Chemoenzymatic Synthesis of Classical and Non-classical Anticoagulant Heparan Sulfate Polysaccharides*

Balagurunathan Kuberan, David L. Beeler, Miroslaw Lech, Zhengliang L. Wu, and Robert D. Rosenberg‡

From the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 and the Department of Molecular Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215

Heparan sulfate (HS) polysaccharides interact with numerous proteins at the cell surface and orchestrate many different biological functions. Though many functions of HS are well established, only a few specific structures can be attributed to HS functions. The extreme diversity of HS makes chemical synthesis of specific bioactive HS structures a cumbersome and tedious undertaking that requires laborious and careful functional group manipulations. Now that many of the enzymes involved in HS biosynthesis are characterized, we show in this study how one can rapidly and easily assemble bioactive HS structures with a set of cloned enzymes. We have demonstrated the feasibility of this new approach to rapidly assemble antithrombin III-binding classical and non-classical anticoagulant polysaccharide structures for the first time.

Heparin and heparan sulfate (HS)

are linear, sulfated polysaccharides that interact with numerous proteins to regulate various biological processes such as the cell cycle, cell growth, cellular differentiation, cell adhesion, anticoagulation, and lipid metabolism (1, 2). Heparin and heparan sulfate are synthesized as proteoglycans that consist of multiple polysaccharide chains attached to various distinct core proteins. The extensive studies of the biosynthesis of heparin and heparan sulfate have delineated the following sequences of events (3, 4): A non-sulfated polysaccharide composed of repeating disaccharide units (4GlcAβ1–4GlcNAcα1) is extended from a common tetrascarhide that is covalently linked to a core protein. In the presence of 3′-phosphoadenosine 5′-phosphosulfate (PAPS), a series of modifications take place, beginning with N-deacetylation and N-sulfation of the N-acetyl glucosamine (GlcNAc) units, followed by epimerization of glucuronic acid (GlcA) to iduronic acid (IdoA), and concluded by stepwise O-sulfation at several positions. Heparin, produced mainly by mast cells, contains predominately N- and O-sulfated, IdoA-rich sequences, whereas heparan sulfate, ubiquitously present on the surface of all cells, possesses domain structures that consist of highly sulfated, IdoA-rich regions separated by more extended, unmodified regions.

Interactions of various proteins with HS are implicated in many physiological and pathological processes (1). Moreover, many pathogens, such as parasites, bacteria, and viruses are known to enter host cells through specific interactions with the cell surface HS. Many of the proteins that interact with HS are well characterized, but the polysaccharide structure that mediates a particular interaction has been defined only in a few cases such as antithrombin III (ATIII) and fibroblast growth factor 2 (FGF2) binding HS structures. This problem is primarily attributed to the structural complexity of HS, which, in turn, arises from a complex biosynthetic pathway (3, 5).

Synthesis of HS fragments would lead to a deciphering of the information encoded within HS molecules that permits these components to regulate many different biological systems as described above and also aid in the design of new drugs based on HS. HS fragments can be synthesized either by chemical routes or enzymatic routes, as demonstrated in this study. Chemical synthesis would allow one to design unusual or non-natural structural motifs to understand enzyme functions and to design drugs with improved pharmacokinetic and pharmacodynamic characteristics. However, the total chemical synthesis of HS fragments is a challenging undertaking and often involves 50 or more individual steps. The stereoselective glycosidic bond formation is a daunting task in carbohydrate chemical synthesis despite several elegant advances in glycosylation procedures. Glucosamine is α-glycosidically linked to adjacent glucuronic acid in HS. It is difficult to form this cis-glycosidic bond (α-linkage) with high specificity and masking of the amino function as a non-participating group is required to generate the α-linkage. In addition, the carboxyl group is generally masked as a protected hydroxyl group during glycosylation, because the presence of this functional group renders GlcA/IdoA a poor glycosyl donor or acceptor.

The enzymatic method for HS assembly could allow for a more rapid synthesis of structures of interest, which would facilitate the establishment of structure-function relationships of this class of molecules. This approach requires at least a dozen enzymes/isoforms involved in HS biosynthesis, and the combination of enzymes needed to generate a specific HS structure is unknown. To examine the potential of this method, we carried out enzymatic synthesis of biologically active HS polysaccharides. Since anticoagulant HS structures are well established, we initiated our explorations to determine the practicality of generating anticoagulant polysaccharides.

Heparin accelerates the inactivation of blood coagulation enzymes by ATIII (6, 7). A unique pentasaccharide domain was found to bind to ATIII in a highly specific way and to promote...
rapid inhibition of the blood coagulation enzymes. The critical structural features of the pentasaccharide required for the anticoagulant function are 3-O and 6-O sulfates of residues C and A, respectively (Fig. 1) (8–12). These two residues are thermodynamically linked and serve as pincer to induce the conformational change in ATIII that is required for accelerating coagulation enzyme inactivation (13).

