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Critical Elements of Oligosaccharide Acceptor Substrates for the *Pasteurella multocida* Hyaluronan Synthase*

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Three-dimensional structures are not available for polysaccharide synthases and only minimal information on the molecular basis for catalysis is known. The *Pasteurella multocida* hyaluronan synthase (PmHAS) catalyzes the polymerization of the alternating β1,3-*N*-acetylglucosamine-β1,4-glucuronic acid sugar chain by the sequential addition of single monosaccharides to the non-reducing terminus. Therefore, PmHAS possesses both GlcNAc-transferase and glucuronic acid (GlcUA)-transferase activities. The recombinant *Escherichia coli*-derived PmHAS enzyme will elongate exogenously supplied hyaluronan chains *in vitro* with either a single monosaccharide or a long chain depending on the UDP-sugar availability. Competition studies using pairs of acceptors with distinct termini (where one oligosaccharide is a substrate that may be elongated, whereas the other cannot) were performed here; the lack of competition suggests that PmHAS contains at least two distinct acceptor sites. We hypothesize that the size of the acceptor binding pockets of the enzyme corresponds to the size of the smallest high efficiency substrates; thus we tested the relative activity of a series of authentic hyaluronan oligosaccharides and related structural analogs. The GlcUA-transferase site readily elongates (GlcNAc-GlcUA)$_2$, whereas the GlcNAc-transferase elongates GlcUA-GlcNAc-GlcUA. The minimally sized oligosaccharides, elongated with high efficiency, both contain a trisaccharide with two glucuronic acid residues that enabled the identification of a synthetic, artificial acceptor for the synthase. PmHAS behaves as a fusion of two complete glycosyltransferases, each containing a donor site and an acceptor site, in one polypeptide. Overall, this information advances the knowledge of glycosaminoglycan biosynthesis as well as assists the creation of various therapeutic sugars for medical applications in the future.

Glycosaminoglycans, linear chains composed of alternating disaccharide repeats containing a hexosamine, are a class of heteropolysaccharides that includes hyaluronan (HA),[2] heparin, chondroitin, and keratan. These carbohydrates serve essential roles in vertebrates, including as intracellular adhesives, signaling molecules, anticoagulants, and structural elements. Certain pathogenic bacteria take advantage of these versatile molecules to produce extracellular glycosaminoglycan capsules, thus constructing molecular camouflage to avoid host defenses and increase virulence.

The bacterial *Pasteurella multocida* synthases are dual action enzymes that add both sugars of the glycosaminoglycan repeat to the non-reducing terminus of the acceptor oligosaccharides (1). The *P. multocida* Type A hyaluronan synthase PmHAS and the *P. multocida* Type F chondroitin synthase PmCS have been amenable to study due to their two active center architectures (2, 3), their ability to polymerize long chains *in vitro* (4, 5), and their ability to elongate certain exogenously added acceptor oligosaccharides (6). The *Escherichia coli* K4 chondroitin polymerase KfoC (7) is ~60% identical to PmHAS and PmCS; therefore, this system probably operates in a similar fashion. The recently described *P. multocida* heparosan synthases, one from Type D (PmHS1) (8) and another cryptic enzyme from Types A, D, and F (PmHS2) (9), promise to be interesting experimental models of dual action enzymes as well, but their overall amino acid sequence appears to differ from PmHAS and PmCS.

In general, enzymological studies of glycosyltransferases have focused on the catalytic residues, the donor binding site, and the acceptor binding site. Structural information on some "simple" glycosyltransferases that add only one sugar to a glycoconjugate has been obtained, but a structure has not been determined for a dual action enzyme or a polysaccharide synthase. PmHAS and PmCS each appear to possess an independent hexosamine donor transfer site and a glucuronic acid donor transfer site (2, 3), but the nature and the number of sugar acceptor sites has not been determined. As a first step to analyze the acceptor site(s), here we have tested a range of acceptor sugars that PmHAS will elongate with the HA chain. We speculate that the size of the synthase acceptor binding pocket corresponds roughly to the size of the smallest high efficiency substrate. Our findings have allowed us to determine the optimal length of the sugar polymer necessary for efficient chain elongation and to design a synthetic, artificial acceptor for the synthase enzyme. In addition, we have indirect evidence for two distinct acceptor sites in PmHAS.

EXPERIMENTAL PROCEDURES

Reagents and Enzyme Preparation—All reagents were of the highest grade available from either Sigma or Fisher, unless otherwise noted. The soluble, truncated dual action PmHAS$^{1-703}$ enzyme was prepared by chromatography as described previously (10). Briefly, the recombinant cells expressing PmHAS$^{1-703}$ were extracted with 1% (w/v) octyl thioglucoside in 1 M ethylene glycol and 50 mM Hepes, pH 7.2. The clarified extract was purified on a Toyopearl Red AF resin (Tosoh, Montgomeryville, PA) column. The protein was eluted with a NaCl gradient (50 mM Hepes, pH 7.2, 1 M ethylene glycol with 0–1.5 M NaCl gradient in 1 h). The peak fractions with PmHAS, as assessed by Coomassie Blue staining of SDS-polyacrylamide gels, were pooled and concentrated by ultra-

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[2] The abbreviations used are: HA, hyaluronan, hyaluronic acid, or hyaluronate; PmHAS, *P. multocida* hyaluronan synthase; PmCS, *P. multocida* Type F chondroitin synthase; GlcUA, glucuronic acid; MP, methoxyphenol.

filtration. The protein content was quantitated by the Bradford assay (Pierce) with a bovine serum albumin standard. The final preparations were typically ~95% pure PmHAS based on staining of the gels.

