Acceptor Specificity of the *Pasteurella* Hyaluronan and Chondroitin Synthases and Production of Chimeric Glycosaminoglycans*\[6\]

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The hyaluronan (HA) synthase, PmHAS, and the chondroitin synthase, PmCS, from the Gram-negative bacterium *Pasteurella multocida* polymerize the glycosaminoglycan (GAG) sugar chains HA or chondroitin, respectively. The recombinant *Escherichia coli*-derived enzymes were shown previously to elongate exogenously supplied oligosaccharides of their cognate GAG (e.g. HA elongated by PmHAS). Here we show that oligosaccharides and polysaccharides of certain noncognate GAGs (including sulfated and iduronic acid-containing forms) are elongated by PmHAS (e.g. chondroitin elongated by PmHAS) or PmCS. Various acceptors were tested in assays where the synthase extended the molecule with either a single monosaccharide or a long chain (~10^2–4 sugars). Certain GAGs were very poor acceptors in comparison to the cognate molecules, but elongated products were detected nonetheless. Overall, these findings suggest that for the interaction between the acceptor and the enzyme (a) the orientation of the hydroxyl at the C-4 position of the hexosamine is not critical, (b) the conformation of C-5 of the hexuronic acid (glucuronic versus iduronic) is not crucial, and (c) additional negative sulfate groups are well tolerated in certain cases, such as on C-6 of the hexosamine, but others, including C-4 sulfates, were not or were poorly tolerated. *In vivo*, the bacterial enzymes only process unsulfated polymers; thus it is not expected that the PmCS and PmHAS catalysts would exhibit such relative relaxed sugar specificity by acting on a variety of animal-derived sulfated or epimerized GAGs. However, this feature allows the chemoenzymatic synthesis of a variety of chimeric GAG polymers, including mimics of proteoglycan complexes.

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*This work was supported in part by National Science Foundation Grant MCB-9876193, Oklahoma Center for Advancement of Science and Technology Health Research Grant HR02-036R (to P. L. D.), and National Institutes of Health Grants GM38060 (to R. J. L.), HL062244 (to R. J. L. and P. L. D.), and Health Grant R24-GM-61894. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.*

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2 The abbreviations used are: GAG, glycosaminoglycan; PmHAS, *P. multocida* hyaluronan synthase; HA, hyaluronan, hyaluronate, or hyaluronic acid; C, chondroitin; PmCS, *P. multocida* chondroitin synthase; HexNAc, N-acetylhexosamine; SEC, size exclusion chromatography; SAX-HPLC, strong anion exchange-high performance liquid chromatography; ESI-MS, electrospray ionization mass spectrometry; MeSO, dimethyl sulfoxide; Hep, heparin; MALLS, multiancele laser light scattering.
**Synthase Acceptor Specificity**

**TABLE 1**

| Carbohydrate nomenclature, code, and structure |

Due to the presence of multiple sulfation isomers within any one commercial chondroitin sulfate preparation (e.g. CSA and CSB, etc.), the simple identification code designates the animal source of polymer (the source is underlined). The manufacturer assay values are presented for polysaccharides. The oligosaccharide structures were verified by mass spectrometry and/or NMR. R indicates functional group on hexosamine.

