High Resolution Structures of the Human ABO(H) Blood Group Enzymes in Complex with Donor Analogs Reveal That the Enzymes Utilize Multiple Donor Conformations to Bind Substrates in a Stepwise Manner*

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Background: Substrate hydrolysis has impeded structural investigation of human ABO(H) glycosyltransferase specificity. Results: Complexes with natural and isosteric non-hydrolyzable donor analogs show multiple stable intermediate donor binding conformations. Conclusion: Subtle stereochemical differences from natural donor prevent isosteric donor analog from displaying full mimicry. Significance: High resolution structural analysis provides insight into inhibitor development and the multistage process of substrate binding.

Homologous glycosyltransferases α-(1→3)-N-acetylgalactosaminyltransferase (GTA) and α-(1→3)-galactosyltransferase (GTB) catalyze the final step in ABO(H) blood group A and B antigen synthesis through sugar transfer from activated donor to the H antigen acceptor. These enzymes have a GT-A fold type with characteristic mobile polypeptide loops that cover the active site upon substrate binding and, despite intense investigation, many aspects of substrate specificity and catalysis remain unclear. The structures of GTA, GTB, and their chimeras have been determined to between 1.55 and 1.39 Å resolution in complex with natural donors UDP-Gal, UDP-Glc and, in an attempt to overcome one of the common problems associated with three-dimensional studies, the non-hydrolyzable donor analog UDP-phosphono-galactose (UDP-C-Gal). Whereas the uracil moieties of the donors are observed to maintain a constant location, the sugar moieties lie in four distinct conformations, varying from extended to the “tucked under” conformation associated with catalysis, each stabilized by different hydrogen bonding partners with the enzyme. Further, several structures show clear evidence that the donor sugar is disordered over two of the observed conformations and so provide evidence for stepwise insertion into the active site. Although the natural donors can both assume the tucked under conformation in complex with enzyme, UDP-C-Gal cannot. Whereas UDP-C-Gal was designed to be “isosteric” with natural donor, the small differences in structure imposed by changing the epimeric oxygen atom to carbon appear to render the enzyme incapable of binding the analog in the active conformation and so preclude its use as a substrate mimic in GTA and GTB.

Glycosyltransferases (GTs)3 mediate glycoside synthesis, a ubiquitous enzymatic process, by catalyzing the transfer of monosaccharides from activated donor to acceptor. GTs generate glycans and glycoconjugates that are critical for cell signaling, immunity, plant cell wall formation, and molecular recognition. These enzymes are essential for normal cell development, and aberrant GT function can result in a number of infections and inflammatory disease states (1). Despite their functional and physiological diversity, GTs exhibit structural and phenotype conservation even in the absence of sequence homology (1–12). There are currently 97 GT families based on sequence identity (2, 13), with almost all of the enzymes falling into two major fold types: GT-A and GT-B.

Two family six GTs with the GT-A fold type (14), α-(1→3)-N-acetylgalactosaminyltransferase (GTA) and α-(1→3)-galactosyltransferase (GTB) perform the final step in human A and B blood group antigen synthesis through monosaccharide transfer to the H antigen acceptor (HA) terminal sequence α-(1→3)-Gal-(1→2)-β-d-Gal-O−R (14–21). As shown in Fig. 1A, GTA catalyzes the transfer of an N-acetylgalactosaminyl (GalNAc) residue from UDP-GalNAc to HA to form the A antigen, and GTB catalyzes the transfer of a galactosyl (Gal) residue from UDP-Gal to HA to form the B antigen (14, 15, 20, 22–25). Blood group O individuals generally express truncated or mutated glycosyltransferases (GTs)3 mediate glycoside synthesis, a ubiquitous enzymatic process, by catalyzing the transfer of monosaccharides from activated donor to acceptor. GTs generate glycans and glycoconjugates that are critical for cell signaling, immunity, plant cell wall formation, and molecular recognition. These enzymes are essential for normal cell development, and aberrant GT function can result in a number of infections and inflammatory disease states (1). Despite their functional and physiological diversity, GTs exhibit structural and phenotype conservation even in the absence of sequence homology (1–12). There are currently 97 GT families based on sequence identity (2, 13), with almost all of the enzymes falling into two major fold types: GT-A and GT-B.

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3 The abbreviations used are: GT, glycosyltransferase; GATA, α-(1→3)-N-acetylgalactosaminyltransferase; GTB, α-(1→3)-galactosyltransferase; HA, H antigen acceptor; UDP-C-Gal, UDP-phosphono-galactose; MPD, 2-methyl-2,4-pentanediol; DI, 3-deoxy inhibitor.
forms of GTA and GTB (16, 25, 26), so biosynthesis terminates at the H antigen.