Sugar Acceptor Substrates—The HA_4 tetrasaccharide (with GlcUA at the non-reducing terminus) was derived from exhaustive digestion of HA (streptococcal) with testicular hyaluronidase (Type V), chloroform solvent extraction, and gel chromatography on P2 resin (Bio-Rad) (11). Longer natural HA oligosaccharides ($HA_{14,\ 15,\ 20,}$ and $_{21}$) were synthesized chemoenzymatically from HA_4 and UDP-sugars using immobilized enzyme reactors (11).

A series of HA-like oligosaccharides were synthesized by organic chemistry methodology; each sugar contained a *para*-methoxyphenyl group at the reducing end (12). For simplicity, a coding scheme was used to designate each monosaccharide: GlcUA, A; GlcNAc, N; methoxyphenyl, MP. For example, the compound AN-MP refers to GlcUA-GlcNAc-MP as read from non-reducing end to reducing end.

Various synthetic glycosides were purchased, and again the simple coding scheme was applied: fluorescein mono-β-D-glucuronide, A-F; fluorescein di-β-D-glucuronide, A-F-A (Molecular Probes, Eugene, OR); fluorescein di-β-D-glucopyranoside, G-F-G (Molecular Probes); fluorescein di-β-D-*N*-acetylgalactosamine, GalNAc-F-GalNAc (Marker Gene Technologies, Eugene, OR); 1-naphthyl β-D-glucuronide, A-NAP; *p*-nitrophenyl-β-D-glucuronide, A-NP (Calbiochem, La Jolla, CA), 4-nitrophenyl-β-D-galacturonide, GalA-NP; 4-methylumbelliferyl β-D-glucuronide, A-MUM; β-trifluoromethylumbelliferyl β-D-glucuronide, A-F_3MUM (Molecular Probes); 3-carboxyumbelliferyl β-D-glucuronide, A-CU (Molecular Probes); 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide, N-MUM; and 4-methylumbelliferyl β-D-glucopyranoside, G-MUM.

Single Sugar Addition Assays—The assays monitored the transfer of either (*a*) a single GlcUA to an acceptor with a non-reducing end terminating in GlcNAc according to the reaction,

$$n\text{UDP-GlcUA} + n\text{GlcNAc-}X \rightarrow n\text{GlcUA-GlcNAc-}X + n\text{UDP}$$

REACTION 1

or (*b*) a single GlcNAc to an acceptor with a non-reducing end terminating in GlcUA according to the reaction,

$$n\text{UDP-GlcNAc} + n\text{GlcUA-}X \rightarrow n\text{GlcNAc-GlcUA-}X + n\text{UDP}$$

REACTION 2

where X = the remainder of the acceptor molecule. PmHAS (0.4 μM) was assayed in 25-μl reactions containing a titration of one of the various acceptors, 50 mM Tris, pH 7.2, 5 mM $MnCl_2$, 1 M ethylene glycol, and either 1 mM UDP-[^{3}H]GlcUA (0.15 μCi) or 1 mM UDP-[^{3}H]GlcNAc (0.15 μCi) (PerkinElmer Life Sciences), respectively. Control assays without any acceptor were also performed in parallel; this background value was subtracted from the value obtained in the acceptor-containing assays. Reactions were incubated at 30 °C for various times ranging from 4 to 1260 min.

Enzyme activity was linear with respect to time, and the reactions consumed <5% of the UDP-sugar substrate. All assay points were performed in duplicate, and the values were averaged. The data were plotted using the Michaelis-Menten equation ($V = V_{max}$ [substrate]/K_m + [substrate]) with Sigma Plot software (Rockware, Golden, CO), where the apparent Michaelis-Menten constants (K_m) were derived from the concentration of sugar that yields 50% of maximal incorporation.

When the acceptor possessed a hydrophobic aglycone (*e.g.* MP, F, etc.), reversed phase solid phase extraction was employed to separate the products from the reactants. Descending paper chromatography was utilized when native HA was longer than HA_{14} (polymer size that is completely retained at the origin, thus facilitating single sugar transfer assays).

For solid phase extraction analysis, reactions were terminated by placing on ice and diluting with 275 μl of ice-cold 1 M NaCl. The free unincorporated UDP-[^{3}H]sugar precursors were separated from the elongated reaction products using reversed phase cartridges (Strata-X polymeric 33-μm resin; 30 mg of sorbent/1-ml cartridge; Phenomenex, Torrance, CA) and a vacuum manifold. When passing solvents through the column, the bed was not allowed to dry until directly before and after the elution step. The columns were sequentially conditioned with 1 ml each of 100% methanol, 50% methanol, and water. The columns were equilibrated with 1 ml of 1 M NaCl, and then samples in 1 M NaCl were added to the sorbent bed. The columns were washed with 7 ml of 1 M NaCl allowing retention of the methoxyphenol or hydrophobic compounds and the removal of UDP-[^{3}H]sugars. After completing the NaCl wash, the column bed was air-dried for 30 s with vacuum suction. To release the acceptors from the sorbent, the columns were eluted with 2 ml of 50% methanol. BioSafe II mixture (4 ml) (Research Products International, Chicago, IL) was added to 1 ml of the eluted sample, and incorporation of the [^{3}H]sugars was quantitated by liquid scintillation counting.

For descending paper chromatography analysis, reactions were terminated by adding SDS to a final concentration of 2% and then spotted onto strips of Whatman No. 3MM paper, and the reaction products at the origin were separated from the free UDP-[^{3}H]sugars by development with 65:35 ethanol/1 M ammonium acetate, pH 5.5, overnight (2). The origins were cut from the paper strips and eluted in 750 μl of water for 1 h. BioSafe II mixture (4 ml) was added, and incorporation of the [^{3}H]sugars was quantitated by liquid scintillation counting.

