Nuclear Magnetic Resonance Spectral Assignments of α-1,4-Galactosyltransferase LgtC from Neisseria meningitidis: Substrate Binding and Multiple Conformational States

Patrick H. W. Chan, Sophie Weissbach, Mark Okon, Stephen G. Withers, and Lawrence P. McIntosh

Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC V6T 1Z3, Canada
Centre for High-throughput Biology, University of British Columbia, Vancouver, BC V6T 1Z4, Canada
Department of Chemistry, University of British Columbia, Vancouver, BC V6T 1Z1, Canada
Michael Smith Laboratories, University of British Columbia, Vancouver, BC V6T 1Z4, Canada

ABSTRACT: Lipopolysaccharide α-1,4-galactosyltransferase C (LgtC) from Neisseria meningitidis is responsible for a key step in lipopolysaccharide biosynthesis involving the transfer of α-galactose from the sugar donor UDP-galactose to a terminal acceptor lactose. Crystal structures of the complexes of LgtC with Mn²⁺ and the sugar donor analogue UDP-2-deoxy-2-fluorogalactose in the absence and presence of the sugar acceptor analogue 4′-deoxylactose provided key insights into the galactosyl-transfer mechanism. Combined with kinetic analyses, the enzymatic mechanism of LgtC appears to involve a “front-side attack” S_{o,b}|-like mechanism with a short-lived oxocarbenium—phosphate ion pair intermediate. As a prerequisite for investigating the required roles of structural dynamics in this catalytic mechanism by nuclear magnetic resonance techniques, the transverse relaxation-optimized amide ¹⁵N heteronuclear single-quantum correlation and methyl ¹³C heteronuclear multiple-quantum correlation spectra of LgtC in its apo, substrate analogue, and product complexes were partially assigned. This was accomplished using a suite of complementary spectroscopic approaches, combined with selective isotopic labeling and mutagenesis of all the isoleucine residues in the protein. Only ~70% of the amide signals could be detected, whereas more than the expected number of methyl signals were observed, indicating that LgtC adopts multiple interconverting conformational states. Chemical shift perturbation mapping provided insights into substrate and product binding, including the demonstration that the sugar donor analogue (UDP-2FGal) associates with LgtC only in the presence of a metal ion (Mg²⁺). These spectral assignments provide the foundation for detailed studies of the conformational dynamics of LgtC.

Received: July 30, 2012
Revised: September 17, 2012
Published: September 19, 2012

Neisseria meningitidis is a Gram-negative bacterium that causes meningitis, an inflammation of the protective membranes covering the central nervous system. The pathogenesis of N. meningitidis, with 10% fatality levels, is dependent upon the bacterium mimicking human lacto-N-neotetraose via its cell wall lipopolysaccharides (LOSs). The LOSs are synthesized in part by glycosyltransferases (GTs) encoded by three genetic loci (lgt-1, -2, and -3), in which seven ORFs (lgtA, lgtB, lgtC, lgtD, lgtE, lgtH, and lgtZ) are in a single lgt-1 locus. By studying the structures and catalytic mechanisms of these GTs, we can gain general insights into therapeutic approaches against N. meningitidis.

Lipopolysaccharide α-1,4-galactosyltransferase (LgtC) is responsible for the transfer of α-galactose from sugar donor UDP-Gal to the LOS terminal sugar acceptor lactose. LgtC belongs to GT family 8 and follows an ordered bi-bi reaction with binding of the donor preceding the acceptor. The X-ray crystal structures of LgtC with Mn²⁺ and the UDP-2-deoxy-2-fluorogalactose (UDP-2FGal) sugar donor analogue in the absence (binary complex) and presence (ternary complex) of the acceptor sugar analogue 4′-deoxylactose have been determined. The two structures are essentially identical and reveal that the monomeric enzyme consists of a large N-terminal mixed α/β domain, containing the active site, and a smaller C-terminal helical domain, which mediates membrane attachment. The full sugar acceptor binding site is proposed to be formed only upon the binding of a sugar donor in the active site and its concomitant burial by the ordering of two potentially flexible loop regions. Although these loops are thought to at least transiently adopt an open conformational state to allow donor binding and product release, there is no structural information for the substrate-free enzyme to support or refute this hypothesis.

On the basis of extensive kinetic analyses and computational calculations, LgtC appears to exploit an S_{o,b}|-like mechanism to catalyze the transfer of galactose to the LOS acceptor with
retention of its anomeric stereochemistry. This “front-side attack” mechanism would require at least localized conformational and electrostatic changes to stabilize a short-lived oxocarbenium–phosphate ion pair intermediate. Hence, the dynamic properties of LgtC and its catalytic mechanism could be highly correlated, at the level of both substrate binding and glycosyl transfer. To provide experimental evidence of this hypothesis, we have used NMR spectroscopy to investigate LgtC along its reaction pathway from the free enzyme to binary and ternary complexes with substrate analogues and finally to the UDP product complex.

NMR spectroscopy is arguably the most informative experimental method for characterizing protein dynamics. However, almost all known structural models of GTs have been obtained by X-ray crystallography. The only two NMR spectroscopically derived structures of partial GTs reported to date are the glycosyl donor binding domain of the bipartite glycosyltransferase (Alg13) and the transmembrane subunit of the oligosaccharyltransferase (OST) complex. In both cases, the catalytic domain was absent. There are several reasons why it is difficult to investigate GTs by NMR spectroscopy. These enzymes are typically large (i.e., >30 kDa and frequently oligomeric) and thus yield complex spectra with broad, overlapping signals. Furthermore, GTs are often membrane-bound and thus have limited solubility, even when their membrane-associating regions are deleted. GTs also appear to bound and thus have limited solubility, even when their oligomeric) and thus yield complex spectra with broad, overlapping signals.

As a first step toward the structure and dynamics of LgtC by NMR spectroscopy is to assign the signals from its apo form. The catalytic domain was absent. There are several reasons why it is difficult to investigate GTs by NMR spectroscopy. These enzymes are typically large (i.e., >30 kDa and frequently oligomeric) and thus yield complex spectra with broad, overlapping signals. Furthermore, GTs are often membrane-bound and thus have limited solubility, even when their membrane-associating regions are deleted. GTs also appear to bind and thus have limited solubility, even when their oligomeric) and thus yield complex spectra with broad, overlapping signals.

Protein Expression and Purification. The unlabeled His6-tagged LgtC proteins were expressed in Escherichia coli BL21(DE3) cells. The cells were grown in 2XYT broth medium at 37 °C to an OD600 of 0.8 and then induced with IPTG at a final concentration of 0.5 mM. After further growth at 16 °C for 16 h, the cells were harvested by centrifugation and lysed by sonication in the presence of 50 μg/mL lysozyme (Sigma). The cell debris was removed by centrifugation at 15,000 rpm in a Sorvall SS32 rotor, and LgtC was isolated from the supernatant using a HisTrap HP column (GE Healthcare). The His6 tag of the purified recombinant protein was then cleaved by His6-tagged TEV protease (8 μM) during a 16 h dialysis against 50 mM Tris, 0.5 mM EDTA, and 1 mM TCEP (pH 8.0) at room temperature. The plasmid encoding the TEV protease construct was provided by the Structural Genomics Consortium, and the enzyme was expressed and purified according to standard protocols. After TEV protease digestion, the HisTrap HP column was used to remove the His6 tag and uncleaved LgtC, as well as the tagged protease.

