Mechanism and Specificity of Human α-1,3-Fucosyltransferase V†

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ABSTRACT: Human α-1,3-fucosyltransferase catalyzes the transfer of the L-fucose moiety from guanosine diphosphate−β-L-fucose (GDP−Fuc) to acceptor sugars to form biologically important fucoglycoconjugates, including sialyl Lewis x (SLe^x). Evidence for a general base mechanism is supported by a pH−rate profile that revealed a catalytic residue with a pK_a of 4.1. The characterized solvent kinetic isotope effect ($D_V = 2.9$, $D_{V/K} = 2.1$) in a proton inventory study indicates that only one-proton transfer is involved in the catalytic step leading to the formation of the transition state. Evidence for Mn^{2+} as an electrophilic catalyst was supported by the observation that the nonenzymatic transfer of L-fucose from GDP−Fuc to the hydroxyl group of water in the presence of 10 mM $MnCl_2$ at 20 °C was accelerated from $k_{obs} = 3.5 \times 10^{-6}$ to 3.8×10^{-5} min^{-1}. Using the GDP−Fuc hydrolysis as the nonenzymatic rate, the enzymatic proficiency of FucT V, $(k_{cat}/K_{i,GDP-fuc} \cdot K_{m,LacNAc})/k_{non}$, was estimated to be 1.2×10^{10} M^{-1} with a transition-state affinity of 8.6×10^{-11} M. The K_m for Mn^{2+} was determined to be 6.1 mM, and alternative divalent metal cofactors were identified as Ca^{2+}, Co^{2+}, and Mg^{2+}. Detailed kinetic characterization of the acceptor sugar specificity indicated that incorporation of hydrophobic functionality [e.g. -O-$(CH_2)_5CO_2CH_3$] to the reducing end of the acceptor sugar substantially decreased the $K_{m,acceptor}$ by over 100-fold. The role of the nucleotide was investigated by studying the inhibition of nucleotides, including the guanosine series. The inhibitory potency trend (GTP ≈ GDP > GMP >> guanosine) is consistent with bidentate chelation of Mn^{2+} by GDP−Fuc. The role of charge and distance in the synergistic inhibitory effect by the combination of GDP, an aza sugar, and the acceptor sugar was probed. A mechanism for fucosyl transfer incorporating these findings is proposed and discussed.

Cell surfaces contain α-1,3-fucosylated oligosaccharide structures, including the trisaccharide Lewis x (Le^x)[1] and sialyl Le^x (SLe^x), that are central to numerous cell−cell interactions (Ichikawa et al., 1994) such as inflammation, tumor development, and blood clotting (Foxall et al., 1992; Parekh & Edge, 1994). Biosynthesis of complex oligosaccharide structures is directed by a family of at least 100 glycosyltransferases, which transfer the sugar moiety from an activated nucleotide−sugar donor to an acceptor sugar (Field & Wainwright, 1995; Paulson & Colley, 1989). Fucosyltransferases utilize guanosine diphosphate−β-L-fucose (GDP−Fuc) as a donor sugar to form α-1,2, α-1,3/4, α-1,3, or α-1,6 linkages (Field & Wainwright, 1995; Palcic, 1994). Five distinct human α-1,3-fucosyltransferases have been cloned (Kukowska-Latallo et al., 1990; Lowe et al., 1991; McCurley et al., 1995; Reguigne-Arnould et al., 1995; Sasaki et al., 1994; Weston et al., 1992a; Weston et al., 1992a,b) and shown to have different acceptor sugar specificity. α-1,3-Fucosyltransferase V (FucT V) is responsible for the terminal step in the biosynthesis of Lewis x (Le^x) and sialyl Lewis x (sLe^x) (Figure 1). DNA sequence analysis of α-1,3-fucosyltransferase III, V, and VI revealed greater than 85% homology, suggesting that these enzymes evolved from common ancestor genes (Reguigne-Arnould et al., 1995). The hypervariable region which codes for 90% of the amino acid differences between FucT III and V (Walz et al., 1990; Weston et al., 1992a,b) is proposed to be responsible for the differential acceptor specificity.

Despite the importance of the reaction that FucT V catalyzes, very little is known about the detailed mechanism. The α-1,3-fucosyltransferase V-catalyzed reaction proceeds with inversion of configuration at the anomeric center of L-fucose (Weston et al., 1992a). Product inhibition studies have been used to establish that FucT V has an ordered, sequential, Bi−Bi mechanism with GDP−Fuc binding first and the product GDP releasing last (Qiao et al., 1996). A related enzyme, α-1,2-fucosyltransferase, has been hypothesized to proceed through a simple ion-pair transition state which results in the inversion of anomeric configuration (Palcic et al., 1989). α-1,3-Fucosyltransferases may operate via a similar mechanism involving a displacement of GDP by the acceptor hydroxyl group assisted by a catalytic active site base. The transition-state structure of glycosyltransferase-catalyzed reactions has been proposed to have a flattened half-chair conformation with substantial oxocarbenium ion character at the anomeric position, analogous to that of the glycosidase reactions (Kim et al., 1988; Look et al., 1993; Sinnott, 1990). This is supported by the observation that aza sugars which mimic the charge distribution of the glycosyl cation are inhibitors of FucT V, and synergistic inhibition by the combination of an aza sugar, GDP, and the acceptor sugar to mimic the transition-state structure has been illustrated (Ichikawa et al., 1992; Look et al., 1993; Qiao et al., 1996).

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[1] Abbreviations: FucT V, α-1,3-fucosyltransferase V; FucT, fucosyltransferase; GDP−Fuc, guanosine diphosphate−β-L-fucose; GDP, guanosine diphosphate; D_V, kinetic isotope effect on V_{max}; $D_{V/K}$, kinetic isotope effect on V_{max}/K_m; Le^x, Lewis x; SLe^x, sialyl Lewis x; LacNAc, *N*-acetyllactosamine, Galβ1,4GlcNAc; DFN, deoxyfuconojirimycin.

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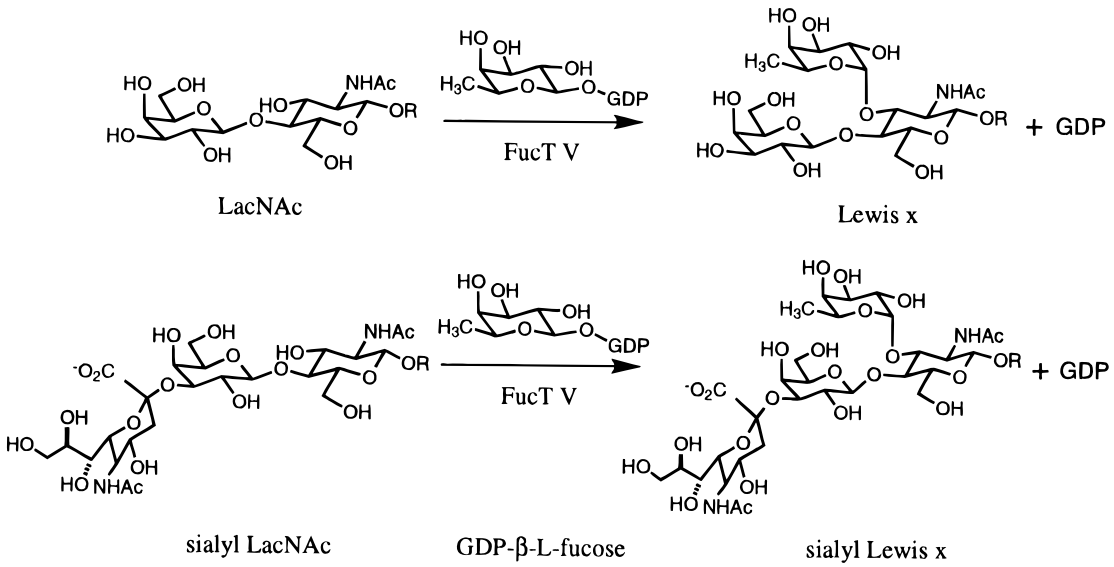

FIGURE 1: Reaction catalyzed by human α-1,3-fucosyltransferase V. The enzyme can utilize both uncharged structures such as *N*-acetyllactosamine (LacNAc) or charged structures such as sialyl LacNAc.

The apparent K_m of GDP−Fuc for FucT V has been reported to be 9 μM with Fucα1,2Galβ1,4GlcNAcβOR as the acceptor sugar (de Vries et al., 1995). Acceptor sugar studies demonstrated that FucT V utilizes either charged or uncharged type 2 sugars, including *N*-acetyllactosamine (LacNAc) and sialyl LacNAc with K_m values of 35 and 100 mM, respectively (de Vries et al., 1995; Ichikawa et al., 1992). Galβ1,4(3-deoxy)GlcNAcβ-O-allyl (3-deoxy-LacNAc) was found to be a very weak inhibitor with a K_i of 710 mM, indicating the importance of this hydroxyl group in substrate recognition (Ichikawa et al., 1992). Further studies on LacNAc derivatives that had either the hydroxyl at C-6 of galactose or the hydroxyl at C-3 of GlcNAc replaced with hydrogen did not function as a substrate or inhibitor, and the two hydroxyl groups were termed "key polar groups" (de Vries et al., 1995). Inactivation of enzymes by *N*-ethylmaleimide (NEM) has been used to discriminate among α-1,3-fucosyltransferases (de Vries et al., 1995), and a detailed study of FucT V inactivation by NEM has concluded that cysteine 156 in the GDP−Fuc binding domain is responsible for the inactivation (Holmes et al., 1995).

In this paper, we further investigate the reaction of FucT V. Evidence is presented for general base catalysis based on pH−rate profiles and solvent kinetic isotope effects. The metal cofactor specificity and its role in catalysis were investigated. The nonenzymatic hydrolysis of GDP−Fuc in the presence of metal ions and detailed kinetic analysis of FucT V were used to calculate the enzymatic proficiency, transition-state affinity, and rate acceleration. In addition, studies of acceptor specificity and of synergistic inhibition by GDP, aza sugars and acceptor sugars, have also been conducted in order to delineate the mechanism of FucT V catalyzed reactions.

MATERIALS AND METHODS

General Methods

N-acetyllactosamine, all nucleotides, guanosine 5′-diphosphate−β-fucose, guanosine-5′-diphospho-β-D-mannose, guanosine-5′-diphosphate−β-D-glucose, Dowex 1 × 8, and cacodylic acid were purchased from Sigma. Guanosine 5′-diphosphate−L-[U-^{14}C]fucose, GDP−[U-^{14}C]-β-L-fucose, GDP−[U-^{14}C]-β-D-mannose, and [U-^{14}C]-β-L-fucose were purchased from Amersham Life Science. ScintiVerseI scintillation cocktail and $MnCl_2 \cdot 4H_20$ were purchased from Fisher Scientific Co. Deoxyfuconojirimycin was purchased from Toronto Research Chemicals, Inc. Protein concentrations were determined with the Coomassie protein staining reagent with albumin standards as purchased from Pierce. The scintillation counter used was the Beckman LS 3801.

