# Ground-State, Transition-State, and Metal-Cation Effects of the 2-Hydroxyl Group on β-D-Galactopyranosyl Transfer Catalyzed by β-Galactosidase (*Escherichia coli, lac Z*)†

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*Received May 20, 2005; Revised Manuscript Received July 12, 2005*

ABSTRACT: Substitution of the C2−OH group by C2−H at 4-nitrophenyl-β-D-galactopyranoside to give 4-nitrophenyl-2-deoxy-β-D-galactopyranoside causes (1) a change in the rate-determining step for β-galactosidase-catalyzed sugar hydrolysis from formation to breakdown of a covalent intermediate; (2) a 14 000-fold decrease in the second-order rate constant $k_3/K_d$ for enzyme-catalyzed transfer of the β-D-galactopyranosyl group from the substrate to form a covalent adduct to the enzyme; and (3) a larger 320 000-fold decrease in the first-order rate constant $k_s$ for hydrolysis of this covalent adduct. Only a small fraction (ca. 7%) of the 2-OH substituent effect is expressed in the ground-state Michaelis complex, so that the (apparent) strong interactions between the enzyme and 2-OH group that stabilize the transition state for β-D-galactopyranosyl transfer only develop upon moving from the Michaelis complex to the transition state. $Mg^{2+}$ activates β-galactosidase for cleavage of both 4-nitrophenyl-β-D-galactopyranoside and 4-nitrophenyl-2-deoxy-β-D-galactopyranoside. This suggests that $Mg^{2+}$ activation does not involve interactions with the 2-OH group. The removal of $Mg^{2+}$ from β-galactosidase causes a change in the rate-determining step for enzyme-catalyzed hydrolysis of 4-nitrophenyl-2-deoxy-β-D-galactopyranoside from breakdown to formation of the covalent intermediate. The observed 2-OH effect would require a very large (10−11 kcal/mol) stabilization of the transition state for β-D-galactopyranosyl group transfer to water by interactions between β-galactosidase and the neutral 2-OH group. We suggest that the apparent effect of the neutral substituent is more simply rationalized by ionization of the 2-OH to form a $2\text{-}O^-$ anion, which provides effective electrostatic stabilization of the cationic transition state for glycoside cleavage at an active site of relatively low dielectric constant.

Enzymes and other catalysts accelerate the rate of uncatalyzed reactions through the stabilization of catalyst-bound transition states (*1*). This stabilization can be quantified as the "transition-state-binding energy", which is the difference in the Gibbs free energy of activation for the catalyzed and uncatalyzed reactions (*2*, *3*). The total binding energy between an enzyme and its transition state may be defined as the *intrinsic* substrate-binding energy, and this may be divided into the binding energy that is expressed in the Michaelis complex and that which is only expressed at the transition state (*4*). The quantification of the intrinsic substrate-binding energy is, in principle, straightforward (*4*−*9*). However, the problem of quantifying the contribution of the intrinsic binding energy of individual fragments of the substrate can be intractable for enzymes that catalyze the reactions of large substrates, where the total binding energy is the sum of many relatively small individual interactions. Enzymes that catalyze the reactions of relatively low molecular weight substrates must focus their transition-state-binding energy on a few substrate fragments. The evaluation of the intrinsic binding energy (*4*) of such *critical* fragments has the potential to provide useful insight into the catalytic reaction mechanism.

We have reported that ca. 80% (14 kcal/mol) of the estimated 16 kcal/mol of the binding energy between triosephosphate isomerase and the transition state for deprotonation of D-glyceraldehyde 3-phosphate is due to interactions with the substrate phosphate group (*10*) and have suggested that this phosphate-binding energy is used to sequester the substrate in a nonpolar environment favorable for proton transfer (*11*, *12*). In this paper, we focus on the apparent strong binding interactions that develop between β-galactosidase and the 2-OH group of β-D-galactopyranosyl derivatives.

D-Galactal (Scheme 1) *acts* as both a slow-binding inhibitor of β-galactosidase-catalyzed cleavage of β-D-galactopyranosyl derivatives (*13*, *14*) and as a poor substrate for β-galactosidase-catalyzed hydration to form 2-deoxygalactose (*14*−*16*). A two-step mechanism was proposed for the hydration reaction, where addition of a basic amino acid side chain to the alkene gives the 2-deoxy analogue of the galactosyl−enzyme intermediate, which then undergoes hydrolysis to **H-1-OH** (Scheme 1) (*14*). The high affinity of D-galactal (low $K_m \approx K_i = 1.4 \times 10^{-5}$ M) for β-galactosidase is due to the great stability of the covalent intermediate ($k_s = 0.0046$ s$^{-1}$, Scheme 1) (*14*).

† This work was supported by GM39754 from the National Institutes of Health.

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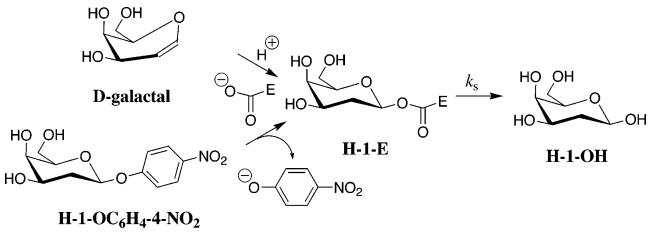
10.1021/bi050936q CCC: $30.25 
Published on Web 08/11/2005

Scheme 1




The $3 \times 10^5$-fold difference in $k_s = 0.0046\ s^{-1}$ (*14*) and 1300 $s^{-1}$ (*17*, *18*) for the hydrolysis of the covalent 2-deoxy-$\beta$-D-galactopyranosyl and $\beta$-D-galactopyranosyl intermediates, respectively, is consistent with at least a 7.4 kcal/mol stabilization of the transition state for hydrolysis of the covalent intermediate of the physiological reaction by interaction of the enzyme with the C-2 hydroxyl group. The inductive effect of the C-2 hydroxyl group causes a ca. 3.1 kcal/mol destabilization of the transition state for nonenzymatic hydrolysis of sugars (*19*).[1] If the 2-OH group caused a similar 3.1 kcal/mol destabilization of the transition state for hydrolysis of the $\beta$-D-galactopyranosyl intermediate, then its binding interaction would need to be $(3.1 + 7.4) \approx 10.5$ kcal/mol to give the observed 7.4 kcal/mol stabilizing effect. The value of $k_{cat}$ ($s^{-1}$) for enzyme-catalyzed hydrolysis of glycosides is ca. $10^{17}$ larger than the first-order rate constant for spontaneous sugar hydrolysis (*7*), which corresponds to ca. 23 kcal/mol stabilization of the transition state for cleavage of simple glycosides (*6*). This suggests that almost half of the total binding energy of $\beta$-galactosidase for its transition state involves, in some sense, interactions with the 2-OH of the substrate. This would represent an extraordinary focus of the catalytic rate acceleration on interactions with a small neutral hydroxyl group.

The magnitude of the interaction between $\beta$-galactosidase and the 2-OH of the substrate has been inferred from studies on enzyme-catalyzed hydration of D-galactal. In addition, this strong interaction has been characterized for $\beta$-glucosidase-catalyzed hydrolysis of $\beta$-D-glucosides (*20*), an enzyme that catalyzes hydration of D-glucal though a stable 2-deoxy-$\beta$-D-glucopyranosyl intermediate (*21*). However, the interaction has not been characterized for $\beta$-galactosidase, an enzyme for which the 2-OH interactions of bound substrate, transition-state analogues, and intermediate analogues have been characterized by X-ray crystallographic analysis (*22*). It is important to characterize kinetically this 2-OH interaction for a $\beta$-galactosidase-catalyzed hydrolysis reaction because this enzyme differs from $\beta$-glucosidase in its subunit structure (*23*), pH-dependence (*24*−*27*), and metal-cation requirements (*24*, *25*) and might therefore show differences in its interaction with the 2-hydroxyl group during catalysis of substrate cleavage. We report here the synthesis of 4-nitrophenyl-2-deoxy-$\beta$-D-galactopyranoside (*28*) and the kinetic parameters for $\beta$-galactosidase-catalyzed cleavage of this substrate in the presence and absence of activation of the enzyme by $Mg^{2+}$ (*25*−*27*).

Our data provide direct evidence that $\beta$-galactosidase-catalyzed hydration of D-galactal and cleavage of 4-nitrophenyl-2-deoxy-$\beta$-D-galactopyranoside proceed through a common $\beta$-D-galactopyranosyl intermediate (Scheme 1), as has been shown previously for $\beta$-glucosidase-catalyzed hydration of D-glucal (*21*) and hydrolysis of 4-methylumbelliferyl-2-deoxy-$\beta$-D-glucopyranoside (*20*). In addition we find that the *observed* binding interactions between the enzyme and the C-2 hydroxyl of the substrate are very small in the ground-state Michaelis complex and only develop with the approach to the transition state for glycoside cleavage. This is in contrast to the observed interactions between $\beta$-galactosidase and C-4 and C-6 sugar hydroxyls, which *are* partly expressed in the ground-state Michaelis complex and then develop fully in the transition state for $\beta$-galactosidase-catalyzed cleavage of 2,4-dinitrophenyl-$\beta$-D-galactopyranoside (*29*).