| Code | Oligosaccharide (source) | Structure |
|------|--------------------------|-----------|
| HA   | Hyaluronan tetrasaccharide (S. zoepidemicus) | (β1,3-GlcNac-β1,4-GlcUA) |
| CSFa | Chondroitin 4-sulfate trisaccharide (bovine trachea) | (β1,3-GlcNac-β1,4-GlcUA) |
| CSb  | Chondroitin 6-sulfate trisaccharide (chark cartilage) | (β1,3-GlcNac-β1,4-GlcUA) |
| CSa  | Chondroitin 6-sulfate pentasaccharide (chark cartilage) | (β1,3-GlcNac-β1,4-GlcUA) |
| CSb13 | C-5-epimerized chondroitin 4-sulfate trisaccharide (porcine intestinal mucosa, Prep II; Celsus) | (β1,3-GlcNac-β1,4-GlcUA) |
| CSpII3 | C-5-epimerized chondroitin 4-sulfate pentasaccharide (porcine intestinal mucosa, Prep II; Celsus) | (β1,3-GlcNac-β1,4-GlcUA) |
| dCSpII3 | Desulfated C-5-epimerized chondroitin sulfate trisaccharide (porcine intestinal mucosa, Prep II; Celsus) | (β1,3-GlcNac-β1,4-GlcUA) |
| Hep  | Synthetic heparin pentasaccharide (Arixtra) | (β1,3-GlcNac-β1,4-GlcUA) |
| Hs   | Heparosan pentasaccharide (E. coli K5) | (β1,3-GlcNac-β1,4-GlcUA) |

*The major disaccharide unit of the polysaccharide. Percentages for purity and/or contaminants were not assessed.

**EXPERIMENTAL PROCEDURES**

Natural GAGs—All GAG polysaccharides, except for HA, are heterogeneous in nature with respect to composition and sulfation pattern. It is currently impossible to isolate GAG polysaccharides from animal sources in one pure isomeric form. Therefore, throughout the text all polysaccharides are defined with respect to their source and are given a simple nomenclature code (Table 1).

A variety of GAGs were obtained from Sigma, including *Streptococcus zoepidemicus* hyaluronan (HA), bovine trachea chondroitin sulfate (CSb), (sold as “chondroitin sulfate A”, mainly composed of chondroitin 4-sulfate), C-5 epimerized porcine intestinal mucosa chondroitin sulfate (CSa), (also referred to as dermatan sulfate and sold as “chondroitin sulfate B”), shark cartilage chondroitin sulfate (CSb) (sold as “chondroitin sulfate C”, mainly composed of chondroitin 6-sulfate), and porcine intestinal heparin (Hep). Shark fin chondroitin sulfate (CSa) (sold as “chondroitin sulfate D”) and squid cartilage chondroitin sulfate (CSa) (sold as “chondroitin sulfate E”) were obtained from Associates of Cape Cod/Seikagaku America (Falmouth, MA). In addition, a highly C-5 epimerized (>95% 4S-GalNac-IdoUA repeats) chondroitin sulfate (CSpII) a high purity dermatan sulfate from porcine intestinal mucosa similar to CSa above, but better characterized) and heparan sulfate (porcine intestinal mucosa) were obtained from Celsus Laboratories (Cincinnati, OH). Unsulfated chondroitin polysaccharide (C) and unsulfated heparan polysaccharide was prepared from cultures of either type F or type D *P. multocida*, respectively (20). The degree of polymerization of the HA and the unsulfated chondroitin was reduced to ~80 and ~20 kDa, respectively, by autohydrolysis (121 °C, 20 p.s.i., 20 min) to be more comparable with the smaller molecular weight chondroitin sulfates (~15–40 kDa) and heparin (~17–19 kDa). In addition, 80-kDa monodisperse HA was prepared by synchronized stoichiometrically controlled chemoenzymatic reactions (12).

To avoid the heterogeneity problem intrinsic to natural GAG polysaccharides, a series of defined oligosaccharides of known...
Oligosaccharide derivative preparation strategy

The various polysaccharides were cleaved by GAG digestive enzymes (HAase, testicular hyaluronidase; ABCase, chondroitin ABC lyase; HepIIIase, heparin lyase III) and/or desulfated by solvolysis. Each oligosaccharide was purified by gel filtration and/or anion exchange chromatography (chrom).

