Structure−Reactivity Relationships for β-Galactosidase (*Escherichia coli, lac Z*). 1. Brønsted Parameters for Cleavage of Alkyl β-D-Galactopyranosides†

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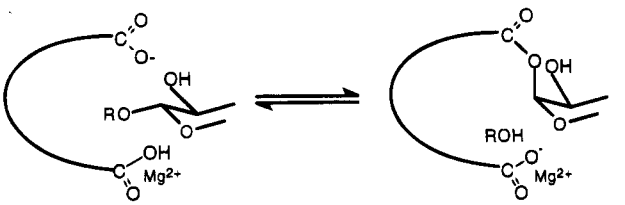
ABSTRACT: Seven substituted alkyl β-D-galactopyranosides **1**-OR have been prepared and shown to be fair to excellent substrates for hydrolysis catalyzed by β-galactosidase (*Escherichia coli, lac Z*). Brønsted parameters of $(\beta_{lg})_{k_3} = -0.49 \pm 0.13$ and $(\beta_{lg})_{k_{cat}/K_m} = -0.75 \pm 0.14$, respectively, were determined at pH 8.6 for k_3 (s^{-1}), the first-order rate constant for cleavage of enzyme-bound **1**-OR, and k_{cat}/K_m (M^{-1} s^{-1}), the second-order rate constant for reaction of the free enzyme and **1**-OR. There is a weak correlation between log K_m and the pK_a of the alkyl alcohol leaving group, which is attributed to stabilization of the Michaelis complex by hydrophobic interactions between the enzyme and electron-withdrawing halogen substituents at the alkoxy leaving group. These binding interactions are probably both productive and expressed in the value of k_{cat}/K_m and nonproductive and expressed in the value of k_3. The negative values of β_{lg} are inconsistent with enzymatic catalysis of endocyclic cleavage of the glycosidic bond. The values of β_{lg} for enzyme-catalyzed cleavage of alkyl β-D-galactopyranosides lie between those observed for the spontaneous ($\beta_{lg} \approx -1.25$) and specific-acid-catalyzed ($\beta_{lg} \approx 0$) cleavage of acetals, and these pathways are therefore excluded for the enzyme-catalyzed reaction. Removal of the metal cofactor Mg^{2+} from the enzyme causes a ~0.2 unit decrease in $(\beta_{lg})_{k_3}$ for β-galactosidase-catalyzed cleavage of **1**-OR. The interpretation of this change in β_{lg} is unclear. The Brønsted coefficients for the β-galactosidase-catalyzed reaction are consistent with participation by an essential catalytic residue in concerted general-acid catalysis of cleavage of the glycosidic bond of **1**-OR and/or stabilization of developing negative charge at the alkoxy oxygen by interaction with the magnesium ion cofactor.

Many of the details of the mechanism for enzymatic catalysis of glycosyl transfer reactions are not well understood. Scheme 1 shows a working model for the first step for catalysis of glycosyl transfer with retention of configuration, which was first proposed after examination of an X-ray crystal structure of a lysozyme−inhibitor complex (Blake et al., 1967). This proposal is now supported by extensive results of model studies of nonenzymatic catalysis of acetal cleavage (Fife, 1975). This reaction mechanism is defined by the imperatives for catalysis of nucleophilic substitution of a poor alkoxide ion leaving group at glycosides, and these imperatives will probably ensure the presence of the following essential catalytic residues or metal cofactors at all enzymes that catalyze glycosyl transfer with retention of configuration at the glycosidic carbon: (1) a nucleophilic residue that participates either by providing assistance to expulsion of the leaving group from the anomeric carbon and/or electrostatic stabilization of an oxocarbenium ion reaction intermediate; (2) an acidic residue (*e.g.*, a carboxylic acid or a metal ion) that provides stabilization of negative charge at the oxygen leaving group.

In the second step for the hydrolysis reaction, the residue that functions in the catalysis of leaving group expulsion may act as a catalyst of the reaction of water (ROH = HOH) with the glycosyl−enzyme intermediate.