Animal-derived heparin has been in use over seven decades as an anticoagulant. Despite having undesirable side effects such as bleeding and heparin-induced thrombocytopenia (HIT) with arterial thrombosis, it is still the drug of choice in combination therapy to treat humans after strokes and heart attacks. Recent concern about the potential spread of bovine spongiform encephalopathy to humans has increased interest in the development of alternatives to animal sources of heparin. Sinay et al. (14) pioneered the original chemical synthesis of the ATIII-binding pentasaccharide. We describe here our enzymatic approach to rapidly synthesize ATIII-binding classical and non-classical anticoagulant polysaccharide structures for the first time. We should like to note our companion investigations, which explored different strategies and resolved different problems of this novel synthetic approach (15, 16). In those studies, we have shown that enzymes whose actions do not interfere with each other can be employed simultaneously in order to shorten the time of synthesis while generating the desired products. Finally, we also demonstrated that the sequential use of enzymes whose actions facilitate each other can not interfere with each other can be employed simultaneously in order to shorten the time of synthesis while generating the desired products.

Separation and characterization of 35S-labeled disaccharides were carried out by HPLC and using a C18-reversed phase column (0.46 × 25 cm) (RPIC-HPLC) (Vydac). Solvent A was double-distilled water containing 10 mM ammonium dihydrophosphate and 1 mM tetrabutylammonium dihydrophosphate. Solvent B was 40% acetonitrile containing 10 mM ammonium dihydrophosphate and 1 mM tetrabutylammonium dihydrophosphate. The RPII-HPLC was eluted at a flow rate of 0.5 mL/min with the following stepwise gradients: 100% solvent A for 15 min; 6% solvent B for 25 min; 12% solvent B for 40 min; 40% solvent B for 60 min; 100% solvent B for 10 min and finally 100% solvent A for 20 min to reequilibrate the column.

**Flow Injection Capillary Liquid Chromatography**—An ultimate capillary HPLC workstation (Dionex) was used for microseparation. UltiChrom software was used in data acquisition and analysis. A gradient elution was performed, using a binary solvent system composed of water (eluent A) and 70% aqueous methanol (eluent B), both containing 8 mM acetic acid and 5 mM dibutylamine as an ion-pairing agent. HPLC separations were performed on a 0.3-mm × 250-mm C18 polymeric silica column (Vydac). The column temperature was maintained at 25 °C, and the flow rate was set to 5 μL min⁻¹. Sample volumes of 6.3 μL were injected. For disaccharide analysis, a 20-μL sample injection loop was used. The chromatographic conditions were optimized for resolution of disaccharides. In brief, non-sulfated disaccharide was eluted with 100% A, single sulfated disaccharides were eluted with 10% B, isotonic elution with 20% B for double-sulfated disaccharides, followed by isotonic elution with 35% B for triple sulfated disaccharide. The column was washed and equilibrated by further elution with 100% B for 10 min, returning to 100% A for 10 min at the end of the run. The absorbance of the column eluate was monitored at 232 nm.

**Mass Spectrometry**

Mass spectra were acquired on a Mariner BioSpectrometry Workstation ESI time-of-flight mass spectrometer (PerSeptive Biosystems, Framingham, MA). In the negative-ion mode, the instrument was calibrated with bis-trifluoromethyl benzoic acid, heptadecafluorononanoic acid, and perfluorotetradecanoic acid. Nitrogen was used as a desolvation gas as well as a nebulizer. Conditions for ESI-MS were as follows: nebulizer flow 1 liter/min, nozzle temperature 140 °C, drying gas (N₂) flow 0.6 liters/min, spray tip potential 2.8 kV, nozzle potential 70 V, and skimmer potential 9 V. Negative ion spectra were generated by scanning the range of m/z 40–2000. During analyses, the indicated vacuum was 2.1 × 10⁻⁶ Torr.

**N-Decacylation/N-Sulfation of K5 Polysaccharide**

30 mg of K5 polysaccharide was dissolved in 7.5 mL of 2 N NaOH, incubated for 24 h at 60 °C, and cooled to room temperature, and was adjusted to pH 7. The solution was warmed to 45–50 °C. 100 mg of sodium carbonate, and 100 mg of trimethylamine-sulfur trioxide complex were added in a single step and incubated for 12 h. An equal portion of sodium carbonate and trimethylamine-sulfur trioxide was added after 12 h and the selective N-sulfation was continued for an additional 12 h at the same temperature. The solution was then brought to room temperature, dialyzed overnight against distilled wa-

FIG. 1. Antithrombin III-binding heparan sulfate structure. Critical functional groups essential for biological activity are shown in red, and those shown in blue are contributing groups.

**Materials and Methods**

HS precursor polysaccharide I was prepared from Escherichia coli K5 strain (17). Heparan sulfate 2-OST-1 sulfotransferase, (18) 3-OST-1 sulfo transferase, (19) 6-OST-1, and 6-OST-2 sulfoo transferase, (20) and C-5 epimerase (21) were all cloned and expressed in the baculovirus system (22). [35S]PAPS and [34S]PAPS were prepared as reported earlier (11). [35S]PAPS was purchased from Calbiochem. All chemicals were purchased from Sigma. Heparitinase I (EC 4.2.2.8), heparitinase II (no EC number), and heparinase (EC 4.2.2.7) were purchased from Seikagaku. ATIII and Factor Xa were from Hematologic Technologies Inc. APS kinase, used in the synthesis of PAPS, was a generous gift from Prof. I. H. Segel (University of California, Davis) (23).