| Polysaccharide | HAase | ABCase | ABCase | ABCase | ABCase | ABCase |
|---------------|-------|--------|--------|--------|--------|--------|
| HA4           | ↓ chrom | ↓ chrom | ↓ NaOH | ↓ chrom | ↓ NaOH | ↓ chrom |
| CSbt3         | ↓ Hg2+ | ↓ chrom | ↓ NaOH | ↓ chrom | ↓ NaOH | ↓ chrom |
| CSbt3         | ↓ Hg2+ | ↓ chrom | ↓ NaOH | ↓ chrom | ↓ NaOH | ↓ chrom |
| HA4           | ↓ Hg2+ | ↓ chrom | ↓ NaOH | ↓ chrom | ↓ NaOH | ↓ chrom |
| CSbt3         | ↓ Hg2+ | ↓ chrom | ↓ NaOH | ↓ chrom | ↓ NaOH | ↓ chrom |
| HA4           | ↓ Hg2+ | ↓ chrom | ↓ NaOH | ↓ chrom | ↓ NaOH | ↓ chrom |
| CSbt3         | ↓ Hg2+ | ↓ chrom | ↓ NaOH | ↓ chrom | ↓ NaOH | ↓ chrom |

| Polysaccharide | HAase | ABCase | ABCase | ABCase | ABCase | ABCase |
|---------------|-------|--------|--------|--------|--------|--------|
| CSbt3         | ↓ chrom | ↓ chrom | ↓ NaOH | ↓ chrom | ↓ NaOH | ↓ chrom |
| CSbt3         | ↓ Hg2+ | ↓ chrom | ↓ NaOH | ↓ chrom | ↓ NaOH | ↓ chrom |
| HA4           | ↓ Hg2+ | ↓ chrom | ↓ NaOH | ↓ chrom | ↓ NaOH | ↓ chrom |
| CSbt3         | ↓ Hg2+ | ↓ chrom | ↓ NaOH | ↓ chrom | ↓ NaOH | ↓ chrom |
| HA4           | ↓ Hg2+ | ↓ chrom | ↓ NaOH | ↓ chrom | ↓ NaOH | ↓ chrom |
| CSbt3         | ↓ Hg2+ | ↓ chrom | ↓ NaOH | ↓ chrom | ↓ NaOH | ↓ chrom |

**Table 2**

**Electrospray ionization mass spectral data for chondroitin sulfate derived oligosaccharides**

| Oligosaccharide | Mass | Parent ion
|-----------------|------|------------------|
| CSbt3, CSbt3, CSbt3 | 826.1 | [M – 3Na + H]^+ = 379.1 (calculated, 379.1) |
| CSbt3, CSbt3, CSbt3 | 1329.1 | [M – 2Na]^+ = 641.5 (calculated, 641.5) |
| CSbt3, CSbt3, CSbt3 | 622.2 | [M – Na]^+ = 599.1 (calculated, 599.2) |

*Highest abundance ion is shown.*
**Synthase Acceptor Specificity**

lyophilized, and desalted on a Bio-Gel P2 column. C-5-epimerized chondroitin tetrasaccharide without sulfate groups at GlcNAc residues (1 mg) was treated with mercuric acetate reagent to allow for normalization. Polymerization reactions contained 0.2 mM UDP-HexNAc (UDP-GlcNAc for PmHAS or UDP-GalNAc for PmCS) and were incubated at 30 °C for either 3 min (polysaccharide acceptors) or 30 min (oligosaccharide acceptors). Reactions were stopped by placing on ice and adding SDS (2% final w/v). Descending paper chromatography was used to separate the unincorporated radiolabel from the elongated acceptors (10). The assays were performed in duplicate and were linear with respect to enzyme concentration and time; less than 5% UDP-sugar substrate was consumed.