The similarity of the GTA and GTB enzyme donor substrates results in the blood group A and B individuals differing only in the replacement of an acetamido with a hydroxyl group on the terminal galactose residue. GTA and GTB are homologous, differing in just four critical amino acid residues: Arg/Gly-176, Gly/Ser-235, Leu/Met-266, and Gly/Ala-268 (16, 25). Prior kinetic and structural studies using GTA/GTB chimeras have provided insight into the roles of these key amino acids. GTA, GTB, and their chimeras can be described by four-letter codes, where each letter corresponds to a critical residue. For example, AAAA refers to GTA, BBBB refers to GTB, and AABB refers to the chimera with the first two critical residues of GTA and the last two critical residues of GTB. Previous findings implicate the first amino acid in enzyme turnover (12, 26), the second and third in acceptor recognition (14, 27), and the third and fourth in donor selection (14, 17, 25, 28, 29).

High resolution structures have revealed two flexible regions near the active site (Fig. 1B): an internal loop (amino acids 176–188) and C-terminal α-helix (amino acids 346–354) (12, 14). In the absence of substrate, these enzymes tend to adopt the “open” conformation, where both regions have higher disorder and/or the internal loop faces away from the active site (12). When UDP and manganese ion (Mn²⁺) bind, there is a shift to the “semi-closed” conformation, where the internal loop is more ordered and oriented toward UDP so as to occlude the active site entrance, whereas the C-terminal loop remains disordered (12). With the addition of UDP or UDP-Gal in combination with acceptor or acceptor analog, GTA and GTB are observed to assume the “closed” state associated with catalytic activity (Fig. 1B), where the internal loop and C-terminal region are both ordered and interacting with donor and acceptor (12).

The challenges associated with soaking or co-crystallizing these enzymes with natural donors and donor analogs (12, 30–33), such as substrate hydrolysis, oxidation, and concomitant conformational changes that lead to destruction of the crystal lattice, have been obstacles to furthering understanding of enzyme action. Although several electrospray ionization-MS crystal lattice, have been obstacles to furthering understanding of enzyme action. Although several electrospray ionization-MS experiments have shown the bound conformations of these nucleotide sugars to be comparable (32), UDP-Glc yields much lower enzymatic activity, 0% compared with UDP-GalNAc for GTA, which has a $k_{cat}$ of 17.5 s⁻¹, and 0.02% compared with UDP-Gal for GTB, which has a $k_{cat}$ of 5.1 s⁻¹ (26, 40).

Here we report the structures to high resolution of GTA, GTB, AABB, ABBA, and ABBB grown in complex with UDP-C-Gal, UDP-Gal, and UDP-Glc with either synthetic H antigen disaccharide α-l-Fuc-(1→2)-β-d-Galp-O(CH₂)₇CH₃, (HA) or the 3-deoxy inhibitor (DI), α-l-Fuc-(1→2)-β-d-(3-deoxy)-Galp-O(CH₂)₇CH₃, providing fresh insight into the glycosyl transfer reaction.

**Experimental Procedures**

**Ligands—** UDP-C-Gal, 1-Fuc-(1→2)-β-d-Galp-O(CH₂)₇CH₃ (HA), and α-l-Fuc-(1→2)-β-d-(3-deoxy)-Galp-O(CH₂)₇CH₃ (DI) were synthesized as reported previously (29, 39, 41). Donor substrates UDP-Gal and UDP-Glc were purchased from Sigma-Aldrich.

**Crystalization—** Chimeric AABB, ABBA, and ABBA proteins were produced as described by Alfaro et al. (12) and crystallized in protein stock solution. Native GTA/GTB proteins were grown at 4 °C from a much higher concentration of protein (30–40 mg ml⁻¹ for GTB) and 16–20 mg ml⁻¹ for GTA) along with 1% polyethylene glycol (PEG) 4000, 4.5–5% 2-methyl-2,4-pentanediol (MPD), 100 mM ammonium sulfate, 70 mM sodium chloride, 50 mM N-[2-acetamido]-2-iminodiacetic acid buffer, pH 7.5, 30 mM sodium acetate buffer, pH 4.6, and 5 mM manganese chloride (MnCl₂) for GTA crystallization and 5–8 mM CoCl₂ for GTB crystallization. Drops of 10–15 μl were placed against a reservoir containing 3.7% PEG 4000, 7% MPD, 0.3 mM ammonium sulfate, 0.25 mM sodium chloride, 0.2 mM N-[2-acetamido]-2-iminodiacetic acid buffer, and 0.1 mM sodium acetate. Crystals grew for 5–10 days at 4 °C.

