Structural Basis of Ordered Binding of Donor and Acceptor Substrates to the Retaining Glycosyltransferase, α-1,3-Galactosyltransferase*

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Bovine α-1,3-galactosyltransferase (α3GT) catalyzes the synthesis of the α-galactose (α-Gal) epitope, the target of natural human antibodies. It represents a family of enzymes, including the histo blood group A and B transferases, that catalyze retaining glycosyltransfer reactions of unknown mechanism. An initial study of α3GT in a crystal form with limited resolution and considerable disorder suggested the possible formation of a β-galactosyl-enzyme covalent intermediate (Gastinel, L. N., Bignon, C., Misra, A. K., Hinds Gaul, O., Shaper, J. H., and Joziassie, D. H. (2001) EMBO J. 20, 638–649). Highly ordered structures are described for complexes of α3GT with donor substrate, UDP-galactose, UDP-glucose, and two acceptor substrates, lactose and N-acetylglucosamine, at resolutions up to 1.46 Å. Structural and calorimetric binding studies suggest an obligatory ordered binding of donor and acceptor substrates, linked to a donor substrate-induced conformational change, and the direct participation of UDP in acceptor binding. The monosaccharide-UDP bond is cleaved in the structures containing UDP-galactose and UDP-glucose, producing non-covalent complexes containing buried β-galactose and α-glucose. The location of these monosaccharides and molecular modeling suggest that binding of a distorted conformation of UDP-galactose may be important in the catalytic mechanism of α3GT.

The oligosaccharide components of glycoproteins, glycolipids, and proteoglycans have major roles in molecular and cellular interactions in eukaryotes. Their importance is highlighted by the recognition that several genes that control developmental processes encode glycosyltransferases (GTs) that catalyze steps in glycoconjugate processing (1). Although GTs catalyze chemically similar reactions in which a monosaccharide is transferred from an activated derivative, such as a UDP-sugar, to an acceptor, few are significantly similar in primary structure. Nevertheless, families of homologous GTs have been identified that are related in sequence and catalyze analogous reactions (2); these appear to merge into larger groups that have similar three-dimensional structures and share conserved sequence motifs (2, 3). Two major subgroups of GTs are those that catalyze reactions in which the anomeric configuration of the transferred monosaccharide is inverted and those in which the configuration is retained. Previously, the three-dimensional structures of four eukaryotic glycosyltransferases from different families have been determined: three inverting transferases, UDP-galactose β-N-acetylglucosaminide β-1,4-galactosyltransferase I (4, 5), UDP-N-acetylgalactosamine α-3-mannoside β-1,2-N-acetylgalactosaminyltransferase I (6), and β-1,3-gluco- nylnyltransferase I (7), and one retaining transference, bovine UDP-galactose β-galactosyl α-1,3-galactosyltransferase (α3GT) (8, 9).

α3GT (EC 2.4.1.151) catalyzes the transfer of galactose from UDP-α-D-galactose into an α-1,3-linkage with non-reducing terminal β-galactosyl moieties in glycoconjugates. This enzyme is expressed in many mammalian species, including most primates and New World monkeys, but is absent from humans, apes, and Old World monkeys (10) because of the mutational inactivation of the gene (11). Humans do not have galactosyl α-1,3-galactosyl β-OR structures (here β is a glycoconjugate of variable structure) on glycoconjugates and produce large amounts of antibody to this structure, the α-Gal epitope, amounting to about 1% of the circulating IgG. The production of this endogenous antibody results from exposure to intestinal bacteria and other antigens (12). α3GT is of medical interest because immune responses to selected cellular targets are enhanced when they are decorated with galactosyl α-1,3-galactosyl structures and retroviruses become sensitive to human serum when produced by cells expressing α3GT (13). Also, the presence of anti-α-Gal antibodies is a barrier to xenotransplantation of organs (14), and with the shortage of some human organs for transplantation, genetic manipulation of the α3GT gene (15, 16) and the development of inhibitors of the enzyme are of interest as complementary approaches to reducing rejec-

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† The atomic coordinates and structure factors (code 1GX4, 1GWY, 1GX0, and 1GWW) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).‡ §§ To whom correspondence may be addressed: Dept. of Biomedical Sciences, Florida Atlantic University, Boca Raton, FL 33431. Tel.: 561-297-0407; Fax: 561-297-2221; E-mail: kbrew@fau.edu.§§§ Present address: European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SD, UK. ¶¶ To whom correspondence may be addressed: Department of Biology and Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, United Kingdom. Tel.: 44-1225-386-238; Fax: 44-1225-386-779; E-mail: K.R.Acharya@bath.ac.uk.