**Digestion of Polysaccharides with Heparitinase I, Heparitinase II, and Heparinase**

Polysaccharides were digested with 1 nU of Hep1, -II, and -III in a total volume of 100 μL of 40 mM ammonium acetate buffer (pH 7.0) containing 3.3 mM calcium chloride at 37 °C overnight.

**High Performance Liquid Chromatography (HPLC)**

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Sulfation with Various Recombinant Sulfotransferases: 2-OST-1, 6-OST-1, 6-OST-2a, and 3-OST-1

The labeling buffer contains 50 mM MES (pH 7.0), 1% (w/v) Triton X-100, 25 mM MgCl₂, 2.5 mM CaCl₂, 0.075 mg/ml potassium chloride, 1.5 mg/ml bovine serum albumin. For a 25-μl reaction, the following were assembled: 1 μg of substrate, 12.5 μl of 2× labeling buffer, 70 ng of the expressed sulfotransferase, 2–10 μl [³⁵S]PAPS (1 μl ~1.0 × 10⁵ cpm) or [³⁴S]PAPS or [³⁵S]PAPS, and the appropriate amount of water. The reaction was incubated at 37 °C for various periods of time ranging from 30 min to overnight. Then diluted to 1 ml with DEAE wash buffer, and purified on a DEAE column. Alternatively, the reaction was stopped by heating at 70 °C, and the reaction mixture was centrifuged at 10,000 g for 3 min, and the supernatant was used for GMSA or polyacrylamide gel analysis. Modified polysaccharide at each stage was digested with heparitinases and analyzed by IRP-IRPLC.

Gel Mobility Shift Assay

Heparin-ATIII binding buffer contained 12% glycerol, 20 mM Tris-HCl (pH 7.9), 100 mM KCl, 1 mM EDTA, and 1 mM dithiothreitol. For a typical 20-μl binding reaction, radiolabeled polysaccharide (1–50,000 cpm) was mixed with ATIII (1 μg) in the binding buffer. The reaction mixture was incubated at room temperature (23 °C) for 20 min and was then applied to a 4.5% native polyacrylamide gel (with 0.1% bis-acrylamide). The gel buffer was 10 mM Tris (pH 7.4) and 1 mM EDTA, and the electrophoresis buffer was 40 mM Tris (pH 8.0), 40 mM acetic acid, 1 mM EDTA. The gel was run at 6 volts/cm for 1–2 h with an S.E. 250 Mighty Small II gel apparatus (Hoefer Scientific Instruments, San Francisco). After electrophoresis, the gel was transferred to 3-mm paper and dried under vacuum. The dried gel was autoradiographed by a PhosphorImager 445SI (Molecular Dynamics, Sunnyvale, CA). The image was analyzed with NIH Image 1.60, and the band intensities were measured at 405 nm.

Factor Xa Inactivation Assay

Purified human factor Xa (Hematologic Technologies, Inc.) was diluted to a concentration of 2.85 mg/ml with 50% glycerol. Human antithrombin III (Cutter Laboratories) was dissolved in PBS at 5.0 mg/ml and frozen in aliquots. Chromogenic substrate S-2765 (Chromogenix) was dissolved in water at 25 mM. Porcine heparin (Sigma, 175 U/mg) was employed as a standard. Protein working stocks were prepared by diluting factor Xa 1:500 in PBS and ATIII 3:1000 in 1× PBS, both containing bovine serum albumin at 5 mg/ml, and were held at 4 °C. Substrate was diluted to 2.25 mM in PBS containing Polybrene at 1.0 mg/ml and held at 37 °C. The assay was initiated by adding 0.1 μl of ATIII (2.6 × 10⁻⁷ M) to 12 μl of heparin standard or test polysaccharide (diluted in distilled water) with incubation at 37 °C for 90 s. Factor Xa stock (20 μl, 1.24 × 10⁻⁷ M) was added and mixed. After 240 s of incubation, 20 μl of substrate was added and incubated for an additional 30 s. The reaction was stopped by the addition of 52 μl of 60% acetic acid, and sample absorbance was measured at 405 nm.

RESULTS

A non-sulfated N-acetyl heparosan 1, a capsular polysaccharide of E. coli strain K5, resembles the unmodified nascent HS chain of proteoglycans (Scheme 1) (17). The production and isolation of bacterial K5 heparosan for use as a starting material in the chemoenzymatic synthesis of bioactive HS polysaccharides is an attractive alternative to the laborious chemical synthesis of the HS backbone. The partial digestion of the isolated polymer with heparitinase enzymes was carried out and analyzed by capillary reverse phase ion-pair high performance liquid chromatography coupled to micro-electrospray ionization time of flight (RP-IP-HPLC-ESI-TOF) mass spectrometry (24). The identity of HS precursor polysaccharide structure 1 was confirmed (Scheme 1).