Single sugar additions were also monitored by reverse phase HPLC ESI-MS (26). Briefly, similar reaction conditions were utilized as described above, except only single unlabeled UDP-sugar (2–6 mM final) was employed. The enzyme adds a monosaccharide unit onto the nonreducing end of various acceptors as shown in Equation 2 or 3.

\[
\text{acceptor} + \text{UDP-GlcUA} \rightarrow \text{UDP} + (\text{GlcUA} - \text{acceptor})
\]

(Eq. 2)

\[
\text{acceptor} + \text{UDP-HexNAc} \rightarrow \text{UDP} + (\text{HexNAc} - \text{acceptor})
\]

(Eq. 3)

**Chimeric GAG Synthesis**—The purified enzymes PmHAS-(1–703) or PmCS-(46–695) (1 mg) were used to add unlabeled HA or chondroitin chains, respectively, to various GAG polysaccharide acceptors (1–8225 μg). Reactions (25 μl) contained the same reaction buffer as above except that 2–4 mM UDP-GlcUA and 2–4 mM UDP-HexNAc (UDP-GlcNAc for PmHAS or UDP-GalNAc for PmCS) were utilized at 30 °C for varying times (see Equation 1, but without the \(^3\)H label).

**Size Analysis of Polysaccharides**—Polymers were analyzed using 1–1.2% 1 TAE-agarose gels (30 V, 5 h, Stains-All detection) (27). The specific Streptomyces hyaluronate lyase from Sigma was employed to destroy and thus identify authentic HA chains. Defined HA molecular weight standards were from (Hyalose L.L.C., Oklahoma City, OK) (12). Kilobase DNA standards were from Stratagene (La Jolla, CA).

Analytical high performance SEC was performed with PLaquagel-60, -H, -OH columns in tandem (15 μm, 7.5 × 300 mm, Polymer Laboratories Amherst, MA) eluted with 50 mM NaPO₄, 150 mM NaCl, pH 7, at 0.4–0.5 ml/min. Multilateral light scattering (MALLS) detection was performed to quantify absolute molecular weights (12).

**RESULTS AND DISCUSSION**

The finding that recombinant PmHAS, and later PmsCS, was able to elongate certain small oligosaccharides, including HA₄ and chondroitin sulfate hexasaccharides, respectively (13, 19), led to a broader investigation of the potential range of acceptor usage (Tables 4 and 5). In contrast, the other distinct bacterial HA synthase enzymes from group A and C Streptococcus cannot be tested at this time because of their inability to elongate exogenously added acceptors.

*E. coli*KfoC, chondroitin polymerase, was shown previously to elongate certain polysaccharides and oligosaccharides, but the polymers were not rigorously defined with respect to sulfation isomers (15). Also, the desulfated chondroitin preparations may have suffered oxidative degradation that hindered enzy-
The conundrum of an impure bulk GAG polymer population found in natural extracts was addressed here by employing defined (i.e. HPLC-purified, mass spectrometry, and NMR-validated) oligosaccharides with known sulfation patterns. The nature of the sulfation pattern seems to be an important characteristic on the extent of utilization of the modified acceptor by both PmCS and PmHAS. For example, the 6-sulfated chondroitin trisaccharide (CS$_{bt3}$) and chondroitin pentasaccharide (CS$_{bt5}$) served as relatively good acceptors for PmHAS. In contrast, the 4-sulfated chondroitin trisaccharide (CS$_{bt4}$) preparation was a poor acceptor (Table 5). In fact, the signals in the radiolabeled incorporation assay of CS$_{bt4}$ appear to be due entirely to a trace amount of pentasaccharide with mixed 4- and 6-sulfates contaminating the preparation based on ESI-MS of the PmHAS-extended mixture. The starting CS$_{bt4}$ trisaccharide peak (calculated 760.12 Da; observed 759.1) was not extended into the predicted tetrasccharide product (calculated 936.15 Da); a major fraction of the pentasaccharide, however, was extended into a hexasaccharide possessing the appropriate mass. The bulk 4-sulfated chondroitin polysaccharide (CS$_{bt4}$) did serve as an acceptor for PmHAS and PmCS, but this polymer is not composed entirely of the 4-sulfated isomer; these preparations are actually a 70:30 mixture of GalNAc-4/6SO$_4$ isoforms (Table 1). The 6-sulfated sequences may be responsible for the signal observed. In summary, as long as the nonreducing terminal GalNAc residue either possesses a 6-sulfate group or lacks a sulfate group, both Pasteurella enzymes should elongate the polymer quite readily. These findings suggest that a bulky negative group at the C-4 position of the hexosamine is causing an unfavorable interaction (steric and/or charge repulsion) with the synthase polypeptide because the HA and unsulfated chondroitin are good acceptors. The 6-sulfate trisaccharide is better tolerated suggesting that a bulky negative group at the C-6 hydroxyl of the HexNAc (Fig. 1) is neither in close proximit or in a bonding interaction with the synthase.