1 The abbreviations used are: GT, glycosyltransferase; α3GT, β-galactosyl α-1,3-galactosyltransferase; ITC, isothermal titration calorimetry; LacNAc, N-acetyllactosamine; Lac, lactose; Gal, galactose; Glc, glucose; MES, 4-(morpholine)ethanesulfonic acid; PEG, polyethylene glycol; ADP, atomic displacement parameter.
Structures of UDP-α3GT Donor and Acceptor Substrate Complexes

**Table I**

| Ligand                  | N-Acetyl-lactosamine | Lactose       | UDP-galactose | UDP-glucose |
|-------------------------|----------------------|---------------|---------------|-------------|
| Cocrystallization       | 10 mM UDP            | 10 mM UDP     | 10 mM UDP     | 10 mM UDP   |
| Soaking conditions      | 50 mM for 24 h       | 100 mM for 3 weeks | 20 mM for 6 h | 50 mM for 2 days |
| Wavelength used for data collection (Å) | 0.87 | 1.488 | 1.488 | 1.488 |
| Resolution range (Å)   | 40.0–1.46            | 39.0–2.5      | 24.0–1.8      | 20.0–1.8    |
| Space group             | P2₁ (2 mol/a.u.)     | P2₁ (2 mol/a.u.) | P2₁ (2 mol/a.u.) | P2₁ (2 mol/a.u.) |
| Cell dimensions (Å)     | a = 45.63, b = 95.24, c = 95.64, β = 99.09° | a = 45.06, b = 94.28, c = 94.36, β = 99.09° | a = 45.16, b = 94.37, c = 94.76, β = 99.4° |
| Number of observations  | 479,901              | 82,283        | 280,613       | 236,674     |
| Number of unique reflections | 132,382          | 27,036        | 72,098        | 65,613      |
| Completeness (%)        | 94.8 (71.7)          | 95.3 (86.7)   | 95.9 (88.8)   | 90.5 (84.8) |
| R/σ(I)                  | 17.6 (1.98)          | 8.76 (2.90)   | 14.0 (2.1)    | 21.5 (2.9)  |
| R cryst (%)             | 6.4 (43.4%)          | 12.6 (36.5%)  | 6.4 (35.8%)   | 5.1 (46.3%) |
| Deviations from ideality| Bond lengths (Å)      | 0.009         | 0.008         | 0.005       | 0.006       |
|                        | Bond angles (°)      | 1.8           | 1.4           | 1.3         | 1.3         |
|                        | Dihedrals angles (°) | 25.4          | 23.3          | 23.0        | 22.9        |
|                        | Improper dihedrals (°) | 1.7       | 0.8           | 0.8         | 0.8         |
|                        | No. of protein atoms | 2423 (mol. A) | 2393 (mol. A) | 2436 (mol. A) | 2414 (mol. A) |
|                        | 2432 (mol. B)       | 2393 (mol. A) | 2402 (mol. B) | 2408 (mol. B) |
|                        | No. of solvent atoms | 777          | 180           | 497         | 445         |
|                        | Glyceroles molecules | 4            | 0             | 2           | 0           |
|                        | B-factor statistics (Å²) |            |               |             |             |
|                        | Overall B-factor    | 18.0          | 21.1          | 24.5        | 30.4        |
|                        | Protein all atoms   | 15.8          | 21.0          | 23.6        | 29.7        |
|                        | Protein main chain  | 13.6          | 20.6          | 22.3        | 28.5        |
|                        | Protein side chain  | 17.9          | 21.4          | 24.8        | 30.9        |
|                        | Solvent atota      | 31.4          | 19.7          | 32.1        | 37.4        |
|                        | UDP atoms*         | 10.7          | 21.8          | 19.6        | 27.7        |
|                        | Ligand atoms        | 19.7*         | 35.7*         | 51.2*       | 59.6*       |
|                        | Mn²⁺ ion*          | 9.8           | 21.1          | 20.0        | 27.7        |
|                        | Mean anisostrity*   | 0.38          |               |             |             |

