Cytosolic β-glucosidase (CBG) from mammalian liver is known for its broad substrate specificity and has been implicated in the transformation of xenobiotic glycosides. CBG also catalyzes a variety of transglycosylation reactions, which have been shown to occur with other glycosylhydrolases to function in synthetic and genetic regulatory pathways. We investigated the catalytic mechanism, substrate specificity, and transglycosylation acceptor specificity of guinea pig liver CBG by several methods. These studies indicate that CBG employs a two-step catalytic mechanism with the formation of a covalent enzyme-sugar intermediate and that CBG will transfer sugar residues to primary hydroxyls and equatorial but not axial C-4 hydroxyls of aldopyranosyl sugars. Kinetic studies revealed that correction for transglycosylation reactions is necessary to derive correct kinetic parameters for CBG. Further analyses revealed that for aldopyranosyl substrates, the activation energy barrier is affected most by the presence of a C-6 carbon and by the configuration of the C-2 hydroxyl, whereas the binding energy is affected modestly by the configuration and substituents at C-2, C-4, and C-5. These data indicate that the transglycosylation activity of CBG derives from the formation of a covalently linked enzyme-sugar intermediate and that the specificity of CBG for transglycosylation reactions is different from its specificity for hydrolysis reactions.

A distinguishing feature of the cytosolic β-glucosidase (CBG) from mammalian liver (EC 3.2.1.21) is its broad substrate specificity. The enzyme hydrolyzes β-D-galactopyranosides, β-D-fucopyranosides, β-D-xlyopyranosides, and α-L-arabinopyranosides, in addition to β-D-glucopyranosides (1). The enzyme also catalyzes transglycosylation reactions in which a sugar residue is transferred from a substrate molecule to an acceptor to form a new glycoside (2). These properties are consistent with the fact that CBG is a configuration-retaining glycosidase (3). Collectively, these data suggest that the catalytic mechanism of CBG consists of a double-displacement reaction involving the formation of a stable enzyme-sugar intermediate, as originally proposed for configuration-retaining glycosidases by Koshland (4).

Additional evidence of a two-step catalytic mechanism for CBG was derived from studies performed with the inhibitor, Br-conduritol-β-epoxide, which is an irreversible, active site-directed inactivator of the enzyme (5). However, studies with Escherichia coli β-galactosidase and human glucocerebrosidase have shown that the mechanism of inactivation by related conduritol epoxide compounds differs from the catalytic mechanisms of these two enzymes. For both glucocerebrosidase and β-galactosidase, the amino acid residue identified as the catalytic nucleophile by labeling with conduritol epoxides was later demonstrated to be incorrect by site-directed mutagenesis experiments (6, 7). The introduction of 2-deoxy-2-fluoro glycoside inhibitors by Withers and co-workers (8, 9) provided true mechanism-based inhibition that correctly identified the catalytic nucleophiles in both E. coli β-galactosidase and glucocerebrosidase, as well as numerous other configuration-retaining glycosidases (reviewed in Ref. 10).

Transglycosylation reactions are intimately involved in the carbohydrate metabolism of numerous species. A large number of well-characterized glycosyltransferases catalyze the transfer of sugars from nucleoside-diphosphate intermediates to monosaccharide, oligo-, and polysaccharide acceptor moieties (11). Less widely appreciated is the metabolic significance of transglycosylation reactions catalyzed by glycosyl hydrolases. In plants, xyloglucan endohydrolase-endotransferase has been implicated in the incorporation of xyloglucan oligomers into cell wall polymers in vivo during remodeling (12). In E. coli, expression of the lac operon is regulated by the product of a transglycosylation reaction. Allolactose, the natural inducer of the lac operon, is produced by the transfer of galactose from the 4-OH of the glucose residue of lactose to the 6-OH of glucose to form β-D-galactopyranosyl-(1→6)-β-D-glucopyranoside. The lac Z β-galactosidase catalyzes the formation of allolactose at a constant fraction of the rate of production of the hydrolysis products glucose and galactose (13). The potential significance of similar transglycosylation reactions in mammalian cells is an area of current investigation.