Scheme 1



Scheme 1 represents a point of departure for development of experiments to provide a more detailed characterization of (1) the sequence of the changes in bonding that occur during enzymatic catalysis of glycosyl transfer reactions, (2) the interactions between the essential catalytic residues and the substrate in the rate-determining transition state, and (3) the effect of such interactions on the structure and the stability of the transition state(s) for the uncatalyzed hydrolysis reactions of acetals in water.

We are interested in characterizing the transition state for the reaction catalyzed by β-galactosidase (*Escherichia coli, lac Z*) and in determining the changes in transition state structure that occur on moving from hydrolysis of glycosides in water to hydrolysis at an enzyme active site. Studies on the effects of systematic changes in structure on the reactivity of acetals toward hydrolysis have provided a detailed picture of the changes in bonding that occur on moving from the reactant to transition state in water (Cordes & Bull, 1974; Fife, 1975). By contrast, there have been fewer studies of isotope effects and electronic substituent effects on the kinetic

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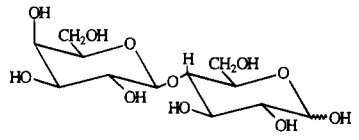
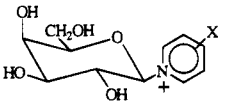
parameters for enzyme-catalyzed cleavage of glycosides, and uncertainty about the interpretation of these experimental results has provided the rational for the present studies on the mechanism of action of β-galactosidase.

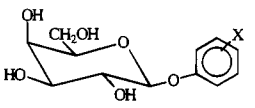
β-Galactosidase catalyzes the physiological hydrolysis of lactose (**1**-glucose) to glucose and galactose by a two-step reaction mechanism, through an intermediate in which the



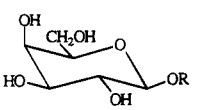
1-Glucose (Lactose)



1-pyr$^+$



1-OAr



1-OR

OR
OCH_2CH_3
OCH_2CH_2OH
$OCH_2CH_2OCH_3$
OCH_2CH_2Cl
OCH_2CH_2F
OCH_2CHCl_2
OCH_2CF_3

galactosyl group is covalently linked (Sinnott & Souchard, 1973) to the enzyme at Glu-537 (Gebler et al., 1992). This enzyme shows a low specificity for the leaving group at the glycoside, which suggests that Brønsted correlations of rate data for enzymatic cleavage of a series of structurally homologous substrates should provide information about the changes in "effective charge" at the leaving group on moving to the reaction transition state (Hupe & Jencks, 1977).

Sinnott and co-workers have determined the kinetic parameters for β-galactosidase-catalyzed cleavage of series of β-D-galactopyranosyl pyridinium ions **1**-pyr$^+$ (Sinnott & Withers, 1974) and of aryl β-D-galactopyranosides **1**-OAr (Sinnott & Souchard, 1973). A good Brønsted correlation with slope $\beta_{lg} = -1.0$ was observed for the values of log k_{cat} for cleavage of **1**-pyr$^+$ (Sinnott & Withers, 1974), but very poor correlations were observed for the values of log k_{cat} and log k_{cat}/K_m for enzyme-catalyzed cleavage of **1**-OAr (Sinnott & Souchard, 1973). The latter poor correlations arise because of a combination of the problems most often encountered in structure−reactivity studies of enzymatic reactions (Jencks, 1971).

(1) Substituted aryl β-D-galactopyranosides **1**-OAr are chemically more reactive than the physiological substrate lactose, and cleavage of the glycosidic bond is not always rate-determining for catalysis. The kinetic parameter k_{cat} for β-galactosidase-catalyzed hydrolysis of substrates with the most weakly basic phenoxide leaving groups (*e.g.*, 2,4-dinitrophenoxide) is limited by the rate of transfer of the galactosyl−enzyme reaction intermediate to water (Sinnott & Souchard, 1973; Sinnott & Viratelle, 1973). This step occurs after cleavage of the bond to the leaving group, and its rate is independent of the leaving group at **1**-OAr.

