Biochemistry

Polysaccharide Sequence Influences the Specificity and Catalytic Activity of Glucuronyl C5-Epimerase

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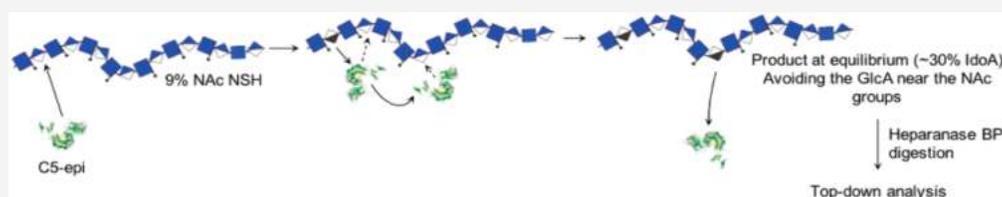
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ABSTRACT: Heparin is a widely used biotherapeutic produced from animal tissues. However, it might be possible to produce a bioengineered version using a multienzyme process, relying on the isolation of the *E. coli* K5 capsule heparosan and its chemical conversion to *N*-sulfoheparosan, NSH. Glucuronyl C5-epimerase, the first enzyme that acts on NSH, catalyzes the reversible conversion of glucuronic acid (GlcA) to iduronic acid (IdoA). Using full-length NSH, containing different amounts of *N*-acetylglucosamine (GlcNAc) residues, we demonstrate that C5-epimerase specificity relates to polysaccharide sequence, particularly the location of GlcNAc residues within the chain. We leveraged the deuterium exchange and the novel *β*-glucuronidase heparanase BP, which cleaves at the GlcA residue. Liquid chromatography−mass spectrometry and gel permeation chromatography of partial/complete heparanase BP digestion products from various NSH substrates treated with C5-epimerase provide information on C5-epimerase activity and action pattern. This study provides insight into optimizing the large-scale production of bioengineered heparin.

Heparin, a highly sulfated glycosaminoglycan (GAG), is a long-chained polydisperse, heterocopolymer comprised of a major disaccharide repeating unit, →4)IdoA2S(1→4) GlcNS6S(1→, with a uronic acid residue, corresponding to α-L-iduronic acid (IdoA) and a 2-*O*-sulfo group (S), and a hexosamine residue corresponding to α-D-glucosamine (GlcN) having *N*- and *O*-S (GlcNS6S).[1] Heparin, a clinical anticoagulant, is biosynthesized in mast cells present within the intestine and lungs of pigs and cows.[2] Heparan sulfate, a related GAG, is comprised of a major disaccharide repeating unit, →4)GlcA(1→4) GlcNAc(1→ with a low level of sulfation, where GlcA is glucuronic acid.[3] Chemoenzymatic synthesis represents an emerging technology to prepare heparan sulfates and bioengineered heparin as an alternative to the animal-sourced anticoagulant drug.[4−6] The capsular polysaccharide, derived from *E. coli* K5, is heparosan, having the structure [→4)GlcA(1→4)GlcNAc(1→]ₙ where GlcA is β-D-glucuronic acid and GlcNAc is *N*-acetyl-β-D-glucosamine.[7,8] Following its partial chemical, de-*N*-acetylation-*N*-sulfonation heparosan affords an *N*-sulfoheparosan (NSH) that still contains ∼10−15% GlcNAc residues,[4] which serves as the substrate for a series of five enzymatic reactions that produce bioengineered heparin. The first enzyme in this pathway is C5-epimerase (C5-epi) that acts on NSH, a key step in the production of bioengineered heparin and in the biosynthesis of animal-sourced heparin.[4,5] C5-epi catalyzes a reversible

reaction converting glucuronic acid (GlcA) to iduronic acid (IdoA), resulting in a polysaccharide with an equilibrium mixture of ∼70% GlcA and 30% IdoA.[9]

Thus, NSH chemically produced from heparosan consists of three different monosaccharide units, GlcA, *N*-sulfoglucosamine (GlcNS), and GlcNAc.[4−6] The polydispersity and structural complexity of NSH offers a significant challenge in understanding the mechanism of action of C5-epi on this substrate, particularly the impact (i.e., the percentage and distribution of GlcNAc residues) on carbohydrate sequence on the epimerization reaction as it proceeds.[10,11] Previous reports, using oligosaccharide substrates, suggest a significant interference by the GlcNAc residues on the action of C5-epi.[11,12] Specifically, Liu et al. evaluated the activity of C5-epi on oligosaccharides containing a distribution of GlcNAc and GlcNS residues.[12] In oligosaccharides with GlcNAc residues, C5-epi catalyzes a reversible reaction, while in the presence of GlcNS residues, an irreversible reaction was observed.[12]
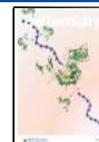
However, there are reported differences in C5-epi action on polysaccharide and oligosaccharide substrates.[11,13] However, there is currently no direct evidence that the NSH polysaccharide substrate, containing GlcNAc residues, shows a restriction on the action of C5-epi, similar to that observed on oligosaccharide substrates.