The intimate relationship between transglycosylation reactions and the catalytic mechanism of configuration-retaining glycosidases prompted us to analyze the catalytic mechanism of
CBG and its acceptor specificity in transglycosylation reactions. In this study, we demonstrate that the catalytic mechanism of CBG involves the formation of a covalent enzyme-sugar intermediate and show that transglycosylation reactions account for a substantial fraction of the catalytic activity at saturating concentrations of substrate. The acceptor specificity of the enzyme in transglycosylation reactions was characterized, and by correcting for transglycosylation reactions in the analysis of a homologous series of substrates, the effects of different sugar epimers on the energies of binding and hydrolysis were quantified.

**EXPERIMENTAL PROCEDURES**

**Materials**

Substrates for β-glucosidase assays, *E. coli* β-galactosidase (grade IX, catalog no. G6512), para-nitrophenyl glycosides, 4-methylumbelliferyl glycosides, and all noncovalent inhibitors were purchased from Sigma. The inhibitor, 2-deoxy-2-fluoro-β-D-glucopyranosyl fluoride (2FβGlcF), was a generous gift of Dr. Steven G. Withers of the University of British Columbia. Cytosolic β-glucosidase was purified from guinea pig liver as described elsewhere (14).

**Kinetic Studies**

β-Glucosidase enzyme assays were performed as described previously (15). All kinetic studies were performed with pure enzyme that had been stored in 50% (v/v) ethylene glycol. Working solutions of enzyme were prepared by removing the ethylene glycol from 1.0 mg aliquots of the stock solution using a Centricon 30 ultrafiltration device. Working solutions of enzyme were prepared as described above. Enzyme aliquots (15 µl) were preincubated with equal volumes of inhibitor or water (for controls) at 25 °C for varying time periods. At the end of the preincubation period, residual enzyme activity was assayed by injecting 29 µl of the preincubation mixture into 3.0 ml of assay solution containing 5 mM MUGlc, 0.2 M sodium citrate buffer, pH 6.0, and 1 mg/ml bovine serum albumin. Prior to addition of the preincubation mixture, the assay solution was equilibrated at 35 °C in the thermostated block of a Perkin-Elmer LS-50 spectrophotometer. Enzyme activity was monitored at a wavelength of 410 nm. Kinetic parameters (K	extsubscript{m} and k	extsubscript{cat}) were determined by fitting the data to the Michaelis-Menten equation using the nonlinear regression program Enzfitter (Elsevier-Biosoft, Cambridge, UK). K	extsubscript{i} values for competitive and noncompetitive inhibitors were determined using standard methods (16).

**Inhibition Studies with 2FβGlcF and 2FβManF**

Inactivation of cytosolic β-glucosidase by the covalent inhibitors 2FβGlcF and 2FβManF was monitored using a continuous fluorimetric assay. Working solutions of enzyme (4 µg) were prepared as described above. Enzyme aliquots (15 µl) were preincubated with equal volumes of inhibitor or water (for controls) at 25 °C for varying time periods. At the end of the preincubation period, residual enzyme activity was assayed by injecting 29 µl of the preincubation mixture into 3.0 ml of assay solution containing 5 mM MUGlc, 0.2 M sodium citrate buffer, pH 6.0, and 1 mg/ml bovine serum albumin. Prior to addition of the preincubation mixture, the assay solution was equilibrated at 35 °C in the thermostated block of a Perkin-Elmer LS-50 spectrophotometer. Enzyme activity was monitored at a wavelength of 410 nm.

