

# Glycosyltransferase Mechanisms: Impact of a 5-Fluoro Substituent in Acceptor and Donor Substrates on Catalysis<sup>†</sup>

Matthew C. T. Hartman,<sup>‡,§</sup> Songmin Jiang,<sup>||</sup> Jeffrey S. Rush,<sup>||</sup> Charles J. Waechter,<sup>||</sup> and James K. Coward<sup>\*,‡</sup>

Departments of Chemistry and Medicinal Chemistry, University of Michigan, Ann Arbor, Michigan 48109-1055, and  
Department of Molecular & Cellular Biochemistry, University of Kentucky College of Medicine,  
Lexington, Kentucky 40536-0001

Received May 7, 2007; Revised Manuscript Received July 7, 2007

**ABSTRACT:** In glycosyltransferase-catalyzed reactions a new carbohydrate–carbohydrate bond is formed between a carbohydrate acceptor and the carbohydrate moiety of either a sugar nucleotide donor or lipid-linked saccharide donor. It is currently believed that most glycosyltransferase-catalyzed reactions occur *via* an electrophilic activation mechanism with the formation of an oxocarbenium ion-like transition state, a hypothesis that makes clear predictions regarding the charge development on the donor (strong positive charge) and acceptor (minimal negative charge) substrates. To better understand the mechanism of these enzyme-catalyzed reactions, we have introduced a strongly electron-withdrawing group (fluorine) at C-5 of both donor and acceptor substrates in order to explore its effect on catalysis. In particular, we have investigated the effects of the 5-fluoro analogues on the kinetics of two glycosyltransferase-catalyzed reactions mediated by UDP-GlcNAc:GlcNAc-P-P-Dol *N*-acetylglucosaminyltransferase (chitobiosyl-P-P-lipid synthase, CLS) and  $\beta$ -*N*-acetylglucosaminyl- $\beta$ -1,4 galactosyltransferase (GalT). The 5-fluoro group has a marked effect on catalysis when inserted into the UDP-GlcNAc donor, with the UDP(5-F)-GlcNAc serving as a competitive inhibitor of CLS rather than a substrate. The (5-F)-GlcNAc  $\beta$ -octyl glycoside acceptor, however, is an excellent substrate for GalT. Both of these results support a weakly associative transition state for glycosyltransferase-catalyzed reactions that proceed with inversion of configuration.

Glycosyltransferases are involved in a wide variety of biological processes ranging from the processing of *N*-linked glycan structures (1) to signal transduction (2) to bacterial cell wall (3) and natural product synthesis (4). A thorough understanding of these enzyme-catalyzed reactions will be important for the design of molecular therapeutics targeting these enzymes. Based on sequence homology, glycosyltransferases have been classified into an expanding list of families (5) (89 as of April 2007; available at <http://afmb.cnrs-mrs.fr/CAZY>). Despite this sequence diversity, glycosyltransferases can be classified into three superfamilies based on current crystal structures (6). Although there are likely to be some subtle differences between the superfamilies, the mechanisms of glycosyltransferase-catalyzed reactions fall into two categories: retaining, where the product glycoside has the same stereochemistry as the activated leaving group, and inverting, where the reaction proceeds with inversion of stereochemistry at C-1.

A number of studies using substrate analogues (7–9) have suggested that inverting glycosyltransferase-catalyzed reactions, like those of glycosidases, proceed *via* an oxocarbenium ion-like (“exploded S<sub>N</sub>2” (10)) transition state (11). This hypothesis suggests a reaction coordinate in which the bond between C-1 of the glycosyl donor and the pyrophosphate leaving group is broken in the transition state, yet little bond formation between the glycosyl acceptor and the oxocarbenium center has occurred (Figure 1).

One clear implication of this hypothesis is that during catalysis the glycosyl donor substrate develops a partial positive charge at C-1 and O-5 due to the formation of the oxocarbenium ion. The amount of negative charge development on the glycosyl acceptor, on the other hand, would be expected to be small, since little deprotonation or bond formation (and thereby stabilization of the developing oxocarbenium ion) occurs in the transition state.

We rationalized that both of these tenets could be investigated through the introduction of a charge-altering 5-fluoro group that would exert an electronic effect on both the developing oxocarbenium ion, if incorporated into the donor, and the C-4 or C-6 hydroxyl group, if incorporated into the glycosyl acceptor. In the realm of carbohydrate enzymology, 5-fluoro glycosyl fluorides have been used extensively for the investigation of glycosidase mechanisms (12), but difficulty in preparing the requisite 5-fluoro substrates has prevented the extension of this approach to the use of 5-fluoro glycosides and glycosylphosphates in the studies of glycosyltransferases.

<sup>†</sup> This research was supported in part by the Vahlteich Research Fund administered by the College of Pharmacy, University of Michigan (J.K.C.), and by NIH Grant GM36065 (C.J.W.). MCTH is the recipient of a Regents Fellowship from the Horace H. Rackham School of Graduate Studies and an Eli Lilly Fellowship from the Department of Chemistry, University of Michigan.

\* To whom correspondence should be addressed. Phone: 734-936-2843. Fax: 734-647-4865. E-mail: [jkcoward@umich.edu](mailto:jkcoward@umich.edu).

<sup>‡</sup> University of Michigan.

<sup>§</sup> Current address: Department of Chemistry, Virginia Commonwealth University, Richmond, VA 23284.

<sup>||</sup> University of Kentucky.

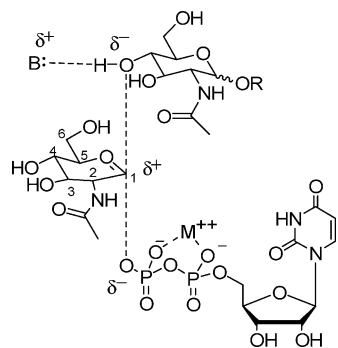
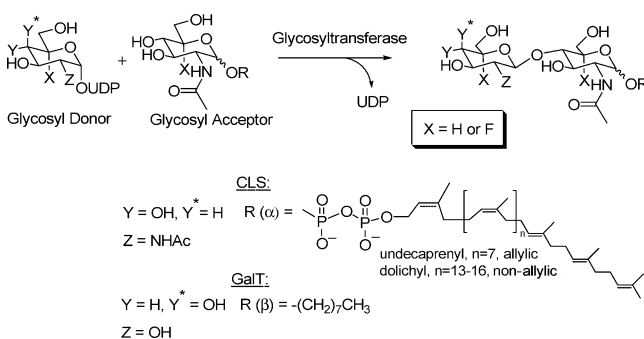


FIGURE 1: Proposed weakly associative transition state for glycosyltransferases involving a UDP-GlcNAc donor and various GlcNAc-containing acceptor substrates.

An efficient route for the synthesis of potential glycosyltransferase acceptors and donors containing 5-fluoro *N*-acetylglucosamine ((5-F)-GlcNAc) has now been developed in our laboratory (13). Because of our ongoing interest in *N*-linked glycosylation (14–16) and the biosynthesis of the oligosaccharyl donor, Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub>-P-P-dolichol (17), we chose UDP-GlcNAc:GlcNAc-P-P-Dol *N*-acetylglucosaminyl transferase (chitobiosyl-P-P-lipid synthase, CLS,<sup>1</sup> EC 2.4.1.141) as an initial target for our mechanistic investigations (Scheme 1). CLS has been shown recently to exist as a heterodimeric complex in yeast consisting of Alg13p and Alg14p in which Alg14p functions as an ER binding partner for the catalytic subunit Alg13p (18–20). It remains to be established conclusively if the mammalian enzyme exists as a similar dimeric complex. In this regard, Gao et al. have reported that although the human ALG13 and ALG14 orthologues apparently do not pair with their respective yeast counterparts, they are able to functionally complement for the loss of either ALG13 or ALG14 when coexpressed in yeast (19). This multi-subunit structure is reminiscent of dolichylphosphomannose synthase (DPM) which has three subunits, DPM1, DPM2, and DPM3 (21). In this mannosyltransferase, the catalytic subunit, DPM1, is associated with the ER via its interaction with DPM3.