*a Outermost shell is 1.51–1.46 Å.
*b Outermost shell is 2.59–2.5 Å.
*c Outermost shell is 1.86–1.80 Å.
*d R cryst = ∑ₙ[I(h)]/∑ₙ[I(h)c], where I(h) is the i-th measurement and I(h)c is the weighted mean of all measurements of I(h).
*e R cryst = ∑ₙ[F(h) - F(h)c]/∑ₙ[F(h)c], where F(h) and F(h)c are the observed and calculated structure factor amplitudes of reflection h.
*f R free is equal to R cryst for a randomly selected 5% subset of reflections not used in refinement.
*g All structures have one UDP molecule and one Mn²⁺ ion per monomer.
*h Two LacNAc molecules (one/monomer).
*i Only one molecule (in mol. B) was built.
*j Mean anisostrity was calculated using the program PARVATI (32).
FIG. 2. A, C, E, and G: diagrams of the $|F_o| - |F_c|$ electron density omit map, contoured at 3.0-$\sigma$ level, of LacNAc (1.46 Å), Lac (2.5 Å), $\beta$-Gal (1.8 Å), and $\alpha$-Glc (1.8 Å), respectively. B, D, F, and H: diagrams showing the interactions of $\alpha$3GT with LacNAc, lactose, $\beta$-Gal, and $\alpha$-Glc, respectively. The protein residues are drawn as ball-and-stick models, water molecules appear as blue spheres, and the ligands are shown in orange. The Mn$^{2+}$ ion is shown as a magenta sphere. H-bonds are indicated by dashed lines. The figures were created with BOBSCRIPT (34).
shows that Mn$^{2+}$ is required for binding donor substrate analogues and that UDP is required for binding acceptor substrate, indicating that donor and acceptor substrates bind in an obligatory ordered manner to the enzyme. This is supported by the present structures of 3GT complexes containing acceptors.

The implications of these results and docking studies with different conformations of UDP-galactose are discussed in relation to the mechanism of the retaining reaction catalyzed by 3GT.

EXPERIMENTAL PROCEDURES

X-ray Crystallography—The catalytic domain of bovine 3GT (residues 80–368) was expressed in Escherichia coli and purified as previously described (20) and stored at $-20^\circ$C in 20 mM MES-NaOH buffer (pH 6) in 50% glycerol. Crystals were grown at 16$^\circ$C by the vapor diffusion, hanging drop method as described previously (9) by mixing 2 ml of the protein at 5 mg/ml in 20 mM MES-NaOH buffer, pH 6.0, 10% glycerol, containing 10 mM UDP and 0.1 mM MnCl$_2$, with an equal volume of a reservoir solution containing 5% PEG 6000 and 0.1 M Tris-HCl, pH 8.0. Single crystals appeared after 1–3 days. The 3GT-N-acetyl lactosamine (LacNAc), lactose (Lac), and UDP-glucose (UDP-Glc) complexes were obtained by soaking the native crystals prior to data collection with a 50–100 mM solution in the crystallization buffer as detailed below in Table I. Co-crystals of the 3GT-UDP-galactose (UDP-Gal) complex were obtained by growing the crystals in the presence of UDP-Gal. In these experiments the protein was diluted in 20 mM MES-NaOH buffer, pH 6.0, 10% glycerol, containing 10 mM UDP-Gal and 0.1 mM MnCl$_2$ and mixed with an equal volume of the crystallization buffer containing 5% PEG 6000 and 0.1 mM Tris-HCl, pH 8.0. Single crystals belonging to the P2$_1$ monoclinic space group (isomorphous with the native crystals) appeared after 3 days. Before data collection, all crystals were flash-cooled at 100 K in a cryoprotectant containing 10%...
**TABLE III**