## EXPERIMENTAL PROCEDURES

Reagent-grade organic chemicals and inorganic salts from commercial sources were used without further purification. Water was distilled and then passed through a Milli-Q water purification system. $\beta$-D-Nicotinamide adenine dinucleotide (NAD), 4-nitrophenyl-$\beta$-D-galactopyranoside, and $\beta$-galactosidase from *Escherichia coli* (grade VIII) were purchased from Sigma.

The solution pH was determined at the end of each kinetic experiment on $\beta$-galactosidase using an Orion Model 601A pH meter equipped with a Radiometer GK2321C combination electrode that was standardized at pH 7.00 and 10.00. The difference in the extinction coefficients at 405 nm for **H-1-OC$_6$H$_4$-4-NO$_2$** and the products of $\beta$-galactosidase-catalyzed hydrolysis was calculated from the change in absorbance observed upon quantitative enzyme-catalyzed hydrolysis. Values of $\Delta\epsilon = 8900$ and 18 300 $M^{-1}\ cm^{-1}$ at pH 7.0 and 8.6, respectively, were the same as determined in an earlier work for enzyme-catalyzed hydrolysis of **HO-1-OC$_6$H$_4$-4-NO$_2$** (*30*). $^1$H NMR spectra at 400 or 500 MHz were recorded in $CDCl_3$ or $D_2O$ on Varian VXR-400 or -500 spectrometers. Chemical shifts in $D_2O$ are reported relative to the chemical shift of 4.67 ppm for DOH of the solvent.

*4-Nitrophenyl-2-deoxy-$\beta$-D-galactopyranoside*. 4-Nitrophenyl-2-deoxy-$\beta$-D-galactopyranoside (**H-1-OC$_6$H$_4$-4-NO$_2$**) was prepared by following a published procedure (*28*). 4-Nitrophenyl-2-deoxy-$\beta$-D-galactopyranoside (**H-1-OC$_6$H$_4$-4-NO$_2$**, recrystallized from methanol) mp, 141−143 °C. $^1$H NMR (500 MHz, $D_2O$) $\delta$: 8.12, 7.08 (4 H, $A_2B_2$, $J = 10.0$ Hz, $C_6H_4$), 5.35 (1 H, dd, H-1, $J = 10.0$, 2.5 Hz), 3.87 (1H, ddd, H-3, $J = 12.0$, 5.0, 3.0 Hz), 3.73 (1H, broad d, H-4, $J = 3$ Hz), 3.67−3.63 (3H, m, H-5, H-6), 2.08 (1H, ddd, H-2, $J = 12.0$, 5.0, 2.5), 1.87 (1H, ddd, H-2, $J = 12.0$, 12.0, 10.0).

*Enzyme Assays.* The activity of $\beta$-galactosidase was routinely determined at 25 °C by monitoring the formation of 4-nitrophenoxide anion at 405 nm for reactions at pH 7.0 (100 mM sodium phosphate) or pH 8.6 (25 mM sodium pyrophosphate) in solutions that contain 1.0 mM $MgCl_2$ and 0.5 mM 4-nitrophenyl-$\beta$-D-galactopyranoside (*31*). Magnesium-free $\beta$-galactosidase was prepared by extensive dialysis against 10 mM EDTA. The activity for the magnesium-free enzyme was determined under similar conditions in solutions that contain no $MgCl_2$ and 10 mM EDTA.

*Enzyme-Catalyzed Reactions of **H-1-OC$_6$H$_4$-4-NO$_2$**.* A stock solution of 0.032 M **H-1-OC$_6$H$_4$-4-NO$_2$** in acetone was used for these experiments. A precipitate was observed when

[1] This substituent effect was calculated from a Hammett-type equation using $\sigma_H = 0$, $\sigma_{OH} = 0.25$, and $\rho_I = -9$ determined for the solvolysis of 2-substituted dinitrophenyl-$\beta$-D-galactopyranosides in water (*19*).

Table 1: Kinetic Parameters for β-Galactosidase-Catalyzed Cleavage of **H-1-OC$_6$H$_4$-NO$_2$** Determined by Steady-State and Pre-Steady-State Analyses at 25 °C

| reaction conditions | $K_d$ (M)[a] | $k_3$ ($s^{-1}$)[a] | $k_s$ ($s^{-1}$)[b] | $k_{cat}$ ($s^{-1}$)[c] | $K_m$ (M)[c] | $k_{cat}/K_m$[c] ($M^{-1}$ $s^{-1}$) |
|---|---|---|---|---|---|---|
| 1 mM $Mg^{2+}$ at pH 8.6[d] | $1.2 \times 10^{-4}$ | 0.017 | 0.0022 (0.0019)[e] | 0.0030 | $1.8 \times 10^{-5}$ ($1.4 \times 10^{-4}$)[f] | 170 |
| 1 mM $Mg^{2+}$ at pH 7.0[g] | | ≫0.0034 | 0.0024 (0.0046)[h] | 0.0034 | $1.2 \times 10^{-5}$ | 280 |
| −$Mg^{2+}$ at pH 7.0[g] | $3.2 \times 10^{-5}$ [i] | 0.0007 | >0.033[j] | 0.0007 | $3.2 \times 10^{-5}$ | 22 |

[a] Obtained by fitting the kinetic data obtained during the approach to steady state (Figure 2) to eq 1. [b] Determined by making a 1000-fold dilution of β-galactosidase that has achieved steady state for the cleavage of **H-1-OC$_6$H$_4$-NO$_2$** and then monitoring the recovery of enzymatic activity (Figure 3). [c] Steady-state kinetic parameters for β-galactosidase-catalyzed cleavage of **H-1-OC$_6$H$_4$-NO$_2$**. [d] In 25 mM sodium pyrophosphate buffer. [e] Determined by making a 1000-fold dilution of β-galactosidase that has achieved steady state for the cleavage of D-galactal and then monitoring the recovery of enzymatic activity (Figure 3). [f] Kinetic parameter calculated from the values of $K_d$, $k_3$, and $k_s$ in this table and the relationship $K_m = K_d[k_s(k_3 + k_s)]$ derived from Scheme 2. [g] In 100 mM sodium phosphate buffer. [h] Rate constant for reactivation of the enzyme inhibited by D-galactal reported in an earlier work (*14*). [i] $K_m \approx K_d$ because $k_3 \gg k_s$. [j] A lower limit set by the failure to observe a lag in the recovery of full enzymatic activity (see Figure 4 and the discussion of this figure in the text).

more concentrated solutions of this substrate in acetone were diluted into water. β-Galactosidase-catalyzed hydrolysis of **H-1-OC$_6$H$_4$-4-NO$_2$** at 25 °C was followed by monitoring the formation of 4-nitrophenoxide anion at 405 nm for reactions at pH 7.0 (100 mM sodium phosphate) or pH 8.6 (25 mM sodium pyrophosphate) in solutions that contain 1.0 mM $MgCl_2$. Similar conditions were used for the magnesium-free enzyme, but 10 mM EDTA was substituted for 1 mM $MgCl_2$. The reactions were initiated by the addition of 1−24 μL of 0.032 M **H-1-OC$_6$H$_4$-4-NO$_2$** in acetone to buffered solutions that contain ca. 1 μM β-galactosidase subunits in a final volume of 1.00 mL. Enzyme-catalyzed cleavage of **H-1-OC$_6$H$_4$-4-NO$_2$** to form 4-nitrophenoxide ion was monitored by following the increase in absorbance at 405 nm. Control experiments to determine the effect of acetone on $k_{cat}$ for β-galactosidase-catalyzed hydrolysis of **HO-1-OC$_6$H$_4$-4-NO$_2$** (0.5 mM ≫ $K_m$) showed that the enzyme activity was reduced by up to 10% for reactions in the presence of 2.4% (0.4 M) acetone.

*Hydrolysis of **H-1-E***. The reaction of β-galactosidase labeled with a 2-deoxy-β-D-galactopyranosyl group (**H-1-E**, Scheme 1) was studied in the following experiments.

(1) β-Galactosidase (0.2 μM enzyme subunits) and **H-1-OC$_6$H$_4$-4-NO$_2$** (0.1 mM) were incubated for 5 min at pH 7.0 (100 mM sodium phosphate) or pH 8.6 (25 mM pyrophosphate) in solutions that contain 1.0 mM $MgCl_2$. A total of 1 μL was withdrawn from each solution and diluted 1000-fold into 1 mL of the same buffer that contains 0.5 mM **HO-1-OC$_6$H$_4$-4-NO$_2$** instead of **H-1-OC$_6$H$_4$-4-NO$_2$**. The increase in absorbance at 405 nm from enzyme-catalyzed hydrolysis of **HO-1-OC$_6$H$_4$-4-NO$_2$** was monitored.

(2) β-Galactosidase (0.4 μM enzyme subunits) and **H-1-OC$_6$H$_4$-4-NO$_2$** (0.1 mM) were incubated for 5 min at pH 7.0 (100 mM sodium phosphate) in a solution that contains 1.0 mM $MgCl_2$. A total of 1 μL was withdrawn and diluted 1000-fold into 1 mL of the same buffer that contains 0.03 mM **HO-1-OC$_6$H$_4$-4-NO$_2$** instead of **H-1-OC$_6$H$_4$-4-NO$_2$** and 10 mM EDTA instead of $MgCl_2$. The increase in absorbance at 405 nm from enzyme-catalyzed hydrolysis of **HO-1-OC$_6$H$_4$-4-NO$_2$** was monitored.