Acceptor Sugar Synthesis

In a typical procedure, crude β-D-galactosidase from *Bacillus circulans* (Daiwa Kasei) (50 mg) was added to a mixture of α-lactose monohydrate (3300 mg, 9.16 mmol) and GlcNAcαOBn (570 mg, 1.83 mmol) in a buffered solution (20 mM pH 7.0 phosphate buffer/acetonitrile, 1/1, 20 mL). The reaction mixture was stirred under an argon atmosphere for 48 h at room temperature, after which it was concentrated *in vacuo*. The residue was chromatographed (silica gel; $CHCl_3$/MeOH/30% NH_4OH_{aq}, 3/1/0.3) to give recovered (446 mg) and the disaccharide Galβ1,4GlcNAcα-O-Bn (107 mg, 14% yield). Further elution (*i*-PrOH/H_2O/30% NH_4OH_{aq}, 7/2/1) afforded the trisaccharide Galβ1,-4Galβ1,4GlcNAcα-O-Bn (**5**) (42 mg, 4% yield).

Galβ1,4GlcNAcα-O-Bn. ^{1}H NMR (400 MHz, D_2O) δ 1.94 (s, 3H, CH_3), 3.53 (dd, 1H, J = 7.8 and 9.8 Hz, 2′-H), 3.65 (dd, 1H, J = 3.2 and 9.9 Hz, 3′-H), 3.68−3.77 (m, 4H), 3.86−3.91 (m, 6H), 4.46 (d, 1H, J = 7.8 Hz, 1′-H), 4.53 (d, 1H, J = 12.0 Hz, benzyl), 4.74 (d, 1H, J = 11.9 Hz, benzyl), 4.93 (d, 1H, J = 1.2 Hz, 1-H), 7.38−7.45 (m, 5H, phenyl); ^{13}C NMR (100 MHz, D_2O) δ 22.2, 53.7, 60.3, 61.4, 68.9, 69.9, 70.1, 71.2, 71.4, 73.0, 75.7, 79.2, 96.0, 103.3, 128.8, 128.9, 129.2, 137.3, 174.6;HRMS calcd for $C_{21}H_{31}O_{11}NCs$ (M + Cs) 606.0951, found 606.0926.

Galβ1,4GlcNAcβ-O-Bn. In a manner similar to the typical procedure, the disaccharide Galβ1,4GlcNAcβ-O-Bn (14%) was obtained from GlcNAcβ-O-Bn (390 mg, 1.2 mmol): ^{1}H NMR (400 MHz, D_2O) δ 1.94 (s, 3H), 3.51 (dd, 1H, J = 7.7 and 9.9 Hz, 2′-H), 3.53−3.59 (m, 1H, 5-H), 3.61−3.78 (m, 7H), 3.84 (dd, 1H, J = 5.1 and 12.3 Hz, 6_a-H), 3.91 (d, 1H, J = 3.3 Hz, 4′-H), 4.00 (dd, 1H, J = 2.1

and 12.4 Hz, 6_b-H), 4.45 (d, J = 7.8 Hz, 1- or 1′-H), 4.53 (d, 1H, J = 8.4 Hz, 1- or 1′-H), 4.66 (d, 1H, J = 12.1 Hz, benzylic), 4.88 (d, 1H, J = 12.1 Hz, benzylic), 7.35−7.46 (m, 5H, phenyl); ^{13}C NMR (100 MHz, D_2O) δ 22.5, 55.5, 60.5, 61.4, 68.9, 71.4, 71.9, 72.8, 72.9, 75.2, 75.8, 78.8, 100.2, 103.3, 128.88, 129.02, 129.14, 137.1, 174.9; HRMS calcd for $C_{21}H_{31}O_{11}NNa$ (M + Na) 496.1795, found 496.1783.

Galβ1,4GlcNAcβ-S-Ph and Galβ1,4Galβ1,4GlcNAcβ-S-Ph. In a manner similar to the typical procedure, the disaccharide Galβ1,4GlcNAcβ-S-Ph (13%) and trisaccharide Galβ1,4Galβ1,4GlcNAcβ-S-Ph (2.4%) were obtained from GlcNAcβ-S-Ph (3.26 g, 10.4 mmol).

Galβ1,4GlcNAcβ-S-Ph. ^{1}H NMR (400 MHz, D_2O) δ 2.04 (s, 3H), 3.55 (dd, 1H, J = 7.8 and 10.0 Hz, 2′-H), 3.60−3.66 (m, 1H, 5-H), 3.67 (dd, 1H, J = 3.4 and 10.0 Hz, 3′-H), 3.71−3.90 (m, 7H), 3.95 (d, 1H, J = 3.4 Hz, 4′-H), 4.01 (dd, 1H, J = 2.2 and 12.4 Hz, 6-H), 4.48 (d, 1H, J = 7.8 Hz, 1′-H), 4.94 (d, 1H, J = 10.3 Hz, 1-H), 7.39−7.42 (m, 3H, phenyl), 7.52−7.55 (m, 2H, phenyl); ^{13}C NMR (100 MHz, D_2O) δ 22.6, 54.5, 60.6, 61.5, 69.0, 71.4, 72.9, 74.2, 75.8, 78.5, 79.3, 86.8, 103.3, 128.6, 129.8, 131.8, 132.7, 174.6; HRMS calcd for $C_{20}H_{30}O_{10}N_1S_1$ (M + H^+) 476.1608, found 476.1608.

Galβ1,4Galβ1,4GlcNAcβ-S-Ph. ^{1}H NMR (400 MHz, D_2O) δ 2.00 (s, 3H, CH_3), 3.58−3.87 (m, 17H), 4.15 (d, 1H, J = 3.0 Hz, 4′-H), 4.48 (d, 1H, J = 7.8 Hz, 1′- or 1″-H), 4.56 (d, 1H, J = 7.8 Hz, 1′- or 1″-H), 4.90 (d, 1H, J = 10.4 Hz, 1-H), 7.32−7.40 (m, 3H, phenyl); 7.48−7.51 (m, 2H, phenyl); ^{13}C NMR (100 MHz, D_2O) δ 22.6, 54.5, 60.5, 61.1, 61.4, 69.0, 71.7, 71.8, 73.2, 73.3, 74.2, 74.9, 75.5, 77.5, 78.6, 79.3, 86.8, 103.3, 104.6, 128.5, 129.7, 131.8, 132.6, 174.7; HRMS calcd for $C_{26}H_{39}O_{15}N_1S_1Cs$ (M + H^+) 770.1095, found 770.1070.

Galβ1,4GlcNAcβ-O-$(CH_2)_5CO_2$Me. In a manner similar to the typical procedure, the disaccharide Galβ1,4GlcNAcβ-O-$(CH_2)_5CO_2$Me (13%) was obtained from GlcNAcβ-O-$(CH_2)_5CO_2$Me (4.85 g, 14.5 mmol): ^{1}H NMR (400 MHz, D_2O) δ 1.32−1.37 (m, 2H), 1.53−1.65 (m, 4H), 2.04 (s, 3H, C(O)CH_3), 2.40 (t, 2H, J = 7.2 Hz, CH_2C(O)), 3.52−3.60 (m, 3H), 3.65−3.93 (m, 13H), 3.99 (d, 1H, J = 8.2 Hz, 6-H), 4.47 (d, 1H, J = 7.8 Hz, 1- or 1′-H), 4.52 (d, 1H, J = 8.4 Hz, 1- or 1′-H); ^{13}C NMR (100 MHz, D_2O) δ 22.6, 24.4, 25.1, 28.7, 34.1, 52.5, 55.6, 60.5, 61.5, 69.0, 70.7, 71.4, 72.9, 73.0, 75.2, 75.8, 78.9, 101.5, 103.3, 174.8, 178.0; HRMS calcd for $C_{21}H_{37}O_{13}NCs$ (M + Cs^+) 644.1319, found 644.1297.

Galβ1,4GlcNAcβ-O-$(CH_2)_7CH_3$. In a manner similar to the typical procedure, the disaccharide Galβ1,4GlcNAcβ-O-$(CH_2)_7CH_3$ (8%) was obtained from GlcNAcβ-O-$(CH_2)_5$-CO_2Me (520 mg, 1.6 mmol): ^{1}H NMR (400 MHz, DMSO-*d*/D_2O = 97/3) δ 0.83 (t, 3H, J = 6.8 Hz, CH_2CH_3), 1.20−1.28 (m, 10H), 1.34−1.48 (m, 2H), 1.81 (s, 3H, C(O)CH_3), 3.25−3.35 (m, 5H), 3.41−3.49 (m, 5H), 3.58−3.61 (m, 2H), 3.67−3.75 (m, 2H), 4.21 (d, 1H, J = 7.5 Hz, 1- or 1′-H), 4.27 (d, 1H, J = 7.8 Hz, 1- or 1′-H); ^{13}C NMR (100 MHz, DMSO-*d*/D_2O = 97/3) δ 14.0, 22.2, 22.9, 25.4, 28.8 (two carbons), 29.1, 31.3, 54.7, 60.38, 60.42, 68.1, 68.5, 70.5, 72.2, 73.1, 75.0, 75.6, 81.4, 100.9, 104.0, 168.8; HRMS calcd for $C_{22}H_{41}O_{11}NCs$ (M + Cs) 628.1734, found 628.1754.

Galβ1,4GlcN$_3$β-S-Ph. In a manner similar to the typical procedure, the disaccharide Galβ1,4GlcN$_3$β-S-Ph (4%) was obtained from GlcN$_3$β-S-Ph (600 mg, 2.0 mmol): ^{1}H NMR (400 MHz, DMSO-*d*/D_2O = 97/3) δ 3.19 (dd, 1H, J = 10.2 and 10.2 Hz, 2-H), 3.28−3.33 (m, 2H), 3.38−3.56 (m, 6H), 3.62−3.64 (m, 2H), 3.74 (d, 1H, J = 12.0 Hz, 6-H), 4.22 (d, 1H, J = 6.6 Hz, 1′-H), 4.71 (d, 1H, J = 10.3 Hz, 1-H), 7.29−7.37 (m, 3H, phenyl), 7.47−7.49 (m, 2H, phenyl). ^{13}C NMR (100 MHz, *d*-DMSO/D_2O=97/3) δ 14.0, 22.2, 22.9, 25.4, 28.8 (two carbons), 60.1, 60.6, 64.9, 68.2, 70.6, 73.3, 74.7, 75.7, 78.8, 79.6, 83.8, 103.8, 127.6, 129.2, 131.2, 132.5; HRMS calcd for $C_{18}H_{25}O_9N_3S_1Cs$ (M + Cs) 592.0366, found 592.0386.

Galβ1,4GlcNH$_2$β-S-Ph. A mixture of Galβ1,4GlcN$_3$β-S-Ph (10 mg) and 5% Rh-carbon (4 mg) in H_2O (0.5 mL) was stirred under an atmospheric pressure of hydrogen gas for 1.5 h at room temperature. The reaction mixture was filtered and concentrated to give Galβ1,4GlcNH$_2$β-S-Ph in quantitative yield: ^{1}H NMR (400 MHz, D_2O) δ 2.76 (dd, 1H, J = 9.7 and 9.7 Hz, 2-H), 3.53 (dd, 1H, J = 7.8 and 10.1 Hz, 2′-H), 3.55−3.79 (m, 7H), 3.81 (dd, 1H, J = 4.6 and 12.3 Hz, 6_a-H), 3.92 (d, 1H, J = 3.4 Hz, 4′-H), 3.96 (dd, 1H, J = 1.8 and 12.2 Hz, 6_b-H), 4.44 (d, J = 7.8 Hz, 1′-H), 4.74 (d, 1H, J = 9.9 Hz, 1-H), 7.40−7.45 (m, 3H, phenyl), 7.58−7.61 (m, 2H, phenyl); ^{13}C NMR (100 MHz, D_2O) δ 55.4, 60.6, 61.5, 69.0, 71.4, 73.0, 75.8, 76.1, 78.8, 79.3, 88.3, 103.4, 128.7, 129.8, 132.3, 135.9; HRMS calcd for $C_{18}H_{27}O_9N_1S_1Cs$ (M + Cs^+) 566.0461, found 566.0475.