Protein crystals were washed with artificial mother liquor containing 3.5% PEG 4000, 50 mM ammonium sulfate, 40 mM sodium chloride, 35 mM N-[2-acetamido]-2-iminodiacetic acid buffer, and 15% MPD prior to substrate addition. Crystals of AAAA, AABB, ABBB, and ABBB in complex with UDP-Gal, UDP-C-Gal, UDP-Glc, and HA were obtained by soaking them in mother liquor-2 with 15% MPD, 60–70 mM UDP-Gal, 60–80 mM UDP-Glc, 40–60 mM UDP-C-Gal, 12–20 mM DI, 20 mM HA, and 10 mM MnCl₂ for 2–5 days at 4 °C. Short soaking times and reduced concentrations of UDP-C-Gal and DI were also used for ABBB and AABB chimeras. Donors, acceptors, and analogs thereof were added incrementally over a period of a few minutes to hours to avoid crystal fracture. The data sets and structures presented were from those crystals that diffracted to highest resolution. Before freezing crystals for data collection, the cryoprotectant concentration was adjusted to 20% MPD.
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A

B

C

Donor | Length A (Å) | Length B (Å) | Angle α (°)
--- | --- | --- | ---
UDP-Gal | 1.43 Å | 1.58 Å | 121 °
UDP-C-Gal | 1.53 Å | 1.81 Å | 110 °
Overall, the resolution limit ranges from 1.55 to 1.39 Å, given in Table 1 for the structures of enzymes in complex with UDP-C-Gal, Table 2 for UDP-Gal, and Table 3 for UDP-Glc.

The closed internal loop (residues 176–188) and C-terminal domain (residues 346–354) are shown in orange.

| Data collection and refinement statistics for GTA, GTB, and their chimeras in complex with UDP-C-Gal |
|---|
| **TABLE 1** |
| **Data collection** |
| **Resolution (Å)** | **R_{work} (%)** | **Completeness (%)** | **Unique reflections** | **Protein Data Bank ID** |
| Di with AAAA | 20–1.55 | 20.1–1.45 | 20.1–1.44 | 1LZ0 |
| Di with AABB | 20–1.40 | 20.1–1.39 | 20.1–1.38 | 1LZ7 |
| Di with ABBA (short soak) | 20–1.40 | 20.1–1.39 | 20.1–1.38 | 1LZ7 |
| Di with ABBA (long soak) | 20–1.39 | 20.1–1.38 | 20.1–1.37 | 1LZ7 |
| Di with ABBB (short soak) | 20–1.39 | 20.1–1.38 | 20.1–1.37 | 1LZ7 |
| Di with ABBB (long soak) | 20–1.39 | 20.1–1.38 | 20.1–1.37 | 1LZ7 |
| Di with BBBBB | 20–1.40 | 20.1–1.39 | 20.1–1.38 | 1LZ7 |

**Refinement**

| **R_{work} (%)** | **R_{free} (%)** | **No. of waters** | **r.m.s. bond (Å)** | **r.m.s. angle (degrees)** |
|---|---|---|---|---|
| **AAAA** | 18.2 | 21.7 | 231 | 0.0105 | 1.516 |
| **ABBA** | 18.1 | 19.5 | 262 | 0.0098 | 1.504 |
| **ABBB** | 20.0 | 21.8 | 259 | 0.0097 | 1.532 |
| **BBBB** | 18.4 | 20.4 | 248 | 0.0094 | 1.553 |

**Data Collection and Reduction—** X-ray diffraction data were collected at −160°C for all crystals using a CryoStream 700 crystal cooler. Each crystal was incubated with a cryoprotectant solution consisting of mother liquor with 20% (v/v) MPD proportionally replacing the volume of water in the initial mother liquor. Data were collected on a Rigaku R-AXIS IV detector at distances of 72 mm and exposure times between 4.0 and 7.0 min for 0.5° oscillations. X-rays were produced by an MM-002 generator (Rigaku/MSC, College Station, TX) coupled to Osmic 9 “Blue” confocal x-ray mirrors (Osmic, Auburn Hills, MI). Data were scaled, averaged, and integrated using d^*trek and CrystalClear (42).

**Structure Determination—** Although the structures were nearly isomorphous, for completeness, they were solved by molecular replacement using the CCP4 module Phaser (43) with the structures of wild type GTA and GTB as starting models (Protein Data Bank accession codes 1LZ0 and 1LZ7, respectively). Structures were then refined using the CCP4 module REFMAC5 (44, 45), and all figures were produced with SetoRibbon.

**Results**

**Loop Ordering—** Data collection and refinement statistics are given in Table 1 for the structures of enzymes in complex with UDP-C-Gal, Table 2 for UDP-Gal, and Table 3 for UDP-Glc. Overall, the resolution limit ranges from 1.55 to 1.39 Å, R_{work} ranges from 17.2 to 20.0%, and R_{free} ranges from 18.6 to 21.8%.

