Heparan/Chondroitin Sulfate Biosynthesis

STRUCTURE AND MECHANISM OF HUMAN GLUCURONYLTRANSFERASE I*

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Human β1,3-glucuronyltransferase I (GlcAT-I) is a central enzyme in the initial steps of proteoglycan synthesis. GlcAT-I transfers a glucuronic acid moiety from the uridine diphosphate-glucuronic acid (UDP-GlcUA) to the common linkage region trisaccharide Galβ1–3Galβ1–4Xyl covalently bound to a Ser residue at the glycosaminoglycan attachment site of proteoglycans. We have now determined the crystal structure of GlcAT-I at 2.3 Å in the presence of the donor substrate product UDP, the catalytic Mn2+ ion, and the acceptor substrate analog Galβ1–3Galβ1–4Xyl. The enzyme is a α/β protein with two subdomains that constitute the donor and acceptor substrate binding site. The active site residues lie in a cleft extending across both subdomains in which the trisaccharide molecule is oriented perpendicular to the UDP. Residues Glu257, Asp259, and Glu281 dictate the binding orientation of the terminal Gal-2 moiety. Residue Glu281 is in position to function as a catalytic base by deprotonating the incoming 3-hydroxyl group of the acceptor. The conserved DXD motif (Asp194, Asp195, Asp196) has direct interaction with the ribose of the UDP molecule as well as with the Mn2+ ion. The key residues involved in substrate binding and catalysis are conserved in the glucuronyltransferase family as well as other glycosyltransferases.

Proteoglycans with side chains such as heparan sulfate and chondroitin sulfate are distributed on the cell surface and in the extracellular matrix and are implicated in various biological processes including cell growth and differentiation, blood coagulation, and viral and bacterial infections. Mutations in the Drosophila homolog of UDP-glucose dehydrogenase, which produce UDP-GlcUA is required for GAG synthesis, result in impaired signaling of Wingless, fibroblast growth factor, and Hedgehog (1–2). More specifically, defects in heparan sulfate impaired signaling of Wingless, fibroblast growth factor, and Notch (3). In addition, heparan sulfate 2-O-sulfotransferase-null mice die neonatally from renal agenesis (4–6). Human EXT1 and EXT2 genes encoding heparan polymerases are linked to hereditary multiple exostoses (7), whereas heparan sulfate 2-O-sulfotransferase-null mice die neonatally from renal agenesis (8).

Following the β1,3-glycosidic bond formation that is catalyzed by β1,3-glucuronyltransferase I (GlcAT-I), the product tetrasaccharide GlcUAβ1–3Galβ1–3Galβ1–4Xylβ1–O-Ser becomes the substrate for α1,4-N-acetylgalcosaminyltransferase (EXTL-2) that adds N-acetylgalcosamine (GlcNAC) to initiate a heparin/heparan chain (9, 10). Subsequently, heparan polymerases elongate the heparan/heparin chain by conjugating alternately β-GlcUA and α-GlcNAC (7). Alternatively, elongation of the chondroitin chain can be initiated by adding GalNAc to the substrate by β-1,4-N-acetylgalactosaminyltransferase I (11), followed by alternate conjugations of β-GlcUA and β-Gal-NAc resulting from the concerted actions of glucuronyltransferase II and β1,4-N-acetylgalactosaminyltransferase II (12) or chondroitin synthetase (13). These glycosyltransferases execute catalysis with a high substrate specificity to produce GAGs. To better understand GAG biosynthesis, we have engineered an active domain of human GlcAT-I, which we have expressed in Escherichia coli cells, crystallized in the presence of UDP-GlcUA, and soaked with the acceptor substrate analog Galβ1–3Galβ1–4Xyl. Here we report the ternary structure of GlcAT-I with both the donor product and the acceptor substrate.

MATERIALS AND METHODS

Protein Expression, Purification, and Enzyme Assay—The coding region of the catalytic domain of GlcAT-I was amplified from human liver cDNA by polymerase chain reaction. The expressed protein contained the following sequence: MGSSHHHHHHSSGLVPRGSMTFDYVNYVNWY AA—V SNW. Sequence analysis revealed the liver enzyme to differ from the placenta enzyme by one substitution Phe at position 204 instead of Ser. The amplified DNA was inserted into the bacterial expression plasmid PET-28a. The PET-28a plasmid was then transformed into BL21(DE3) cells. To express selenomethionyl GlcAT-I, B834(DE3) cells were used. Expression protein was purified using Ni2+-agarose and eluted with a gradient of imidazole. The eluted fractions containing GlcAT-I were pooled, dialyzed against 25 mM HEPES, pH 7.5, and 50 mM NaCl and concentrated to 30.8 mg of protein/ml. For enzyme assay, UDP-[14C] GlcUA (320 mCi/mmol) was obtained from American Radiolabeled Chemicals, Inc. Unlabeled UDP-GlcUA and ATP were obtained from Sigma. The glucuronyltransferase activity was determined as described previously using Galβ1–3Galβ1–4Xyl or asialoorosomucoid (Galβ1–4GlcNAC-R, representing the remainder of the N-linked oligosaccharide chain) as substrate (14). The substrate Galβ1–3Galβ1–4Xyl was obtained by Dr. N. B. Schwartz (University of Chicago).