Formation of the galactosyl−enzyme reaction intermediate is rate-determining for cleavage of **1**-OC_6H_4-4-NO_2; but, the observation of a large ^{18}O (Rosenberg & Kirsch, 1981) and a small α-deuterium isotope effect on k_{cat} (Sinnott & Souchard, 1973) for the reaction of this substrate is not consistent with simple rate-determining cleavage of the glycosidic bond to form the covalent reaction intermediate. A simple explanation for these isotope effects is that the rate-determining step for k_{cat} occurs after the cleavage of the bond to the leaving group and formation of the covalent galactosyl reaction intermediate, but before irreversible loss of leaving group to solution (Selwood & Sinnott, 1990). The solvent deuterium isotope effect of $k_{HOH}/k_{DOD} = 1.7$ on k_{cat} for this reaction is consistent with rate-determining protonation of the 4-nitrophenoxide anion that may be required to promote its release from the enzyme (Selwood & Sinnott, 1990).

(2) Different pathways may be followed for β-galactosidase-catalyzed hydrolysis of **1**-OAr, in which the leaving group is a weakly basic aryl oxide anion, and for hydrolysis of the physiological substrate lactose, where the leaving group is a strongly basic alkoxide anion.

(a) Concerted general-acid catalysis of leaving group expulsion may be important for cleavage of the physiological substrate lactose, where the leaving group is a strongly basic alkoxide anion (Fife, 1975). However, a change to a stepwise mechanism involving spontaneous expulsion of the leaving-group oxyanion *must occur* when the leaving group anion is so stable that its protonation by the enzyme is uphill thermodynamically, *i.e.*, when the pK_a of the leaving group conjugate acid is lower than that of the catalytic acid residue (Jencks, 1972).

(b) There may be a change from cleavage of the exocyclic glycosidic bond, which will be favored by the choice of weakly basic leaving groups, to a more baroque mechanism for substrates with stronger exocyclic glycosidic bonds to basic leaving groups, in which the ring first opens by endocyclic C−O bond cleavage, and cleavage of the bond to the formal leaving group occurs at a later step (Franck, 1992; Sinnott, 1993).

(3) The observations of significant changes in K_m with changing aromatic ring substituent at **1**-OAr and of a tighter overall binding of **1**-OAr ($K_m = 0.05-0.5$ mM) (Sinnott & Souchard, 1973) than of physiological substrate lactose ($K_m = 1.4$ mM) (Bock & Adelhorst, 1992) in their β-galactosidase-catalyzed cleavage reactions are consistent with a binding pocket for the aromatic ring of **1**-OAr which shows differential interactions with phenyl ring substituents. Brønsted correlations of values of k_{cat} and/or k_{cat}/K_m for β-galactosidase-catalyzed hydrolysis of **1**-OAr may be affected by these differences in the binding affinity of different substituted phenyl rings for the enzyme.

Alkyl β-D-galactopyranosides are superior to **1**-OAr as model substrates for β-galactosidase-catalyzed hydrolysis of the physiological substrate lactose, because the pK_a values of the alkyl alcohol leaving groups (12.4−16) bracket the pK_a of glucose. A systematic determination of the kinetic parameters for enzyme-catalyzed hydrolysis of these substrates is likely to give linear, readily interpretable, Brønsted correlations for the following reasons: (1) The velocity of cleavage of **1**-OR should be reduced compared to cleavage of **1**-OAr due to the greater basicity of alkoxide leaving groups, and this will favor rate-determining substrate cleavage for the enzymatic reaction. (2) The similarity in the pK_as of simple alkyl alcohols and of glucose eliminates questions about the effect of changing leaving group pK_a on the mechanism of the physiological reaction and allows for an evaluation of the contribution of electrophilic catalysis by the enzyme to cleavage of glycosides with strongly basic leaving groups. (3) The structural variations across this series of alkyl alcohols are relatively small, and their effect on substrate binding will be minimal for a study of this type.

We report here the synthesis of a series of alkyl β-D-galactopyranosides and the kinetic parameters for the hydrolysis of these substrates catalyzed by β-galactosidase. Fair to good Brønsted correlations, with negative slopes of β_{lg}, are obtained for plots of log k_3 and log k_{cat}/K_m against the pK_a of alkyl alcohol leaving group. The results show that **1**-OR are useful probes for the mechanism of β-galactosidase-catalyzed hydrolysis of glycosides and the structure of the reaction transition state.

