Structure−Reactivity Relationships for β-Galactosidase (*Escherichia coli, lac Z*). 2. Reactions of the Galactosyl−Enzyme Intermediate with Alcohols and Azide Ion†

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ABSTRACT: Velocities for the synthesis of β-D-galactopyranosyl derivatives by transfer of the galactosyl group from β-galactosidase to seven alkyl alcohols, glucose, and azide ion have been determined as the difference in the velocities for β-galactosidase-catalyzed cleavage of 4-nitrophenyl β-D-galactopyranoside to give 4-nitrophenoxide anion (ν_{PNP}) and hydrolysis of this compound to give D-galactose (ν_{Gal}). Rate constant ratios k_{ROH}/k_s (M^{-1}) for partitioning of the galactosylated enzyme between reaction with alkyl alcohols and solvent determined by this method are in good agreement with values of k_{ROH}/k_s (M^{-1}) determined by analysis of alcohol inhibition of enzyme-catalyzed hydrolysis of the corresponding alkyl β-D-galactopyranosides. Absolute rate constants k_{ROH} (M^{-1} s^{-1}) for reaction of alkyl alcohols with the galactosylated enzyme intermediate were calculated from the corresponding rate constant ratio k_{ROH}/k_s (M^{-1}) and $k_s = 710$ s^{-1}. A Brønsted parameter of $(\beta_{nuc})_{ROH} = -0.19 \pm 0.10$ was determined from the second-order rate constants for the reactions of alcohols with the galactosylated enzyme. The large difference between $(\beta_{lg})_{k_{cat}/K_m} = -0.75 \pm 0.14$ for cleavage of alkyl β-D-galactopyranosides to form the galactosylated enzyme and $(\beta_{nuc})_{ROH} = -0.19$ for the reverse synthesis reaction requires that the equilibrium constants for galactosyl group transfer from alkyl β-D-galactopyranosides to the enzyme increase sharply with decreasing pK_a of the alkyl alcohol leaving group. These data give $\beta_{eq} = -0.56 \pm 0.05$ for the reaction of alkyl β-D-galactopyranosides with ethanol to form ethyl β-D-galactopyranoside and alkyl alcohol. Several effects that lead to this increased ease of cleavage of alkyl β-D-galactopyranosides with decreasing basicity of the alkoxy group are discussed. A second-order rate constant of $k_{Glc} = 1.2 \times 10^4$ M^{-1} s^{-1} was determined for reaction of glucose with the galactosylated enzyme. The relatively low reactivity of glucose is surprising, because an earlier observation that the galactosylated enzyme complex generated by the cleavage of lactose undergoes release of glucose and synthesis of allolactose at nearly equal rates suggests that the binding of glucose to the galactosylated enzyme should be partly irreversible and that it takes place near the encounter-controlled limit. The data suggest a significant stabilization of nonproductive complexes formed by binding of glucose to the galactosylated enzyme. β-Galactosidase catalyzes the hydrolysis of β-D-galactopyranosyl azide, but not the synthesis of this compound by reaction of azide ion with the galactosylated enzyme. This suggests that different forms of β-galactosidase catalyze the cleavage and synthesis of β-D-galactopyranosyl azide. This may correspond to a change in the state of ionization of the residue that participates in acid−base catalysis of the reaction in the cleavage and synthesis directions.

β-Galactosidase catalyzes the hydrolysis of lactose and other β-D-galactopyranosyl derivatives by a two-step mechanism through a galactosyl−enzyme reaction intermediate. The large secondary α-deuterium isotope effect of $k_H/k_D = 1.25$ (Sinnott & Souchard, 1973) for the enzyme from *Escherichia coli* (lac Z) on the rate constant k_s (s^{-1}) for transfer of the galactosyl group from enzyme to water shows that there is a large change from sp^3 to sp^2 hybridization at carbon-1 of the galactosyl moiety on moving to the transition state for hydrolysis of the intermediate, which requires sp^3-hybridized covalent attachment of the intermediate to the enzyme. The point of covalent attachment of the galactosyl group was identified as Glu-537, by degradation and amino acid analysis of a stable 2-deoxy-2-fluoro-β-D-galactopyranosyl enzyme intermediate generated by enzyme-catalyzed cleavage of the corresponding 2,4-dinitrophenyl β-D-galactopyranoside (Gebler et al., 1992). The absolute rate constant of $k_s = 1300$ s^{-1} for transfer of the galactosyl group to water at 25 °C and pH 7 was determined as the turnover number k_{cat} when hydrolysis of the intermediate is rate-determining for reaction of β-D-galactopyranosyl derivatives (Sinnott & Souchard, 1973). These data show that β-galactosidase causes a profound change in the reactivity of the acylal linkage, the cleavage of which is more than 10^8-fold faster than that of simple glycosidic acylals in water (Brown & Bruice, 1973).

The reaction of the galactosyl−enzyme intermediate in water containing 1 M methanol results in a 70% yield of methyl β-D-galactopyranoside (Viratelle & Yon, 1973; Sinnott & Viratelle, 1973). A rate constant ratio of $k_{MeOH}/k_s = 2.2$ M^{-1} for partitioning of the intermediate, and an

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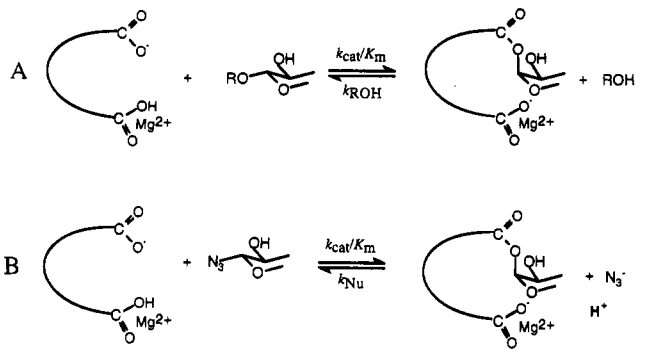
Scheme 1



absolute rate constant $k_{MeOH} = 2900\ M^{-1}\ s^{-1}$ for its reaction with methanol, were calculated from these results (Sinnott & Viratelle, 1973). In this paper, we expand upon the methanol trapping experiments and report the results of a study of the effect of changing the pK_a of the alcohol nucleophile on the reactivity of the alcohol toward the galactosyl–enzyme intermediate and an attempt to trap the intermediate with the highly reactive nucleophile azide ion. These experiments were designed to address several questions.

(1) How does the value of the Brønsted coefficient β_{nuc} for transfer of the galactosyl group from the enzyme to external alcohols compare with related values determined for nucleophilic substitution reactions in water, which proceed by well-defined chemical mechanisms (Jencks & Jencks, 1977; Jencks, 1985; Richard & Jencks, 1984a; Ta-Shma & Jencks, 1986)? This comparison may exclude some mechanisms for the galactosyl transfer reaction and provide information about the nature of the rate-determining transition state.

(2) What is the effect of changing alkoxy substituents on the equilibrium constants for transfer of the galactosyl group from alkyl β-D-galactopyranosides to β-galactosidase? These equilibrium constants may be calculated from a Haldane relationship for reversible cleavage and formation of alkyl β-D-galactopyranosides: $K_T = (k_{cat}/K_m)/k_{ROH}$ (Scheme 1A), using kinetic data for the cleavage reaction reported in the previous paper (Richard et al., 1995).

(3) Does β-galactosidase catalyze the transfer of the galactosyl group to nucleophilic anions? β-Galactosidase is a reasonably efficient catalyst of cleavage of β-D-galactopyranosyl azide (Sinnott, 1971), and a superficial application of the principal of microscopic reversibility would appear to require that the enzyme also catalyze the reverse nucleophilic reaction of azide ion with the galactosyl–enzyme intermediate (Scheme 1B).

We report here the effect of changing pK_a on the second-order rate constants for the reaction of short-chain alkyl alcohols with the galactosyl–enzyme intermediate of the β-galactosidase-catalyzed cleavage of 4-nitrophenyl β-D-galactopyranoside. There is a modest increase in k_{ROH} ($M^{-1}\ s^{-1}$) with decreasing alcohol pK_a, which is correlated by the Brønsted parameter $\beta_{nuc} = -0.19 \pm 0.10$. This is consistent with a mechanism involving general-base catalysis of the addition of these alcohols to an oxocarbenium ion reaction intermediate. By contrast, a significantly more negative value of $(\beta_{lg})_{k_{cat}/K_m} = -0.75 \pm 0.14$ was determined for the enzyme-catalyzed cleavage of alkyl β-D-galactopyranosides, so that there must be a large increase in the equilibrium constant $K_T = (k_{cat}/K_m)/k_{ROH}$ for transfer of the galactosyl group from alkyl β-D-galactopyranosides to the enzyme with decreasing basicity of the alkoxy leaving group, which is correlated by $\beta_T = -0.56$. This is a surprising result with important consequences for the interpretation of these Brønsted parameters.

There is no detectable trapping of the galactosyl–enzyme intermediate by the very good nucleophile azide ion at concentrations of up to 0.5 M. The observation of enzymatic catalysis of the cleavage, but not the synthesis, of β-D-galactopyranosyl azide has interesting implications with respect to the nature of the interactions between the enzyme catalyst and anionic nucleophiles/leaving groups.

MATERIALS AND METHODS

The sources for most of the chemicals and enzymes used in this work are given in the preceding paper (Richard et al., 1995). β-D-Galactopyranosyl azide was synthesized by a published procedure (Sinnott, 1971). Methyl β-D-galactopyranoside and D-galactose were purchased from Sigma and used without further purification. Deuterium oxide (99.9% D) and methanol-d_4 (99.8% D) were purchased from Cambridge Isotope Laboratories.

^1H NMR spectra at 400 MHz were recorded in D_2O on a Varian VXR-400 spectrometer. Chemical shifts are reported relative to HOD at $\delta = 4.65$ ppm.

Enzyme Assays. The methods for routine assays of the activity of β-galactosidase and galactose dehydrogenase were described in the preceding paper (Richard et al., 1995). Magnesium-free β-galactosidase was prepared by extensive dialysis against 10 mM EDTA, and the magnesium-free enzyme was assayed in the presence of 10 mM EDTA (Tenu et al., 1972). β-Galactosidase-catalyzed hydrolysis of β-D-galactopyranosyl azide was monitored by coupling the formation of D-galactose to the reduction of NAD^+ using galactose dehydrogenase (Richard et al., 1995).