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**Fig. 1.** A, inactivation of CBG by 2FβGlcF. The enzyme was incubated with differing concentrations of 2FβGlcF and the residual activity measured at successive time points after the start of the incubation. Pseudo-first order rate constants were determined from the slope of the resulting regression lines. The concentrations of 2FβGlcF employed were as follows: ●, 0.5 mM; ▲, 0.4 mM; □, 0.3 mM; △, 0.2 mM; ○, 0.1 mM; ■, 50 µM. B, plot of pseudo-first order rate constants (k	extsubscript{obs}) versus the concentration of 2FβGlcF. The value of k	extsubscript{inact} was determined from the slope of the regression line obtained at each concentration of 2FβGlcF as shown in A. C, reactivation of enzyme inactivated with 2FβGlcF. Inactivated enzyme was incubated with 0.25 M 1-butanol and aliquots were assayed for enzymatic activity at various times after starting the incubation. D, dependence of the reactivation rate on [1-butanol]. β-Glucosidase inactivated with 2FβGlcF was incubated for 30 min in the presence of varying concentrations of 1-butanol and assayed for recovery of enzymatic activity.
zyme activity was quantitated by monitoring the release of 4-methylumbelliferyl, using an excitation wavelength of 325 nm and an emission wavelength of 437 nm, with slit widths set at 10 nm. Fluorescence readings were taken at 1 s intervals for 300 s and converted to an ASCII data file. Data files were exported and analyzed by linear regression using Enzfitter. Enzyme activity was proportional to the slope of the regression line. Preliminary experiments determined that this assay procedure yields a linear response with variations in both enzyme and substrate concentration. The enzyme was stable for over 1 h under the conditions of preincubation (using water instead of inhibitor). Pseudo-first order rate constants of inactivation were determined by measuring the time-dependent loss of enzyme activity at each inhibitor concentration. At each concentration of inhibitor, preincubation of enzyme with inhibitor was performed for five or more intervals of increasing duration. The natural logarithm of the residual enzyme activity (\(\ln(v/v_0)\)) was plotted versus time, and the pseudo-first order rate constant was determined from the slope of the resulting regression line. Values for the slopes were accepted if the standard error of the slope was less than or equal to 10% of the magnitude of the slope. Rate constants thus determined (\(k_{\text{obs}}\)) were used to calculate the maximal rate constant for inactivation (\(k_{\text{inact}}\)) and the inhibitor binding constant (\(K_I\)) using the following equation.

\[
k_{\text{obs}} = \frac{k_{\text{inact}}[2F\beta\text{GlcF}]}{K_I + [2F\beta\text{GlcF}]} \quad \text{(Eq. 1)}
\]

Reactivation of Inactivated Enzyme

An aliquot of the working dilutions of enzyme (4 \(\mu\)g), prepared as described above, was diluted with an equal volume of 0.5 M 1-butanol and assayed for activity using the continuous fluorimetric assay. The enzyme solution was then incubated with 0.5 mM 2F\(\beta\)GlcF for 100 min at 25 \(^\circ\)C. Excess inhibitor was removed by dilution with 2.0 ml of 10 mM sodium phosphate buffer, pH 6.0, followed by concentration using a Centricon 30 filter. The dilution and concentration steps were repeated once, and the retentate was brought to a final volume of 250 \(\mu\)l in 10 mM sodium citrate buffer, pH 6.0. Aliquots of the enzyme-inhibitor complex thus prepared were diluted with an equal volume of 0.5 M 1-butanol and incubated at 37 \(^\circ\)C for various time intervals prior to assaying for recovered activity. There was no activity in control aliquots of enzyme incubated with water instead of 1-butanol. The recovery of activity was plotted as a function of time, and the first-order rate constant was calculated using Enzfitter. In separate experiments, enzyme was prepared and inactivated with 2F\(\beta\)GlcF as described above, and the recovery of activity was measured after incubation for 20 min at 37 \(^\circ\)C with 0, 50, 100, 150, 200, and 250 mM 1-butanol. The association constant for 1-butanol was calculated by fitting the data to the Michaelis-Menten equation.