HA Polymerization Assays—The PmHAS-catalyzed polymerization assay measured the incorporation of both GlcUA and GlcNAc onto acceptors to form longer HA chains as in

$$n\text{UDP-GlcNAc} + n\text{UDP-GlcUA} + nX \rightarrow n(\text{GlcNAc-GlcUA})_n\text{-}X + 2n\text{UDP}$$

or

$$n\text{UDP-GlcNAc} + n\text{UDP-GlcUA} + nX \rightarrow n(\text{GlcUA-GlcNAc})_n\text{-}X + 2n\text{UDP}$$

REACTIONS 3 AND 4

where X = the acceptor (exact product depends on the identity of the acceptor non-reducing terminus). PmHAS polymerization activity was assayed under the identical conditions as the single sugar addition assay, except 1 mM UDP-[^{3}H]GlcUA (0.15 μCi) and 1 mM UDP-GlcNAc were present simultaneously. Descending paper chromatography was used to measure incorporation as described above. The experiments were performed in duplicate, and data points were averaged, unless otherwise noted.

Sugar Competition Assays—To assess the number of acceptor sites within the PmHAS polypeptide, we devised competition experiments

between two distinct oligosaccharides, each having a different non-reducing terminal sugar; HA_{14} terminates in GlcUA, whereas HA_{15} terminates in GlcNAc. Both oligosaccharides were present simultaneously in a 3–4-min reaction, with only one type of radiolabeled UDP-sugar nucleotide. In this situation, one oligosaccharide served as the acceptor substrate molecule and the other as the potential competitor molecule that cannot be extended. For example, in one experiment with UDP-[^{3}H]GlcNAc, HA_{14} (0.25 mM) functioned as the acceptor for the addition of the GlcNAc moiety, whereas HA_{15} served as the potential competitor. In the converse experiment with UDP-[^{3}H]GlcUA, HA_{15} (0.15 mM) served as the acceptor for the addition of the GlcUA monosaccharide, whereas HA_{14} functioned as the potential competitor. The reactions without a potential competitor (only the acceptor with the appropriate non-reducing terminus) were run in parallel and served as the "100% activity" value. The products of the reactions were analyzed by paper chromatography.

Analysis of in Vitro Synthesized HA—The synthetic molecule A-F-A was used as the acceptor to synchronize the synthesis of HA. Reaction conditions were 50 mM Tris, pH 7.2, 5 mM $MnCl_2$, 1 M ethylene glycol, 12.2 mM UDP-GlcUA, 12.2 mM UDP-GlcNAc, and 14 μM PmHAS plus A-F-A acceptor at three different concentrations (8, 80, and 800 μM) in a total volume of 25 μl. The reactions were incubated at 30 °C overnight. The size of the products was analyzed using agarose gel electrophoresis (1.2%; 1× TAE buffer (40 mM Tris acetate, 2 mM EDTA); 30 V) and Stains-All dye detection (0.005% w/v in ethanol) (13). Select-HA Lo and Hi Ladders composed of monodisperse HA polymers (10) were used as standards for size estimates (Hyalose, Oklahoma City, OK). To assess the authenticity of the HA linkages, portions of the reactions were treated with *Streptomyces* HA lyase, an enzyme that degrades no other glycosaminoglycan except HA. The pH value for the reaction was adjusted to pH 6 by the addition of sodium acetate (50 mM final). The reaction was boiled for 1 min at 95 °C and centrifuged to remove PmHAS. After overnight incubation with *Streptomyces* lyase, the sample was loaded onto the agarose gel.

To ascertain the presence of the aglycone in the product polymer chains, the reactions were adjusted to 0.2 M sodium nitrate and analyzed by high performance gel filtration chromatography on a Polysep 4000 column (1 ml/min, 0.2 M sodium nitrate; Phenomenex, Torrance, CA) with UV light absorbance detection at 272 nm for the A-F-A glycone. Fluorescent dextran standards with molecular masses of 4, 12, 50, and 580 kDa were used as calibrants (detection 490 nm).

The masses of products formed after reaction of A-F-A with PmHAS and UDP-GlcNAc were measured by mass spectrometry. Matrix-assisted laser desorption ionization time-of-flight spectra were obtained using a Voyager Elite DE mass spectrometer. The sample in water (1 μl of ~0.01 μg/μl oligosaccharide) was spotted on a target plate followed by 1 μl of matrix solution (10 mg/ml 6-aza-2-thiothymine in 50% acetonitrile, 49.9% water, 0.1% trifluoroacetic acid) and then mixed and vacuum-dried. The samples were analyzed using a negative ion reflectron mode with the following parameters: acceleration, 20 kV; low mass gate, 400 Da; and delayed extraction, 200 ns.

RESULTS

Catalytic Activities of PmHAS Transferase Sites—PmHAS has been shown previously to possess two relatively independent glycosyltransferase activities (UDP-*N*-acetylglucosamine, glucuronic acidyl: β(1,4)-N-acetylglucosaminyl transferase = GlcNAc-transferase and UDP-glucuronic acid, *N*-acetylglucosaminyl: β(1,3)glucuronic acidyltransferase = GlcUA-transferase) within a single polypeptide chain (2, 3). To directly compare the catalytic activities of the PmHAS GlcNAc-transferase site and GlcUA-transferase site, we performed parallel time course experiments monitoring the two single sugar addition reactions. For GlcNAc-transferase activity, HA_{22}, an acceptor that possesses a GlcUA at the non-reducing terminus, was employed with the UDP-[^{3}H]GlcNAc donor. HA_{21}, an acceptor that terminates in GlcNAc, was utilized with the UDP-[^{3}H]GlcUA donor for the GlcUA-transferase activity. The HA_{21} and HA_{22} oligosaccharides were chosen to mimic a long nascent HA polysaccharide chain but not cause the viscosity of the reaction mixture to increase. Portions of the reactions were removed at various times, quenched, and analyzed by descending paper chromatography (Fig. 1). The rate of each transferase activity corresponds to the slope (average $\Delta V/\Delta$time) at the initial phase of the reaction. The initial velocity of the GlcUA-transferase activity (6.5 nmol/min) was much more rapid (~20-fold) than the GlcNAc-transferase activity (0.32 nmol/min). These values translate to an enzyme turnover number of 10 and 0.5 substrate molecules/s, respectively; for comparison, the turnover numbers for two well studied enzymes, DNA polymerase and hen egg lysozyme, are 15 and 0.5, respectively.