The reaction of 2-nitrophenyl β-D-galactopyranoside in the presence of increasing concentrations of trifluoroethanol was monitored by following the formation of 2-nitrophenoxide anion ($\Delta\epsilon_{410} = 4300\ M^{-1}\ cm^{-1}$, pH 8.6) in a solution containing 25 mM sodium pyrophosphate (pH 8.6), 1.0 mM $MgCl_2$, and 2.2 mM 2-nitrophenyl β-D-galactopyranoside at 25 °C.

β-Galactosidase-catalyzed hydrolyses of fixed concentrations of alkyl β-D-galactopyranosides in the presence of increasing concentrations of the corresponding alkyl alcohol leaving group were monitored by the galactose dehydrogenase-coupled enzyme assay (Richard et al., 1995). The following concentrations of alkyl β-D-galactopyranosides were used for these experiments: ethyl β-D-galactopyranoside, 1.43 mM; 2-chloroethyl β-D-galactopyranoside, 0.033 mM; trifluoroethyl β-D-galactopyranoside, 0.042 mM.

The initial velocities of formation of 4-nitrophenoxide anion and of D-galactose in the β-galactosidase-catalyzed cleavage of 4-nitrophenyl β-D-galactopyranoside were determined at 25 °C in a single-assay solution that contained 25 mM sodium pyrophosphate (pH 8.6), 1.0 mM $MgCl_2$, 0.7 mM NAD^+, 0.5 mM 4-nitrophenyl β-D-galactopyranoside, and 0.3 unit of galactose dehydrogenase in a total volume of 1.0 mL. The absorbance at 340 nm was monitored until it was constant with time (~3 min), and

β-galactosidase was then added. The formation of D-galactose was monitored for 4 min by following the increase in absorbance at 340 nm (ΔA_{340}), and the formation of 4-nitrophenoxide anion was then monitored for 4 min, by following the increase in absorbance at 405 nm (ΔA_{405}). Control experiments showed the following: (1) Identical results were obtained when ΔA_{405} was monitored for 4 min first, followed by ΔA_{340}. (2) There is no significant change in absorbance at 405 nm associated with the reduction of NAD^+. (3) The change in absorbance at 340 nm due to the formation of 4-nitrophenoxide is 25-fold smaller than the change in absorbance at 405 nm. The initial velocity (<10% consumption of substrate) of formation of 4-nitrophenoxide anion (v_{PNP}) was calculated from ΔA_{405} using $\Delta\epsilon = 18\,300$ M^{-1} cm^{-1} at pH 8.6, determined from the absorbance change observed for quantitative conversion of substrate to D-galactose and 4-nitrophenoxide anion catalyzed by β-galactosidase. The initial velocity of formation of D-galactose (v_{Gal}) was calculated from the observed change in absorbance at 340 nm $(\Delta A_{340})_{obsd}$, with a small correction for the contribution to this absorbance change from formation of 4-nitrophenoxide ion (eq 1).

$$(\Delta A_{340})_{Gal} = (\Delta A_{340})_{obsd} - [(\Delta A_{405})/25] \quad (1)$$

Generally, these experiments were performed at concentrations of the alkyl alcohol that lead to less than 5% inhibition of the cleavage of 4-nitrophenyl β-D-galactopyranoside. Controls were routinely performed to show that the velocity ratio v_{PNP}/v_{Gal} at the largest concentration of each alkyl alcohol used in these experiments is independent of the concentration of the galactose dehydrogenase coupling enzyme.

A similar procedure was employed to determine v_{PNP} and v_{Gal} for β-galactosidase-catalyzed cleavage of 4-nitrophenyl β-D-galactopyranoside in the presence of increasing concentrations of azide ion, except that a substrate concentration of 0.07 mM was used.

Equilibrium Constant for Interconversion of D-Galactose and Methyl β-D-Galactopyranoside. Solutions in D_2O were prepared to contain 50 mM sodium phosphate (pD 7.4), 1.0 mM $MgCl_2$, and 20 mM of either methyl β-D-galactopyranoside or D-galactose. These were freed of residual H_2O by the repeated (three times) evaporation of D_2O and addition of fresh solvent. A measured amount of the solution in D_2O was transferred to an NMR tube, and sufficient methanol-d_4 was added to give a 1.0 M solution of CD_3OD in a total volume of 1 mL. The enzyme-catalyzed reaction was initiated by the addition of 14.8 units of β-galactosidase. The relative concentrations of reactant and products at various times were determined by ^1H NMR spectroscopy at 400 MHz, by integration of the peaks for the anomeric protons of methyl β-D-galactopyranoside (δ = 4.13 ppm, A_{GalOCD_3} (eq 2)) and D-galactose (δ = 4.40 and 5.07 ppm, A_{GalOD} (eq 2)). The equilibrium constant, K_{MeOD}, for interconversion of these compounds was calculated from their relative concentrations at chemical equilibrium using eq 2 with $[D_2O]$ = 55 M.

$$K_{MeOD} = A_{GalOCD_3}[D_2O]/A_{Gal}[CD_3OD] \quad (2)$$

Data for the effect of increasing concentrations of trifluoroethanol on the velocity of β-galactosidase-catalyzed cleavage of 2-nitrophenyl β-D-galactopyranoside ([S] = 2.2 mM ≫ K_m) were fit to eq 5 derived for Scheme 4, using SigmaPlot from Jandel Scientific.

The slopes of the Brønsted correlations were determined by linear least-squares analysis. The quoted errors are standard deviations.

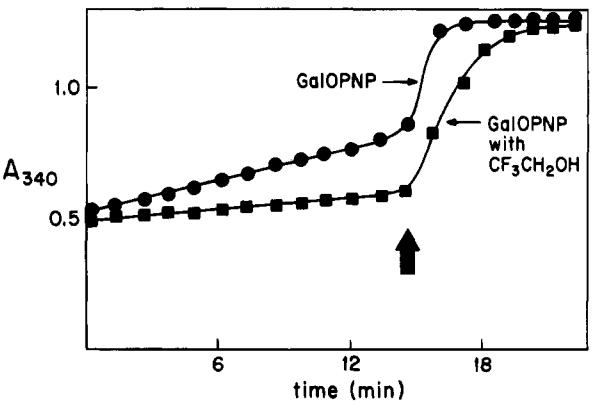
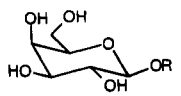


FIGURE 1: Change in A_{340} with time due to the formation of galactose coupled to the reduction of NAD^+ using galactose dehydrogenase in the β-galactosidase-catalyzed reaction of 4-nitrophenyl β-D-galactopyranoside at pH 8.6 (25 mM sodium pyrophosphate) in the absence (●) and in the presence (■) of 0.35 M trifluoroethanol. The heavy arrow indicates the time of addition of additional β-galactosidase to give a ~50-fold increase in enzyme concentration.

RESULTS

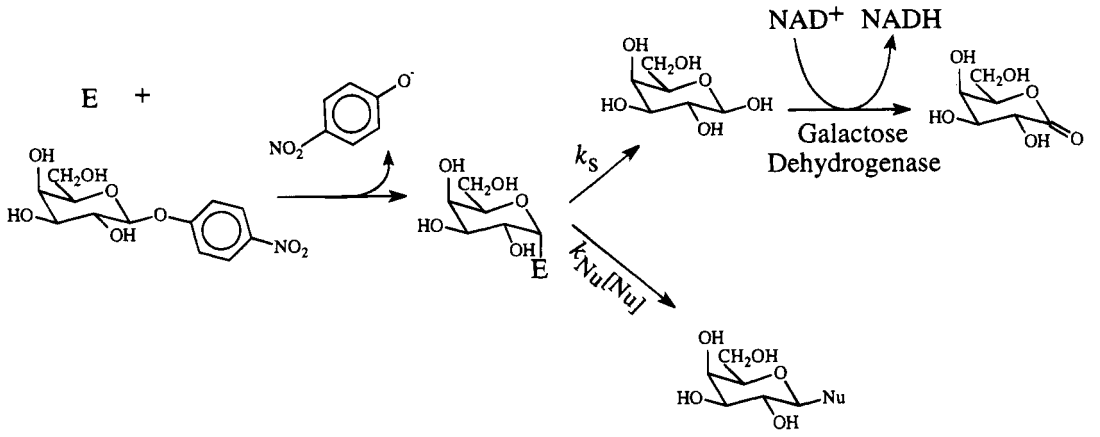
Figure 1 shows the time course, monitored using a galactose dehydrogenase-coupled enzyme assay (Richard et al., 1995), for the formation of D-galactose in the β-galactosidase-catalyzed (*E. coli*, lac Z) cleavage of 0.12 mM 4-nitrophenyl β-D-galactopyranoside at pH 8.6 (25 mM sodium pyrophosphate) in the absence (●) and in the presence (■) of 0.35 M trifluoroethanol. The slower velocity of formation of D-galactose in the presence of trifluoroethanol is not due to inhibition of the cleavage of the substrate by trifluoroethanol, because the overall velocity of formation of 4-nitrophenoxide anion, determined by following the change in absorbance at 405 nm, is independent of $[CF_3CH_2OH]$. A rapid, quantitative, production of D-galactose was observed in both assay mixtures when the concentration of β-galactosidase is increased by ~50-fold at the time indicated by the arrow. The data in Figure 1 show that trifluoroethanol reacts with the galactosyl–enzyme intermediate to form trifluoroethyl β-D-galactopyranoside (**1**-OCH_2CF_3, Scheme 2), which is not detected initially by the coupled enzyme assay for D-galactose, but which undergoes β-galactosidase-catalyzed hydrolysis to give, eventually, a quantitative yield of D-galactose.



1-OR

The initial velocities of β-galactosidase-catalyzed cleavage of 4-nitrophenyl β-D-galactopyranoside (**1**-OC_6H_4-4-NO_2, 0.5 mM) to give 4-nitrophenoxide anion (v_{PNP}) and D-galactose (v_{Gal}) were determined at pH 8.6 (25 mM pyrophosphate) in a single cuvette by monitoring the formation of 4-nitrophenoxide anion at 405 nm and the formation of D-galactose

Scheme 2



coupled to the reduction of NAD^+ at 340 nm in a coupled enzyme assay using galactose dehydrogenase. The UV spectra of 4-nitrophenoxide ion and NADH are well resolved, but there is considerable overlap between the UV spectra of 4-nitrophenol and NADH. Therefore, these experiments were conducted at pH 8.6 where 4-nitrophenol is largely ionized. The data from experiments at pH 7.0 (not shown) yield much less accurate velocities of formation of NADH.