Crystallization and Data Collection—Crystals of GlcAT-I were obtained by the vapor diffusion hanging drop method. 4 μl of 15 mg/ml
GlcAT-1 in 25 mM HEPES, pH 7.5, 50 mM NaCl, and 4 mM UDP-GlcUA were mixed with 4 μl of the reservoir solution containing 840 mM NaH₂PO₄, 560 mM K₂HPO₄, 140 mM LiSO₄, and 70 mM CHAPS, pH 6.2. Average crystals grew to the dimensions of 0.5 mm × 0.5 mm × 0.05 mm after 2 weeks. For data collection, crystals were transferred in 4 steps from the crystallization conditions to the cryo-protectant solution of 900 mM NaH₂PO₄, 600 mM K₂HPO₄, 150 mM LiSO₄, 75 mM CAPS pH 6.2, 4 mM UDP-GlcUA, and 10% ethylene glycol. Crystals of the ternary complex were obtained using a reservoir solution of 21% monomethyl-ether polyethylene glycol 2000 (MME-PEG-2000), 0.1M MES, pH 6.0, and a protein solution of 15 mg/ml GlcAT-I in 25 mM HEPES, pH 7.5, 50 mM NaCl, 5 mM MnCl₂, 5 mM MgCl₂, and 10 mM UDP-GlcUA. Crystals were then transferred in multiple steps into a solution containing 23% MME-PEG-2000, 20 mM MnCl₂, 10% ethylene glycol, 0.1 M MES, pH

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**Table 1**

**Data collection and statistics**

| Source                           | NSLS X9b                  | RUH3R rotating anode |
|----------------------------------|---------------------------|-----------------------|
| Wavelength (Å)                   | 0.97892                   |                       |
| Space group                      | P2₁                       |                       |
| Unit cell                        | a = 50.40 b = 98.58 c = 58.73 | a = 57.84 b = 48.22 c = 102.20 |
| Res. (Å)                         | 1.6                       | 1                     |
| No. of crystals                  | 1                         | 1                     |
| No. of observations              | 262,022                   | 69,398                |
| No. of unique refl.             | 135,003                   | 24,675                |
| Redundancy                       | 1.9                       | 2.8                   |
| % Completeness (last shell)     | 90.6 (56.6)               | 97.3 (82.5)           |
| R_{ave} (last shell)a           | 0.03 (0.29)               | 0.06 (0.24)           |
| I/0 (last shell)                | 14.1 (1.7)                | 11.1 (2.5)            |
| Resolution (Å)                  | 20–1.6                    | 50–2.3                |
| R_{cryst}/R_{free} (%)          | 21.7/24.6                 | 18.8/21.5             |
| No. of waters                    | 411                       | 221                   |
| RMS deviation from ideal values | Mean B value (Å²)         | 23.6                   | 29.8
|                                 | Bond length (Å)           | 0.007                  | 0.006
|                                 | Bond angle (°)            | 1.4                    | 1.3
|                                 | Dihedral angle (°)        | 23.9                   | 22.5
|                                 | Improper angle (°)        | 0.98                   | 0.89

**Ramachandran statistics**

Residues in:

- Most favored regions (%) | 89.5 | 88.8
- Additionally allowed regions (%) | 10.5 | 11.0
- Generously allowed regions (%) | 0.0 | 0.2
- Disallowed regions (%) | 0.0 | 0.0

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**Fig. 1.** Gel-filtration chromatography of bacterial-expressed GlcAT-I. One milligram of the purified recombinant enzyme was applied on a Superdex 200 column (1.0 × 30 cm) equilibrated with 50 mM MES/NaOH buffer, pH 6.5 containing 150 mM NaCl and was eluted with the same buffer solution. Fractions (0.25 ml each) were collected at a flow rate of 0.5 ml/min. Activity assays of GlcAT-I were performed on each of the fractions by measuring the formation of the radioactive product in dpm (closed circles) as described under "Materials and Methods." The dotted line represents A₂₈₀ absorption for protein concentration. The molecular mass markers (vertical bars) used were cytochrome c (12 kDa), ovalbumin (43 kDa), and bovine serum albumin (67 kDa). In addition, the position of the serum albumine dimer is indicated by a vertical bar.
Crystal Structure of Human GlcAT-I