α-*1,3-Fucosyltransferase V*

Soluble FucT V was expressed in the filamentous fungus, *Aspergillus niger* var. *awamori*. The strain was chromosomally integrated with a gene construct containing the FucT V catalytic domain (Weston et al., 1992a) fused to the glucoamylase promoter and coding region (Dunn-Coleman et al., 1991) and expressed as a fusion protein. The fusion protein has a kex 2 proteolytic site that is cleaved in the secretory pathway to liberate the catalytic domain and the stem region of FucT V. The enzyme purity was greater than 90%. Approximately 300 units of FucT V (1 unit = 1 μmol of GDP−Fuc consumed per minute with LacNAc acceptor) can be produced per liter. FucT V was purified from fungal supernatants by 20−60% ammonium sulfate precipitation and phenyl-Sepharose column chromatography. The enzyme was stored at −20 °C in 150 mM NaCl, 50 mM MOPS (pH 7.0), 10 mM $MnCl_2$, and 50% glycerol.

The activity of FucT V was detected by the assay described previously (Sarnesto et al., 1992). Typically, assays contained 10 mM $MnCl_2$, 0.2 milliunit of purified FucT V, and 25 mM cacodylate buffer (pH 6.2) in a total assay volume of 0.05 mL. Assays were performed at 25 °C. Reactions were halted with the addition of 0.5 mL of distilled, deionized water. GDP−Fuc was separated from the product, Lewis x, with a 1.0 mL Dowex 1 × 8 pipette column. The reaction mixtures were applied to the column and washed with 0.3 mL of distilled, deionized water three times. The flow-through and the column washes were collected in 10 mL of ScintiVerse I scintillation cocktail. Control reactions, without enzyme, were used to establish the background, nonenzymatic cleavage rate. A typical control reaction of 32 672 cpm guanosine 5′-diphosphate-L-[U-^{14}C]fucose would result in 173 cpm of nonenzymatic column flow-through.

An initial experiment with 24 mM LacNAc and 0.05 mM GDP−Fuc (0.67 Ci/mol) was used to define the time course of the reaction. Aliquots (0.05 mL) of the 0.25 mL enzymatic reaction mixture were taken at 5, 10, 15, 30, 60,

90, 155, and 330 min. Time points at 30 min or less were judged to be in the linear portion of the velocity−time profile. All subsequent data were obtained with 1 h of reaction time. Kinetic parameters for α-1,3-fucosyltransferase V were in agreement with the literature values.

Data Analysis

Precise kinetic constants for FucT V were determined with a nonlinear, least-squares fit of the kinetic data to the equation for an ordered sequential, Bi−Bi mechanism with the Sequenl FORTRAN program of Cleland (eq 1) (Cleland, 1979). Apparent kinetic parameters were determined by subjecting the data to nonlinear least-squares fit of the Michaelis−Menten equation with the Hypero FORTRAN program of Cleland (eq 2) (Cleland, 1979). The pK_a determination used the Bell (eq 3) and Habell (eq 4) FORTRAN nonlinear, least-square programs adapted for the Apple Macintosh (Cleland, 1979). There are Gross−Butler equations for one proton (eq 5) and two protons (eq 6) in flight in the transition state. F_i is the mole fraction of D_2O and ϕ_n^T values are the isotopic fractionation factors of individual hydrogenic positions (transferred protons) in the transition state in which $n = 1$ and 2. V_{F_i} and V_{F_0} are values of V measured at a mole fraction of D_2O of F_i or 0, respectively (Schowen & Schowen, 1982; Venkatasubban & Schowen, 1985). The Arrhenius equation (eq 7) was used to determine the nonenzymatic rate constant for the hydrolysis of GDP−Fuc. A steady-state derivation for the synergistic inhibition of FucT V has been reported (eq 8) (Qiao et al., 1996)

$$v = V_{max}[\text{LacNAc}][\text{GDP−Fuc}]/(K_{i,\text{GDP−Fuc}}K_{m,\text{LacNAc}}/K_{m,\text{GDP−Fuc}} + [\text{GDP−Fuc}]K_{m,\text{LacNac}} + [\text{LacNac}]/K_{m,\text{GDP−Fuc}} + [\text{LacNAc}][\text{GDP−Fuc}]) \quad (1)$$

$$v = V_{max}*[S]/([S] + K_m*) \quad (2)$$

$$\log v = \log(C/(1 + [H^+]/K_a + K_b/[H^+])) \quad (3)$$

$$\log v = \log[C/(1 + [H^+]/K_a)] \quad (4)$$

$$V_{F_i} = V_0(1 - F_i + F_i\phi^T) \quad (5)$$

$$V_{F_i} = V_0(1 - F_i + F_i\phi_1^T)(1 - F_i + F_i\phi_2^T) \quad (6)$$

$$k = Ae^{-E^*/RT} \quad (7)$$

$$v = V_{max}[\text{GDP−Fuc}]/[K_{m,\text{GDP−Fuc}}I^*[1 + K_{i,\text{GDP−Fuc}}K_{m,\text{LacNAc}}/(K_{m,\text{GDP−Fuc}}[\text{LacNac}])] + [\text{GDP−Fuc}](1 + K_{m,\text{LacNac}}/[\text{LacNac}])]$$

$$I^* = 1 + \frac{[\text{GDP}]}{K_{i,\text{GDP}}} + \frac{[\text{aza,sugar}]}{K_{i,\text{aza sugar}}} + \frac{[\text{GDp}][\text{aza sugar}]}{\alpha K_{i,\text{GDP}}K_{i,\text{aza sugar}}} \quad (8)$$

where the asterisks represent apparent kinetic constants in eqs 1 and 2.

pH−Rate Studies

The stability of FucT V in the 3−10 pH range was determined. FucT V (0.2 milliunit, 5 μL) was incubated with an equal volume of 25 mM phosphate buffer at variable pH values. A 30 min incubation was followed by a 5-fold dilution with a 100 mM pH 6.0 buffer containing enzyme substrates and cofactors to a final volume of 50 μL. The final concentrations were as follows: 10 mM Mn^{2+}, 20 mM LacNAc, and 0.1 mM GDP−Fuc. Phosphate buffers 25 mM at either pH 3 or 10 quenched with 4 times the volume of a 100 mM pH 6.0 phosphate buffer was found to have a final pH value of 6.0. A study that monitored the relative enzyme velocity as a function of pH was initially conducted in two different buffer systems: 25 mM cacodylate and 25 mM phosphate. All reactions were for 30 min, and all mixtures contained 0.2 milliunit of FucT V, 10 mM $MnCl_2$, 20 mM LacNAc, and 0.050 mM GDP−Fuc in 0.050 mL. A full pH−rate profile was then undertaken. All reactions were for 30 min in a 25 mM phosphate buffer which contained 0.2 milliunit of FucT V, 10 mM $MnCl_2$, and 0.050 mM GDP−Fuc in 0.050 mL. LacNAc concentrations typically were varied from 5 to 80 mM. Kinetic data were analyzed with the Hypero FORTRAN program (eq 2), and evaluation of the kinetic parameters as a function of pH was done, the Habell FORTRAN program (eq 4) (Cleland, 1979).

Solvent Isotope Effect Studies

^2H_2O (99.96% ^2H; Cambridge Isotope Laboratories) was used to make the 25 mM cacodylate buffer. The pD was adjusted to 5.8 (pH = pD + 0.4) with ^2HCl (99.5% ^2H, Aldrich). LacNAc (200 mM) and $MnCl_2$ (100 mM) solutions were made with ^2H_2O (99.96% ^2H). All 50 μL assay reaction mixtures contained 1 μL of a 50%/50% glycerol/H_2O FucT V solution (0.313 milliunit/assay) and 2.5 μL of a 1 mM GDP−[U-^{14}C]fucose solution prepared in H_2O. Two 225 μL reaction mixtures (H_2O-type and D_2O-type) containing 4.5 μL of FucT V (313 milliunits/mL), 11.25 μL of GDP−Fuc (1 mM), 22.5 μL of LacNAc (200 mM), and 22.5 μL of $MnCl_2$ (100 mM) and 168.8 μL of cacodylate buffer was used to compare the velocity of FucT V in H_2O to D_2O with 50 μL aliquots taken at the following time points: 5, 10, 15, 30, and 70 min. A control experiment was performed to eliminate the possibility that the slower reaction in D_2O was due to its viscosity of 1.24 (Karsten et al., 1995). A 9% glycerol solution has a density of 1.24 and was used as a microviscogen control reaction. All of the 225 μL reaction mixtures each contained 4.5 μL of FucT V (313 milliunits/mL), 11.25 μL of GDP−Fuc (1 mM), 22.5 μL of LacNAc (200 mM), and 22.5 μL of $MnCl_2$ (100 mM). The reaction mixture with the glycerol microviscogen had 45 μL of glycerol (45%) and 117.8 μL 25 mM of cacodylate buffer, while the normal reaction mixture contained only 168.8 μL of cacodylate buffer. Aliquots (50 μL) were taken and assayed for product formation at the following times: 5, 15, 30, and 50 min.

Proton Inventory

A proton inventory experiment was conducted on FucT V (Venkatasubban & Schowen, 1985). Identical reaction mixtures, independently prepared with 100% H_2O and 100% D_2O components and variable LacNAc concentrations (4, 8, 20, and 48 mM), were combined to give 47.5 μL reaction mixtures with variable mole fractions of D_2O. The reactions were initiated with the addition of 2.5 μL of 1 mM GDP−Fuc. As stated previously, both FucT V (1 μL) and GDP−

Fuc (2.5 μL) contained H_2O such that the final mole fractions of D_2O were as follows: 0, 23.5, 47.0, 70.5, and 94%. Reactions were carried out at room temperature for 30 min. Nonlinear, least-squares analyses of the enzyme velocity data were performed with the Hypero FORTRAN program of Cleland (eq 2) (Cleland, 1979). Replots of the kinetic parameters (V_{max} and V_{max}/K_m) vs the mole fraction of D_2O were judged to be linear through the inspection of the residuals of the linear, least-squares fit and by analysis of the least-squares correlation coefficients to the Gross−Butler equations for one- (eq 5) and two-proton (eq 6) transfers in the transition state. F tests of the chi-squared values for the fits of the V_{max} and V_{max}/K_m data to eqs 5 and 6 were performed to determine which equation the data fit best.

