beta,3-Glucuronyltransferase (GlcAT-I) is an essential enzyme involved in heparan sulfate and chondroitin sulfate biosynthesis. GlcAT-I is an inverting glycosyltransferase that catalyzes the transfer of glucuronic acid (GlcUA) to the common growing linker region Gal beta 1-3Galbeta1-4Xyl that is attached to a serine side chain of a core protein. Previously the structure of GlcAT-I has been solved in the presence of the donor product UDP and an acceptor analog Galbeta1-3Galbeta1-4Xyl (Pedersen, L. C., Tsuchida, K., Kitagawa, H., Sugahara, K., Darden, T. A. & Negishi, M. (2000) J. Biol. Chem. 275, 34580–34585). Here we report the x-ray crystal structure of GlcAT-I in complex with the complete donor UDP-GlcUA, thereby providing structures of an inverting glycosyltransferase in which both the complete donor and acceptor substrates are present in the active site. This structure supports the in-line displacement reaction mechanism previously proposed. It also provides information on the essential amino acid residues that determine donor substrate specificity.

Glycosaminoglycans such as heparan sulfates and chondroitin sulfates are attached to proteoglycans that are distributed on the cell surface and extracellular matrix (2). These molecules have been implicated in a variety of biological processes such as cell growth and differentiation, blood coagulation, and viral and bacterial infection (3, 4). Both heparan sulfate and chondroitin sulfate utilize the common linker region GlcUAbeta1-3Galbeta1-3Galbeta1-4Xyl-Ser to attach to the core protein at specific serine residues. GlcAT-I catalyzes the transfer of the GlcUA from the donor substrate UDP-GlcUA to the linker region Galbeta1-3Galbeta1-4Xyl-Ser (5, 6). In heparan biosynthesis this step is followed by the addition of N-acetylgalactosamine (GlcNAc) by alpha1,4-N-acetylgalactosaminyltransferase (EXTL-2) to the GlcUA of the linker (7). The heparan polymerases (EXTLs) elongate the polysaccharide chain by alternating the addition of GlcUA and GlcNAc (8). The bifunctional N-deacetylase/N-sulfotransferases (NDSTs) initiate sulfation of heparan by first deacetylation of certain GlcNAc moieties at the N2 position followed by sulfation of the nitrogen (9). This in turn allows for epimerization of certain GlcUA residues to iduronic acid by C5-epimerase followed by sulfation by specific sulfotransferases such as the 3-O-sulfotransferase, the 2-O-sulfotransferase, and the 6-O-sulfotransferase (3, 4).

Disruption of the enzymes involved in glycosaminoglycan biosynthesis has severe biological consequences in mammals. In humans, mutations in the genes EXT1 and EXT2 encoding for heparan polymerases lead to a disease known as hereditary multiple exostoses, which is characterized by cartilage-capped tumors (8). Mice deficient in NDST-1 die in the neonatal state and exhibit phenotypes for pulmonary hypoplasia (10). Mice deficient in NDST-2 exhibit abnormal mast cells (11, 12). In addition mice deficient of another heparan sulfotransferase, heparan sulfate 2-O-ST, die in the neonatal state from renal agenesis (13). Because GlcAT-I lies before these enzymes in the biosynthesis cascade, it appears that its function is essential for proper growth and development as well. To better understand the mechanistic properties of enzymes involved in glycosaminoglycan biosynthesis we have used x-ray crystallography to examine amino acid residues required for substrate recognition as well as catalysis for the GlcAT-I enzyme. The enzyme GlcAT-I can be divided into three regions: a membrane-spanning region (residues 8–25), a proline-rich stem region (residues 26–74), and a catalytic domain (residues 75–335). We previously solved the structure of the catalytic domain GlcAT-I in complex with the donor product UDP and an acceptor analog Galbeta1-3Galbeta1-4Xyl (1).