RESULTS AND DISCUSSION

To crystallize GlcAT-I, the N-terminal 75 residues including the proposed cytoplasmic, transmembrane, and stem regions were removed. The truncated GlcAT-I-(Thr76–Val335) was expressed in E. coli. The GlcAT-I-(Thr76–Val335) protein and activity co-eluted as a large single peak with an apparent molecular mass of 43 kDa from the gel filtration column (Fig. 1). The GlcAT-I-(Thr76–Val335) did not exhibit activity toward asialoglycoprotein, the specific acceptor substrate of GlcAT-P (data not shown). Thus, the recombinant enzyme lacking the N-terminal region appeared to be monomeric in solution and retained significant specific GlcAT-I activity.

The present crystal structure has revealed that GlcAT-I-(Thr76–Val335) is approximately 40 × 40 × 50 Å3 in size with an extended C terminus (Fig. 2A). The enzyme contains a seven-stranded mixed β-sheet, which can be divided into two subdomains. The active site is found in a cleft of the molecule that extends across both subdomains. The N-terminal subdomain is an α/β motif with alternating β-strands and α helices. The β-strands form a parallel β-sheet with strand order 3, 2, 1, and 4. This subdomain contains the majority of the residues associated with donor substrate binding. The C-terminal subdomain, which contains the acceptor substrate binding site, includes β-strands 9, 6, and 10 that form a continuous β-sheet with strands 3, 2, 1, and 4 from the N-terminal subdomain. The C-terminal subdomain is largely a mixed β-sheet with strand order 5, 11, 6, 7, and 8 followed by the C terminus of the molecule extending away from the core along another molecule in the crystal lattice.

Crystals grown from both phosphate and MME-PEG contain the same dimer in the asymmetric unit (Fig. 2, B and C). The overall buried surface area for the dimer is 4000 Å2, representing 6.0, 10 mM UDP, and ~20 mM Galβ1–3Galβ1–4Xyl.

Data for the selenomethionine GlcAT-I crystal were collected on Beamline X9B at Brookhaven National Laboratories. Phases for the electron density map were obtained from data collected at a single wavelength equal to 0.97892 Å representing the peak of the anomalous dispersion. All data was processed using DENZO and SCALEPACK (15).

Positions for the selenium atoms were defined using SHELX (16). Phases were calculated in MLPHARE (17) and then improved using DM (17). WARP (18) was employed to fit approximately half the backbone and several side chains, to the electron density. Following multiple rounds of model building and refinement in O (19) and refinement in CNS version 0.5 (20), a final R factor of 21.7% and Rfree of 24.6% was obtained at 1.6 Å resolution (Table I). This model includes residues Met75–Arg144, Glu149–Val180 of molecule A, and Met75–Val335 of molecule B.

To determine the structure of the ternary complex, one molecule from the acceptor-unbound structure was used as a search model in molecular replacement using the program AMoRe (17). Two solutions with correlation coefficients of 17.0 and 12.1 were obtained for the rotation function. The correct solution for two molecules in the translation function gives a correlation coefficient of 61.1 and a R factor of 37.8%. After multiple cycles of model building and refinement, a final R factor of 18.8% and a Rfree of 21.5% were obtained. The final model contains residues Met75–Pro140 and Trp152–Val335 of molecule A and Met75–Pro150, His154–Glu244, and Arg247–Val335 of molecule B. In addition, each molecule contains a bound UDP molecule, one Mn2+ ion, and an unidentified metal. The Galβ1–3Galβ1–4Xyl substrate is bound to molecule A. Attempts to determine the location of the glucuronic acid by binding UDP-GlcUA present showed no density for the molecule, probably because of competition with the phosphate buffer present. Electron density for only the UDP segment of the UDP-GlcUA was observed (data not shown) for crystals grown in MME-PEG-2000.

FIG. 2. A, secondary structure elements of the catalytic domain (residues 75–335) of the GlcAT-I enzyme. B, scheme of the crystallographic dimer of GlcAT-I with monomers A (orange) and B (seagreen). The UDP molecule is colored yellow, the Mn2+ ion chartreuse, and the substrate in dark green. C, rotation of the crystallographic dimer by approximately 90° with respect to panel B, along a horizontal axis. In this panel residue Gln318 of one molecule extends into the active site region of the other. Panels A and B were created using MOLSCRIPT (31) and Raster3D (32).
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**Fig. 3. The active site of GlcAT-I.** Residues involved in substrate and cofactor binding are pictured. The Mn\(^{2+}\) ion is colored chartreuse and the unidentified metal ion *tan* (P-Fo) omit map of UDP, Gal\(^{1–3}\)Gal\(^{1–4}\)Xyl, and metal ions (with chelating waters) contoured at 3 sigma is shown in *light blue*. Possible hydrogen bonding partners are connected with *dashed black lines* and metal interactions are shown in *sold black lines*. This figure was created using MOLSCRIPT (31) and Raster3D (32).