The control of *N*-deacetylation and *N*-sulfonation steps can result in improved bioengineered heparin products and a higher similarity to animal-sourced heparins.[4,6] This chemical control results in different GlcNAc contents and different molecular weight properties of the resulting NSH.[14] We hypothesized that the GlcNAc content of the resulting NSH also exerts control on C5-epi catalysis, and hence, the content and distribution of IdoA residues.[15] These studies are relevant to the preparation of bioengineered heparins and heparan sulfates for biological and pharmacological evaluation. Hydrogen−deuterium exchange was used to determine the activity of C5-epi. Furthermore, heparanase BP, a *β*-endoglucuronidase, which selectively cleaves at the GlcA residues within the polysaccharide chain, was used for the first time as a direct method to evaluate the impact of the GlcNAc content of the polysaccharide substrate on C5-epi catalysis.[16,17]

■ MATERIALS AND METHODS

Preparation of NSH. Heparosan was purified from the fermentation of *E. coli* K5 as described previously.[8] Once purified, heparosan was then *N*-deacetylated using aqueous NaOH (Sigma-Aldrich, St. Louis, MO) and chemically *N*-sulfonated using equal portions of sodium carbonate and trimethylamine-sulfur trioxide for varying times to produce NSH with various percentages of NS within the chain. Although the placement of these groups cannot be controlled and is assumed to be random, the total amount present on the chain can be well-controlled (Table 1).[14,18]

Table 1. NSH Substrates Used in This Study and Their Composition

NSH substrate			
% NAc	% NS	Mw	PD
52	48	11 300 ± 3800	1.5
35	65	25 700 ± 4300	1.2
24	76	17 500 ± 3000	1.2
9.0	91	21 100 ± 4600	1.1
1.0	99	16 000 ± 600	1.1

Engineered C5-Epimerase Expression, Purification, and Assay. We have used a previously reported engineered human C5-epi that is transmembrane domain-truncated and has an MBP fusion tag. The accession identification number of the wild-type enzyme is UniProt O94923. The expression system used is *E. coli* Origami B (DE3) using a pMAL expression vector. Purification was performed using an amylose column as previously described.[9] C5-epi was buffer-exchanged in 99.9% D$_2$O (Sigma-Aldrich) using 200 *μ*L each of C5-epi (0.5 mg/mL), NSH (various % NS, redissolved in D$_2$O at 10 mg/mL), and 50 mM MES (Sigma-Aldrich) at pH 7.0. The mixture was incubated for 17 h to allow for the epimerization reaction to attain apparent equilibrium.[9] The reaction was then terminated by heat shocking the enzyme at 95 °C for 10 min and centrifuged at 12 000 rcf for 10 min to separate the aggregated enzyme, and the product was lyophilized for further analysis. The assay was run in biological quintuplicates.

NMR Analysis of Products. The lyophilized products of the C5-epi reaction were dissolved in 500 *μ*L of D$_2$O and were prepared for NMR analysis using a 5 mm tube (Norell, Morganton, NC). The 1D ^{1}H spectrum was acquired using a Brüker Advance II 800 MHz spectrometer (Brüker BioSpin, Billerica, MA) run on Topspin 3.2.1. The 1D ^{1}H spectra were based on an average of 64 scans, with 4 dummy scans, and an acquisition time of 0.991 s with a relaxation delay of 11.5 s at 310 K.

Heparanase BP Treatment for Partial and Complete Depolymerization. The products and the substrate of the epimerization reaction (10 *μ*g) were treated with 10 *μ*L of heparanase BP (0.7 mg/mL) in 200 *μ*L of digestion buffer (50 mM ammonium acetate, pH 4.5) at 37 °C for 4 h (partial digestion) and 18 h (complete digestion).[16]

Carbohydrate Gel Analysis. Carbohydrate gel analysis was performed using a resolving gel of 22.5% acrylamide and a stacking gel of 4.5% acrylamide prepared at pH 6.3. The gel was resolved with an upper chamber buffer of 1 M glycine, 0.2 M tris(hydroxymethyl)aminomethane (Tris), and a resolving gel buffer of 0.1 M boric acid, 0.1 M Tris, and 0.01 M ethylenediaminetetraacetic acid (EDTA). The gel and chamber, once assembled, were prerun for 5 min at 100 V and then for 45 min at 200 V. A standard mixture of heparin oligosaccharides was used to determine the size of the oligosaccharide products obtained on heparanase BP treatment. Once run, the gel was stained with Alcian blue for 30 min. The gel was then destained using DI water by periodically changing to fresh water.

LC−MS Top-down Analysis. A Luna HILIC column was used for the separation of the partially depolymerized samples using heparanase BP. Mobile phase A was 5 mM ammonium acetate, and mobile phase B was 5 mM ammonium acetate in 98% acetonitrile and 2% water. The injection volume was 5 *μ*L on an LTQ Orbitrap XL FT-MS instrument (Thermo Fisher Scientific, San Jose, CA) with an ESI source.[19,20] The biological quintuplicate samples were run in technical triplicates. Raw data were visualized using the Thermo Scientific, Xcalibur software. Oligosaccharides of specific chain length and composition were first located using their retention time. The molecular weights at the various ionizable states were identified on the MS. For each ionizable state, isotopic peaks were identified, and the exact mass was determined and exported to HDX (Thermo Scientific). Deuterium incorporation was then calculated from the centroid provided by HDX as a function of the total number of available GlcA residues present on the chain.

Analytical Gel Permeation Chromatography to Understand the Processivity of C5-epi. A Shimadzu HPLC (Kyoto, Japan) with a refractive index detector was used. Two types of columns were used for efficient separation; a guard column 6 mm × 40 mm connected to two 7.8 mm × 300 mm columns (Waters). The mobile phase was 0.5 M lithium nitrate with a flow rate of 0.6 mL/min, and the injection volume was 20 *μ*L.