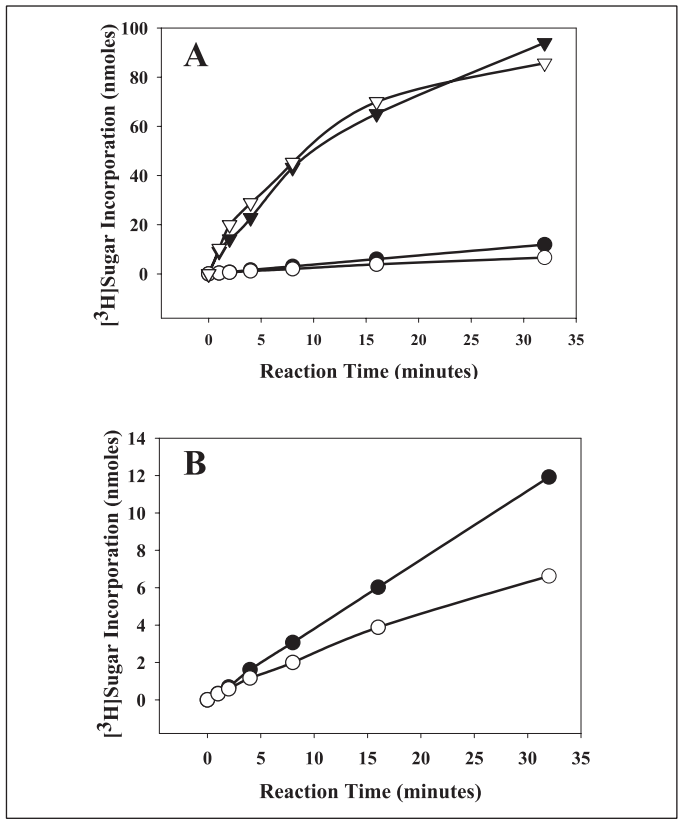

FIGURE 1. **Time course of single sugar addition to native HA oligosaccharides**. The reactions were carried out as described for single sugar addition with a 1 mM acceptor and analyzed using descending paper chromatography. *A*, two independent reactions (*open* or *solid symbols*) were monitored over time. Under virtually identical conditions, the GlcUA-transferase activity of PmHAS (*triangles*) was ~20-fold more rapid than that of the GlcNAc-transferase activity (*circles*) *in vitro*. *B*, magnified scale to depict the linearity of GlcNAc-transferase activity.

Evidence for Two Acceptor Binding Sites—Although there is information regarding the PmHAS UDP-sugar donor binding sites, the number of acceptor binding sites was not known, and there is no precedent in the literature on any dual action glycosyltransferases. We performed a series of competition assays to detect the presence of single or multiple acceptor binding sites within the PmHAS polypeptide. In these reactions, one oligosaccharide (HA_{14} or $_{15}$) served as the acceptor for the appropriate radiolabeled UDP-sugar (UDP-GlcNAc or UDP-GlcUA for even length or odd length HA polymers, respectively). The HA_{14} and

HA_{15} oligosaccharides are convenient acceptors for paper chromatography assays that remain at the origin, allowing the facile monitoring of the single monosaccharide addition. The potential competitor oligosaccharide, which is incapable of being extended due to the lack of the appropriate UDP-sugar in the reaction, was introduced into the reaction at equimolar or 10-fold higher concentrations. For example, the PmHAS GlcNAc-transferase activity was measured for (i) HA_{14} alone (defined as 100% activity), (ii) 1:1 HA_{14} to HA_{15} (a potential competitor that ends in a GlcNAc and therefore cannot be extended), and (iii) 1:10 HA_{14} to HA_{15}. Conversely, the PmHAS GlcUA-transferase activity was measured for (i) HA_{15} alone (again 100% activity), (ii) 1:1 HA_{15} to HA_{14}, and (iii) 1:10 HA_{15} to HA_{14}. Essentially, in these reactions, the competitor oligosaccharide could potentially bind to an acceptor site, but elongation by the supplied UDP-sugar is impossible. In Table 1, the lack of inhibition by the oligosaccharide with the inappropriate non-reducing termini suggests that PmHAS possesses at least two independent acceptor binding sites.

TABLE 1

Competition studies of GlcUA- or GlcNAc-terminated acceptors with PmHAS

Single sugar addition assays where one oligosaccharide served as the acceptor (*e.g.* $[GlcUA\text{-}GlcNAc]_{14}$ = HA_{14} in reaction with UDP-GlcNAc), whereas the other oligosaccharide (*e.g.* GlcNAc-$[GlcUA\text{-}GlcNAc]_{14}$ = HA_{15}) served as a potential competitor that may bind but cannot be elongated. Data from averaged duplicates are shown from two independent experiments (I and II). The absence of inhibition by the second oligosaccharide suggests that at least two distinct acceptor binding sites exist for the PmHAS enzyme. NA, not applicable.