15N-labeled proteins were expressed in M9 minimal medium containing 1 g/L 15NH4Cl and purified as described above. Proteins selectively labeled with [15N]alanine, [15N]leucine, [15N]tyrosine, [15N]valine, [15N]glutamate, or [15N]aspartate were produced using auxotrophic strain BL21(DE3) ibvE, tyrB, espC, avaT, and trpB, grown in a medium containing a mixture of unlabeled amino acids along with one of the following labeled amino acids: 100 mg/L [15N]-i-alanine, 100 mg/L [15N]-i-leucine, 50 mg/L [15N]-i-tyrosine, 50 mg/L [15N]-i-valine, 500 mg/L [15N]-i-glutamate, or 200 mg/L [15N]-i-aspartate (Sigma-Aldrich).

Proteins selectively labeled with [13CH3]Ile, [13CH3,12CD3]-Leu, and [13CH3,12CD3]-Val in an otherwise deuterated background were expressed essentially according to published protocols. The salts, antibiotics, and IPTG were dissolved in 99% D2O and lyophilized to remove the exchangeable proton atoms. The cells were first grown in 2XYT/H2O broth at 37 °C to an OD600 of 0.8 and then pelleted and resuspended in standard M9/H2O medium to an OD600 of 0.2. Next, the cells were grown at 37 °C to an OD600 of 0.7 and then pelleted and resuspended in standard M9/D2O medium containing 1 g/L 15NH4Cl and 3 g/L [D2]-α-glucose in 99% D2O (Cambridge Isotope Laboratories) to an OD600 of 0.1. The D2O culture was grown until OD600 reached 0.5, diluted with the latter medium to an OD600 of 0.25, grown to an OD600 of 0.5, and again diluted to an OD600 of 0.1. After further growth to an OD600 of 0.25, 70 mg/L 2-keto-3-deoxy-4-[13C]butyrate and 120 mg/L of 2-keto-3-methyl-4,4-[13C]butyrate (that is, α-ketoisovalerate deuterated at the β-position with one of the two methyl groups being 13CH3 and the other being 12CD3) were added (Cambridge Isotope Laboratories).

Precursor 2-keto-3-deoxy-4-[13C]butyrate was prepared from the 2-keto-3-H2-4-[13C]butyrate (Cambridge Isotope Laboratories) by incubation in 50 mM Na2DPO4 (pH 10).

---

**Materials and Methods**

Cloning and Site-Directed Mutagenesis of LgtC. The previously described gene encoding LgtC, with the C128S and C174S mutations and a deletion of the C-terminal 25-residue membrane association sequence, was used as the starting point for this project. Using directed evolution, the additional T273A mutation and two other silent mutations (GTC → GGT for Val133 and CAG → CAA for Gln189) were found to help increase the level of expression of LgtC without affecting its enzymatic activity (R. Kittl, personal communication).
in 99% D₂O at 45 °C for 16 h to exchange the β-proton atom with deuterium. D₂O-exchanged IPTG (final concentration of 0.5 mM) was added when the culture OD₆₀₀ reached 0.8. The cells were then grown at 16 °C for 8 h postinduction before being harvested, and LgtC was purified as described above.

Protein selectively labeled with [¹³CH₃]Ile, [¹³CH₃,¹²CD₃]-Leu, and [¹³CH₃,¹²CD₃]Val in an otherwise deuterated and ¹³C-labeled background was expressed using the same method as described above except 3 g/L D-[D₇]-[¹³C₆]glucose and 70 mg/L methyl labeling precursors 2-keto-3-d₇-1,2,3,4-[¹³C]butyrate and 120 mg/L 2-keto-3-methyl-d₇-1,2,3,4-[¹³C]butyrate (Cambridge Isotope Laboratories) were used.

Unless stated otherwise, all purified proteins were concentrated to ~500 μM and exchanged into 20 mM Tris and 5 mM TCEP (pH 8.5) or 20 mM d₁₁-Tris and 5 mM TCEP (pH 8.5) in D₂O, using an Amicon Ultra-15 centrifugal filter device. High concentrations of MgCl₂ (final concentration of 10 mM), UDP (final concentration of 1 mM), UDP-2FGal (final concentration of 1 mM), and TCEP were included in the exchange buffer to preserve protein stability.

Figure 1. (A) Overlaid ¹⁵N-TROSY-HSQC spectra of LgtC in the absence (red) and presence (blue) of 1 mM MgCl₂ and 1.5 mM UDP-2FGal show extensive perturbations caused by substrate analogue binding. Aliased peaks are colored green. (B) Partially assigned ¹⁵N-TROSY-HSQC spectra of uniformly deuterated ¹³C- and ¹⁵N-labeled apo LgtC. The crowded central region is redrawn in the expanded, dashed box. (C) Strips from ¹⁵N planes of three-dimensional TROSY-HN(COCA)CA (i)/HNCA (ii) spectra show the backbone assignments of I59, R60, and F61 obtained by linking signals from ¹³Cα nuclei of residue i − 1 (strip i) and both residues i − 1 and i (strip ii). Similar patterns were observed for the ¹³Cα signal from complementary HN(CA)CO/HNCO spectra and the ¹³Cβ signals from HN(COA)CB/HN(CA)CB spectra (not shown). (D) The assigned amides are mapped in red on the crystal structure of the LgtC ternary complex, and the active site loops are colored green. The active site residues could not be assigned because of their absence from the ¹⁵N-TROSY-HSQC spectra.
Activity Assays. The activities of LgtC and its mutants (1 μM) in a buffer of 50 mM HEPES and 10 mM MnCl₂ (pH 7.0) were determined qualitatively by a TLC assay with fluorescent detection using UDP-Gal (1 mM) and bodipy-lactose (0.5 mM) as substrates. The TLC solvent consists of ethyl acetate, methanol, H₂O, and acetic acid in a 7:2:1:0.1 ratio. Steady-state kinetic parameters for LgtC were determined quantitatively by a continuous coupled enzyme assay in which the time-dependent formation of NAD⁺ was monitored by absorption spectroscopy at 340 nm. The assay utilized 0.1 μg/mL LgtC in a buffer consisting of 20 mM HEPES, 50 mM KCl, 15 mM MnCl₂, 0.5 mM NADH, 0.1% bovine serum albumin (BSA), 0.7% phenylenediamine (PDP), and 5 mM DTT (pH 7.5) with 2.7 μM of lactate dehydrogenase (LDH) and 2 μM of pyruvate kinase (PK) (Sigma-Aldrich). Depending on which parameter was being measured, saturated amounts of either UDP-Gal (3 mM) or lactose (160 mM) were also included, and the concentration of the other substrate was varied.