■ RESULTS

The C5-epi reaction is a key step in the synthesis of heparan sulfates and heparin. Understanding the substrate-based factors that can impact this reaction should afford an improved ability to manipulate the production of the large scale control of these

GAGs. We hypothesized that GlcNAc residues present in NSH interfere with the action of C5-epi on the GlcA residues adjacent to these GlcNAc residues. In contrast, the GlcNS residues promote C5-epi catalysis to more efficiently form IdoA residues. Herein, we investigate the impact of GlcNAc/GlcNS composition within the polysaccharide on the apparent equilibrium position of the epimerase reaction. We also probe further the effect of GlcNAc residues on the action pattern of C5-epi, using polymeric substrates with various GlcNAc/GlcNS compositions. Finally, we examine the sequential processing activity of C5-epi, i.e., the number of consecutive GlcA residues on which C5-epi was capable of acting, to test these hypotheses.

Influence of GlcNAc Residues on the Apparent Equilibrium Constant of C5-epi Catalysis. NSH, chemically synthesized from heparosan, with a range of GlcNAc residue content, was used to evaluate the influence of GlcNAc content on the apparent equilibrium constant of the C5-epi-catalyzed reaction. The apparent equilibrium constant was determined as it relates to the limit of C5-epi catalysis and not the actual thermodynamic equilibrium between the GlcA and its IdoA epimer. The content of GlcNAc residues in NSH was varied from 1 to 52%, and the molecular weight properties of these polymeric substrates are shown in Table 1. These NSH substrates were treated with 0.5 mg/mL C5-epi in D_2O and allowed to react for 17 h to reach apparent equilibrium. An additional 0.5 mg/mL C5-epi was added after 17 h to ensure that equilibrium had been reached. In no case was additional IdoA generated, indicating that the apparent equilibrium had been reached within 17 h. This results in the maximum number of IdoA residues generated in each NSH chain as a function of its GlcNAc residue content. As expected, the IdoA content decreased as the GlcNAc content of the NSH substrate increased. This result demonstrates that higher fractions of GlcNAc residues prevent C5-epi from catalyzing the epimerization reaction (Figure 1). NSH with 1 and 9% GlcNAc residues resulted in ~32% IdoA residues, consistent with the "true" thermodynamic equilibrium between GlcA and IdoA.[9,10,13] When the GlcNAc residue content was increased to 52%, the product of C5-epi catalysis contained only 8% IdoA residues. Moreover, C5-epi was inactive on the fully *N*-acetylated heparosan, consistent with the literature.[21]

C5-epi Catalysis on NSH Probed Using Heparanase BP. An NSH chain containing ~1% GlcNAc residues essentially represents a consecutive grouping of GlcA residues on which C5-epi is able to act. This NSH substrate almost completely eliminates interference on C5-epi activity by GlcNAc residues within the chain. The NSH containing ~1% GlcNAc residues was treated with 0.5 mg/mL C5-epi in D_2O for 17 h to reach apparent equilibrium, resulting in the maximum number of IdoA residues in each NSH chain. The resulting product was completely depolymerized using heparanase BP, ensuring that every GlcA residue present within each chain was cleaved. Heparanase BP is a hydrolase that cleaves the linkage between GlcA and either GlcNS or GlcNAc, but it does not cleave the linkage between IdoA and either GlcNS or GlcNAc. A schematic of this cleavage strategy is shown in Figure 2.

Analytical GPC was used to analyze the products of C5-catalyzed epimerization and heparanase BP-catalyzed depolymerization reactions (Figure 3). We expected heparanase BP-catalyzed depolymerization of NSH containing ~1% GlcNAc residues to afford monosaccharide, disaccharide, and trisac-
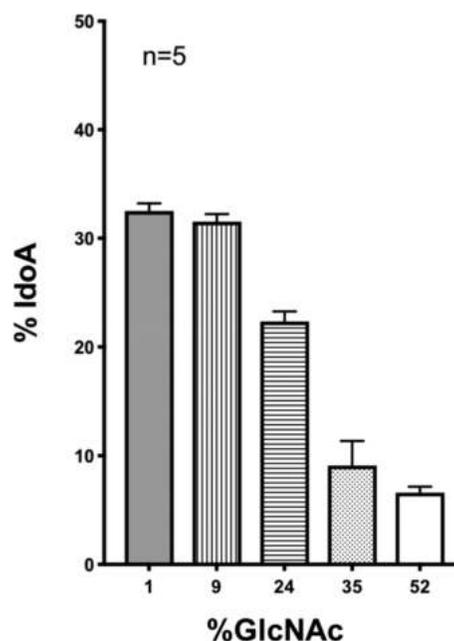

Figure 1. Influence of GlcNAc NSH content on the apparent equilibrium of C5-epi catalysis.

charide products (monosaccharides and trisaccharides from the ends of chains having an odd number of saccharide residues) (Figure 3). However, on C5-epi treatment, heparanase BP-catalyzed hydrolysis of NHS containing ~1% GlcNAc residues should form higher oligosaccharides due to the presence of recalcitrant IdoA residues within the chain (Figure 2). As anticipated, a mixture of oligosaccharides was produced from C5-epi-treated NSH containing GlcNAc residues (Figure 3A). Most of the products were GlcNS(1→4) GlcA disaccharide (~95 mol %). The remaining ~4 mol % consisted of tetrasaccharides with an internal IdoA residue (~4 mol %) with the structure GlcNS(1→4)IdoA(1→4)-GlcNS(1→4)GlcA and hexasaccharides (~1 mol %) with the structure GlcNS(1[→4]IdoA(1→4)GlcNS(1→]₂4)GlcA, having two consecutive disaccharides containing IdoA residues (Figure 3).