These experiments give a velocity ratio of $v_{PNP}/v_{Gal} = 1.0$ for reaction in water, where the formation of 4-nitrophenoxide anion and D-galactose are stoichiometric, but this ratio increases as the intermediate is trapped by added alkyl alcohols to form alkyl β-D-galactopyranosides at the expense of D-galactose. The data provide a quantitative measure of the velocity of formation of the alkyl β-D-galactopyranosides, as the difference between the overall velocity of cleavage of **1**-OC_6H_4-4-NO_2 to give 4-nitrophenoxide (v_{PNP}) and the velocity of formation of D-galactose (v_{Gal}).

Figure 2 shows the linear increases in v_{PNP}/v_{Gal} for reaction of **1**-OC_6H_4-4-NO_2 in the presence of increasing concentrations of alkyl alcohols ROH. The rate constant ratios k_{ROH}/k_s (M^{-1}, Table 1) for partitioning of the galactosyl−enzyme intermediate between reaction with ROH and with water were obtained as the slopes of these linear correlations, according to eq 3, derived for Scheme 2. The rate constant ratio for

$$v_{PNP}/v_{Gal} = 1 + k_{ROH}[ROH]/k_s \quad (3)$$

partitioning of the galactosyl−enzyme intermediate between reaction with methanol and with water determined by this method is $k_{MeOH}/k_s = 2.3\ M^{-1}$.

The rate constant ratio $k_{Glc}/k_s = 17\ M^{-1}$ for partitioning of the galactosyl−enzyme intermediate between reaction with glucose and solvent was determined from the increase in v_{PNP}/v_{Gal} for reaction of **1**-OC_6H_4-4-NO_2 in the presence of increasing concentrations of glucose (eq 3). The products of the reaction of glucose were not identified, but were assumed to be a mixture of lactose, allolactose, and possibly other disaccharides, arising from nonspecific reaction of the ring hydroxyls of glucose with the galactosyl−enzyme intermediate (Jobe & Bourgeois, 1972; Huber et al., 1976, 1983).

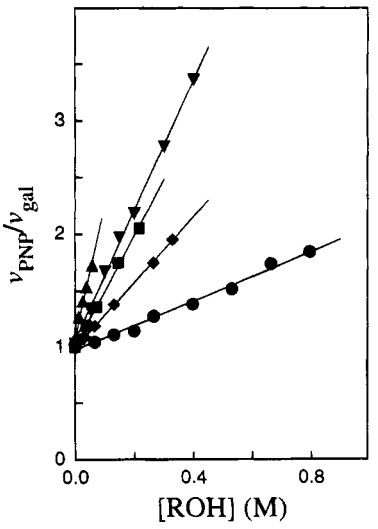


FIGURE 2: Effect of increasing concentrations of alkyl alcohols on the ratio of initial velocities of formation of 4-nitrophenoxide anion (v_{PNP}) and galactose (v_{Gal}) in the β-galactosidase-catalyzed reactions of 4-nitrophenyl β-D-galactopyranoside at pH 8.6 (25 mM sodium pyrophosphate). Key: ▲, 2-chloroethanol; ▼, 2,2,2-trifluoroethanol; ■, 2-hydroxyethanol; ◆, 2-methoxyethanol; ●, ethanol.

Figure 3A shows the change in v_{obsd}/v_o with increasing concentrations of the corresponding alkyl alcohols ROH for the reactions of **1**-OR at pH 8.6 (25 mM sodium pyrophosphate), where v_{obsd} is the observed initial velocity for cleavage of **1**-OR and v_o is the initial velocity when [ROH] = 0. The rate constant ratios k_{ROH}/k_s (M^{-1}, Table 1) were determined as the slopes of reciprocal replots of the data according to eq 4 derived for Scheme 3 (Figure 3B). The rate constant

$$v_o/v_{obsd} = k_{ROH}[ROH]/k_s \quad (4)$$

ratios k_{ROH}/k_s (M^{-1}) determined in this experiment showed satisfactory agreement (±10%) with the ratios determined from the slopes of the correlations in Figure 2. However, a bias was detected toward determination of slightly larger values k_{ROH}/k_s (M^{-1}) by the leaving group inhibition experiments (Table 1). This bias is probably due to weak inhibition of the β-galactosidase-catalyzed cleavage of **1**-OR by alkyl alcohols, which leads to increases in v_o/v_{obsd} that are unrelated to the alcohol trapping reaction. The rate constant ratios obtained from the data in Figure 2 are insensitive to such inhibition, which would cause the same proportional decrease in v_{PNP} and v_{Gal} but no change in the ratio of these velocities. Therefore, rate constant ratios k_{ROH}/k_s (M^{-1}) obtained from indirect product analysis (Figure 2) were used for all of the calculations described in this paper.

Table 1: Rate Constant Ratios for Partitioning of the Galactosyl−Enzyme Intermediate between Reaction with Alkyl Alcohols and Water and Absolute Rate and Equilibrium Constants for Galactosyl Transfer Reactions[a]

alcohol	pK_a[b]	k_{ROH}/k_s[c] (M^{-1})	k_{ROH}[d] (M^{-1} s^{-1})	$k_{ROH}K_d$[e] (s^{-1})	$K_T = (k_{cat}/K_m)/k_{ROH}$	$K_{eq} = (K_T)_{ROH}/(K_T)_{EtOH}$[f]
CH_3CH_2OH	16.0	1.1[g] 1.3[h]	780[i]	4.3	1.79	1.0
$HOCH_2CH_2OH$	15.1	4.9[g]	3500	30	4.3	2.4
$CH_3OCH_2CH_2OH$	14.8	2.9[g]	2060	4.9	7.8	4.4
$ClCH_2CH_2OH$	14.3	11.7[g] 13.0[h]	8300[i]	12.4	30	17
FCH_2CH_2OH	14.2	2.0[g]	1420	5.0	12.0	6.7
Cl_2CHCH_2OH	12.9	10.2[g]	7200	3.3	103	58
CF_3CH_2OH	12.4	6.0;[g] 7.1;[h] 6.0[g,j] ($-Mg^{2+}$); 6.5[h,k] (pH 7.0)	4200[i]	6.0	160	89

[a] At 25 °C in 25 mM sodium pyrophosphate buffer (pH 8.6) containing 1.0 mM $MgCl_2$, unless noted otherwise. [b] Jencks and Regenstein (1976). [c] Rate constant ratio for partitioning of the galactosyl−enzyme intermediate between reaction with the alkyl alcohol and with water (Scheme 2). [d] Second-order rate constant for reaction of the alkyl alcohol with the galactosylated enzyme, calculated from k_{ROH}/k_s (M^{-1}) and k_s = 710 s^{-1} (Selwood & Sinnott, 1990). [e] K_d (M) is the dissociation constant for β-galactosidase-catalyzed hydrolysis of the corresponding alkyl β-D-galactopyranoside (Richard et al., 1995). [f] The equilibrium constant for exchange of an alkoxy group between the β-D-galactopyranosyl moiety and the proton (Scheme 6A). [g] Determined by analysis of the effect of increasing concentrations of alkyl alcohol on the relative velocities of formation of 4-nitrophenoxide anion and D-galactose in the β-galactosidase-catalyzed reaction of 4-nitrophenyl β-D-galactopyranoside (Figure 2). [h] Determined by analysis of leaving group inhibition of β-galactosidase-catalyzed hydrolysis of the corresponding alkyl β-D-galactopyranoside. [i] Calculated using the value of k_{ROH}/k_s (M^{-1}) determined by analysis of the effect of increasing concentrations of alkyl alcohol on the relative velocities of formation of 4-nitrophenoxide anion and D-galactose in the β-galactosidase-catalyzed reaction of 4-nitrophenyl β-D-galactopyranoside (see text). [j] For reaction of the Mg^{2+}-free enzyme in the presence of 10 mM EDTA. [k] For reaction at pH 7.0 (25 mM sodium pyrophosphate).

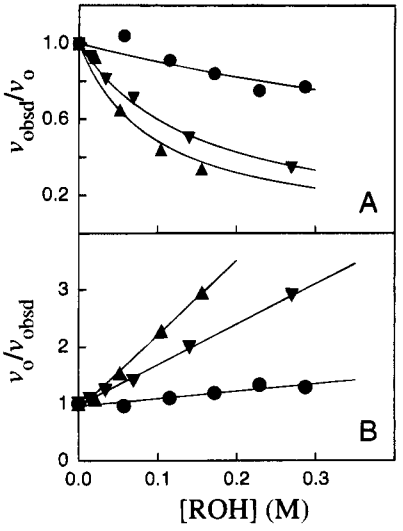


FIGURE 3: (A) Decrease in v_{obsd}/v_o for the β-galactosidase-catalyzed cleavage of alkyl β-D-galactopyranosides (**1**-OR) in the presence of increasing concentrations of the corresponding alkyl alcohols ROH at pH 8.6 (25 mM sodium pyrophosphate). (B) Reciprocal replot of the data from (A), according to eq 4 derived for Scheme 3. Key: ▲, 2-chloroethanol; ▼, 2,2,2-trifluoroethanol; ●, ethanol.

Scheme 3

$$\mathbf{E} + \mathbf{1\text{-}OR} \underset{k_{ROH}[ROH]}{\overset{k_{cat}/K_m}{\rightleftharpoons}} \mathbf{E\text{-}1} \xrightarrow{k_s} \mathbf{E} + \mathbf{1\text{-}OH}$$

Table 1 also gives values of k_{ROH}/k_s (M^{-1}) for partitioning of the galactosyl−enzyme intermediate with ROH = CF_3CH_2OH, determined under the following reaction conditions: (1) At pH 8.6 (25 mM sodium pyrophosphate, 10 mM EDTA, and no Mg^{2+}) for an enzyme preparation that had been freed of magnesium ion by extensive dialysis. This ratio was determined from the increase in v_{PNP}/v_{Gal} for enzyme-catalyzed cleavage of **1**-OC_6H_4-4-NO_2 in the presence of increasing concentrations of trifluoroethanol (eq 3). (2) At pH 7.0 (25 mM sodium pyrophosphate and 1.0 mM Mg^{2+}) determined by analysis of leaving group inhibition of β-galactosidase-catalyzed cleavage of **1**-OCH_2CF_3 (eq 4).