NMR Spectroscopy. NMR spectra were recorded at 25 °C on Varian Inova 600 MHz and Bruker Avance III 600 and 850 MHz spectrometers equipped with cryogenic probes. Spectra were processed using NMRpipe and analyzed with SPARKY-3. One-bond sensitivity-enhanced 15N-HSQC and 15N-TROSY-HSQC spectra were recorded for 15N-labeled LgtC and its variants. The sequence-specific backbone assignments of 2H-, 13C-, and 15N-labeled LgtC [500 μM protein, 20 mM Tris, and 5 mM TCEP (pH 8.5)] were achieved using three-dimensional 1Hβ-detected TROSY-HNCA, TROSY-HN(CO)CA, TROSY-HN(CA)CB, TROSY-HN(CA)CO, and TROSY-HN(CA)CO spectra. Methyl-TROSY experiments were recorded with [13CH₃]methyl-deuterated LgtC [500 μM protein in 99% D₂O with 20 mM d₅-Tris and 5 mM TCEP (pH 8.5)]. Partial assignments of methyl-protonated [Ile(1-13CH₃) only], Leu(13CH₃,15CD₃), and Val(13CH₃,15CD₃)] in otherwise U-1H,13C-,15N-labeled LgtC were obtained using Ile-Leu-(HM)-CM(CGCBCA)NH, Val-(HM)CM(CBCCNAH, Ile-Leu-HMCM(CGCBCA)CO, Val-HMCM(CBCCNA)CO, and HMCM(CG)CBCA experiments. In addition, a complete set of LgtC mutants with systematic isoleucine to alanine or valine substitutions were generated and selectively labeled with [13CH₃]methyl deuterium for analysis by methyl-TROSY spectroscopy.

UDP-2FGal Synthesis. Initial samples of UDP-2FGal were obtained via chemical synthesis. Subsequently, this donor analogue was generated enzymatically from chemically synthesized 2-deoxy-2-fluorogalactose by sequential use of galactokinase and galactose-1-phosphate uridyltransferase (Galk/GalT), as summarized in Figure S1 of the Supporting Information. Structural Analyses and Molecular Graphics. LgtC structural figures were rendered with PyMol. The coordinates for the complexes of LgtC with Mn²⁺-UDP-2FGal in the absence [Protein Data Bank (PDB) entry 1G9R] and presence of 4'-deoxy lactose (PDB entry 1G8A) were used, with substrates shown only as appropriate.

Optimizations of Experimental Conditions. Although an initial 15N-HSQC spectrum of LgtC was promising, only ~120 of 300 expected 1H-15N signals were detected (not shown). This behavior is both consistent with the size of the LgtC construct (32 kDa, 292 residues) and suggestive of aggregation or conformational exchange broadening. Therefore, we conducted an extensive screen of conditions to increase the solubility and stability of LgtC for long-term analyses by NMR spectroscopy. As summarized in Table S1 of the Supporting Information, LgtC was least prone to aggregation under basic conditions at pH 8.0–9.0. An elevated ionic strength and additives, such as UDP, UDP-2FGal, and lactose, did not help significantly. Although Mn²⁺ is required for the activity of LgtC and has been suggested to stabilize its substrate-bound structure, Mg²⁺ can replace this paramagnetic metal ion with an only 50% reduction in activity. Unfortunately, Mg²⁺ promotes slow (~1–2 days) precipitation of the enzyme, and we were unable to find conditions to prevent this behavior. In the end, to also maximize cryoprobe sensitivity, a simple buffer of 20 mM Tris (pH 8.5) with 5 mM TCEP reducing agent was selected as optimal for all subsequent studies. Spectra were recorded at 25 °C as a compromise between long-term stability, which was best for LgtC at lower temperatures, and signal line width, which generally becomes sharper at higher temperatures.

We also examined conditions for exchanging the backbone amide deuteration atoms of LgtC produced in D₂O medium to proton atoms for 1H detection. Despite exhaustive trials with several different methods, including thermal unfolding and refolding on-column refolding of His₆-tag-immobilized LgtC, and dilution of GdnHCl-denatured LgtC into 96 different conditions (Table S2 and Figure S2 of the Supporting Information, modified from ref 32), active LgtC could not be recovered in any practical yield. Fortunately, because LgtC is stable and soluble under alkaline conditions, we found that the amides of the folded protein could be reprotinated simply by incubation in H₂O buffer at pH 8.5 and 25 °C for 1–2 days (Figure S3 of the Supporting Information).

15N-TROSY-HSQC Spectra and Assignments. The 15N-TROSY-HSQC spectrum of uniformly deuterated 15N-labeled apo LgtC is shown in Figure 1B. Although improved greatly relative to the conventional 15N-HSQC spectrum of the nondeuterated protein recorded at pH 7.5, the spectrum exhibited only ~210 of ~300 expected peaks. Upon formation of a binary complex with Mg²⁺ and UDP-2FGal, many amides showed chemical shift perturbations, indicative of a change in the structure and dynamics of LgtC (Figure 1A). Importantly, over the course of a titration experiment, only signals from the free or bound LgtC were detected. Thus, the binding of this donor analogue occurs in the slow-exchange regime on the chemical shift time scale (i.e., kex < Δω, where Δω is the chemical shift difference between the free and bound states).

TROSY-based 1H, 13C, 15N correlation experiments (Figure 1C) were undertaken to help assign as well as possible the amide 15N and 1Hα signals of LgtC. Along with selective 15N amino acid labeling (Figure S4 of the Supporting Information) and site-directed mutagenesis (Figure S5 of the Supporting Information), 146 of ~210 observable peaks in the 15N-TROSY-HSQC spectrum of apo LgtC were assigned confidently (Table S3 of the Supporting Information). Unfortunately, this corresponds to only ~50% of the total number of residues in LgtC. Furthermore, when they are mapped onto the structure of...
the protein (Figure 1D), it is apparent that the assigned amides are distal from the active site, whereas those within the active site region of LgtC remained essentially unassigned. It is unlikely that this is simply a result of spectral overlap, incomplete reprotonation, or rapid amide hydrogen exchange at pH 8.5, as none of these factors should be unusually pronounced for this particular region of the protein. Instead, the lack of assignments likely reflects line broadening caused by millisecond to microsecond time scale conformational dynamics of the active site of LgtC. Indeed, we speculate that such backbone dynamics are linked to catalysis and are currently investigating this hypothesis using NMR relaxation experiments.

Parenthetically, of the assigned amides, the 15N signals of Ile40, Arg53, Leu74, and Leu112 are unusually downfield-shifted, whereas that of Asp2 is unusually upfield-shifted (Figure 1B). On the basis of the X-ray crystal structure of substrate analogue-bound LgtC, the amide protons of Ile40 and Arg53 are hydrogen bonded with the carboxylate oxygens of the side chains of Asp37 and Gln23, respectively. This could lead to their downfield signals. In contrast, the amide protons of Asp2 and Leu112 are close to the indole ring of Trp116 and imidazole ring of His26, respectively, and thus are likely perturbed by ring current effects. Interestingly, the amide proton of Leu74, in one of the active site loops, appears to be solvent-exposed and is neither involved in an intramolecular hydrogen bond nor adjacent to an aromatic ring. Thus, the origin of its unusually downfield-shifted signal is unclear and suggests that the conformation of Leu74 might differ in apo LgtC versus that determined for the LgtC complexes by X-ray crystallography.

