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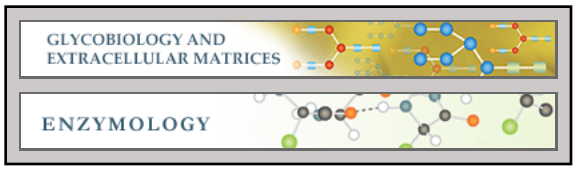

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Structure/Function Analysis of *Pasteurella multocida* Heparosan Synthases

TOWARD DEFINING ENZYME SPECIFICITY AND ENGINEERING NOVEL CATALYSTS[*S]

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Background: The *Pasteurella* heparosan synthase isozymes are highly homologous yet have different catalytic properties.
Results: Chimeric enzymes bearing combinations of desirable traits were produced, and structure/function relationships were studied.
Conclusion: Distinct regions of the isozymes are important for high specific activity, sugar analog usage, and chain initiation and elongation.
Significance: New catalysts with utility for production of defined heparan sulfate polysaccharides were created.

The *Pasteurella multocida* heparosan synthases, PmHS1 and PmHS2, are homologous (~65% identical) bifunctional glycosyltransferase proteins found in Type D *Pasteurella*. These unique enzymes are able to generate the glycosaminoglycan heparosan by polymerizing sugars to form repeating disaccharide units from the donor molecules UDP-glucuronic acid (UDP-GlcUA) and UDP-*N*-acetylglucosamine (UDP-GlcNAc). Although these isozymes both generate heparosan, the catalytic phenotypes of these isozymes are quite different. Specifically, during *in vitro* synthesis, PmHS2 is better able to generate polysaccharide in the absence of exogenous acceptor (*de novo* synthesis) than PmHS1. Additionally, each of these enzymes is able to generate polysaccharide using unnatural sugar analogs *in vitro*, but they exhibit differences in the substitution patterns of the analogs they will employ. A series of chimeric enzymes has been generated consisting of various portions of both of the *Pasteurella* heparosan synthases in a single polypeptide chain. *In vitro* radiochemical sugar incorporation assays using these purified chimeric enzymes have shown that most of the constructs are enzymatically active, and some possess novel characteristics including the ability to produce nearly monodisperse polysaccharides with an expanded range of sugar analogs. Comparison of the kinetic properties and the sequences of the wild-type enzymes with the chimeric enzymes has enabled us to identify regions that may be responsible for some aspects of both donor binding specificity and acceptor usage. In combination with previous work, these approaches have enabled us to better understand the structure/function relationship of this unique family of glycosyltransferases.

The GAG[2] synthases that are responsible for forming the polysaccharide capsule of different serotypes of the pathogenic bacteria *Pasteurella multocida* have been identified. The enzymes PmHAS (Carter Type A), PmCS (Type F), and PmHS1 (Type D) are responsible for forming hyaluronan, chondroitin, or heparosan capsule, respectively (1). PmHS2, which synthesizes heparosan *in vitro*, is also found in multiple serotypes of *Pasteurella* (Types A, D, and F) and has been hypothesized to be responsible for capsule switching (2). In all cases identified thus far, the disaccharide repeats that constitute these GAGs are synthesized by glycosyltransferases, which use UDP-sugar nucleotide donor molecules according to the reaction

$$n\ \text{UDP-GlcUA} + n\ \text{UDP-HexNAc} \rightarrow 2n\ \text{UDP} + (\text{GlcUA-HexNAc})_n$$

where HexNAc = *N*-acetyl-glucosamine (GlcNAc) or *N*-acetyl-galactosamine (GalNAc).

Each of the GAGs generated by these synthases are identical or similar to the GAGs found in vertebrates and are postulated to act as a form of "molecular camouflage" for enhancing infection of *Pasteurella* (1). Typically, anti-capsule antibodies are effective host weapons, but GAGs are a difficult target to defend against due to their "self" nature.

The *Pasteurella* heparosan synthases are bifunctional glycosyltransferases that consist of two domains that have significant similarity to the *Escherichia coli* K5 monofunctional glycosyltransferases, KfiA and KfiC, which together are responsible for generating the heparosan (*N*-acetylheparosan or the unmodified heparin backbone) capsule of *E. coli* K5 (3, 4). KfiC is a CAZy (Carbohydrate Active enZymes database)

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[S] This article contains supplemental Figs. 1–4 and Tables 1 and 2.
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[2] The abbreviations used are: GAG, glycosaminoglycan; $6N_3$-GlcNAc, 6-deoxy, 6-azido-*N*-acetylglucosamine; PmCS, *P. multocida* chondroitin synthase; HA, hyaluronan; PmHAS, *P. multocida* hyaluronan synthase; PmHS, *P. multocida* heparosan synthase; HexN, hexosamine; GlcN-TFA, *N*-trifluoroacetyl-glucosamine.

GT2 family GlcUA-transferase, whereas KfiA is a GT45 family GlcNAc-transferase.

PmHAS and PmCS are also bifunctional glycosyltransferases, each consisting of two CAZy GT2 family domains (containing 22,514 predicted and 255 characterized members as of 2011) (5). Each of these synthases have similarity to the *E. coli* K4 chondroitin polymerase for which there is a crystal structure available (6). The structure and activity of the *Pasteurella* heparosan synthases are less well studied. There are only five biochemically characterized members with the GT45 family GlcNAc-transferase component (containing 15 total members) found in their domains. The GlcUA-transferase domain, located at the N terminus of the PmHS synthases, is a GT2 family member. The corresponding, but rather dissimilar (~30% protein sequence identity), functional domains of both PmHAS and PmCS reside at the C terminus.

In lieu of specific three-dimensional structural information for PmHS1 or PmHS2, we explored the possibility of threading the *Pasteurella* heparosan synthase sequences onto existing protein structures, but appropriate candidates with suitable sequence similarity are not apparent. Our attempts to dissect the bifunctional heparosan synthases into two polypeptides each with an active glycosyltransferase component (*i.e.* either GT2 or GT45) by molecular genetic means or partial proteolysis were unsuccessful; therefore, our annotation of the boundaries of the domains are approximate. The high degree of sequence identity between PmHS1 and PmHS2 (supplemental Fig. 1), along with their distinct catalytic abilities to initiate polysaccharide chains and utilize unnatural UDP-sugar analogs (7), affords a unique opportunity to study the structure/function relationship of these synthases.