Nonenzymatic Hydrolysis of GDP−Fuc

A common solution of GDP−β-L-[U-^{14}C]fucose was prepared: 37.5 μL of 1 mM GDP−Fuc and 713 μL of 25 mM cacodylate (pH 6.0). Aliquots (50 μL) were put in 0.5 mL plastic tubes and placed in heat blocks at the following temperatures: 50, 75, and 92 °C. Each sample initially contained 7791 cpm of GDP−β-L-[U-^{14}C]fucose. At fixed time periods, the reactions were halted with the addition of 0.5 mL of ice cold distilled, deionized water and the mixtures immediately loaded onto 1 mL Dowex 1 (chloride form) columns. The column load effluent and the subsequent three column washes were put directly into 10 mL of scintillation cocktail. For 50 °C, samples were taken at 65 (122 cpm), 75 (170 cpm), 454 (307 cpm), 1392 (887 cpm), and 2772 (1611 cpm) min. For 75 °C, samples were taken at 79 (610 cpm), 123 (876 cpm), 192 (1247 cpm), and 454 (2864 cpm) min. For 92 °C, samples were taken at 20 (597 cpm), 65 (1625 cpm), 75 (1771 cpm), and 123 (2940 cpm) min. Background radioactivity was determined to be 100 cpm by passing a sample of GDP−Fuc (7791 cpm) through a Dowex 1 column. A room-temperature control sample (2772 min) was subjected to the Dowex 1 chromatography and found to have only a background level of radioactivity (104 cpm). To eliminate the possibility that L-fucose was retained by the Dowex 1 column, L-[U-^{14}C]fucose (10 μL, 20 839 cpm) was loaded onto the column and subsequently washed with distilled, deionized water. The effluent contained 20 345 cpm which showed that no significant amount of L-fucose is retained by the Dowex 1 column. This protocol was repeated except that all reactions were performed in the presence of 10 mM $MnCl_2$.

Precise Kinetic Analysis of FucT V

A 3 × 4 matrix of GDP−Fuc and LacNAc concentrations was constructed to obtain the absolute kinetic parameters for the ordered, Bi−Bi mechanism of FucT V. FucT V velocity was measured as a function of LacNAc concentration (5, 10, 20, and 40 mM) at fixed GDP−Fuc concentrations (0.0250, 0.0500, and 0.100 mM). Data were initially evaluated by the method of Eadie−Hofstee ([LacNAc]/v vs [LacNAc]), and precise kinetic constants were derived from the Sequenl FORTRAN program of Cleland (eq 1) that performed a nonlinear least-squares fit to the kinetic equation for an ordered, sequential, Bi−Bi mechanism (Cleland, 1979). A 4 × 4 matrix of GDP−Fuc and LacNAcβ-O-$(CH_2)_5CO_2CH_3$ was used to determine the precise kinetic constants for this acceptor sugar. GDP−Fuc concentrations were as follows: 0.0125, 0.025, 0.050, and 0.10 mM. The LacNAcβ-O-$(CH_2)_5CO_2CH_3$ concentrations used were as follows: 0.10, 0.25, 0.50, and 1.0 mM. Hydrolysis of GDP−Fuc was measured as a function of pH in 0.050 mL of 25 mM phosphate buffers. GDP−Fuc (0.050 mM) was reacted at pH values of 3−9 at 78 °C for 60 min. The hydrolysis of 0.05 mM GDP−Fuc was monitored at 90 °C for 5, 10, and 15 min in 25 mM phosphate buffers (pH 4−8).

Metal Cofactor Studies

The manganese divalent ion concentration was varied (0.1, 0.2, 0.5, 2, 5, and 10 mM) at fixed concentrations of GDP−[U-^{14}C]fucose (0.0025 mM) and LacNAc (20 mM). Reaction mixtures were in 50 μL and each contained 0.31 milliunit of FucT V and 5.0 μL of 200 mM LacNAc. $MnCl_2$ stock solutions (100, 10, and 1 mM) were prepared in the assay buffer and used to achieve the desired metal ion concentration. The activity of FucT V was monitored by the Dowex 1 column method. Kinetic data were subjected to a nonlinear, least-squares fit to Michaelis−Menten equation with the Hypero FORTRAN program of Cleland (eq 2) (Cleland, 1979).

Alternative metal cofactors at a 5 mM level were investigated by observing the amount of enzymatic activity relative to a 5 mM concentration of $MnCl_2$. Stock solutions (100 mM) of $MgCl_2$, $CaCl_2$, $FeCl_2$, $CuCl_2$, $CoSO_4$, $NiCl_2$, $ZnCl_2$, and $MnCl_2$ were made in a 25 mM cacodylate buffer (pH 6.2). A stock solution containing 22.5 μL of 1 mM GDP−[U-^{14}C]fucose (73 267 cpm), 9 μL of FucT V, and 351 μL of 25 mM cacodylate buffer (pH 6.2) was prepared and separated into 42.5 μL aliquots. The metals were then added (2.5 μL, 100 mM), and the reactions were initiated with the addition of 5 μL of 200 mM LacNAc. The reactions were terminated after 30 min of reaction time with the addition of 500 μL of distilled, deionized water. To eliminate the possibility that a metal·GDP−Fuc would allow unreacted GDP−Fuc to pass through the Dowex 1 column, control reactions were performed in a manner identical to that of the enzymatic reactions except FucT V was omitted. Each reaction mixture contained 7991 cpm of GDP−[U-^{14}C]-fucose, and only a background level of radioactivity was observed to have passed through the Dowex 1 column (103−121 cpm).

Acceptor Specificity

Each 50 μL assay contained the following: 0.025 mM GDP−Fuc, 10 mM $MnCl_2$, and 0.1 milliunit of enzyme. Assays were performed in a 25 mM cacodylate solution (pH 6.2). Initial studies were conducted to find the range in which the acceptor sugar K_m could be found. The enzyme velocity was measured for assays containing 0.1, 1, and 10 mM acceptor sugar. Then, acceptor sugar concentrations were chosen to be above and below the K_m concentration. Nonlinear least-squares analysis was performed with the Hypero FORTRAN program of Cleland (eq 2) (Cleland, 1979) to yield accurate kinetic parameters. The acceptor sugar concentration ranges were as follows: LacNAc (5, 10, 20, 40, and 80 mM), Galβ1,4GlcNAcβ-O-$(CH_2)_5CO_2CH_3$ (0.125, 0.250, 0.500, 1.00, and 2.00 mM), Galβ1,4GlcNAcβ-O-$(CH_2)_7CH_3$ (0.200, 0.500, 1.00, and 2.00 mM; poor solubility at higher concentrations), GlcNAcβ-O-$(CH_2)_5CO_2$-CH_3 (20, 40, 80, 120, and 160 mM), Galβ1,4GlcNAcβ-S-

phenyl (0.100, 0.250, 0.500, 1.25, and 2.50 mM), Galβ1,-4Galβ1,4GlcNAcβ-S-phenyl (4.00, 8.00, 12.0, and 16.0 mM), Galβ1,4Glc$N_3\beta$-S-phenyl (2.50, 5.00, 10.0, and 20.0 mM), Galβ1,4Glc$NH_2\beta$-S-phenyl (5.00, 10.0, 20.0, and 40.0 mM), and Galβ1,4GlcNAcα-O-benzyl (0.100, 0.250, 0.500, 1.00, and 2.00 mM). FucT V-mediated hydrolysis of GDP−Fuc was performed in the absence of an acceptor sugar and with the following GDP−Fuc concentrations: 0.005 00, 0.01 00, 0.0250, 0.0500, and 0.100 mM.

Nucleotide Inhibition Studies

All FucT V reaction mixtures were 50 μL and contained 0.025 mM GDP−Fuc, 20 mM LacNAc, 10 mM $MnCl_2$, and 0.20 milliunit of FucTV in a 25 mM cacodylate buffer (pH 6.2). The V_{max} for LacNAc was determined by varying LacNAc and using the Hypero nonlinear, least-squares fit of the Michaelis−Menten equation (eq 2). Dixon analysis was employed utilizing $1/V_{max}$. The inhibitor concentrations used were as follows: L-Fucal (0, 10, 20, 50, 75, and 100 mM), DFN (0, 2.0, 15.0, 18.4, 25.0, and 40.0 mM), GDP−α-D-mannose (0, 0.10, 0.20, 0.50, 1.0, and 2.0 mM), GDP−α-D-glucose (0.0, 0.20, 0.50, 0.75, and 1.00), GMP (0, 0.25, 0.50, 1.0, 2.0, and 3.0 mM), GTP (0, 0.20, 0.050, 0.10, 0.20, and 0.250 mM), UDP (0, 0.5, 1.0, 2.0, and 4.0 mM), ADP (0, 0.25, 0.50, 1.0, and 2.0 mM), ATP (0, 1.0, 2.0, 4.0, 8.0, and 10.0 mM), CMP (0, 2.0, 5.0, and 10.0 mM), CDP (0, 2.0, 5.0, and 10.0 mM), CTP (0, 1.0, 2.5, 5.0, and 10.0 mM), TDP (0, 2.0, 5.0, and 10.0 mM), IDP (0, 0.075, 0.15, 0.30, 0.60, and 2.0 mM), and XDP (0, 0.15, 0.30, 0.60, 1.50, and 2.00 mM).

Donor Sugar Analog Studies

All FucT V reaction mixtures were 50 μL and contained 0.025 mM GDP−Fuc, 20 mM LacNAc, 10 mM $MnCl_2$, and 0.10 milliunit of FucT V in a 25 mM cacodylate buffer (pH 6.2). The V_{max} for LacNAc was determined by varying LacNAc and using the Hypero nonlinear least-squares fit of the Michaelis−Menten equation (eq 2). Dixon analysis was employed utilizing $1/V_{max}$. The inhibitor concentrations used were as follows: L-Fucal (0, 10, 20, 50, 75, and 100 mM), DFN (0, 2.0, 15.0, 18.4, 25.0, 40.0 mM), GDP−α-D-mannose (0, 0.10, 0.20, 0.50, 1.0, and 2.0 mM), GDP−α-D-glucose (0.0, 0.20, 0.50, 0.75, and 1.00).

Since GDP−α-D-mannose inhibited FucT V, GDP−[U-^{14}C]-α-D-mannose was examined as a possible alternative substrate to replace GDP−β-L-fucose. A 100 μL solution containing 2 mM GDP−[U-^{14}C]-α-D-mannose, 10 mM $MnCl_2$, 20 mM LacNAc, and 1.9 milliunit of FucT V was prepared in 25 mM cacodylate buffer (pH 6.2) and compared to an identical solution without FucT V as a control reaction. Aliquots (50 μL) were taken for each solution at 1 and 5 h and run on a Dowex 1 column. The column was eluted directly into 10 mL of scintillation cocktail. The reaction mixture with FucT V had 535 cpm, while the control reaction mixture without FucT V had 475 cpm. Within experimental error, these values are indistinguishable.

Synergistic Inhibition

Synergistic inhibition was initially probed by monitoring the percent inhibition of FucT V (0.05 mM GDP−Fuc, 24 mM LacNAc) with K_i levels of the individual inhibitors and combination of a nucleotide and a donor sugar mimetic at their K_i levels. Deoxyfuconojirimycin was varied (0, 5, 15, and 30 mM) at fixed GDP concentrations (0, 0.025, and 0.10 mM) or at fixed GMP concentrations (0, 0.5, 1.0, and 2.0 mM). Yonetani−Theorell analysis was used next to address synergism of multiple inhibitors (Segal, 1975; Yonetani & Theorell, 1964). FucT V, LacNAc, and GDP−Fuc were held constant at 0.30 milliunit, 24 mM, and 0.05 mM, respectively, and the donor sugar was varied at fixed concentrations of the nucleotide. A steady-state derivation for the synergistic inhibition of FucT V has been reported (eq 8) (Qiao et al., 1996).