The second step is synthesis of N-sulfated polysaccharide 2, as it is essential for subsequent enzymatic transformations. N-Deacetylase-N-sulfotransferase is a single protein that catalyzes the two initial modifications, N-deacetylation and N-sulfation, of the polysaccharide precursor in the biosynthesis of heparin and heparan sulfate (25, 26). This enzyme exists as four isoforms in humans. The ratio of N-deacetylase to...
$N$-sulfotransferase activities differ dramatically among the four isoforms. For these reasons, we utilized the established simple chemical approaches to prepare the $N$-sulfated polysaccharide 2 (Scheme 1). $N$-deacetylation of polysaccharide 1 was accomplished by hydrazinolysis at 100 °C or alkaline treatment with 2 M NaOH at 60–65 °C. The resulting free amino groups were selectively $N$-sulfated using trimethylamine sulfur trioxide, a chemoselective sulfonating agent, under controlled reaction conditions (27). Polysaccharide 2 was partially digested using heparitinases and analyzed by LC/MS to confirm its identity. Next, synthesis of polysaccharide 3 was undertaken, in which $N$-sulfated polysaccharide 2 was treated with heparan sulfate glucuronyl C-5 epimerase, resulting in inversion of stereochemical configuration at the C-5 carbon of many uronic acids along the chain. To date there are no efficient chemical strategies available to selectively epimerize glucuronic acid to iduronic acid. In fact the presence of both epimers along the polysaccharide adds further complexity and challenges in chemical synthesis and structure-function relationship studies (21, 28).

Epimerization proceeds only at residues located at the reducing side of $N$-sulfated glucosamine residues, and only with uronic acids that are neither O-sulfated nor adjacent to O-sulfated glucosamine residues (21, 29). Therefore, epimerase treatment was carried out before any further modifications with sulfotransferases. The epimerization process has been monitored in the past by measuring the release of $^3$H$_2$O from $^3$H-labeled (at C-5) polysaccharide 2 (21, 28, 30). We devised a new strategy in which we monitored the epimerization of polysaccharide 2 by determining selective incorporation of sulfate at the 2-O position of the newly generated iduronic acid using limiting amounts of $[^{35}S]$PAPS and HS 2-OST. The selective

**Scheme 1.** Enzymatic synthesis of antithrombin III-binding classical heparan sulfate polysaccharides.
2-Ο sulfation of iduronic acid on polysaccharide 3 was observed to be predominant whereas incorporation of sulfate on polysaccharide 2 was observed to be minor (Fig. 2). Synthesis of polysaccharide 3 containing iduronic acid was confirmed on 5% native polyacrylamide gel through selective radioactive 2-Ο sulfation of iduronic acid. This approach is better than traditional approaches, which involve measuring the release of tritium as $^3$H$_2$O to monitor the epimerization process. Our approach is superior because, in our opinion, the classical approach may be biased by isotope effects, and furthermore the assay is relatively slow and therefore could not be employed in real-time monitoring of our synthetic strategy. Next the extent of epimerization was determined by treating the polysaccharide 3 with $[^{35}S]$PAPS in the presence of 6-ΟSTs to prepare radioactive 6-Ο sulfated polysaccharide 7, which was then subjected to low pH nitrous acid and sodium borohydride treatment (31) to obtain two disaccharides, GluA-anMan$_6$S$[^{35}S]$ and IdoA-anMan$_6$S. These two disaccharides were then resolved on C18 reverse phase HPLC to determine the percentage of each epimer (Fig. 3). Normally one quantifies the percentage of iduronic acid and glucuronic acid by nitrous degradation coupled to $[^3]$H borohydride end labeling. However, the traditional approach can be utilized only in the analysis of polymers that are fully N- and O- sulfated. In the case of polysaccharide 3, the above approach generated two disaccharides, which do not contain any sulfate groups and hence bind very weakly to C18 columns. On the other hand, the nitrous acid degradation of polysaccharide 3 after 6-Ο sulfation generates two disaccharides, each containing one sulfate group, which allowed them to bind more tightly to the C18 column. Furthermore, we confirmed that the 6-Ο sulfation is complete using LC/MS analysis, which eliminated the potential errors of not taking into account the presence of non-6-Ο-sulfated disaccharides. We further validated the results by co-injection of standard disaccharides, as previously reported. The ratio of iduronic to glucuronic acid was found to be ~85:15. In previous studies, epimerization of the polysaccharide 2 by epimerase resulted in generation of about 20% iduronic acid only (21). This is a striking result. We speculate that our baculovirus expression and purification system provides C5-epimerase with improved functional characteristics as compared with enzyme preparation utilized by other investigators.

It was suggested that 2-Ο sulfation of iduronic acid within the ATIII-binding pentasaccharide (Unit D, Fig. 1) limits 3-ΟST-1 mediated 3-Ο sulfation of glucosamine residues at the reducing end of IdoA(2S) residue, while it has no effect at its non-reducing end (32). The polysaccharide 4 was prepared from polysaccharide 3 in the presence of PAPS or radioactive $[^{35}S]$PAPS, catalyzed by 2-ΟST-1 (Scheme 1). The radioactive polysaccharide 4 was analyzed by 5% native polyacrylamide gel to confirm the action of 2-ΟST-1 (Fig. 2).