Stoichiometry of 2F\(\beta\)GlcF Incorporation

An aliquot (20 \(\mu\)l) of stock \(\beta\)-glucosidase was concentrated using a Centricon 30 filter to remove ethylene glycol and diluted in 10 mM sodium phosphate buffer, pH 6.0, in a final volume of 30 \(\mu\)l. An equal volume of 4 mM 2F\(\beta\)GlcF was added, and the mixture was incubated at 25 \(^\circ\)C for 60 min. A control sample of enzyme was incubated with water instead of inhibitor. Following incubation, the enzyme samples were lyophilized to a final volume of 20 \(\mu\)l using a SpeedVac (Savant, Farmingdale, NY), and stored at −20 \(^\circ\)C. Immediately prior to analysis, samples were thawed, and a 2.5-\(\mu\)l aliquot was withdrawn and added to 7.5 \(\mu\)l of 80% CH\(_3\)CN (v/v), 19.9% H\(_2\)O, and 0.1% (v/v) formic acid. The sample was introduced by direct infusion into a Fisons Quattro mass spectrometer (Fisons Instruments, Manchester, United Kingdom) equipped with an Analytica electrospray source (Analytica, Inc., Branford, CT). Data were analyzed using the Fisons Masslynx software.

Analysis of Transglycosylation Products

**HPLC**—Transglycosylation products generated by CBG were assayed by reversed-phase HPLC. Enzyme assays were performed as described above and terminated by adding 10 \(\mu\)l of 50% trifluoroacetic acid (v/v). Samples were injected onto a C\(_18\) column (8 \(\times\) 100 mm, Waters Delta Pak, Milford, MA). The glycosides of 4-methylumbelliferyl-one were eluted using a Waters 600E gradient controller according to the following linear gradient program: 0–5 min, 100% solvent A (0.1% trifluoroacetic acid (v/v) in \(d\)H\(_2\)O); 5–30 min, 0–35% solvent B (0.1% trifluoroacetic acid (v/v) in acetonitrile); 30–40 min, 35–100% solvent B; 40–42 min, 100% solvent B. The flow rate was 1.0 ml/min. The column effluent was monitored at 314 nm using a Waters 484 variable absorbance detector. Glycosides of \(p\)-nitrophenol were analyzed using the same system with slightly modified gradient conditions: 0–5 min, 100% solvent A; 5–25 min, 0–20% solvent B; 20–25 min, 20–80% solvent B; 28–30 min, 80–100% solvent B; 30–32 min, 100% solvent B. The column effluent was monitored at 310 nm for \(p\)-nitrophenol glycosides. Disaccharides identified by this procedure were purified for further analysis using identical separation conditions, with manual collection of the appropriate fractions.

**NMR Spectroscopy**—\(^1\)H NMR spectra (600 MHz) and \(^1\)H-decoupled \(^13\)C NMR spectra (125 MHz) were obtained on a Varian UnityPlus 600 MHz NMR spectrometer equipped with a 3-mm microprobe (Nalorac). Samples were dissolved in \(^2\)H\(_2\)O solvent (Cambridge Isotope Laboratories) in 3-mm NMR tubes, and spectra were obtained at 25 \(^\circ\)C. \(^1\)H spectra were referenced (internal) to the HOD signal (8.000 ppm), and \(^13\)C spectra were referenced (external) to the C-1 signal of \(\alpha\)-[\(^1\)\(^3\)C]mannopyranose (95.5 ppm). \(^1\)H and \(^13\)C chemical shifts are accurate to ±0.001 and 0.1 ppm, respectively, unless otherwise noted. \(^1\)H-\(^1\)H spin couplings (\(J\) values) were extracted directly from the spectra and are accurate to ±0.1 Hz. Two-dimensional heteronuclear multiple-quantum coherence spectra (17) were obtained as 2K \(\times\) 2K matrices after
data processing; gaussian exponential functions were applied to the F1 and F2 dimensions prior to zero-filling and Fourier transformation.

Thermodynamic Calculations

Binding energies were calculated using the Gibbs equation,

$$
\Delta G^0 = -RT \ln K_{eq}
$$

(Eq. 2)

where $K_{eq} = 1/K_m$ or $1/K_u$, $T = 310$ K, and $R = 8.3144$ J mol$^{-1}$ K$^{-1}$. Similarly, changes in the free energies of activation ($\Delta G^\circ$) were calculated using the following relationship,

$$
\Delta G^\circ = -RT \ln \left( \frac{k_{cat1}}{k_{cat2}} \right)
$$

(Eq. 3)

where the subscripts 1 and 2 refer to the $K_m$ and $k_{cat}$ values of the two substrates being compared.