The mammalian CLS has the added advantage that both the donor and acceptor contain GlcNAc, allowing the investigation of charge buildup in both substrates for a single enzyme-catalyzed reaction. However, only limited quantities of the CLS acceptor substrates, GlcNAc-P-P-Dol (and 5-fluoro GlcNAc-P-P-Dol), are available *via* biosynthesis. Therefore, to corroborate the tentative conclusions reached with the biosynthetic CLS acceptor substrates, we also chose to study  $\beta$ -1,4-galactosyltransferase (GalT, EC 2.4.1.38, family GT7) which utilizes synthetically accessible GlcNAc-containing acceptors. The effects of the 5-fluoro substrate

Scheme 1



analogues on these enzyme-catalyzed reactions support a weakly associative transition state for inverting glycosyltransferase-catalyzed reactions.

## EXPERIMENTAL PROCEDURES

**Materials.** Unless otherwise indicated, all chemicals were purchased from Aldrich or Sigma and were used without further purification. Pyridine and CH<sub>3</sub>OH were distilled from CaH<sub>2</sub> prior to use. [<sup>3</sup>H]Ac<sub>2</sub>O (100 mCi/mmol, lot #011126) and UDP-[6-<sup>3</sup>H]galactose ([<sup>3</sup>H]UDP-Gal, 40–60 Ci/mmol) were purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). GalT from bovine milk (10 IU, MW 44,000) was purchased from Sigma, dissolved in water, and divided so as to provide aliquots containing 0.5 unit. These aliquots were lyophilized and stored at –80 °C prior to use. 5-Fluoro-glucosamine 1-phosphate, UDP-(5-F)-GlcNAc, and octyl 5-fluoro-2-deoxy-2-acetamido- $\beta$ -D-glucopyranoside ((5-F)-GlcNAc  $\beta$ -octyl glycoside) were synthesized as previously described (13). GlcNAc  $\beta$ -octyl glycoside was synthesized as described in the literature (22). Pyridinium acetate solutions were prepared by adding the requisite amount of acetic acid followed by addition of pyridine to pH 4.5 and dilution with water to provide the desired concentration. Baker Si250 thin layer plates were from VWR Scientific Products (Willard, OH). Analytical HPLC was carried out on a Waters 996 photodiode array system running Millennium<sup>32</sup> software. Econo-safe biodegradable scintillation counting mixture is a product of Research Products International (Mount Prospect, IL). EDTA-free protease inhibitor cocktail was purchased from Roche. Reactive Blue 4 immobilized on cross-linked 4% agarose was from Sigma Chemical Co. (St. Louis, MO). Tn-10 CHO cells were generously provided by Dr. Mark Lehrman (UT-Southwestern, Dallas, TX).

**Synthesis of UDP-(5-F)-[<sup>3</sup>H]GlcNAc.** Et<sub>3</sub>N (11  $\mu$ L, 0.080 mmol) was added to a stirred suspension of crude 5-fluoro glucosamine 1-phosphate (0.010 mmol) in dry CH<sub>3</sub>OH (100  $\mu$ L) in a sealable glass tube. The supplied vial with [<sup>3</sup>H]Ac<sub>2</sub>O (5 mCi, 100 mCi/mmol) was cooled to –78 °C to prevent any escape of volatile [<sup>3</sup>H]Ac<sub>2</sub>O, and 50  $\mu$ L CH<sub>3</sub>OH was added. This solution was added to the 5-fluoro glucosamine 1-phosphate-containing tube to initiate the reaction; quantitative transfer was ensured by rinsing the vial containing residual [<sup>3</sup>H]Ac<sub>2</sub>O with an additional 50  $\mu$ L of CH<sub>3</sub>OH. The tube was sealed and stirred at room temperature for 4 h. The reaction vessel was opened and the mixture was evaporated under high vacuum *via* a line that contained a series of four traps to prevent radioactive contamination

<sup>1</sup> Abbreviations: BME,  $\beta$ -mercaptoethanol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CLS, UDP-GlcNAc:GlcNAc-P-P-Dol *N*-acetylglucosaminyltransferase (EC 2.4.1.141); Dol, dolichyl; Dol-P, dolichyl phosphate; GalT,  $\beta$ -*N*-acetylglucosaminyl-glycopeptide  $\beta$ -1,4-galactosyltransferase (EC 2.4.1.38); GlcNAc-P-P-Dol, GlcNAc-P-P-dolichol; GlcNAc-P-P-Undec, GlcNAc-P-P-undecaprenol; Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub>-P-P-Dol, Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub>-P-P-dolichol; GPT, UDP-GlcNAc:Dol-P-GlcNAc 1-phosphate transferase (EC 2.7.8.15); octyl GlcNAc or GlcNAc  $\beta$ -octyl glycoside, octyl 2-deoxy-2-acetamido- $\beta$ -D-glucopyranoside; octyl (5-F)-GlcNAc or (5-F)-GlcNAc  $\beta$ -octyl glycoside, octyl 5-fluoro-2-deoxy-2-acetamido- $\beta$ -D-glucopyranoside; P-P, pyrophosphoryl linkage (–O–P(O)(OH)–O–P(O)(OH)–O–); Undec, undecaprenyl; Undec-P, undecaprenyl phosphate.

(dry ice/acetone (2), liquid N<sub>2</sub> (1), and K<sub>2</sub>CO<sub>3</sub> (1)). The resulting brown oil was dissolved in CH<sub>3</sub>OH (1 mL) and cooled to -10 °C. NH<sub>3</sub> was bubbled through the solution, and the flow out of the flask was passed through a check valve (to prevent reverse flow) and two aqueous NaHCO<sub>3</sub> traps. After 1 h, the solvent was removed under a stream of N<sub>2</sub> (passing through the same traps). The resulting brown residue was dissolved in water (~10 mL) and passed through a Bio-Rad AG 1 × 8 anion exchange column (1 × 6 cm, acetate form). The column was washed with water (20 mL) followed by elution of the desired product with 500 mM pyridinium acetate (40 mL). The pyridinium acetate effluent was collected as a single fraction and evaporated using a Speed-Vac vacuum centrifuge to provide (5-F)-[<sup>3</sup>H]GlcNAc 1-phosphate pyridinium salt. The resulting oil was passed through a cation exchange column (Dowex 50WX8-200, Et<sub>3</sub>-NH<sup>+</sup> form), eluting with several column volumes of CH<sub>3</sub>-OH. The fractions with significant radioactivity were pooled and evaporated leaving (5-F)-[<sup>3</sup>H]GlcNAc 1-phosphate triethylammonium salt as an oil (240 μCi). To this oil were added UMP-morpholidate (4.9 mg, 0.0072 mmol) and pyridine (200 μL). The resulting yellow solution was evaporated and then coevaporated with pyridine (2 × 200 μL). To this yellow oil were added 1*H*-tetrazole (0.33 mg, 0.0047 mmol) and pyridine (200 μL). The reaction was monitored by TLC analysis (3:1 CH<sub>3</sub>CN:H<sub>2</sub>O). After 8 days the reaction was quenched by addition of 50 mM aqueous pyridinium acetate (100 μL) and evaporated. The resulting yellow oil was purified by chromatography on Bio Gel P-2 (80 cm × 1.5 cm) with 50 mM pyridinium acetate as the eluant. Fractions (100 drops) were collected and analyzed by TLC (UV absorbance). Fractions that contained the desired product were pooled, giving the title compound contaminated with [<sup>3</sup>H]acetate. The acetate was removed by HPLC purification in several portions on a C<sub>18</sub> Varian Microsorb-MV 300 Å column (4 mm × 25 cm) eluting with 400 mM aqueous hexafluoro-2-propanol adjusted to pH 7.8 with Et<sub>3</sub>N at 1 mL/min. The fractions containing the desired product were pooled and evaporated on a Speed-Vac vacuum centrifuge yielding 5 μCi of UDP-(5-F)-[<sup>3</sup>H]GlcNAc.

Each reaction in the synthesis of UDP-(5-F)-[<sup>3</sup>H]GlcNAc (Scheme 2) was first examined on an identical small scale using unlabeled precursors. The purity and chemical identification of each intermediate were ascertained using standard methods (TLC, NMR, MS). The final product, UDP-(5-F)-[<sup>3</sup>H]GlcNAc, was chromatographically identical to unlabeled UDP-(5-F)-GlcNAc by both size exclusion chromatography and HPLC. In addition, this product was shown to be a WecA substrate in the biosynthesis of (5-F)-[<sup>3</sup>H]GlcNAc-P-P-Undec which, in turn, is a CLS substrate (*vide infra*).

