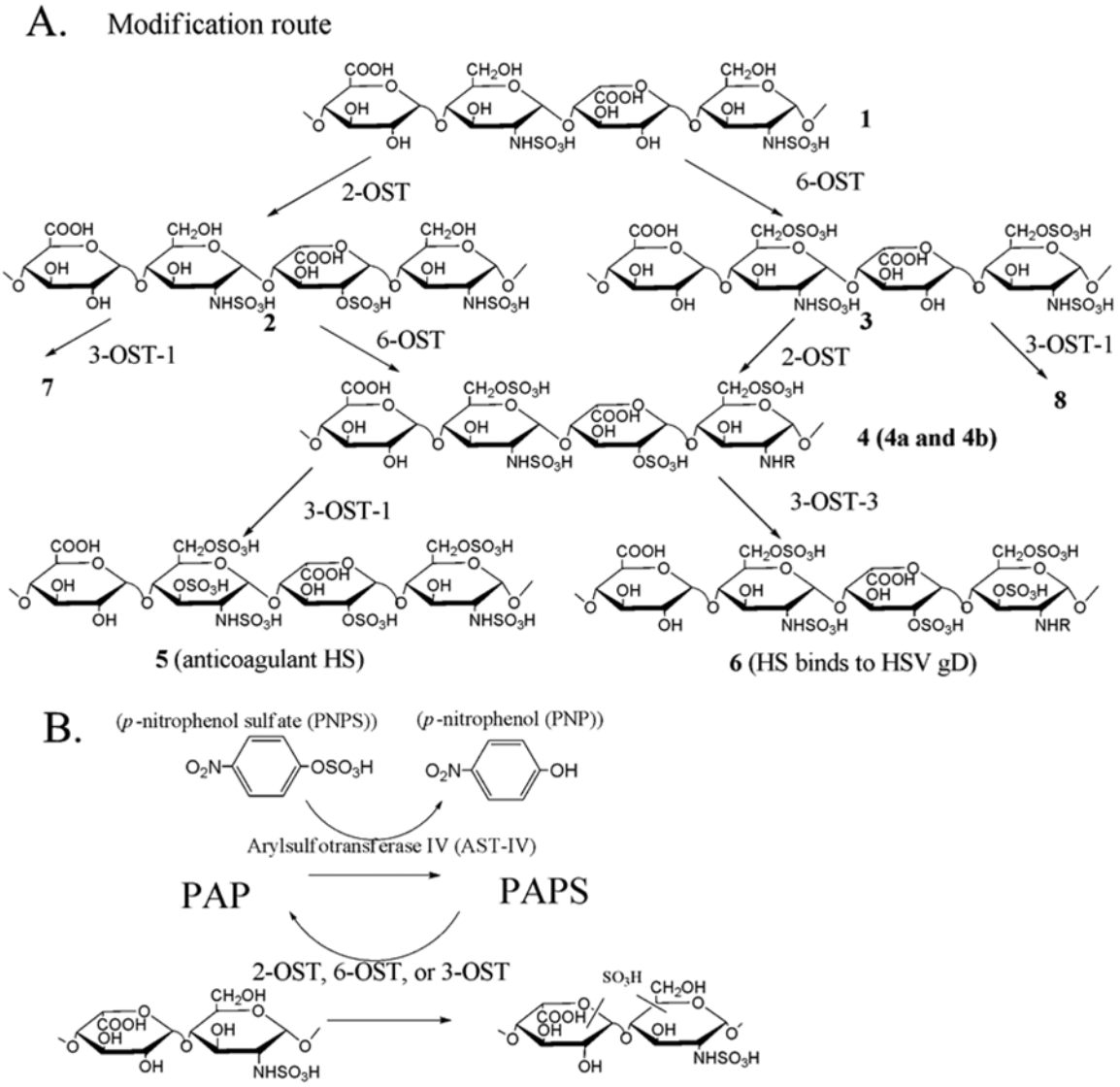

Figure 6. Pathways for Synthesis of su lfated polysaccharides and the PAPS regeneration system

Panel A shows the stepwise enzymatic synthesis of sulfated polysaccharides using HS sulfotransferases. Compounds **4a** and **4b** are prepared by inverting the order of sulfation steps: **4a** is prepared by incubating compound **1** with 2-OST followed by 6-OST, whereas **4b** is prepared by incubating compound **1** with 6-OST followed by 2-OST. Panel B shows the reaction catalyzed by arylsulfotransferase IV (AST-IV) to generate PAPS. R represents −H or $-SO_3$.