RESULTS

Inactivation by 2FβGlcF—Inactivation of CBG by 2FβGlcF followed pseudo-first order kinetics (Fig. 1A). We could not calculate the inactivation rate constant ($k_{inact}$) or the binding affinity ($K_j$) for 2FβGlcF because the rate of inactivation at high concentrations of inhibitor was too rapid to permit the reliable measurement of the pseudo-first order rate constants. The plot of the pseudo-first order rate constant $k_{inact}$ versus [2FβGlcF] was linear, however (Fig. 1B), which allowed calculation of the ratio of $k_{inact}/K_j$ (0.00122 s$^{-1}$) according to Equation 1. The competitive inhibitor, N-octyl β-D-glucopyranoside, protected the enzyme against inactivation by 2FβGlcF, thus demonstrating that the inhibitor is active site-directed. To estimate the stoichiometry of inhibitor incorporation, we determined the mass of the enzyme before and after inhibition with 2FβGlcF. The mass of the control enzyme was 53,710 Da, which was consistent with the mass estimated by SDS-polyacrylamide gel electrophoresis (53 ± 1 kDa) (14). The difference of 34 Da between the mass predicted from the cDNA sequence (53,744 Da) and the mass of the purified enzyme is greater than the error for the mass estimation (> 20 Da), which suggests the presence of a posttranslational modification. Incubation of the enzyme with 2FβGlcF resulted in the shift of the major mass peak to 53,875 Da (Fig. 2). The difference of 165 Da represents the incorporation of one 2FGlc residue per enzyme molecule.

To determine whether the inactivated enzyme retained catalytic activity, we assayed for the recovery of enzyme activity by incubation with established transglycosylation acceptors (2). No reactivation was observed in the presence of 5 mM octyl β-D-glucopyranoside or with 50 mM pNPGlc. However, the 2FβGlcF-inactivated β-glucosidase was reactivated by addition of 1-butanol, a potent transglycosylation acceptor for CBG (2). Incubation of the completely inactivated enzyme with 0.25 mM 1-butanol resulted in exponential recovery of activity, with a rate constant of 0.028 s$^{-1}$ (Fig. 1C). Reactivation by 1-butanol showed a saturating dependence on 1-butanol concentration (Fig. 1D), with an association constant of 0.34 mM.

Effects of Transglycosylation Reactions on Kinetic Parameters—CBG exhibits a biphasic pattern of $v_0$ versus [S] data with the substrates MUGal and MUGlc but not with MUFuc (Fig. 3). A previous study demonstrated that CBG forms disaccharides by transferring a sugar moiety to an appropriate acceptor (2). Therefore, to determine whether the biphasic kinetics were due to transglycosylation reactions, we analyzed the reaction products by HPLC. A disaccharide product was detected at concentrations of MUGal greater than 0.1 mM (Fig. 4); similar data were obtained with MUGlc (data not shown). In contrast, no disaccharide was produced when MUFuc was the substrate.
These data indicated that transglycosylation competes with hydrolysis at high concentrations of MUGal or MUGlc. The inflection point in the $v_0$ versus $[S]$ data for MUGal suggests that a second substrate molecule binds to the enzyme at concentrations above 0.1 mM and serves as the acceptor for the sugar moiety of the substrate, in lieu of water. The $K_m$ and $k_{cat}$ values for hydrolysis and transglycosylation reactions with MUGal and MUGlc were estimated by fitting the data at the extremes of the $v_0$ versus $[S]$ curves to the Michaelis-Menten equation. The $K_m$ and $k_{cat}$ values for the hydrolysis of both substrates were significantly lower when estimated in this manner than when the correction for transglycosylation was not made (Table I).