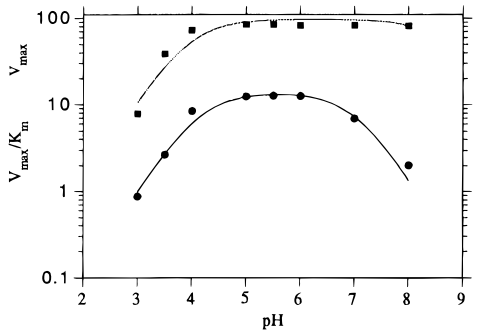

FIGURE 2: pH−rate profile for FucT V. V_{max} data were fit to the nonlinear, least-squares equation for one ionizable group and yielded a pK_a of 4.1 ± 0.2. The V_{max}/K_m data were fit to the equation for two ionizable groups and yield pK_a values of 4.2 ± 0.3 and 6.9 ± 0.3. The concentration of LacNAc was varied from 5 to 80 mM at 0.05 mM GDP−Fuc in 25 mM phosphate buffers. The units picomoles per minute; are as follows: V_{max}, and V_{max}/K_m, picomoles per minute per millimolar.

RESULTS

pH−Rate Studies. The rate of the FucT V-catalyzed reaction was measured as a function of pH in two buffers at constant GDP−Fuc and LacNAc concentrations in an initial study designed to identify the mechanistically relevant functional groups. A plot of the relative enzyme velocity (V_{rel}) at 20 mM LacNAc and 0.05 mM GDP−Fuc has demonstrated that maximal FucT V activity is in the 5−7 pH range in both phosphate and cacodylate buffers. The stability of FucT V in the 3−10 pH range maintained by a 25 mM phosphate buffer was established by incubating of FucT V at different pH conditions for 30 min, then shifting the pH to 6.0 by diluting the enzyme solution 5-fold with a 100 mM phosphate buffer (pH 6.0), and determining the activity. This study confirmed that FucT V was stable toward irreversible inactivation in the range of pH 3.5−8.0 with a sharp decrease in activity between pH 3.5−3.0 and a gradual decrease in activity between pH 8 and 10. A full pH−rate profile as a function of both pH and substrate concentration was performed to identify the precise pH sensitivity of the individual kinetic parameters (Figure 2). At a constant GDP−Fuc concentration of 0.050 mM, LacNAc was varied from 5 to 80 mM. A nonlinear least-squares fit of the data to the Michaelis−Menten equation was used to determine the apparent kinetic parameters (eq 2). The apparent V_{max}/K_m decreased at pH values greater than 7 and less than 5. The V_{max}/K_m data were fit to the equation for two ionizable groups (eq 3) with the nonlinear, least-squares FORTRAN program Bell and resulted in two pK_a values: 4.2 ± 0.3 and 6.9 ± 0.3. The pH−V_{max} profile is invariant for the pH values greater than 5 and decreased at values lower than 5. The apparent V_{max} data were fit to

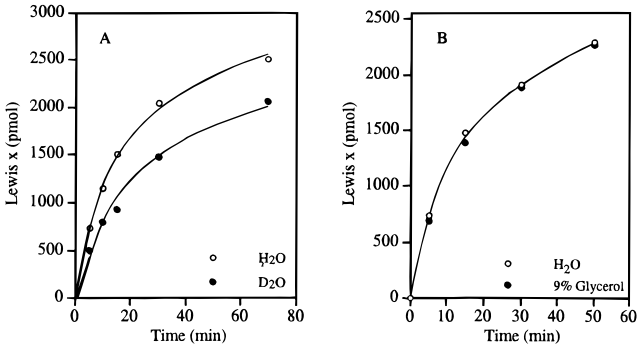

FIGURE 3: (A) Solvent kinetic isotope effect on FucT V. Time profile for the formation of product, Lewis x, in either H_2O (○) or D_2O (●). Both reaction mixtures contained 0.05 mM GDP−Fuc, 20 mM LacNAc, and 6.3 milliunits/mL FucT V. (B) Viscosity effect on FucT V. Glycerol (9%) was used as microviscogen control to demonstrate that the effect of D_2O was not viscosity-based. LacNAc and GDP−Fuc were 20 and 0.05, respectively.

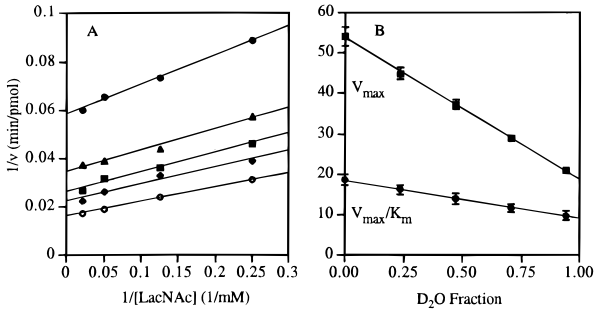

FIGURE 4: Proton inventory on FucT V reaction. (A) Kinetic constants were measured for FucT V as a function of LacNAc and percent D_2O. LacNAc was varied from 4 to 48 mM at a constant GDP−Fuc concentration of 0.05 mM. The percent D_2O used was as follows: 0% (○), 23.5% (◆), 47% (■), 70.5% (▲), and 94% (●). (B) The kinetic constants derived from the double-reciprocal plot (A) were plotted as a function of the D_2O fraction: V_{max} (■) and V_{max}/K_m (●).

the equation for one ionizable group (eq 4) with the nonlinear, least-squares program Habell and resulted in a pK_a of 4.1 ± 0.2.

A D_2O solvent kinetic isotope effect for FucT V was determined for V_{max} to be 2.9 ± 0.1 and for V_{max}/K_m to be 2.1 ± 0.2 (Figures 3A and 4B). The LacNAc concentration was varied from 4 to 48 mM at 0.05 mM GDP−Fuc, and the data were fit to the Michaelis−Menten equation (eq 2) with the nonlinear least-squares FORTRAN program Hypero to provide the apparent kinetic parameters. The densities of D_2O and 9% glycerol/H_2O solutions have been reported to be 1.24 (Karsten et al., 1995). A 9% glycerol microviscogen control reaction (0.2 milliunit of FucT V, 0.05 mM GDP−Fuc, and 20 mM LacNAc) in H_2O demonstrated that the observed effect was not due to the effect of the denser D_2O on a physical step (Figure 3B).

A proton inventory relates the effect of hydrogen being replaced by deuterium in the medium on the observed solvent isotope effect. The inverse of the solvent isotope effect (k_D/k_H) is equal to the isotopic fractionation factor (ϕ_T/ϕ_R). The individual fractionation factors (ϕ_T and ϕ_R) measure the deuterium preference of a particular site (in the reactant or transition state) relative to the deuterium preference for bulk water (Venkatasubban & Schowen, 1985). Bonds to neutral oxygen or nitrogen are essentially equivalent to those in bulk water and often lead to a fractionation factor of unity for ϕ_R. A single proton being transferred in the transition state would yield a linear relationship between the fraction of deuterium in the solvent and the observed solvent isotope effect. If two protons are being transferred in the transition state, then the change of solvent isotope effect with respect to deuterium fraction will be a quadratic function. A linear dependence of the solvent isotope effect of FucT V on deuterium mole fraction was observed in a proton inventory experiment (Figure 4B). Kinetic parameters for FucT V were determined at a constant GDP−Fuc concentration (0.050 mM), variable LacNAc concentrations (4−48 mM), and variable percent D_2O (0 - 94%). The kinetic data were subjected to a least-squares fit to the Gross−Butler equations for one- (eq 5) or two-proton (eq 6) transfer in the transition state (Venkatasubban & Schowen, 1985). Statistical analysis of the fits was performed by chi-square and F tests (Shoemaker et al., 1989). The isotopic fractionation factor for V_{max} was determined to be 0.35 ± 0.01 from the linear equation and 0.34 ± 0.01 and 0.15 ± 0.00 for the quadratic form. The isotopic fractionation factor for V_{max}/K_m was determined to be 0.48 ± 0.01 from the linear form of the Gross−Butler equation and 0.64 ± 0.12 and 0.77 ± 0.14 for the quadratic form with two fractionation factors. The *F* test was used to discriminate between the two mathematical models, and it revealed that there was a greater than 95% confidence that the true fit of the data is to the linear form of the Gross−Butler equation (eq 5). This is consistent with a one-proton transfer in the rate-determining catalytic step leading to the transition state.

Table 1: Kinetic Parameters for FucT V Derived from a Best Fit to the Equation for an Ordered, Sequential, Bi−Bi Mechanism (eq 1)

α-1,3-fucosyltransferase V	LacNAc	LacNAcβ-O-$(CH_2)_5CO_2CH_3$
k_{cat} (min^{-1})	40 ± 3	10 ± 1
$K_{m,GDP-Fuc}$ (mM)	0.060 ± 0.007	0.025 ± 0.006
$K_{i,GDP-Fuc}$ (mM)	0.0062 ± 0.0027	0.031 ± 0.011
$K_{m,acceptor}$ (mM)	8.8 ± 1.3	0.17 ± 0.05
$k_{cat}/K_{i,GDP-fuc}K_{m,acceptor}$ (min^{-1} mM^{-2})	740	1900
$(k_{cat}/K_{i,GDP-Fuc}K_{m,acceptor})/k_{non}$ (M^{-1})	1.2×10^{10}	3.0×10^{10}
transition-state affinity (M)	8.6×10^{-11}	3.4×10^{-11}

Precise Kinetic Parameters. The mechanism of FucT V has been determined to be an ordered, sequential, Bi−Bi mechanism with GDP−Fuc bound first followed by the acceptor sugar and oligosaccharide product released first followed by GDP (Qiao et al., 1996). Precise kinetic parameters for FucT V were derived from velocity data that were a function of GDP−Fuc and either LacNAc or LacNAcβ-O-$(CH_2)_5CO_2CH_3$ (Table 1). In separate experiments, at fixed concentrations of GDP−fucose (0.025, 0.050, and 0.10 mM), the LacNAc concentration was varied from 5 to 40 mM or LacNAcβ-O-$(CH_2)_5CO_2CH_3$ was varied from 0.10 to 1.0 mM. Figure 5 shows a Hanes−Woolf analysis of FucT V as a function of GDP−Fuc and LacNAc. The FucT V concentration was determined to be 0.137 mg/mL by the Bradford method (Bradford, 1976), and the molecular mass is 40 kDa (Jody Schultz, personal communication). A typical 0.050 mL assay contained 0.137 μg of FucT V. A nonlinear, least-squares fit of the rate equation for an ordered, sequential, Bi−Bi mechanism (eq 1) was used to derive the kinetic parameters of FucT V (Table 1). The catalytic efficiency of an ordered, sequential, Bi−Bi enzyme is defined as $k_{cat}/(K_{i,GDP-fuc}K_{m,acceptor})$ (Burgner & Ray, 1984) and was