Each of the PmHS1 and PmHS2 glycosyltransferases possess a desirable catalytic property or "phenotype" that the other enzyme lacks (7). In addition to the structure/function insights gained in this work, our chimeric polypeptide approach to studying these enzymes has also enabled us to generate mutant enzymes possessing multiple desirable traits for the chemoenzymatic synthesis of GAGs. We present here the generation of novel catalysts that are able to transfer two useful UDP-sugar analogs, as well as possessing enhanced transferase activity and the ability to generate longer, more monodisperse polysaccharides containing these sugar analogs. As a demonstration, we have also synthesized polysaccharides containing both natural and analog sugar "blocks" with potential utility for producing mimics of heparan sulfate/heparin polysaccharides.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were purchased from Sigma-Aldrich or Fisher Scientific unless otherwise noted. Custom synthetic DNA oligonucleotides were from Integrated DNA Technologies (Coralville, IA). Various HA molecular weight standards were obtained from Hyalose, LLC (Oklahoma City, OK) (8).

Cloning and Expression of PmHS Chimeras—Chimeric enzymes were generated using gene splicing by overlap extension (9) using *Pfu* Ultra DNA polymerase (Agilent Technologies, Santa Clara, CA). A previously generated construct containing maltose-binding protein fusion of PmHS2 (7) and an *E. coli* codon-optimized PmHSI (Genscript) were used for the template DNAs, and constructs were produced using the primers noted (supplemental Tables 1 and 2). PCR products resulting from the gene splicing reactions (supplemental Fig. 2) were cloned into the pMAL-c4e vector (New England Biolabs, Ipswich, MA) according to manufacturer's instructions and transformed into *E. coli* TOP-10F′ cells with selection on LB/ampicillin plates at 30 °C. The plasmids of transformants were screened by restriction digest, and those with the correct insert were confirmed by DNA sequencing of both strands (Oklahoma Medical Research Foundation sequencing facility). Plasmids were then transformed into the production host, phage lysin-expressing freeze/thaw lysis *E. coli* XJa cells (Zymo Research, Orange, CA).

For protein production, cultures of recombinant *E. coli* XJa in Superior Broth (AthenaES, Baltimore, MD) with ampicillin (50 μg/ml), carbenicillin (50 μg/ml), and L-arabinose (3.25 mM final; to induce the lysin enzyme) were grown at 30 °C. Expression of target protein was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (0.2 mM final) when the culture A_{600} reached 0.35. At 1 h after induction, growth was supplemented with fructose (12.8 mM final), and growth proceeded for ~16 h before cells were harvested by centrifugation (3000 × *g*, 30 min at 4 °C). The cell pellet was resuspended in 50 mM Tris, pH 7.2, with protease inhibitors *p*-(4-2-aminoethyl)benzenesulfonyl fluoride, pepstatin, benzamidine, *N*-(*N*-(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl)-agmatine, and leupeptin on ice and subjected to two freeze/thaw cycles to allow the phage lysin to degrade the cell walls. The lysates were then clarified by centrifugation (20,000 × *g*, 30 min at 4 °C). Protein content was measured by the Bradford assay with a BSA standard (Thermo Fisher Scientific).

The maltose-binding protein-tagged chimeric proteins were purified by affinity chromatography on immobilized amylose beads according to the manufacturer's guidelines (New England Biolabs, Ipswich, MA). After ultrafiltration (Amicon Ultra 10 kDa, Millipore, Billerica, MA) and buffer exchange into 20 mM Tris, pH 7.2, the purified proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by staining with Coomassie Blue dye or by Western blotting with detection using an anti-PmHS primary antibody that recognizes a common PmHS1/PmHS2 sequence (2).

Synthesis of UDP-Sugar Analogs—UDP-*N*-trifluoroacetylglucosamine (UDP-GlcN-TFA) was synthesized using chemoenzymatic methods as described (10). The synthesis of UDP-6-deoxy, 6-azido-*N*-acetylglucosamine (UDP-6N_3-GlcNAc), began with a selective tosylation at the C-6 position of GlcNAc α-benzyl glycoside (11). Acetylation followed by hydrogenolysis generated the free anomeric hydroxyl, which was phosphorylated using phosphoramidite chemistry. Removal of all protecting groups gave 6-tosyl-α-GlcNAc 1-phosphate, and treatment with sodium azide in *N*,*N*-dimethylformamide introduced the 6-azido functionality. A final coupling with UMP-morpholidate gave the product UDP-6-deoxy, 6-azido-*N*-acetylglucosamine, which was stored as the disodium salt.

Glycosyltransferase Activity Assays—Radiolabeled sugar incorporation assays (25-μl reactions, 30 °C for the times noted) were performed using purified enzymes (2–12 μg of

protein, as noted) in the presence of 10 mM UDP-GlcUA with radioactive precursor (UDP-[^{3}H]GlcUA, 0.2 μCi per assay, PerkinElmer Life Sciences) and various amounts as noted of UDP-GlcNAc, UDP-GlcN-TFA, or UDP-6N_3-GlcNAc. An assay with no second hexosamine precursor was used as a negative control. The reaction buffer was 50 mM Tris, pH 7.2, 1 mM $MnCl_2$. As noted, extensively sonicated Type D *P. multocida*-derived heparosan (sonicated 60 min on ice to increase the number of termini; average size ~30 kDa) was used as an acceptor.

Reactions were incubated at the indicated temperatures for the times noted and then stopped with 2% final SDS and separated by descending paper chromatography (overnight in 65:35 ethanol/1 M ammonium acetate buffer, Whatman 3MM paper). This method enables the separation of GAG polysaccharides with greater than ~14 sugar units, which remain at the origin, from smaller oligomers and unincorporated nucleotide sugars that migrate down the strip. The origin of the strip was cut out and subjected to liquid scintillation counting (Biosafe, RPI Corp., Mount Prospect, IL).

Single Sugar Addition Reactions—Single sugar incorporation assays were performed using 2 mM heparosan tetrasaccharide as an acceptor (GlcUA-GlcNAc-GlcUA-anhydromannitol; prepared as in Ref. 12). Donor nucleotide sugars (UDP-GlcNAc, UDP-6N_3-GlcNAc, or UDP-GlcN-TFA) were used at 10 mM. Reactions were performed in the presence of 50 mM Tris, pH 7.2, 1 mM $MnCl_2$, and 0.2 μg/μl of the enzyme noted for 16 h at 30 °C. Reaction products were assessed by matrix-assisted laser desorption/ionization time of flight (MALDI-ToF) mass spectrometry (reflector negative mode using an Ultraflex II instrument, Bruker Daltonics, Billerica, MA), with the matrix 6-aza-2-thiothymine at a concentration of 5 mg/ml in 50% acetonitrile, 0.1% trifluoroacetic acid (TFA). HA oligosaccharides were employed as mass calibrants (Hyalose, LLC, Oklahoma City, OK). Spectra were analyzed using flexAnalysis version 2.4 (Bruker Daltonics, Billerica, MA).