Two other sulfated chondroitin polysaccharides containing GalNAc-6SO$_4$, C$_S$ and C$_S$ also show some activity as acceptors (Table 4), but both C$_S$ and C$_S$ also have an additional sulfate group; C$_S$ contains GlcUA-2SO$_4$, whereas C$_S$ contains GalNAc-6/4-disulfate. These polymers serve as modest acceptors (2.4 and 5.1%, respectively, for PmHAS) but are not as good as C$_S$ (12%) and C$_S$ (10%). Again, the relative activity of the various natural chondroitin preparations to HA may be affected by the fact that these polymers are not 100% pure and can contain additional minor sequences at the nonreducing termini.

### TABLE 4

| Oligosaccharide | Relative PmHAS activity normalized to HA | Relative PmCS activity normalized to HA |
|-----------------|----------------------------------------|----------------------------------------|
| HA              | 100**                                  | 100**                                  |
| C$_S$           | 3.6**                                  | 5.0**                                  |
| C$_S$           | 61*                                    | 140**                                  |
| C$_S$           | 0.3*                                   | 3.9*                                   |
| C$_S$           | 0.4*                                   | 4.1*                                   |
| H$_{bt3}$       | -0.1**                                 | ND                                     |
| H$_{bt5}$       | 1.6                                   | ND                                     |

PmHAS and PmCS polysaccharide acceptor specificity

Polymerization assays with each acceptor (at least three experiments in duplicate, except for H$_{bt5}$) were performed. The value of no acceptor control (~300–600 dpm) was subtracted from each point. The averaged values for PmHAS or PmCS are presented. The ratio (nanomoles of test acceptor/nmol of HA$_4$) was employed to normalize to the HA signal (30,000 dpm for 0.02 nmol of HA$_4$), which was set to 100%. For all results, * = $p < 0.05$ and ** = $p < 0.01$ in a Student’s one-tailed t test for comparison of a given test acceptor to the no acceptor control assay. ND indicates not determined. Both enzymes may utilize either HA or chondroitin polymers, but heparin and heparosan are very poor acceptors or slightly inhibitory.

### TABLE 5

| Oligosaccharide | Relative PmHAS activity normalized to HA$_4$ | Relative PmCS activity normalized to HA$_4$ |
|-----------------|-----------------------------------------------|-----------------------------------------------|
| HA              | 100**                                         | 100**                                         |
| C$_S$           | 3.6**                                         | 5.0**                                         |
| C$_S$           | 61*                                           | 140**                                         |
| C$_S$           | 0.3*                                          | 3.9*                                          |
| C$_S$           | 0.4*                                          | 4.1*                                          |
| H$_{bt3}$       | -0.1**                                        | ND                                            |
| H$_{bt5}$       | 1.6                                           | ND                                            |