MATERIALS AND METHODS

Reagent grade organic chemicals and inorganic salts from commercial sources were used directly without further purification. Water was first distilled and then passed through a Milli-Q water purification system. Precoated thin-layer chromatography plates (0.2 mm) of Alufolien-Kieselgel 60 were from EM separations. 2,3,4,6-Tetra-*O*-acetyl-α-galactosyl bromide was prepared by a published procedure (Bárczay-Marcos & Kõrösy, 1950). β-D-Nicotinamide adenine dinucleotide (NAD^+), 4-nitrophenyl β-D-galactopyranoside, and 2-nitrophenyl β-D-galactopyranoside were purchased from Sigma and were used without further purification. β-Galactosidase from *E. coli* (Grade VIII) was purchased from Sigma. A homogeneous preparation of β-galactosidase, purified by a procedure similar to that of Brake et al. (1978), was used in some experiments. Galactose dehydrogenase from *E. coli* that contains the gene for the *Pseudomonas fluorescens* enzyme on a plasmid was purchased from Boehringer Mannheim or Sigma.

^1H NMR spectra at 400 MHz were recorded in D_2O on a Varian VXR-400 spectrometer. Chemical shifts are reported as δ ppm downfield of an internal 3-(trimethylsilyl)-propionate (TMSP) standard at 0 ppm.

Alkyl β-D-galactopyranosides were detected during purification by column chromatography either by application of a small amount of the column eluant to a thin-layer chromatography plate followed by staining with 5% (w/v) phosphomolybdic acid in 100% ethanol or enzymatically by coupling β-galactosidase-catalyzed hydrolysis of the alkyl β-D-galactopyranoside to reduction of NAD^+ using galactose dehydrogenase.

Syntheses of Alkyl β-D-Galactopyranosides. The following procedure was used for conversion of tetra-*O*-acetyl-α-galactosyl bromide to alkyl β-D-galactopyranosides (Schroeder & Green, 1966). $CaSO_4$ (10 g, 10−20 mesh), HgO (3.2 g), and $HgBr_2$ (0.25 g) were added to 100 mL of a 1:1 (v/v) solution of the alkyl alcohol and chloroform. The mixture was stirred for 1 h at room temperature, after which 2,3,4,6-tetra-*O*-acetyl-α-galactosyl bromide (7.5 g, 0.018 mol) was added and the mixture stirred overnight at room temperature. The solid inorganic residue was removed by filtration through Celite, and the organic solvent was removed by evaporation at reduced pressure to give the tetra-*O*-acetyl-β-D-galactopyranosides as oils. These were dissolved in 25 mL of 0.022 M sodium methoxide in methanol, and the solution was stirred at room temperature for 1 h. The pH of the solution was then adjusted to ~6 with Amberlite H^+-resin, and the solution was clarified by filtration over 5 g of decolorizing charcoal. The solvent was then removed under reduced pressure to give oils which were shown by ^1H NMR to be the respective alkyl β-D-galactopyranosides, with variable amounts (up to 10%) of the α-isomer as a contaminant.

1-OCH_2CF_3 and **1**-OCH_2CH_2Cl were purified by recrystallization from absolute ethanol. **1**-OCH_2CH_3 was purified by recrystallization from methanol. The other alkyl β-D-galactopyranosides were purified by anion exchange chromatography over Dowex-1 (HO^-) by the following procedure (Austin et al., 1963). Crude **1**-OR (~0.15 g) was dissolved in 1 mL of water and the solution applied to a 2 × 20 cm column of Dowex-1 (HO^-). The column was washed with water, and the β-isomer of the respective **1**-OR was observed to elute just before the α-isomer, at an elution volume of ~100 mL. Evaporation of water under reduced pressure gave **1**-OR as crystalline solids except for **1**-$OCH_2CH_2OCH_3$, which was obtained as an oil.