Experiment	Acceptor	Competitor	Ratio	GlcNAc-transferase activity	GlcUA-transferase activity
				%	
I	HA_{14}	None		100	NA
	HA_{14}	HA_{15}	1:1	110	NA
	HA_{14}	HA_{15}	1:10	150	NA
	HA_{15}	None		NA	100
	HA_{15}	HA_{14}	1:1	NA	93
	HA_{15}	HA_{14}	1:10	NA	99
II	HA_{14}	None		100	NA
	HA_{14}	HA_{15}	1:1	140	NA
	HA_{14}	HA_{15}	1:10	160	NA
	HA_{15}	None		NA	100
	HA_{15}	HA_{14}	1:1	NA	98
	HA_{15}	HA_{14}	1:10	NA	82

Analysis of HA Sugars as Acceptors for PmHAS—To determine the minimal acceptor structure necessary for efficient HA elongation, we investigated two series of authentic, synthetic hyaluronan ($[\beta4GlcUA\text{-}\beta3GlcNAc]_n$ = $[AN]_n$ or $[\beta3GlcNAc\text{-}\beta4GlcUA]_n$ = $[NA]_n$) oligosaccharides containing a MP group at the reducing termini (Fig. 2). The use of sugars with (*a*) a non-reducing termini ending in GlcUA or (*b*) a non-reducing termini ending in GlcNAc allows the probing of both putative acceptor sites in the model. The relative activity of the MP sugars, including AN-MP, ANA-MP, ANAN-MP, ANANAN-MP, N-MP, NA-MP, NAN-MP, NANA-MP, and NANAN-MP, were tested. The hydrophobicity of the methoxyphenol group of these HA-related oligosaccharides permits the use of solid phase extraction with a reverse phase sorbent for facile analysis of single sugars to small molecule acceptors.

Fig. 3 depicts a uniform, representative data set of all of the methoxyphenol sugars from two independent experiments. For the GlcUA-transferase activity, the tetrasaccharide NANA-MP and longer served as efficient acceptors for PmHAS-catalyzed elongation (*i.e.* rapid reac-

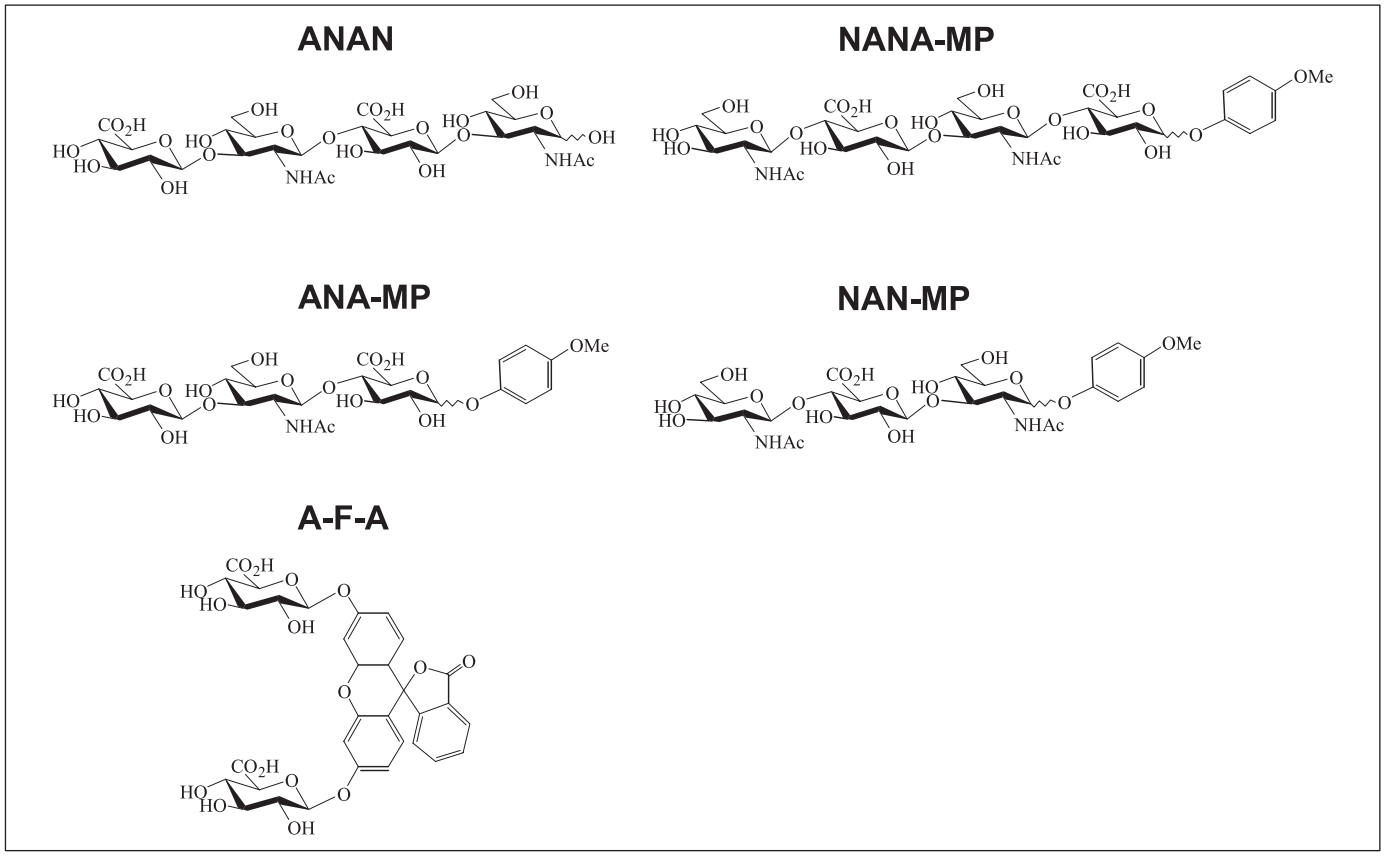

FIGURE 2. **Structures of various HA oligosaccharides and a glycoside analog.** Various synthetic analogs of the functional native HA tetrasaccharide ANAN were tested for activity. The trisaccharide ANA-MP and the tetrasaccharide NANA-MP were the minimal high efficiency acceptors for the PmHAS GlcNAc-transferase and GlcUA-transferase activities, respectively. On the other hand, the trisaccharide NAN-MP was a poor acceptor. The synthetic glycoside mimic A-F-A functioned almost as well as ANA-MP.