(3) β-Galactosidase (0.2 μM enzyme subunits) and D-galactal (0.01 mM) were incubated for 5 min at 25 °C and pH 8.6 (25 mM pyrophosphate) in a solution that contains of 1 mM MgCl. A total of 1 μL was withdrawn and diluted 1000-fold into 1 mL of the same buffer that contains 0.5 mM **HO-1-OC$_6$H$_4$-4-NO$_2$** instead of D-galactal, and the increase in absorbance at 405 nm from enzyme-catalyzed hydrolysis of **HO-1-OC$_6$H$_4$-4-NO$_2$** was monitored.

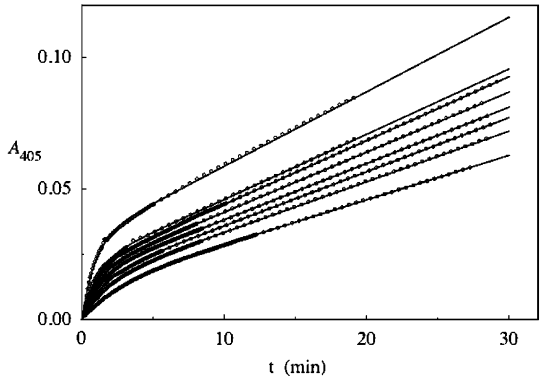



FIGURE 1: Increase in $A_{405}$ with time during the approach to steady state for β-galactosidase-catalyzed hydrolysis of **H-1-OC$_6$H$_4$-4-NO$_2$** at pH 8.6 and 25 °C. The individual curves, running from the bottom to the top of the figure, were obtained for reactions of the following concentrations of **H-1-OC$_6$H$_4$-4-NO$_2$**: 0.032, 0.064, 0.096, 0.128, 0.192, 0.384, 0.576, and 0.768 mM.

*Data Analysis.* Nonlinear least-squares fits of kinetic data to the appropriate kinetic equation were performed using SigmaPlot from Jandel Scientific.

## RESULTS

Figure 1 shows representative data for β-galactosidase-catalyzed hydrolysis of **H-1-OC$_6$H$_4$-4-NO$_2$** at 25 °C and pH 8.6 in the presence of 1.0 mM $Mg^{2+}$. A burst of 4-nitrophenoxide ion is observed at early reaction times during the approach to steady state followed by a linear *initial* steady-state velocity ($v_{ss}$). These data were evaluated separately during the pre-steady-state and steady-state time regimes.

The steady-state kinetic parameters $V_{max}$ and $K_m$ for β-galactosidase-catalyzed hydrolysis of **H-1-OC$_6$H$_4$-4-NO$_2$** at 25 °C and in the presence of 1 mM $Mg^{2+}$ at pH 8.6 were determined from the nonlinear least-squares fit of the steady-state velocities $v_{ss}$ to eq 1. Values of $k_{cat}$ for hydrolysis of **H-1-OC$_6$H$_4$-4-NO$_2$** were calculated from the relative values of $V_{max}$ determined for β-galactosidase-catalyzed hydrolysis of **HO-1-OC$_6$H$_4$-4-NO$_2$** and **H-1-OC$_6$H$_4$-4-NO$_2$** by identical concentrations of enzyme, and $k_{cat} = 120$ $s^{-1}$ for hydrolysis of **HO-1-OC$_6$H$_4$-4-NO$_2$** at pH 8.6 (*31*). The same procedure was followed in determining values of $k_{cat}$ and $K_m$ for β-galactosidase-catalyzed hydrolysis of **H-1-OC$_6$H$_4$-4-NO$_2$** at pH 7.0 but using $k_{cat} = 156$ $s^{-1}$ for enzyme-catalyzed hydrolysis of **HO-1-OC$_6$H$_4$-4-NO$_2$** at the lower pH (*17*). The values of $k_{cat}$ and $K_m$ determined in these experiments are summarized in Table 1.

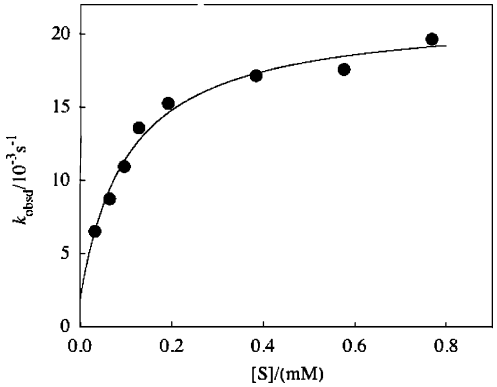
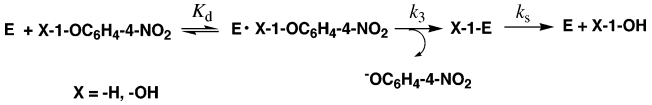



FIGURE 2: Effect of increasing [**H-1-OC$_6$H$_4$-4-NO$_2$**] on the observed first-order rate constant for the buildup of the 2-deoxy-$\beta$-D-galactopyranosyl intermediate during the approach to steady state for the $\beta$-galactosidase-catalyzed reaction at pH 8.6 and 25 °C.

Scheme 2




The data from Figure 1 for formation of 4-nitrophenoxide ion during the approach to steady state for $\beta$-galactosidase-catalyzed hydrolysis of **H-1-OC$_6$H$_4$-4-NO$_2$** at pH 8.6 were fit to eq 2 derived from Scheme 2 where $v_{ss}$ is the steady-state velocity for the change in absorbance at 405 nm [$(dA_{405}/dt)_{ss}$], $v_o$ is the reaction velocity at $t = 0$ [$(dA_{405}/dt)_o$], and $k_{obsd}$ is the apparent first-order rate constant for the approach to the steady-state concentration of the covalent intermediate. The solid lines in Figure 1 show the nonlinear least-squares fits of the data to eq 2 derived from Scheme 2 that were obtained using the values of $v_{ss}$ determined from the constant slopes of the reaction profiles at long reaction times and treating $v_o$ and $k_{obsd}$ ($s^{-1}$) as variable parameters. Small corrections for the effect of acetone added with **H-1-OC$_6$H$_4$-4-NO$_2$** ($\leq$2.4% of the final volume) on $k_{obsd}$ were made by assuming that acetone has the same effect ($\leq$10%) on $k_{obsd}$ and $k_{cat}$ for $\beta$-galactosidase-catalyzed hydrolysis of **HO-1-OC$_6$H$_4$-4-NO$_2$**. Note that the amount of acetone added with substrate only becomes sufficient to cause small changes in the activity of $\beta$-galactosidase as the concentration of the substrate approaches saturation ([S] $\gg$ $K_d$, below)

$$v_{ss} = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

$$A_{405} = v_{ss}t - \left[\frac{v_{ss} - v_o}{k_{obsd}}\right](1 - e^{-k_{obsd}t}) \quad (2)$$

$$k_{obsd} = k_s + \frac{k_3[S]}{K_d + [S]} \quad (3)$$

Figure 2 shows the effect of increasing concentrations of **H-1-OC$_6$H$_4$-4-NO$_2$** on $k_{obsd}$ ($s^{-1}$) for the approach to steady-state concentrations of **H-1-E**. The solid line in Figure 2 shows the fit of the data to eq 3 derived from Scheme 2 using $k_s = 0.0022$ $s^{-1}$ determined as described below (Table 1) and treating $k_3$ and $K_d$ as variable parameters. The nonlinear least-squares fit of the data from Figure 2 to eq 3 gave the kinetic parameters $K_d = 0.12$ mM and $k_3 = 0.017$ $s^{-1}$ (Table 1).

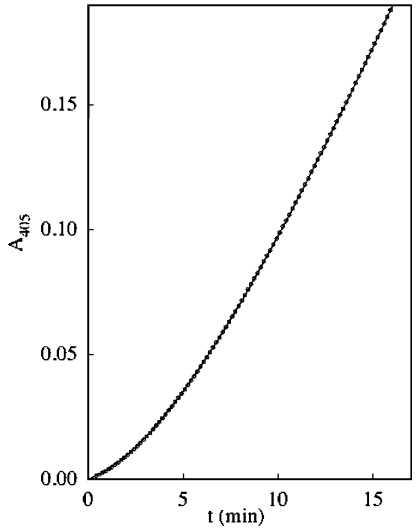
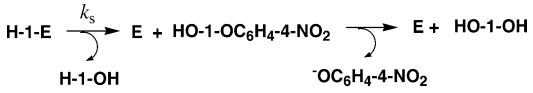



FIGURE 3: Time course for the recovery of activity of $\beta$-galactosidase labeled with a 2-deoxy-$\beta$-D-galactopyranosyl group. The enzyme was incubated with 0.1 mM **H-1-OC$_6$H$_4$-4-NO$_2$** at pH 8.6 (1 mM $Mg^{2+}$) and diluted by 1000-fold into an identical assay solution, except that 0.5 mM **HO-1-OC$_6$H$_4$-4-NO$_2$** was substituted for **H-1-OC$_6$H$_4$-4-NO$_2$**, and the recovery of activity was monitored by following the formation of 4-nitrophenoxide ion at 405 nm.