The purified alkyl β-D-galactopyranosides were shown to undergo hydrolysis catalyzed by β-galactosidase to release 1 mol equiv of D-galactose, which was quantified by coupling its formation to the reduction of NAD^+ using galactose dehydrogenase. Further characterization of these galactopyranosides was provided by ^1H NMR spectroscopy and elemental analysis (for all new compounds except **1**-OCH_2CH_2Cl).

*Ethyl β-D-Galactopyranoside (**1**-OCH_2CH_3):* mp 158−159 °C [159−161 °C (Austin et al., 1963)]; ^1H NMR (D_2O) δ 1.23 (3 H, t, CH_3, J = 7.1 Hz), 3.50 (1 H, dd, H-2, J = 7.9, 10 Hz), 3.67 (1 H, dd, H-3, J = 3.5, 10 Hz), 3.70 (1 H, ddd, H-5, J = 1.0, 4.4, 7.8), 3.73, 3.93 (2 H, m, CH_2CH_3), 3.78 (2 H, m, H-6), 3.92 (1 H, dd, H-4, J = 1.0, 3.5 Hz), 4.41 (1 H, d, H-1, J = 7.9 Hz). Anal. ($C_8H_{16}O_6$) C, H.

*2,2,2-Trifluoroethyl β-D-Galactopyranoside (**1**-OCH_2CF_3):* mp 127 °C; ^1H NMR (D_2O) δ 3.57 (1 H, dd, H-2, J = 7.7, 9.9 Hz), 3.67 (1 H, dd, H-3, J = 3.4, 9.9 Hz), 3.72 (1 H, ddd, H-5, J = 1.0, 4.2, 7.9 Hz), 3.78 (2 H, m, H-6), 3.94 (1 H, dd, H-4, J = 1.0, 3.4 Hz), 4.24 (1 H, dq, $CH_AH_BCF_3$, J = 8.9, 12.4 Hz), 4.33 (1 H, dq, $CH_AH_BCF_3$, J = 8.9, 12.4 Hz), 4.54 (1 H, d, H-1, J = 7.7 Hz); ^{19}F NMR (D_2O) δ −73.8 (t, CF_3, J = 8.9 Hz). Anal. ($C_8H_{13}F_3O_6$) C, H.

*2,2-Dichloroethyl β-D-Galactopyranoside (**1**-OCH_2CHCl_2):* mp 105−106 °C; ^1H NMR (D_2O) δ 3.57 (1 H, dd, H-2, J = 7.8, 9.8 Hz), 3.67 (1 H, dd, H-3, J = 3.4, 9.8), 3.71 (1 H, m, H-5), 3.78 (2 H, m, H-6), 3.94 (1 H, m, H-4), 4.15, 4.27 (2 H, m, CH_2CHCl_2), 4.54 (1 H, d, H-1, J = 7.8 Hz), 6.10 (1 H, t, CH_2CHCl_2 J = 5.6 Hz). Anal. ($C_8H_{14}Cl_2O_6$) C, H.

*2-Hydroxyethyl β-D-Galactopyranoside (**1**-OCH_2CH_2OH):* mp 136−137 °C; ^1H NMR (D_2O) δ 3.56 (1 H, dd, H-2, J = 7.8, 9.9 Hz), 3.67 (1 H, dd, H-3, J = 3.5, 9.9 Hz), 3.70 (1 H, m, H-5), 3.78 (2 H, m, H-6), 3.8−4.1 (5 H, m, H-4, CH_2CH_2OH), 4.43 (1 H, d, H-1, J = 7.8 Hz). Anal. ($C_8H_{16}O_7$) C, H.

*2-Methoxyethyl β-D-Galactopyranoside (**1**-$OCH_2CH_2OCH_3$):* oil; ^1H NMR (D_2O) δ 3.41 (3 H, s, OCH_3), 3.54 (1 H, dd, H-2, J = 7.8, 9.9 Hz), 3.65 (1 H, dd, H-3, J = 3.5, 9.9 Hz), 3.69 (3 H, m, H-5, CH_2OCH_3), 3.78 (2 H, m, H-6), 3.82, 4.08 (2 H, m, $CH_2CH_2OCH_3$), 3.93 (1 H, dd, H-4, J = 0.6, 3.5 Hz), 4.42 (1 H, d, H-1, J = 7.8 Hz). Anal. ($C_9H_{18}O_7$) C, H.