Next, synthesis of polysaccharide 5 from polysaccharide 4, as catalyzed by 6-Ο sulfotransferase, was carried out (Scheme 1). There are three heparan sulfate 6-Ο sulfotransferase isoforms (20). These are 6-ΟST-1, 6-ΟST-2, and 6-ΟST-3, while 6-ΟST-2 possesses two splice variants, namely 6-ΟST-2a and 6-ΟST-2b. The results produced from the current in vitro studies suggest that these isoforms do not seem to have significant differences in their substrate preferences for polysaccharides 1, 2, 3, or 4. The 6-Ο sulfation of polysaccharides was carried out in the presence of radioactive $[^{35}S]$PAPS or PAPS using 6-ΟST-1 or 6-ΟST-2a. The radiolabeled 6-Ο-sulfated polysaccharide 5 was digested to disaccharides by heparitinases. Disaccharide analysis by C18 HPLC confirmed the incorporation of 6-Ο sulfate group as they eluted at the expected position along with standards. The 6-ΟST-1 and 6-ΟST-2a gave equivalent results.

The final step in our synthetic strategy was the 3-Ο sulfation of polysaccharide 5, catalyzed by 3-ΟST-1 sulfotransferase, to generate anticoagulant HS polysaccharide 6 (Scheme 1). There are as many as six isoforms of HS 3-Ο sulfotransferases, namely 3-ΟST-1, 3-ΟST-2, 3-ΟST-3, 3-ΟST-4, 3-ΟST-5, and 3-ΟST-6 (19, 22, 33–35). Substrate specificity of these isoforms is not well characterized. However, it was demonstrated that 3-ΟST-1 is primarily involved in generating anticoagulant HS polysaccharide, whereas the 3-ΟST-3a sulfotransferase prefer-
entially produces the gD-binding HS polysaccharide, which is involved in cellular entry of herpes simplex virus I (36). The recently cloned 3-OST-5 was shown to generate ATIII-binding HS as well as gD-binding HS (34). The functional significances of 3-OST-2 and 3-OST-4 modified HS are still unknown. However, it is important to note that these isoforms are exclusively expressed in human brain, suggesting a role for HS in neurobiology (19, 37).

It was shown earlier that 3-OST-1 generally acts on glucosamine units located between GlcA (at the non-reducing side of glucosamine) and IdoA (at the reducing side of glucosamine) and generates ATIII binding structures. Polysaccharide 6 was successfully prepared from polysaccharide 5 using 3-OST-1 and radioactive [34S]PAPS. The purified polysaccharide 6, containing radiolabeled sulfate at the 3-O position, was digested with heparitinases and analyzed by C18 HPLC (data not shown). This radiolabeled polysaccharide was used in gel mobility shift analysis to test its ability to bind to ATIII. Polysaccharide 5 was treated with [34S]PAPS in the presence of enzyme 3-OST1 analysis to test its ability to bind to ATIII. Polysaccharide 6 that is devoid of IdoA(2S) should be more effective in areas with platelet enriched thrombi (arterial side) which release PF4 and generate a protected sanctuary for blood clotting. In addition, it should be resistant to heparanase cleavage and hence effective at lower dosages (40). This hypothesis can only be substantiated by experimental studies using thrombotic models in different vascular beds of primates. Polysaccharide 8 was prepared by 3-O sulfation of polysaccharide 7, which in turn was prepared from 6-O sulfation of polysaccharide 3 (Scheme 2). Polysaccharide 7 was also treated with [34S]PAPS in the presence of enzyme 3-OST-1 to prepare 3-O [34S]sulfate containing polysaccharide 8, which was then digested with heparitinases and analyzed by LC/MS system (Fig. 5) (24). The disaccharide analysis of the products was then digested with a mixture of heparitinases. The resulting disaccharides were separated on a capillary HPLC C18 column. The extractive ion chromatogram (XIC) for trisulfated disaccharide profile is given (Fig. 4a). Peak A corresponds to a single charged molecular ion with m/z of 707.14 [M-H+1DBA]-1 indicating 3-O sulfated disaccharides, △UA-GlcNS34S6S (Fig. 4b) whereas peak B corresponds to single charged molecular ion with m/z of 705.15 [M-H+1DBA]-1 suggesting trisulfated disaccharides without a 3-O sulfate, △UA2S-GlcNS6S (Fig. 4c).

![Disaccharide analysis of polysaccharide 6 using IPRP-capillary HPLC coupled to micro ESI-TOF-MS.](image)

On the other hand, this new non-classical anticoagulant should be more effective in areas with platelet enriched thrombi (arterial side) which release PF4 and generate a protected sanctuary for blood clotting. In addition, it should be resistant to heparanase cleavage and hence effective at lower dosages (40). This hypothesis can only be substantiated by experimental studies using thrombotic models in different vascular beds of primates. Polysaccharide 8 was prepared by 3-O sulfation of polysaccharide 7, which in turn was prepared from 6-O sulfation of polysaccharide 3 (Scheme 2). Polysaccharide 7 was also treated with [34S]PAPS in the presence of enzyme 3-OST-1 to prepare 3-O [34S]sulfate containing polysaccharide 8, which was then digested with heparitinases and analyzed by LC/MS system (Fig. 5) (24). The disaccharide analysis of the products was carried out by total enzymatic degradation and subsequently identified by LC/MS (Table I). The data shows the presence of expected disaccharides. Gel mobility shift assay (GMSA) was carried out to determine the ability of radiolabeled polysaccharide 6 and radiolabeled polysaccharide 8 to bind to ATIII. Radiolabeled polysaccharide 5, lacking critical 3-O sulfate residue essential for binding to ATIII and creating anticoagulant activity, failed to bind to ATIII and hence was not
shifted (Fig. 6a) whereas polysaccharide 6 and polysaccharide 8 both specifically bound to ATIII (Fig. 6, b and c). The factor Xa assay confirmed the ability of polysaccharide 6 and polysaccharide 8 to bind to and accelerate the action of ATIII.