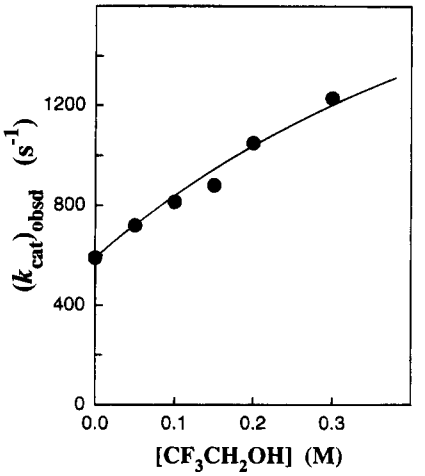


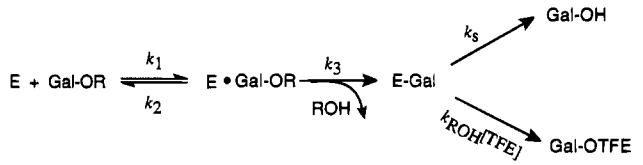
FIGURE 4: Effect of increasing concentrations of trifluoroethanol on $(k_{cat})_{obsd}$ for the β-galactosidase-catalyzed hydrolysis of 2-nitrophenyl β-D-galactopyranoside at 25 °C in 25 mM sodium pyrophosphate buffer at pH 8.6. The solid line shows the nonlinear least-squares fit of the data to eq 5.

The relatively large reactivity of trifluoroethanol toward addition to the galactosylated enzyme was also detected as an effect of increasing concentrations of trifluoroethanol on $(k_{cat})_{obsd}$ for enzyme-catalyzed hydrolysis of a saturating concentration (2.2 mM) of **1**-OC_6H_4-2-NO_2 (Figure 4), a substrate for which breakdown of the galactosyl−enzyme intermediate is partly rate-determining for turnover (Viratelle & Yon, 1973; Sinnott & Souchard, 1973). The solid line through the data in Figure 4 shows the theoretical fit of the experimental data to eq 5, derived for Scheme 4, using $(k_{cat})_o$

$$(k_{cat})_{obsd} = \frac{1 + k_{ROH}[TFE]/k_s}{1/(k_{cat})_o + k_{ROH}[TFE]/k_3k_s} \quad (5)$$

= 590 s^{-1} observed for reaction in the absence of trifluoroethanol, k_{ROH}/k_s = 6.0 M^{-1} for partitioning of the galac-

Scheme 4



tosyl–enzyme intermediate (Table 1), and $k_3 = 2800\ s^{-1}$ determined from the nonlinear least-squares fit of the data. The value of $k_3 = 2800\ s^{-1}$ estimated here is in fair agreement with the value of $2100\ s^{-1}$ estimated from the effect of increasing methanol concentrations on $(k_{cat})_{obsd}$ for reaction of **1**-OC_6H_4-2-NO_2 (Viratelle & Yon, 1973). There is a large uncertainty in our estimate for k_3, because it is based on a large extrapolation of the experimental data to the limiting value of $(k_{cat})_{obsd}$ at high concentrations of trifluoroethanol.

An increase from 0 to 0.50 M sodium azide causes a 33% reduction in both v_{PNP} and v_{Gal} for the β-galactosidase-catalyzed reaction of **1**-OC_6H_4-4-NO_2 (0.07 mM). However, the ratio of these velocities (v_{PNP}/v_{Gal}) remains constant and equal to 1.0. Our experimental methods could have detected a 10% decrease in v_{Gal} relative to v_{PNP}. Therefore, $k_{az}/k_s \leq 0.1\ M^{-1}$ (eq 3) and $k_{az} \leq 71\ M^{-1}\ s^{-1}$, since $k_s = 710\ s^{-1}$ at pH 8.6 (Selwood & Sinnott, 1990).

Kinetic parameters of $k_{cat} = 25\ s^{-1}$ and $K_m = 1.6$ mM were determined for β-galactosidase-catalyzed cleavage of **1**-N_3 at pH 8.6 (25 mM sodium pyrophosphate) in the presence of 1.0 mM Mg^{2+}. These data are in fair agreement with values of $k_{cat} = 26\ s^{-1}$ (Sinnott, 1971), $k_{cat} = 44\ s^{-1}$ (Sinnott et al., 1978), and $K_m = 2.8$ mM (Sinnott, 1971) determined for reactions at pH 7.0. A value of $k_{cat} = 0.124\ s^{-1}$, determined for the enzyme-catalyzed cleavage at pH 8.6 in the absence of Mg^{2+}, is also in fair agreement with $k_{cat} = 0.22\ s^{-1}$ determined at pH 7.0 (Sinnott et al., 1978).

Figure 5 shows the change in the relative concentration of methyl β-D-galactopyranoside, determined by ^1H NMR, as it approaches chemical equilibrium with D-galactose, catalyzed by β-galactosidase, in D_2O containing 1.0 M CD_3OD starting with either D-galactose (▲) or methyl β-D-galactopyranoside (●). The change in the relative concentration of D-galactose was also determined in this experiment. The data give $K_{MeOD} = 6.7$ as the equilibrium constant for the interconversion of D-galactose and methyl β-D-galactopyranoside (eq 2, Scheme 5). An attempt to determine the equilibrium constant for β-galactosidase-catalyzed interconversion of D-galactose and ethyl β-D-galactopyranoside in D_2O failed, because the enzyme was inactivated over a period of hours by the concentration of deuterated ethanol needed to give a detectable amount of ethyl β-D-galactopyranoside at chemical equilibrium.

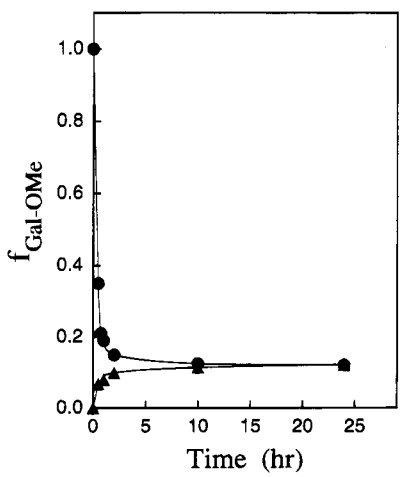
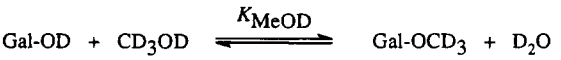


FIGURE 5: Change in the relative concentration of methyl β-D-galactopyranoside with time determined by ^1H NMR for β-galactosidase-catalyzed reactions in D_2O containing 1.0 M CD_3OD at pD 7.4 (50 mM sodium phosphate) starting either with 20 mM methyl β-D-galactopyranoside (●) or with 20 mM D-galactose (▲).

Scheme 5



DISCUSSION

A simple method (Scheme 2) has been developed to monitor, in a single reaction mixture, the initial velocity of β-galactosidase-catalyzed cleavage of **1**-OC_6H_4-4-NO_2 to form 4-nitrophenoxide anion (v_{PNP}) and the initial velocity of the hydrolysis reaction to form D-galactose (v_{Gal}). The velocity of galactosyl transfer to added alkyl alcohols ROH to form **1**-OR can then be calculated as the difference between v_{PNP} and v_{Gal}. The ratio v_{PNP}/v_{Gal} is equal to 1.0 when water is the only nucleophilic acceptor for the intermediate, and rate constant ratios k_{ROH}/k_s (M^{-1}) for partitioning of the galactosyl–enzyme intermediate can be conveniently calculated from the linear increases in v_{PNP}/v_{Gal} for reactions in the presence of increasing concentrations of alkyl alcohols (eq 3 derived for Scheme 2).

Huber et al. have determined kinetic parameters for reaction of the galactosyl–enzyme intermediate with methanol, ethanol, 2-hydroxyethanol, and 2-methoxyethanol by examining the effect of increasing concentrations of these alcohols on k_{cat} for hydrolysis of **1**-OC_6H_4-2-NO_2, a substrate for which reaction of the galactosyl–enzyme intermediate is partially rate-determining for turnover (Huber et al., 1984). The values of k_{ROH}/k_s (M^{-1}) for these alcohols reported in Table 1 are 1.7–3.7-fold smaller than the corresponding ratios calculated from the data of Huber; however, there is fair agreement between the relative reactivities of the alkyl alcohols determined in these two sets of experiments. The value of $k_{Glc}/k_s = 17\ M^{-1}$ determined here for the reaction of glucose is significantly larger than $k_{Glc}/k_s = 9.2\ M^{-1}$ calculated from the kinetic data of Huber et al. (1984).[1] We are uncertain of the explanation for the differences in the rate constant ratios obtained by these different experimental methods; however, the following observations provide strong evidence that the slopes of the correlations in Figures 2 and 3B provide an accurate measure of the relative reactivity of alcohols and water toward the galactosyl–enzyme intermediate under our reaction conditions:

(1) There is good agreement between the ratio $k_{MeOH}/k_s = 2.3\ M^{-1}$ at pH 8.6 determined in this work and an earlier literature value of $k_{MeOH}/k_s = 2.2\ M^{-1}$ at pH 7.0 determined by direct analysis of product yields (Sinnott & Viratelle, 1973). Both of these values are smaller than the ratio of

[1] The rate constant ratio $k_{Glc}/k_s = 9.2\ M^{-1}$ can be calculated from the second-order rate constant $k_{cat}/K_i'' = 380\ s^{-1}/0.34\ mM = 1.1 \times 10^4\ M^{-1}\ s^{-1}$ for the reaction of glucose with the galactosyl–enzyme intermediate, and the first-order rate constant $k_s = 1200\ s^{-1}$ for the reaction of water used by these investigators (Huber et al., 1984).

k_{MeOH}/k_s = 4.0 M^{-1} determined from kinetic data alone (Huber et al., 1984).[2]

(2) There is acceptable agreement between the ratios k_{ROH}/k_s (M^{-1}) determined by analysis of the relative velocities of the cleavage and hydrolysis reactions of **1**-OC_6H_4-4-NO_2 (Figure 2) and those determined by analysis of leaving group inhibition of β-galactosidase-catalyzed cleavage of **1**-OR (Figure 3).

(3) The increase in velocity observed for the β-galactosidase-catalyzed reaction of **1**-OC_6H_4-2-NO_2 in the presence of increasing concentrations of trifluoroethanol (Figure 4) is consistent with k_{ROH}/k_s = 6.0 M^{-1} determined in this work for partitioning of the galactosylated enzyme between reaction with trifluoroethanol and water.

The pH–rate profile of k_s (s^{-1}) for hydrolysis of the galactosylated enzyme (Selwood & Sinnott, 1990) shows a downward break at pH 8.8. The observation that the rate constant ratios k_{ROH}/k_s (M^{-1}) for partitioning of the galactosylated enzyme between reaction with trifluoroethanol and water at pH 8.6 and 7.0 are identical within the experimental error (Table 1) is consistent with similar dependencies of the two reactions on the state of protonation of the galactosylated enzyme.