Table 2: Acceptor specificity for FucT V[a]

	acceptor substrates	$K_{m,acceptor}$[b] (mM)	k_{cat}[b] (min^{-1})	$k_{cat}/K_{i,GDP-Fuc}K_{m,acceptor}$
1	Galβ1,4GlcNAc	31 ± 6	17 ± 1	100
2	Galβ1,4GlcNAcβ-O-$(CH_2)_5CO_2CH_3$	0.36 ± 0.08	3.13 ± 0.24	290
3	Galβ1,4GlcNAcβ-O-$(CH_2)_7CH_3$	3.1 ± 1.5	4.7 ± 1.8	51
4	GlcNAcβ-O-$(CH_2)_5CO_2CH_3$	60 ± 1	0.91 ± 0.01	0.50
5	Galβ1,4GlcNAcβ-S-phenyl	0.37 ± 0.05	4.4 ± 0.2	400
6	Galβ1,4Galβ1,4GlcNAcβ-S-phenyl	9.4 ± 2.1	2.2 ± 0.2	7.7
7	Galβ1,4GlcN$_3\beta$-S-phenyl	5.5 ± 1.9	6.5 ± 2.0	40
8	Galβ1,4GlcN$H_2\beta$-S-phenyl	18 ± 5	4.7 ± 0.2	8.9
9	Galβ1,4GlcNAcα-O-benzyl	0.30 ± 0.04	4.4 ± 0.2	500
10	H_2O	–	0.5 ± 0.0	–
11	Galβ1,4Glc	500	26	1.7
12	Galβ1,4(5-thio)Glc	12	8.9	25
13	Galβ1,4GlcNAcβ-O-allyl	16	11	23
14	Galβ1,3GlcNAc	600	23	1.3
15	Galβ1,4Glucal	34	1.7	1.7
16	NeuAcα2,3Galβ1,4GlcNAc	100	110	37
17	NeuAcα2,3Galβ1,4GlcNAcβ-O-allyl	280	66	8.0
18	NeuAcα2,3Galβ1,4Glucal	64	57	29

[a] The acceptor sugar concentrations were chosen to be around their respective K_m values. The GDP−Fuc concentration was held constant at 0.05 mM, and $MnCl_2$ was constant at 10 mM. Kinetic constants were derived from the nonlinear, least-squares fit of the Michaelis−Menten equation (eq 2) with the Hypero FORTRAN program. [b] Apparent kinetic values. LacNAc is Galβ1,4GlcNAc. The first two entries were subjected to precise kinetic investigation, and the same relationship was observed (Table 1). Catalytic efficiencies were relative to LacNAc, 739 min^{-1} mM^{-2}. The $K_{i,GDP-Fuc}$ value of 0.0325 mM was used for all calculations of catalytic efficiency except for LacNAc. Data for entries 11−19 were from a previously reported study (Ichikawa et al., 1992).

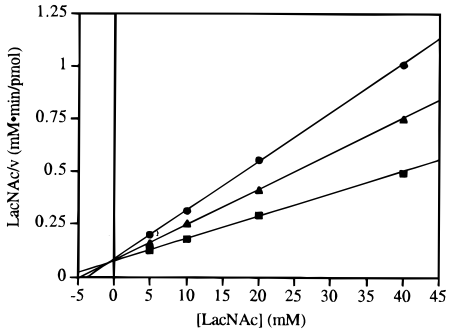

FIGURE 5: Hanes−Woolf analysis of FucT V as a function of both GDP−Fuc and LacNAc for the determination of precise kinetic values for FucT V. The concentrations were as follows: 0.025 mM GDP−Fuc (●), 0.05 mM GDP−Fuc (s), and 0.1 mM GDP−Fuc (n). The LacNAc concentration was varied from 5 to 40 mM for each GDP−Fuc concentration.

calculated to be 740 $min^{-1}mM^{-2}$ for LacNAc and 1900 min^{-1} mM^{-2} for LacNAcβ-O-$(CH_2)_5CO_2CH_3$ (Table 1).

Acceptor Specificity. The acceptor sugar specificity was subjected to detailed kinetic investigations. The relative kinetic values for FucT V were determined at a fixed GDP−Fuc concentration and variable acceptor concentrations, and the data were fit to the Michaelis−Menten equation (eq 2) with the nonlinear, least-squares program Hypero (Table 2). Entries 1 and 2 of Table 2 correlate with the precise values for these two acceptor sugars (Table 1). The K_m of acceptor sugars with a hydrophobic group at the reducing end decreased 100-fold, while the k_{cat} only decreased 3-fold. Since disaccharides containing a hydrophobic group at the reducing end were bound to the enzyme more strongly, the monosaccharide GlcNAc was functionalized with the same series of hydrophobic groups. Only GlcNAc-β-O-$(CH_2)_5$-CO_2CH_3 (entry 4) had a solubility in the assay buffer at concentrations above 5 mM. The k_{cat} (0.91 min^{-1}) was 22-fold lower than that of LacNAc (entry 1, 17 min^{-1}), and the K_m (60 mM) was comparable to that of LacNAc (31 mM). The 100-fold change in affinity observed in substrate K_m is also observed for oligosaccharide product inhibition. The inhibition constant for Lewis x is 52 mM, and the inhibition constant for Lewis x with a β-O-$(CH_2)_5CO_2CH_3$ functionality at the reducing end is 0.12 ± 0.01 mM. This constitutes a 400-fold increase in binding affinity of Lewis x with the hydrophobic moiety at the reducing end of the trisaccharide (Qiao et al., 1996).

GDP−β-L-Fucose Hydrolysis. GDP−Fuc hydrolysis was studied at 50, 75, and 92 °C in a 25 mM cacodylate buffer at pH 6.0. GDP−[U-^{14}C]-β-L-fucose was used to quantify the amount of hydrolysis. HPLC studies of GDP−Fuc hydrolysis have shown that the reaction products are GDP and fucose (Nunez et al., 1981). The unreacted GDP−Fuc can be separated from the product fucose with a Dowex 1 anion exchange column in a manner similar to the enzymatic assay. In the accompanying control experiments, no appreciable amount of unreacted GDP−Fuc was detected to pass through the anion exchange column. [U-^{14}C]-L-Fucose was found to have no significant affinity for the Dowex 1 anion exchange column. The volume of the sealed reaction vials before and after the exposure to elevated temperatures was unchanged. Using the Arrhenius equation (eq 7), the observed hydrolysis at 20 °C was calculated to have a $t_{1/2}$ of 139 ± 3 days and a k_{obs} = (3.48 ± 0.09) × 10^{-6} min^{-1} (Figure 6A). The rate of GDP−Fuc hydrolysis at pH 6.0 was invariant in both phosphate and cacodylate buffers varying from 20 to 500 mM. Hydrolysis of GDP−Fuc in 25 mM phosphate buffers was biphasic and marginally dependent on pH at pH 6 and greater. The second-order rate constant for the hydrolysis of GDP−Fuc at pH 6.0, k_{non}, was estimated by $k_{obs}/[H_2O]$ (6.33 × 10^{-8} min^{-1} M^{-1}) (Figure 6B). Barker and co-workers also reported a similar behavior for GDP−Fuc hydrolysis; GDP−Fuc was degraded to GDP at 37 °C with a half-life of 7 h at pH 3, but at pH 5−8, there was no detectable hydrolysis after 1 week (Nunez et al., 1981). The hydrolysis of nucleotides is multiphasic due to the multiple ionization states of the leaving group (Benkovic & Schray, 1973). The specificity constant for the ordered, sequential, Bi−Bi mechanism such as with FucT V is defined as $k_{cat}/K_{i,GDP-Fuc}K_{m,LacNAc}$ (Burgner & Ray, 1984)

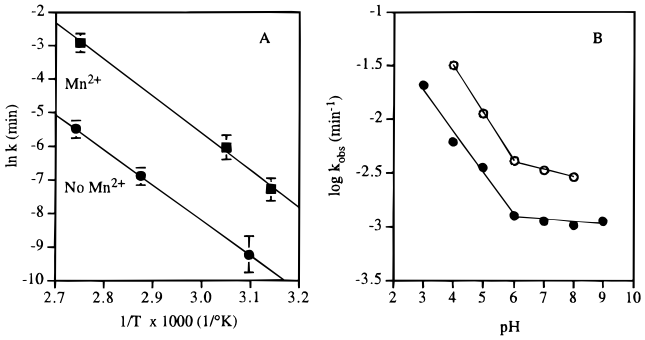

FIGURE 6: (A) Arrhenius plots of the hydrolysis of GDP−β-L-fucose with (■) and without (●) 10 mM $MnCl_2$. The rates of GDP−Fuc (0.05 mM) hydrolysis in a pH 6.0, 25 mM phosphate buffer at temperatures ranging from 45 to 90 °C were determined. The observed rate constant for GDP−Fuc hydrolysis at 20 °C in the absence of $MnCl_2$ was $(3.48 \pm 0.09) \times 10^{-6}$ min^{-1}, while the rate in the presence of 10 mM $MnCl_2$ was $(3.83 \pm 0.36) \times 10^{-5}$ min^{-1}. (B) The effect of pH on the hydrolysis of 0.05 mM GDP−Fuc at 78 °C (●) and 90 °C (○) in 25 mM phosphate buffers.

and determined to be 740 min^{-1} mM^{-2} (Table 1). The enzymatic proficiency, $(k_{cat}/K_{i,GDP-Fuc}k_{m,acceptor})/k_{non}$, of FucT V is estimated to be 1.2×10^{10} M^{-1}, and the transition-state affinity is then 8.6×10^{-11} M. A crude estimate of the rate enhancement of FucT V can be made by comparing the k_{cat} of FucT V to the hydrolysis rate of GDP−Fuc in the absence of FucT V (k_{non}). The rate enhancement of FucT V, k_{cat}/k_{non}, is 6.4×10^8 M for LacNAc as the acceptor sugar. In the presence of 10 mM $MnCl_2$, the half-life of GDP−Fuc at 20 °C was calculated to decrease by 1 order of magnitude to $t_{1/2} = 12.6 \pm 1.2$ days (Figure 6A).

Metal Cofactor Studies. The concentration of divalent manganese needed to achieve half-maximal activity was determined to be 6.1 ± 0.5 mM at fixed concentrations of both GDP−Fuc (0.050 mM) and LacNAc (20 mM) with the nonlinear least-squares fit to the Michaelis−Menten equation (eq 2) with the Hypero FORTRAN program. Alternative metal cofactors were investigated and evaluated. The relative velocity of FucT V in the presence of various divalent metals (5 mM) was investigated at fixed concentrations of GDP−Fuc (0.025 mM) and LacNAc (20 mM). The counterions for all of the metals were chloride ions. Divalent calcium, cobalt, and magnesium had 63, 56, and 53% of the enzymatic rate of divalent manganese. Next, the relative kinetic parameters for FucT V were determined at constant concentrations of 0.050 mM, 20 mM, and 0.3 milliunit for GDP−Fuc, LacNAc, and FucT V, respectively. Apparent kinetic parameters were determined by nonlinear, least-squares analysis with the FORTRAN program Hypero (eq 2) (Table 3).

The enzymatic assay is designed to measure the incorporation of L-[U-^{14}C]fucose into LacNAc. Reactant is separated from product with a strong anion exchange column. The possibility that the metal chelates GDP−Fuc and prevents it from binding to the anion exchange resin was eliminated by performing control reactions that contained substrates, buffer, and metal but no enzyme. In this case, only a background radioactivity was measured. ADP has been reported to form 1:1 complexes with divalent metals, and the stabilities of these complexes have been reported to increase in the order $Ba^{2+} < Sr^{2+} < Ca^{2+} < Mg^{2+} < Co^{2+} < Mn^{2+} < Zn^{2+} < Ni^{2+} < Cu^{2+}$ (Taqui & Martell, 1967). GDP−Fuc should form a tighter complex with Mn^{2+} than with either Ca^{2+}, Co^{2+}, or Mg^{2+}.