Scheme 3




$Mg^{2+}$ activates $\beta$-galactosidase (*25*, *27*) for cleavage of $\beta$-galactopyranosyl derivatives with oxygen leaving groups (*26*). There is no burst in the formation of 4-nitrophenoxide ion during $\beta$-galactosidase-catalyzed hydrolysis of **H-1-OC$_6$H$_4$-4-NO$_2$** at 25 °C and pH 7.0 in a magnesium-free solution that contains 10 mM EDTA. The steady-state kinetic parameters $V_{max}$ and $K_m$ (Table 1) for $\beta$-galactosidase-catalyzed hydrolysis of **H-1-OC$_6$H$_4$-4-NO$_2$** at 25 °C were determined from the nonlinear least-squares fit of the initial velocities $v_{ss}$ to eq 1. The value of $k_{cat}$ (Table 1) was determined as described for the reactions in the presence of $Mg^{2+}$ using $k_{cat} = 26$ $s^{-1}$ for $Mg^{2+}$-free enzyme-catalyzed hydrolysis of **HO-1-OC$_6$H$_4$-4-NO$_2$** at pH 7.0 (*32*).

$\beta$-Galactosidase was labeled with a 2-deoxy-$\beta$-D-galactopyranosyl group (**H-1-E**, Scheme 3) by incubation of the enzyme with 0.1 mM **H-1-OC$_6$H$_4$-4-NO$_2$** at pH 8.6 (1 mM MgCl). The labeled enzyme was then diluted 1000-fold into a solution that contains 0.5 mM **HO-1-OC$_6$H$_4$-4-NO$_2$** at the same pH and [$Mg^{2+}$] as for the labeling reaction. The recovery of activity from hydrolysis of **H-1-E** was followed by monitoring the increase in absorbance at 405 nm from enzyme-catalyzed hydrolysis of **HO-1-OC$_6$H$_4$-4-NO$_2$** (Figure 3). Reactivation is due to the hydrolysis of the enzyme rather than to transglycosylation, because the concentration of the sugar derivative (0.5 mM) used in this assay is far too small to give transglycosylation for the reaction of the native substrate (*33*) and because the 2-deoxy-$\beta$-D-galactopyranosyl enzyme shows a lower selectivity than the intermediate of the physiological reaction for transfer to methanol (*13*) and

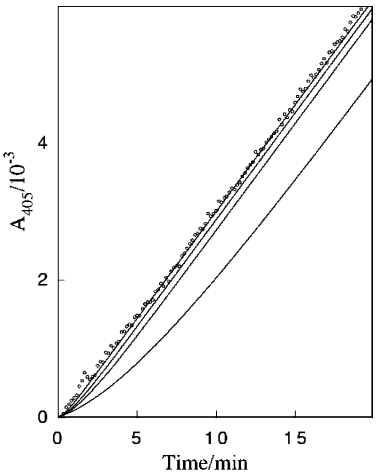



FIGURE 4: Time course for the recovery of activity of $\beta$-galactosidase labeled with a 2-deoxy-$\beta$-D-galactopyranosyl group. The enzyme was incubated with 0.1 mM **H-1-OC$_6$H$_4$-4-NO$_2$** at pH 7.0 (1 mM $Mg^{2+}$) and then diluted by 1000-fold into a solution at the same pH that contains 0.03 mM **HO-1-OC$_6$H$_4$-4-NO$_2$**, no $Mg^{2+}$, and 10 mM EDTA. The recovery of activity was monitored by following the formation of 4-nitrophenoxide ion at 405 nm. The solid lines, running from the bottom to the top of the graph, show theoretical curves calculated as described in the text for reactivation of $\beta$-galactosidase using the following hypothetical rate constants $k_s$ for the addition of water to the 2-deoxy-$\beta$-D-galactopyranosyl enzyme intermediate: 0.003, 0.01, 0.017, and 0.033 $s^{-1}$.

other alkyl alcohols.[2] The solid line in Figure 3 shows the fit of the experimental data to eq 4 derived from Scheme 3, where $v_{ss}$ is the steady-state velocity $[(dA_{405}/dt)_{ss}]$, $v_o$ is the reaction velocity at $t = 0$ $[(dA_{405}/dt)_o]$, and treating $v_o$ and $k_s$ as variable parameters. This fit gives $k_s = 0.0022$ $s^{-1}$ (Scheme 3) for the reactivation of $\beta$-galactosidase (Table 1). A similar experiment at pH 7.0 gave $k_s = 0.0024$ $s^{-1}$ (Scheme 1) for hydrolysis of **H-1-E** at the lower pH (Table 1).

$$A_{405} = v_{ss}t - \left[\frac{v_{ss} - v_o}{k_s}\right](1 - e^{-k_s t}) \qquad (4)$$

A reaction time course similar to that in Figure 3 was observed for the recovery of activity of $\beta$-galactosidase previously inactivated by a 5 min incubation with 0.01 mM D-galactal at 25 °C and pH 8.6 (25 mM pyrophosphate) and in the presence of 1 mM MgCl (*14*). The fit of this experimental data (not shown) to eq 4 gives $k_s = 0.0019$ $s^{-1}$ for the reactivation of $\beta$-galactosidase (Table 1). This value is in fair agreement with $k_s = 0.0046$ $s^{-1}$ determined in an earlier work (*14*).

$\beta$-Galactosidase labeled with a 2-deoxy-$\beta$-D-galactopyranosyl group (**H-1-E**, Scheme 3) by incubation of the enzyme with 0.1 mM **H-1-OC$_6$H$_4$-4-NO$_2$** at pH 8.6 (1 mM MgCl) was diluted into a solution at pH 7.0 (100 mM sodium phosphate) that contains 0.03 mM **HO-1-OC$_6$H$_4$-4-NO$_2$** and 10 mM EDTA. Figure 4 shows that there is no lag in the formation of 4-nitrophenoxide ion following dilution.[3] The solid lines in Figure 4 were calculated using eq 4, with the steady-state velocity $v_{ss}$ determined at long reaction times, the increasing hypothetical values of $k_s$ given in the caption of Figure 4, and a value of $v_o$ calculated from $v_{ss}$ and the fraction (0.2) of enzyme that is active in the presence of **H-1-OC$_6$H$_4$-4-NO$_2$** determined by examining reactivation of $\beta$-galactosidase in the presence of $Mg^{2+}$ (Figure 3). This figure shows the estimated curvature that would have been observed for values of $k_s$ (Scheme 2) that range from 0.003 to 0.0033 $s^{-1}$. Because no curvature is observed, we conclude that $k_s > 0.033$ $s^{-1}$ for hydrolysis of the 2-deoxy-$\beta$-D-galactopyranosyl intermediate at the magnesium-free enzyme (Table 1).

## DISCUSSION

*Kinetic Parameters for $\beta$-Galactosidase-Catalyzed Hydrolysis of **H-1-OC$_6$H$_4$-4-NO$_2$**.* The kinetic parameters for $\beta$-galactosidase-catalyzed hydrolysis of **H-1-OC$_6$H$_4$-4-NO$_2$** at pH 7.0 and 8.6 are reported in Table 1. The value of $k_{cat}/K_m = 280$ $M^{-1}$ $s^{-1}$ for $\beta$-galactosidase-catalyzed hydrolysis of **H-1-OC$_6$H$_4$-4-NO$_2$** at pH 7.0 is similar to $k_{cat}/K_m = 270$ $M^{-1}$ $s^{-1}$ reported for hydration of D-galactal (*14*). The values of $k_{cat}$ and $k_s$ for $\beta$-galactosidase-catalyzed hydrolysis of **H-1-OC$_6$H$_4$-4-NO$_2$** at pH 7.0 (Table 1) are similar to the corresponding values reported for $\beta$-galactosidase-catalyzed hydration of D-galactal (*14*). This is direct evidence that the two reactions proceed with the common rate-limiting step of addition of water to a 2-deoxy-$\beta$-D-galactopyranosyl intermediate (Scheme 1).

The kinetic parameters $K_d$ and $k_3$ (Scheme 2) that define the time course for the buildup of the 2-deoxy-$\beta$-D-galactopyranosyl intermediate of $\beta$-galactosidase-catalyzed hydrolysis of **H-1-OC$_6$H$_4$-4-NO$_2$** at pH 8.6 are also reported in Table 1. The value of $K_d = 1.2 \times 10^{-4}$ M (Table 1) for formation of a Michaelis complex to **H-1-OC$_6$H$_4$-4-NO$_2$** is similar to $K_m \approx K_d = 6 \times 10^{-5}$ M (*31*) for enzyme-catalyzed hydrolysis of **HO-1-OC$_6$H$_4$-4-NO$_2$**, for which the formation of the covalent intermediate is rate-determining. This suggests that there is only a weak interaction of the 2-OH group with $\beta$-galactosidase in the Michaelis complex to **HO-1-OC$_6$H$_4$-4-NO$_2$**. The large difference in the values of $k_3 = 0.017$ $s^{-1}$ (Table 1) and 140 $s^{-1}$ (*31*) for enzyme-catalyzed reactions of **H-1-OC$_6$H$_4$-4-NO$_2$** and **HO-1-OC$_6$H$_4$-4-NO$_2$**, respectively (Scheme 2), suggests that strong binding interactions between $\beta$-galactosidase and the C-2 oxygen develop with the approach to the transition state for transfer of the $\beta$-D-galactopyranosyl group from **HO-1-OC$_6$H$_4$-4-NO$_2$** to Glu-537, the active-site nucleophile (*34*).

The effect of the 2-OH substituent on the kinetic parameters for $\beta$-galactosidase-catalyzed hydrolysis of **HO-1-OC$_6$H$_4$-4-NO$_2$** is similar to that reported by Roesler and Legler for $\beta$-glucosidase-catalyzed hydrolysis of 4-methylumbelliferyl-$\beta$-D-glucosides (*20*). In the case of $\beta$-glucosidase, the 2-H for the 2-OH substitution causes a change in the rate-determining step for enzyme-catalyzed glucoside cleavage from formation to breakdown of a $\beta$-D-glucopyra-

[2] M. M. Toteva and J. P. Richard, unpublished results.