**Biosynthesis of [<sup>3</sup>H]GlcNAc-P-P-Undec and (5-F)-[<sup>3</sup>H]-GlcNAc-P-P-Undec for Use as Chitobiosyl-P-P-lipid Synthase (CLS) Acceptor Substrates.** Each reaction solution contained *Escherichia coli* membranes (8 μg total protein) prepared as described by Rush et al. (23), UDP-(5-F)-[<sup>3</sup>H]-GlcNAc (215,000 cpm, 97 cpm/pmol) or UDP-[<sup>3</sup>H]GlcNAc (858,000 cpm, 58,500 cpm/pmol), 52 mM Tris-HCl (pH 8), 11 mM sucrose, 7.4 mM 2-mercaptoethanol, 0.5 mM EDTA, 40 mM MgCl<sub>2</sub>, and 0.58% CHAPS in a total volume of 50 μL. The reactions were incubated for 30 min at 37 °C. After extraction with CHCl<sub>3</sub>:CH<sub>3</sub>OH (2:1), the organic layer

was dried by a stream of N<sub>2</sub> and the radiolabeled glycolipid substrate was dispersed in 1% Triton X-100 (200 μL for [<sup>3</sup>H]-GlcNAc-P-P-Undec or 22 μL for (5-F)-[<sup>3</sup>H]GlcNAc)-P-P-Undec to be tested as substrates in CLS reactions.

**Biosynthesis of [<sup>3</sup>H]GlcNAc-P-P-Dol.** [<sup>3</sup>H]GlcNAc-P-P-Dol was synthesized enzymatically and purified as described elsewhere (24) except microsomes from Tn-10 CHO cells overexpressing GPT (25) were used.

**Solubilization and Partial Purification of UDP-GlcNAc:GlcNAc-P-P-Dol N-Acetylglucosaminyltransferase (Chitobiosyl-P-P-lipid Synthase, CLS) from CHO Cells.** CHO cells were grown to confluence in F-12 media with 9% bovine serum and suspended in 0.9% NaCl, 3 mM EDTA (pH 8). The suspended cells were homogenized by sonication (15 s), and a crude particulate fraction was obtained by centrifugation (3,000 rpm, 4 °C, 15 min). Crude microsomes were sedimented from the low-speed supernatant fluid by centrifugation (60,000 rpm, 4 °C, 40 min) in a Beckman TL-100 ultracentrifuge. The membranous pellet was washed and resuspended in 1 mL of sonication buffer. CLS activity was solubilized by extraction of the membrane fraction (2 mg/mL protein) in 10% glycerol, 25 mM Tris-HCl (pH 8.0), 0.65% Triton X-100, 5 mM β-mercaptoethanol, and 1x proteinase inhibitor cocktail (EDTA-free) for 60 min on ice with stirring. The solubilized CLS activity was recovered in the supernatant fluid after centrifugation of the detergent-extract (46,000 g, 40 min, 4 °C).

The solubilized CLS activity (specific activity = 5,400 cpm/min/mg) was then partially purified by ion-exchange chromatography on a TSK-Gel Toyopearl DEAE-650M column (40–90 microns) washed by 15 vol of 25 mM Tris-HCl (pH 8.0) and equilibrated with 5 vol of 20% glycerol, 25 mM Tris-HCl (pH 8.0), 0.05% Triton X-100, and 5 mM β-mercaptoethanol. Approximately 6 mg of membrane protein was applied to a 1 mL column at a rate of 30 mL/h. The column was then washed with 2 vol of 20% glycerol, 25 mM Tris-HCl (pH 8.0), 0.05% Triton X-100, 0.2 mM PMSF, 2 μg/mL leupeptin, 1x proteinase cocktail for mammalian tissues (Sigma), and 5 mM β-mercaptoethanol. The TSK-Gel Toyopearl DEAE-650M column was eluted with a linear NaCl gradient (0–0.3 M NaCl in the equilibration buffer), eluting at a rate of 35 mL/hr. The fractions from a single peak of CLS activity were pooled and then desalted and concentrated using an Amicon filtration cell fitted with a YM100 membrane. The desalted fraction was applied to a butyl-Sepharose column and eluted with a decreasing linear salt gradient (1.0 M –0.0 M NaCl). Fractions corresponding to the peak of CLS activity were pooled and applied to a second TSK-Gel Toyopearl DEAE-650M column. The column was eluted with a linear NaEDTA gradient (0–5 mM). The fractions containing CLS activity were pooled and concentrated as above, applied to a Reactive Blue 4-agarose column, and eluted with a linear NaCl gradient (0–0.8 M). Although it was estimated that this fraction was 730-fold purified relative to the crude microsomes, 10 polypeptide bands were still visualized by SDS–PAGE. CLS activity is based on the conversion of exogenous acceptor substrate, [<sup>3</sup>H]GlcNAc-P-P-Dol, to [<sup>3</sup>H](GlcNAc-β-(1-4)-GlcNAc)-P-P-Dol (cpm/mg protein) in the standard assay described below. It is not possible to determine the specific activity of biosynthetic [<sup>3</sup>H]GlcNAc-P-P-Dol because of the presence of an unknown amount of endogenous GlcNAc-



P-P-Dol in the substrate preparation. The highly purified fractions from the Reactive Blue 4-agarose column were used for the enzymatic studies described in the Results Section.

**Enzyme Assays.** *Chitobiosyl-P-P-lipid Synthase.* For routine assay during purification of CLS, each reaction mixture contained partially purified fractions of CLS (3.6  $\mu$ g protein), 55 mM Tris-HCl (pH 8), 0.25 mM sucrose, 4% glycerol, 1 mM 2-mercaptoethanol, 8 mM NaCl, 5 mM EDTA, 5 mM AMP, 0.5% Triton X-100, 0.1 mM UDP-GlcNAc, and [ $^3$ H]-GlcNAc-P-P-Dol (4,000 cpm) in a total volume of 100  $\mu$ L. This assay method is a slight modification of that described previously (24). After incubation for 20 min at 37 °C unless indicated otherwise, the reactions were terminated by the addition of 20 vol of  $\text{CHCl}_3$ : $\text{CH}_3\text{OH}$  (2:1) and washed with 0.9% NaCl (0.4 mL). The lower phase was washed once with an equal volume of  $\text{CHCl}_3$ : $\text{CH}_3\text{OH}$ :0.9% NaCl, (3:48:47), and the lower organic layer was removed. Brain phosphatidylcholine (75  $\mu$ g), was added and the organic solvent was evaporated under a stream of  $\text{N}_2$  at ambient temperature. The resulting residue was redissolved in  $\text{CHCl}_3$ : $\text{CH}_3\text{OH}$  (2:1) (12  $\mu$ L) and loaded onto a Baker Si250 TLC plate eluting with  $\text{CHCl}_3$ : $\text{CH}_3\text{OH}$ : $\text{H}_2\text{O}$  (65:25:4). The radioactive zones corresponding to  $^3\text{H}$ -labeled GlcNAc-P-P-Dol substrate and GlcNAc- $\beta$ -(1,4)-GlcNAc-P-P-Dol product were located using a BioScan System 200 imaging scanner (1 h scan/lane), and the percentage of the glycolipid substrate converted to the chitobiosyl-P-P-lipid product was determined.

To compare the ability of CLS to catalyze the reaction of fluorinated *vs* non-fluorinated substrates, partially purified CLS (600 ng protein) was assayed with either 0.5 mM UDP-GlcNAc or UDP-(5-F)-GlcNAc as the nucleotide sugar donor substrate and [ $^3\text{H}$ ]GlcNAc-P-P-Undec (7,000 cpm, 58,500 cpm/pmol) or (5-F)-[ $^3\text{H}$ ]GlcNAc-P-P-Undec (7,000 cpm, 97 cpm/pmol) as the glycolipid acceptor substrate in a total volume of 25  $\mu$ L. Reaction conditions and product analysis were carried out as described above.

**$\beta$ -1,4-Galactosyltransferase. General Procedure.** For assays, a concentrated (162.5 $\times$ ) stock solution (25 munits/ $\mu$ L) of GalT containing 20 mM Tris-HCl, 2 mM BME, 2 mM EDTA, 0.5 mg/mL BSA, pH 7.5 was prepared and stored at -20 °C in 4  $\mu$ L aliquots. In this form the enzyme was stable for several months. GlcNAc  $\beta$ -octyl glycoside and (5-F)-GlcNAc  $\beta$ -octyl glycoside solutions were prepared in DMSO and stored at -20 °C. (5-F)-GlcNAc  $\beta$ -octyl glycoside solutions decomposed over several weeks at rt in DMSO and were prepared fresh weekly. UDP-Gal (Sigma) was prepared as a 10 mM stock solution (determined gravimetrically) and stored at -20 °C. UDP-[ $^3\text{H}$ ]Gal was purchased as a 1 mCi/mL solution in 70% aq. EtOH and was diluted 40-fold with water to prepare a 20 $\times$  solution for assays. This solution was stored at -20 °C.