**Methyl-TROSY Spectra Reveal Multiple Conformations.** Because the amide signals of active site residues could not be identified in 15N-TROSY-HSQC spectra, we turned our attention to the methyl groups of LgtC. Figure 2A shows the methyl-TROSY spectrum of LgtC selectively labeled with [13CH3]Ile, [13CH3,12CD3]Leu, and [13CH3,12CD3]Val in an otherwise deuterated background. The signals are generally well dispersed, especially in the upfield 13C region corresponding to the isoleucine residues. We expected to observe 15 peaks from the 15 isoleucine δ1-methyls and 86 peaks from the 24 leucine and 19 valine methyls (Figure 2B). Somewhat surprisingly, approximately 21 and 95 peaks could be counted in the isoleucine and leucine/valine regions, respectively, of the methyl-TROSY spectrum of the apoenzyme. Most of these peaks were of comparable intensity, yet a subset were weaker. Furthermore, as demonstrated below, the relative intensities of these peaks change with mutation and with substrate–product binding and thus arise from multiple conformations of LgtC that are in slow exchange on the chemical shift time scale.

**Methyl-TROSY Assignments by Scalar Correlations.** Using a suite of methyl-directed multidimensional 1H, 15N, and 13C scalar coupling experiments,60 the signals from nine isoleucine, five leucine, and seven valine residues in apo LgtC were assigned (Figure 2A and Figure S6 of the Supporting Information). Hence, their structures should be similar to that of the wild-type enzyme. Not surprisingly, mutation of Ile79, which directly contacts the bound sugar donor, had the greatest detrimental effect on catalysis. Ideally, when this spectrum is compared to the spectrum of wild-type LgtC, one peak should be absent because of an isoleucine

![Figure 2](image-url)

**Figure 2.** (A) Methyl-TROSY spectrum of apo LgtC. Partial assignments were obtained using magnetization-transfer experiments as summarized in Figure S6 of the Supporting Information. (B) Cartoon of the LgtC ternary complex with the isoleucine (red) and leucine/valine (cyan) residues identified by spheres. Selected residues are labeled; the proposed active site flexible loops are colored green, and UDP-2FGal and 4′-deoxylactose are shown as sticks (carbon, gray; oxygen, red; nitrogen, blue; phosphorus, orange).
Table 1. Steady-State Kinetic Parameters for the IA and VA Mutants of LgtC

<table>
<thead>
<tr>
<th></th>
<th>UDP-2FGal$^a$</th>
<th>lactose$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>kcat (μM)</td>
<td>kcat (μM)</td>
<td>kcat (μM)</td>
</tr>
<tr>
<td>WT LgtC</td>
<td>29</td>
<td>16</td>
</tr>
<tr>
<td>LgtC-I76A</td>
<td>35</td>
<td>5</td>
</tr>
<tr>
<td>LgtC-I79A</td>
<td>19</td>
<td>1</td>
</tr>
<tr>
<td>LgtC-I104A</td>
<td>18</td>
<td>3</td>
</tr>
<tr>
<td>LgtC-V106A</td>
<td>32</td>
<td>26</td>
</tr>
</tbody>
</table>

$^a$Data for sugar donor UDP-Gal in the presence of 160 mM acceptor lactose at pH 7.5 and 25 °C. $^b$Data for sugar acceptor lactose in the presence of 10 mM donor UDP-Gal at pH 7.5 and 25 °C.

Mutation and two because of a valine mutation. However, multiple spectral changes were observed upon mutation with both the apoenzymes and, to a lesser extent, their UDP-2FGal-Mg$^{2+}$ binary complexes (Figures S8 and S9 of the Supporting Information). This made it difficult to confidently identify which signal was indeed absent and which were shifted due to conformational perturbations resulting from the mutation. In the end, only the two methyl signals from Val133 in apo LgtC, identified previously from scalar correlation spectra, could be confirmed. Because of improved spectral dispersion with UDP-2FGal-Mg$^{2+}$ bound, the signals from Ile76, Ile81, Ile104, and Val133 in the binary complex could be assigned on the basis of alanine mutagenesis (Figure S9 of the Supporting Information).

**Methyl-TROSY Assignments of Apo LgtC via Isoleucine to Valine Mutagenesis.** Recently, Amero et al. successfully assigned signals from the Ala and Ile $\delta_1$-methyl groups of the 468 kDa multimeric aminopeptidase PhTET2 by generating a complete set of single mutants with every isoleucine and alanine in the protein changed to leucine and valine, respectively. By considering the patterns of methyl-TROSY spectral perturbations for the entire set, these researchers were able to confidently distinguish absent versus shifted peaks. Inspired by this approach, we generated a set of LgtC mutants in which each isoleucine has been individually substituted with a valine. Note that valine was chosen, rather than alanine as we used initially or leucine as used previously, because of improved spectral dispersion with UDP-2FGal-Mg$^{2+}$ bound, the signals from Ile76, Ile81, Ile104, and Val133 in the binary complex could be assigned on the basis of alanine mutagenesis (Figure S9 of the Supporting Information).

**Methyl-TROSY Assignments of Apo LgtC via Isoleucine to Valine Mutagenesis.** The signals from the isoleucine $\delta_1$-methyl groups in the LgtC binary complex were also assigned by comparing the methyl-TROSY spectra of the 13 IV mutants saturated with 10 mM Mg$^{2+}$ and 1 mM UDP-2FGal (Figure 4). As with the apoenzyme samples, the IV mutations generally caused spectral perturbations, some of which can be rationalized by the proximity of interacting residues in the crystal structure of the enzyme. For example, substitution of Ile81 influences the chemical shifts of Ile76 and Ile79 in the adjacent active site loop (Figure 3O). Similarly, the $\delta_1$-methyls of Ile62 and Ile93 are in van der Waals contact (Figure 3P), and indeed, the chemical shift of one changes upon the mutation of the other, albeit by different amounts. However, Ile159 is also near Ile93 and within the same $\beta$-strand as Ile62, yet neither pair appears to be coupled. More perplexing are the nonreciprocal changes in the signals of Ile31 and Ile104 caused by the mutation of the distal Ile129 (Figure 3J). This reflects the difficulty in rationalizing chemical shift perturbations on the basis of simple expectations from a static crystal structure. Furthermore, the available structures are of the LgtC binary and ternary complexes, which may differ from that of apo LgtC.