In the space group in which all of the enzymes crystallized, C222_1, there is a solvent channel that allows considerable mobility to the two loops of mobile polypeptide that surround the active site. Observed electron density surrounding the internal loop (residues 176–188) and the C-terminal loop (residues 346–354) for all structures is summarized in Table 4. With the exception of these sometimes ordered and sometimes disordered regions, the entire length of the polypeptide chains had excellent electron density. Table 4 notes which residues in the loops have ordered atoms, which have interpretable density for the main chain atoms only, and which residues are completely disordered. Generally, when only main chain atoms are observed, the relative temperature factors are higher than the proximal protein atoms, indicating that there is still considerable lability in such a region of polypeptide.

When in complex with UDP-C-Gal, GTA, GTB, and all chimeras have disordered C termini, even with acceptor or acceptor analog UDP-C-Gal. Donor analog UDP-C-Gal (6) only differs from UDP-Gal in replacement of the anomeric oxygen with a methylene group, whereas UDP-Glc (7) differs in the equatorial position of Gal-C4-OH. Labeled UDP-C-Gal carbon-carbon (6A) and carbon-phosphorus (6B) bonds are longer than UDP-Gal carbon-oxygen (2A) and oxygen-phosphorus (2B) bonds, and the UDP-C-Gal C-C-P bond angle (6a) is much smaller than the UDP-Gal C-O-P bond angle (2a), B, superimposition of unliganded ABBB (Protein Data Bank code 2RIZ) in the open state with AABB (Protein Data Bank code 2RJ7) in the closed state. The closed internal loop (residues 176–188) and C-terminal domain (residues 346–354) are shown in red, whereas the open loops of ABBB are shown in yellow.

The location of Arg-176 is indicated, and the C-terminal loop is labeled C. The dashed arrow indicates the movement of the internal loop toward the donor in going from the open state to both the semi-closed and closed states. Mn2+ is shown as a magenta sphere, donor is shown in blue, and acceptor is shown in orange.
In this set of structures, only GTA is in the semi-closed state with a structured internal loop. The remaining complexes are in the open state, although ABBB becomes more ordered in the long soak condition. Due to weak or ambiguous density, the UDP-C-Gal sugar moiety was not modeled for GTA and GTB (with HA) complexes.

Structures in complex with the native donor UDP-Gal generally show much greater order than those in complex with UDP-C-Gal or UDP-Glc. GTA and ABBA adopt the closed state with structured internal and C-terminal loops (Table 4) when in complex with UDP-Gal and DI, similar to the previously published AABB structure (12). ABBB is in the semi-closed state with a flexible C terminus, and GTB is in the open state with both domains disordered.

When compared to native donor UDP-Gal, structures in complex with UDP-Glc generally have more disordered C-terminal and internal loops except for GTA and GTB (the latter in complex with HA instead of DI). GTA is in the semi-closed state with flexible C terminus, and GTB is in the open state with both domains disordered.

Table 3: Data collection and refinement statistics for GTA, GTB, and their chimera in complex with UDP-Glc

| Data collection | DI with AAAA | DI with ABBB | DI with ABBA | DI with ABBB | DI with BBBBB | HA with BBBBB |
|-----------------|--------------|--------------|--------------|--------------|--------------|--------------|
| Resolution (Å)  | 20.1-1.54    | 20.1-1.41    | 20.1-1.45    | 20.1-1.43    | 20.1-1.40    | 20.1-1.55    |
| Rmerge (%)      | 4.2 (35.1)   | 3.6 (32.8)   | 3.0 (22.1)   | 3.2 (26.3)   | 3.3 (27.8)   | 4.6 (27.3)   |
| Completeness (%)| 94.4 (89.1)  | 97.2 (82.0)  | 97.9 (98.8)  | 97.7 (96.3)  | 98.1 (87.1)  | 98.5 (99.7)  |
| Unique reflections | 44,020       | 58,865       | 54,336       | 56,554       | 60,723       | 45,267       |

Table 4: Loop ordering in AAAA, BBBB, and chimeric enzyme complexes

| Enzyme+substrates | Donor sugar density | Donor conformation | Acceptor sugar density | Internal loop | C-terminus |
|-------------------|---------------------|--------------------|------------------------|---------------|------------|
| UDP-Gal complexes |                     |                    |                        |               |            |
| AAAA+UDP-C-Gal+DI | None                | n/a                | Good                   | 176           | 346        |
| ABBB+UDP-C-Gal+DI | Good                | #2                 | Good                   | 181           | 351        |
| ABBA+UDP-C-Gal short soak +DI | #3 (2) | Poor | EV Raykr wgdv MR | VP knhqa vrn p |
| ABBA+UDP-C-Gal long soak +DI | #2 (3) | Poor | EV Raykr wgdv MR | VP knhqa vrn p |
| ABBB+UDP-C-Gal short soak +DI | #3 | Poor | EV Raykr wgdv MR | VP knhqa vrn p |
| ABBB+UDP-C-Gal long soak +DI | Partial | #2 | EV Raykr wgdv MR | VP knhqa vrn p |
| BBBB+UDP-C-Gal +DI | Good                | #2                 | Good                   | 186           |            |
| BBBB+UDP-C-Gal +HA | Poor                | n/a                | Good                   |               |            |