PmHAS and PmCS oligosaccharide acceptor specificity

Polymerization assays with each acceptor (at least three experiments in duplicate, except for H$_{bt5}$) were performed. The value of no acceptor control (~300–600 dpm) was subtracted from each point. The averaged values for PmHAS or PmCS are presented. The ratio (nanomoles of test acceptor/nmol of HA$_4$) was employed to normalize to the HA$_4$ signal (62,000 dpm for 0.15 nmol of HA$_4$), which was set to 100%. For all results, * = $p < 0.05$ and ** = $p < 0.01$ in a Student’s one-tailed t test for comparison of a given test acceptor to the no acceptor control assay. ND indicates not determined. Both enzymes efficiently utilize the 6-sulfated chondroitin oligosaccharide but not the 4-sulfated isomer. Again, the heparin and heparosan oligosaccharides are very poor acceptors or slightly inhibitory.
FIGURE 2. PmHAS-catalyzed addition of HA chains onto various sulfated chondroitin acceptors. Reactions with various acceptors (CS<sub>mt</sub>, chondroitin sulfate, bovine trachea; CS<sub>pl</sub>, C-5-epimerized chondroitin sulfate, porcine intestinal mucosa, preparation I; CS<sub>s</sub>, chondroitin sulfate, shark cartilage) were incubated for various times (2, 4, and 6 h or overnight) and separated on a 1% agarose gel with Stains-All detection. Additional aliquots of each UDP-sugar (2 mM final) were added at the 2- and 4-h time points. The chondroitin sulfate starting materials (S) for each reaction were run on both sides of the 2-h and overnight time points (migration distance indicated with an arrowhead). A no acceptor control denoting de novo initiation of HA is shown as well; only a small amount of high molecular weight HA polysaccharide forms after extended incubation times without acceptor present. D, kb DNA ladder, 12, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1.5, 1, 0.75, 0.5, 0.25 kb from top to bottom.

Chondroitin sulfate B polysaccharides (CS<sub>pl</sub> and CS<sub>pl13</sub>) contain IdoUA, the C-5 epimer of GlcUA (Fig. 1), and contain GalNAc-4SO₄ residues; these polymers possess very low activity (0.4 and 0.5%; see Table 4). Both polymers also contain a low percentage (4%) of an additional sulfate, IdoUA-2SO₄, which may contribute to their reduced activity as acceptors (Table 4). To further assess the roles of IdoUA and 4-SO₄, pure chondroitin sulfate B oligosaccharides with specified modification patterns were analyzed as acceptors. CS<sub>pl13</sub> (C-5-epimerized chondroitin 4-sulfate trisaccharide) and CS<sub>pl15</sub> (C-5-epimerized chondroitin 4-sulfate pentasaccharide) oligosaccharides verified the results seen with CS<sub>pl</sub> and CS<sub>pl13</sub> (Table 5); these oligosaccharides are poor acceptors.

Desulfated C-5-epimerized chondroitin sulfate B (dCS<sub>pl</sub>) was also assayed to determine whether the IdoUA or sulfate groups (Fig. 1) were the problematic structures for the syntheses. The desulfated C-5-epimerized polysaccharide, dCS<sub>pl</sub>, showed 2.4% activity relative to HA, higher than that of the 4-sulfated epimerized chondroitin polymers (CS<sub>pl</sub> and CS<sub>pl13</sub>, 0.4 and 0.5%, respectively; see Table 4) but lower than that of the unsulfated C polymer (54%). We hypothesized that PmHAS and PmCS may be able to tolerate IdoUA to some degree due to the increase in signal in dCS<sub>pl13</sub> (after de-sulfation) when compared with CS<sub>pl</sub> and CS<sub>pl13</sub>. There is a chance, however, that IdoUA was not well tolerated. The observed signal may have actually been because of the nonreducing terminus of some chains in the polysaccharide population containing an accessible GlcUA, the native uronic acid for the synthase, instead of IdoUA (i.e. CS<sub>pl</sub>; the original material that was de-sulfated to make dCS<sub>pl13</sub> contains ~95% IdoUA, and thus there is an ~5% chance of the GlcUA residing at the nonreducing terminus of the polymers). To further address this issue, a defined IdoUA-containing trisaccharide, dCS<sub>pl13</sub>, was subjected to a single sugar addition reaction (Eq. 2) with PmHAS. Reverse phase HPLC-ESI-MS analysis verified that the trisaccharide (calculated 600.2 Da; observed 599.2 Da) was converted to the expected tetrasaccharide (calculated 776.23 Da; observed 775.1 Da). Therefore, IdoUA is tolerated by PmHAS.