We have shown for the first time that enzymatic synthesis of bioactive HS polysaccharide structures with anticoagulant properties can be accomplished in a relatively simple and expeditious manner as compared with classical chemical synthesis. This synthetic strategy, in conjunction with exoglycosidases, which remove terminal sugar residues at the non-reducing end, can be applied to synthesize bioactive heparan sulfate of any size or structure and should also permit us to identify proteins that recognize the various HS structures. These structures can also be used as a diagnostic probe to detect functional abnormalities in a specific protein implicated in human disease, such as detection of antibodies that cause heparin induced thrombosis, detection of tumor associated heparanases or tumor suppressor gene products that are implicated in tumor metastasis, bone growth plate abnormalities, and detection of pathological alterations in growth factor-growth factor receptor interactions that are implicated in various developmental abnormalities.

**DISCUSSION**

In this study, we described a novel method for synthesizing bioactive HS structures using a panel of recombinant HS biosynthetic enzymes, which act upon bacterial product consisting of -GlcNAc-GlcA repeating units. This method mimics, under *in vitro* conditions, the synthesis of HS polymers within the Golgi. We note that our other companion investigations dealt with various strategies to optimize this novel synthetic approach (15, 16). The bacterial starting material represents the unmodified HS precursor structure, whose generation constitutes the first step in the biosynthesis of HS catalyzed by EXT polymerase. The major problem that remains unsolved in HS biology is how structures of HS proteoglycans regulate interactions with a specific biological target. It has been tacitly assumed that the sequence within heparan sulfate/heparin is unique and provided HS chains with the ability to interact with a specific protein. This idea is analogous to a similar paradigm widely held for DNA and proteins. Unfortunately there is little data to support this concept. Bernfield et al. (1) pointed out that heparan sulfate exhibiting differences in sequences may have similar biological functions. One can infer this concept by examining the extensive data obtained with the ATIII-binding pentasaccharides, generated over 3 decades, in which it has been shown that only 3-0 and 6-O groups and their spacing are critical for the activation of ATIII. Changes outside these critical groups have little effect on the biological activity of ATIII-binding pentasaccharides.

A series of reports have been published by both our and other laboratories that attempt to solve this problem of defining structural parameters on HS chains, which are responsible for interactions with biological targets other than ATIII. This problem can now be addressed by using our novel enzymatic synthetic methodology. Underlying these differences in approach between various laboratories is a major difference in the philosophic approach of how structural differences in HS chains allow them to interact with many biological targets. We have emphasized that differences in sequences of the HS chains, which possess the same biological function, is due to the presence of common critical groups with identical spacing and that this parameter permits specific interactions with biological targets. It is perhaps worth emphasizing the difference between critical groups and critical sequences. In the case of sequences, biological activity requires alignment of multiple groups whereas in the case of critical groups, biological activity requires less stringent condition in which a fewer groups must be aligned. The difference in these two concepts has important implications for both elucidations of structure-function relationship and the manner by which respective biosynthetic pathways functions.

In addition, there are important differences between our
PAPS. Purified polysaccharide mine unit enriched with stable 34S isotope at the 3-lysaccharide, was prepared from polysaccharide 7 using 3-OST-1 and 34SiPAPS. Purified polysaccharide 8 contained a 3-O-sulfated glucosamine unit enriched with stable 34S isotope at the 3-O position. The polysaccharide 8 was then digested with a mixture of heparitinases. The resulting disaccharides were separated on a capillary HPLC C18 column, and the extractive ion chromatogram showed a trisulfated disaccharide (Fig. 5a) with a single peak suggesting the presence of only one trisulfated disaccharide. Peak A corresponds to a single charged molecular ion with m/z of 707.14 [M-1H+1DBA]- indicating of 3-O-sulfated disaccharide, △UA-GlcNS334S6S (Fig. 4b).

**Fig. 5.** Disaccharide analysis of polysaccharide 8 using IPRP-capillary HPLC coupled to micro ESI-TOF-MS. 3-O-sulfated polysaccharide 8 was prepared from polysaccharide 7 using 3-OST-1 and 34SiPAPS. Purified polysaccharide 8 contained a 3-O-sulfated glucosamine unit enriched with stable 34S isotope at the 3-O position. The polysaccharide 8 was then digested with a mixture of heparitinases. The resulting disaccharides were separated on a capillary HPLC C18 column, and the extractive ion chromatogram showed a trisulfated disaccharide (Fig. 5a) with a single peak suggesting the presence of only one trisulfated disaccharide. Peak A corresponds to a single charged molecular ion with m/z of 707.14 [M-1H+1DBA]- indicative of 3-O-sulfated disaccharide, △UA-GlcNS334S6S (Fig. 4b).