Block Polysaccharide Synthesis—Polysaccharides containing alternating blocks of GlcNAc- and GlcN-TFA-based repeats were synthesized by a series of successive addition reactions. Reactions contained 50 mM Tris, pH 7.2, 1 mM $MnCl_2$ buffer. Each stage was incubated at 30 °C for 16 h. The first reaction had 400 μM heparosan tetrasaccharide (GlcUA-GlcNAc-GlcUA-anhydromannitol) as an acceptor. All reactions received 10 mM UDP-GlcUA and, depending on the desired block, either 10 mM UDP-GlcNAc or 10 mM UDP-GlcN-TFA. Each reaction received 0.5 μg/μl Chimera G enzyme. After each step, polysaccharide products were purified from the unincorporated UDP-sugars and UDP using ultrafiltration (Amicon Ultra 3 kDa, three times with 500-μl water rinses). These polysaccharide intermediates were then used as acceptor (as noted) in reactions where the hexosamine donors were alternated (*e.g.* after GlcNAc was added in first block, UDP-GlcN-TFA was then employed) as indicated to produce bipartite or tripartite polysaccharide products.

RESULTS AND DISCUSSION

Chemoenzymatic Synthesis of Novel GAG Structures—We were able to generate unique GAG pentasaccharides by extending tetrasaccharides using unnatural UDP-sugar analog donor molecules (Fig. 1). PmHS1 was able to utilize the donor molecule UDP-6N_3-GlcNAc to add 6N_3-GlcNAc to GlcUA-terminated heparosan tetrasaccharides. PmHS2 could transfer GlcN-TFA from the donor UDP-GlcN-TFA to the same heparosan tetrasaccharides. In contrast, PmHS1 incorporates the GlcN-TFA analog very poorly, whereas PmHS2 is unable to use the 6N_3-GlcNAc analog (as shown later). Chimera G is able to transfer both GlcN-TFA and 6N_3-GlcNAc as well as the natural hexosamine sugar. These unnatural sugar analogs have functional chemical groups, making them useful for a wide range of applications ranging from biomaterials to therapeutics (Fig. 2). Demonstrations of the utility of analog functionalities in novel heparosan oligosaccharides are shown in supplemental Figs. 3 and 4. For the TFA analog-containing oligosaccharide, the protecting group was removed to expose the free amine (while preserving other existing acetyl groups), which was subsequently reacted with an isothiocyanate dye (supplemental Fig. 3). For the azide analog-containing oligosaccharide, a biotin click reagent was installed (supplemental Fig. 4).

Specific Activity of Chimeric Synthases—The specific activity of the wild-type PmHS2 synthase for polymerization of heparosan in the presence of an exogenous acceptor is ~50–100-fold lower than that of PmHS1 (Fig. 3). It is of interest to determine which regions of these synthases account for this difference. Previous work has indicated that both the PmHS1 and PmHS2 GlcUA-transferase components operate at a higher rate than their GlcNAc-transferase counterparts (13, 14). Of the chimeras that are active, enzymes containing portions of the PmHS1 GlcUA-transferase domain (PmHS1$^{1-318}$) exhibit markedly higher rates of polymerization than those with portions of the PmHS2 GlcUA-transferase, although none of the chimeric enzymes achieved the rate of polymerization of wild-type PmHS1. Three of the 10 chimeric enzymes failed to have detectable activity (Chimeras C, F, and H); this observation may be due to stability issues for those constructs or the loss of important residues or structures required for function. Notably, two of the inactive chimeras contained the same splice site at the C-terminal of the protein where the amino acid sequence of PmHS1 switches to PmHS2 (Chimeras C and H).

Acceptor Usage by Chimeric Synthases—The minimal oligosaccharide required for binding as an efficient acceptor for PmHS1 is not well characterized, but PmHAS employs hyaluronan trisaccharide and tetrasaccharide acceptors rather efficiently (15). However, it is clear that heparosan tetrasaccharide acceptors increase the overall rate of PmHS1-catalyzed polymer formation. This acceptor-stimulating effect on PmHAS and PmHS1 is postulated to be due to bypassing the relatively slow initiation phase of synthesis (coupling the first monosaccharide to a UDP-sugar) and allowing the much more rapid elongation phase to proceed.

The initial polymerization rate of PmHS1 in the presence of exogenous acceptor is ~350-fold higher than in reactions without acceptor. PmHS2 activity is also boosted by the presence of acceptor, but to a much lower extent (~4-fold). Previous work using electrophoretic gel analysis of *in vitro* polymerization reactions has shown that, in the presence of acceptor, PmHS1 appears to prefer to elongate existing polysaccharide chains