Each enzyme assay (100  $\mu$ L) contained 100 mM Na cacodylate (pH 7.5), 10 mM  $\text{MnCl}_2$ , 0.5 mg/mL BSA, GlcNAc  $\beta$ -octyl glycoside (various concentrations in DMSO), 100  $\mu$ M UDP-Gal, UDP-[ $^3\text{H}$ ]Gal (ca. 0.4  $\mu$ Ci), and  $\beta$ -GalT from bovine milk (11.3 ng), in an aqueous solution containing 5% DMSO. The assay solutions were preincubated for 9 min, at 37 °C prior to addition of the enzyme (5  $\mu$ L in a stock solution of 20 mM Tris-HCl, 2 mM BME, 2 mM EDTA, 0.5 mg/mL BSA, pH 7.5). After 5 min incubation at 37 °C, the reaction was stopped by dilution with ice-cold deionized

water (500  $\mu$ L) or ice-cold 100 mM EDTA (500  $\mu$ L). The assay solution was passed through a Sep-Pak cartridge either immediately (water quench) or after storage on ice (EDTA quench). The assay tube was rinsed with water (2  $\times$  500  $\mu$ L), and each aqueous rinse was applied to the same Sep-Pak cartridge. The Sep-Pak was washed with 20 mL of  $\text{H}_2\text{O}$  followed by 4  $\times$  2.5 mL of  $\text{CH}_3\text{OH}$ . The  $\text{CH}_3\text{OH}$  eluant was analyzed *via* liquid scintillation counting. Background values were determined by addition of DMSO without any acceptor.

The assays for (5-F)-GlcNAc- $\beta$ -octyl glycoside were completed in the identical manner as above, except (5-F)-GlcNAc- $\beta$ -octyl glycoside was added after the preincubation step, 30 s prior to the addition of enzyme. The assay solutions were incubated for 9 min at 37 °C, and were passed through the Sep-Pak immediately after quenching to minimize decomposition of the disaccharide product.<sup>2</sup>

**Gal T. Steady-State Kinetic Analysis.** The assays followed the general procedure with UDP-Gal (19, 24, and 33  $\mu$ M) and GlcNAc  $\beta$ -octyl glycoside (0, 17, 21, 30, 50, and 75  $\mu$ M). The reactions were performed on the same day and were quenched with ice-cold 100 mM EDTA. For (5-F)-GlcNAc- $\beta$ -octyl glycoside, the assays followed the general procedure with UDP-Gal (27.3, 33.3, 42.8, 60, 100, and 300  $\mu$ M) and GlcNAc- $\beta$ -octyl glycoside (0, 100, 129, 180, 300, and 900  $\mu$ M). The 100  $\mu$ M UDP-Gal assay utilized a 2 $\times$  enzyme stock, and the 300  $\mu$ M UDP-Gal assay utilized a 5 $\times$  enzyme stock prepared from a fresh enzyme aliquot (see General Procedure).

**Data Collection and Analyses.** Unless indicated otherwise each assay was performed in duplicate and the error bars signify the range of the data collected (varied time or concentration). The kinetic constants were determined by least-squares fitting to either the Michaelis-Menten equation using Kaleidagraph (CLS) (Synergy Software) or eq 1 using the Leonora program (27), and the errors reported are standard deviations. The standard errors for  $k_{\text{cat}}$  and  $K_{\text{m}}$  were not propagated in the  $k_{\text{cat}}/K_{\text{m}}$  calculations.

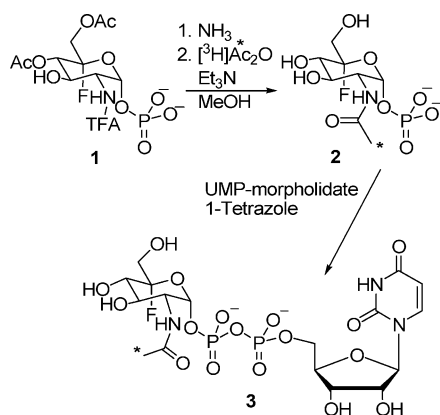
## RESULTS

**Synthesis of UDP-(5-F)-[ $^3\text{H}$ ]GlcNAc.** To facilitate biochemical assays with UDP-(5-F)-GlcNAc, a tritium label (represented by \*) was incorporated in the penultimate acetylation step (Scheme 2). Precursor **1** was prepared as previously described (13). The acetate and trifluoroacetyl (TFA) groups were removed with methanolic ammonia, and the radiolabel was then introduced using [ $^3\text{H}$ ]Ac<sub>2</sub>O. Following nucleotide sugar formation with UMP-morpholidate (28), purification by HPLC provided the desired product **3** in radiochemically pure form.

**Synthesis of (5-F)-GlcNAc-P-P-lipid.** Initially, synthesis of a (5-F)-GlcNAc-containing acceptor substrate for CLS was attempted using methods described in the literature for the synthesis of substituted pyrophosphates. Two routes were

<sup>2</sup> The experiment in which GlcNAc- $\beta$ -octyl glycoside was used as the acceptor substrate was completed using a single stock solution of reagents for all of the experiments, and all of the assays were quenched in a single day. However, assays with (5-F)-GlcNAc- $\beta$ -octyl glycoside were completed over several days from separate, freshly prepared reagent stock solutions. This precautionary method was employed due to concerns about the stability of (5-F)-GlcNAc- $\beta$ -octyl glycoside, and perhaps the disaccharide product, in aqueous buffer.

Scheme 2



investigated involving reacting either **1** or **2** with a lipid morpholidate (28, 29) or imidazolide (30) intermediate. The latter method has been used in our lab (14, 31) and by others (32–35) for the synthesis of (GlcNAc- $\beta$ -(1-4)GlcNAc)-P-P-Dol and lipid analogues. Unfortunately, neither method was effective in yielding the (5-F)-GlcNAc-containing analogue (36). Given these difficulties, a biosynthetic route to (5-F)-GlcNAc-P-P-lipid was pursued using UDP-GlcNAc: Dol-P-GlcNAc 1-phosphate transferase (GPT). Enzymatic synthesis of the 5-fluoro substrate, (5-F)-GlcNAc-P-P-Dol, containing the lipid found in mammalian cells, is not possible because UDP-(5-F)-GlcNAc is not a substrate for the mammalian GPTs (data not shown). Thus, an alternate strategy using a truncated, unsaturated lipid, undecaprenol, to afford (5-F)-GlcNAc-P-P-Undec was undertaken. This strategy appeared promising because CLS had been shown previously to accept a completely unsaturated GlcNAc-P-P-lipid substrate (37), and it was determined that UDP-(5-F)-GlcNAc is a substrate for a bacterial GPT, WecA (data not shown). Fortunately, this approach allowed for the synthesis of [ $^3$ H]GlcNAc-P-P-Undec and (5-F)-[ $^3$ H]GlcNAc-P-P-Undec by WecA-catalyzed *N*-acetylglucosaminylphospho transfer. Each product was isolated and then tested as a CLS acceptor substrate.

**Solubilization, Partial Purification, and Properties of CLS Associated with CHO Microsomes.** To resolve CLS from other membrane-associated GlcNAc-transferases, the enzyme was solubilized and partially purified from CHO membranes. A preliminary screen of detergents revealed that stable fractions could be effectively solubilized by extraction with Triton X-100. Approximately 56% of the membrane protein and 80% of the enzyme activity were solubilized by extraction of CHO microsomes with 0.5% Triton X-100. The activity was also fairly stable in Nonidet P-40 extracts, but Tween 20, Tween 80, CHAPS, sodium taurocholate, and sodium deoxycholate markedly inhibited the activity. The effects of the detergents on CLS activity were virtually the same for microsomal fractions from both CHO cells and bovine brain.