In the completely assigned methyl-TROSY spectrum of apo LgtC, Ile3, Ile31, Ile76, Ile104, Ile129, and Ile143 each yielded two peaks, with the weaker denoted as “a” and the more intense as “b” (Figure 3N). The ratios of the “a” to “b” peak intensities varied between residues, which could result from differential relaxation or from sensitivity to multiple conformational states of LgtC with differing populations. Furthermore, the relative intensities of the “a” versus “b” peaks from each of these isoleucines generally increased with the various IV mutants relative to the wild-type enzyme. This observation, which suggests that the mutants favor the conformations leading to the “a” peaks, will be discussed further below.

**Methyl-TROSY Assignments of the UDP-2FGal-Mg$^{2+}$ Binary Complex via Isoleucine to Valine Mutagenesis.** The signals from the isoleucine $\delta_1$-methyl groups in the LgtC binary complex were also assigned by comparing the methyl-TROSY spectra of the 13 IV mutants saturated with 10 mM Mg$^{2+}$ and 1 mM UDP-2FGal (Figure 4). As with the apoenzyme samples, the IV mutations generally caused spectral perturbations, some of which can be rationalized on the basis of the known structure of the complex. Fortunately, the spectra of the binary complexes of the IV (and initial “IA”) mutants showed better peak dispersion than those of the corresponding apo samples, making it easier to discriminate between absent and shifted signals. This was particularly true for the isoleucines closest to the active site because these residues are most influenced by substrate analogue binding.

A comparison of the spectra of LgtC in its apo versus binary complex forms is provided in Figures 5 and 6. When UDP-2FGal-Mg$^{2+}$ binds, the $\delta_1$-methyl signals of Ile76, Ile79, Ile81, Ile104, and Ile191 were the most substantially perturbed. As expected, these isoleucines generally clustered near the donor binding site and thus may experience chemical shift changes because of conformational changes because of the sugar donor binding and/or because of electrostatic and ring current effects
from the metal ion and sugar donor. In the cases of Ile81 and Ile191, which are more distal from the active site, the chemical shift perturbations upon substrate binding might result from structural changes of adjacent aromatic residues and hence altered ring current effects. It is also noteworthy that the introduction of mutations I40V and I79V both altered the chemical shift of Ile104 and caused visible line broadening (Figure 4B,F). Mutation of Ile79 to a valine may alter the interaction of the donor analogue with Ile104, both structurally and dynamically, thereby yielding the observed spectral changes. In contrast, Ile40 is approximately 8 Å from Ile104 yet more distant from the active site of LgtC (Figure 4O). Mutation of this residue might alter packing within the hydrophobic core of the enzyme and thus indirectly perturb the interaction of Ile104 with the bound UDP-2FGal. In the spectrum of LgtC-I104V, the signals of Ile40 and Ile79 are not significantly changed (Figure 4I). However, chemical shifts depend upon the environment of each residue within the protein, and there is no reason to expect reciprocal spectral perturbations because of the mutations of these three isoleucine residues.

Figure 3. Assignment of the isoleucine $\delta_1$-methyl signals of apo LgtC. Methyl-TROSY spectra of uniformly deuterated and selectively $[^1H,^{13}C]$methyl-labeled apo (A) LgtC-I31V, (B) LgtC-I40V, (C) LgtC-I59V, (D) LgtC-I62V, (E) LgtC-I76V, (F) LgtC-I79V, (G) LgtC-I81V, (H) LgtC-I93V, (I) LgtC-I104V, (J) LgtC-I1129V, (K) LgtC-I1143V, (L) LgtC-I1169V, and (M) LgtC-I191V (green) overlaid with that of wild-type LgtC (red). Assigned peaks from the mutated residues are labeled in black. The peaks that are most significantly shifted in the mutant spectra are labeled in purple, whereas those with changes in the relative intensities of their "a" and "b" peaks are labeled in cyan. (N) Assigned methyl-TROSY spectrum of apo wild-type LgtC. (O and P) Expanded regions of LgtC showing isoleucines (cyan; C$\delta_1$-methyl, red), as discussed in the text.
When UDP-2FGal·Mg^{2+} binds, the magnitudes of the δ_{1}-methyl signals from the isoleucine “a” peaks decreased in relative intensity, whereas those from the “b” peaks both increased in intensity and shifted in spectral position (Figures 5 and 6). This suggested that the sugar donor analogue bound only to the form of LgtC giving rise to the “b” peaks, and that the “a” peaks remained detectable because of incomplete saturation with the donor analogue or perhaps a small population of inactive enzyme with an apo-like spectrum. However, the methyl signals of Ile93, Ile159, and Ile169 also split slightly, indicating possible conformational heterogeneity in the binary complex. Furthermore, Ile79 in the active site loop yielded three peaks with distinct chemical shifts, suggesting possible multiple conformers of LgtC.

Figure 4. Assignment of the isoleucine δ_{1}-methyl signals in the UDP-2FGal·Mg^{2+} binary complex of LgtC. Methyl-TROSY spectra of the uniformly deuterated and selectively [¹H,¹³C]methyl-labeled LgtC IV mutants (A) LgtC-I31V, (B) LgtC-I40V, (C) LgtC-I59V, (D) LgtC-I62V, (E) LgtC-I76V, (F) LgtC-I79V, (G) LgtC-I81V, (H) LgtC-I93V, (I) LgtC-I104V, (J) LgtC-I129V, (K) LgtC-I143V, (L) LgtC-I169V, and (M) LgtC-I191V (green) overlaid with that of wild-type LgtC (blue). All samples were saturated with 10 mM Mg^{2+} and 1 mM UDP-2FGal. Assigned peaks from the mutated residues are labeled in black. The peaks that are most significantly shifted in the mutant spectra are labeled in purple, whereas those with changes in the relative intensities of their “a” and “b” peaks are labeled in cyan. The “c” peak of Ile79 represents a possible third state of LgtC. (N) Overall assigned methyl-TROSY spectrum of the isoleucine residues of wild-type LgtC binary complex. (O) Crystal structure of the LgtC binary complex showing the active site isoleucine residues (cyan; Cδ_{1}-methyl, red) and UDP-2FGal (carbon, gray; oxygen, red; nitrogen, blue; phosphorus, orange; Mn^{2+}, magenta).
relative intensities that changed upon mutation (Figures 4 and 5). Thus, LgtC exhibits multistate conformational behavior.

**Methyl-TROSY Assignments of the UDP-2FGal-Mg<sup>2+</sup>-Lactose Ternary Complex.** The addition of lactose to the preformed complex of LgtC with UDP-2FGal·Mg<sup>2+</sup> led to only modest methyl-TROSY spectral changes. This allowed us to readily extend the assignments of the isoleucine δ₁-methyl signals from the binary to the resulting ternary complex by monitoring these changes as a function of added lactose (Figure 5). Most significantly, the signals from Ile<sub>76</sub> and Ile<sub>104</sub> were no longer observed, possibly because of conformational exchange broadening. Otherwise, only minor chemical shift perturbations were observed for Ile<sub>81</sub> and Ile<sub>191</sub> (Figure 6). The smaller spectral effects of lactose binding to form the ternary complex relative to UDP-2FGal·Mg<sup>2+</sup> binding to form the binary complex are consistent with the nearly identical crystal structures of the two complexes, as well as the smaller interface of LgtC with the sugar acceptor, and its neutral, non-aromatic character.