The removal of Mg^{2+} from the enzyme causes similar reductions in the rate constants for transfer of the galactosyl group to trifluoroethanol and water, since there is no detectable change in the ratio k_{ROH}/k_s = 6.0 M^{-1} for reaction of the Mg^{2+}-free galactosylated enzyme (Table 1). We expect a small Mg^{2+} activation of the reaction of trifluoroethanol compared with that of ethanol (Selwood & Sinnott, 1990), because a small activation was observed for the reverse cleavage reaction of trifluoroethyl compared with ethyl β-D-galactopyranoside (Richard et al., 1995).

Binding Affinities of Alkyl Alcohols and the Rate-Determining Step. Absolute second-order rate constants for the reactions of alkyl alcohols with the galactosylated enzyme at pH 8.6, calculated from the rate constant ratios k_{ROH}/k_s (M^{-1}) and k_s = 710 s^{-1} for the reaction of water (Selwood & Sinnott, 1990), are reported in Table 1. The 11-fold variation in k_{ROH} (M^{-1} s^{-1}, Table 1) with changing structure of the alkyl alcohol shows that the overall rates of these reactions are limited by the rate of binding and chemical reaction of the alcohol with the galactosyl–enzyme intermediate rather than by a step that involves the enzyme alone, such as a conformational change or a change in the state of protonation of the catalyst.

The second-order rate constants k_{ROH} (M^{-1} s^{-1}) for reaction of the galactosylated enzyme with alkyl alcohols ROH are equal to $(k_{cat}/K_m)_{ROH}$ for the binding and chemical reaction of the alcohol. If it is assumed that the binding interactions between the alkoxy side chain and β-galactosidase are similar for the alcohols HOR and the corresponding alkyl β-D-galactopyranosides (**1**-OR), then changes in the affinity of the alkoxy side chain for the free enzyme can be estimated from the variations in K_d determined from the kinetic parameters for enzyme-catalyzed hydrolysis of alkyl β-D-galactopyranosides (Richard et al., 1995). Figure 6 shows

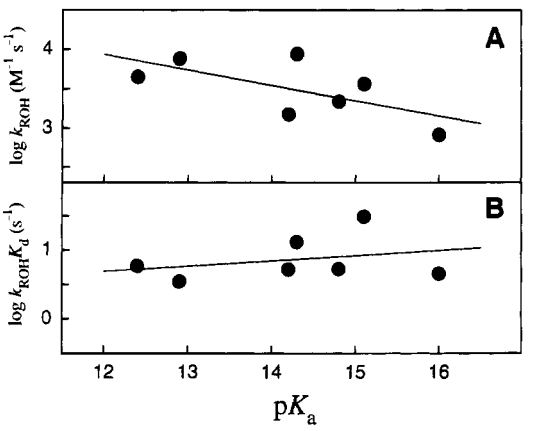


FIGURE 6: (A) Brønsted correlation with slope β_{nuc} = −0.19 ± 0.10 of log k_{ROH} (M^{-1} s^{-1}) against the pK_a of the alkyl alcohol for reaction of the galactosylated enzyme with alkyl alcohols at pH 8.6 (25 mM sodium pyrophosphate). (B) Brønsted correlation with slope β_{nuc} = 0.08 ± 0.10 of log $k_{ROH}K_d$ (s^{-1}) against the pK_a of the alkyl alcohol, where K_d is the dissociation constant for the β-galactosidase-catalyzed hydrolysis of the corresponding alkyl β-D-galactopyranosides (see text).

Brønsted plots of log k_{ROH} (M^{-1} s^{-1}, Figure 6A) and log $k_{ROH}K_d$ (s^{-1}, Figure 6B) against the pK_a of the alkyl alcohol. The latter attempts to correct the slope of the former correlation for the effect of changing affinity of the alkoxy side chain for the enzyme. This correction does not significantly improve the correlation of the data with the pK_a of the alkyl alcohol nucleophile, but it does lead to an increase in the slope from $(\beta_{nuc})_{ROH}$ = −0.19 ± 0.10 (Figure 6A) to $(\beta_{nuc})_{ROH} + \beta_d$ = 0.08 ± 0.10 (Figure 6B), which reflects the weak correlation observed for the Brønsted plot for log K_d (Richard et al., 1995).

The simplest explanation for the observed deviations from the Brønsted correlations in Figure 6A,B, discussed in greater detail in the previous paper (Richard et al., 1995) is that they represent stabilization of the Michaelis complex between the enzyme and the alkyl alcohol by productive binding interactions with hydrophobic halogen substituents in one case (A) and by nonproductive binding interactions in the other (B) (Jencks, 1971). We are unable to separate the relative contributions of productive and nonproductive binding interactions to the observed dissociation constants for the reactions of **1**-OR, so that the difference in the slopes of the correlations in Figure 6A [$(\beta_{nuc})_{ROH}$ = −0.19 ± 0.10] and B [$(\beta_{nuc})_{ROH} + \beta_d$ = 0.08 ± 0.10] represents the range of uncertainty in the effects of polar substituents on the reactivity of alkyl alcohols.

Chemical and Enzymatic Selectivity of Glycosyl Derivatives toward Reaction with Methanol. Nonenzymatic hydrolysis of α-D-glucopyranosyl fluoride in solvents of up to 90% methanol in water gives exclusively glucose and no detectable methyl D-glucopyranoside (Banait & Jencks, 1991). By contrast, a 70% yield of methyl β-D-galactopyranoside is obtained from reaction of the galactosyl–enzyme intermediate of β-galactosidase in just 4% methanol in water (Sinnott & Viratelle, 1973). We do not fully understand the explanation for this striking change in chemical selectivity associated with moving the glycosyl moiety from solution to the active site of β-galactosidase, but we can suggest several effects that may contribute to this change.

(1) The high selectivity of β-D-glucopyranosyl fluoride toward reaction with water is probably due to (a) ionization

[2] The rate constant ratio k_{MeOH}/k_s = 4.0 M^{-1} can be calculated from the second-order rate constant k_4/K_i'' = 10 500 s^{-1}/2.2 M = 4800 M^{-1} s^{-1} for the reaction of methanol with the galactosyl–enzyme intermediate, and the first-order rate constant k_s = 1200 s^{-1} for reaction of water used by these investigators (Huber et al., 1984).

of the substrate within a solvation pool composed nearly exclusively of water to form a glucosyl oxocarbenium ion and (b) reaction of the oxocarbenium ion with water in the initial solvation shell faster ($k_s \approx 10^{12}$ s^{-1}) (Amyes & Jencks, 1989) than its equilibration with methanol in bulk solvent (Banait & Jencks, 1991). Any oxocarbenium ion intermediate formed at the enzyme should be more stable than the free oxocarbenium ion formed in solution, so that its lifetime might be long enough to allow for its equilibration with bulk solvent [see (3) below].

(2) The equilibrium constant for interconversion of D-galactose and methyl β-D-galactopyranoside is $K_{MeOD} = 6.7$ (Scheme 5, Figure 5).[3] This shows that the preferential reaction of methanol, compared to water, with the galactosylated enzyme is due in part to the greater thermodynamic stability of the methyl β-D-galactopyranoside product.

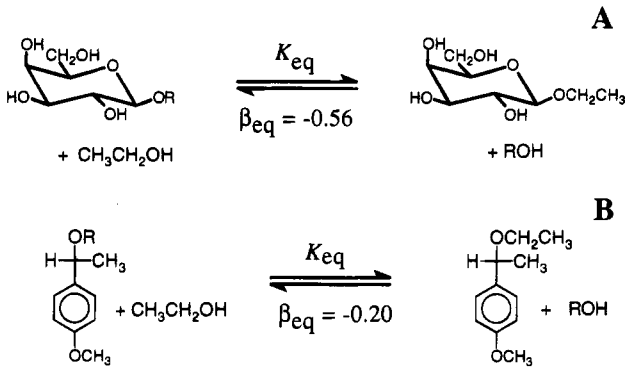
(3) The large selectivity of the galactosylated enzyme for reaction with methanol is consistent with ionization of this acylal intermediate to form a relatively stable oxocarbenium ion which partitions between reaction with methanol and water. The hydrolysis of the enzyme-bound acylal (Sinnott & Souchard, 1973) and of glycosyl acylals in water (Brown & Bruice, 1973) both proceed through cationic transition states, but the enzymatic reaction is accelerated by more than 10^8-fold. A rate acceleration that results from stabilization of a cationic transition state must also lead to stabilization of the putative oxocarbenium ion intermediate and a reduction in its reactivity toward water to below $k_s \approx 10^{12}$ s^{-1} that was estimated for the nonenzymatic reactions of glycosyl oxocarbenium ions (Amyes & Jencks, 1989). There is a large selectivity of $k_{MeOH}/k_{HOH} = 70$ for partitioning of the relatively stable 1-[(4-dimethylamino)phenyl]ethyl carbocation in aqueous trifluoroethanol (Richard & Jencks, 1984b). The even larger selectivity of $k_{MeOH}/k_{HOH} = k_{MeOH}[55.5]/k_s = 130$ for partitioning of the galactosyl−enzyme intermediate is therefore consistent with trapping of a very stable oxocarbenium ion.

(4) The large selectivities for the reactions of alcohols with the galactosylated enzyme may represent the preferential binding and catalysis of the reaction of alkyl alcohols or the exclusion of water from a hydrophobic binding pocket for the leaving group/nucleophile.

Enzymes that catalyze glycosyl transfer to water with retention of configuration at carbon often show a relatively large selectivity for reaction with the small organic alcohol methanol (Viratelle & Yon, 1973; Sinnott & Viratelle, 1973; Schuber et al., 1976; Rupley et al., 1968; Sinnott, 1990), while enzymes that catalyze glycosyl transfer with inversion of configuration may show a very small methanol selectivity (Parkin et al., 1991; Sinnott, 1990). For example, no detectable (≤5%) methylribose is formed as a product of the nucleoside hydrolase-catalyzed reaction of inosine in a solvent of 20% methanol in water (Parkin et al., 1991), which shows that $k_{MeOH}/k_s \leq 0.45$ for this enzyme. The large methanol selectivity observed for retaining enzymes must reflect, in part, the presence of a single binding pocket which *accommodates* both water in the hydrolysis reaction of the

[3] In this experiment, the methyl D-galactopyranoside is formed as a single β-anomer, while D-galactose is obtained as a mixture of α- and β-anomers. A somewhat larger value of $K_{MeOD} = 10.6$ can be calculated for the equilibrium constant for interconversion of only the β-anomers of D-galactose and methyl D-galactopyranoside.