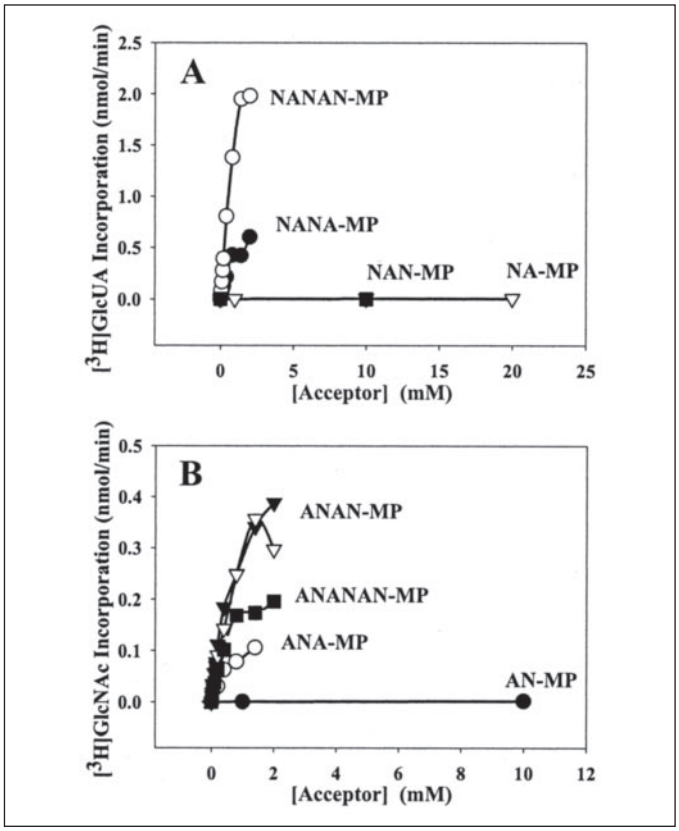

FIGURE 3. **Michaelis-Menten analysis of methoxyphenol sugars as acceptors.** Single sugar addition reactions were performed in duplicate using MP-oligosaccharides and then purified using solid phase extraction; averaged data minus the "no acceptor control" (<0.1 pmol/min) is shown. *A*, GlcUA-transferase activity assays with GlcNAc-terminated acceptors: NANAN-MP (*open circles*), NANA-MP (*solid circles*), NAN-MP (*solid squares*), and NA-MP (*open triangles*). *B*, GlcNAc-transferase activity assays with GlcUA-terminated acceptors: ANAN-MP (*open* and *solid triangles*), ANANAN-MP (*solid squares*), ANA-MP (*open circles*), and AN-MP (*solid circles*).

tions (4 min) and low concentrations (1–2 mM) (Fig. 3*A*). Conversely, for the GlcNAc-transferase activity, the trisaccharide ANA-MP and longer were efficient acceptors (Fig. 3*B*). In contrast, the two possible methoxyphenol disaccharides and NAN-MP were poor substrates that required longer times (30–60 min) and higher concentrations (10–30 mM) to detect sugar transfer. However, the worst acceptor, N-MP, may be elongated after extensive reactions (Table 2); thin-layer chromatography analysis of the solid phase extraction-purified product verified that this low level of activity was indeed true sugar addition and not simply an assay background problem (data not shown).

Analysis of Synthetic Analogs as Acceptors for PmHAS—The data generated from the activity of the methoxyphenol sugars in elongation assays suggested a requirement of two GlcUA sugars for the high efficiency acceptors and thus enzyme recognition and/or utilization. Recently, with characterization of the PmHAS enzyme's usage of HA-like analogs with unnatural hexosamine sugars, the hydrophobic interaction appears to be involved in binding or in catalysis.[3] To confirm the significance of these putative critical structural elements of acceptors for PmHAS, multiple GlcUA groups, and a hydrophobic moiety on the hexosamine, we tested a variety of commercially available, synthetic analogs.

A collection of hydrophobic glycosides were tested as PmHAS acceptors: A-F, A-F-A, G-F-G, GalNAc-F-GalNAc, A-NAP, A-NP, GalA-NP, A-MUM, N-MUM, Gluc-MUM, A-F_3MUM, and A-CU. Most of the

[3] K. J. Williams, F. M. Haller, and P. L. DeAngelis, unpublished data.

TABLE 2

PmHAS velocities (*V*) for methoxyphenol sugars and synthetic acceptors

Single sugar addition assays were performed where the next appropriate sugar necessary for HA chain elongation was incorporated (*e.g.*, AN-MP + UDP-GlcNAc). The velocities at different acceptor concentrations (2 or 10 mM) were compared. Comparison of the trisaccharide efficiencies (ANA-MP at 2 mM and NAN-MP at 10 mM) indicate that ANA-MP is much more efficient at a lower substrate concentration. PmHAS efficiency for A-F-A, the simple glycoside, is similar to ANA-MP at equivalent concentrations.

Sugar	*V* (2 mM)	*V* (10 mM)
	nmol/min	
AN-MP		0.0013 ± 0.0005
ANA-MP	0.11	
ANAN-MP	0.34	
ANANAN-MP	0.22	
A-F-A	0.091	
N-MP		0.000055
NA-MP		0.0043
NAN-MP		0.0056 ± 0.002
NANA-MP	0.47 ± 0.14	
NANAN-MP	1.8	

substrates tested were poor PmHAS acceptors, as seen by the production of no or small amounts of elongation products, even after utilizing extensive reaction times and/or high concentrations. However, A-F-A (Fig. 2) generated a signal similar to ANA-MP (V = ~0.1 nmol/min at 2 mM) (Table 2). Although A-F elongation was detected (~9% of the A-F-A value), the addition of the second GlcUA to produce A-F-A greatly boosted the velocity.