**Table I**

Disaccharide types present in polysaccharides 6 and 8

| Polysaccharide | △UA2S-GlcNS6S | △UA-GlcNS334S6S | △UA-GlcNS3S6S |
|---------------|--------------|-----------------|----------------|
| 6             | ✓            |                 |                |
| 8             | ✓            | ✓               | ✓              |

a ✓ indicates the presence of disaccharide.
b x indicates the absence of disaccharide.

current experimental approach and those of others. Reports by other laboratories have used heparin oligosaccharides and a combination of chemical de-sulfation and enzymatic re-sulfation to generate heterogeneous oligosaccharide libraries, which were then employed to screen for interacting with growth factors (41). On the other hand, our laboratory utilized heparin oligosaccharides in conjunction with enzymatic sulfation and gel mobility shift analysis to identify critical groups on the oligosaccharides which permitted interactions with specific biological targets (11). Thus, our study provided a novel approach to obtain critical functional groups on HS oligosaccharides. A subsequent report from our laboratory analyzed in a greater detail those critical groups on HS oligosaccharide and heparan sulfate, which are involved in growth factor/growth factor receptor interactions. This information was corroborated with somatic cell mutants and mitogenic activity in BAF cell systems to demonstrate those critical groups involved in binary complex (HS/growth factor or HS/growth factor receptor) and ternary complex formation (HS/growth factor/growth factor receptor) and evaluation of the biological significance of binary and ternary complex formation with regard to mitogenic activity (42). Comparison of our approach to define critical groups on heparan sulfate involved in growth factor-growth factor receptor interactions should be compared with that of Jemth et al., (41) who only considered binary interactions and failed to define experimentally those critical groups required for mitogenic activity.

We utilized both philosophical insights as outlined above and an extensive panel of isomeric specific recombinant heparan sulfate biosynthetic enzymes to generate HS-like polysaccharides that bind specifically to ATIII. The enzymes employed included not only sulfotransferases and their various isoforms, but also C5 epimerase. Various steps in our synthetic process were monitored by a powerful new LC/MS technology developed by us. Our approach permitted us to generate ATIII binding polysaccharide without 2-O sulfate groups, which change the sequence of polysaccharide but which lie outside those groups required for biological activity. Our demonstration that polysaccharides that lack 2-O sulfate groups, but which exhibits biological activity, emphasizes the importance of critical groups, but not sequence, to biological activity. Finally the absence of 2-O sulfate groups may impart valuable therapeutic characteristics to the final product. These benefits include enhanced activity on the arterial side as well as prolonged in vivo half-life and decreased ability to bind PF4 with consequent absence or reduction in HIT. Our enzymatic synthetic approach can be extended to generate libraries of homogeneous oligosaccharides, which will allow the definition of critical functional groups and ultimately the design of heparin/HS based drugs for different pathological states.

One can envision that our approach can be adopted to synthesize different HS structures by adding different isoforms, or by changing the order of the addition of enzymes or omitting enzymes and by altering the structures at the non-reducing end with catabolic enzymes in conjunction with EXT polymerase. Finally, we would like to point out that this strategy can be
employed in a stepwise manner with complete control over the production of homogeneous products (16). This stepwise enzymatic approach could be tailored such that one can synthesize structures with a specific N-sulfation pattern and restricted epimerization (15). Alternatively, enzymes can produce heterogeneous products in a combinatorial fashion (15). As noted above, simple stepwise or contemporaneous modifications can produce homogeneous or heterogeneous products, respectively, and hence our strategy can be expanded to accomplish the production of oligosaccharides or polysaccharides as desired.