At apparent equilibrium, C5-epi acting on NSH containing ~1% GlcNAc results in ~30% of its sequence, comprising IdoA residues, as determined using NMR. For C5-epi-treated NSH (16 400 Da, Table 1), with a chain length of 21 disaccharide repeating units within the polymeric chain, this would, on average, result in ~6 disaccharide repeating units containing an IdoA residue. Based on GPC, this should result in three tetrasaccharides per chain, each containing a single IdoA residue, and one hexasaccharide per chain, each containing two IdoA residues. An additional monosaccharide trisaccharide may also arise from the terminus of chains having an odd number of saccharide residues. Thus, the complete heparanase BP treatment and subsequent GPC analysis are consistent with the IdoA content at apparent equilibrium. Such analysis, however, does not provide sufficient information on the polysaccharide sequence obtained following C5-epi catalysis nor insight into the action pattern of the C5-epimerase, which acts reversibly on the uronic acid residues.
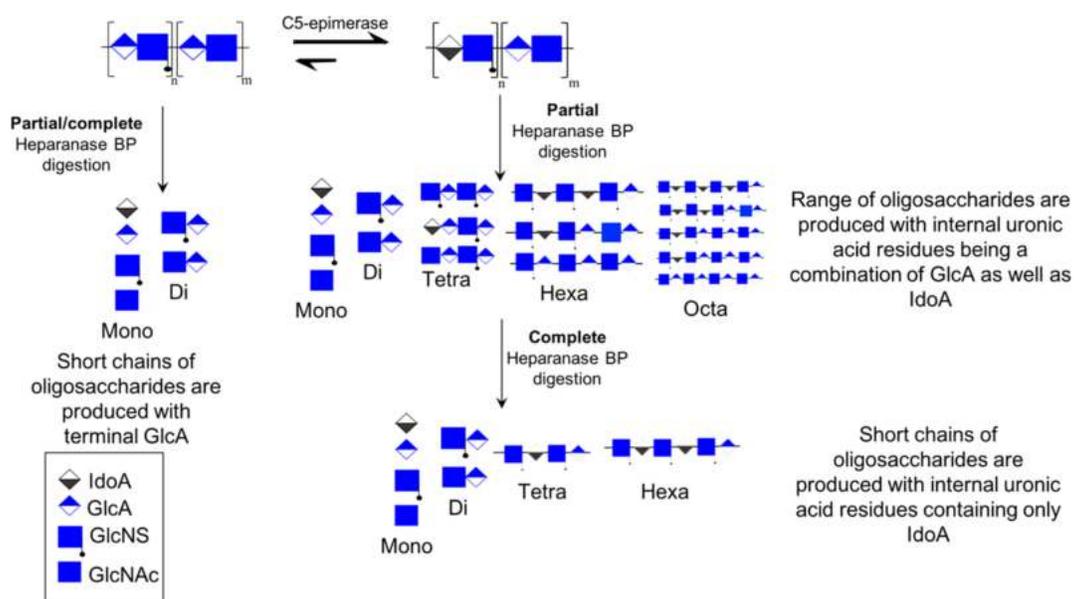
Figure 2. Proposed schematic indicating heparanase BP-catalyzed hydrolysis of NSH before and after C5-epi catalysis.
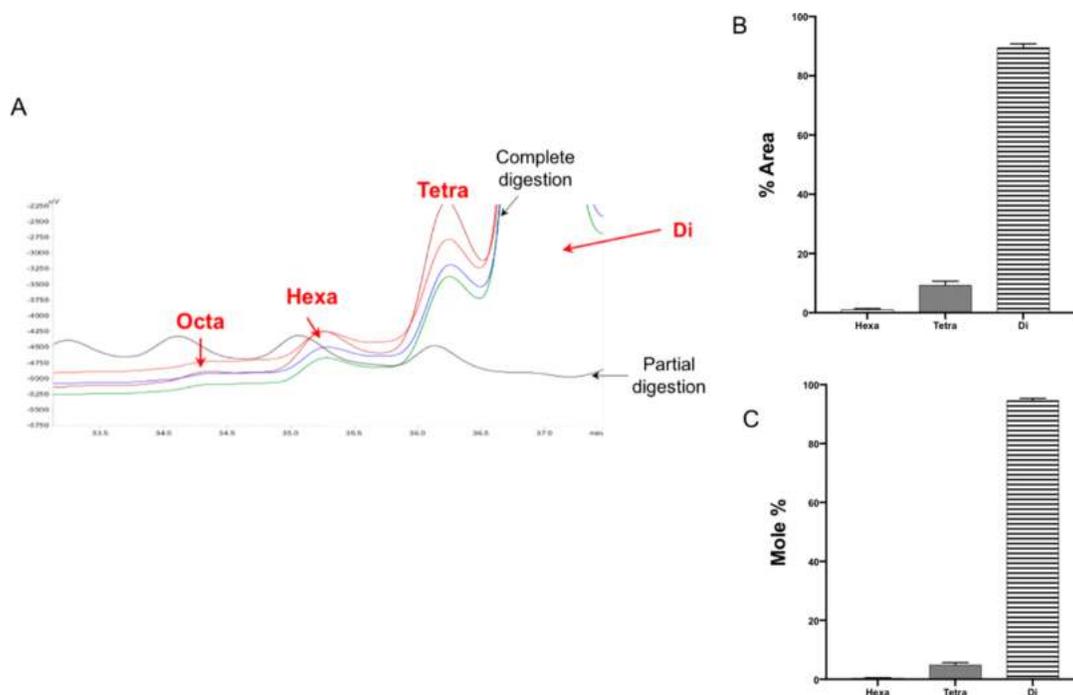