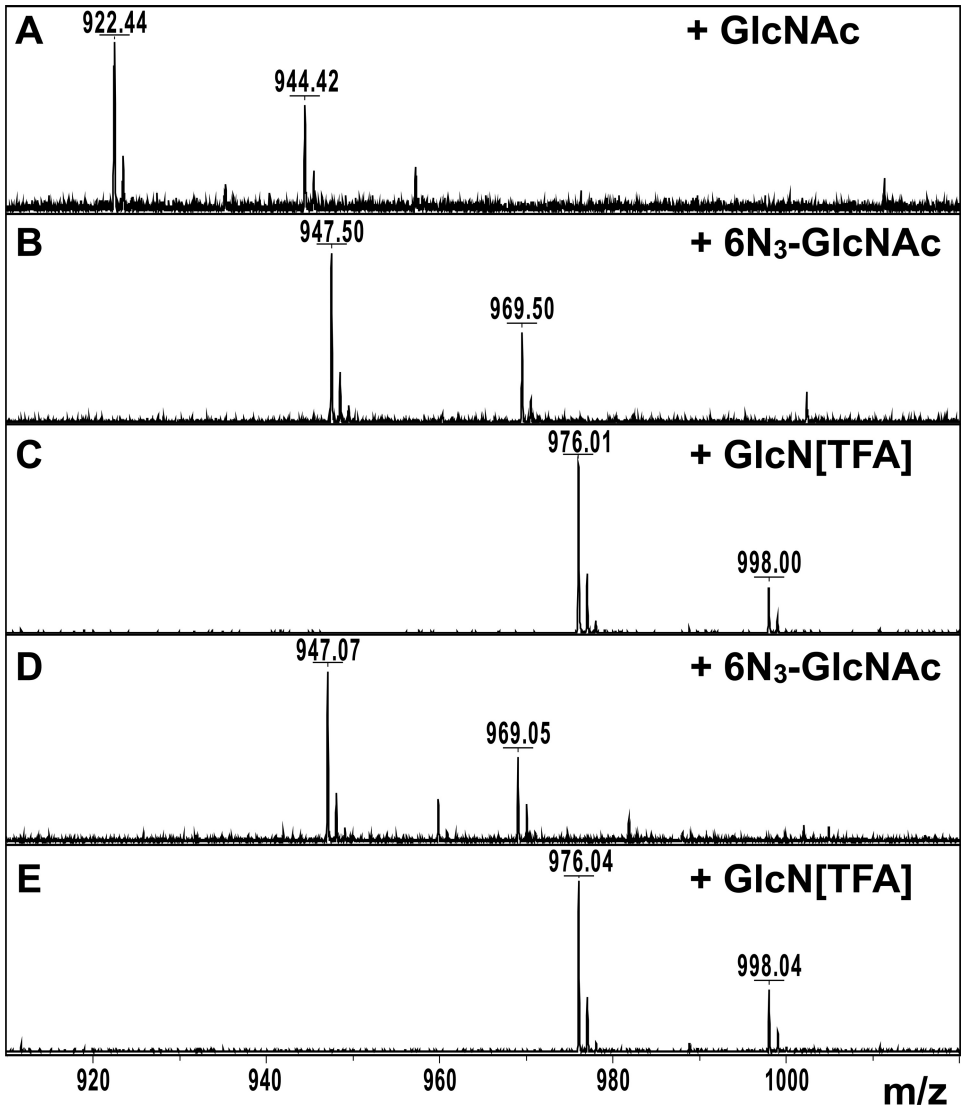


FIGURE 1. **MALDI-ToF mass spectra of single sugar addition reaction products.** The starting heparosan tetrasaccharide (molecular mass = 718.90 Da) was extended *in vitro* with GlcNAc (+ 203.08 Da), $6N_3$-GlcNAc (+ 228.14 Da), or GlcN-TFA (+ 257.13 Da) residues using the synthases as noted (note: the minor peak at 22 Da greater than the major peak is the sodium form). *A* and *B*, PmHS1 is able to extend heparosan tetrasaccharide with either GlcNAc (*A*) or $6N_3$-GlcNAc (*B*). *C*, PmHS2 is able to add GlcNAc (not shown) or GlcN-TFA. *D* and *E*, Chimera G discussed in this work is able to add either $6N_3$-GlcNAc or GlcN-TFA, as well as GlcNAc (not shown).

rather than start new chains (7). This circumstance allows synchronization of polymerization resulting in the production of heparosan with a narrow size distribution (quasi-monodisperse) due to virtually simultaneous extension. Conversely, PmHS2 is better at initiating synthesis of new polysaccharide chains, so the resulting heparosan is much more polydisperse and potentially of lower size (*i.e.* chains are not extensively elongated) (7, 13). The ability to control the size distribution of the polysaccharide product through synchronized reactions is a desirable property; therefore, it is of interest to determine which portions of PmHS1 and PmHS2 are responsible for their differential abilities to initiate *de novo* synthesis of heparosan. Work is in progress to address this question, but based on our chimeric enzyme study (Fig. 3), we predict that residues in the heparosan synthases GlcUA-transferase domain acceptor binding site play a role in promoting new chain initiation by promoting binding of UDP-GlcNAc as an acceptor as in Ref. 13.

Sugar Analog Usage by Chimeric Synthases—Unnatural UDP-sugar donor molecules have the promise of generating GAG structures with utility for orthogonal chemistry. UDP-$6N_3$-GlcNAc is the source of an azide group known to be useful for efficient click chemistry. UDP-GlcN-TFA is the source of a hexosamine that may be deprotected, leaving a free amine group that has utility for further reaction with NHS-esters, isothiocyanates, etc. (Fig. 2). In the case of GAGs, the normal acetyl groups on glucosamine residues are unaffected by mild base treatment (16), but the TFA group on the glucosamine is removed, revealing the underlying free amine. This procedure allows site-specific reactions including installation of *N*-sulfo groups (16). By comparing the ability of the various chimera constructs to transfer two hexosamine UDP-sugar analogs, we have been able to tentatively identify regions of the respective polypeptides that impact analog specificity (Fig. 3).

As evident in Reactions 1–3 below, the monosaccharide unit of a UDP-hexosamine (HexN) plays multiple roles in polymerization catalyzed by a GAG synthase. First, it binds and is transferred by the hexosamine-transferase component (Reaction 1). Second, the residue binds to the GlcUA-transferase site and serves as part of an acceptor (Reaction 2). As the chain is extended with a second hexosamine, the original unit now serves as part of an acceptor in the HexN-transferase site (Reaction 3). Therefore, an unnatural analog has to interact with multiple binding sites and to be compatible with various catalytic processes to support repetitive polymerization by a GAG synthase.

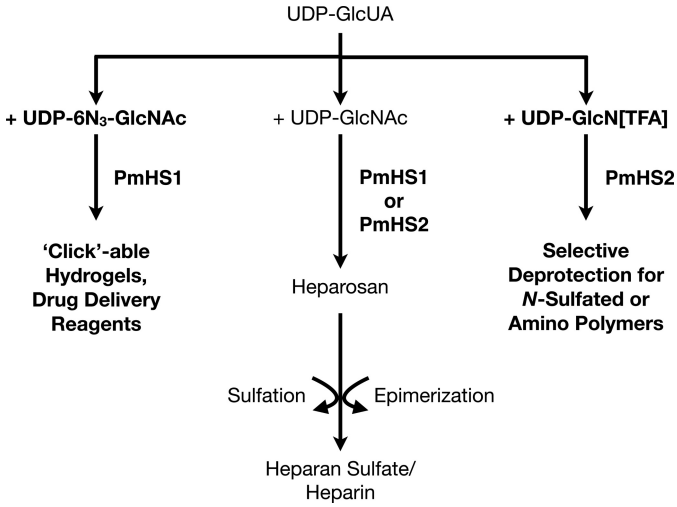


FIGURE 2. **Utility of unnatural UDP-sugar donors for synthesis of novel polysaccharides.** GAGs with new functional chemical groups may be prepared by chemoenzymatic synthesis with the appropriate precursors and catalysts. PmHS1 is able to use the analog donor UDP-6N_3-GlcNAc, but uses UDP-GlcN-TFA poorly. PmHS2 is able to use UDP-GlcN-TFA, but is not able to use UDP-6N_3-GlcNAc. Another level of *N*-sulfation control is possible using Chimera G and UDP-GlcN-TFA.