An exact comparison of the GlcUA versus IdoUA utilization rates will need to await the preparation of larger amounts of defined dCS<sub>pl13</sub> oligosaccharide. The difference between the GlcUA and the IdoUA epimers is the conformation of the sugar ring. Even though both monosaccharide units have the carboxylate in the equatorial position, two different chair forms ([C<sub>i</sub> versus C<sub>4</sub> for GlcUA or IdoUA, respectively) are present and changes the linkage from β to α upon epimerization.

Two antithrombotics, the synthetic heparin pentasaccharide, Arixtra (Hep<sub>p</sub>); and bulk heparin polysaccharide (Hep), show very low (0.03%, Hep<sub>p</sub>) relative acceptor activity to HA or were inhibitory (−0.1%, Hep<sub>p</sub>) with PmHAS (Tables 4 and 5). The unsulfated heparan oligosaccharide (1.6%, H<sub>5</sub>) and polysaccharide (0.1%, H), tested show slightly more activity than the sulfated heparin oligosaccharide and polysaccharide, respectively. It may be that the repeated GlcUA groups among all acidic GAGs can partially compensate for the improper sugar backbone linkages, albeit poorly. However, too many additional negative groups (i.e. sulfate groups) may also be detrimental.

Overall, PmCS exhibited similar acceptor specificity relative to PmHAS (Tables 4 and 5) except that the various chondroitin oligosaccharides were better acceptors for the chondroitin synthase, perhaps reflecting its optimization for chondroitin biosynthesis. A few minor potential differences among the various acceptors were noted, but at this time limiting amounts of sugar reagents precluded further ranking.

Perhaps it is unexpected that the PmHAS and PmCS enzymes would elongate noncognate or sulfated GAGs, but it is not illogical. Some glycosyltransferases have been reported to be promiscuous with respect to their substrate recognition. Also, in living bacteria, the synthase enzymes in the cell interior are not expected to be confronted with noncognate acceptor molecules; thus these catalysts do not need to be selective. On the other hand, the synthase donor sites (especially the UDP-hexosamine pocket) are exposed to structurally similar UDP-sugars, and thus more stringent selectivity for the authentic substrate is expected and is observed.

A potential biotechnological utility of the relaxed acceptor usage (i.e. the ability of these particular synthases/polymerases to accept unnatural substrates) is the creation of GAG molecules with two or more types of sugar repeating units. A variety of chimERIC polymers consisting of two distinct GAG polysaccharides fused together were produced (Figs. 2 and 3). The synthase-catalyzed reactions were rapid (complete within 2–6 h). The length of the added HA or chondroitin chain may be con-
In Fig. 3, polydispersity appears to be relatively monodisperse populations (for example, donor/GAG acceptor ratio. In addition, the chimeric polymers Fig. 3) to controlled by altering the stoichiometry of UDP-sugar to acceptor (12). For example, chondroitin sulfate was extended by PmHAS with HA chains ranging from ~400 kDa (~2,000 saccharides; Fig. 3) to ~1,600 kDa (~8,000 saccharides) depending on the donor/GAG acceptor ratio. In addition, the chimeric polymers appear to be relatively monodisperse populations (for example, in Fig. 3, polydispersity Mw/Mn values: HA-CSbt, 1,006 ± 0.02; C-HA, 1,003 ± 0.02; for reference, “1” is the ideal polymer preparation).

We verified that HA was not contaminating the chondroitin sulfate preparations by pre-treating with HA lyase; this enzyme produces HA oligosaccharides with defective unsaturated non-reducing ends that cannot be extended by Pasteurella synthases (data not shown). In addition, the Stains-All detection method for agarose gels can distinguish chondroitin sulfate from HA. The sulfated polymers yield a purple or yellow color (depending on sulfation level), whereas the HA is blue; the chimeric molecules have a purple color readily distinguishable from the HA alone (supplemental data, Fig. S1). Furthermore, the chimeric nature of the new GAGs is demonstrated by treatment with specific HA lyase (Fig. 3). For example, an HA chain was extended with a chondroitin chain via the action of PmCS. The chimeric molecule was then treated with HA lyase, digesting the HA chain, leaving the chondroitin chain component intact.