Figure 3. NSH containing ~1% GlcNAc was subject to complete depolymerization using heparanase BP. (A) Raw chromatogram showing the separation of the oligosaccharides by size. Relative amounts of oligosaccharides produced represented in (B) area % and in (C) mole %.

These results do not provide sufficient information on interference from the GlcNAc residues as this NSH substrate contained only ~1% GlcNAc residues. Nonetheless, this experiment does provide important information on the action pattern or the sequential nature of C5-epi catalysis along the polysaccharide chain. Specifically, in a substrate nearly devoid of GlcNAc residues, there would be almost no interference by these residues. Thus, all GlcA residues within a given chain

should be equally accessible to C5-epi. The observation of a tetrasaccharide being a major product of heparanase BP-catalyzed cleavage suggests that C5-epi generally only catalyzes the epimerization of a single GlcA residue to an IdoA residue before falling off the NSH substrate chain and then either finds another uronic acid residue on a second chain or a distant residue on the same chain before acting again. Since a small amount of hexasaccharide is also observed after complete
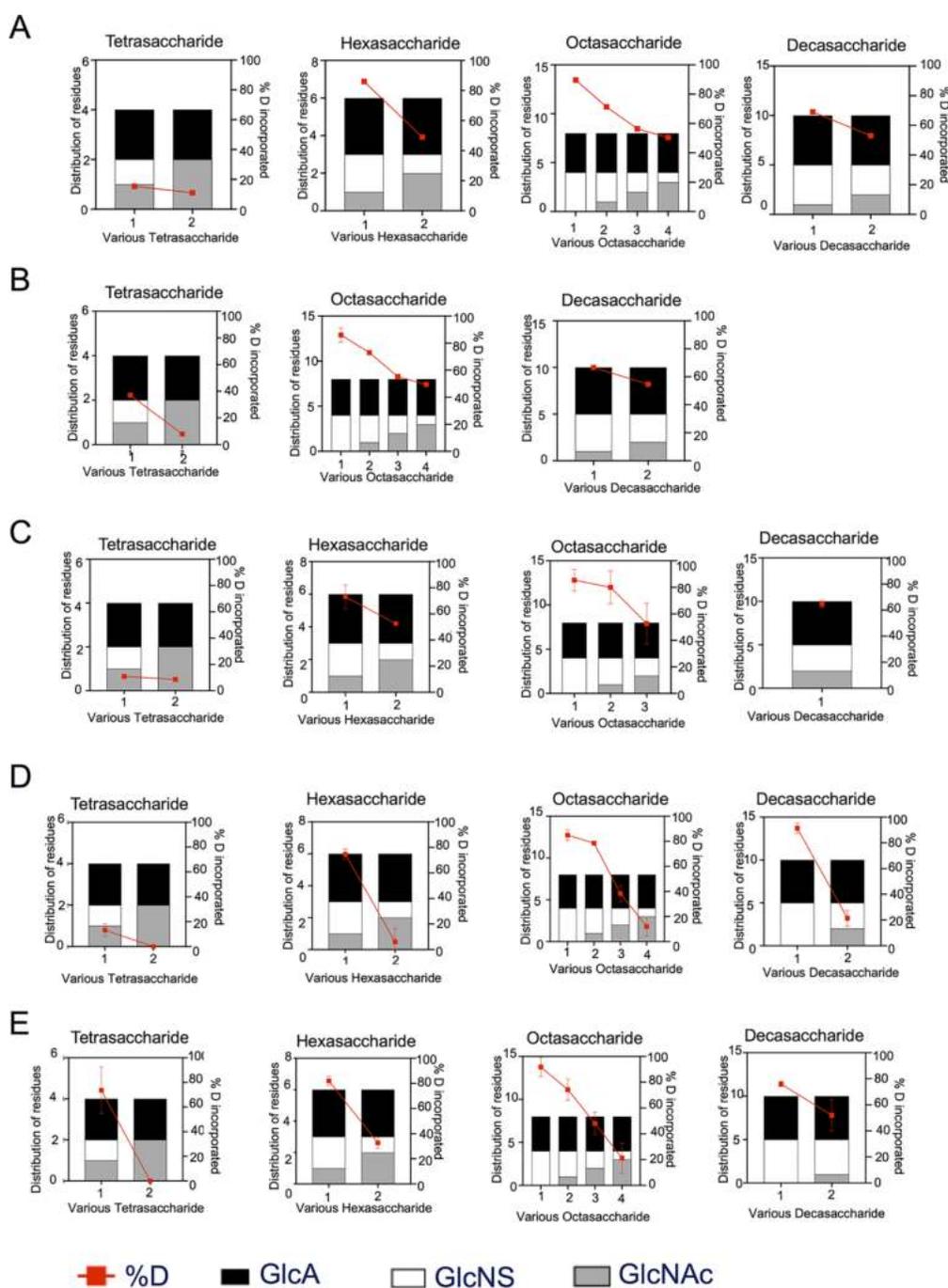
Figure 4. Oligosaccharides produced after partial heparanase BP depolymerization further analyzed using LC−MS and epimerase-mediated deuterium incorporation. (A) 52% GlcNAc, (B) 35% GlcNAc, (C) 24% GlcNAc, (D) 9% GlcNAc, and (E) 1% GlcNAc NSH substrate.

heparanase BP-digestion of NSH containing 1% GlcNAc residues, C5-epi can also bind to an NSH chain, act on a GlcA residue, and then occasionally move down the chain to act on an adjacent GlcA residue, affording two IdoA residues in a row to give rise to the small fraction of hexasaccharide observed.