UDP-**HexN** (donor) + GlcUA-R → **HexN**-GlcUA-R + UDP

REACTION 1

UDP-GlcUA + **HexN**-GlcUA-R (GlcUA-T acceptor)

→ GlcUA-**HexN**-GlcUA-R + UDP

REACTION 2

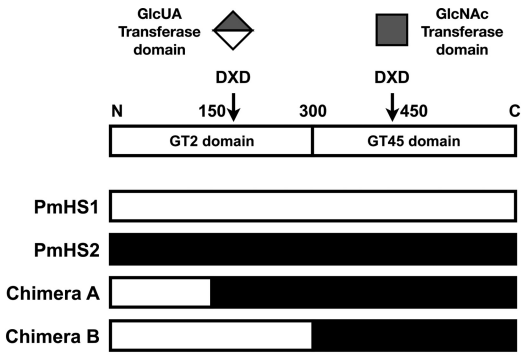
UDP-HexN + GlcUA-**HexN**-GlcUA-R (HexN-T acceptor)

→ HexN-GlcUA-**HexN**-GlcUA-R + UDP

REACTION 3

It should be noted that the acceptors are hypothesized to make a series of contacts with each of the glycosyltransferases of a bifunctional synthase. If the acceptor pocket interacts with a tetrasaccharide or greater size portion of the nascent chain, then an acceptor containing multiple hexosamine analogs making multiple potentially unfavorable contacts will become an increasingly poor acceptor as more analog units are added.

In all cases where the chimeric enzyme was active, polypeptides containing the putative PmHS1 GlcUA-transferase site region (PmHS1$^{134-318}$) had the ability to transfer 6N_3-GlcNAc



	Specific Activity (pmol/min/μg)	Acceptor Stimulation Type	UDP-GlcN[TFA] Usage	UDP-6N_3-GlcNAc Usage
PmHS1	1700 ± 90	"PmHS1"	+	++++
PmHS2	60 ± 2	"PmHS2"	++++	-
Chimera A	220 ± 10	PmHS2	++	-
Chimera B	300 ± 20	PmHS1/2	++++	++++
Chimera C	*No Detectable Activity*			
Chimera D	300 ± 50	PmHS1	+	++
Chimera E	10 ± 2	-	-	-
Chimera F	*No Detectable Activity*			
Chimera G	600 ± 20	PmHS1/2	++++	++++
Chimera H	*No Detectable Activity*			
Chimera I	100 ± 20	PmHS1	-	-
Chimera J	700 ± 30	PmHS1	-	+++

FIGURE 3. **Chimeric synthase schematic and catalytic properties.** Ten PmHS1/PmHS2 chimeras were generated, and their catalytic properties were compared with that of each of the parental wild-type synthases. The specific activity using authentic UDP-sugar precursors in polymerization assays for each of the active enzymes is noted. Three of the 10 chimeras had no detectable activity using UDP-GlcNAc (Chimeras C, F, and H) and were not used for further study with the UDP-sugar analogs. Acceptor stimulation for a given enzyme is presented as the -fold change in activity in the presence *versus* absence of exogenous acceptor (the latter is *de novo* synthesis); the resemblance to the parental acceptor usage is noted as *PmHS1* (>50-fold) or *PmHS2* (<5-fold) or intermediate *PmHS1/2* (~10–50-fold). Usage of each of the UDP-sugar analogs is relative to the activity of wild-type enzyme (normalized with PmHS1 for UDP-6N_3-GlcNAc or PmHS2 for UDP-GlcN-TFA; ++++ = 75–100%, +++ = 50–75%, ++ = 25–50%, + = 1–25%, − = no detectable activity). Chimeras B and G will utilize either UDP-hexosamine analog. For reactions containing acceptor, ~30-kDa heparosan was used.

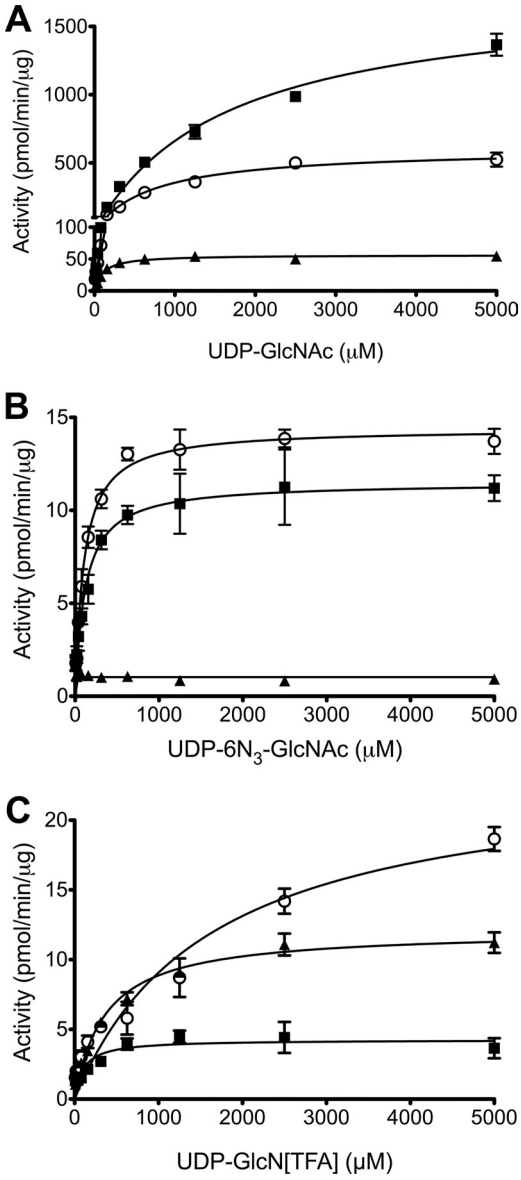


FIGURE 4. **Michaelis-Menten plots for PmHS1, PmHS2, and Chimera G.** Reaction rates were determined for usage of UDP-GlcNAc, UDP-6N_3-GlcNAc, or UDP-GlcN-TFA. *A*, PmHS1 and Chimera G have much higher activity with UDP-GlcNAc than PmHS2 (note the split *y* axis). *B* and *C*, Chimera G is able to utilize both of the UDP-sugar analogs, whereas the parental synthases can only efficiently employ one or the other analogs (*i.e.* PmHS1 has poor activity using UDP-GlcN-TFA and PmHS2 is unable to use UDP-6N_3-GlcNAc). All reactions contained ~30-kDa heparosan as an acceptor. ■ = PmHS1, ▲ = PmHS2, ○ = Chimera G. *Error bars* indicate S.E.