[3] There is also no lag in the time course for formation of 4-nitrophenoxide when **H-1-E** is diluted in a solution that contains no $Mg^{2+}$ but a saturating concentration of 0.5 mM **HO-1-OC$_6$H$_4$-4-NO$_2$**. Under these conditions, the initial reaction velocity was similar to that observed when $Mg^{2+}$ is present in solution, and the velocity decreases with time to that observed for the $Mg^{2+}$-free enzyme (C. K. Heo, unpublished results). The difference in the time course for reactivation of enzyme in the presence of low and high concentrations of **HO-1-OC$_6$H$_4$-4-NO$_2$** is because the binding and release of $Mg^{2+}$ from the free enzyme is much faster than the binding and release of $Mg^{2+}$ from the enzyme that is saturated with substrate (*25*).

nosylated intermediate and a 250 000-fold reduction in $k_{cat} = k_s$ for hydrolysis of the sugar derivative.

*Hydrolysis of 2-Deoxy-β-D-galactopyranosylated Enzyme.* The same value of $k_s = 0.0020 \pm 0.002$ s$^{-1}$ (Table 1) at pH 8.6 was determined by monitoring the recovery of the activity of β-galactosidase that had been incubated with **H-1-OC$_6$H$_4$-4-NO$_2$** (Figure 3) or with D-galactal. This is required by Scheme 1, where the reactions of these two substrates proceed through a common covalent intermediate. The value $k_{cat} = 0.0030$ s$^{-1}$ at pH 8.6 determined for β-galactosidase-catalyzed hydrolysis of **H-1-OC$_6$H$_4$-4-NO$_2$** by steady-state kinetic analysis is larger than $k_{cat} = 0.0018$ s$^{-1}$ that may be calculated from the values of $k_s$ and $k_3$ (Table 1) and the expression $k_{cat} = k_3k_s/(k_3 + k_s)$ for Scheme 2. This difference reflects experimental error and, possibly, the uncertainty in $k_{cat}$ for β-galactosidase-catalyzed hydrolysis of **HO-1-OC$_6$H$_4$-4-NO$_2$**. Our practice is to use $k_{cat} = 156$ s$^{-1}$ reported for β-galactosidase-catalyzed cleavage of **HO-1-OC$_6$H$_4$-4-NO$_2$** at pH 7 (*17*) when calculating $k_{cat}$ for other enzyme-catalyzed hydrolysis reactions. A smaller value of $k_{cat} = 0.0017$ s$^{-1}$ for enzyme-catalyzed hydrolysis of **H-1-OC$_6$H$_4$-4-NO$_2$** would have been obtained using the smaller value of $k_{cat} = 90$ s$^{-1}$ for β-galactosidase-catalyzed cleavage of **H-1-OC$_6$H$_4$-4-NO$_2$** that has also been reported in the chemical literature (*35*).

*Effects of Mg$^{2+}$.* Removal of Mg$^{2+}$ from β-galactosidase results in a 13-fold decrease in $k_{cat}/K_m$ for the reaction of **HO-1-OC$_6$H$_4$-4-NO$_2$** at pH 7. This is smaller than the 25-fold decrease in $k_{cat}/K_m$ reported for the reaction of **HO-1-OC$_6$H$_4$-4-NO$_2$** at the same pH (*32*), but the difference is not striking. The observation of similar effects of Mg$^{2+}$ on the kinetic parameters for cleavage of **H-1-OC$_6$H$_4$-4-NO$_2$** and **HO-1-OC$_6$H$_4$-4-NO$_2$** shows that there is relatively little stabilization of the transition state for enzyme-catalyzed cleavage of **HO-1-OC$_6$H$_4$-4-NO$_2$** from interactions between Mg$^{2+}$ and the C-2-hydroxyl group. This is consistent with the results of X-ray crystallographic analysis of β-galactosidase, which shows that there are no contacts between Mg$^{2+}$ and the C-2 hydroxyl of the β-D-galactopyranosyl group at enzyme complexes to a variety of ligands (*22*).

The observation of burst kinetics for enzyme-catalyzed hydrolysis of **H-1-OC$_6$H$_4$-4-NO$_2$** in the presence of Mg$^{2+}$ (Figure 1) shows that the hydrolysis of the covalent intermediate ($k_s$, Scheme 1) is the rate-determining step for the enzyme-catalyzed reaction. No burst is observed for the enzyme-catalyzed reaction of **H-1-OC$_6$H$_4$-4-NO$_2$** in the absence of Mg$^{2+}$, so that removal of Mg$^{2+}$ causes a change in the rate-determining step. The same effect of Mg$^{2+}$ on the rate-determining step has been observed for β-galactosidase-catalyzed hydrolysis of **HO-1-OC$_6$H$_4$-2-NO$_2$** (*o*-nitrophenyl leaving group), where breakdown of the intermediate is partly rate-determining for the reaction in the presence of Mg$^{2+}$ and intermediate formation becomes strongly rate-determining upon removal of Mg$^{2+}$ (*36*).

Incubation of β-galactosidase with **H-1-OC$_6$H$_4$-4-NO$_2$** in the presence of Mg$^{2+}$ to label the enzyme with a 2-deoxy-β-D-galactopyranosyl group, followed by a 1000-fold dilution into an assay solution that contains **HO-1-OC$_6$H$_4$-4-NO$_2$** and Mg$^{2+}$, gives a "lag" in recovery of fully active enzyme, because of the slow hydrolysis of the covalent intermediate (Figure 3). There is no lag in the recovery of the activity of β-galactosidase labeled with a 2-deoxy-β-D-galactopyranosyl group by the same procedure, upon dilution into a solution that contains 0.03 mM **HO-1-OC$_6$H$_4$-4-NO$_2$** but no Mg$^{2+}$ and 10 mM EDTA. This shows that removal of Mg$^{2+}$ from β-galactosidase causes an increase in $k_s$ for hydrolysis of **H-1-E** (Scheme 3). The lower limit of $k_s > 0.033$ s$^{-1}$ for hydrolysis of the intermediate at the magnesium-free enzyme, estimated on the basis of the shortest lag that could been observed for the experiment shown in Figure 4, is greater than $k_s = 0.002$ s$^{-1}$ determined for the hydrolysis of the labeled enzyme in the presence of Mg$^{2+}$. We conclude that Mg$^{2+}$ has an opposite stabilizing effect on the transition state for formation of **H-1-E** by cleavage of **HO-1-OC$_6$H$_4$-4-NO$_2$** and a destabilizing effect on the transition state for hydrolysis of this intermediate. We are not able to offer an interpretation for this result, and note that the mechanism for Mg$^{2+}$ activation of β-galactosidase for hydrolysis of sugars is not well-understood (*13*, *22*, *26*, *27*, *30*, *37*, *38*).

Table 2: Effect of the 2-OH Substrate on the Kinetic Parameters for the β-Galactosidase-Catalyzed Hydrolysis of **X-1-C$_6$H$_4$-4-NO$_2$** at 25 °C

| kinetic parameter[a] | C-2 substituent[b] | | ratio | ΔΔ*G* or ΔΔ*G*$^{\dagger}$[c] |
|---|---|---|---|---|
| $K_d$ (M) | 2-OH | $6.0 \times 10^{-5}$ | 0.50 | 0.4[d] |
| | 2-H | $1.2 \times 10^{-4}$ | | |
| $k_3$ (s$^{-1}$) | 2-OH | 140 | 7000 | −5.2[e] |
| | 2-H | 0.020 | | |
| $K_d/k_3$ (M$^{-1}$ s$^{-1}$) | 2-OH | $2.3 \times 10^6$ | 14 000 | −5.6[f] |
| | 2-H | 170 | | |
| $k_s$ (s$^{-1}$) | 2-OH | 710 | 320 000 | −7.5[g] |
| | 2-H | 0.0022 | | |

[a] Kinetic parameters defined by Scheme 2. [b] Data for the 2-OH-substituted sugar derivative is from ref *32*, and data for the 2-H group is from Table 1. [c] Difference in the change in Gibbs free energy observed for the reaction of C-2 hydroxyl and C-2 hydrogen-substituted sugars. [d] Difference in the change in Gibbs free energy observed upon transfer of the sugar from solution to the β-galactosidase. [e] Difference in the change in Gibbs free energy observed on proceeding from the Michaelis complex to the transition state for formation of the covalent intermediate. [f] Difference in the change in Gibbs free energy observed on proceeding from solution to the transition state for formation of the covalent intermediate. [g] Difference in the change in Gibbs free energy observed upon moving from the covalent intermediate to the transition state for transfer of the intermediate to water.

*Magnitude of the 2-OH Substituent Effect.* The observed effect of the 2-OH group on the rate constants for formation and hydrolysis of the covalent intermediate of β-galactosidase-catalyzed hydrolysis of **HO-1-OC$_6$H$_4$-4-NO$_2$** may be due to direct stabilization of the respective transition states by interactions with the 2-OH group or to relief of destabilizing ground-state interactions with the approach to the transition state. The stabilization of the transition state for transfer of the β-D-galactopyranosyl group from the enzyme to water relative to the covalent intermediate ($k_s$, Scheme 2) is estimated to be $7.5 + 3.1 \approx 10.6$ kcal/mol, where (a) 7.5 kcal/mol is the observed substituent effect (Table 2) and (b) 3.1 kcal/mol is the *destabilization* of this transition state from the inductive effect and the 2-OH substituent effect that must be balanced by other interactions to give the observed effect (*19*). Interactions between β-galactosidase and the 2-OH group are estimated to provide a somewhat smaller $5.3 + 3.1 \approx 8.4$ kcal/mol (Table 2) stabilization of the transition state for $k_3$ relative to the Michaelis complex.