**Methyl-TROSY Assignments of the UDP-Mg<sup>2+</sup> Product Complex.** The addition of UDP-Mg<sup>2+</sup> to apo LgtC also resulted in modest spectral changes. This was also straightforward to assign the isoleucine δ₁-methyl signals in the product complex by direct spectral comparisons (Figure 5). The smaller chemical shift perturbations resulting from UDP-Mg<sup>2+</sup> binding relative to the larger effects of UDP-2FGal-Mg<sup>2+</sup> suggest that the product complex is more similar structurally to apo LgtC than to the binary substrate complex (Figure 6).

**The Sugar Donor Requires Mg<sup>2+</sup> for Binding to LgtC.** On the basis of crystallographic studies and molecular dynamics simulations, it was suggested that LgtC binds Mn<sup>2+</sup> prior to the sugar donor UDP-Gal. However, there was no clear experimental test of this binding order. Therefore, NMR spectroscopy was used to monitor the titration of LgtC with Mn<sup>2+</sup> and the donor analogue UDP-2FGal (Figure 7). The addition of either 10 mM Mn<sup>2+</sup> or 1 mM UDP-2FGal independently did not perturb the methyl-TROSY spectrum of methyl-labeled LgtC or the ¹⁵N-TROSY-HSQC spectrum of [¹⁵N]tyrosine-labeled LgtC. In contrast, when both Mn<sup>2+</sup> and UDP-2FGal were present, signals from several methyls and tyrosine amides were significantly shifted. Therefore, within these concentration ranges, it is clear that UDP-2FGal binds LgtC only when Mn<sup>2+</sup> (and, by inference, Mn<sup>2+</sup>) is present. It is also likely that Mn<sup>2+</sup> binds LgtC cooperatively with the sugar donor, rather than independently. However, the latter conclusion is less certain as it is based on the assumption the binding of Mn<sup>2+</sup> would have at least altered the chemical shifts of Leu<sub>102</sub>, Ile<sub>104</sub>, Val<sub>156</sub>, and Tyr<sub>245</sub>, which are reporter groups in the proximity of the crystallographically defined catalytic metal binding site of LgtC. Unfortunately, the methyl signals of Leu<sub>102</sub> and Val<sub>156</sub> were not assigned, and the amide signal of Tyr<sub>245</sub> was not detected. Thus, the insensitivity of the Ile<sub>104</sub> methyl signal to the presence of Mn<sup>2+</sup> provides the main evidence of the simultaneous binding of UDP-2FGal·Mg<sup>2+</sup> to LgtC.

**Multiple Conformations Probed by Mn<sup>2+</sup> Binding.** The binding of Mn<sup>2+</sup> to the multiple conformational states of LgtC was investigated by monitoring the loss of isoleucine δ₁-methyl signal intensity in methyl-TROSY spectra because of paramagnetic relaxation. To avoid complications due to the spectral changes accompanying substrate binding, a preformed LgtC binary complex saturated with Mn<sup>2+</sup> and UDP-2FGal was titrated with Mn<sup>2+</sup>. Because the <i>Kₘ</i> value of Mn<sup>2+</sup> (27 μM) is 13-fold lower...
lower than that of Mg\(^{2+}\) (370 \(\mu\)M),\(^{21}\) we assumed that Mn\(^{2+}\) will displace Mg\(^{2+}\) and alter the intensities, but not chemical shifts, of nearby isoleucine methyl groups. As summarized in Figure 8, this indeed occurred. Qualitatively, the signals from residues closest to the metal binding sites, including Ile76, Ile79, and Ile104, disappeared at lower Mn\(^{2+}\) concentrations than those from more distal residues. However, a clear correlation of intensity loss with distance was not observed, possibly because of nonspecific effects from the relatively high concentrations of Mn\(^{2+}\) in the bulk solvent and differential nonparamagnetic relaxation rates of the methyl groups. Most strikingly, in the presence of 400 \(\mu\)M Mn\(^{2+}\), only signals from “a” peaks were observed in methyl-TROSY spectra, albeit at a reduced intensity. This result also indicates that UDP-2FGal-Mn\(^{2+}\) binds only to the form of the protein giving rise to the “b” peaks, and that the residual “a” peaks originate from a population of protein not saturated with the sugar donor analogue.

**DISCUSSION**

**Spectral Assignments.** Using a combination of mutagenesis, isotope labeling strategies, and TROSY-based pulse sequences, we have made substantial progress toward assigning the NMR spectra of LgtC along its reaction pathway. Initially, we focused on its amide \(^{15}\)N-TROSY-HSQC spectrum as this can provide a probe of each non-proline residue in the backbone of a protein. Although the assignments were facilitated by deuteration and amino acid selective \(^{15}\)N labeling, we were able to detect only \(\sim 70\%\) and assign only \(\sim 50\%\) of the expected \(^{1}H\)-\(^{15}\)N signals from apo LgtC with TROSY-based \(^{1}H\), \(^{13}\)C, \(^{15}\)N correlation experiments. Unfortunately, these assigned signals corresponded to amides that are remote from the active site. A similar result occurred in a related study of *Campylobacter jejuni* sialyltransferase CstII\(^{38}\) and is suggestive of active site backbone dynamics in glycosyltransferases on a millisecond to microsecond time scale that led to conformational exchange broadening. However, such motions were not substantially dampened in either protein upon addition of a sugar donor
analogue, which caused extensive chemical shift changes but not the appearance of the numerous missing amide signals. In contrast to amide-directed approaches, the methyl-TROSY spectra of selectively 13CH3-labeled LgtC in an otherwise deuterated background yielded excellent quality spectra. Because of the limited main chain assignments, combined with the aggregation of LgtC in the presence of Mg2+, only a subset of these methyl signals could be assigned using multidimensional 1H, 13C, 15N correlation experiments. Accordingly, a mutational approach was required. However, initial attempts to assign selected active site residues by alanine substitutions were hampered by chemical shift perturbations, which precluded the unambiguous identification of the one absent peak in the methyl-TROSY spectrum of each mutant. Thus, a more laborious approach of mutating each isoleucine to a valine was undertaken. Via comparison of the rapidly measured spectra from the entire set of mutants, it was possible to fully assign all of the isoleucine δ1-methyl signals of LgtC in its apo form, as well as with substrate analogues and product UDP bound (Figure 5). As expected, the complicating spectral perturbations resulting from the valine mutations were generally smaller than those observed with the initial alanine substitutions. In some cases, the spectral changes could be rationalized, whereas in others, an obvious explanation was not readily apparent on the basis of simple expectations from the available static crystal structures. This suggests the latter may not completely reflect the solution conformation of LgtC at all locations and that mutations may have subtle, long-range effects on its structure and dynamics.