Partial NSH Digestion to Elucidate the Action Pattern of C5-epi. Heparanase BP was next used to partially digest the

NSH product chain and provide insight into the influence of the percentage of GlcNAc residues in an NSH chain on the action of C5-epi (Figure 2). In partial digestion, heparanase BP affords longer product chains in which the internal uronic acid residues could be either GlcA or IdoA with the reducing terminal residue always being GlcA. Carbohydrate gel analysis was first performed to assess the impact of C5-epi catalysis on

different NSH substrates, containing varying levels of GlcNAc residues, followed by partial hydrolysis using heparanase BP. In each case, oligosaccharides of various sizes were produced on partial digestion with heparanase BP, as shown by initial carbohydrate gel analysis and confirmed by LC−MS analysis. NSH having different percentages of GlcNAc residues not treated with C5-epi and partially digested with heparanase BP were run as a control. Gel electrophoresis could not resolve monosaccharides and disaccharides due to their small molecular size (Figure S1). The partial heparanase BP-digested samples produced a range of oligosaccharide sizes as observed in carbohydrate gel analysis (Figure S1).

The partially heparanase BP-digested, C5-epi-treated, and untreated samples were next analyzed using top-down LC−MS analysis, in which carbohydrate sequence data could be obtained. This analysis is capable of providing a better understanding of the influence of GlcNAc content on C5-epi activity. Following partial digestion with heparanase BP, a range of oligosaccharides was produced with internal GlcA or IdoA residues as well as a reducing-end terminal GlcA residue (Figure 2). The oligosaccharides that can be produced with all possible combinations of GlcA, GlcNS, and GlcNAc residues, the molecular weights of these oligosaccharides, and their molecular weights in various ionizable states are provided in Table S1. The C5-epi reaction was performed in D_2O to quantify the activity of C5-epi by relying on epimerase-mediated deuterium incorporation, again followed by partial heparanase BP treatment and LC−MS analysis, and the amount of deuterium incorporated was calculated for each oligosaccharide produced (Figure 4).

Each panel within Figure 4 represents the analysis for the NSH substrate having a different percentage of GlcNAc residues. For example, Figure 4A shows the analysis of the NSH with the highest content of GlcNAc residues (52% GlcNAc residues), treated with C5-epi, and then subject to partial depolymerization with heparanase BP. Within each panel of Figure 4A, four oligosaccharides of different sizes (tetrasaccharides to decasaccharides) were analyzed in five experimental and five technical (analytical) replicates. Oligosaccharides of a specific size were analyzed, maintaining a constant number of GlcA residues. For example, when tetrasaccharides were analyzed, only those tetrasaccharides with two GlcA residues were considered. Similarly, for hexasaccharides, only those with three GlcA residues were analyzed, and so forth. This was done to maintain a constant number of available GlcA residues with respect to C5-epi and, therefore, to allow any resulting product to be a factor of the glucosamine residues, either GlcNAc or GlcNS, at each oligosaccharide chain length. Within each plot, the x-axis represents the various oligosaccharides of a specific size available for analysis. The bar graphs represent the composition of a given oligosaccharide, indicated in the left y-axis. Since the number of GlcA residues remain constant in all size groups, the types of glucosamine residues (i.e., GlcNS or GlcNAc) vary. The red line in the plot represents the percent deuterium incorporated as indicated in the right y-axis.

In a second example, the analysis of the 9% GlcNAc NSH substrate was treated with C5-epi and partially digested with heparanase BP (Figure 4D). In this figure, four oligosaccharides of different sizes were considered (tetrasaccharide to decasaccharides). The bar graphs at each chain length again represent various combinations of GlcNS and GlcNAc analyzed using analysis. In this example, all octasaccharides

with four GlcA residues were analyzed, but each had varying contents of GlcNS and GlcNAc. A downward trend was observed with increasing GlcNAc residues present within a chain of a given size. Similar trends were observed in the other oligosaccharide sizes produced on partial depolymerization of NSH substrate 9% GlcNAc.