| UDP-Glc complexes |                     |                    |                        |               |            |
| AAAA+UDP-Glc+DI | Good                | #4                 | Good                   | 176           | 346        |
| ABBB+UDP-Glc+DI | Good                | #4                 | Good                   | 181           | 351        |
| ABBA+UDP-Glc+DI | Good                | #1                 | Good                   | 186           |            |
| BBBB+UDP-Glc+DI | Good                | #4                 | Good                   |               |            |
| BBBB+UDP-Glc+HA | None                | n/a                | Good                   |               |            |

a: Value in parentheses indicates a lower occupancy alternate conformation.
b: As reported by Alfaro et al. (12); Protein Data Bank code 2RJ7.
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Donor Sugar Occupancy—In four of the structures, unexplained electron density was observed in the active sites surrounding the donor sugar moiety (Fig. 5, left). Inspection showed that the density was explained by the donor sugar (and sometimes the donor phosphate groups) being disordered over two conformations, with the second conformation lying in the difference density (Fig. 5, right). The occupancy of the primary conformation in all four of these structures was adjusted to yield temperature factors comparable with adjacent protein atoms. The difference electron densities in Fig. 5 are unbiased because they do not include refinement of the alternate conformation.

Specifically, in each of the ABBA (short and long soak) structures, UDP-C-Gal was found to be disordered over conformations 2 and 3 (Fig. 5). In the short soak structure, the extended conformation predominates in a ratio of 80:20, whereas in the long soak structure, conformation 2 predominates in a ratio of 70:30. In contrast, UDP-C-Gal was observed to be 100% occupied when in complex with AABB, ABBB (short soak), ABBB (long soak), and GTB (with DI) and completely disordered when in complex with GTA and GTB (with HA).

Also, natural donor UDP-Gal was found to be disordered over conformations 1 and 2 in an approximate ratio of 70:30 in the active site of ABBA and with an approximate ratio of 40:60 in the active site of ABBB (Fig. 5). Again in contrast, UDP-Gal was 100% occupied when in complex with GTB and completely disordered when in complex with GTA. All enzymes were soaked with the same batch of donor, so the disorder observed in the GTA+UDP-Gal structure was probably not due to hydrolyzed donor. UDP-Glc was ordered when in complex with GTA, AABB, ABBB, and GTB (with DI) and disordered when in complex with GTB (with HA).

Discussion

Four distinct donor conformations were observed in total using three different donors or donor analogs (Figs. 2 and 3 and Table 4). GTA, GTB, and their chimeras and mutants have only been observed in complex with intact donor in two previous structures, most notably AABB+UDP-Gal+DI (12), where UDP-Gal is observed in the classic tucked under conformation (Table 4, conformation 1); a second UDP-Gal conformation (conformation 2) was observed in GTB Cys/Ser mutant structures (48). The observation of several new structures with intact bound donor represents a significant advance in understanding donor recognition in this class of enzymes.

UDP-C-Gal Cannot Fully Mimic Bound Donor—There is a paucity of glycosyltransferase structures determined in complex with donor substrates, which has been attributed partly to the enzymes’ ability to hydrolyze donor in the absence of acceptor and partly to the enzymes’ tendency to undergo conformational rearrangement upon binding substrate and so shatter the crystal lattice (12, 30–33).

The introduction of non-hydrolyzable donors like UDP-C-Gal provides an opportunity to negate the first barrier but, because the analog necessarily contains structural differences from the natural donors, does not allow complete mimicry (Fig. 1A and Table 4). For example, the anomeric C–O bond length of 1.4 Å changes to 1.55 Å for the C–C bond. More significantly,
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A
ABBA
UDP-Gal

B
GTB
UDP-Gal

C
ABBB short
UDP-C-Gal

D
AABB
UDP-Glc

E
ABBA
UDP-Glc

F
#1
#2
#3
#4
whereas the C-O-P bond angle is close to a trigonal $123 \pm 11^\circ$ (averaged from UDP-sugar structures in the RCSBD Protein Data Bank) because of the ability of the anomeric oxygen to assume partial sp$^2$ character, the purely sp$^3$ methylene group in UDP-C-Gal would be expected to be closer to a tetrahedral $109^\circ$. These small changes lead to significant differences in binding.

UDP-C-Gal is observed in two distinct conformations in the active sites of six of the eight structures in which it was soaked. The analog displays the greatest order in the chimera (Table 4) because the galactosyl residue is completely disordered in GTA and GTB. In four of the chimeras, it is observed in a single conformation (either conformation 2 or 3), whereas in two of the structures, there is clear evidence that it is disordered over these two distinct conformations. In none of the structures does UDP-C-Gal assume the tucked under conformation associated with catalysis. The inability of UDP-C-Gal to assume the catalytically competent conformation is probably due to the small differences in bond angles and lengths compared with the native donor.