*Origin of the 2-OH Substituent Effect.* The data from Table 2 show the following:

(1) The 2-OH group at the β-D-galactopyranosyl enzyme causes a ca. 7.5 kcal/mol stabilization of the transition state for transfer of the sugar from the enzyme to water relative to the covalent reaction intermediate but a smaller 5.3 kcal/mol stabilization of the transition state for transfer of the sugar from **HO-1-OC$_6$H$_4$-4-NO$_2$** to β-galactosidase ($k_3$, Table 2) relative to the bound substrate. This is consistent with the notion that there are significant differences in the transition states for transfer of the β-D-galactopyranosyl from the substrate to the enzyme and for group transfer from the enzyme to water (*22*) that have not been fully rationalized.

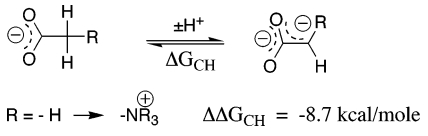
(2) The deletion of the 2-OH group causes just a 2-fold increase in the dissociation constant $K_d$ of substrate **HO-1-OC$_6$H$_4$-4-NO$_2$** for β-galactosidase and much larger 7000- and 320 000-fold changes, respectively, in the rate constants $k_3$ and $k_s$ (Scheme 1). This suggests that the interactions between the 2-OH and enzyme are weak in the Michaelis complex and only develop at the transition states for enzyme-catalyzed β-D-galactopyranosyl group transfer. It is consistent with the notion that there is no significant stabilization of the Michaelis complex by interactions between the enzyme and 2-OH group of substrate but does not exclude the possibility that such stabilizing interactions are balanced by compensating stabilizing interactions.

We conclude that the interaction of the 2-OH substituent with β-galactosidase is unusual because of its magnitude (10.6 kcal/mol) and suggest that, in fact, almost *none* of this effect is expressed at the Michaelis complex (Table 2). We propose that the 2-OH substituent effect on transition-state stability is large because it is, in fact, the effect of the ionized anionic 2-O$^-$ substituent and that the interactions between the enzyme and neutral 2-OH group are weak because this group only ionizes after formation of the Michaelis complex. The following are consistent with this proposal:

(1) The rate constant $k_s = 4.4 \times 10^{-6}$ s$^{-1}$ for reactivation of 2-fluoro-β-D-galactopyranosylated enzyme at 25 °C (*34*) is 450-fold smaller than $k_s = 2.0 \times 10^{-3}$ s$^{-1}$ for reactivation of 2-deoxy-β-D-galactopyranosylated enzyme (Table 1). This shows that the inductive effect of the neutral 2-OH group should cause a substantial *stabilization* of the covalent intermediate (see above), but that the inductive effect of the putative 2-O$^-$ would cause an even larger *destabilization* of the intermediate.

(2) The apparent contribution of the binding energy of single −OH groups to catalysis of glycoside hydrolysis is typically 3−6 kcal/mol (*29*, *39*, *40*). We are not aware of *any* literature precedent for a 10.6 kcal/mol stabilizing interaction between a protein and a neutral hydroxyl. The effect of replacement of neutral −OH by −H on enzymatic activity for glycosyl transfer is often used to estimate the transition-state-binding energy for the hydroxyl fragment (*29*, *39*−*42*). However, if ionization of the C-2 hydroxyl is a step in enzyme-catalyzed cleavage of β-D-galactopyranoside derivatives, then the large effect of substitution of the 2-OH by −H cannot be used to estimate the binding energy of this fragment. This is because (a) a large effect of a 2-O$^-$ group on the stability of the transition state for glycoside cleavage because of electrostatic interactions with the oxocarbenium ion-like transition state is expected, even if there are no strong specific interactions between the substituent and the protein catalyst, and (b) the observed effect of the 2-OH group may be caused, in part, by using the binding interactions between β-galactosidase and other nonreacting hydroxyls of the β-D-galactopyranosyl group to move the sugar substrate into a nonpolar environment that enhances the stabilizing intramolecular interactions at the putative zwitterionic transition (see below).

Scheme 4

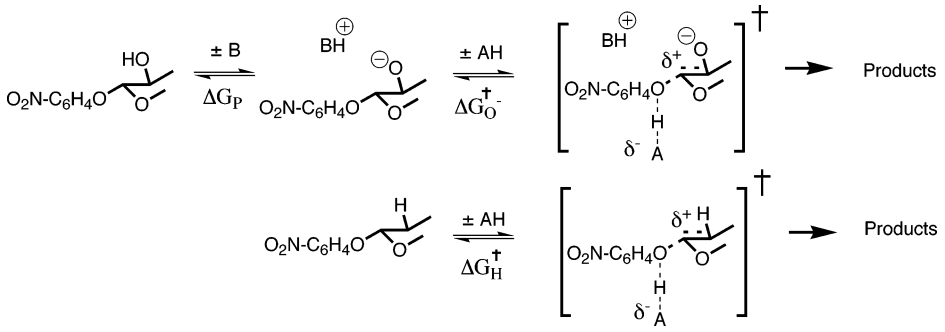



(3) The stabilizing intramolecular interaction between closely spaced positive and negative charges at organic zwitterions is large. The enolate dianion that forms by carbon deprotonation of acetate ion is stabilized by ca. 9 kcal/mol by the interaction with an α-NMe$_3^+$ (Scheme 4) (*43*). This stabilizing interaction in kcal/mol should increase by more than 10-fold (!), with transfer of the carbon acid from aqueous solution to the gas phase (*44*, *45*). The stabilizing interaction between anionic 2-O$^-$ and cationic C-1 at the transition state for enzyme-catalyzed heterolytic bond cleavage (Scheme 5) will also be enhanced by transfer of reactants from aqueous solution to a nonpolar active site (*46*, *47*) with a dielectric constant of 10−20 typically observed for protein interiors (*48*−*50*). The observed 7.5 kcal/mol 2-OH substituent effect will be smaller than the hypothetical 2-O$^-$ effect ($\Delta G^{\dagger}_{O-}$, Scheme 5), because part of the stabilizing electrostatic interaction is used to *drive* unfavorable proton transfer ($\Delta G^{\dagger}_{p}$, Scheme 5).

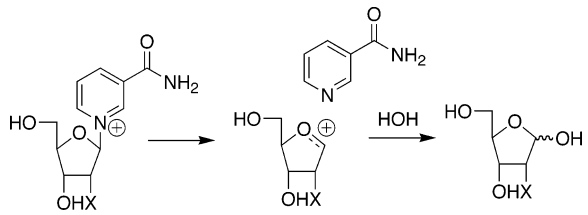
(4) The demonstration that solvolysis of sugars in water proceeds with ionization of the C-2 hydroxyl would favor observation of a similar pathway for enzymatic reactions, because enzymes generally follow one of the mechanisms observed for the uncatalyzed reaction. There is an upward break at pH 8.5 in the pH rate profile for uncatalyzed hydrolysis of 2,4-dinitrophenyl-β-D-galactopyranoside and evidence that this break is due to ionization of the 2-OH group of the sugar substrate (*51*). The higher reactivity of the O-ionized substrate compared with neutral substrate might be due to concerted intramolecular displacement of the leaving-group anion by the 2-O$^-$. However, only very small rate accelerations are observed for intermolecular-concerted bimolecular nucleophilic substitution at sugars (*52*). We suggest that this upward break is due to a ca. $10^4$-fold effect of ionization of the 2-OH on the rate constant for heterolytic bond cleavage at the sugar to form an oxocarbenium ion intermediate with a finite lifetime in water (*53*).

(5) There is evidence that spontaneous and enzyme-catalyzed hydrolysis of NAD occurs by a stepwise mechanism though an oxocarbenium ion-like transition state (Scheme 6) (*54*, *55*). The good linear correlation, with slope $\rho = -9.4$, between log $V_{max}$ and the Taft substituent constant $\sigma_i$ for hydrolysis of 2-X-substituted ribosides (X = −NH$_2$, H, N$_3$, and F) catalyzed by NAD glycohydrolase from calf liver is consistent with this mechanism (*56*). NAD (X = −OH) shows a $10^4$-fold positive deviation from this linear Hammett correlation, as expected for the physiological substrate. However, the linear correlation is observed for reactions where the substrate 2-X is either a good (X = NH$_2$)

Scheme 5




Scheme 6




or poor (X = H, $N_3$, and F) hydrogen-bond acceptor. This provides evidence that there is little specific stabilization of the transition state by hydrogen-bonding interactions between the enzyme and 2-X (*56*) and suggests that the enhanced enzymatic activity for hydrolysis of NAD is due to ionization of the 2-OH to 2-$O^-$ rather than to the development of binding interactions to 2-OH. The value of log $V_{max}$ for the 2-OH-substituted substrate (NAD) does show a good fit to the correlation for reaction of other 2-X-substituted ribosides when the Taft substituent constant for 2-$O^-$ is used (*56*).