Similar analyses were performed using each of the NSH substrates having various percentages of GlcNAc residues (Figure 4). An overall trend was observed of decreasing deuterium incorporation with increasing GlcNAc content within oligosaccharide chains produced after partial depolymerization using heparanase BP. This was the case for all of the substrates studied and for oligosaccharide sizes analyzed for each of these substrates (Figure 4). It is noteworthy that the fraction of deuterium incorporated does not reflect the fraction of IdoA in each chain, since C5-epi catalyzes a reversible reaction. Thus, it is possible that C5-epi may catalyze the conversion of GlcA to IdoA and then catalyze the conversion of IdoA to GlcA in the same position. This would result in a GlcA residue with an incorporated deuterium at its C5 position. This could be considered as a reversible action. Therefore, the total IdoA residues produced on a chain may be constant at apparent equilibrium (as shown in Figure 1), but the % of deuterium incorporated within this chain can be considerably higher due to the reversible conversion of the uronic acid residues within the chain. Nonetheless, since C5-epi is unable to act on heparosan, these results demonstrate that GlcNAc residues interfere with the action of C5-epi on NSH, as those oligosaccharides with a greater number of GlcNAc residues (or no GlcNS residues) have reduced (<8%) deuterium incorporation. The results from LC−MS analysis depicted in Figure 4 indicate that, after partial digestion with heparanase BP, the short stretches of GlcNAc residues within the polysaccharide chain interfere with the activity of C5-epi, perhaps even promoting the reversible generation of GlcA from an initially generated IdoA. This is directly reflected in the low % deuterium incorporated in the oligosaccharides of various chain lengths from various substrates (range of % GlcNAc residues) but with the same number of uronic residues. Although these analyses do not consider the number of irreversible IdoA residues produced, as previously reported for oligosaccharide substrates,[14] the results do suggest that C5-epi is unable to react with such uronic acid residues even to reversibly catalyze their conversion from GlcA to IdoA.

■ DISCUSSION

We have investigated the processivity of C5-epi on polymeric NSH substrates with different percentages of GlcNAc residues. We have also investigated the impact of GlcNAc residues within a substrate chain on the action of C5-epi. Finally, catalysis was also examined using C5-epi-mediated deuterium incorporation using 1D ^1H NMR to obtain the IdoA and GlcA content of the polysaccharide products. Heparanase BP, a recently discovered β-glucuronidase[17] that cleaves specifically at GlcA residues,[16] was used to understand the influence of NSH sequence and composition on the processivity of C5-epi. Partial and complete digestion of products with heparanase BP afforded sequence-specific information on C5-epi catalysis.

The apparent equilibrium constant of the reaction, determined using 1D ^1H NMR, was found to decrease with an increased percentage of GlcNAc residues within the NSH substrate. This result is in agreement with previous studies performed on oligosaccharide substrates that suggest GlcNAc
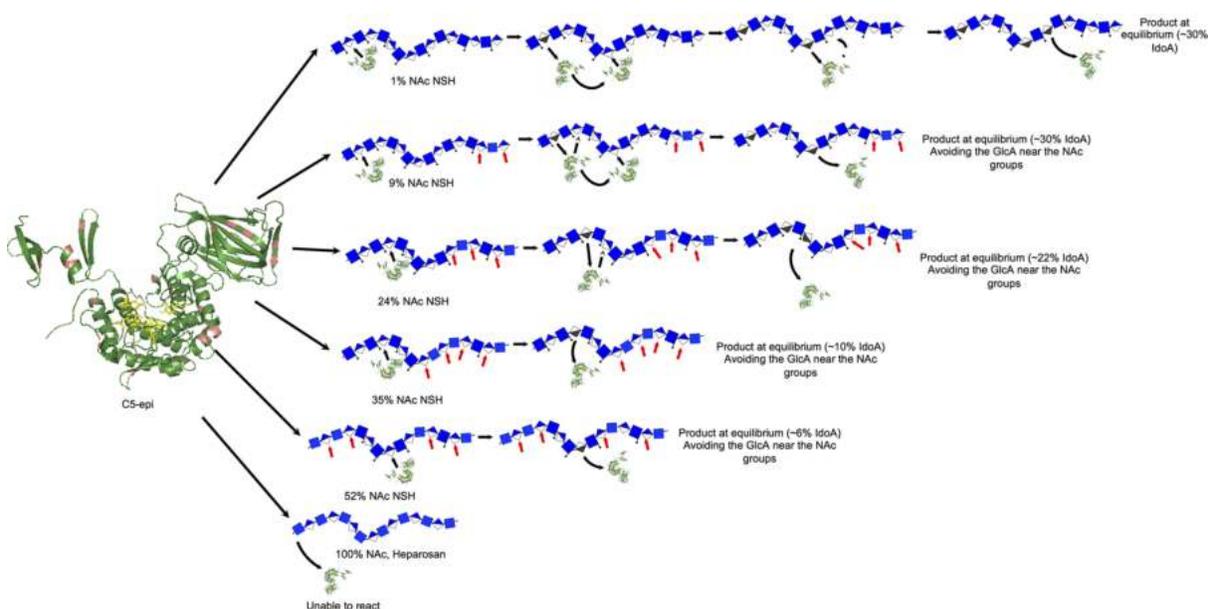
Figure 5. Proposed mechanism of C5-epi processivity on NSH substrates. NSH with 1% NAc to produce ~30% IdoA at equilibrium; 9% GlcNAc NSH to produce ~30% IdoA at equilibrium, 24% GlcNAc NSH to produce ~20% IdoA; 52% GlcNAc NSH to produce ~10% IdoA; 100% GlcNAc, heparosan to produce ~10% IdoA. Mechanism of the reaction avoiding the GlcA residues adjacent to or near the GlcNAc groups within the chain (red arrows). Dashed arrows provide an infrequent sliding of C5-epi acting on two GlcA residues.

residues interfere with the action of C5-epi.[22] The NSH substrate with ~1% GlcNAc residues was used to eliminate most of the interference from GlcNAc residues to further elucidate the sequential action pattern of C5-epi. The product of the C5-epimerization reaction was completely digested with heparanase BP. This ensured that all oligosaccharides produced contained a reducing terminal GlcA and that all the internal uronic acid residues were IdoA. These experiments provide information on the number of consecutive GlcA residues that could be irreversibly converted to IdoA. While this analysis does not afford information on the number of residues that are reversibly acted upon by C5-epi, some information can be gleaned on the sequential action of C5-epi. Our analysis suggests that C5-epi can act on, at most, two consecutive disaccharide sequences containing GlcA before releasing its substrate chain. The action at two adjacent sites, however, is less frequent than C5-epi, acting at a single, isolated site containing a GlcA residue.