When soaking times are short and substrate concentrations are low, the substrate primarily assumes an extended conformation (conformation 3 in Fig. 2C). In this conformation, UDP-C-Gal utilizes almost none of the stabilizing donor-enzyme interactions of the tucked under conformation, and the galactosyl moiety makes enzyme contacts normally reserved for acceptor, including His-233 and Glu-303 hydrogen bonds to C-Gal hydroxyl groups O-2, O-3, and O-4, which explains the poor or absent acceptor electron density in the two structures where the donor has assumed conformation 3. The rotated donor sugar makes space for two new water molecules in the active site to form bridging hydroxyl bonds with residues Asp-302, Arg-188, and Asp-211 normally used to form direct hydrogen bonds to the donor sugar (Fig. 2A).

**UDP-C-Gal Biases Loop Structures toward the Open Conformation**—Previous studies have documented that the GTA/GTB internal loop generally becomes increasingly ordered in the presence of both donor and acceptor (12), with a tendency for enzymes with greater GTA character to order more readily than enzymes with greater GTB character (12, 46, 47). The UDP-Gal-bound structures presented in this study obey this trend (Table 4, middle). GTA + UDP-Gal is in the closed form, ABBA + UDP-Gal is nearly closed, ABBB + UDP-Gal is semi-closed, and BBBB + UDP-Gal is open.

In contrast, enzymes in complex with UDP-C-Gal and DI do not obey this trend but are consistently biased toward the open form (Table 4, top). GTA is well established as the least labile of all wild type and chimera, and whereas it is observed in a closed form in complex with UDP-Gal, it can only achieve the semi-closed form in complex with UDP-C-Gal. All of the remaining UDP-C-Gal structures are observed to lie in the open conformation. This can be directly attributed to the inability of UDP-C-Gal to assume the tucked under conformation, which leads to a steric clash with Trp-181 of the internal loop and with the acceptor (evidenced by weaker electron density), which itself impacts the ability of the C-terminal tail to adopt the closed state. Trp-181 was noted in a previous study to form a stabilizing interaction with Arg-352 of the terminal loop (12), explaining why internal loop order is a prerequisite to C-terminal loop order.

**UDP-Gal Is Observed Ordered in the Active Site of Wild Type GTB**—The search for GTA, GTB, and other GT structures in complex with UDP-Gal has been ongoing, involving various preparations of donor and acceptor and different soaking conditions, to yield finally this report of the crystal structure of GTB in which unambiguous electron density is seen for its natural donor UDP-Gal.

Interestingly, the substrate adopts the same conformation (number 2) in the wild-type GTB structure that was observed in the crystal structure of GTB Cys/Ser mutants (48) (Fig. 2C) but without achieving the closed state. Although their conformations may appear to be similar (Fig. 2), the donor-enzyme contacts in conformations 1 and 2 are significantly different. Relative to the tucked under donor (Figs. 2A and 3A), the enzyme no longer interacts with Gal-O-6, and Arg-188 and Asp-211 no longer interact with the galactosyl moiety directly but make bridging contacts through a newly introduced water molecule. Both differ from the conformation for bound donor first predicted by Heissigerova et al. (49).

**UDP-Glc Can Adopt the Tucked under Conformation in ABBA**—The structures reported here show UDP-Glc in two positions. The fourth distinct conformation (Figs. 2D and 3D) is observed only for UDP-Glc. As seen for other conformations with UDP-Gal, five hydrogen bonds stabilize the glucosyl moiety (Figs. 2D and 3D) with an additional contact between DI and Glc-O-2.

UDP-Glc is observed to assume the tucked under conformation (conformation 1; Figs. 2E and 3E) in ABBA, which was not expected, given that UDP-Glc is known to be a poor donor for these enzymes. The observed $k_{cat}$ for GTA is too low to measure; however, the small transfer for GTB of $k_{cat} = 0.001 s^{-1}$ (50) is well above background.