We now report the structure of the enzyme in the presence of the active donor UDP-GlcUA. This structure not only reveals the residues involved in recognizing the GlcUA but also supports the mechanism previously proposed for inverting glycosyltransferases. This provides insight for glycosaminoglycan biosynthesis as well as for many processes that utilize UDP-sugar-dependent glycosyltransferases.

MATERIALS AND METHODS

Protein of the recombinant human GlcAT-I catalytic domain was cloned, expressed, and purified as previously described (1). Crystals of the enzyme were obtained by the vapor diffusion hanging drop method. 4 μl of 15 mg/ml GlcAT-I in 25 mM HEPES pH 7.5, 50 mM NaCl, 10 mM MnCl2, and 10 mM UDP-GlcUA was mixed with 4 μl of the reservoir solution containing 21% monomethyl ether polyethylene glycol 2000 (MME-PEG-2000) and 100 mM MES, pH 6.0. Crystals were grown at 23 °C.

Although the protein was crystallized in the presence of UDP-GlcUA, only the UDP portion of the molecule was visible in the electron density when data were collected on these crystals (1). Two possibilities exist for the lack of electron density. The first is that the GlcUA portion of the molecule is highly mobile and not fixed in a single conformation. The other possibility is that on the time scale of the crystallization the bond in UDP-GlcUA is hydrolyzed releasing the GlcUA. The latter case has been demonstrated for previous crystallization attempts of T4 phage beta-glucosyltransferase with UDP-Glc (14).

To overcome this problem, crystals were transferred to 4 °C in four steps from a solution containing 21% MME-PEG-2000, 100 mM MES, pH 6.0, 10 mM MnCl2, and 10 mM UDP-GlcUA to a solution containing 23% MME-PEG-2000,
UDP-GlcUA Binding to GlcAT-I

RESULTS AND DISCUSSION

UDP-GlcUA Binding—The catalytic domain of GlcAT-I crystallizes with two molecules in the asymmetric unit. These two molecules have been suggested to represent the physiological dimer (1). The catalytic domain of GlcAT-I is made up of two subdomains. Residues 75–197 comprise the NTP–sugar donor subdomains. Residues 198–305 define the acceptor substrate binding subdomain. The UDP–GlcUA binding subdomain consists of an α/β Rossmann-like motif. The position of the uridine base is fixed through a hydrogen bond with conserved residue Asp113 (3.0 Å). The nucleophile then attacks the C1 carbon of the GlcUA from the left. In the superposition the 3-hydroxyl on the acceptor Gal is in-line with the UDP to a β linkage with respect to the Galβ[3Galβ1–4Xyl] product. This would result in an inversion of the stereochemistry with respect to the C-1 bond converting it from an α linkage with the UDP to a β linkage with respect to the Galβ[3Galβ1–4Xyl]. Previously it was assumed that the C1 carbon would be in-line with the leaving oxygen on the β phosphate of UDP and the 3-oxygen of the terminal Gal. However, there were no data to indicate this was true. Therefore it became imperative to obtain the crystal structure of GlcAT-I not only of the UDP[Galβ1–3Galβ1–4Xyl] complex but also with the entire UDP–GlcUA donor bound.

Superposition of the ternary complex with UDP[Galβ1–3Galβ1–4Xyl] and the UDP–GlcUA structures reveals the relative orientation of the donor to the acceptor substrate (Fig. 2). We find that the acceptor substrate binds in a groove in the acceptor binding domain with the Xyl portions of the Galβ[3Galβ1–4Xyl] analog extending away from the protein and with the 3-hydroxyl of the terminal Gal 5.1 Å from the β phosphate of the UDP molecule. Conserved residues Glu227 and Arg247 form hydrogen bonds with the 6-hydroxyl of the terminal Gal whereas Asp252 forms a hydrogen bond with the 4-hydroxyl. Glu281 forms a hydrogen bond with the 3-hydroxyl of the terminal Gal moiety, and therefore it has been proposed that this residue may function as the catalytic base.
other NTP-sugar-dependent inverting glycosyltransferases with a similar fold may share a similar mechanism.