Kinetic Effects Caused by Single-Site Mutations. The effects of mutations on the activity of LgtC were examined either through quantitative kinetic measurements or via a qualitative fluorescence-based TLC assay. On the basis of a detailed kinetic analysis conducted with the initial set of alanine mutants (Table 1), substitution of Ile79 had the greatest detrimental effect on catalysis, reducing $k_{cat}/K_m$ for UDP-Gal and lactose to ~5% of this wild-type level. This is likely due to the direct contact of its side chain with UDP-Gal. More modest effects were observed upon mutation of Ile76 and Ile104, both of which also contact the substrate. In contrast, mutation of Val106 to alanine increased $k_{cat}/K_m$ toward lactose by ~5-fold for reasons that are not immediately obvious as its side chain is >7 Å from any substrate atom. Furthermore, from qualitative assays, the substitution of Ile40 and, more so, Ile31 impaired LgtC activity (Figure S7 of the Supporting Information). Both are quite remote from the active site of LgtC and appear to exert long-range effects on the structure or dynamics of the enzyme. However, such effects were not manifest via any unusual methyl-TROSY spectral perturbations, except for a pronounced change in the chemical shift of the active site Ile104 in the UDP-2F Gal·Mg2+ complex of the LgtC-I40V mutant.

Donor Substrate Binding. Using both methyl-TROSY and 15N-TROSY-HSQC measurements, we demonstrated unambiguously that UDP-2F Gal binds LgtC only in the presence of Mg2+. This is consistent with both the mandatory requirement of the enzymatic activity of LgtC for a divalent metal ion and the crystallographic structures of its complexes in which a Mn2+ bridges two phosphate oxygens of the UDP moiety with the side chains of Asp103, Asp105, and His244. These two aspartates form the conserved DxD motif that is found in a wide range of glycosyltransferases. In the absence of a metal ion, an unfavorable electrostatic interaction between this motif and the negatively charged sugar donor would certainly arise. The lack of spectral perturbations upon addition of Mg2+ to apo LgtC also suggests that the sugar donor and the metal ion bind the enzyme

Figure 7. UDP-2F Gal binding by LgtC requires Mg2+. (A, C, and D) Overlaid methyl-TROSY and 15N-TROSY-HSQC spectra of LgtC in the absence (red) and presence of 10 mM Mg2+ (green) or 1 mM UDP-2F Gal (yellow) show no significant differences. Thus, UDP-2F Gal alone does not bind LgtC. Assuming that metal binding should cause some detectable spectral perturbations, the same conclusion likely holds for Mg2+. (B and E) The clear differences between the overlaid methyl-TROSY and 15N-TROSY-HSQC spectra of LgtC in the absence (red) and presence (blue) of 10 mM Mg2+ and 1 mM UDP-2F Gal confirm that the sugar donor binds as a metal complex.
cooperatively as a UDP-2FGal·Mg\(^{2+}\) complex. Indeed, it is well-established that Mg\(^{2+}\) interacts directly with nucleotides,\(^{39,40}\) and previous kinetic measurements showed that LgtC has similar \(K_m\) values for UDP-Gal and Mn\(^{2+}\).\(^{4,21}\) However, it is possible that the chemical shifts of the methyl and tyrosine amide reporter groups are insensitive to the presence of bound Mg\(^{2+}\). If so, then the metal ion could bind LgtC either independently before or simultaneously with UDP-Gal. Regardless of pathway, by thermodynamic linkage, the affinities of both the metal and sugar donor for the enzyme will be enhanced in the presence of each other.

NMR-monitored titration experiments also demonstrated that UDP-2FGal·Mg\(^{2+}\) binds LgtC in the slow-exchange limit such that distinct signals from the free and bound protein were observed. This indicates that the exchange rate \(k_{ex} = k_{on}[UDP-2FGal\cdot Mg^{2+}] + k_{off}\) is smaller than the chemical shift difference for both amides and methyl groups in the apo versus binary complex states of LgtC. Extrapolating from previously reported kinetic data [metal ions Mg\(^{2+}\) \((K_m = 370 \mu M\) with UDP-Gal) and Mn\(^{2+}\) \((K_m = 27 \mu M\) with UDP-Gal); sugar donors UDP-Gal \((K_m = 30 \mu M\) with Mn\(^{2+}\)) and UDP-2FGal \((K_i = 2 \mu M\) with Mn\(^{2+}\))],\(^{4,21}\) we think it is likely that UDP-2FGal·Mg\(^{2+}\) binds LgtC with a \(K_d\) value on the order of \(\sim 25 \mu M\). Given that this modest affinity usually results in binding in the fast- to intermediate-exchange limit,\(^{10}\) it appears that both the association \((k_{on})\) and dissociation \((k_{off})\) rate constants for the sugar donor analogue with LgtC are slower than those typically found for simple protein–ligand complexes with comparable \(K_d\) values. On the basis of the X-ray crystal structure of the LgtC binary and ternary complexes, a plausible explanation for this result is that the two loops flanking the active site loops must undergo a conforma-

Figure 8. Analysis of Mn\(^{2+}\) binding by LgtC. (A) Methyl-TROSY spectrum of the LgtC binary complex (200 \(\mu M\) protein) in the presence of 10 mM MgCl\(_2\) and 1 mM UDP-2FGal. The complex was titrated with (B) 25, (C) 50, (D) 100, (E) 200, and (F) 400 \(\mu M\) MnCl\(_2\). (G) Histogram showing the relative intensity change of each isoleucine \(\delta_1\)-methyl group with respect to increasing concentrations of Mn\(^{2+}\) due to paramagnetic relaxation.
tional change to allow access of the UDP-2FGal-Mg\(^{2+}\) to an otherwise buried position within the enzyme’s active site.

**Acceptor Lactose Binding.** Relatively small spectral changes occurred when lactose was added to the binary complex of LgtC with UDP-2FGal-Mg\(^{2+}\), indicating that acceptor binding does not substantially perturb the structure of the enzyme. This is consistent with the highly similar crystal structures of its binary and ternary substrate complexes. Surprisingly, however, in methyl-TROSY-monitored titrations, the “b” δ-δ-methyl signals of the three isoleucines (Ile76, Ile79, and Ile104) that contact the bound UDP-2FGal all disappeared upon the addition of lactose. Such exchange broadening (i.e., interconversion between states with different chemical shifts at a rate \(k_{\text{ex}} \sim \Delta \omega\)) is unexpected given the very low affinity of lactose for the LgtC binary complex \((K_a = 13 \text{ mM})^4\) and hence predicted fast-exchange (\(k_{\text{ex}} > \Delta \omega\)) behavior. Possible explanations for this result are that the exchange rate for lactose is slowed to the millisecond to microsecond time scale typically associated with exchange broadening because of a requisite structural or dynamic change for binding or that, once bound, lactose induces conformational motions within the ternary complex on this time scale.