NMR studies have shown that UDP-Gal and UDP-Glc bind GTB with the same affinity, but in the latter case, binding is

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**FIGURE 2. Four donor conformations and key enzyme-substrate interactions.** UDP-Gal is tucked under in ABBA + UDP-Gal + DI (A) and is in an alternate conformation in GTB + UDP-Gal + DI (B). In 8, relative to A, the Gal hydroxyl groups have changed binding partners, but DI-enzyme interactions are the same. Donor analog UDP-C-Gal is in an extended conformation in ABBA + UDP-C-Gal + DI short soak (C). Here acceptor is absent, and donor extends into the acceptor binding site. Glu-303 and His-233 interact with C-Gal-O-2, -O-3, and -O-4 instead of DI, and Arg-188, Asp-211, and Asp-302 contact Gal via two water molecules. UDP-Glc is in a fourth conformation in D, ABBB + UDP-Glc + DI. DI makes a new contact to His-233, Glu-303 interacts with Glc-O-3, Arg-188 and Asp-211 interact with Glc-O-5, and Asp-302 interacts with Glc-O-6. In E, ABBA + UDP-Glc + DI, Glc is in the same conformation as in A but with equatorial Glc-O-4. Here, Asp-302 contacts Glc-O-4, and there is an additional interaction between Arg-188. In both D and E, the contact between Glc and His-301 is lost, and in A–E Glu-303 consistently makes two contacts to DI. Donor substrate has excellent electron density in all five structures. F, stereoview of overlapped donor conformations shows movement of the saccharide moiety and the β-phosphate. ABBA + UDP-Gal is in green, GTB + UDP-Gal is in yellow, ABBA short + UDP-C-Gal in orange, and ABBB + UDP-Glc is in magenta, with conformation numbers indicated in the corresponding color. Electron density diagrams are $F_o - F_c$ maps contoured at 1.0σ. Protein atoms are colored by element with red for oxygen, yellow for phosphorus, white for carbon, and blue for nitrogen. Waters are shown as red spheres, and Mn$^{2+}$ is shown as a magenta sphere. Green dotted spheres represent hydrogen bonds.
unproductive, because transfer to the H antigen acceptor does not readily occur (31, 32). On the basis of NMR data for UDP-Glc bound to GTB, Angulo et al. (32) proposed a “tweezers” mechanism where Asp-302 and Glu-303 lock Gal in the tucked under conformation to facilitate formation of the transition state. They suggest that, unlike the natural donor, UDP-Glc...
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**FIGURE 4. UDP-Gal conformations influence enzyme ordering.** UDP-Gal is in conformation 1 (yellow), and the enzyme is in the closed state in ABBA + UDP-Gal + DI. Here Trp-181 is distant from Gal-O-6, and Lys-346 interacts with the β-phosphate oxygen. When UDP-Gal is in conformation 2 (cyan), as seen in complex with GTB, the enzyme is in the semi-closed state with a less ordered internal loop and a disordered C-terminal tail. Here Trp-181 is near Gal-O-6, and Asp-211 interacts with the β-phosphate oxygen. Mn^{2+} is shown as a magenta sphere. Green dotted spheres represent hydrogen bonds, and black dashed lines indicate measured distances that are not hydrogen bond interactions.

cannot undergo this conformational transition because GlcO-4 is unable to interact with Asp-302. However, the structure of ABBA shows clearly that GlcO-4 can and does form a strong hydrogen bond (2.62 Å) to Asp-302 (Figs. 2E and 3E).

These structures show that the ability of the enzymes to distinguish between UDP-Gal and UDP-Glc more likely depends on their internal and C-terminal loop organization. The structure of ABBA in complex with UDP-Glc (conformation 1) displays considerably more disorder than the corresponding complex with UDP-Gal (Table 4). Further, ABBA in complex with UDP-Glc shows that residues Met-186 and Ser-185 are offset significantly compared with their position in the ordered Alfaro et al. (12) AABB + UDP-Gal structure. The resulting disorder and displacement of the internal loop and C-terminal tail mean that key stabilizing residues, including Trp-181, Lys-346, and Arg-352 are no longer positioned to stabilize substrate for transfer. Although the orientation of C4-OH seems compatible with conformation 1, it has a significant effect on mobile loop organization and the enzymes’ ability to adopt the catalytically active closed state.

**Insight into Substrate Binding**—Angulo et al. (32) observed conformation 1 in the active site of GTB using NMR methods, prompting the hypothesis that the enzymes selected this catalytically competent conformation despite its sparse population in solution. However, the second UDP-Gal orientation is actually similar to the dominant low energy conformer observed in solution (32), and these data suggest that the enzyme binds the abundant low energy UDP-Gal conformer and shifts it to the catalytically competent conformation.

Indeed, the selection of the extended conformer (i.e. approximately conformation 3) from solution offers an explanation of the kinetically observed phenomenon that donor binding precedes acceptor binding because the structures with the bound donor in conformation 3 clearly show that this conformation sterically impedes the binding of acceptor (29).

**Observed Donor Sugar Disorder Supports the Mechanism of Substrate Binding**—Four of the structures show clear evidence that the donor sugar is disordered over two conformations (Fig. 5), indicating that the enzymes do indeed bind the substrates in the predominant extended conformation 3 and shift it in a step-wise fashion to the tucked under conformation 1.