**Transglycosylation Acceptor Specificity**—The results described above were consistent with a previous demonstration that CBG will transfer sugars to alkyl primary hydroxyl acceptors but not to secondary or tertiary alkyl hydroxyls (2). We therefore hypothesized that this enzyme would form disaccharide transglycosylation products only with acceptor sugars having a primary hydroxyl. To test this hypothesis, we determined the structures of disaccharide products generated from pNPGal and from mixtures of pNPGal with either pNP-α-L-arabinofuranoside, pNP-α-L-arabinopyranoside, or pNPXyl. Transglycosylation products were purified by HPLC, and their structures were determined by NMR spectroscopy.

The $^1$H NMR spectrum of the disaccharide 1 in $^2$H$_2$O formed from pNPGal alone was identical to that obtained for authentic pNP β-D-galactopyranosyl-(1→6)-β-D-galactopyranoside, thus establishing its structure (Scheme 1).

The $^1$H NMR spectrum of the disaccharide 1 in $^2$H$_2$O formed from pNPXyl was essentially identical to that obtained for authentic pNP β-D-galactopyranosyl-(1→5)-α-L-arabinofuranoside (Table II), thus establishing its structure (Scheme 1).

The reaction of pNPGal with pNP α-L-arabinopyranosidase did not produce a disaccharide product, but the reaction of pNPGal with pNP α-L-arabinofuranosidase yielded a novel disaccharide, pNP β-D-galactopyranosyl-(1→5)-α-L-arabinofuranoside 2. The $^{13}$C spectrum (125 MHz) of 2 in $^2$H$_2$O contained 11 nonaromatic signals, which is consistent with a disaccharide composed of hexose (Gal) and pentose (Ara) units (Table III). Characteristic signals at 82.5 and 85.6 ppm indicate the presence of an α-arabinofuranosyl ring (Tables III and IV) (18). The C-5 signal of the Ara residue of 2 is observed at 70.3 ppm, which is significantly downfield of the signal found in methyl α-arabinofuranoside (62.4 ppm, Table IV). The latter shift, and the lack of a second hydroxymethyl carbon signal at ~62 ppm in the $^{13}$C spectrum of 2, indicates that the Gal residue is linked at C-5 of Ara. Further evidence of the α-arabinofuranosyl ring derives from the $^1$H NMR spectrum of 2 in $^2$H$_2$O, which is essentially first-order at 600 MHz (Table II). Endocyclic $^1$H-$^1$H spin-couplings observed in the Ara component (Table III) are very similar to those found in the authentic pNP α-L-arabinofuranosidase (Table V).

The reaction of pNPGal with pNPXyl gave the disaccharide, pNP β-D-galactopyranosyl-(1→4)-β-D-xylopyranoside 3. The $^1$H NMR spectrum of 3 at 600 MHz was essentially first-order, and signals were assigned based on the assumption that 3 contained Gal and Xyl monomers (Table VI). $^2$H$_{1H,1H}$ values (7.6 and 7.8 Hz) indicated that both residues have the β-anomeric configuration (Table VII). $^1$H signal assignments for 3 were

**Table I**

Kinetic constants for substrates and inhibitors

| Substrate (inhibitor) | $K_m$ (Km) | $k_{cat}$ | $k_{cat}/K_m$ |
|-----------------------|-----------|----------|--------------|
| MUGlc, uncorrected    | 0.082     | 239      | 2910         |
| Hydrolysis            | 0.031     | 147      | 4740         |
| Transglycosylation    | 0.105     | 250      | 2380         |
| MUGal, uncorrected    | 0.185     | 188      | 1020         |
| Hydrolysis            | 0.062     | 96       | 1550         |
| Transglycosylation    | 0.213     | 196      | 920          |
| MUGlc + 1-butanol     | 0.373     | 485      | 1300         |
| MUFuc                 | 0.035     | 170      | 4860         |
| MUXyl                 | 0.028     | 4.4      | 157          |
| MUAra                 | 0.0048    | 5.8      | 1210         |
| MUMMan                | 0.0065    | ND       | ND           |

* The notation "hydrolysis" indicates substrate concentrations below 0.1 mM.
* Transglycosylation indicates substrate concentrations greater than 0.1 mM. ND = not determined.
The formation of pNP β-D-galactopyranosyl-(1→4)-β-D-xylopyranoside indicates that the C-4 secondary hydroxyl of xylose can serve as a transglycosylation acceptor.