**Hydrogen bonds and van der Waals interactions in a3GT acceptor complexes**

| Hydrogen bond ligand atom, LacNAc | Interacting atoms | Distance (Å) | Ligand atom, Lac | Interacting atoms | Distance (Å) |
|----------------------------------|-------------------|--------------|-----------------|------------------|--------------|
| O2                              | Wat148            | 2.72         | O2              | Wat11            | 2.95         |
| O2                              | Wat155            | 3.13         | O2              | Wat13            | 2.83         |
| O3                              | Wat296            | 2.39         | O3              | Wat187           | 3.29         |
| O3                              | UDP O2 PB         | 2.64         | O3              | UDP O2 PB        | 2.71         |
| O3                              | Wat155            | 2.89         | O3              | Wat14            | 2.85         |
| O3                              | Wat355            | 3.22         | O3              | UDP O1 PB        | 3.30         |
| O4                              | Glu 247 Ne2       | 3.01         | O4              | Glu147 Ne2       | 2.78         |
| O4                              | Glu 317 Oe1       | 2.75         | O4              | Glu137 Oe1       | 2.69         |
| O4                              | Wat296            | 2.87         | O5              | Glu147 Ne2       | 3.03         |
| O5                              | Wat341            | 3.10         | O5              | Wat19            | 2.79         |
| O6                              | Wat169            | 2.51         | O6              | Thr259 Oy1       | 2.56         |
| O6                              | Wat454            | 2.93         | O6              | Thr259 Oy1       | 2.56         |
| O6'                             | Wat167            | 2.78         | O6'             | Wat200           | 2.68         |
| O5'                             | Wat777            | 3.19         | O5'             | Wat200           | 2.68         |
| O5'                             | Wat421            | 3.26         | O5'             | Wat200           | 2.68         |
| O3'                             | Wat169            | 3.04         | O3'             | Wat200           | 2.68         |
| O3'                             | Wat336            | 3.03         | O3'             | Wat200           | 2.68         |
| O5'                             | Wat177            | 3.19         | O5'             | Wat200           | 2.68         |
| O6'                             | Wat356            | 3.25         | O6'             | Wat200           | 2.68         |
| O5'                             | Wat553            | 3.01         | O5'             | Wat200           | 2.68         |
| O7'                             | Wat476            | 3.08         | O7'             | Wat200           | 2.68         |
| O6'                             | Wat356            | 3.08         | O7'             | Wat200           | 2.68         |
| O7'                             | Wat356            | 3.08         | O7'             | Wat200           | 2.68         |

Hydrophobic interactions

|          | Gln147            | 8 contacts  | Glu147            | 9 contacts  |
|----------|-------------------|-------------|-------------------|-------------|
| Trp320   |                   | >10 contacts | Trp320            | 10 contacts |
| Thr259   |                   | 3 contacts  | Thr259            | 3 contacts  |
| Tyr278   |                   | 2 contacts  | Tyr278            | 2 contacts  |
| Trp314   |                   | >10 contacts | Trp314            | >10 contacts|
| Glu317   |                   | 5 contacts  | Glu317            | 3 contacts  |
| Trp256   |                   | 6 contacts  | Trp256            | 7 contacts  |
| Lys259   |                   | 2 contacts  | Lys259            | 2 contacts  |
| UDP (PB) |                   | 3 contacts  | UDP (PB)          | 2 contacts  |

* These residues have several atoms that are between 3.0 and 4.1 Å from the ligand atom.

PEG 6000, 0.1 M Tris-HCl, pH 8.0, 25% glycerol, and appropriate ligands. High resolution data sets (space group P21, with two molecules in the asymmetric unit) for the four complexes, LacNAc (1.46 Å), Lac (2.5 Å), UDP-Gal (1.8 Å), and UDP-Glc (1.8 Å), were collected at the Synchrotron Radiation Source, Daresbury, UK (station PX9.6, using an Area Detector Systems Corp. detector system for Lac, UDP-Gal, and UDP-Glc complexes and for the LacNAc complex station PX 14.1 with an MAR 345 image plate system). Raw data images were indexed and scaled using the DENZO and SCALEPACK modules of the HKL suite (Ref. 21, see Table I).

Because the cell dimensions for all the complex data sets were isomorphic with the previously determined a3GT-UDP structure (form II (9), Protein Data Bank entry 1K4V) (Table I), these coordinates were used as a starting model. Crystallographic refinement was performed using the program package CNS (22). Several rounds of bulk solvent correction, energy minimization, individual R-factor refinement, simulated annealing, and model building using the "O" program (23) were performed until the R的能量 value could not be improved. Water molecules were gradually included into the model at positions corresponding to peaks in the |Fo| − |Fc| electron density map with heights greater than 3σ and at H-bond distance from appropriate atoms. Waters with a temperature factor higher than 65 Å² were excluded from subsequent refinement steps.

For the LacNAc complex structure at 1.46 Å, further refinement was carried out using SHELXL-97 (24). Initial conjugate gradient least squares refinement in SHELXL was carried out restraining all the 1.2- and 1.3-distances with the Engh and Huber (25) restraints. Initially all the atomic displacement parameters (ADPs) were kept isotropic. The $R_\text{free}$ value after isotropic refinement of the ADPs was 0.24, and $R_\text{free}$ could not be refined beyond 0.23. The data to parameter ratio, $>2$, enabled us to carry out anisotropic refinement on ADPs that reduced both $R$-factors by at least 4.5% thereby justifying the use of this method of refinement. All the alternate conformations were added after initial anisotropic refinement. Any new atoms added to the molecule were isotropically refined for at least two cycles before they were refined anisotropically. The multiple conformation site occupation factors were refined constraining their sum to be unity. The average anisotropy value of 0.38 is between accepted values for structure refined at 1.5 Å and at borderline between isotropic and anisotropic refinement. Analysis of the Ramachandran (φ-ψ) plot showed that all residues lie in the allowed regions for all the refined structures. The final refinement statistics are included in Table I.