Table 3: Alternative Metal Cofactors for FucT V[a]

metal	V_{max}[b] (pmol/min)	K_m[b] (mM)	V_{max}/K_m[b] (pmol min^{-1} mM^{-1})
Mn^{2+}	75	6.1	12
Ca^{2+}	35	6.7	5.2
Co^{2+}	36	1.2	30
Mg^{2+}	56	8.6	6.0
Cu^{2+}, Fe^{2+}, Ni^{2+}, and Zn^{2+}	no activity	−	−

[a] The metal concentrations were chosen to be around the K_m value. GDP−Fuc, LacNAc, and FucT V were held constant at the following concentrations: 0.050 mM, 20 mM, and 0.3 milliunit. [b] Apparent values.

Synergistic Inhibition. Synergisitic inhibition of FucT V with an aza sugar and GDP has been observed (Ichikawa et al., 1992; Wong et al., 1992) and kinetically analyzed (Qiao et al., 1996). The role of the nucleotide charge and distance between the aza sugar and the nucleotide in the proposed synergisitic effect were investigated. The following nucleotides were evaluated, and their K_i values were determined: GDP ($K_i = 0.029$ mM) and GMP ($K_i = 0.70$ mM). L-Fucal and deoxyfuconojirimycin (DFN) were found to be modest inhibitors with K_i values of 71 and 45 mM, respectively. The combination of donor sugar mimic and nucleotide was analyzed at their K_i levels with K_m levels of substrates GDP−Fuc and LacNAc. Individually, DFN caused a 21% inhibition and GDP was responsible for a 22% inhibition. The combination of DFN and GDP at their K_i levels resulted in 80% inhibition. L-Fucal had a 26% inhibition, yet the combination with GDP only produced 40% inhibition. GMP inhibited FucT V to 23%, and the combination of GMP and DFN only produced 50.4% inhibition. These results are consistent with the proximal ionic interaction between the charged donor sugar mimic and the negatively charged nucleotide.

Yonetani−Theorell analysis was used to define the synergistic effect (eq 8) (Qiao et al., 1996; Yonetani & Theorell, 1964). This analysis is a type of Dixon analysis ($1/v$ vs [I]) at multiple fixed concentrations of a second inhibitor. Intersecting lines is indicative of nonexclusive binding of two inhibitors. The projection of the intersection points on the abscissa is the value αK_i. The α value is a measure of the interaction of the two inhibitors. The projection of the intersection points (αK_i) revealed that α was less than 1. An α of <1 is indicative of synergistic inhibition. Intersecting lines with an α of 1 is the hallmark of nonexclusive additive inhibition. The combination of GDP and DFN resulted in intersecting lines with $\alpha = 0.12$, consistent with synergistic inhibition, while the combinations of GMP/deoxyfuconojirimycin and GDP/L-Fucal both had α values of 1 (Figure 7). No synergism was detected for the combination of L-fucal and GDP. These studies support the proposed synergism model with a positively charged donor sugar interacting with a proximal phosphate of a nucleotide.

Nucleotide Inhibition Studies. The specificity of FucT V for the nucleoside portion of the donor sugar was probed by measuring the inhibition of the naturally occurring nucleotide diphosphates with Dixon analysis ($1/v$ vs [I]) at the following concentrations of GDP−Fuc and LacNAc: 0.025 and 20

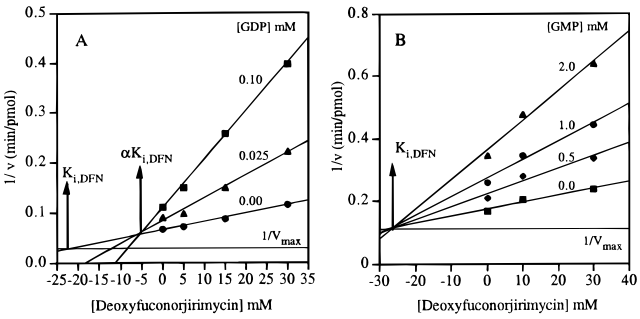

FIGURE 7: Multiple-inhibitor kinetics of the combination of deoxyfuconojirimycin and either GDP or GMP. All assays contained 0.025 mM GDP−Fuc and 20 mM LacNAc. (A) Synergism is observed for the combination of GDP and deoxyfuconojirimycin with 1.9 milliunits of FucT V in the Yonetani−Theorell plot. (B) No synergism is observed for the combination of GMP and deoxyfuconojirimycin with 7.5 milliunits of FucT V. The inhibition is additive because the family of lines is intersecting but the interaction constant is one ($\alpha = 1$).

Table 4: Investigation of the Nucleotide Requirement for FucT V[a]

nucleotide	K_i (mM)
guanosine triphosphate	0.030
guanosine diphosphate	0.029
guanosine monophosphate	0.70
guanosine	>10
inosine diphosphate	0.069
xanthine diphosphate	0.30
adenosine diphosphate	0.58
adenosine triphosphate	1.7
cytosine diphosphate	3.5
uridine diphosphate	8.2
thymine diphosphate	21
GDP−α-D-mannose	0.29
GDP−α-D-glucose	0.38

[a] The importance of two phosphates can be observed from the guanosine nucleotide series. Inosine diphosphate is a potent inhibitor of FucT V. Although GDP−α-mannose is bound by FucT V, no turnover is observed.

mM, respectively (Table 4). FucT V has a strong preference for purine diphosphates compared to pyrimidine diphosphates. The simpler structure of inosine diphosphate (K_i = 0.069 mM) might be useful in the synthesis of inhibitors. Both GDP−α-D-mannose and GDP−α-D-glucose are inhibitors of FucT V (K_i = 0.29 and 0.38 mM, respectively), but no reaction of GDP−α-D-mannose was detected after 5 h of reaction (10 times the usual reaction time).

DISCUSSION

pH−Rate Studies. FucT V was found to be stable toward inactivation in solutions with pH values ranging from 3.5 to 10. A catalytic base has been identified for FucT V that has a pK_a comparable to that of carboxylic acids. Also, a mechanistically relevant acidic residue was identified in both the pH−activity and pH−V_{max}/K_m profiles but not in the pH−V_{max} profile. The GDP−Fuc binding site of FucT V has an essential cysteine residue (Holmes et al., 1995). Furthermore, FucT VI, which is extremely homologous to FucT V, has both cysteine and histidine residues of catalytic importance (Britten et al., 1995). α-1,3-Fucosyltransferase from human small cell lung carcinoma also has an essential lysine residue in the GDP−Fuc binding site. (Holmes, 1992). These studies show that FucT V may have mechanistically relevant residues with a pK_a of ∼6.9 which interacts with GDP−Fuc and is not involved in the transition state.

Proton Inventory Studies. Deuterium oxide can have a number of effects on a protein in solution, including a kinetic isotope effect upon transfer of hydrogen in the transition state (Schowen & Schowen, 1982). We have observed a significant solvent isotope effect on both V_{max} and V_{max}/K_m and ruled out the possibility that the effect is due to the change of viscosity (Karsten et al., 1995). Alternatively, hydrogen bonds in proteins are quite important to proper folding so D_2O may have an effect on the protein stability. However, a recent finding showed that, although D_2O increases the transition temperature and decreases the enthalpy of unfolding, the stability of proteins has been reported to be largely unchanged due to entropic compensation for the decrease in enthalpy from changes in the hydration (Makhatadze et al., 1995). The proton inventory technique has been used widely to characterize the transition-state nature of many enzymes (Venkatasubban & Schowen, 1985). Enzymes as varied as HIV protease (Hyland et al., 1991), β-galactosidase (Selwood & Sinnott, 1990), phosphotyrosyl protein phosphatase (Zhang & Etten, 1991), and galactose oxidase (Driscoll & Kosman, 1987) have been studied by this technique. The proton inventory of FucT V is consistent with only one proton "in flight" in the transition state through statistical analysis of the fit of the data to the Gross−Butler equations for one- or two-proton transfer reactions (eqs 5 and 6). However, a precaution must be taken regarding the interpretation of a linear proton inventory. Kresge showed that a linear proton inventory could be due to multiple protonic sites. For the deacylation of acetylchymotrypsin, it was mathematically calculated that two dominant sites and between 6 and 20 other sites together could possibly give the linear effect (Kresge, 1973; Pollock et al., 1973). Although unlikely, the linear proton inventory may be due to multiple protonic sites. This possibility is not consistent with the emerging picture of the transition state of FucT V. A 3-deoxy-LacNAc has been shown to be a very weak inhibitor of FucT V (de Vries et al., 1995; Ichikawa et al., 1992) which indicates that the active site base forms a tight complex with the 3-hydroxyl group of LacNAc. The proton inventory technique is diagnostic of the number of proton transfers involved in the transition state; thus, it is feasible to have two mechanistically relevant ionizable residues with only one involved in proton transfer in the transition state. The magnesium-dependent *Escherichia coli* β-galactosidase has two catalytically relevant ionizable residues but has a linear proton inventory (Selwood & Sinnott, 1990). In addition, *E. coli* β-galactosidase has a metal cofactor proposed to be an electrophilic catalyst and an active site residue as a proton donor to assist the product release of the aglycone (Selwood & Sinnott, 1990).

GDP−Fuc Hydrolysis. GDP−Fuc hydrolysis at pH 6 and greater was shown to be relatively slow, invariant on buffer concentration, and modestly dependent on buffer pH. GDP−Fuc stability has been studied in an acidic medium (Nunez et al., 1981). At pH 5−8, Barker and co-workers found that there was neither detectable hydrolysis of GDP−Fuc at 37 °C in 1 week nor detectable hydrolysis catalyzed by buffer, Mn^{2+}, or Mg^{2+}. At pH 3, GDP−Fuc was found to be degraded to L-fucose and GDP with a $t_{1/2}$ of 7 h at 37 °C and 52 h at 4 °C. The hydrolysis of GDP−Fuc is thus considered to proceed through the glycosidic cleavage, similar to the case of glycoside hydrolysis. Hydrolysis of glucopyranosides is currently thought to undergo rate-limiting exocyclic C−O bond cleavage of the protonated glucoside

with nucleophilic assistance (Amyes & Jencks, 1989; Banait & Jencks, 1991; Huang et al., 1995; Jencks, 1980). The mechanism of hydrolysis of glucopyranosides is dependent on the leaving group. For example, the hydrolysis of (2-deoxy-β-D-glucopyranosyl)pyridinium salts is pH-independent (Huang et al., 1995). GDP−Fuc hydrolysis in the absence of FucT V is subject to a rate acceleration with the addition of divalent manganese ions. Classic studies of ATP have shown that the hydrolysis is accelerated by the presence of divalent metal ions (Alberty, 1968; Miller & Westheimer, 1966). In the active site of FucT V, divalent manganese may stabilize the transition state through electrophilic catalysis and facilitate the departure of the leaving group.