Scheme 6



glycosylated enzyme intermediate and alkyl alcohols in the cleavage reaction of the corresponding alkyl glycosides. By contrast, there are separate binding sites for the leaving group and the nucleophile at the inverting enzymes, and the small methanol selectivities observed for these enzyme-catalyzed reactions are consistent with a preference for the binding of water at the nucleophile site and the catalysis of its reaction.

Substituent Effects on Equilibrium Constants for Galactosyl Group Transfer. There is an apparent contradiction between the large negative value of $(\beta_{lg})_{k_{cat}/K_m} = -0.75$ for cleavage of alkyl β-D-galactopyranosides (Richard et al., 1995), which suggests a large buildup in negative charge at the alkoxy leaving group when the transition state is approached from neutral **1**-OR, and $(\beta_{nuc})_{ROH} = -0.19$ for the reaction of alkyl alcohols (Figure 6A), which suggests a much smaller buildup in negative charge when the same transition state is approached from the neutral alkyl alcohol. The large difference in these Brønsted parameters requires that the equilibrium constants $K_T = (k_{cat}/K_m)/k_{ROH}$ (Table 1) for reversible formation of the galactosyl−enzyme intermediate increase sharply with decreasing pK_a of the leaving alcohol (Scheme 1A).

It is convenient to eliminate the galactosyl−enzyme from these chemical equilibria by reporting the ratio of equilibrium constants K_T for reaction alkyl β-D-galactopyranosides and ethyl β-D-galactopyranosides with β-galactosidase. The ratios $K_{eq} = (K_T)_{ROH}/(K_T)_{EtOH}$ for reaction of alkyl β-D-galactopyranosides with ethanol to form ethyl β-D-galactopyranoside and alkyl alcohols (Scheme 6A) are reported in Table 1. The Brønsted plot for K_{eq} then has the same slope $\beta_{eq} = -0.56 \pm 0.05$ (Figure 7) as that for K_T ($\beta_T = -0.56$, not shown), and the explanation for these large negative slopes is also the same. The largest deviations from the Brønsted plots for the kinetic parameters k_{cat}/K_m (M^{-1} s^{-1}) (Richard et al., 1995) and k_{ROH} (M^{-1} s^{-1}, Figure 6) are not manifested in the Brønsted plot of the ratio of these values (Figure 7). This provides strong evidence that K_{eq} is indeed a measure of the chemical equilibrium constant for the alkoxy group exchange. Brønsted correlations of rate or equilibrium data for changing polar substituents provide a measure of the change in "effective" charge at a reacting atom on proceeding from reactant to transition state or product (Hupe & Jencks, 1977; Williams, 1992). The large negative value of $\beta_{eq} = -0.56$ shows that the alcohol exchange reaction (Scheme 6A) behaves as though there were a net decrease in positive charge of 0.56 units at the alkoxy oxygen on moving from **1**-OR to H-OR. Large negative values of β_{eq} have been observed for a variety of group transfer reactions

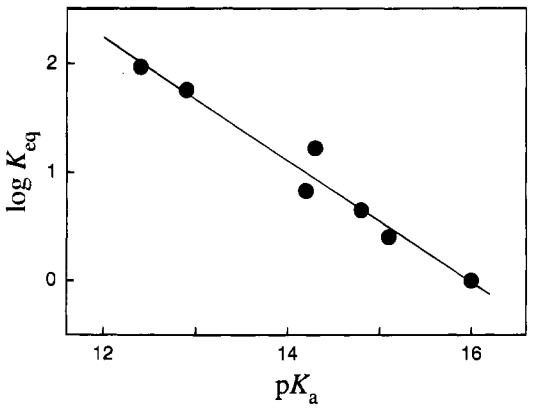
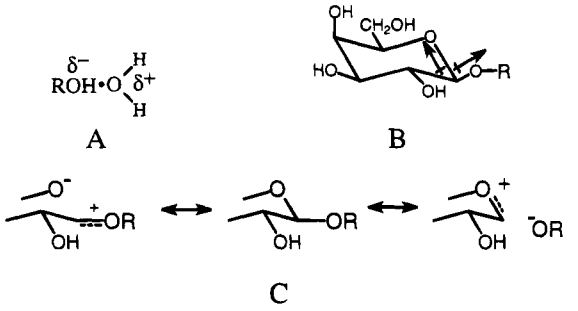


FIGURE 7: Brønsted correlation with slope $\beta_{eq} = -0.56 \pm 0.05$ of log K_{eq} = log $(K_T)_{ROH}/(K_T)_{EtOH}$ (Table 1) for alkoxy group exchange between the β-D-galactopyranosyl moiety and the proton (Scheme 6A).

Scheme 7

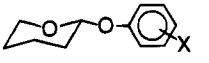


in water, including the hydrolysis of ring-substituted phenyl acetates to form acetic acid and phenols, for which $\beta_{eq} = -0.70$ (Ba-Saif et al., 1987), and hydrolysis of ring-substituted phenyl diethylphosphates to form diethylphosphoric acid and phenols, for which $\beta_{eq} = -0.87$ (Ba-Saif & Williams, 1988). These Brønsted parameters have been attributed to interactions between strongly electron-withdrawing groups (*i.e.,* acetyl or diethylphosphoryl) and polar substituents on the phenol leaving groups which cause the reactants to behave as though there were a large "effective" positive charge at the aryl oxygen, which becomes neutralized on moving to the products (Williams, 1992) independent of the presence of the enzyme catalyst.

By contrast, a relatively small value of $\beta_{eq} = -0.20$ has been determined for the interconversion of alkyl 1-(4-methoxyphenyl)ethyl ethers (Scheme 6B) (Rothenberg et al., 1985). The 0.20 unit decrease in "effective" positive charge at the alkoxy group that is observed when this group is exchanged between the 1-(4-methoxyphenyl)ethyl moiety and the proton (Scheme 6B) may be due partly or entirely to partial negative charge at the alkyl alcohol product as a result of its hydrogen bonding to water (Scheme 7A) (Rothenberg et al., 1985). The even more negative value of $\beta_{eq} = -0.56$ for alkoxy group exchange between the galactosyl moiety and the proton (Scheme 6A) requires a significantly larger "effective" positive charge at the alkoxy oxygen of **1**-OR than at that of 4-$MeOC_6H_4CH(CH_3)OR$. This cannot represent differences in the *formal* charge at these oxygens, which are both neutral. Rather, the change must reflect differences in the intramolecular interactions of the alkoxy groups with the 1-(4-methoxyphenyl)ethyl and galactosyl moieties, which lead to the same changes in reaction equilibria as would variations in formal charge at the alkoxy oxygens. There are at least two interactions between the galactosyl group and electron-withdrawing alkoxy groups which would favor cleavage of a covalent linkage between the two.

(1) A direct, destabilizing, dipole−dipole interaction between the galactosyl group and electron-withdrawing alkoxy groups (Scheme 7B), which is relieved upon transfer of the alkoxy group to the proton. The difference between the Taft σ* values for CH_2OCH_3 (σ* = 0.52) and $CH(CH_3)$-Ph (σ* = 0.11) shows that a glycosyl ring is more strongly electron-withdrawing than the 1-(4-methoxyphenyl)ethyl group (Leffler & Grunwald, 1963). Therefore, destabilizing dipole−dipole interactions will be more severe at **1**-OR than at 4-$MeOC_6H_4CH(CH_3)OR$, and relief of these interactions on moving to the alkyl alcohol product will make a larger contribution to the Brønsted coefficient for the alkoxy exchange reaction of the former compounds (Scheme 6) (Rothenberg et al. 1985; Jencks & Jencks, 1977). However, it is difficult to rationalize the value of $\beta_{eq} = -0.56$ for Scheme 6A by a simple consideration of the polar effect of the glycosyl group alone, because β_{eq} is similar in magnitude to values observed for group exchange at highly electronegative centers. For example, a value of $\beta_{eq} = -0.70$ has been determined for hydrolysis of ring-substituted phenyl acetates (Ba-Saif et al., 1987), a reaction in which substituted phenoxy groups are exchanged between the proton and the acetyl group (σ* = 1.65), which is substantially more electronegative than the methoxymethylene group (σ* = 0.52).

(2) The change in "effective" charge at the alkoxy group on moving from reactant to product can be estimated from the value of β_{eq}, provided the Brønsted correlation includes only polar substituents which have negligible effects on the intrinsic strengths of bonds within the molecule (Hine, 1975). X-ray crystallographic analysis reveals a significant shortening of the endocylic C−O bond and lengthening of the exocyclic bond of **2** with decreasing basicity of the aryl oxide



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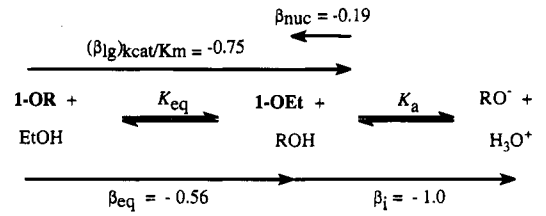
leaving group, which results in the placement of increasing negative charge at the oxygen best able to accommodate such charge (Scheme 7C) (Jones & Kirby, 1979, 1984). Smaller related changes in bond lengths with changing exocyclic substituent have been detected in the crystal structures of alkyl and aryl α-D-glucopyranosides and β-D-glucopyranosides (Briggs et al., 1984). These structural changes may reveal an effect which causes a significant change in the Brønsted parameter for reaction of **1**-OR, if they correlate with an intrinsic weakening of the acetal linkage. For example, a difference of 0.1 unit in β_{eq} for the reactions of **1**-OR and 4-$MeOC_6H_4CH(CH_3)OR$ could be explained if there were a 0.5 kcal/mol greater weakening of the C−O bond on moving from **1**-OCH_2CH_3 to **1**-OCH_2CF_3 compared with 4-$MeOC_6H_4CH(CH_3)OCH_2CH_3$ and 4-$MeOC_6H_4CH(CH_3)OCH_2CF_3$.