*2-Fluoroethyl β-D-Galactopyranoside (**1**-OCH_2CH_2F):* mp 124−125 °C; ^1H NMR (D_2O) δ 3.55 (1 H, dd, H-2, J = 7.9, 9.9 Hz), 3.67 (1 H, dd, H-3, J = 3.4, 9.9 Hz), 3.71 (1 H, m, H-5), 3.78 (2 H, m, H-6), 3.94 (1 H, dd, H-4, J = 0.8, 3.4 Hz), 4.03, 4.11 (2 H, m, CH_2CH_2F), 4.47 (1 H, d, H-1, J = 7.9 Hz), 4.68 (2 H, m, CH_2F). Anal. ($C_8H_{15}FO_6$) C, H.

2-Chloroethyl β-D-Galactopyranoside (1-OCH_2CH_2Cl): mp 110−112 °C; ^{1}H NMR (D_2O) δ 3.56 (1 H, dd, H-2, J = 7.9, 9.9 Hz), 3.66 (1 H, dd, H-3, J = 3.4, 9.9 Hz), 3.71 (1 H, m, H-5), 3.78 (2 H, m, H-6), 3.8−4.2 (4 H, m, CH_2CH_2-Cl), 3.93 (1 H, dd, H-4, J = 1.0, 3.4 Hz), 4.48 (1 H, d, H-1, J = 7.9 Hz).

Enzyme Assays. The commercial preparations of galactose dehydrogenase were freed of ammonium sulfate by dialysis against 25 mM sodium pyrophosphate (pH 8.6) that contained 1 mM EDTA. The dialyzed enzyme was stable at 5 °C for ~1 week. The enzyme activity was assayed by following the formation of NADH at 340 nm in a solution that contained sodium pyrophosphate (25 mM, pH 8.6), 0.9 mM NAD^+, and 16.5 mM D-galactose at 25 °C.

Commercial β-galactosidase (lyophilized powder) was dissolved in 25 mM sodium pyrophosphate (pH 8.6) to give a final concentration of ~1 mg/mL. This preparation was stable for several months at 5 °C. The magnesium-free enzyme was prepared by dialysis against 10 mM EDTA (Tenu et al., 1972). The changes in molar absorbtivity upon hydrolysis of aryl β-D-galactopyranosides were calculated from the change in absorbance upon quantitative β-galactosidase-catalyzed hydrolysis of a known concentration of substrate.

The activity of β-galactosidase was assayed by following the formation of 2-nitrophenoxide anion at 410 nm in a solution that contained 100 mM sodium phosphate ($\Delta\epsilon_{410}$ = 3500 M^{-1} cm^{-1}, pH 7.3), 1.0 mM $MgCl_2$, and 2.2 mM 2-nitrophenyl β-D-galactopyranoside at 25 °C.

β-Galactosidase-catalyzed hydrolysis of 4-nitrophenyl β-D-galactopyranoside was monitored by following the increase in absorbance at 405 nm ($\Delta\epsilon$ = 18 300 M^{-1} cm^{-1}, pH 8.6) in a solution that contained 25 mM sodium pyrophosphate (pH 8.6), 1.0 mM $MgCl_2$, and a specified concentration of 4-nitrophenyl β-D-galactopyranoside at 25 °C.

The initial velocity of β-galactosidase-catalyzed hydrolysis of the alkyl β-D-galactopyranosides was determined by coupling the formation of D-galactose to the reduction of NAD^+ using galactose dehydrogenase. The standard assay solution contained 25 mM sodium pyrophosphate (pH 8.6 or 7.0), 1.0 mM $MgCl_2$, 0.7 mM NAD^+, and 0.3 unit of galactose dehydrogenase in a total volume of 1.0 mL. The same conditions were used to assay the magnesium-free enzyme, except that the solution contained 10 mM EDTA (Tenu et al., 1972). After the addition of galactose dehydrogenase, the absorbance at 340 nm was monitored for ~3 min, during which time a small increase in absorbance was sometimes observed due to oxidation of the small amount of galactose (<1%) present as a contaminant in the substrate. After stabilization of the absorbance reading, β-galactosidase was added and the reduction of NAD^+ was monitored by following the increase in absorbance at 340 nm. It was necessary to increase the concentration of the galactose dehydrogenase coupling enzyme for reactions in the presence of nearly saturating concentrations of alkyl β-D-galactopyranoside, in order to overcome inhibition of the coupling enzyme by the alkyl β-D-galactopyranosides.