To verify that PmHAS was incorporating the GlcNAc sugar onto A-F-A, a single sugar addition reaction was analyzed by mass spectrometry. After a 2-h reaction incubation period, the major reaction product was a compound formed by the addition of a single GlcNAc residue (experimental = 683 Da; theoretical = 684 Da). Upon longer incubation, PmHAS added a GlcNAc to both sides of the substrate (experimental = 886 Da; theoretical = 887 Da), but the single addition product was still more abundant. Thus the increase in activity of A-F-A over A-F was not because of a simple doubling of the number of usable termini. The ability of PmHAS to extend both GlcUA groups of A-F-A (although at low levels in these experimental conditions) is probably the result of the initial NA-F-A product molecule rebinding to the enzyme and being elongated to form NA-F-AN.

Two other structurally similar compounds, G-F-G and GalNAc-F-GalNAc, however, did not show high activity (<2% of the A-F-A value). Therefore, the substrate containing both the hydrophobic component (potentially mimicking the acetyl moiety of the GlcNAc saccharide and/or hydrocarbon face of the pyranose ring) as well as two GlcUA sugars elicited the best enzyme activity, reinforcing the importance of both characteristics. The predicted spacing between the two carboxylate groups of the ANA and the A-F-A molecules are similar (estimated distances from C6-GlcUA to C6-GlcUA: ANA, 9.0 ± 0.8 Å in aqueous solution or 10.7 Å if totally extended; A-F-A ~12–20 Å),[4] but the actual bound conformation of the saccharides in the acceptor site is not yet known.

Synthesis of HA Polymers with Artificial Acceptors—The success of A-F-A as an acceptor substrate initiated the exploration of its utility as a primer for the synthesis of HA. An acceptor molecule will bypass the slow PmHAS initiation step, resulting in synchronized reactions that yield relatively monodisperse polymer products. Polymerization reactions with A-F-A at three different concentrations (8, 80, and 800 μM) were performed and analyzed by gel electrophoresis (Fig. 4). The size of

[4] A. Almond, estimates from preliminary molecular modeling, dynamic simulations; personal communication.

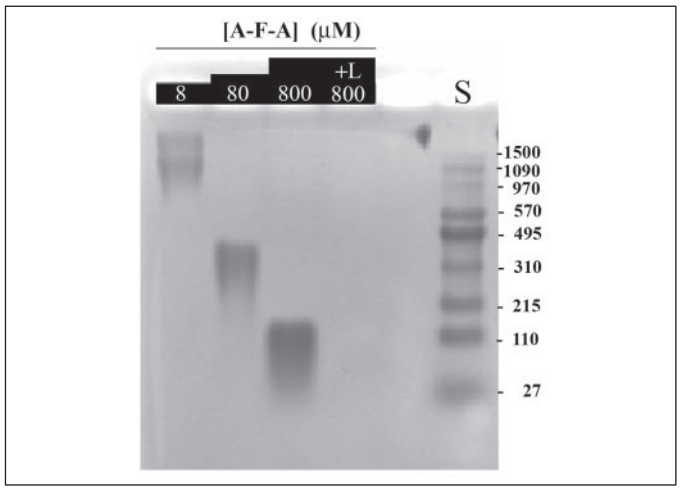

FIGURE 4. **Gel Analysis of HA polymer products using the A-F-A acceptor.** Three parallel chemoenzymatic HA synthesis reactions with different concentrations of A-F-A were separated on a 1.2% agarose gel with Stains-All detection. The material is authentic HA as shown by its sensitivity to HA lyase treatment (+*L*). The UDP-sugar/A-F-A acceptor stoichiometry of the reaction controls the size of the HA polymer product. (*S* = HA standards: ranging from 27–1500 kDa from *bottom* to *top*)

the products were ~1,500, ~400, or ~110 kDa for various reactions where higher concentrations of A-F-A yielded smaller chains as expected. Essentially, the presence of a low concentration of acceptor with a finite amount of UDP-sugars will synthesize large HA products; conversely, the presence of a high concentration of acceptor and the same finite amount of UDP-sugars will synthesize smaller HA products (10). Gel filtration analysis with ultraviolet absorbance detection proved that the polymer contained the fluorescein aglycone (not shown). Lyase degradation of the A-F-A reaction products proved that authentic HA chains were produced.

Monodisperse HA built on A-F-A as a primer will not fluoresce until hyaluronidases remove the HA chains and β-glucuronidase cleaves the GlcUA groups proximal to the fluoresceine moiety. These degradation enzymes co-exist in the lysosome; thus, such probes should be suitable for tracking HA degradation following uptake via receptors in liver sinusoidal cells or lymph node cells.

DISCUSSION

Disparity among Transferase Site Catalytic Activities—Previous PmHAS investigations demonstrate that two relatively independent active sites on one polypeptide are responsible for the alternating GlcUA and GlcNAc addition to form the HA polymer (2, 3); molecular genetic disruption of one transferase domain does not affect the catalytic activity of the other domain. Under identical reaction conditions, the time course data indicates that the GlcUA-transferase activity is ~20-fold faster than the GlcNAc-transferase activity; GlcNAc-transfer does indeed occur, although at a much lower rate. This finding is interesting in light of the similar K_m values of the UDP-sugar precursors for PmHAS (UDP-GlcNAc, 160 ± 60 μM; UDP-GlcUA, 140 ± 40 μM) (2).

There may be two possible explanations for this disparity between the two transferase activities based on (*a*) biochemical or (*b*) evolutionary rationales. In the first explanation, the synthesis of the HA chain is driven by the hydrolysis of UDP-sugar precursors in which the activated bond between the oxygen and the sugar to be transferred is broken, and the sugar is then added to the growing polymer chain. The terminal phosphate of the UDP by-product gains an additional negative charge (two other negative groups are present in the original UDP-sugar, but these groups are probably chelated by the Mn^{2+} metal ion). In the case of UDP-GlcUA addition, it is possible that this negative charge on the UDP by-product repels the glucuronic acid sugar moiety on the terminus of the nascent HA chain because of the similar charge of the carboxylate group, thus enhancing reaction rate and/or aiding product departure from the enzyme active site. On the other hand, in the case of UDP-GlcNAc addition, the neutral hexosamine is at a relative disadvantage, because it is not subject to electrostatic repulsion with the UDP by-product.