Transition State for β-Galactosidase-Catalyzed Galactosyl Group Transfer. An important conclusion from this work is that the Brønsted coefficients β_{lg} and β_{nuc} obtained from the kinetic parameters for enzyme-catalyzed hydrolysis of glycosides provide limited information about the change in

Scheme 8: Changes in Effective "Charge" at the Alkoxy Oxygen of OR on Proceeding from **1**-OR to the Product or Transition State for the Reversible Nucleophilic Substitution Reaction of Ethanol with Alkyl β-D-Galactopyranosides To Form the Alkyl Alcohol or Alkoxide Anion



formal charge at the alkoxy oxygen on moving to the reaction transition state. Net development of negative charge at the alkoxy group on moving to the transition state certainly contributes to the negative Brønsted coefficients of $(\beta_{lg})_{k_{cat}/K_m} = -0.75$ and $(\beta_{nuc})_{ROH} = -0.19$ for the reactions in the cleavage and synthesis directions, respectively (Scheme 8, where β_i is the Brønsted coefficient for the ionization of alkyl alcohols). These Brønsted coefficients set broad limits on the charge developed at the alkoxy oxygen in the transition state. The former coefficient will overestimate charge development, if the cleavage reaction is favored by the relief of destabilizing intramolecular electrostatic interactions between the galactosyl and alkoxy groups (Scheme 7B) or by weakening of covalent bonds to weakly basic groups (Scheme 7C). However, the latter coefficient will underestimate charge development, if the synthesis reaction is sensitive to dipole−dipole interactions that develop on moving from the alkyl alcohol and galactosylated enzyme to the reaction transition state (Scheme 7B).

There are similar uncertainties in the interpretation of Brønsted coefficients for the nonenzymatic hydrolysis of glycosides. The rate constants for the spontaneous hydrolysis of **2** (Craze & Kirby, 1978) and of β-D-galactopyranosyl pyridinium ions in water (Jones et al., 1977) are correlated by Brønsted coefficients of $\beta_{lg} = -1.28$ and -1.26, respectively. It is generally assumed that these Brønsted coefficients require full cleavage of the glycosidic bond in the rate-determining transition state, which closely resembles an ion-pair or ion−dipole reaction intermediate (Young et al., 1980; Craze & Kirby, 1978). However, these Brønsted coefficients may be less negative than the maximum possible for full cleavage of the bond to the leaving group if, for instance, there were weak nucleophilic participation by solvent at the transition state for a concerted A_ND_N (S_N2) (Guthrie & Jencks, 1989) reaction, because of the large negative Brønsted coefficient for the reaction of **1**-OR to form **1**-OCH_2CH_3 and an alkoxide ion ($\beta = \beta_{eq} + \beta_i = -1.56$, Scheme 8).

The negative average value of β_{lg} for the β-galactosidase-catalyzed cleavage of **1**-OR ($\beta_{lg} \approx -0.6$)[4] is consistent with general-acid catalysis by the enzyme of formation of a relatively stable oxocarbenium ion (Richard et al., 1995). Similarly, the value of $\beta_{nuc} \approx -0.06$ for the synthesis reaction [the average of $(\beta_{nuc})_{ROH} = -0.19 \pm 0.10$ (Figure 6A) and $(\beta_{nuc})_{ROH} + \beta_d = 0.08 \pm 0.10$ (Figure 6B)] is consistent with general-base catalysis of the addition of alkyl alcohols to a relatively stable oxocarbenium ion. For example, a value of $\beta_{nuc} = 0.04$ has been determined for acetate ion-catalyzed addition of alkyl alcohols to the relatively stable 1-[(4-dimethylamino)phenyl]ethyl carbocation (Ta-Shma & Jencks, 1986).

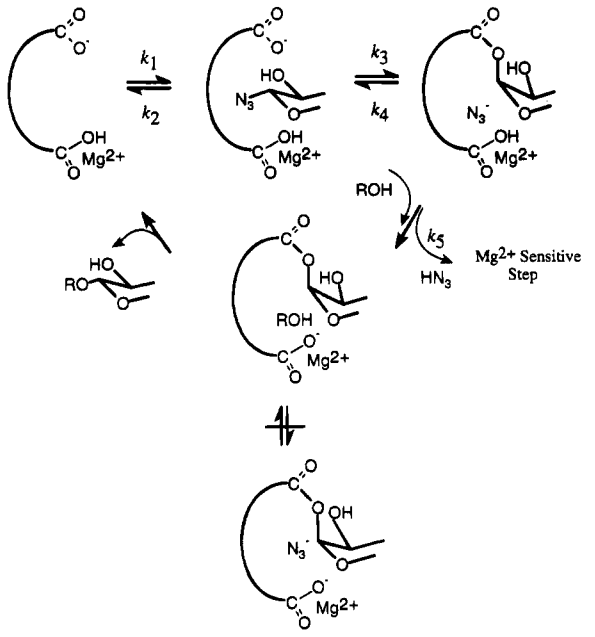
Reaction of the Galactosyl−Enzyme Intermediate with Glucose. A second-order rate constant of $k_{Glc} = 1.2 \times 10^4$ M^{-1} s^{-1} (pH 8.6) for the reaction of glucose with the galactosyl−enzyme intermediate can be calculated from the product rate constant ratio $k_{Glc}/k_s = 17$ M^{-1} (results) and $k_s = 710$ s^{-1} (Selwood & Sinnott, 1990). This relatively small second-order rate constant for the reaction of glucose is surprising, in light of the observation that β-galactosidase catalyzes both the hydrolysis of lactose and the isomerization of this compound to allolactose [6-O-(β-D-galactopyranosyl)-D-glucose] (Jobe & Bourgeois, 1972; Huber et al., 1976, 1983). This observation shows that the complex of glucose with the galactosylated enzyme undergoes release of glucose to the solution and reaction to form allolactose at similar rates, so that the release of glucose is partly rate-determining for the β-galactosidase-catalyzed hydrolysis of lactose. Now, if the release of glucose is partly rate-determining for the hydrolysis of lactose, then the binding of glucose is expected to be partly rate-determining in the direction of lactose/allolactose synthesis. However, the observed second-order rate constant for this reaction, $k_{Glc} = 1.2 \times 10^4$ M^{-1} s^{-1}, lies far below 10^7-10^9 M^{-1} s^{-1} for encounter-limited reactions (Hammes & Schimmel, 1970), and the first-order rate constant for reaction of the galactosylated enzyme with bound glucose is 3-fold smaller than reaction with water alone (Huber et al., 1984). The low observed first- and second-order rate constants for the reaction of glucose with the galactosylated enzyme suggest that most complexes of glucose with the galactosyl−enzyme are nonproductive and undergo fast dissociation to release glucose, so that the observed reaction of glucose occurs from the small fraction of productive complexes.

The observation of a ~20-fold larger rate constant for cleavage of enzyme-bound **1**-OC_6H_4-2-NO_2 ($k_3 = 2800$ s^{-1}) than of **1**-OC_6H_4-4-NO_2 ($k_3 = 120$ s^{-1}) at pH 8.6, but only a 2-fold difference in k_{cat}/K_m for these substrates (Sinnott & Souchard, 1973), is also consistent with differential stabilization of the Michaelis complexes of these substrates by nonproductive binding. These observations of nonproductive binding suggest that there are significant binding interactions between β-galactosidase and the leaving group/nucleophile but that the interactions provide relatively little stabilization of the transition state for the glycoside cleavage or synthesis reactions.

Reaction of β-Galactosidase in the Presence of Azide Ion. Trapping studies with azide ion show that the yield of D-galactose from the β-galactosidase-catalyzed cleavage of **1**-OC_6H_4-4-NO_2 remains at 100% as the concentration of sodium azide is increased from 0 to 0.50 M. Therefore, there is no detectable trapping of the galactosylated enzyme by the good nucleophile azide ion, which shows that $k_{az} \leq 71$ M^{-1} s^{-1} (see results). There is an apparent contradiction between the failure to observe the synthesis of β-D-galactopyranosyl azide (**1**-N_3) by reaction of 0.50 M azide ion with the galactosylated enzyme and the following: (a) the relatively efficient enzymatic catalysis of the reverse cleavage of **1**-N_3 to form the galactosylated enzyme (Sinnott, 1971) and (b) the relatively efficient synthesis of alkyl β-D-

[4] The average of $(\beta_{lg})_{k_3} = -0.49 \pm 0.13$ and $(\beta_{lg})_{k_{cat}/K_m} = -0.75 \pm 0.14$ for β-galactosidase-catalyzed cleavage of **1**-OR (Richard et al., 1995).

Scheme 9



galactopyranosides by transfer of the galactosyl group from the galactosylated enzyme to alkyl alcohols (Table 1), which are much less nucleophilic than azide ion. For example, the second-order rate constant for galactosyl transfer from the galactosylated enzyme to trifluoroethanol is ≥60-fold larger than for transfer to azide ion.

The results of the azide ion trapping studies suggest that the form of the galactosylated enzyme that is competent to undergo reversible return to **1**-N_3 is different from the free galactosylated enzyme, which reacts with water and alcohols but not azide anion. This difference may correspond to different states of ionization of the enzyme, if the residue involved in proton transfer at the leaving group/nucleophile undergoes a large change in pK_a and loss of a proton on moving from the Michaelis complex to the galactosylated enzyme. This is shown in Scheme 9, where Glu-537 is the galactosyl group acceptor (Gebler et al., 1992) and Glu-461 may be the catalytic acid–base (Herrchen & Legler, 1984). If Glu-461 undergoes a large decrease in pK_a, then the formal product of cleavage of **1**-N_3 will be azide ion plus a proton which is lost from the enzyme, *i.e.*, HN_3. The synthesis **1**-N_3 by the microscopic reverse of this mechanism is not observed, presumably because the concentration of hydrazoic acid (pK_a = 4.7) (Jencks & Regenstein, 1976) present at pH 8.6 is extremely low. Very efficient trapping of the galactosylated enzyme by azide ion is observed for the Gly-461 mutant, where the propionate side chain of Glu-461 has been excised and replaced by hydrogen to give Gly-461 (Cupples et al., 1990). In fact, the second-order rate constant for reaction of azide ion with the galactosylated mutant enzyme, 7500 M^{-1} s^{-1},[5] is ≥100-fold larger than that for reaction of the native enzyme. These data show that binding and reaction of azide ion at the wild-type enzyme is inhibited by interactions, which may be electrostatic in nature, with the carboxylate side chain of Glu-461 (Scheme 9).

Withers and co-workers have observed effective azide ion trapping of the glycosylated enzyme intermediate of Glu-127 mutant forms of exoglucanase/xylanase from *Cellulomonas fimi*. They have also suggested that excision of an essential glutamate, which probably functions in acid–base catalysis, facilitates access of azide to the active site (McLeod et al., 1994).