The most significant evidence comes from the two structures of ABBA soaked in UDP-C-Gal for a shorter and longer time, respectively. The short soak structure shows the donor analog predominantly in the extended conformation (number 3), with a small proportion in conformation 2. The longer soaking time shows that a greater proportion is observed in conformation 2.

Further evidence of the stepwise insertion of donor comes in the structures of ABBA and ABBB in complex with UDP-Gal, which show disorder over conformations 1 and 2, with ABBA being predominantly conformation 1 and ABBB being predominantly conformation 2. Together, these structures show how the enzyme facilitates the rotation of the donor into the catalytically active tucked under conformation 1.

**Donor Selectivity and Acceptor Influence in the Wild Type Enzymes**—GTB in complex with UDP-Gal and UDP-C-Gal shows unambiguous density for UDP bound to the donor-binding site but weak or absent density for the sugar moiety, indicating, certainly for the non-hydrolyzable UDP-C-Gal, that the moiety is disordered in the active site. This is interesting in the case of GTA + UDP-Gal + DI because the enzyme achieves the fully closed state although GTA displays a very low transfer rate of UDP-Gal to the natural acceptor (HA). However, the corresponding complexes with GTB show the sugar to be fully ordered. These observations speak directly to the specificity of these enzymes toward their cognate donor substrates.

Whereas the selectivity of the smaller GTB active site against the larger blood group A-donor (UDP-GalNac) could always be explained on steric grounds, the reverse was not true because the GTA active site could easily accommodate the smaller UDP-Gal donor. These structures show that the selectivity of GTA against the B-donor (UDP-Gal) may simply depend on the inability of the GTA enzyme to stabilize the B-donor in a catalytically active conformation.

Finally, the UDP-C-Gal donor was designed in an effort to allow the visualization of the donor sugar residue by preventing donor hydrolysis, so it is somewhat paradoxical that the sugar moiety is not observed in two of the structures (Table 4). In both wild type enzymes, GTA and GTB, soaked with donor analog and intact HA, there is unambiguous density for UDP bound to the donor-binding site but weak or absent density for the C-Gal moiety, indicating that the moiety is disordered in the active site. This is understandable for GTA, because its active site accommodates the much larger N-acetylgalatosamine residue, and the UDP-Gal substrate is also observed to be disordered.

The wild type GTB is also interesting, because the complex with the DI shows an ordered active site with the donor analog UDP-C-Gal in conformation 2, whereas the presence of the 3-OH group on the natural acceptor (HA) galactosyl residue causes the donor analog sugar group to become completely
This missing hydroxyl group clearly results in large steric clashes, because a superposition of the two structures would put the 3-OH group only \( \sim 2.5 \) Å away from C-1 of the ring, \( \sim 3.3 \) Å from the methylene carbon, and \( \sim 2.89 \) Å from one of the phosphate oxygen atoms.

**Conclusions**—Despite being soaked under identical conditions, the UDP-C-Gal donor analog in the active site of GTA, GTB, or their chimera approaches but cannot achieve the tucked under conformation associated with catalysis. This is probably due to the differences in chemical geometries imposed when substituting the glycosidic oxygen atom for carbon to make a non-hydrolyzable donor analog. Although the enzymes do bind the UDP moiety of UDP-C-Gal in the same manner as the native donors, the different conformation of the altered sugar moiety does not permit formation of the semi-closed or closed states, and its utility as a probe of the GTA/GTB transfer mechanism may be limited. The exquisite specificity of these enzymes is remarkable because even a substrate analog designed to be isosteric can encounter major structural barriers.

It was also observed that the donors and donor analogs bind these enzymes in more than one conformation and, further, that these multiple conformations have similar free energies of binding in the active site. UDP-Glc binds in an orientation distinct from those previously observed with significantly different enzyme-donor sugar hydrogen bond interactions. It also binds in the tucked under conformation associated with catalysis, but the slight differences in binding induced by the epimeric position of the 4-OH group prevent loop closure and so allow the enzymes to distinguish between epimers.

Together, these structures provide insight into the development of specific GT inhibitors and, in particular, calls into...
question the potential utility of C-phosphate analogs as biologically relevant mimics of sugar nucleotides. In addition, the multiple observed conformers suggest that donor binding to GTA and GTB is a multistage process that implements significant stepwise shifts to achieve the catalytically active conformation.

Author Contributions—S. M. L. G. performed some of the experiments, processed and analyzed the data, made the figures, and wrote a major part of the paper. P. J. M., R. B. Z., and T. L. L. performed the synthesis of the non-hydrolyzable donor analog and contributed to writing the paper. O. H. G. processed some of the data and contributed to Fig. 1 and Fig. 3A. A. R. J. processed and analyzed data and contributed to the paper. S. N. B. performed many of the experiments. S. V. E. conceived many of the experiments and wrote a major part of the paper. All authors reviewed the results and approved the final version of the manuscript.

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