(Chimeras B, D, G, and J, Fig. 3). None of the constructs that lacked this region of PmHS1 had the ability to transfer the 6N_3-GlcNAc sugar analog. In summary, usage of this hexosamine analog tracks with the uronic acid-transferase domain of PmHS1. We hypothesize that for PmHS2 and chimeras lacking the PmHS1 GlcUA-transferase region (PmHS1$^{134-318}$), the inability to generate polysaccharide using the UDP-6N_3-GlcNAc donor is probably due to an as yet unidentified deficiency in acceptor binding or positioning in the PmHS2 GlcUA-transferase domain.

Although we do observe low activity by wild-type PmHS1 toward the transfer of GlcN-TFA (Fig. 4*C*), we have been unable to readily generate long polysaccharide chains containing this sugar analog (data not shown). It appears that PmHS1 may be able to transfer one or two of these sugar units, but the subsequent transfer of sugar units falters. This result indicates that incorporation of GlcN-TFA by PmHS1 does not fail due to donor binding. Instead, it is possible that more than one or two of these unnatural sugar units in the nascent acceptor polysaccharide are enough to either disrupt or inhibit synthesis at one or both of the acceptor sites as discussed later. In most cases, the ability to use the UDP-GlcN-TFA sugar analog appears to track with chimeras that contain the GlcNAc-transferase domain of PmHS2 (PmHS2$^{353-651}$). There are two exceptions that may be informative. (i) Chimera D has low activity with UDP-GlcN-TFA that is comparable with that seen with PmHS1 and only contains the N-terminal region of PmHS2 (PmHS2$^{1-167}$), and (ii) Chimera J contains the GlcNAc-transferase region of PmHS2 (PmHS2$^{353-501}$) and does not have any detectable activity with UDP-GlcN-TFA. These results indicate that specificity toward usage of UDP-GlcN-TFA may reside in the C terminus of the GlcNAc-transferase domain (PmHS2$^{502-651}$). The observed bottleneck may be due to acceptor specificity rather than donor specificity. Further studies will be required to determine whether this is the case.

Chimeras with the PmHS1 GlcUA-transferase region (PmHS1$^{134-318}$) and the entire GlcNAc-transferase domain of PmHS2 (PmHS2$^{353-651}$) can transfer both sugar analogs. Fortunately, the GlcUA-transferase region of PmHS1 (PmHS1$^{134-318}$) is also associated with higher specific activity as described earlier. Therefore, we have been able to generate two promiscuous chimeric enzymes (Chimeras B and G) that combine the desirable attributes of high specific activity and the ability to use both of the UDP-sugar analogs discussed.

Comparison of Kinetic Parameters of PmHS Wild-type Synthases and Promiscuous Chimera—We employed enzyme kinetics to probe the differential abilities of PmHS1, PmHS2, and Chimera G (PmHS2$^{1-167}$PmHS1$^{134-318}$PmHS2$^{353-651}$) to use the donor molecules UDP-GlcNAc, UDP-6N_3-GlcNAc, or UDP-GlcN-TFA (Fig. 4). Chimera G contains the putative GlcUA-transferase site of PmHS1 (PmHS1$^{134-318}$), whereas the rest of the polypeptide chain is derived from PmHS2. Chimera G was selected for these studies due to its ability to generate polysaccharide using both hexosamine analogs and its high specific activity in the presence of exogenous acceptors.

To employ the Michaelis-Menten model to determine the kinetic properties of these synthases (Table 1), UDP-GlcUA was used at a saturating concentration of 10 mM, and the concentration of the hexosamine donors was varied from 0.01 to 5 mM. PmHS1 had a V_{max} of 1700 pmol/min/μg using the natural donor UDP-GlcNAc in the presence of exogenous acceptor. Using the same UDP-GlcNAc donor, PmHS2 had a V_{max} more than 30-fold lower, at 50 pmol/min/μg, whereas Chimera G had a V_{max} of 600 pmol/min/μg (Fig. 4*A*).

The Michaelis-Menten constant, K_m, obtained by kinetic studies can yield information related to substrate binding by enzymes. However, it must be noted that in the case of GAG synthases polymerizing chains, the calculated apparent K_m values for both the natural and the analog UDP-sugar precursor molecules for each of these enzymes are potentially a combina-

TABLE 1

Kinetic parameters for wild-type and chimeric heparosan synthases

Values were determined by three replicate kinetics experiments using a saturating concentration of UDP-GlcUA and titrating the indicated hexosamine donors. Error is expressed as the S.E. ND = no detectable activity. Data were analyzed using the Michaelis-Menten model from GraphPad Prism 5.0d (GraphPad Software Inc., San Diego, CA).

	Apparent K_m (μM)		
	UDP-GlcNAc	**UDP-GlcN-TFA**	**UDP-6N_3-GlcNAc**
PmHS1	1650 ± 200	100 ± 40	120 ± 15
PmHS2	100 ± 10	350 ± 50	ND
Chimera G	710 ± 70	1600 ± 600	105 ± 7
	V_{max} (pmol/min/μg)		
	UDP-GlcNAc	**UDP-GlcN-TFA**	**UDP-6N_3-GlcNAc**
PmHS1	1700 ± 90	4 ± 0.5	12 ± 0.3
PmHS2	60 ± 2	12 ± 0.5	ND
Chimera G	600 ± 20	24 ± 4	14 ± 0.2
	K_{cat} (min^{-1})		
	UDP-GlcNAc	**UDP-GlcN-TFA**	**UDP-6N_3-GlcNAc**
PmHS1	130 ± 6	0.30 ± 0.01	0.85 ± 0.03
PmHS2	4 ± 0.1	0.90 ± 0.03	ND
Chimera G	45 ± 2	1.75 ± 0.2	1.05 ± 0.02

tion of donor binding as well as acceptor binding at both of the acceptor binding sites (*i.e.* three different interactions). Because of this circumstance, the synthase K_m values are perhaps less informative than for other simplistic enzyme models characterized by a single unique binding and catalytic event. However, it is interesting to note that for PmHS1, lower apparent K_m values appear to correlate with lower V_{max} values for UDP-GlcN-TFA relative to the natural UDP-GlcNAc. In contrast, PmHS2 has a higher K_m for UDP-GlcN-TFA relative to UDP-GlcNAc. We hypothesize that the slower rate of dissociation of either the analog donor or the acceptor molecules (or both) may be due to a stronger substrate/enzyme interaction actually impeding the rate of polymerization *in vitro*. Based on comparing the natural and analog K_m values, perhaps the fluorines of GlcN-TFA are interacting more strongly via hydrophobic interactions with the PmHS1 enzyme than the hydrogen atoms of the acetyl group of the authentic precursor. It is likely that PmHS2 and Chimera G interact less strongly with the GlcN-TFA unit, allowing more rapid polymerization.