The pH–rate profile for the reaction of enzyme-bound **1**-OC_6H_4-4-NO_2 shows a downward break at pH 9.2 at the high end, while the pH–rate profile for hydrolysis of the galactosylated enzyme shows a downward break at pH 5.5 at the low end (Selwood & Sinnott, 1990). These data are consistent with a pK_a = 9.2 for an essential catalytic acid that assists in the cleavage of **1**-OC_6H_4-4-NO_2, and pK_a = 5.5 for the catalytic base that assists in the reaction of water with the galactosylated enzyme (Selwood & Sinnott, 1990). The profiles require: (a) that different residues participate in the catalysis of leaving group expulsion and of the reaction of the water nucleophile, (b) that there is no such acid–base catalysis because Mg^{2+} functions in this respect (Selwood & Sinnott, 1990), or (c) that a single residue undergoes a large change in pK_a upon conversion of the Michaelis complex with **1**-OC_6H_4-4-NO_2 to the galactosyl–enzyme intermediate (Scheme 9).

The 200-fold decrease in k_{cat} for the β-galactosidase-catalyzed cleavage of **1**-N_3 at pH 8.6 upon removal of Mg^{2+} is surprising, because azide ion is a weakly basic leaving group (pK_a = 4.7) (Jencks & Regenstein, 1976), and there is a sharp falloff in the activation by Mg^{2+} of the β-galactosidase-catalyzed cleavage of **1**-OR with decreasing pK_a of the oxygen leaving group. For example, the effect of Mg^{2+} on k_{cat} for cleavage of **1**-OR decreases from 230-fold to only 5-fold as the pK_a of the oxygen leaving group is reduced from 16.0 (**1**-OCH_2CH_3) to 7.1 (**1**-OC_6H_4-4-NO_2) (Richard et al., 1995). We suggest that β-galactosidase-catalyzed cleavage of **1**-N_3 at the protonated galactosylated enzyme is fast and reversible, so that the rate-determining step for its reaction is loss of the nucleophilic azide anion from this form of the enzyme. The metal ion might promote the loss of this leaving group to solution by formation of a chelate with the nucleophile, which would decrease its nucleophilicity (Selwood & Sinnott, 1990) or, more likely, by causing an increase in the acidity of the essential catalytic acid which increases the fraction of leaving group present in the weakly nucleophilic protonated form (Richard et al., 1995).

Metal ion-promoted dissociation of the leaving group as illustrated in Scheme 9 would only be observed when the leaving group is strongly nucleophilic and the cleavage of **1**-X is readily reversible. The α-deuterium (k_H/k_D = 1.04) (Sinnott & Souchard, 1973) and ^{18}O-kinetic isotope effects ($k^{18}O/k^{16}O$ = 1.022) (Rosenberg & Kirsch, 1981) on k_{cat} for β-galactosidase-catalyzed hydrolysis of **1**-OC_6H_4-4-NO_2 are consistent with reversible transfer of the galactosyl group

[5] This second-order rate constant was calculated as the ratio of the intercept (13.5 s^{-1}) and slope (0.0018 M) from Figure 2 and Table 1 in published work of Huber and Chivers (1993) by assuming that $k_3 \gg k_s$ (Scheme 4) for reactions of 2-nitrophenyl β-D-galactopyranoside with the E461G mutant enzyme. This assumption is justified by the observation of a 17-fold acceleration in the velocity of hydrolysis of this substrate in the presence of increasing concentrations of azide ion (Huber & Chivers, 1993).

[6] We are not aware of data pertaining to the relative nucleophilicity of azide ion and substituted pyridines toward carbenium ions. However, azide ion shows an unusually large nucleophilic reactivity toward triarylmethyl carbocations and other charged electrophiles (*e.g.*, it is 10 000-fold more reactive than trifluoroethylamine, a primary amine of pK_a = 5.9 similar to that of pyridine) (Ritchie, 1986), and there is no evidence that the nucleophilicity of pyridine is abnormally large.

from substrate to enzyme to form a 4-nitrophenoxide ion, while the modest solvent deuterium isotope effect of 1.7 on k_{cat} may represent rate-determining protonation of the enzyme-bound leaving group anion (Selwood & Sinnott, 1990). Metal ion assistance to the reaction of **1**-OC_6H_4-4-NO_2 (5-fold activation) is smaller than that for reaction of **1**-N_3 (200-fold activation), possibly because 4-nitrophenoxide ion is a weaker nucleophile than azide ion, so that there is less internal return from the galactosylated enzyme to give **1**-OC_6H_4-4-NO_2, and a smaller effect of the metal ion which eliminates such return. The lack of activation by Mg^{2+} of the β-galactosidase-catalyzed hydrolysis of substituted β-D-galactopyranosyl pyridinium ions (Sinnott et al., 1978) is consistent with rate-determining irreversible cleavage of this substrate by the apoenzyme and rapid release of the weakly nucleophilic pyridine leaving group to solution.[6]

REFERENCES

Amyes, T. L., & Jencks, W. P. (1989) *J. Am. Chem. Soc. 111*, 7888−7900.

Banait, N. S., & Jencks, W. P. (1991) *J. Am. Chem. Soc. 113*, 7951−7958.

Ba-Saif, S., & Williams, A. (1988) *J. Org. Chem. 53*, 2204−2209.

Ba-Saif, S., Luthra, A. K., & Williams, A. (1987) *J. Am. Chem. Soc. 109*, 6362−6368.

Briggs, A. J., Glenn, R., Jones, P. G., Kirby, A. J., & Ramaswamy, P. (1984) *J. Am. Chem. Soc. 106*, 6200−6206.

Brown, A., & Bruice, T. C. (1973) *J. Am. Chem. Soc. 95*, 1593−1601.

Craze, G.-A., & Kirby, A. J. (1978) *J. Chem. Soc., Perkin Trans. 2* 354−356.

Cupples, C. G., Miller, J. H., & Huber, R. E. (1990) *J. Biol. Chem. 265*, 5512−5518.

Gebler, J. C., Aebersold, R., & Withers, S. (1992) *J. Biol. Chem. 267*, 11126−11130.

Guthrie, R. D., & Jencks, W. P. (1989) *Acc. Chem. Res. 22*, 343−349.

Hammes, G. G., & Schimmel, P. R. (1970) *Enzymes (3rd Ed.) 2*, 67−114.

Herrchen, M., & Legler, G. (1984) *Eur. J. Biochem. 138*, 527−531.

Hine, J. (1975) *Structural Effects on Equilibria in Organic Chemistry*, pp 58−65, Wiley-Interscience, New York.

Huber, R. E., & Chivers, P. T. (1993) *Carbohydr. Res. 250*, 9−18.

Huber, R. E., Kurz, G., & Wallenfels, K. (1976) *Biochemistry 15*, 1994−2001.

Huber, R. E., Gaunt, M. T., Sept, R. L., & Babiak, M. J. (1983) *Can. J. Chem. Cell Biol. 61*, 198−206.

Huber, R. E., Gaunt, M. T., & Hurlburt, K. L. (1984) *Arch. Biochem. Biophys. 234*, 151−160.

Hupe, D. J., & Jencks, W. P. (1977) *J. Am. Chem. Soc. 99*, 451−464.

Jencks, D. A., & Jencks, W. P. (1977) *J. Am. Chem. Soc. 99*, 7948−7960.

Jencks, W. P. (1971) *Cold Spring Harbor Symp. Quant. Biol. 36*, 1−11.

Jencks, W. P. (1985) *Chem. Rev. 85*, 511−527.

Jencks, W. P., & Regenstein, J. (1976) *Handbook of Chemistry and Biochemistry, Physical and Chemical Data*, 3rd ed. (Fasman, G. D., Ed.), Vol. 1, pp 305−351, CRC Press, Cleveland, OH.

Jobe, A., & Bourgeois, S. (1972) *J. Mol. Biol. 69*, 397−408.

Jones, C. C., Sinnott, M. L., & Souchard, I. J. L. (1977) *J. Chem. Soc., Perkin Trans. 2* 1191−1198.

Jones, P. G., & Kirby, A. J. (1979) *J. Chem. Soc., Chem. Commun.* 288−289.

Jones, P. G., & Kirby, A. J. (1984) *J. Am. Chem. Soc. 106*, 6207−6212.

Leffler, J. E., & Grunwald, E. (1963) *Rates and Equilibria of Organic Reactions*, p 222, John Wiley & Sons, New York.

McLeod, A. M., Lindhorst, T., Withers, S. G., & Warran, R. A. J. (1994) *Biochemistry 33*, 6371−6376.

Parkin, D. W., Horenstein, B. A., Abdulah, D. R., Estupinan, B., & Schramm, V. L. (1991) *J. Biol. Chem. 266*, 20658−20665.

Richard, J. P., & Jencks, W. P. (1984a) *J. Am. Chem. Soc. 106*, 1396−1401.

Richard, J. P., & Jencks, W. P. (1984b) *J. Am. Chem. Soc. 106*, 1373−1383.

Richard, J. P., Westerfeld, J. G., & Lin, S. (1995) *Biochemistry 34*, 11703−11712.

Ritchie, C. D. (1986) *Can. J. Chem. 64*, 2239−2250.

Rosenberg, S., & Kirsch, J. F. (1981) *Biochemistry 20*, 3189−3196.

Rothenberg, M. E., Richard, J. P., & Jencks, W. P. (1985) *J. Am. Chem. Soc. 107*, 1340−1346.

Rupley, J. A., Gates, V., & Bilbrey, R. (1968) *J. Am. Chem. Soc. 90*, 5633−5635.

Schuber, F., Travo, P., & Pascal, M. (1976) *Eur. J. Biochem. 69*, 593−602.

Selwood, T., & Sinnott, M. L. (1990) *Biochem. J. 268*, 317−323.

Sinnott, M. L. (1971) *Biochem. J. 125*, 717−719.

Sinnott, M. L. (1990) *Chem. Rev. 90*, 1171−1202.

Sinnott, M. L., & Souchard, I. J. L. (1973) *Biochem. J. 133*, 89−98.

Sinnott, M. L., & Viratelle, O. M. (1973) *Biochem. J. 133*, 81−87.

Sinnott, M. L., Withers, S. G., & Viratelle, O. M. (1978) *Biochem. J. 175*, 539−546.

Ta-Shma, R., & Jencks, W. P. (1986) *J. Am. Chem. Soc. 108*, 8040−8050.

Tenu, J.-P., Viratelle, O. M., & Yon, J. (1972) *Eur. J. Biochem. 26*, 112−118.

Viratelle, O. M., & Yon, J. M. (1973) *Eur. J. Biochem. 33*, 110−116.

Williams, A. (1992) *Adv. Phys. Org. Chem. 27*, 1−55.

Young, P. R., Bogseth, R. C., & Rietz, E. G. (1980) *J. Am. Chem. Soc. 102*, 6268.

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