**Isothermal Titration Calorimetry**—The thermodynamics of binding of inhibitors and substrates to a3GT and mutants were characterized using a VP-ITC microcalorimeter (Microcal Inc.) at 30 °C. The enzyme was dialyzed extensively against 20 mM Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl and 10% glycerol and degassed prior to use. Enzyme solution (1.4 ml, 25–40 μM) was titrated with 25–30 additions of ligand (8 μl each). For the titrations with UDP (2 mM each) and UDP-glucose (5 mM), 2 mM MnCl₂ was included in the enzyme and ligand solutions, and for titrations with lactose (100 mM) or N-acetyllactosamine (10 mM), 2 mM MnCl₂ was present in the enzyme solution with or without UDP (2 mM). Each injection of ligand lasted 16 s with 120-s intervals between successive injections. During the titration, the enzyme was stirred continuously at 200 rpm. Heats of binding were determined by integrating the signal from the calorimeter. The heat of dilution of the enzyme complex was precluded by co-crystallization, and the UDP-Glc complex was prepared by soaking crystals initially grown in the presence of 5 mM UDP and at borderline between isotropic and anisotropic refinement. Analysis of the Ramachandran (φ-ψ) plot showed that all residues lie in the allowed regions for all the refined structures. The final refinement statistics are included in Table I.

**RESULTS**

**Structures of a3GT Complexes with Donor Substrate, UDP-Gal, and UDP-Glc**—The UDP-Gal-enzyme complex was prepared by co-crystallization, and the UDP-Glc complex was prepared by soaking crystals initially grown in the presence of UDP with UDP-Glc, yet the structures are closely similar to each other and to the a3GT-Mn⁺-UDP complex. The refined

PEG 6000, 0.1 M Tris-HCl, pH 8.0, 25% glycerol, and appropriate ligands. High resolution data sets (space group P21, with two molecules in the asymmetric unit) for the four complexes, LacNAc (1.46 Å), Lac (2.5 Å), UDP-Gal (1.8 Å), and UDP-Glc (1.8 Å), were collected at the Synchrotron Radiation Source, Daresbury, UK (station PX9.6, using an Area Detector Systems Corp. detector system for Lac, UDP-Gal, and UDP-Glc complexes and for the LacNAc complex station PX 14.1 with an MAR 345 image plate system). Raw data images were indexed and scaled using the DENZO and SCALEPACK modules of the HKL suite (Ref. 21, see Table I).
Structures of UDP-α3GT Donor and Acceptor Substrate Complexes

A

\[
\begin{array}{c}
\text{Time (min)} \\
0 & 10 & 20 & 30 & 40 & 50 & 60 \\
\mu\text{cal/sec} \\
0 & 0.0 & 0.5 & 1.0 & 1.5 & 2.0 & 2.5
\end{array}
\]

\[
\begin{array}{c}
\text{Molar Ratio} \\
0 & 20 & 40 & 60 & 80 \\
k\text{cal/mole of injectant} \\
0 & 0.3 & 0.6 & 0.9 & 1.2
\end{array}
\]

B

\[
\begin{array}{c}
\text{Time (min)} \\
0 & 10 & 20 & 30 \\
\mu\text{cal/sec} \\
0 & 0.5 & 1.0 & 1.5 & 2.0
\end{array}
\]

\[
\begin{array}{c}
\text{Molar Ratio} \\
0 & 30 & 60 & 90 & 120 \\
k\text{cal/mole of injectant} \\
0 & 0.01 & 0.02 & 0.03 & 0.04
\end{array}
\]