(6) X-ray crystal structures have been determined for complexes between $\beta$-galactosidase and the following ligands (*22*): (a) Substrates **HO-1-OC$_6$H$_4$-4-NO$_2$** and **HO-1-OC$_6$H$_4$-2-NO$_2$** complexed to the E537G mutant enzyme, which lacks the side chain that forms a covalent adduct to the $\beta$-D-galactopyranosyl group (*34*); (b) the transition-state analogues galactonolactone (*58*) and galactotetrazole (*59*) complexed to the wild-type enzyme; and (c) the covalent adducts of Glu-537 to carbon 1 of 2-deoxy-$\beta$-D-galactopyranose and 2-deoxy-2-fluoro-$\beta$-D-galactopyranose. These data show that that the $\beta$-D-galactopyranosyl group moves deep into the active-site pocket as the reaction proceeds from the Michaelis complex to the covalent intermediate (*22*). This penetration of the substrate is accompanied by movement of the side chain of Phe-601 and a loop running from residues 794−804 (*22*). These results are consistent with the notion that the structure of the major Michaelis complex is different from the structures of the complexes that undergo heterolytic bond cleavage. One role for conformational changes that bury the $\beta$-D-galactopyranosyl group in the protein would be to sequester the substrate from the solvent at a nonpolar active site that enhances the stabilizing interactions between opposing charges at the putative zwitterionic transition state (Scheme 5) (*47*).

(7) X-ray crystal structures for covalent adducts of Glu-537 to 2-deoxy-$\beta$-D-galactopyranoside and to 2-deoxy-2-fluoro-$\beta$-D-galactopyranoside suggest that the imidazole group of His-391 interacts with the 2-OH of the $\beta$-D-galactopyranosyl intermediate (*22*, *60*). This imidazole, which is essential for the observation of robust enzymatic activity (*60*), is one candidate for the base to accept a proton from the 2-OH group.

We cannot exclude the possibility that the large effect of the 2-OH substituent on the kinetic parameters for formation and cleavage of the covalent intermediate of $\beta$-galactosidase-catalyzed hydrolysis of **HO-1-OC$_6$H$_4$-4-NO$_2$** is due to strong stabilization of the transition state by interactions between the enzyme and 2-hydroxyl group. For example, these interactions might develop during large conformational changes of the enzyme that occurs with the approach to these transition states.

It is needlessly controversial to generalize to all retaining $\beta$-1,4-glycanases the above proposal that enzyme-bound sugars are activated for hydrolysis by ionization of the C-2 hydroxyl. However, we are not aware of evidence that rigorously excludes this mechanism for any $\beta$-1,4-glycanase. The covalent enzyme−cellobiose intermediate of an E127A/H205N double mutant form of the retaining $\beta$-1,4-glycanase Cex from *Cellumonas fimi*, a 47 100-D cellulase, shows an unusually short 2.37 Å distance between the C-2 hydroxyl and the carboxylate group of Glu-233, which is attached covalently to the C-1 glucosyl residue (*61*). It has been proposed that the cleavage of this covalent linkage to form an enzyme-bound oxocarbenium ion intermediate is accompanied by a strengthening of the hydrogen bond between the developing carboxylate ion of Glu-233 and the C-2 hydroxyl (*61*, *62*) and that the stabilization of the oxocarbenium ion intermediate by this short hydrogen bond may be as large as 10−20 kcal/mol (*63*, *64*). This proposal is consistent with the crystal structure for the double-mutant enzyme. However, it may be significant that the hydrogen bond between Glu-233 and His-205 at the wild-type enzyme is lost for the H205N mutant. This competing hydrogen bond at the wild-type enzyme may act to substantially weaken the hydrogen bond between Glu-233 and the C-2 hydroxyl observed at the double-mutant enzyme. It is interesting that a longer and presumably weaker 2.8 Å hydrogen bond is observed between the O$\epsilon$2 of Glu233 of wild-type $\beta$-1,4-glycanase Cex and the C-2 hydroxyl of deoxynojirimycin, a putative transition-state analogue (*61*).

## REFERENCES

1. Pauling, L. (1946) Molecular architecture and biological reactions, *Chem. Eng. News 24*, 1375−1377.
2. Wolfenden, R. (1969) Transition state analogues for enzyme catalysis, *Nature 223*, 704−705.
3. Lienhard, G. E. (1973) Enzymic catalysis and transition-state theory, *Science 180*, 149−154.
4. Jencks, W. P. (1975) Binding energy, specificity, and enzymic catalysis: The circe effect, *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 219−410.