**Product UDP Binding.** As typically observed with glycosyltransferases, LgtC is subject to product inhibition by UDP \((K_i = 80 \text{ \mu M})^4\). When apo LgtC was titrated with UDP-Mg\(^{2+}\), only minor isoleucine δ-δ-methyl chemical shift perturbations were observed in the slow-exchange regime, with the largest effect seen for Ile104 and Ile191. It is noteworthy that these chemical shift perturbations were substantially smaller than those accompanying UDP-2FGal-Mg\(^{2+}\) binding, despite the fact that several isoleucines are expected to contact the UDP moiety in both cases. Furthermore, the signals of Ile76 and Ile79, which are within one of the active site loops, disappeared upon UDP-Mg\(^{2+}\) binding. This is suggestive of conformational exchange broadening. To exclude alternative possibilities, such as aggregation, UDP was converted “in situ” to UTP by the addition of pyruvate kinase and phosphoenolpyruvate (Figure S10 of the Supporting Information). Indeed, after this treatment, the methyl-TROSY spectrum of the LgtC sample returned to that observed with the apoenzyme as UTP no longer binds. Although a crystal structure of LgtC with UDP-Mg\(^{2+}\) has not been determined, these results indicate that conformational and dynamic differences exist between its substrate and product complexes.

**Multiple Conformational States of LgtC.** More peaks than expected were observed in the methyl-TROSY spectra of apo LgtC and its substrate and product complexes. This suggested two general hypotheses. First, the sample used in the NMR experiments could be heterogeneous, perhaps containing chemically modified forms of LgtC (e.g., oxidized or proteolyzed) or other unrelated *E. coli* contaminants. However, the fact that mutations and substrate or product binding alter the relative intensities of the “a” and “b” peaks argues against any predominant irreversible chemical modification. Nevertheless, although the LgtC samples appeared to be >90% pure via sodium dodecyl sulfate—polyacrylamide gel electrophoresis, a signal of ~10% relative intensity was observed in a MALDI-TOF mass spectrum with a mass ~130 Da greater than that measured for apo LgtC (Figure S11 of the Supporting Information). We speculated initially that this could be due to a galactose or glucose covalently linked with Asp190 in the active site during protein expression because a similar phenomenon had previously been observed with the Q189E mutant of LgtC.\(^7\) However, despite many efforts to cleave the proposed saccharide from the active site of LgtC by the addition of UDP, Mn\(^{2+}\), and lactose to complete the glycosyl-transfer reaction, the higher-molecular mass species remained in the sample. This minor species is thus assumed to be an unknown modification of the enzyme.

An alternative and more consistent hypothesis is that apo LgtC exists in at least two conformational states that interconvert slowly enough to yield distinct NMR signals, denoted as “a” and “b” peaks in a methyl-TROSY spectrum. Indeed, preliminary measurements have detected magnetization transfer between the corresponding “a” and “b” peaks of several methyl groups in LgtC with rate constants on the order of ~0.2 s\(^{-1}\).\(^1\) These states are in equilibrium when no substrate is present, and their relative populations vary with mutation. When the enzyme was titrated with UDP-2FGal-Mg\(^{2+}\) or UDP-Mg\(^{2+}\), the intensities of the “a” peaks diminished, whereas the intensities of the “b” peaks both grew in intensity and, in several cases, changed in chemical shift. Furthermore, the “b,” but not “a,” peaks disappeared upon replacement of Mg\(^{2+}\) with paramagnetic Mn\(^{2+}\). Collectively, these data suggest that LgtC exists in at least two interconverting conformations, of which only the form giving rise to “b” peaks is competent for substrate and product binding.

Although the differences between the putative active and inactive conformations of LgtC are currently unknown, one plausible albeit purely speculative explanation is a slow cis—trans isomerization of one of the two proline residues in an active site loop. It is likely that this loop transiently opens to allow binding and release of UDP-2FGal or UDP from an otherwise buried position in the active site of LgtC. However, the splitting of the signals from Ile159 and Ile169 and the appearance of peak “c” for Ile79 in the UDP-2FGal-Mg\(^{2+}\) binary complex, as well as the fact that different residues in apo LgtC yield different “a” versus “b” methyl-TROSY peak intensity ratios, indicate that such a simple two-state model is inadequate. Furthermore, although reduced in intensity, “a” peaks are still observed when LgtC is titrated with what should be saturating amounts of substrate analogues or UDP product, as well as excess paramagnetic Mn\(^{2+}\). This suggests that a fraction of the “a”-state population cannot interconvert to the “b” state and bind these species, possibly because of the unknown modification detected by mass spectrometry.

Collectively, these studies reveal that LgtC exhibits complex conformational and motional properties that extend beyond what has been observed in the static crystal structures of its binary and ternary substrate complexes. With spectral assignments in hand, we are currently using a range of NMR techniques to examine the behavior of LgtC along its reaction pathway and thereby gain further insights into the structural and dynamic mechanisms underlying glycosyl transfer.

### ASSOCIATED CONTENT

#### Supporting Information

Four tables of screening conditions and spectral assignments and 11 figures of activity assays, NMR spectra, and a mass spectrum. This material is available free of charge via the Internet at http://pubs.acs.org.

#### AUTHOR INFORMATION

**Corresponding Author**

*Department of Biochemistry and Molecular Biology, Life Sciences Centre, University of British Columbia, Vancouver, BC, Canada V6T 1Z3. E-mail: mcintosh@chem.ubc.ca. Phone: (604) 822-3341. Fax: (604) 822-5227.

8290 dx.doi.org/10.1021/bi3010279 Biochemistry 2012, 51, 8278—8292
Biochemistry

Funding
This research was supported by grants from the Natural Sciences and Engineering Research Council of Canada (L.P.M. and S.G.W.) and the Canadian Institutes for Health Research (S.G.W.). S.G.W. is the recipient of a Canada Research Chair in Chemical Biology. Instrument support was provided by the Canadian Institutes for Health Research, the Canada Foundation for Innovation, the British Columbia Knowledge Development Fund, the University of British Columbia Blusson Fund, and the Michael Smith Foundation for Health Research.

Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS
We thank Roman Kitit for providing the LgtC (C128S, C174S, and T273A) construct.

ABBREVIATIONS
GT, glycosyltransferase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HSQC, heteronuclear single-quantum correlation; IPTG, isopropyl β-D-thiogalactopyranoside; kcat catalytic rate constant; kcat/Km second-order rate constant; Ks equilibrium dissociation constant; Kd inhibitor equilibrium dissociation constant; Km Michaelis constant; LgtC, lipopolysaccharide α-1,4-galactosyltransferase C; LOS, lipooligosaccharide; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; NMR, nuclear magnetic resonance; pH*, pH meter reading without correction for isotope effects; TLC, thin layer chromatography; TROSY, transverse relaxation-optimized spectroscopy; UDP, uridine-5′-diphosphate; UDP-2FGal, uridine-5′-diphosphate 2-deoxy-2-fluorogalactose; UDP-Gal, uridine-5′-diphosphate galactose.

REFERENCES
(32) Vincentelli, R., Canaan, S., Campanacci, V., Valencia, C., Maurin, D., Frassinetti, F., Scappucci-Calvo, L., Bourne, Y., Cambillau, C., and...