Fig. 5. Calorimetric profiles of the binding of UDP-Glc and lactose to wild-type α3GT. A, the reaction cell contained a solution of α3GT (1.433 ml, 18.5 μM) in 20 mM Tris-Cl buffer, pH 7.4, plus 2 mM MnCl₂, 0.1 M NaCl, and 10% glycerol, and the syringe contained 284 μl of 7.5 mM UDP-Glc in the same buffer. Top, calorimetric data obtained from the injection of 10-μl aliquots of UDP-Glc at 2-min intervals. B, the reaction cell contained a solution of α3GT (1.433 ml, 25.8 μM) in 20 mM Tris-Cl buffer, pH 7.4, plus 2 mM UDP, 2 mM MnCl₂, 0.1 M NaCl, and 10% glycerol, and the syringe was filled with 100 μl lactose in the same buffer. Top, data obtained for 15 injections of 8-μl aliquots of lactose at 2-min intervals (bold line) and for the injection of lactose in the absence of UDP (light line). In both A and B, the lower plot shows the integrated binding isotherm with the experimental points (■) and best fit.
which packs against Trp250 (Fig. 4). Both acceptors are bound through interactions that are similar to those observed in previously described protein–carbohydrate complexes (26). All hydroxyl groups of the two pyranose rings of the acceptors engage in H-bonds with side-chain groups of the enzyme or with solvent molecules (Table III) while several aromatic side-chains form H-bonds and non-polar interactions. Tryptophans 249, 250, 314, and 356 (also Tyr278) make the majority of non-polar van der Waals contacts (stacking interactions) with the two pyranose rings, particularly the primed monosaccharide. UDP also interacts with the unprimed monosaccharide, forming an H-bond with the 3-hydroxyl group (to which the α-galactosyl moiety is transferred during catalysis) and non-polar contacts with both acceptors. In contrast to the binding site for the donor substrate, the acceptor binding site is largely solvent-accessible (Fig. 3) as reflected in the considerable number of H-bonding interactions with water molecules (Table III).

Thermodynamics of Substrate and Inhibitor Binding—The binding of the substrates, UDP-Gal, lactose, and LacNAc, and the inhibitors, UDP and UDP-Glc, to wild-type α3GT was investigated by isothermal titration calorimetry (ITC). The heat released or absorbed was measured during successive additions of ligand to a solution of enzyme (25–40 μM), and the integrated heats of binding were plotted against ligand concentration to generate a binding isotherm. From this, the stoichiometry, affinity (Kd), and enthalpy and entropy of the binding can be determined provided the value of ε (ε = [M]i/[Kd], where [M]i is the total concentration of enzyme in the titration cell) is between 1 and 1000 (27). For most of the experiments reported here, this condition was not achieved (ε ≤ 1) owing to the limited solubility of the enzyme under the experimental conditions (~40 μM) relative to its affinity for the various ligands. For this reason, it is not possible to measure the binding stoichiometry from these experiments. However, an assumption of 1:1 for all complexes can be justified by structural data (Fig. 2, with good density for all the carbohydrates), and in all experiments it was possible to reach sufficiently high ligand concentrations to produce saturation. Consequently, the affinity (Kd) and enthalpy of binding (ΔH) could be measured and the entropy of binding (ΔS) calculated with reasonable confidence. In binding studies with UDP in the absence of other ligands, heat release or uptake was not observed, but when Mn2+ (2 mM) was present in both ligand and enzyme solutions, an exothermic binding profile was obtained (Fig. 5A). Similarly, binding of lactose to the enzyme was not detected in the presence of Mn2+ alone, but, in the presence of UDP and Mn2+, binding with a negative enthalpy change was observed (Fig. 5B). A similar result was obtained with the other acceptor substrate, N-acetyllactosamine. The binding of UDP-Gal (in the presence of Mn2+) was also exothermic.

The signal from the calorimeter did not return to the initial baseline after each addition of substrate, particularly for the initial injections. The baseline signal from the calorimeter reflects the energy added or removed from the reaction to maintain constant temperature, and, in this case, the changed baseline during successive additions of substrate appears to result from the low level of UDP-galactose hydrolase activity displayed by α3GT (20). This baseline was subtracted manually to generate a binding isotherm; this process adds a slight uncertainty to the value calculated for the enthalpy of binding, but the Kd value is reasonably consistent with the Kd value determined from kinetics (20). No baseline shift was observed for the binding of UDP-Glc, which is not a substrate for α3GT.