A highly purified fraction of CLS (730-fold relative to CHO microsomes) was obtained by a combination of chromatographic steps using TSK-Gel Toyopearl-DEAE 650M, butyl-agarose, and Reactive Blue 4-agarose as the chromatographic supports (Table 1). Although the CLS activity was enriched 730-fold relative to CHO microsomes, the pooled fractions still contained approximately 10 polypep-

Table 1: Partial Purification of CLS Activity from CHO Microsomes

purification step	protein recovered (mg)	enzyme activity ( $\times 10^{-5}$ )	specific activity <sup>a</sup>	fold purification
microsomes	192	4.6	2,396	1
detergent extracts	120	4.3	3,583	1.5
Toyo-DEAE-I	9.1	4.0	43,956	18.3
butyl-agarose	2.0	3.9	195,000	81.4
Toyo-DEAE-II	1.2	3.85	320,833	134
Reactive Blue 4-agarose	0.12	2.1	1,750,000	730.4

<sup>a</sup> Specific activity is based on the amount of [ $^3$ H]GlcNAc-P-P-Dol converted to [ $^3$ H]GlcNAc<sub>2</sub>-P-P-Dol (cpm/mg protein) as described in Experimental Procedures.

tides as visualized by Coomassie blue staining of SDS-PAGE gels. Additional studies will clearly be required to identify the polypeptide(s) that constitute the active enzyme (18–20). The partially purified preparation of CLS exhibited a broad pH optimum between 8.0 and 8.5. Partially purified CLS also does not appear to have a divalent cation requirement, atypical of most *N*-acetylglucosaminyl transferases, and was actually stimulated by 10 mM EDTA. The partially purified fractions from the Reactive Blue 4-agarose column were used for the enzymology studies described below. It is not known if these partially purified fractions contained the mammalian homologues of both Alg13 and Alg14 (18–20).

**Analysis of UDP-(5-F)-GlcNAc as a Donor Substrate for CLS.** The first enzyme of interest, CLS (38), was partially purified from CHO cells as described above and used to evaluate the effect of a 5-F substituent in either the nucleotide sugar donor or the GlcNAc-lipid acceptor substrate. Using the natural acceptor substrate, [ $^3$ H]GlcNAc-P-P-Dol, typical saturation kinetics data were obtained with the natural donor substrate, UDP-GlcNAc to provide a  $K_m$  of  $392 \pm 83 \mu\text{M}$  and a  $V_{\max}$  of  $27.5 \pm 1.9 \text{ cpm/min}/\mu\text{g protein}$  (Figure S1, Supporting Information). In contrast, UDP-(5-F)-GlcNAc was completely inactive as a glycosyl donor for CLS even under extended incubation conditions that led to complete conversion of [ $^3$ H]GlcNAc-PP-Dol to a disaccharide product using UDP-GlcNAc as the donor substrate (data not shown). Moreover, UDP-(5-F)-GlcNAc apparently bound to the enzyme based on its ability to inhibit catalysis. Increasing the concentration of UDP-(5-F)-GlcNAc at a constant concentration of UDP-GlcNAc ( $300 \mu\text{M}$ ,  $\sim K_m$ ) led to a marked reduction in rate with an  $\text{IC}_{50}$  of approximately 1.25 mM for UDP-(5-F)-GlcNAc (data not shown). As shown in Figure 2, inhibition at two different concentrations of UDP-(5-F)-GlcNAc could be overcome by increasing the amount of UDP-GlcNAc, thus indicating that UDP-(5-F)-GlcNAc acts as a competitive or mixed inhibitor of the CLS-catalyzed reaction. Limited availability of biosynthetic [ $^3$ H]GlcNAc-P-P-Dol acceptor substrate prevented more extensive kinetic analysis of the inhibition of CLS by UDP-(5-F)-GlcNAc and a precise determination of the concentration of the glycolipid substrate used in these studies.

**5-Fluoro GlcNAc Lipid Pyrophosphate as a CLS Acceptor Substrate.** CLS-catalyzed glycosylation of (5-F)-GlcNAc-P-P-Undec ( $2.8 \mu\text{M}$ ) acceptor substrate by UDP-GlcNAc for a period of 20 min led to acid-labile products (characteristic of the polyisoprenol phosphate linkage), which were chromatographically identical to (GlcNAc- $\beta$ -(1,4)-GlcNAc)-P-P-lipids (data not shown). Thus, the presence of a 5-fluoro

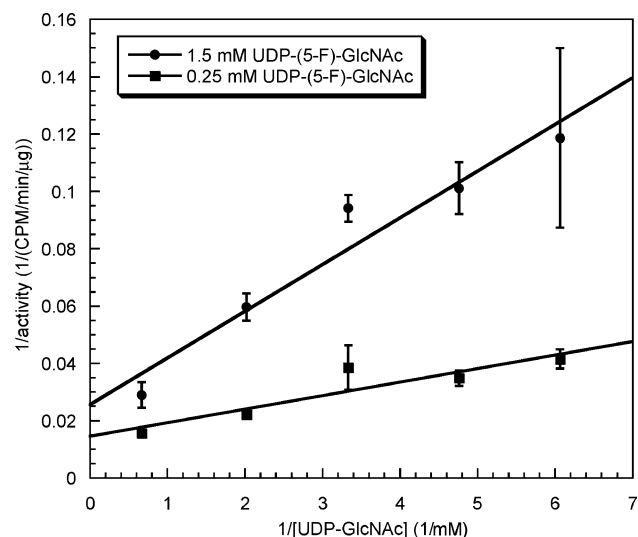


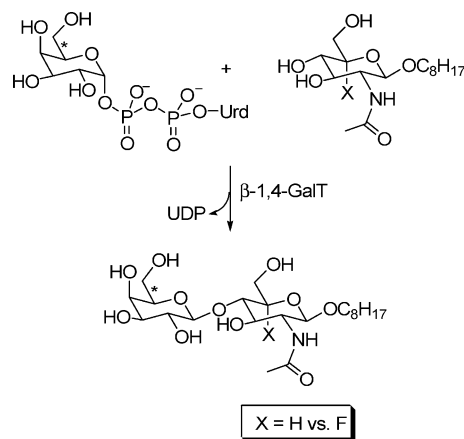
FIGURE 2: Inhibition of CLS by UDP-(5-F)-GlcNAc. Each assay was carried out for 5 min as described in Experimental Procedures using partially purified CLS (3.6  $\mu$ g), UDP-(5-F)-GlcNAc (0.25 mM or 1 mM), and UDP-GlcNAc (165 mM–1.5 mM).

substituent does not interfere with the ability of (5-F)-GlcNAc-P-P-Undec to act as a glycosyl acceptor. Unfortunately, limited availability of the radiolabeled acceptors, [ $^3$ H]GlcNAc-P-P-Undec and (5-F)-[ $^3$ H]GlcNAc-P-P-Undec, available only *via* biosynthesis, prevented further kinetic analysis. UDP-(5-F)-GlcNAc was also tested as a glycosyl donor with the acceptor, (5-F)-[ $^3$ H]GlcNAc-P-P-Undec. As expected, UDP-(5-F)-GlcNAc did not serve as a glycosyl donor under these conditions (data not shown).

**5-Fluoro GlcNAc  $\beta$ -Octyl Glycoside as a GalT Acceptor Substrate.** Because [ $^3$ H]GlcNAc-P-P-Undec and (5-F)-[ $^3$ H]GlcNAc-P-P-Undec were available in only small quantities, full kinetic analysis of (5-F)-GlcNAc-P-P-Undec as a CLS acceptor was not feasible. Thus, another enzyme,  $\beta$ -1,4-galactosyltransferase (GalT, EC 2.4.1.38) (39–41), that catalyzes a mechanistically similar reaction with readily available substrates, was investigated. To measure rates of the GalT-catalyzed reaction, a Sep-Pak assay (42) was used with both GlcNAc  $\beta$ -octyl glycoside (22) and (5-F)-GlcNAc  $\beta$ -octyl glycoside as glycosyl acceptors (Scheme 3). A preliminary comparison of octyl GlcNAc and octyl (5-F)-GlcNAc at near saturating concentrations of UDP-Gal (100  $\mu$ M) was reported in a previous publication from this laboratory (13). These data indicated that the  $K_m$  for the octyl (5-F)-GlcNAc was increased, whereas the  $k_{cat}$  slightly decreased, thus leading to a ca. 8-fold decrease in  $k_{cat}/K_m$ . Here we report a full kinetic analysis of the GalT-catalyzed reactions with octyl GlcNAc and octyl (5-F)-GlcNAc as acceptor substrates.