In the second potential explanation, during evolution, PmHAS may have been forged by gene duplication and fusion events. Potential evidence for a duplication event is found within the PmHAS protein sequence itself; two tandem domains of ~100 residues with similar motifs are present (2). Later, mutation of one transferase site into a new activity rendered the glycosyltransferase capable of producing a heteropolymer. It is not readily apparent if the fusion occurred before or after the mutation event. Essentially, it is possible that the adaptation of the transferase site to the new function is not complete and therefore non-optimal, resulting in the observed disparate reaction speeds of the GlcUA-transferase and the GlcNAc-transferase activities.

Postulation of a Two-site Acceptor Binding Model—Competition between GlcUA- and GlcNAc-terminated oligosaccharides for PmHAS-mediated elongation was not observed. Therefore, Occam's razor (*i.e.* the simplest explanation is usually correct) was invoked to consider the possibility that PmHAS functions by utilizing at least two independent acceptor binding sites.

Several general hypothetical models of dual action glycosyltransferase structure are obvious including: 1) the "two acceptor binding sites/two donor binding sites" model, where the acceptor itself transitions from one binding site to the other site after each monosaccharide is added during elongation, (2) the "one acceptor binding site/two donor binding sites" model, where repetitive, cycling conformational change of the enzyme positions the non-reducing terminal sugar of the acceptor in the oligosaccharide binding site in close proximity to the appropriate transferase site, or (3) the "one acceptor binding site/one donor binding site" model, where repetitive conformational change creates/locates the appropriate catalytic machinery within the acceptor and donor binding sites for catalytic activity. Model 1 best depicts the observed PmHAS characteristics based on our new data and previous work by Jing and DeAngelis (2, 3).

Interestingly, the HA_{15} competitor appears to cause a slight increase in the GlcNAc-transferase activity (but the converse competitor HA_{14} does not stimulate GlcUA-transferase activity), suggesting potential conformational feedback or cooperativity between the two putative acceptor sites. This issue will require further study.

On basic physical premises, it is expected that polymerization catalyzed by a model 1 enzyme is intrinsically non-processive (*i.e.* the nascent chain is repeatedly released and rebound before the polymer is completed), whereas a model 2 or 3 enzyme is potentially processive (*i.e.* the nascent chain is not released until the polymer is completed). In several tests, including *in vitro* syntheses of oligosaccharides (11) and polysaccharides (10), PmHAS appears to operate in a non-processive fashion. The prototypical streptococcal HA synthase enzyme SpHAS (or HasA), which possesses a very different amino acid sequence, exhibits processive behavior *in vitro* (14).

Proposed Model of a Sugar Binding Pocket with Important Carboxylate Contacts—At this time, three-dimensional structures are not available for polysaccharide synthases. However, crystal structures have been solved in the presence of both donor and acceptor substrates for two non-homologous single action glycosyltransferases, GlcUAT1 and EXTL2 (15, 16); their amino acid sequences are not similar to the

PmHAS sequence, but their transferase activities are similar. The natural acceptor substrates are the trisaccharide Gal-Gal-Xyl-*O*-Ser-protein for GlcUAT1 and the tetrasaccharide GlcUA-Gal-Gal-Xyl-*O*-Ser-protein (but a GlcUA-Gal-glycoside will work *in vitro*) for EXTL2, a GlcNAc transferase. The structures of these acceptors are not identical to the repeating glycosaminoglycan backbone; however, the structural information indicates that interactions with the non-reducing disaccharide portion of the acceptor substrates determine acceptor specificity for these two enzymes (17). Essential oligosaccharide acceptor substrate binding sites have been determined for a number of carbohydrate-active enzymes. For example, the rate of lysozyme-catalyzed hydrolysis of oligomers of GlcNAc increases distinctively as the number of residues is increased from five to six; the substrate binding pocket accommodates a hexasaccharide as determined by crystallography (18, 19). A maltononaose binding site was observed in the crystal structure of the cyclodextrin transferase of *Bacillus circulans* strain 251 (20), illustrating that acceptors of nine sugars in length are not outside the realm of possibility.

Our experimental model system of PmHAS has allowed us to analyze protein-oligosaccharide interactions indirectly. Probing the active site of PmHAS with the series of methoxyphenol sugars of different lengths and other various acceptor substrates potentially reveals information about the acceptor specificity of PmHAS in the absence of a crystal structure. The kinetic data allow the ranking of various lengths of sugar acceptors to determine the optimal length of the sugar polymer necessary for efficient PmHAS chain elongation. The PmHAS GlcUA-transferase site efficiently elongates the tetrasaccharide NANA-MP at a low concentration during short incubation periods, whereas the PmHAS GlcNAc-transferase site efficiently elongated the trisaccharide ANA-MP. Therefore, our data suggest the acceptor binding sites of PmHAS contain pockets that can bind at least three or four monosaccharides for the GlcNAc-transferase or the GlcUA-transferase, respectively.

The minimal length acceptors demonstrating efficient elongation are oligosaccharides that contain the trisaccharide element ANA. The predilection for ANA-MP over NAN-MP suggests there are important contacts between the carboxylate groups of the two GlcUA sugars and the acceptor binding site of PmHAS. The substantial increase in the PmHAS elongation efficiency for the A-F-A acceptor, the simple proxy for ANA-MP, in comparison to the A-F acceptor also supports the hypothesis that the two GlcUA groups provide important enzyme contacts. Overall, this study should lead to synthesis of better analogs (*e.g.* higher efficiency, less expensive, animal-free manufacture) in the future.

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Critical Elements of Oligosaccharide Acceptor Substrates for the *Pasteurella multocida* Hyaluronan Synthase

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