We did note that the entire bulk chondroitin sulfate polymer starting material was not incorporated into chimeric molecules even after repeated addition of synthase and UDP-sugars; this observation suggests that certain nonreducing termini (especially the 4-sulfated chondroitin units) cannot be efficiently extended by native sequence PmHAS or PmCS enzymes. At the start of a reaction utilizing a poorly functioning noncognate acceptor, the Pasteurella synthase slowly extends the polymer, but once the catalyst adds on a short cognate sugar extension to the nonreducing terminus, the nascent molecule is transformed rapidly into an excellent acceptor. At longer times (e.g. approximately >2 h), the differences because of the initial lag period are obscured, and thus the differences among the various chondroitin sulfate preparations are less apparent.

The only other reported methods for chemoenzymatically constructing chimeric GAGs involved the use of testicular hyaluronidase to (a) transglycosylate GAG chains (28, 29) or (b) couple oxazoline monomers (30). Testicular hyaluronidase catalyzes the cleavage of HA, chondroitin, or chondroitin sulfate using a water hydroxyl with an optimum at pH 5. However, the hyaluronidase can also use a polymer hydroxyl group thus adding saccharide units instead of cleaving in a reaction called transglycosylation with an optimum at pH 7. Unfortunately, the transglycosylation method is difficult to control and has low yields; a mixture of products (i.e. a series of structurally similar chimeric GAGs with ~6–22 saccharide units) result, and the degrading enzyme will actually cleave the products. In the latter elegant method, oxazoline sugar analogs (e.g. employing mixtures of HA, chondroitin, or chondroitin sulfate disaccharides, etc.) mimicking the transition state are coupled by the hyaluronidase. However, these analogs are unstable in water resulting in a potential overabundance of “dead-end” acceptors (the oxazoline analog degrades to an ordinary disaccharide) compared with the activated donor that drives down the size distribution of the final products (~10–20 kDa or ~50–100 sugars reported). In addition, the product degradation by hyaluronidase problem mentioned above in method a also exists. Also, the exact placement of desired sugars within a given target structure (especially for small chains) is difficult or impossible for the oxazoline process. On the other hand, the HA and chondroitin synthases and their mutants may be utilized in a stepwise fashion to make defined GAG oligosaccharides (14).

Proteoglycan-like molecules with two distinct high molecular weight GAG components, but missing the protein bridge (e.g. core and link proteins), can be assembled by our method. These molecules may potentially serve the field of tissue engineering as scaffolds to assemble various cell types into tissues or organs. The lack of any polypeptide in these artificial chimeric molecules removes the issues of protease susceptibility and concerns of both immunogenicity and allergenicity (e.g. when an animal-derived product is used in humans). Furthermore, the threat of adventitious agents (e.g. prions, virus) from animal sources may be diminished or eliminated.

On a smaller scale, novel oligosaccharides with HA-like and/or chondroitin-like structures may also be constructed using the Pasteurella synthases.3 We are exploring if these molecules bind with different selectivity or affinity to hyaladherins, HA-binding proteins, or receptors. In addition to mapping out the oligosaccharide binding requirements of hyaladherins, we

3 B. S. Tracy, A. E. Sismey, and P. L. DeAngelis, unpublished observations.
may be able to generate compounds that will selectively inhibit the binding of HA to one particular hyaladherin species without perturbing other species. Such sugar molecules may have future utility as selective therapeutics with minimal side effects for diseases such as cancer, autoimmune disease, inflammation, and infection.

Acknowledgments—We thank Tasha A. Kane, Dixy E. Green, Dr. F. Michael Haller, Dr. Wei Jing, Leonard C. Oatman, Dr. Phillip Pummill, Alison E. Sismey, Carissa L. White, Bruce Baggenstoss, Lianli Chi, and Craig Jewett for technical assistance.

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