5. Bearne, S. L., and Wolfenden, R. (1997) Mandelate racemase in pieces: Effective concentrations of enzyme functional groups in the transition state, *Biochemistry 36*, 1646−1656.
6. Wolfenden, R., and Snider, M. J. (2001) The depth of chemical time and the power of enzymes as catalysts, *Acc. Chem. Res. 34*, 938−945.
7. Wolfenden, R., Lu, X., and Young, G. (1998) Spontaneous hydrolysis of glycosides, *J. Am. Chem. Soc. 120*, 6814−6815.
8. Radzicka, A., and Wolfenden, R. (1995) A proficient enzyme, *Science 267*, 90−93.
9. Richard, J. P. (1984) Acid−base catalysis of the elimination and isomerization reactions of triose phosphates, *J. Am. Chem. Soc. 106*, 4926−4936.
10. Amyes, T. L., O'Donoghue, A. C., and Richard, J. P. (2001) Contribution of phosphate intrinsic binding energy to the rate acceleration of triosephosphate isomerase, *J. Am. Chem. Soc. 123*, 11325−11326.
11. O'Donoghue, A. C., Amyes, T. L., and Richard, J. P. (2005) Hydron transfer catalyzed by triosephosphate isomerase. The products of isomerization of dihydroxyacetone phosphate in $D_2O$, *Biochemistry 44*, 2622−2631.
12. O'Donoghue, A. C., Amyes, T. L., and Richard, J. P. (2005) Hydron transfer catalyzed by triosephosphate isomerase. The products of isomerization of (*R*)-glyceraldehyde 3-phosphate in $D_2O$, *Biochemistry 44*, 2610−2621.
13. Viratelle, O. M., and Yon, J. M. (1980) Comparison of the $\beta$-galactosidase conformations induced by D-galactal and by magnesium ions, *Biochemistry 19*, 4143−4149.
14. Wentworth, D. F., and Wolfenden, R. (1974) Slow binding of D-galactal, a "reversible" inhibitor of bacterial $\beta$-galactosidase.
15. Lehmann, J., and Zieger, B. (1977) The stereochemistry of the addition of glycerol to D-galactal, catalyzed by $\beta$-D-galactosidase, *Carbohydr. Res. 58*, 73−78.
16. Lehmann, J., and Schroter, E. (1972) Reaktionen enolischer zuckerderivate, *Carbohydr. Res. 23*, 359−368.
17. Sinnott, M. L., and Souchard, I. J. L. (1973) The mechanism of action of $\beta$-galactosidase, *Biochem. J. 133*, 89−98.
18. Sinnott, M. L., and Viratelle, O. M. (1973) The effect of methanol and dioxan on the rates of the $\beta$-galactosidase-catalysed hydrolyses of some $\beta$-D-galactopyranosides: Rate-limiting degalactosylation, *Biochem. J. 133*, 81−87.
19. Namchuk, M. N., McCarter, J. D., Becalski, A., Andrews, T., and Withers, S. G. (2000) The role of sugar substituents in glycoside hydrolysis, *J. Am. Chem. Soc. 122*, 1270−1277.
20. Roeser, K. R., and Legler, G. (1981) Role of sugar hydroxyl groups in glycoside hydrolysis. Cleavage mechanism of deoxyglucosides and related substrates by $\beta$-glucosidase A3 from *Aspergillus wentii*, *Biochim. Biophys. Acta 657*, 321−333.
21. Legler, G., Roeser, K. R., and Illig, H. K. (1979) Reaction of $\beta$-D-glucosidase A3 from *Aspergillus wentii* with D-glucal, *Eur. J. Biochem. 101*, 85−92.
22. Juers, D. H., Heightman, T. D., Vasella, A., McCarter, J. D., Mackenzie, L., Withers, S. G., and Matthews, B. W. (2001) A structural view of the action of *Escherichia coli* (*lac Z*) $\beta$-galactosidase, *Biochemistry 40*, 14781−14794.
23. Legler, G., von Radloff, M., and Kempfle, M. (1972) Composition, N-terminal amino acids, and chain length of a $\beta$-glucosidase from *Aspergillus wentii*, *Biochim. Biophys. Acta 257*, 40−48.
24. Legler, G. (1967) Studies on the mechanism of action of glycoside-splitting enzymes. II. Isolation and enzymatic properties of 2 $\beta$-glucosidases from *Aspergillus wentii*, *Hoppe-Seyler's Z. Phys. Chem. 348*, 1359−1366.
25. Tenu, J.-P., Viratelle, O. M., and Yon, J. (1972) Kinetic study of the activation process of $\beta$-galactosidase from *Escherichia coli* by $Mg^{2+}$, *Eur. J. Biochem. 26*, 112−118.
26. Sinnott, M. L., Withers, S. G., and Viratelle, O. M. (1978) The necessity of magnesium cation for acid assistance of aglycone departure in catalysis by *Escherichia coli* (*lac Z*) $\beta$-galactosidase, *Biochem. J. 175*, 539−546.
27. Case, G. S., Sinnott, M. L., and Tenu, J.-P. (1973) The role of magnesium ions in $\beta$-galactosidase catalyzed hydrolyses, *Biochem. J. 133*, 99−104.
28. Bielawska, H., and Michalska, M. (1986) First stereospecific synthesis of nitrophenyl-2-deoxy-$\beta$-D-glycosides, *J. Carbohydr. Chem. 5*, 445−458.
29. McCarter, J. D., Adam, M. J., and Withers, S. G. (1992) Binding energy and catalysis, *Biochem. J. 286*, 721−727.
30. Richard, J. P., Huber, R. E., Lin, S., Heo, C., and Amyes, T. L. (1996) Structure−reactivity relationships for $\beta$-galactosidase (*Escherichia coli*, *lac Z*). 3. Evidence that Glu-461 participates in Brønsted acid−base catalysis of $\beta$-D-galactopyranosyl group transfer, *Biochemistry 35*, 12377−12386.
31. Richard, J. P., Westerfeld, J. G., and Lin, S. (1995) Structure−reactivity relationships for $\beta$-galactosidase (*Escherichia coli*, *lac Z*). 1. Brønsted parameters for cleavage of alkyl $\beta$-D-galactopyranosides, *Biochemistry 34*, 11703−11712.
32. Selwood, T., and Sinnott, M. L. (1990) A solvent-isotope-effect study of proton-transfer during catalysis by *Escherichia coli* (*lac Z*) $\beta$-galactosidase, *Biochem. J. 268*, 317−323.
33. Huber, R. E., Kurz, G., and Wallenfels, K. (1976) A quantitation of the factors which affect the hydrolase and transglycolase activities of $\beta$-galactosidase (*E. coli*) on lactose, *Biochemistry 15*, 1994−2001.
34. Gebler, J. C., Aebersold, R., and Withers, S. (1992) Glu-537, not Glu-461, is the nucleophile in the active site of (*lac Z*) $\beta$-galactosidase from *Escherichia coli*, *J. Biol. Chem. 267*, 11126−11130.
35. Tenu, J.-P., Viratelle, O. M., Garnier, J., and Yon, J. (1971) pH dependence of the activity of $\beta$-galactosidase from *Escherichia coli*, *Eur. J. Biochem. 20*, 363−370.
36. Viratelle, O. M., and Yon, J. M. (1973) Nucleophilic competition in some $\beta$-galactosidase-catalyzed reactions, *Eur. J. Biochem. 33*, 110−116.
37. Richard, J. P. (1998) The enhancement of enzymatic rate accelerations by Brønsted acid−base catalysis, *Biochemistry 37*, 4305−4309.
38. Richard, J. P., Huber, R. E., and McCall, D. A. (2001) Effect of an E461G mutation of $\beta$-galactosidase (*Escherichia coli*, *lac Z*) on pL rate profiles and solvent deuterium isotope effects, *Bioorg. Chem. 29*, 146−155.
39. Street, I. P., Armstrong, C. R., and Withers, S. G. (1986) Hydrogen bonding and specificity. Fluorodeoxy sugars as probes of hydrogen bonding in the glycogen phosphorylase−glucose complex, *Biochemistry 25*, 6021−6027.
40. Street, I. P., Rupitz, K., and Withers, S. G. (1989) Fluorinated and deoxygenated substrates as probes of transition-state structure in glycogen phosphorylase, *J. Am. Chem. Soc. 28*, 1581−1587.
41. Roth, N. J., Rob, B., and Huber, R. E. (1998) His-357 of $\beta$-galactosidase (*Escherichia coli*) interacts with the C3 hydroxyl in the transition state and helps to mediate catalysis, *Biochemistry 37*, 10099−10107.
42. Roth, N. J., and Huber, R. E. (1996) The $\beta$-galactosidase (*Escherichia coli*) reaction is partly facilitated by interactions of His-540 with the C6 hydroxyl of galactose, *J. Biol. Chem. 271*, 14296−14301.
43. Rios, A., Amyes, T. L., and Richard, J. P. (2000) Formation and stability of organic zwitterions in aqueous solution: Enolates of the amino acid glycine and its derivatives, *J. Am. Chem. Soc. 122*, 9373−9385.
44. Patrick, J. S., Yang, S. S., and Cooks, R. G. (1996) Determination of the gas-phase basicity of betaine and related compounds using kinetic methods, *J. Am. Chem. Soc. 118*, 231−232.
45. Price, W. D., Jockusch, R. A., and Williams, E. R. (1998) Binding energies of protonated betaine complexes: A probe of zwitterion structure in the gas phase, *J. Am. Chem. Soc. 120*, 3474−3484.
46. Williams, G., Maziarz, E. P., Amyes, T. L., Wood, T. D., and Richard, J. P. (2003) Formation and stability of the enolates of N-protonated proline methyl ester and proline zwitterion in aqueous solution: A nonenzymatic model for the first step in the racemization of proline catalyzed by proline racemase, *Biochemistry 42*, 8354−8361.
47. Richard, J. P., and Amyes, T. L. (2004) On the importance of being zwitterionic: Enzymic catalysis of decarboxylation and deprotonation of cationic carbon, *Bioorg. Chem. 32*, 354−366.
48. Antosiewicz, J., McCammon, J. A., and Gilson, M. K. (1996) The determinants of p$K_a$'s in proteins, *Biochemistry 35*, 7819−7833.
49. Sham, Y. Y., Muegge, I., and Warshel, A. (1998) The effect of protein relaxation on charge−charge interactions and dielectric constants of proteins, *Biophys. J. 74*, 1744−1753.
50. Simonson, T., Carlsson, J., and Case, D. A. (2004) Proton binding to proteins: p$K_a$ calculations with explicit and implicit solvent models, *J. Am. Chem. Soc. 126*, 4167−4180.
51. Cocker, D., and Sinnott, M. L. (1975) Generation of glycopyranosyl cations in the spontaneous hydrolyses of 2,4-dinitrophenyl-glycopyranosides. General intermediacy of glycopyranosyl cations in the acid-catalyzed hydrolyses of methyl glycopyranosides, *J. Chem. Soc., Perkin Trans. 2*, 1391−1395.

52. Banait, N. S., and Jencks, W. P. (1991) Reactions of anionic nucleophiles with α-D-glucopyranosyl fluoride, *J. Am. Chem. Soc. 113*, 7951−7958.
53. Amyes, T. L., and Jencks, W. P. (1989) Lifetimes of oxocarbenium ions in aqueous solution from common ion inhibition of the solvolysis of α-azido ethers by added azide ion, *J. Am. Chem. Soc. 111*, 7888−7900.
54. Bull, H. G., Ferraz, J. P., Cordes, E. H., Ribbi, A., and Apitz-Castro, R. (1978) Concerning the mechanism of the enzymatic and nonenzymatic hydrolysis of nicotinamide nucleotide coenzymes, *J. Biol. Chem. 253*, 5186−5192.
55. Tarnus, C., and Schuber, F. (1987) Application of linear free-energy relationships to the mechanistic probing of nonenzymatic and $NAD^+$-glycohydrolase-catalyzed hydrolysis of pyridine dinucleotides, *Bioorg. Chem. 15*, 31−42.
56. Handlon, A. L., Xu, C., Muller-Steffner, H. M., Schuber, F., and Oppenheimer, N. J. (1994) 2′-Ribose substituent effects on the chemical and enzymic hydrolysis of $NAD^+$, *J. Am. Chem. Soc. 116*, 12087−12088.
57. Cupples, C. G., Miller, J. H., and Huber, R. E. (1990) Determination of the roles of Glu-461 in β-galactosidase (*Escherichia coli*) using site-specific mutagenesis, *J. Biol. Chem. 265*, 5512−5518.
58. Huber, R. E., and Brockbank, R. L. (1987) Strong inhibitory effect of furanose and sugar lactones on β-galactosidase of *Escherichia coli*, *Biochemistry 26*, 1526−1531.
59. Heightman, T. D., Ermert, P., Klein, D., and Vasella, A. (1995) Synthesis of galactose- and *N*-acetylglucosamine-derived tetrazoles and their evaluation as β-glycosidase inhibitors, *Helv. Chim. Acta 78*, 514−532.
60. Huber, R. E., Hlede, I. Y., Roth, N. J., McKenzie, K. C., and Ghumman, K. K. (2001) His-391 of β-galactosidase (*Escherichia coli*) promotes catalysis by strong interactions with the transition state, *Biochem. Cell. Biol. 79*, 183−193.
61. Notenboom, V., Williams, S. J., Hoos, R., Withers, S. G., and Rose, D. R. (2000) Detailed structural analysis of glycosidase/inhibitor interactions: Complexes of Cex from *Cellulomonas fimi* with xylobiose-derived aza-sugars, *Biochemistry 39*, 11553−11563.
62. White, A., Tull, D., Johns, K., Withers, S. G., and Rose, D. R. (1996) Crystallographic observation of a covalent catalytic intermediate in a β-glycosidase, *Nat. Struct. Biol. 3*, 149−154.
63. Gerlt, J. A., Kreevoy, M. M., Cleland, W. W., and Frey, P. A. (1997) Understanding enzymic catalysis: The importance of short, strong hydrogen bonds, *Chem. Biol. 4*, 259−267.
64. Cleland, W. W., Frey, P. A., and Gerlt, J. A. (1998) The low barrier hydrogen bond in enzymatic catalysis, *J. Biol. Chem. 273*, 25529−25532.

BI050936Q