In the case of 6N_3-GlcNAc, the extended N_3 group at the 6 position of the pyranose ring bears a resonance structure with a partial positive and negative charge (C6–N=N^+=N^- or C6–N^-–N^+≡N) and is clearly bulkier than the natural hydroxyl found on GlcNAc. Either of these properties could affect the interactions with the synthase, and this analog may interact more strongly with the enzymes (as suggested by lower K_m, Table 1) than the natural precursor. Unlike the case of UDP-GlcN-TFA, the azide analog does not display notably different binding between the PmHS1 parent and Chimera G (as measured by K_m values, Table 1). For the donor hexosamine analogs, it is clear that PmHS1 prefers UDP-6N_3-GlcNAc (Fig. 4*B*), whereas PmHS2 prefers to use UDP-GlcN-TFA (Fig. 4*C*). Chimera G is able to use both of these hexosamine donors and in fact uses both at least as well, if not better, than each of the wild-type parental synthases (Fig. 4, *B* and *C*).

Molecular Models for Synthase Analog Selectivity—There are multiple models that could explain the failure of a given synthase to generate polysaccharide using unnatural sugar analogs as we discussed earlier. In the first most simplistic case, polysaccharide synthesis cannot occur if the synthase is unable to bind the UDP-sugar donor analog. In the second case, the donor sugar may bind, but in a mode in which nucleophilic attack of the anomeric group cannot occur, thus preventing formation of a covalent bond with the growing acceptor polysaccharide. In the third case, it is possible that the donor sugar is able to be successfully transferred to the growing acceptor polysaccharide, but in the next step at the other glycosyltransferase site, an acceptor containing a sugar analog at the nonreducing end binds relatively poorly, or in a mode of binding that is not conducive to efficient extension. Another possibility is an extension of this third case; one single unnatural monosaccharide unit may not disrupt acceptor binding, but as more sugars are added (2, 3, or more analogs), the nascent acceptor is no longer able to bind in a manner conducive to efficient polymerization.

The simplest hypothesis for explaining the observed selectivity of PmHS1 and PmHS2 is that specificity for each of these hexosamine donor analogs would track with the hexosamine transferase domain of each of the wild-type enzymes as in the first or second cases above. Specifically, for these cases, chimeras containing the PmHS1 GlcNAc-transferase domain would have the ability to transfer 6N_3-GlcNAc, whereas chimeras containing the PmHS2 GlcNAc-transferase domain would have the ability to transfer GlcN-TFA. If these hypotheses were true, then it would be unlikely that we could generate chimeric synthases with the ability to transfer both of these sugar analogs. The observation that Chimera G is able to use both analogs rules out this model.

Alternately, as in the third case above, specificity for these two hexosamine sugar analogs may be determined at sites within different transferase domains (Fig. 5). For example, for one hexosamine analog, specificity is determined by the acceptor binding site component of the GlcUA-transferase site during the second step in polymerization. For the other analog, the specificity is determined by either the acceptor binding site or the hexosamine donor pocket of the GlcNAc-transferase site. Based on the observation that Chimeras B and G possess the ability to transfer both sugar

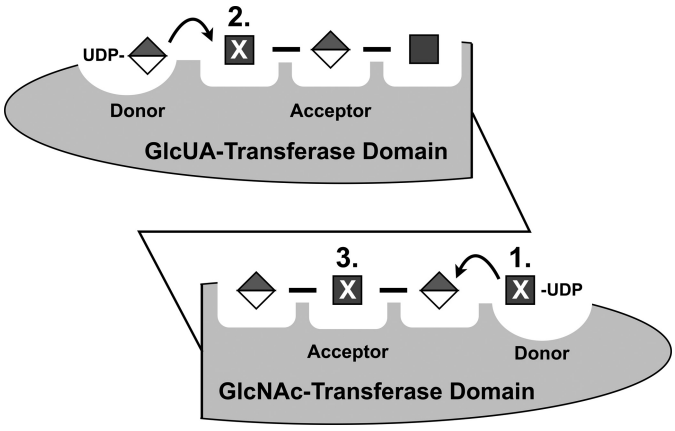


FIGURE 5. **Bifunctional synthase model for polymerization using synthetic analogs.** The ability to incorporate the hexosamine analog $6N_3$-GlcNAc tracks with the GlcUA-transferase domain of PmHS1 between residues 134 and 318. This observation indicates that synthesis using $6N_3$-GlcNAc fails in chimeras (and PmHS2) lacking this region because of a deficiency in *acceptor* binding or conformation at that site (Reaction 2). GlcN-TFA usage tracks with the GlcNAc-transferase domain of PmHS2 between residues 502 and 651, and specificity could be due to *donor* binding and/or *acceptor* binding (Reactions 1 and/or 3) (analogs marked with *white X*).

analogs, we can make the prediction that this third model appears most likely.

Chimeric Synthase Donor and Acceptor Specificity—In the absence of crystal structures for the bacterial heparosan synthases, we are not able to evaluate the PmHS1 and PmHS2 structures and make rational predictions about which residues are responsible for donor and acceptor specificity. The current results using these chimeric heparosan synthases suggest that extension of the growing polysaccharide chain with the UDP-sugar analogs UDP-GlcN-TFA and UDP-$6N_3$-GlcNAc fails not due to an inability to utilize the donor molecules, but rather an inability for the growing polysaccharide chain containing these sugar analogs to act as an efficient acceptor for further extension. In the case of polysaccharides containing GlcN-TFA, the residues responsible for acceptor binding specificity appear to be in the carboxyl-terminal region of the PmHS2 GlcNAc-transferase domain between amino acid residues 502 and 651. For production of polysaccharides containing $6N_3$-GlcNAc, the residues responsible for acceptor binding specificity appear to reside in the PmHS1 GlcUA-transferase domain between amino acid residues 134–318. Chimeras B and G contain both of these regions, and in each case, these chimeric constructs have the ability to transfer $6N_3$-GlcNAc as well as GlcN-TFA. This model is consistent with others who have hypothesized or observed that discrete amino acid sequences within glycosyltransferases can be responsible for acceptor specificity (17–19). Each of the regions in our chimera swapping locations correspond to ~150-amino acid stretches, with only ~30 differences between PmHS1 and PmHS2 (supplemental Fig. 1). These observations narrow the search space for the residues responsible for this acceptor specificity, and potentially, in the future should assist in the design of synthases with an increased synthetic repertoire.