Kinetic Characterization of FucT V. Precise kinetic constants for FucT V were determined by a nonlinear, least-squares fit of the data to the rate equation for an ordered, sequential, Bi−Bi mechanism (eq 1). The observed k_{cat} of 40 min^{-1} for LacNAc and 10 min^{-1} for LacNAcβ-O-$(CH_2)_5$-CO_2CH_3 was in the range of glycosyltransferase enzymes: 10.1 min^{-1} for human β-1,4-galactosyltransferase, 0.2 min^{-1} for xylosyltransferase (Kearns et al., 1991), and 1200 min^{-1} for GlcNAc transferase II (Bendiak & Schachter, 1987). We report a 6.6-fold higher K_m value for GDP−Fuc with LacNAc as the acceptor sugar and only 2.8-fold higher with the better acceptor [LacNAc-β-O-$(CH_2)_5CO_2CH_3$] compared with the literature value of 9 μM with an α-1,2-fucosylated acceptor sugar (de Vries et al., 1995). The difference in K_m values may be due to the use of different acceptor sugars and also the fact that the initial reports for the K_m values were apparent values. Our current and past acceptor specificity studies provide a more complete picture of the scope of the acceptor specificity of FucT V. The hydrophobic moiety at the reducing end of the acceptor sugar has a striking influence on the acceptor K_m. The inhibition of the product inhibitor Lewis x was shown to improve 400-fold with the addition of a $(CH_2)_5CO_2CH_3$ group at the reducing end. This strategy allowed for the fucosylation of the monosaccharide GlcNAc (entry 4, Table 2). The role of the *N*-acetyl group was shown to have a major impact on the K_m and only a minor impact on the k_{cat}. Comparison of entry 5 (NHAc) to entry 7 (N_3) and entry 8 (NH_2) in Table 2 showed a 15−48- fold increase in the K_m going from entry 5 to 7 or 8 while the k_{cat} was essentially invariant. A similar result was reported for the comparison of LacNAc (K_m = 35 mM, V_{rel} = 100%) to lactose (K_m = 500 mM, V_{rel} = 150%) (Ichikawa et al., 1992). These data are in agreement with the relative velocity studies in which LacNAc with a $(CH_2)_8CO_2CH_3$ tail on the reducing end had 28% of the V_{rel} of LacNAc (de Vries et al., 1995). The lack of α/β discrimination at the reducing end of the acceptor sugar LacNAc glycosides is interesting (entries 5 and 9, Table 2). This acceptor specificity study should provide a new strategy for the synthesis of more potent inhibitors through the incorporation of the hydrophobic moiety at the reducing end. The ability of FucT V to utilize azido-oligosaccharides can be exploited in the chemoenzymatic synthesis of other Le^x derivatives such as photoaffinity labels for the study of their interaction with protein receptors. Nucleotide sugars other than GDP−Fuc were shown to bind to FucT V through inhibition studies (Table 4). Both GDP−α-mannose and GDP−α-glucose inhibited FucT V, but there was no detectable activity with GDP−α-D-[U-^{14}C]mannose as the donor sugar. This is an interesting observation because the wrong anomer of the proper nucleotide sugar is commonly thought not to contribute significantly to glycosyltransferase inhibition (Arlt & Hindsgaul, 1995).

Metal Cofactor Studies. α-1,3-Fucosyltransferase has an essential metal cofactor. Studies presented here indicate that FucT V has a preference for metal ions that can adopt an octahedral geometry: Mn^{2+}, Mg^{2+}, Co^{2+}, and Ca^{2+} (Table 3). FucT V has been known to be manganese ion-dependent. Under V_{max} conditions, FucT V operates optimally with Mn^{2+}. A surprising result is that the observed specificity constant of Co^{2+} is 2-fold lower than for Mn^{2+}. In the case of galactosyltransferase enzymes, the optimal Mn^{2+} concentrations have been reported to range from 2 to 43 mM and other divalent metals (Mg^{2+}, Ca^{2+}, Ba^{2+}, and Co^{2+}) have been reported to be partially effective (Ram & Munjal, 1985). Oligosaccharide transferase has been reported to be a manganese-dependent enzyme. Divalent calcium and magnesium could replace manganese for oligosaccharide transferase, but only 50% of the relative activity could be achieved compared to an equal concentration of manganese (Breuer & Bause, 1995). A more comprehensive investigation of the metal requirement of oligosaccharyltransferase suggested that the enzyme has a preference for metal ions that can adopt an octahedral coordination geometry (Mn^{2+}, Fe^{2+}, Mg^{2+}, and Ca^{2+}) (Hendrickson & Imperiali, 1995). Manganese divalent ions have been shown to be bound to galactosyltransferase before UDPgalactose which binds before GlcNAc (Ram & Munjal, 1985). Manganese is reported to depart from galactosyltransferase as a UDP·Mn^{2+} complex (Tsopanakis & Herries, 1978). Thus in galactosyltransferase, and possibly FucT V, the leaving group departure is facilitated by a metal cofactor. Support of the hypothesis that the metal cofactor chelates the nucleotide phosphates can be derived from the fact that the nucleotide inhibition follows the following order for both galactosyltransferase (Ram & Munjal, 1985) and FucT V: NDP > NMP > N (Table 4).

Transition-State Affinity. Enzyme proficiency has been defined as the ratio of the enzymatic specificity constant to the nonenzymatic rate because the specificity constant relates the reaction rate to free enzyme and free substrate (Radzicka & Wolfenden, 1995; Wolfenden, 1976). Enzyme proficiency can be used to estimate the transition-state affinity of the enzyme (Radzicka & Wolfenden, 1995). Although this calculation is more commonly used for single-substrate enzymes, the transition-state affinity of multisubstrate enzymes can be calculated provided that the enzyme does not proceed by a double-displacement mechanism (Wolfenden, 1976). The proficiency of FucT V can be estimated by assigning the nonenzymatic reaction to the transfer of L-fucose from GDP−Fuc to H_2O (k_{non}). The enzymatic proficiency of FucT V was estimated to be 1.2×10^{10} M^{-1}, and thus the transition-state affinity is 8.6×10^{-11} M. A crude estimate of the rate enhancement of FucT V can be made by comparing the k_{cat} of FucT V to the hydrolysis rate of GDP−Fuc in the absence of FucT V (k_{non}). The rate enhancement of FucT V (k_{cat}/k_{non}) is approximately 6.4×10^8 M. Statistically, the nonenzymatic Mn^{2+}-catalyzed GDP−Fuc hydrolysis will not contribute to the enzymatic rate of hydrolysis. The hydrolysis of GDP−Fuc in the absence of Mn^{2+} was chosen as the nonenzymatic rate because Mn^{2+} catalyzes the hydrolysis, possibly in a mode that also contributes to enzymatic catalysis. The enzymatic proficiency of a small population of single-substrate enzymes has been determined to range from 5.3×10^8 M^{-1} (cyclo-

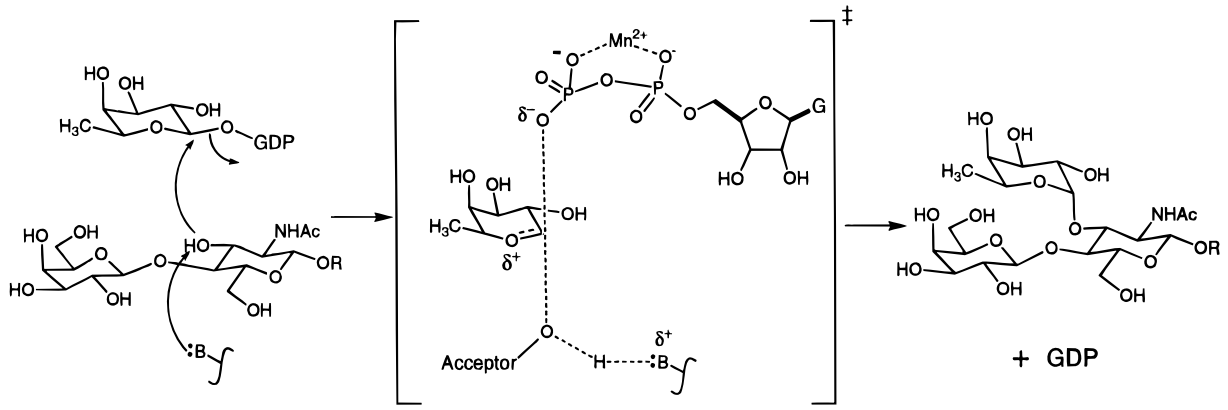

FIGURE 8: Proposed mechanism of α-1,3-fucosyltransferase V.

philin) to 2.0×10^{23} M^{-1} (orotidine-5-phosphate decarboxylase) (Radzicka & Wolfenden, 1995). A few multisubstrate enzymes have been investigated. For example, lactate dehydrogenase has a proficiency of 10^{19} M^{-1} and a rate acceleration of 10^{14} M (Burgner & Ray, 1984). Catechol *O*-methyltransferase has been reported to have a rate acceleration of 3.2×10^{16} M (Mihel et al., 1979). This value may be useful in the evaluation of FucT V inhibitors.

Synergism with Aza Sugars. The synergistic interaction between a positively charged aza sugar, GDP, and an acceptor sugar observed previously (Ichikawa et al., 1992; Wong et al., 1992) has been further characterized in this and other studies (Qiao et al., 1996). The synergistic interaction has been proposed to mimic the structure and charge distribution in the transition state. The synergistic effect enhances the inhibitory potency of aza sugars by 2 orders of magnitude (Qiao et al., 1996). The aza sugar deoxyfuconojirimycin (DFN) had the expected synergism with GDP but not with GMP. Yonetani−Theorell analysis showed that, although GMP and DFN can bind to FucT V, no synergism is observed. This result is consistent with the proposed model for the synergistic interaction and the proposed transition-state structure. GMP is an effective inhibitor of FucT V ($K_i = 0.70$ mM). Thus, the change from GDP to GMP shifts the negatively charged phosphate two bond lengths from the aza sugar which is critical to the synergistic interaction. L-Fucal and GDP can also bind simultaneously to FucT V, yet there is no observed synergism. This result is consistent with the charge−charge interaction of the aza sugar and GDP in the proposed model of the synergistic interaction. A catalytic base is proposed to facilitate the acceptor sugar hydroxyl group attack on the anomeric position of the donor sugar, GDP−Fuc. Evidence for possible hydrogen-bonding interaction between the catalytic base and the hydroxyl group can be found in studies that show that Galβ1,4(3-deoxy)GlcNAc is a very poor inhibitor of the enzyme ($K_i = 71$ mM) (de Vries et al., 1995; Ichikawa et al., 1992). In addition, FucT V will process acceptor sugars with either oxygen or sulfur in the GlcNAc ring, but a similar sugar structure with nitrogen in the GlcNAc ring, is an inhibitor. Perhaps the catalytic base forms a hydrogen bond with the ring NH group, thereby weakening its basicity (Ichikawa et al., 1992).

Mechanism. From the studies presented in this paper and a previous paper (Qiao et al., 1996), a mechanism of the enzymatic reaction is proposed (Figure 8). The synergistic inhibitory effect is thought to mimic the charge distribution and structure in the transition state. The proton inventory study indicates that a single-proton transfer occurs in the transition state. The pH−rate profile shows that FucT V has a catalytic base with a pK_a of 4.1, presumably a carboxylic acid residue. The guanosine series of nucleotides as inhibitors of FucT V shows that only GTP and GDP are potent inhibitors of FucT V, GMP is a moderate inhibitor, and guanosine is a noninhibitor. This result is consistent with bidentate chelation of an enzyme-bound, divalent manganese atom by GDP−Fuc. FucT V can use calcium, cobalt, and magnesium as metal cofactors. In the nonenzymatic hydrolysis of GDP−Fuc, divalent manganese was shown to accelerate the hydrolysis reaction, presumably through electrophilic catalysis. This mode of action may also occur in the active site of FucT V in the transferase reaction.

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