Thermodynamic Comparisons of Sugar Epimers—After applying the correction for transglycosylation as appropriate, we calculated the effects of different substituents at the C-4 and C-5 positions of the pyranose ring on the energies of binding and transition state stabilization using Equations 2 and 3. Data in Table VIII reveal that the presence of a C-6 hydroxymethyl group, an axial O-4 (MUGal) is less favorable than an equatorial O-4 (MUGlc); a larger activation energy barrier (2.9 kJ/mol) and a lower binding energy (1.8 kJ/mol) are observed for the axial epimer. This effect is absent when a CH₃ group replaces the hydroxymethyl group, with nearly identical binding energies and activation energy barriers for MUFuc and MUGlc (Table VIII). These results indicate that the configurations of the hydroxyls at C-4 and C-6 exert subtle effects on binding and hydrolysis, whereas the presence of a C-6 carbon greatly enhances the rate of hydrolysis.

To determine the effects of configuration at C-2, we compared a homologous series of β-D-glucopyranosides and β-D-mannopyranosides. Two inhibitors, 1-deoxynorjirimycin and 1-deoxymannorjirimycin, showed Kᵢ values of 0.2 and 0.67 mM, respectively, which suggested that the axial O-2 has a more favorable interaction than an equatorial O-4 (MUGlc); an axial O-4 (MUGal) is less favorable than an equatorial O-4 (MUGlc), with activation energy barriers (2.9 kJ/mol) and lower binding energy (1.8 kJ/mol) relative to their respective equatorial counterparts. There was no detectable hydrolysis of MUFuc by cytosolic β-glucosidase. As was the case for MUFuc, we were unable to achieve saturating concentrations of 2F GlcF (Table I), but there was no increase in the rate of hydrolysis of MUMan under the same conditions. These data were consistent with the norjirimycin inhibitor results and suggested that the rate-limiting step for hydrolysis of MUMan by cytosolic β-glucosidase is the enzyme glycosylation step (Fig. 5). We therefore surprised to find that 2FβManF rapidly and completely inactivated cytosolic β-glucosidase. As was the case with 2FβGlcF, we were unable to achieve saturating concentrations of 2FβManF and thus could not calculate reliable kᵢ and Kᵢ values. However, the plot of the pseudo-first order rate constants versus [2FβManF] was linear (Fig. 6A) and yielded an estimate of kᵢ/Kᵢ of 1.95 × 10⁻⁴. In contrast to the fluorglucosidase inhibitor, CBG inactivated with 2FβManF showed a much greater rate of reactivation, with a half-life for reactivation of 22.9 min (Fig. 6A), indicating that the enzyme-inhibitor complex formed with 2FβManF is much less stable than that formed from 2FβGlcF.

**DISCUSSION**

The results of this study show that the catalytic mechanism of cytosolic β-glucosidase involves the formation of a covalently linked enzyme-sugar intermediate. The enzyme generates disaccharide transglycosylation products with either β-(1→6) orβ-(1→3) linkages.
As we were unable to detect significant hydrolysis by MUMan, this was calculated using Equations 2 and 3 as described in the text. All values used in the calculations are as reflected in Table I. For estimation of binding energies and changes in activation energies (ΔG° and ΔDDG°), we took data from Ref. 19. Methyl a-D-[1-13C]mannopyranoside (95.5 ppm) was used as an internal reference.

Data taken from Ref. 19. Methyl a-D-[1-13C]mannopyranoside (95.5 ppm) was used as an internal reference.

Assignments may be reversed.