REFERENCES
1. Bernfield, M., Gotte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Lincecum, J., and Zako, M. (1999) *Annu. Rev. Biochem.* 68, 729–777
2. Capila, I., and Linhardt, R. (2002) *Annu. Rev. Biochem.* 71, 435–471
3. Rosenberg, R. D., Shworak, N. W., Liu, J., Schwartz, J. J., and Zhang, L. J. (1997) *J. Clin. Investig.* 99, 2062–2070
4. Esko, J. D., and Selseck, S. B. (2002) *Annu. Rev. Biochem.* 71, 435–471
5. Salmivirta, M., Lidholt, K., and Lindahl, U. (1996) *FASEB J.* 10, 1270–1279
6. Damus, P. S., Hicks, M., and Rosenberg, R. D. (1973) *Nature* 246, 355–357
7. Rosenberg, R. D., and Damas, P. S. (1973) *J. Biol. Chem.* 248, 6489–6505
8. Ather, D. H., Lermane, J. C., Petitou, M., Rosenberg, R. D., and Choay, J. (1987) *Biochemistry* 26, 6454–6461
9. Ather, D. H., Stephens, A. W., Rimon, A., and Rosenberg, R. D. (1984) *Biochemistry* 23, 5801–5812
10. Ather, D. H., Stephens, A. W., and Rosenberg, R. D. (1984) *Proc. Natl. Acad. Sci. U. S. A.* 81, 1030–1034
11. Wu, Z. L., Zhang, L., Beeler, D. L., Koven, B., and Rosenberg, R. D. (2002) *FASEB J.* 16, 539–545
12. Hermann, M., Liu, J., Rosenberg, R. D., and Linhardt, R. J. (2000) *Biochem. Biophys. Res. Commun.* 276, 292–297
13. Desai, U. R., Petitou, M., Bjork, I., and Olson, S. T. (1998) *J. Biol. Chem.* 273, 7478–7487
14. Sinay, P., Jacquinet, J. C., Petitou, M., Douchaussay, P., Lederman, L., Choo, J., and Torri, G. (1984) *Carbohydr. Res.* 132, C3–C9
15. Kuberan, B., Beeler, D. L., Lawrence, R., Lech, M., and Rosenberg, R. D. (2003) *J. Am. Chem. Soc.* 125, 12424–12425
16. Kuberan, B., Lech, M. Z., Beeler, D. L., Wu, Z. L., and Rosenberg, R. D. (2003) *Nat. Biotechnol.* 21, 1343–1346
17. Vann, W. F., Schmidt, M. A., Jann, B., and Jann, K. (1981) *Eur. J. Biochem.* 116, 359–364
18. Rong, J., Habuchi, H., Kimata, K., Lindahl, U., and Kusche-Gullberg, M. (2001) *Biochemistry* 40, 5548–5555
19. Shworak, N. W., Liu, J. A., Petros, L. M., Zhang, L. J., Kobayashi, M., Copeland, N. G., Jenkins, N. A., and Rosenberg, R. D. (1999) *J. Biol. Chem.* 274, 5170–5184
20. Habuchi, H., Tanaka, M., Habuchi, O., Yashida, K., Suizki, H., Ban, K., and Kimata, K. (2000) *J. Biol. Chem.* 275, 28529–2868
21. Li, J., Hapner-McWharter, A., Kjellon, L., Pulji, J., Jalkanne, M., and Lindahl, U. (1997) *J. Biol. Chem.* 272, 28158–28163
22. Liu, J. A., Shworak, N. W., Sinay, P., Schwartz, J. J., Zhang, L. J., Fritze, L. M. S., and Rosenberg, R. D. (1999) *J. Biol. Chem.* 274, 5185–5192
23. MacRae, I. J., Rose, A. B., and Segel, I. H. (1998) *J. Biol. Chem.* 273, 28583–28589
24. Kuberan, B., Lech, M., Zhang, L. J., Wu, Z. L., Beeler, D. L., and Rosenberg, R. D. (2002) *J. Am. Chem. Soc.* 124, 8707–8718
25. Orellana, A., Hirschberg, C. B., Wei, Z., Swiedler, S. J., and Ishihara, M. (1994) *J. Biol. Chem.* 269, 2270–2276
26. Akizawa, J., Grobe, K., Tsujimoto, M., and Esko, J. D. (2001) *J. Biol. Chem.* 276, 5876–5882
27. Lloyd, A. G., Embery, G., and Fowler, L. J. (1971) *Biochem. Pharmacol.* 20, 637–648
28. Campbell, P., Hennerson, H. H., Sandbeck, D., Roden, L., Lindahl, U., and Li, J. P. (1994) *J. Biol. Chem.* 269, 26953–26958
29. Kusche, M., Hennerson, H. H., and Lindahl, U. (1991) *Biochem. J.* 275, 151–158
30. Crawford, B. E., Olson, S. K., Esko, J. D., and Pinhal, M. A. (2001) *J. Biol. Chem.* 276, 21538–21543
31. Shively, J. E., and Conrad, H. E. (1970) *Biochemistry* 9, 35–43
32. Zhang, L., Lawrence, R., Schwartz, J. J., Bai, X., Wei, G., Esko, J. D., and Rosenberg, R. D. (2001) *J. Biol. Chem.* 276, 28806–28813
33. Liu, J., Shworak, N. W., Fritze, L. M. S., Edelberg, J. M., and Rosenberg, R. D. (1996) *J. Biol. Chem.* 271, 27072–27082
34. Xia, G., Chen, J., Tiwari, J., Wu, L., Li, J. P., Malstrom, A., Shukla, D., and Liu, J. (2002) *J. Biol. Chem.* 277, 37912–37919
35. Liu, J. A., Shrimer, Z., Blaklock, P., Yoshida, K., Sasisekharan, R., and Rosenberg, R. D. (1999) *J. Biol. Chem.* 274, 38155–38162
36. Shukla, D., Liu, J., Blaklock, P., Shworak, N. W., Bai, M. X., Esko, J. D., Cohen, G. H., Eisenberg, R. J., Rosenberg, R. D., and Spear, P. G. (1999) *Science* 285, 13–22
37. Herndon, M. E., Stupp, C. S., and Lander, A. D. (1999) *Glycobiology* 9, 143–155
38. Stephan, D., and Nabel, E. G. (1997) *Fundam. Clin. Pharmacol.* 11, 97–110
39. Stringer, S. E., and Gallagher, J. T. (1997) *J. Biol. Chem.* 272, 20508–20514
40. Bame, K. J. (2001) *Glycoconjugate J.* 18, 91B–98B
41. Jemth, P., Kreuger, J., Kusche-Gullberg, M., Sturiale, L., Gimenez-Gallego, G., and Lindahl, U. (2003) *J. Biol. Chem.* 278, 30567–30573
42. Wu, Z. L., Zhang, L., Yabe, T., Kuberan B., Beeler, D. L., Love, A., and Rosenberg, R. D. (2003) *J. Biol. Chem.* 278, 17121–17129