| Ligand bound | Other components | Kd \(10^{-3} M\) | ΔH \(\text{kJ/mol}\) | ΔS \(\text{J/K mol}\) |
|--------------|-----------------|----------------|----------------|----------------|
| UDP          | Mn2+            | 0.053 ± 0.004  | -5.93 ± 0.01   | -17.40 ± 0.14 |
| UDP          | Not detected    |                |                |                |
| UDP-Glc      | Mn2+            | 0.258 ± 0.008  | -4.96 ± 0.04   | -8.38 ± 0.16   |
| UDP-Gal      | Mn2+            | 0.060 ± 0.002  | -5.86 ± 0.01   | -13.81 ± 0.22 |
| Lac          | Mn2+            | Not detected   |                |                |
| Lac          | UDP, Mn2+       | 2.68 ± 0.06    | -3.57 ± 0.02   | -9.47 ± 0.13   |
| LacNAc       | UDP, Mn2+       | 0.800 ± 0.013  | -4.49 ± 0.01   | -11.25 ± 0.15  |

Table IV

Thermodynamic parameters for the binding of substrates and inhibitors to α3GT

Fig. 6. A, the active site of α3GT showing the locations of UDP, Gal, Lac, and the docked UDP-Gal derived from the LgtC structure (Protein Data Bank code 1G9R (28)). The Mn2+ ion is shown as a purple ball, Lac is pink, modeled UDP-Gal is green, and Glu337 is parrot green. B, a possible transition state for the non-covalent mechanism for α3GT.
The binding of both donor substrate analogues and acceptors is driven by a negative enthalpy change, which is partially countered by a positive (unfavorable) entropy change. The magnitude of ΔH for the binding of UDP-Gal is less than that for UDP by about 3 kcal/mol, possibly reflecting fewer electrostatic interactions between the enzyme and UDP-Gal, but the free energy of binding is similar to that for UDP, because of a compensating change in entropy (Table IV). The weaker binding of UDP-Glc reflects its lower enthalpy of binding, presumably arising from less favorable interactions between the glucose moiety and α3GT (Table II). The lower Km of LacNac for the enzyme-Mn2+·UDP complex, as compared with lactose, is consistent with its lower Km value as a substrate; the increased affinity arises from a more negative enthalpy of binding, presumably arising from the more extensive interactions between the 2-acetamido group and enzyme, as compared with the 2-hydroxyl group of lactose.

**Molecular Modeling**—The locations of UDP and β-Gal in the active site of α3GT in the enzyme complex formed by co-crystallization with UDP-Gal suggests that, in the Michaelis complex, UDP-Gal may not bind to the enzyme in the extended, low energy conformation found in most UDP-sugar complexes of known structure, e.g., rabbit N-acetylglucosaminyltransferase I (6) and β-1,4-galactosyltransferase I (4). When we attempted to dock UDP-Gal in an extended conformation with the α3GT structure using the docking module of Insight II, there were steric clashes between the galactose ring and the side chain of Glu317 and with the acceptor substrate when this was included in the structure. Energy minimization of this structure did not resolve these problems. As an alternative, we used a different conformation found in an uncleavable analogue of UDP-Gal, UDP-2F-galactose (UDP-2F-Gal), when bound to the bacterial retaining galactosyltransferase, LgtC from Neisseria meningitidis (28); in this conformation, the UDP component is extended but the pyranose ring is bent back under the phosphates and is almost parallel to the plane of the diphosphate (28), the galactose-phosphate having a similar conformation to glucose 1-phosphate bound to glycogen phosphorylase (29). UDP-Gal in this conformation could be docked readily with the enzyme, and, when the complex was optimized by energy minimization, it produced a structure that was devoid of steric clashes with enzyme groups or bound acceptor (Fig. 6). In this model, the UDP moiety of the substrate binds similarly to UDP, and the galactose moiety makes H-bonds and other interactions with protein groups (Table V).

**DISCUSSION**

The results presented here identify the binding site for acceptor substrates in α3GT and provide other information relevant to donor substrate binding and catalytic mechanism. We have previously reported that, although a double-displacement mechanism, in which UDP is released prior to acceptor substrate binding, is a likely mechanism for a retaining glycosyltransferase reaction, α3GT utilizes a sequential mechanism in which both substrates bind before any product is released. Ordered binding of donor and acceptor was suggested by the low UDP-Gal hydrolase activity of the enzyme (20). The lack of any calorimetric response to the addition of lactose or LacNac, in the absence of UDP, also supports the hypothesis that the donor substrate binds prior to the acceptor substrate in an obligatory order. Although it is theoretically possible that acceptor binding occurs in the absence of a donor substrate analogue but is not detected by calorimetry because of a minimal change in enthalpy under these experimental conditions, the extensive contacts observed in the α3GT-acceptor complexes make this seem extremely implausible. Also, two features of the enzyme-UDP-acceptor complexes provide a structural rationale for ordered binding of donor substrate (or analogue) and acceptor. First, the conformational change in the C-terminal 10 residues induced by UDP binding (9) reorients the aromatic ring of Trp316 and side chain of Lys315 to form multiple interactions with acceptor; second, UDP interacts directly with the acceptor through O2 of the β-phosphate and multiple non-polar contacts (Table III).