For 3, relative to the HOD signal (4.800 ppm); for pNPXyl, relative to C1 of a-D-[1-13C]mannopyranose (95.5 ppm); for 6, relative to C1 of methyl a-D-xylopyranose. (95.5 ppm): NMR data for disaccharide 3 and reference compounds

| Disaccharide | Nucleus | 1H shifts | 1H-1H spin coupling constants
|--------------|---------|-----------|----------------------------|
|              | ppm     | Hz        |                           |
| 3: Gal residue | H1      | 4.596     | H1,H2 7.8                 |
|              | H2      | 3.635     | H2,H3 10.0                |
|              | H3      | 3.752     | H3,H4 3.4                 |
|              | H4      | 4.024     | H4,H5 1.0                 |
|              | H5      | 3.809     | H5,H6 8.2                 |
|              | H6      | 3.914     | H5,H6′ 3.9                |
|              | H6′     | 3.856     | H6,H6′ 11.9               |
| 3: Xyl residue | H1      | 5.355     | H1,H2 7.6                 |
|              | H2      | 3.773     | H2,H3 9.4                 |
|              | H3      | 3.849     | H3,H4 8.9                 |
|              | H4      | 4.062     | H4,H5 5.3                 |
|              | H5      | 4.296     | H4,H5′ 10.2               |
|              | H5′     | 3.731     | H4,H5′ 11.8               |

| 3: pNP Reference compounds |
|----------------------------|
| pNPXyl H1 | 5.297 | C1 | 101.5 |
| pNPXyl H2 | ~3.713 | C2 | 74.1 |
| pNPXyl H3 | ~3.680 | C3 | 76.8 |
| pNPXyl H4 | 3.826 | C4 | 70.4 |
| pNPXyl H5 | 4.143 | C5 | 66.8 |
| pNPXyl H5′ | 3.629 |     |      |

a For 3, relative to the HOD signal (4.800 ppm); ±0.001 ppm; for pNPXyl: ±0.002.
b ±0.1 Hz.
c For 3 and pNPXyl, relative to C1 of a-D-[1-13C]mannopyranose (95.5 ppm); ±0.1 ppm.
d Assignments may be reversed.

FIG. 5. Reaction mechanism proposed for CBG. R-OH, the departing aglycone with its free alcohol; A-OH, an incoming acceptor that can be either an organic compound with a free hydroxyl or a water molecule; Enz–Glc, the covalent enzyme-substrate intermediate; ES, the precatalytic enzyme substrate complex.

The differences in the binding energies for MUGlc, MUGal, and MUFuc likely reflect subtle differences in their interactions with active site residues (Table VII). The major effect of the C-6 carbon on the energy of transition state stabilization,
Our data indicate that the transglycosylation specificity of cytosolic β-glucosidase is broader than its disaccharide hydro-

lase specificity. The generation of β-(1→6)-linked disaccharides from MUGlc is consistent with the exo
dlytic hydrolysis of pNP-

tentiobiose by this enzyme (15). However, the enzyme also

generates pNP β-D-galactopyranosyl-β-(1→6)-D-galactopyra-

nose, pNP β-D-galactopyranosyl-(1→5)-α-L-arabinofuranoside,

and pNP β-D-galactopyranosyl-(1→4)-β-D-xylpyranoside; none

of these disaccharides are hydrolyzed by cytosolic β-glucosi-

dase. These results indicate that, unlike secondary alkyl alco-

hols (e.g. isopropanol (21)), secondary alcohols of sugars may

serve as transglycosylation acceptors if they are appropriately

configured. Moreover, disaccharide formation did not occur

with pNPXyl alone. Therefore, for this enzyme, disaccharide

formation is not simply the reverse of disaccharide hydrolysis.

Furthermore, the determination of the specificity for transgly-

cosylation reactions is a prerequisite for accurate interpreta-

tion of kinetic data obtained with CBG. The ability of CBG to

form novel disaccharides via transglycosylation reactions might prove useful for synthetic purposes.

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diagram

caption: Fig. 6. Inactivation of CBG by 2FβManF. A, plot of pseudo-first

order rate constants (kobs) versus [2FβManF]. Measurements and cal-

culation were as described for Fig. 1. B, reactivation of cytosolic β-glucosi-

dase in aqueous solution after complete inactivation with 2FβManF.

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Catalytic Mechanism and Specificity for Hydrolysis and Transglycosylation Reactions of Cytosolic β-Glucosidase from Guinea Pig Liver

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