Saturation with both octyl-GlcNAc and octyl (5-F)-GlcNAc could not be achieved due to pronounced substrate inhibition. Double reciprocal plots at constant UDP-Gal concentrations (71.4  $\mu$ M for octyl GlcNAc and 41  $\mu$ M for octyl (5-F)-GlcNAc) show upward curvature at high substrate concentrations (see Supporting Information for details). Therefore, for determination of the kinetic parameters, both acceptor and donor substrates were varied in a noninhibitory range. The non-fluorinated glycoside, octyl GlcNAc, was evaluated as a GalT acceptor substrate with UDP-Gal as the donor, and double reciprocal plots of the resulting data are

Scheme 3



shown in Figure 3A and 3B. In a similar way, the fluorinated glycoside, octyl (5-F)-GlcNAc, was evaluated with UDP-Gal as the donor, and double reciprocal plots of the resulting data are shown in Figure 3C and 3D. Using these data, kinetic constants were calculated for both acceptors based on least-squares fitting to eq 1, the rate equation for a steady-state ordered bi bi mechanism that has been demonstrated for a number of glycosyltransferases, including GalT (27, 39). The parameters  $V_{max}$ ,  $K_{iA}K_B$ ,  $K_B$ , and  $K_A$ , derived from this fit, were inserted into eq 1, and the predicted double reciprocal linear transforms were obtained (Figure 3A–D, solid lines).

$$\text{rate} = \frac{V_{max}[A][B]}{K_{iA}K_B + K_A[B] + K_B[A] + [A][B]} \quad (1)$$

Visual inspection indicates that the data obtained for GlcNAc- $\beta$ -octyl glycoside as the acceptor substrate result in a better fit to the equation than data for the 5-fluoro analogue. This is presumably due to an apparent slight instability of the fluorinated acceptor substrate and/or product (see Experimental Procedures). Based on analysis of these data, the value of  $k_{cat}/K_B$  for octyl (5-F)-GlcNAc is 4.3-fold less than the value for octyl GlcNAc (Table 2), due primarily to a significant effect on  $K_B$  (5-fold increase) rather than  $k_{cat}$  ( $\sim$ 20% increase). Within error, the  $k_{cat}$  values for these two substrates are the same, signifying that the 5-fluoro group has little effect on the chemical step of the GalT reaction.

## DISCUSSION

In order to investigate the effect of fluorine substitution in glycosyltransferase donor and acceptor substrates, an isotopically labeled donor, UDP-(5-F)-[ $^3$ H]GlcNAc, has been synthesized *via* acetylation of 5-fluoro glucosamine-1-phosphate with [ $^3$ H]Ac<sub>2</sub>O to provide a radiolabeled 5-fluoro GlcNAc-1-phosphate, **2**, which was converted to UDP-(5-F)-[ $^3$ H]GlcNAc, **3**, by standard methods available for the synthesis of nucleotide sugars. (5-F)-GlcNAc- $\beta$ -octyl glycoside (13) was employed as the fluorinated acceptor substrate. The glycosyltransferases used in this study were UDP-GlcNAc:GlcNAc-P-P-Dol *N*-acetylglucosaminyl transferase (EC 2.4.1.141, chitobiosyl-P-P-lipid synthase, CLS), partially purified as described herein, and commercially available *N*-acetylglucosamine synthase (galactosyltransferase, GalT, EC 2.4.1.38). It is not clear if the partially purified fractions of CLS contained mammalian homologues to the



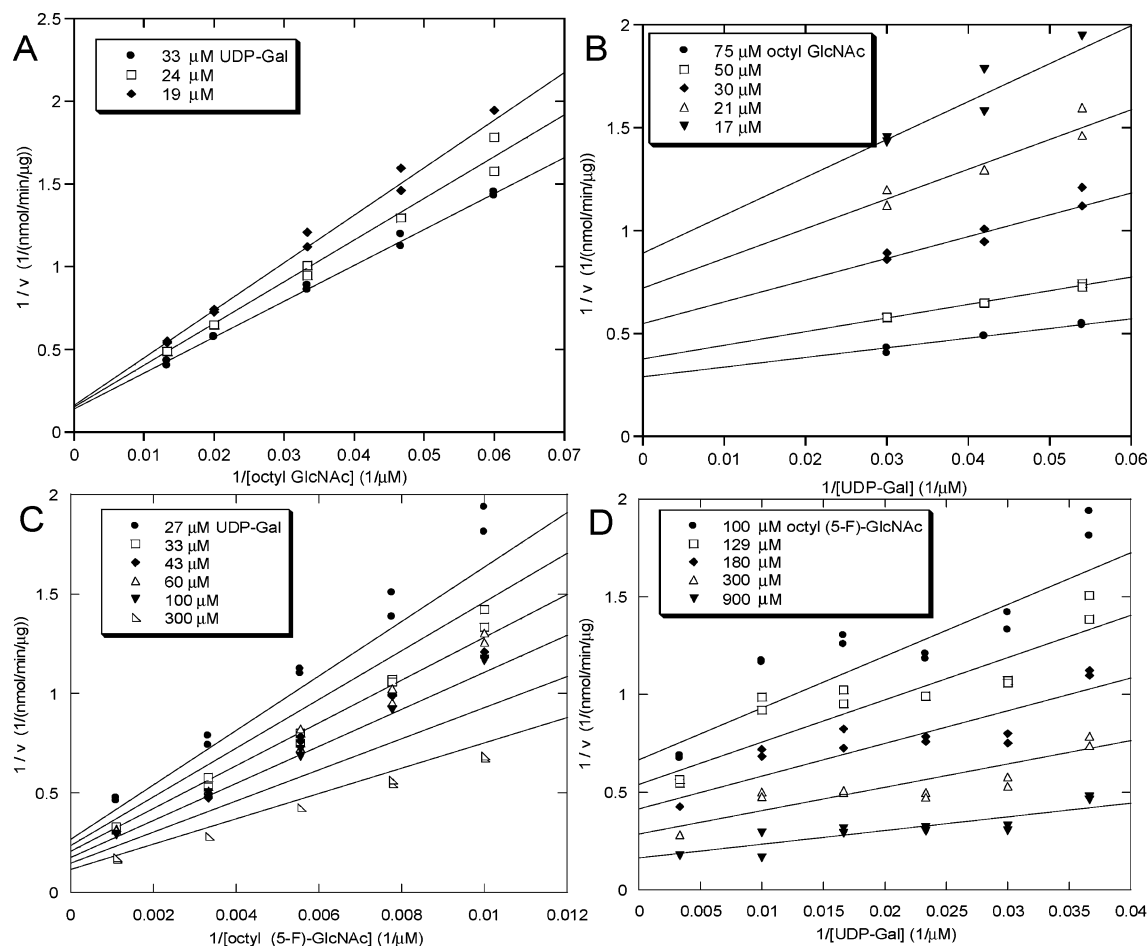


FIGURE 3: Double reciprocal plots of GalT-catalyzed glycosylation of GlcNAc- $\beta$ -octyl glycoside (3A, 3B) and (5-F)-GlcNAc- $\beta$ -octyl glycoside (3C, 3D). The data were fit to eq 1 (see text for details), and the lines shown are derived from inserting the fitted parameters into eq 1.

Table 2: GalT Kinetics Data: GlcNAc- $\beta$ -octyl Glycoside vs (5-F)-GlcNAc- $\beta$ -octyl Glycoside

acceptor substrate	$K_{iA}K_B (\mu\text{M})^2$	$K_A (\mu\text{M})$ (donor)	$K_B (\mu\text{M})$ (acceptor)	$k_{\text{cat}} (\text{s}^{-1})$	$k_{\text{cat}}/K_B (10^4 \text{ M}^{-1} \text{ s}^{-1})$
octyl GlcNAc	$2500 \pm 290$	$6.5 \pm 6.6$	$110 \pm 34$	$6.1 \pm 1.3$	$5.7 \pm 0.6$
octyl (5-F)-GlcNAc	$22000 \pm 3600$	$46 \pm 20$	$560 \pm 156$	$7.3 \pm 1.5$	$1.31 \pm 0.13$

Alg13 and Alg14 subunits of the heterodimeric complex of the yeast enzyme (18–20).

**5-Fluorinated GlcNAc Carbohydrates as Donor Substrates.** Studies of CLS with UDP-(5-F)-GlcNAc as a donor clearly show that it serves as a competitive inhibitor of UDP-GlcNAc (Figure 2) rather than a substrate. Thus, a very strong inactivating effect is exerted simply by addition of the small but strongly electron-withdrawing 5-fluoro group. The simplest explanation of the magnitude of this effect is that the enzyme-catalyzed reaction proceeds through an oxocarbenium ion-like transition state which is destabilized by the presence of the electron-withdrawing fluorine at the neighboring 5-position. This type of mechanism has been supported by other studies of glycosyltransferases (7–9), but the experiments described herein provide the first evidence in support of an oxocarbenium ion-like transition state in a glycosyltransferase utilizing UDP-GlcNAc as a donor.