Block Polysaccharide Synthesis toward Defined GAG Structures—Heparan sulfate chains found in nature are heterogeneous polysaccharides produced by the Golgi apparatus. Thus a goal of chemoenzymatic synthesis is to prepare more defined GAGs for structure/function studies as well as new potential therapeutic leads (20).

We were able to take advantage of the novel properties of Chimera G as demonstrated by the synthesis of polysaccharides that contain stretches of GlcNAc with interspersed blocks containing GlcN-TFA. Heparan sulfate is known to contain highly sulfated domains interspersed with tracts containing little or no modification (*i.e.* heparosan or low sulfation, respectively) (21–24). From knock-out studies of the *N*-deacetylase/*N*-sulfotransferase enzymes, it is known that *N*-sulfation is a prerequisite for all subsequent modifications of heparosan (25, 26). Recently, it was observed that the presence and locations of *N*-sulfation modifications in oligosaccharides (in this case, 8–12 sugar units) have an important role in forming the domain structure of heparan sulfate (16).

By deprotecting and *N*-sulfating the GlcN-TFA analog-containing heparosan polysaccharide, we would accomplish an important step toward the goal of generating high molecular weight heparan sulfate that more closely resembles polysaccharides found in nature. Specifically, after creating regions of either high or zero *N*-sulfation, various *O*-sulfotransferases could be used to modify the synthetic polymers in a fashion to mimic natural heparan sulfate. Additionally, this analog provides a route to synthesis of polysaccharides containing *N*-unsubstituted groups that may also play important biological roles (27).

In vitro, the wild-type PmHS2 enzyme (which is able to use the UDP-GlcN-TFA donor) tends to yield more polydisperse products as well as multiple species corresponding to elongated acceptor products and *de novo* synthesis products. Spontaneously occurring initiation activity previously confounded our efforts to produce defined polysaccharides with alternating natural and analog hexosamine blocks and is thus undesirable here, especially when UDP-sugar concentrations are high (*i.e.* favorable conditions for the initiation step).

Chimera G was selected as the catalyst for block polysaccharide synthesis because it exhibits at least 10- and 2-fold higher specific activity using UDP-GlcNAc and UDP-GlcN-TFA when compared with PmHS2, respectively. Also, Chimera G is roughly twice as acceptor-dependent as PmHS2 due to a lower level of *de novo* synthesis. We hypothesize that a potential cause for the altered acceptor dependence observed with Chimera G when compared with PmHS2 may be due to its relative inability to use the hexosamine donor sugar as an acceptor for new chain initiation. PmHS2 has been observed to use UDP-GlcNAc as an acceptor molecule producing disaccharides consisting of GlcUA-GlcNAc-UDP (13), whereas PmHS1 is less able to initiate new polysaccharide chains in such a fashion. Chimera G consists of a portion of the PmHS1 GlcUA-transferase site ($PmHS1^{134-318}$) with the rest of the synthase coming from PmHS2. For new chain initiation to occur, the hexosamine donor must first bind to the GlcUA-transferase acceptor site for the GlcUA monosaccharide to be transferred. Further studies will be required to determine the precise regions responsible for *de novo* synthesis of new GAG chains.

We have been able to generate analog-containing heparosan polysaccharides ~24 kDa in size consisting of alternating

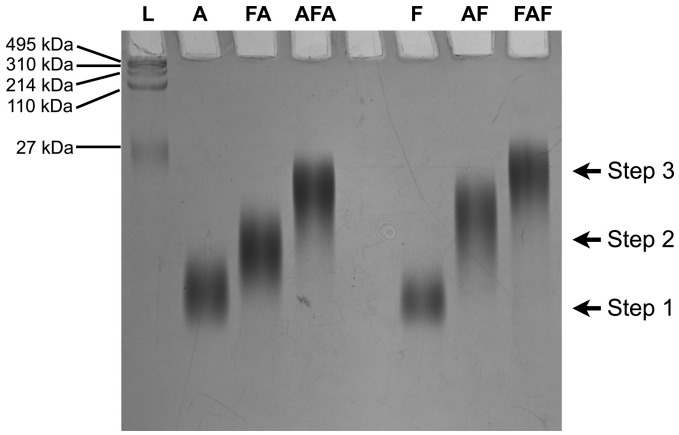


FIGURE 6. **Block polysaccharide synthesis steps.** Samples from each successive step of the block polysaccharide synthesis reactions (using Chimera G for all steps) were separated on 8% polyacrylamide gel and stained with Alcian blue dye. *L* = HA Loladder (Hyalose LLC, Oklahoma City, OK). *A* = GlcNAc-containing block; *F* = GlcN-TFA-containing block. Each block added increased the size of the polysaccharide by ~8 kDa based on this gel and those with other HA size standards (not shown).

~8-kDa blocks of either $(GlcUA\text{-}GlcNAc)_n$ or $(GlcUA\text{-}GlcN\text{-}TFA)_n$ by controlling the donor/acceptor stoichiometry of the reactions and the choice of UDP-hexosamine present (Fig. 6). The efficient production of these polysaccharides has not previously been possible using either of the wild-type synthases; PmHS1 is not able to generate polysaccharides using UDP-GlcN-TFA, and PmHS2 is less able to use existing acceptor molecules and produces more polydisperse polysaccharides due to a higher rate of *de novo* synthesis.

Our chimeric approach has enabled the generation of novel catalysts that are able to generate polysaccharide products that would not have been possible using either of the native synthases alone. The chimera containing the PmHS1 GlcUA-transferase site (Chimera G) in the background of PmHS2 is able to produce polysaccharides useful for making more defined heparin analogs and heparan sulfate. The implications of this strategy have yet to be fully explored, but such synthetic polysaccharides may enable the study of biological effects such as cytokine or growth factor binding by chains containing defined regions of variable sulfation. This work describes one methodology toward the production of defined sulfated polysaccharides, which has to date precluded the ability to study the roles of sulfation domains.

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