The sequential mechanism ascribed to α3GT indicates that UDP is not released but does not preclude a covalent mechanism. If a β-galactosyl-enzyme intermediate is formed in the catalytic mechanism of α3GT, it is not necessary that UDP is released after cleavage of the galactose-UDP bond; the binding studies and structures reported here indicate that the presence of UDP is necessary for acceptor binding. If UDP remains bound, its location must allow the acceptor to approach in the correct orientation for the second transfer reaction. Gastinel et al. (8) observed electron density adjacent to the side chain of Glu317 in the structure of an Hp-UDP-Gal·enzyme complex that they interpret as a covalent galactosyl-enzyme intermediate. In the high resolution structure of the UDP-Gal complex reported here, the UDP to galactose bond is cleaved but no covalent link is present between galactose and enzyme; instead, a β-linked OH group can be identified at C1 of the galactose ring. The ability of the enzyme to hydrolyze donor substrate is consistent with the low UDP-Gal hydrolase activity of α3GT (20), and the β-anomeric configuration of the galactose product suggests direct hydrolysis of UDP-Gal rather than formation of a β-galactosyl-enzyme intermediate. The cleavage of the UDP to glucose bond in the crystals of enzyme soaked with UDP-Glc indicates that this bond is also labile in the enzyme complex on the time scale of soaking (2 days, Table I). The retention of the monosaccharides galactose and glucose in the donor substrate-binding site following cleavage emphasizes the lack of solvent exposure of this region. Although the orientations of the two sugars are different, they interact with a similar subset of amino acid residues, suggesting that their binding sites identify a region with high affinity for monosaccharides. Modeling studies indicate that UDP-Gal cannot readily bind to α3GT in an extended conformation but can bind in a distorted conformation similar to that adopted by UDP-2F-Gal when bound to the bacterial retaining glycosyltransferase LgtC (Fig. 6A) (28). Two features of this structure suggest that it may be a useful model of the Michaelis complex of the donor substrate. First, many of the atoms of the enzyme that interact with galactose and glucose in the UDP-sugar complexes interact with the galactose component of this model (Tables II and V). Second, in the model, O2 of the galactose forms an H-bond with the side chain of His280 of α3GT; this residue corresponds to Leu266 and Met266 of human blood group A and B glycosyltransferases, respectively, which have a major influence on the speci-
ficity of these two enzymes for N-acetylgalactosamine and galactose as donor sugar (30). Blood group A and B enzymes differ in sequence at only four sites, of which residues 266 and 268 have the major influence on donor substrate specificity. Kinetic parameters reported for recombinant blood group A enzyme with B-like substitutions reveal that specificity for the donor substrate is not determined by a change in affinity for UDP-Gal relative to UDP-GalNAc but by changes in catalytic efficiency ($k_{cat}/K_m \times \text{rate}$), a parameter that reflects transition state stabilization (20). The stabilization of the distorted conformation of UDP-Gal by an interaction of the 2-OH with His$_{280}$ lends support to the hypothesis that substrate distortion has a role in the catalytic mechanism of α3GT.

A model for the ternary complex of α3GT with lactose and the bent conformer of UDP-Gal (plus metal cofactor) is shown in Fig. 6A. Examination of this complex suggests that the stereochemistry of the galactose-phosphate linkage would allow the O3 of the acceptor to displace the UDP on the same side of the galactose ring to produce an α-galactosyl product without formation of a β-galactosyl-enzyme intermediate. At the same time, O2 of the β-phosphate of the UDP is positioned to deprotonate the acceptor O3. Fig. 6B provides a schematic view of the transition state for this mechanism. An S$_{N}$2 type mechanism has been proposed for the retaining glycosyltransferase, glycosphenylase, and its relatives (29) and is consistent with current structural data for LgtC (28, 31). However, at this time, a covalent mechanism for α3GT cannot be totally excluded. The oxocarbenium ion character of the acceptor O3. Fig. 6 supports to the hypothesis that substrate distortion has a role in the catalytic mechanism of α3GT.

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