**Comparison to Retaining Glycosyltransferases**—The donor binding subdomain of GlcAT-I shows a great deal of similarity to other glycosyltransferases such as galactosyltransferase LgtC from *Neisseria meningitidis* (25) and bovine 1,3-galactosyltransferase (26) despite little apparent sequence identity. In addition to the present GlcAT-I structure, four of these structures (GnT1, 1,3GalT, LgtC, and 4GalT1) have been solved with a complete donor substrate in place (21, 24–26). Three of these glycosyltransferase structures contain an acceptor substrate: GlcAT-I, 4GalT1, and LgtC. The first two glycosyltransferases catalyze an inverting transfer reaction and therefore are often called inverting glycosyltransferase, whereas the last one is a retaining glycosyltransferase that catalyzes retaining transfer reaction.

To better understand the differences between retaining and inverting transfer reactions we have superimposed the ternary complex structure of LgtC to the previously described ternary complex of GlcAT-I (Fig. 4). Despite the fact that GlcAT-I and LgtC are inverting and retaining enzymes, respectively, the UDP-sugar molecules superimpose well. Interactions of the donor substrates with the enzymes are conserved. For example, the corresponding aspartic acids in the DXD motif superimpose well. The first position of the DXD motif (Asp194 and Asp103 of GlcAT-I and LgtC, respectively) interacts with the donor sugar, and the third position (Asp196 and Asp105, respectively) coordinates with the Mn2\(^{2+}\) ion. Arg161 forms a hydrogen bond with the side chain of position 1 in the DXD motif as well as with the donor sugar in the GlcAT-I structure. Arg86 is the equivalent residue found in the LgtC structure, thereby indicating that these interactions are conserved in both structures. Asn153 and Asp252 are also superimposed in the LgtC and GlcAT-I structures, respectively, and both residues are in position to form a hydrogen bond with the O-2 oxygen of the donor sugar substrate (Fig. 4). In addition, His244 of LgtC is in a similar location as His308 of GlcAT-I with respect to the donor sugar, although it does not appear to form an interaction with the sugar like His308 but rather interacts with the Mn2\(^{2+}\) ion. As a result of these similarities, the orientation and conformation of the donor substrate in the active site are conserved in both the inverting GlcAT-I and retaining LgtC enzymes.

Interestingly, the superposition of the retaining enzyme LgtC with the inverting enzyme GlcAT-I reveals the position of the acceptor substrates to be different with respect to the C-1 carbon of the donor sugar. As a result, the acceptor 4-hydroxyl for LgtC would not be in-line to the leaving UDP (Fig. 4). It has been suggested that the retaining enzymes proceed by a double displacement reaction in which a covalent intermediate to the protein might be formed prior to final transfer of the galactose sugar. The superposition of LgtC to GlcAT-I positions Glc189 of LgtC in a similar position to the O-3 acceptor of GlcAT-I,
suggesting it as a catalytic nucleophile in the first transfer (data not shown). Gln189 has been mutated to examine its role as a catalytic residue in the transfer reaction (25). Gln189 mutations decreased but did not abolish activity of LgtC, indicating another mechanism other than a double displacement mechanism may be involved for the retaining enzymes (25). From this analysis it is clear that striking similarities exist between the retaining and inverting glycosyltransferases in the UDP-sugar binding subdomain. However these similarities do not necessarily extend to the acceptor binding subdomain.

Conclusion—We have obtained the crystal structure of GlcAT-I in the presence of the donor substrate UDP-GlcUA. This structure has revealed that side chains from conserved residues Arg161, Asp194, Asp252, and His308 as well as backbone interactions from conserved residue Arg156 orient the donor substrate for catalysis. This position of the donor with respect.
to the acceptor substrate in the superposition supports the previously proposed in-line displacement mechanism for GlcAT-I. Other NTP-dependent inverting glycosyltransferases that share a similar structure may function by an analogous mechanism.

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Crystal Structure of β1,3-Glucuronyltransferase I in Complex with Active Donor Substrate UDP-GlcUA
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