**Fig. 4. Schematic diagram of the proposed catalytic mechanism of GlcAT-I.** In this mechanism Glu\(^{291}\) acts as the catalytic base deprotonating the 3-hydroxyl on the terminal Gal of the linker region. This would allow for nucleophilic attack on the C-1 position of the glucuronic acid of the UDP-GlcUA molecule followed by dissociation of the UDP leaving group. This would result in the conversion of an \(\alpha\)-linked UDP-GlcUA reactant to a \(\beta\)-1–3 linked GluUA\(\beta\)-1–3Gal\(\beta\)-1–4Xyl\(\beta\)-1-O-Ser product.

...ing 16\% of the total surface area of the two molecules. Residues Leu\(^{87}\)–Leu\(^{102}\), Asp\(^{196}\)–Arg\(^{201}\), Leu\(^{200}\)–Phe\(^{206}\), His\(^{301}\)–Trp\(^{304}\), Val\(^{327}\)–Leu\(^{335}\) line the dimer interface. Residues Glu\(^{312}\)–Gln\(^{318}\) of one monomer extend into the active site of the other. This orientation positions both N termini on one face of the dimer with the active sites on the opposite face in which the N-terminal membrane-binding domains would be directed in the same orientation. Type II Golgi membrane enzymes such as \(\beta\)-1,4-galactosyltransferase have been reported to be homodimers (21, 22). Thus, GlcAT-I, also a type II membrane-associated protein, is likely to be a homodimer in the Golgi membrane.

The UDP molecule binds across a long cleft on the surface of the molecule with the uridine and ribose rings mainly centered in the N-terminal subdomain of the molecule. The OD2 oxygen of D113 from strand 3 (Fig. 3) is within hydrogen bonding distance of atom N-3 (2.9 Å) of the uridine ring. The side chain of D113 from strand 3 (Fig. 3) is within hydrogen bonding distance of atom N-3 (2.9 Å) of the uridine ring. The side chain of D113 from strand 3 (Fig. 3) is within hydrogen bonding distance of atom N-3 (2.9 Å) of the uridine ring. The side chain of D113 from strand 3 (Fig. 3) is within hydrogen bonding distance of atom N-3 (2.9 Å) of the uridine ring.

The Mn\(^{2+}\) atom is in an approximately octahedral coordination state in which two of the coordination atoms, O1B (2.1 Å) and O2A (2.1 Å), are from the \(\beta\)- and \(\alpha\)-phosphates of the UDP molecule. Asp\(^{196}\) forms a bidentate interaction through OD1 (2.2 Å) and OD2 (2.2 Å). The remaining two ligands are from two water molecules. One water molecule is found 2.3 Å from the Mn\(^{2+}\) and is also hydrogen bound to OD1 of Asn\(^{197}\) (2.8 Å) and O of Thr\(^{309}\) (2.8 Å). The other water molecule is found 2.1 Å from the Mn\(^{2+}\) and 2.6 Å from OD1 of Asp\(^{194}\). Thus, residues Asp\(^{194}\), Asp\(^{196}\), and Asp\(^{196}\) of the conserved DXD motif (23), are involved in ribose interactions as well as direct and indirect interactions with the Mn\(^{2+}\) ion. A second unidentified metal ion is found in a tetrahedral geometry with ligands O3B of UDP (1.9 Å), NE2 of His\(^{301}\) (2.0 Å), and two possible water molecules (2.0 Å and 2.2 Å). However, it is less certain whether this metal ion is physiological. This metal ion is not present in either the unbound or the UDP/Mn\(^{2+}\)/no acceptor substrate structures (data not shown), suggesting that the metal may have come from the trisaccharide solution.