NSH substrates with a range of GlcNS and GlcNAc residues were used to understand the impact of GlcNAc residues on the C5-epi catalysis. The epimerization reaction was performed in D_2O to determine C5-epi-mediated deuterium incorporation. Partial digestion was then performed using heparanase BP, affording a distribution of oligosaccharides of different sizes with reducing terminal GlcA residues and internal uronic acid residues consisting of either GlcA or IdoA. Analysis performed by gel electrophoresis showed the size range of the oligosaccharides produced. LC−MS top-down analysis was performed on products from each NSH substrate with varying content of GlcNAc residues. Oligosaccharides of various sizes afforded by partial digestion were analyzed, at various ionizable states, to determine the incorporation of deuterium as a function of oligosaccharide composition. Decreasing deuterium incorporation was observed with an increasing fraction of

GlcNAc residues within these oligosaccharide chains. This trend was observed for all NSH substrates examined in this study. Since C5-epi catalyzes a reversible reaction, the percentage of deuterium does not correspond to the conversion of GlcA into IdoA, yet there was a trend showing decreasing deuterium incorporation with increasing GlcNAc content. This is the case even with the same number of available GlcA residues within the chain on which C5-epi can act. It should be noted that those oligosaccharide chains with almost no GlcNS residues and only GlcNAc residues have <5% deuterium incorporation irrespective of chain length or overall composition of the oligosaccharide chain (Figure 4). These results confirm that GlcNAc residues present within a chain interfere with C5-epi catalysis, consistent with reports in the literature.[12] While the mechanism underlying the lack of reactivity of uronic acid residues adjacent to GlcNAc residues remains unclear, we hypothesize that this interference may be sterically driven by restricting substrate availability to the active site of the C5-epi.

Herein, we have uncovered the action pattern of C5-epi acting on a polymeric NSH substrate, where only a small number of adjacent GlcA residues can react. Our complete heparanase BP digestion studies that rely on gel permeation chromatography suggest that C5-epi most frequently acts only on a single GlcA residue before disengaging with its substrate. Occasionally, two adjacent GlcA residues are converted to IdoA by C5-epi. On the basis of these results, we propose a model for C5-epi processivity on the polymeric NSH substrate (Figure 5). All of the substrates used in this study are displayed in Figure 5 to represent the impact of GlcNAc residues on the action pattern of C5-epi. The C5-epi shown in Figure 5 is based on a homology model of the engineered enzyme used in this study against the 3D structure of C5-epi in zebra fish.[9] In the case of NSH without GlcNAc residues, C5-epi acts on one

GlcA residue at a time converting it to IdoA but infrequently acts on two adjacent GlcA residues. This information obtained using gel permeation chromatography after complete heparanase BP digestion results in hexasaccharides, represented by the dashed arrows present in Figure 5. Most frequently, C5-epi acts on a single GlcA converting to IdoA and either disengages with this chain or moves further away to act on the next GlcA residue. Next, the NSH substrate containing 9% GlcNAc residues is represented, affording ∼30% IdoA at equilibrium. A similar action pattern is shown (Figure 5), and again C5-epi avoids GlcA residues near or adjacent to the GlcNAc residues within the chain (red arrows). Analysis after partial digestion with heparanase BP also demonstrates the infrequent action of C5-epi on adjacent GlcA residues. This action pattern is observed through all the substrates used in this study.

In summary, we exploited the unique specificity of the heparanase BP enzyme to elucidate the processivity of C5-epi as well as the influence of GlcNAc residues within an NSH substrate. Carbohydrate gel analysis, gel permeation chromatography, and LC−MS analysis afforded sequence information on the activity of C5-epi. The information obtained on polysaccharide substrates provides additional insight into the mechanism of C5-epi, which may aid in optimizing the *in vitro* large-scale production of bioengineered heparin and heparan sulfate.

◼ ASSOCIATED CONTENT

🔵Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biochem.0c00419.

> Possible oligosaccharides detected using top-down LC−MS analysis; carbohydrate gel analysis of NSH untreated with C5-epi (PDF)

Accession Codes

Accession identification number for C5-epimerase: UniProt O94923.

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Author Contributions

The study was performed by D.V., X.K., and Y.Y., and the manuscript was written by D.V., J.S.D., and R.J.L. All authors have given their approval to the final version of this manuscript.

Notes

The authors declare no competing financial interest.

◼ ABBREVIATIONS

C5-epi, glucuronyl C5-epimerase; 2OST, 2-*O*-sulfotransferase; S, sulfo; NSH, *N*-sulfoheparosan; LC−MS, liquid chromatography−mass spectrometry; GlcA, glucuronic acid; IdoA, iduronic acid; GlcNAc, *N*-acetylglucosamine; GlcNS, *N*-sulfoglucosamine; GAG, glycosaminoglycan; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; D-MES, deuterated 2-(*N*-morpholino) ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid

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