**5-Fluorinated GlcNAc Glycans as Acceptor Substrates.** Analogues with 5-fluoro substituents are competent acceptor substrates for both CLS and GalT. Due to limitations in the quantity of biosynthetic substrates, GlcNAc-P-P-lipid, or the 5-fluoro analogue, a full kinetic study of the CLS-catalyzed reaction could not be undertaken. With GalT, however, octyl

5-fluoro GlcNAc is clearly a good substrate, with  $k_{\text{cat}}/K_B$  decreased by only 4.4-fold relative to the non-fluorinated substrate (Figure 3). Moreover, the modest reduction in catalytic efficiency results almost entirely from a higher  $K_B$ ; the values of  $k_{\text{cat}}$  for the two substrates are within experimental error (Table 2).

There are several possible explanations for why the values of  $k_{\text{cat}}$  for the non-fluorinated and fluorinated substrates are nearly the same. First, if the bond-forming step of the GalT-catalyzed reaction is not rate limiting, the effect of the 5-fluoro group will be masked. Among many earlier studies on the kinetics of GalT-catalyzed glycosylation, a significant secondary isotope effect at C-1 was observed ( $^{\text{D}}V_{\text{max}}/^{\text{H}}V_{\text{max}} = 1.21$ ) (43). A secondary isotope effect of this magnitude provides strong support for a reaction mechanism in which the chemical step of disaccharide formation, and not substrate binding or product release, is rate limiting.

A second possibility is that the 5-fluoro group is too distal to exert an electronic effect on the 4-OH. By analogy with the effect of a  $\beta$ -fluoro group on 2-fluoroethanol, we expect that the  $\text{p}K_{\text{a}}$  of octyl-(5-F)-GlcNAc should be reduced by 1.6 units relative to octyl GlcNAc (44, 45). Electronic effects on general base-catalyzed nucleophilic substitution reactions

can be expressed using Hammett plots to give a nucleophilic correlation constant ( $\beta_{\text{nuc}}$ ) which estimates the amount of charge build up on the nucleophilic atom in the transition state of the reaction (46). Due to the complexity in synthesizing the required fluorinated substrates, it was not feasible to do a full analysis of  $\beta_{\text{nuc}}$  for the GalT reaction to verify that the expected linear free energy relationship holds. Therefore, we cannot be certain that the transition state remains the same when the  $\text{pK}_{\text{a}}$  of the acceptor is altered over a wide range. However, there is precedent that a linear free energy relationship holds for mechanistically similar enzyme-catalyzed reactions. For example, the deglycosylation of the galactosyl enzyme intermediate in  $\beta$ -galactosidase, a general base-catalyzed reaction with an oxocarbenium-like transition state, was shown to have a small and slightly negative  $\beta_{\text{nuc}}$  ( $-0.06$ ) (47). Assuming the linear free energy relationship holds for GalT, even a reaction with a small  $\beta_{\text{nuc}}$  of 0.2, a change in  $\text{pK}_{\text{a}}$  of  $-1.6$  units would predict decrease in rate of 2-fold. This clearly was not observed in our experiments (Table 2). Our kinetics data are consistent with other studies suggesting that the GalT reaction proceeds via an oxocarbenium ion-like transition state consistent with a small  $\beta_{\text{nuc}}$ . First, the significant secondary isotope effect observed for the GalT-catalyzed reaction (*vide supra*) led Raushel and co-workers to conclude that "substantial  $\text{sp}^2$  character develops on the anomeric carbon in the transition state." Second, the fact that UDP-(2-F)-Gal acts as a competitive inhibitor of UDP-Gal and not an alternate substrate is consistent with an oxocarbenium ion-like transition state that is destabilized by the adjacent electron-withdrawing fluorine substituent at C-2 (48).

Taken together, the data for the 5-fluoro acceptors and donors support a transition state with significant bond breaking of the anomeric sugar-UDP bond yet little bond formation with the GlcNAc acceptor for the CLS and GalT-catalyzed reactions. Through the use of 5-fluoro glycosyltransferase donors and acceptors, the involvement of such a weakly associative transition state can be investigated for both substrates. The usefulness of nucleotide 5-fluoro sugars should extend well beyond the current study. In particular, other GlcNAc-transferases which catalyze reactions with retention of configuration, for which mechanistic data is fairly unclear, would be particularly amenable to this nondisruptive approach (11). 5-Fluoro nucleotide sugars could be used for many other carbohydrate processing enzymes that catalyze chemistry at or near the C-5 position including enzymes involved in nucleotide sugar epimerization and dehydration (13). Similarly, we envision 5-fluoro glycosides as useful tools for the investigation of glycosyltransferases that form bonds at the C-3 or C-6 OH positions.

## ACKNOWLEDGMENT

We thank Prof. Stephen Withers for stimulating discussions and encouragement.

## SUPPORTING INFORMATION AVAILABLE

Saturation curves for UDP-GlcNAc and GlcNAc-P-P-Dol with CLS and evidence for uncompetitive substrate inhibition of GalT with octyl GlcNAc. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## REFERENCES

- Sears, P., and Wong, C. H. (1998) Enzyme action in glycoprotein synthesis, *Cell. Mol. Life. Sci.* 54, 223–252.
- Slawson, C., Housley, M. P., and Hart, G. W. (2006) O-GlcNAc cycling: how a single sugar post-translational modification is changing the way we think about signalling networks, *J. Cell. Biochem.* 97, 71–83.
- Hancock, I. C. (1997) Bacterial cell surface carbohydrates: structure and assembly, *Biochem. Soc. Trans.* 25, 183–187.
- Vogt, T., and Jones, P. (2000) Glycosyltransferases in plant natural product synthesis: characterization of a supergene family, *Trends Plant Sci.* 5, 380–386.
- Coutinho, P. M., Deleury, E., Davies, G. J., and Henrissat, B. (2003) An evolving hierarchical family classification for glycosyltransferases, *J. Mol. Biol.* 328, 307–317.
- Breton, C., Snajdrova, L., Jeanneau, C., Koca, J., and Imberty, A. (2006) Structures and mechanisms of glycosyltransferases, *Glycobiology* 16, 29R–37R.
- Murray, B. W., Takayama, S., Schultz, J., and Wong, C.-H. (1997) Mechanism of Human  $\alpha$ -1,3-Fucosyltransferase V: Glycosidic Cleavage Occurs Prior to Nucleophilic Attack, *Biochemistry* 36, 823–831.
- Burkart, M. D., Vincent, A., Duffels, B. W., Ley, S. V., and Wong, C.-H. (2000) Chemo-enzymatic synthesis of fluorinated sugar nucleotide: Useful mechanistic probes for glycosyltransferases, *Bioorg. Med. Chem.* 8, 1937–1946.
- Compain, P., and Martin, O. R. (2003) Design, Synthesis and Biological Evaluation of Iminosugar-Based Glycosyltransferase Inhibitors, *Curr. Top. Med. Chem.* 3, 541–560.
- Banait, N. S., and Jencks, W. P. (1991) Reactions of anionic nucleophiles with  $\alpha$ -D-glucopyranosyl fluoride in aqueous solution through a concerted,  $\text{A}_{\text{N}}\text{D}_{\text{N}}$  ( $\text{S}_{\text{N}}2$ ) mechanism, *J. Am. Chem. Soc.* 113, 7951–7958.
- Lairson, L. L., and Withers, S. G. (2004) Mechanistic analogies amongst carbohydrate modifying enzymes, *Chem. Commun.* 2243–2248.
- Williams, S. J., and Withers, S. G. (2000) Glycosyl fluorides in enzymatic reactions, *Carbohydr. Res.* 327, 27–46.
- Hartman, M., and Coward, J. K. (2002) Synthesis of 5-Fluoro N-Acetylglucosamine Glycosides and Pyrophosphates via Epoxide Fluoridolysis: Versatile Reagents for the Study of Glycoconjugate Biochemistry, *J. Am. Chem. Soc.* 124, 10036–10053.
- Lee, J., and Coward, J. K. (1992) Enzyme-catalyzed glycosylation of peptides using a synthetic lipid disaccharide substrate, *J. Org. Chem.* 57, 4126–4135.
- Lee, J., and Coward, J. K. (1993) Oligosaccharyltransferase: Synthesis and use of deuterium-labeled peptide substrates as mechanistic probes, *Biochemistry* 32, 6794–6801.
- Xu, T., and Coward, J. K. (1997)  $^{13}\text{C}$ - and  $^{15}\text{N}$ -labeled peptide substrates as mechanistic probes of oligosaccharyltransferase, *Biochemistry* 36, 14683–14689.
- Schenk, B., Fernandez, F., and Waechter, C. J. (2001) The inside and outside of dolichyl phosphate biosynthesis and recycling in the endoplasmic reticulum, *Glycobiology* 11, 61R–70R.
- Chantret, I., Dancourt, J., Barbat, A., and Moore, S. E. H. (2005) Two proteins homologous to the N- and C-terminal domains of the bacterial glycosyltransferase MurG are required for the second step of dolichyl-linked oligosaccharide synthesis in *Saccharomyces cerevisiae*, *J. Biol. Chem.* 280, 9236–9242.
- Bickel, T., Lehle, L., Schwarz, M., Aebi, M., and Jakob, C. A. (2005) Biosynthesis of lipid-linked oligosaccharides in *Saccharomyces cerevisiae* - Alg13p AND Alg14p for a complex required for the formation of GlcNAc<sub>2</sub>-PP-dolichol, *J. Biol. Chem.* 280, 34500–34506.
- Gao, X. D., Tachikawa, H., Sato, T., Jigami, Y., and Dean, N. (2005) Alg14 recruits Alg13 to the cytoplasmic face of the endoplasmic reticulum to form a novel bipartite UDP-N-acetylglucosamine transferase required for the second step of N-linked glycosylation, *J. Biol. Chem.* 280, 36254–36262.
- Ashida, H., Maeda, Y., and Kinoshita, T. (2006) DPM1, the catalytic subunit of dolichol-phosphate mannose synthase, is tethered to and stabilized on the endoplasmic reticulum membrane by DPM3, *J. Biol. Chem.* 281, 894–904.
- Boullanger, P., Chevalier, Y., Croizier, M.-C., Lafont, D., and Sancho, M.-R. (1995) Synthesis and surface-active properties of some alkyl 2-amino-2-deoxy- $\beta$ -D-glucopyranosides, *Carbohydr. Res.* 278, 91–101.