Residues involved in acceptor substrate binding are isolated to the C-terminal subdomain of the GlcAT-I molecule. The trisaccharide Gal\(^{1–3}\)Gal\(^{1–4}\)Xyl binds with the O3 of the terminal galactose (Gal-2) 5.1 Å from the O3B atom of the UDP molecule (Fig. 3). Electron density exists only for the Gal\(^{1–3}\)Gal portion of the trisaccharide Gal\(^{1–3}\)Gal\(^{1–4}\)Xyl. Atom OE1 of Glu\(^{277}\) is within hydrogen bonding distance to O-6 of Gal-2 (2.5 Å). NH1 of Arg\(^{247}\) is also found 2.8 Å from O-6. The O-4 oxygen of Gal-2 is found 2.6 Å from OD2 of Asp\(^{202}\) and O-3 of Gal-2 is 2.7 Å from OE2 of Glu\(^{281}\). The Gal-1 moiety is positioned near the surface of the molecule. Residue Gln\(^{318}\) of the second monomer is in position to form a hydrogen bond with O-6 of Gal-1. Atoms OE1 and NE2 are positioned 2.6 Å and 3.0 Å, respectively from O-6 of Gal-1. Although Trp\(^{243}\) does not appear to form hydrogen bonds with the acceptor substrate, the plane of the side chain is parallel to the ring of the Gal-1 molecule.

Based on the present binding orientation of the acceptor, the Xyl moiety would be expected to reside outside the substrate-binding cavity. However, no density was observed for the Xyl moiety of the trisaccharide. Xyl may simply act as a spacer between the Gal\(^{1–3}\)Gal disaccharide in the substrate cavity, and the protein to which the linker region is attached and consequently would have no specific interactions with the enzyme.
The majority of the hydrogen bond interactions between GlcAT-I and the acceptor substrate are through Gal-2. The three-dimensional orientation of residues Glu281, Asp252, and Glu281 in the active site may dictate the specificity for the acceptor substrate through interactions with O-6, O-4, and O-3 hydroxyl groups of Gal-2. These three residues are highly conserved in all glucuronyltransferases.

The reaction catalyzed by GlcAT-I is the transfer of glucuronic acid from UDP-GlcUA to the O-3 oxygen of the terminal Gal-2 of the growing linkage region Galβ1-3Galβ1-4Xylβ1-O-Ser. A reasonable mechanism involves attack by a deprotonated form of the incoming 3-hydroxy group of the acceptor oligosaccharide, on the C-1 position of the glucuronic acid (Fig. 4). Formation of a GlcUAβ1-3Gal linkage and subsequent dissociation of the UDP molecule follows. This reaction would require a catalytic base to deprotonate the hydroxyl group at the C-3 position of Gal-2. Atom OE2 of Glu281 is located 2.7 Å from the nucleophilic O-3 of the 3-hydroxyl group. This suggests that Glu281 could play a key role as the catalytic base in the transfer reaction. The histidine at position 308 is found located near the β-phosphate of UDP and the O-3 hydroxyl of Gal-2. His308 may interact with the glucuronic acid portion of the UDP-GlcUA molecule either by orienting the glucuronic acid for catalysis or by stabilizing the transition state. Both Glu281 and His308 are totally conserved in all glucuronyltransferases. The present GlcAT-I structure supports the proposed catalytic mechanism for NDP-sugar dependent glycosyltransferases (24).

To date three crystal structures of NDP-sugar dependent-glycosyltransferases have been reported: T4 phage β-glucosyltransferase (25), bovine β-α-galactosyltransferase (26) and SpSA from Bacillus subtilis (17). However, none of these structures have acceptor substrate bound. The present structure of GlcAT-I shows high similarities in the tertiary fold and NDP binding with SpSAa, despite only a 7.3% sequence identity (Fig. 5A). Insightfully, the catalytic base Glu281 of GlcAT-I superimposes with Asp191 of the SpSA structure, which has been proposed as the catalytic base in SpSA (27) (Fig. 5B). In addition, Asp252 of GlcAT-I that interacts with O-4 of Gal-2, superimposes well with Asp158 in the SpSA structure. This data suggest that the reaction mechanism and the catalytic site structure may also be applicable to other families of glycosyltransferases.

Critical roles of GAGs, especially heparan sulfate, in developmental processes and specific signaling pathways have recently been demonstrated by the identification of mutations in biosynthetic enzymes for heparan sulfate synthesis in the Drosophila, mouse, and human (28). The structural biology of GAGs and enzymes involved in GAG biosynthesis is evolving rapidly (29). The ternary structure of GlcAT-I is the first, not only for glucuronyltransferases but also for glycosyltransferases that are involved in heparan/heparin biosynthesis. The structure has revealed the basis for understanding glucuronic acid transfer to the linkage region at a branching point common to various GAGs. This structure, along with the crystal structure of the sulfotransferase domain of heparan sulfate N-deacetylated/N-sulfotransferase (30), opens a new era of structural biology of heparan/chondroitin sulfate biosynthesis and GAG biology.

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