23. Rush, J. S., Rick, P. D., and Waechter, C. J. (1997) Polyisoprenyl phosphate specificity of UDP-GlcNAc:undecaprenylphosphate N-acetylglucosaminyl 1-P transferase from *E. coli*, *Glycobiology* **7**, 315–322.
24. Waechter, C. J., and Harford, J. B. (1979) A dolichol-linked trisaccharide from calf brain: biosynthesis and structure, *Arch. Biochem. Biophys.* **192**, 380–390.
25. Zhu, X., Zeng, Y., and Lehrman, M. (1992) Evidence that the hamster tunicamycin resistance gene encodes UDP-GlcNAc-dolichol phosphate N-acetylglucosamine-1-phosphate transferase, *J. Biol. Chem.* **267**, 8895–8902.
26. This reference was deleted on revision.
27. Cornish-Bowden, A. (1995) *Analysis of Enzyme Kinetic Data*, Oxford University Press, Oxford.
28. Wittmann, V., and Wong, C.-H. (1997) 1*H*-tetrazole as a catalyst in phosphomorpholidate coupling reactions: efficient synthesis of GDP-fucose, GDP-mannose, and UDP-galactose, *J. Org. Chem.* **62**, 2144–2147.
29. Moffatt, J. G., and Khorana, H. G. (1961) Nucleoside polyphosphates. X. The synthesis and some reactions of nucleoside-5'-phosphomorpholidates and related compounds. Improved methods for the preparation of nucleoside-5' polyphosphates, *J. Am. Chem. Soc.* **83**, 649–658.
30. Hoard, D., and Ott, D. G. (1965) Conversion of mono- and oligodeoxyribonucleotides to 5'-triphosphates, *J. Am. Chem. Soc.* **87**, 1785–1788.
31. Fang, X., Gibbs, B. S., and Coward, J. K. (1995) Synthesis and evaluation of synthetic analogues of dolichyl-P-P-chitobiose as oligosaccharyltransferase substrates, *Bioorg. Med. Chem. Lett.* **5**, 2701–2706.
32. Tai, V. W. F., and Imperiali, B. (2001) Substrate specificity of the glycosyl donor for oligosaccharyl transferase, *J. Org. Chem.* **66**, 6217–6228.
33. Tai, V. W. F., O'Reilly, M. K., and Imperiali, B. (2001) Substrate specificity of N-acetylglucosaminyl(diphosphodolichol) N-acetylglucosaminyl transferase, a key enzyme in the dolichol pathway, *Bioorg. Med. Chem.* **9**, 1133–1140.
34. Ye, X. Y., Lo, M. C., Brunner, L., Walker, D., Kahne, D., and Walker, S. (2001) Better substrates for bacterial transglycosylases, *J. Am. Chem. Soc.* **123**, 3155–3156.
35. Zhang, Y., Fechter, E. J., Wang, T. S., Barrett, D., Walker, S., and Kahne, D. E. (2007) Synthesis of heptaprenyl-lipid IV to analyze peptidoglycan glycosyltransferases, *J. Am. Chem. Soc.* **129**, 3080–3081.
36. Hartman, M. C. T. (2002) Ph.D. Thesis, University of Michigan, Ann Arbor.
37. McLachlan, K. R., and Krag, S. S. (1994) Three enzymes involved in oligosaccharide-lipid assembly in Chinese hamster ovary cells differ in the lipid substrate preference, *J. Lipid. Res.* **35**, 1861–1838.
38. Kean, E. L., and Niu, N. (1998) Kinetics of formation of GlcNAc-GlcNAc-P-P-dolichol by microsomes from the retina of the embryonic chick, *Glycoconjugate J.* **15**, 11–17.
39. Morrison, J. F., and Ebner, K. E. (1971) Studies of galactosyltransferase - kinetic investigations with N-acetylglucosamine as galatosyl group acceptor, *J. Biol. Chem.* **246**, 3971–3984.
40. Bell, J. E., Beyer, T. A., and Hill, R. L. (1976) The kinetic mechanism of bovine milk galactosyltransferase - role of lactalbumin, *J. Biol. Chem.* **251**, 3003–3013.
41. Ramakrishnan, B., and Qasba, P. K. (2001) Crystal structure of lactose synthase reveals a large conformational change in its catalytic component, the  $\beta$ -1,4-galactosyltransferase-1, *J. Mol. Biol.* **310**, 205–218.
42. Palcic, M. M., Heerze, L. D., Pierce, M., and Hindsgaul, O. (1988) The use of hydrophobic synthetic glycosides as acceptors in glycosyltransferase assays, *Glycoconjugate J.* **5**, 49–53.
43. Kim, S. C., Singh, A. N., and Raushel, F. M. (1988) Analysis of the galactosyltransferase reaction by positional isotope exchange and secondary deuterium isotope effects, *Arch. Biochem. Biophys.* **267**, 54–59.
44. Takahashi, S., Cohen, L. A., Miller, H. K., and Peake, E. G. (1971) Calculation of the pK<sub>a</sub> values from sigma constants and from the carbonyl frequencies of their esters, *J. Org. Chem.* **36**, 1205–1209.
45. Timperley, C. M., and White, W. E. (2003) The steric and electronic effects of aliphatic fluoroalkyl groups, *J. Fluorine Chem.* **123**, 65–70.
46. Ta-Shma, R., and Jencks, W. P. (1986) How does a reaction change its mechanism? General base catalysis of the addition of alcohols to 1-phenylethyl carbocations, *J. Am. Chem. Soc.* **108**, 8040–8050.
47. Richard, J. P., Westerfeld, J. G., Lin, S., and Beard, J. (1995) Structure-reactivity relationships for beta-galactosidase (*Escherichia coli*, lac Z). 2. Reactions of the galactosyl-enzyme intermediate with alcohols and azide ion, *Biochemistry* **34**, 11713–11724.
48. Hayashi, T., Murray, B. W., Wang, R., and Wong, C.-H. (1997) A chemoenzymatic synthesis of UDP-(2-deoxy-2-fluoro)galactose and evaluation of its interaction with galactosyltransferase, *Bioorg. Med. Chem. Lett.* **5**, 497–500.

BI700863S