Values of K_m and V_{max} for β-galactosidase-catalyzed hydrolysis of alkyl β-D-galactopyranosides were determined from the nonlinear least-squares fit of the initial velocity data to eq 1, using SigmaPlot from Jandel Scientific.

$$v = V_{max}[S]/(K_m + [S]) \quad (1)$$

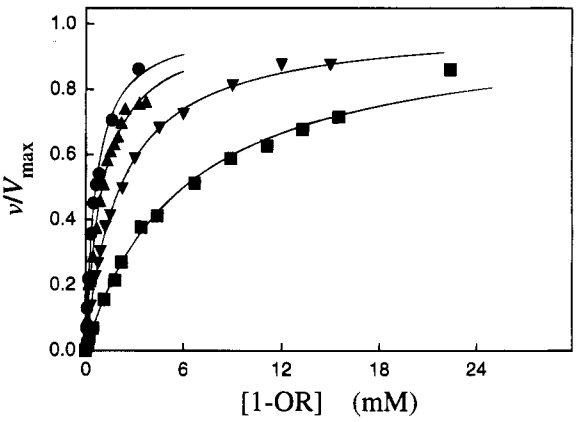


FIGURE 1: Effect of increasing concentrations of alkyl β-D-galactopyranosides **1**-OR on the normalized initial velocities v/V_{max} for the β-galactosidase-catalyzed hydrolysis at 25 °C in 25 mM sodium pyrophosphate buffer at pH 8.6. The solid lines show the nonlinear least-squares fit of the data to eq 1. Key: (●) **1**-OCH_2CH_2Cl; (▲) **1**-OCH_2CF_3; (▼) **1**-$OCH_2CH_2OCH_3$; (■) **1**-OCH_2CH_2OH.

Initial velocities for hydrolysis of alkyl β-D-galactopyranosides and of 4-nitrophenyl β-D-galactopyranoside at pH 8.6 and 7.0 were determined for the reactions catalyzed by a *fixed concentration* of β-galactosidase at nearly saturating concentrations of substrate (≥5.5 K_m), and relative values of V_{max} were calculated from the initial velocity and the Michaelis constant K_m for the respective substrates using eq 1. Values of k_{cat} were calculated from the relative values of V_{max} and k_{cat} = 156 s^{-1} for the cleavage of **1**-OC_6H_4-4-NO_2 at pH 7.0 (Sinnott & Souchard, 1973).

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

RESULTS

Alkyl β-D-galactopyranosides **1**-OR were prepared from tetra-*O*-acetyl-α-galactosyl bromide and the corresponding alkyl alcohol by adaptation of a published procedure (Schroeder & Green, 1966). The structures of **1**-OR were verified by ^{1}H NMR spectroscopy, elemental analyses (for all new compounds except **1**-OCH_2CH_2Cl), and the observation that 1 mol equiv of D-galactose is released upon hydrolysis catalyzed by β-galactosidase.

The effect of increasing concentrations of **1**-OR on the normalized velocity v/V_{max} for their hydrolysis catalyzed by β-galactosidase at pH 8.6 and 25 °C is shown in Figure 1, where v is the initial velocity of the reaction and V_{max} is the initial velocity at saturating concentrations of substrate, calculated as described in the experimental section. The values of K_m for β-galactosidase-catalyzed hydrolysis of **1**-OR at pH 8.6 or 7.0, determined from the fit of the initial velocity data to eq 1, are reported in Table 1.

Values of k_{cat} for β-galactosidase-catalyzed hydrolysis of **1**-OR, calculated as the product of the ratio of values of V_{max} for hydrolysis of **1**-OR and **1**-OC_6H_4-4-NO_2 (pH 7.0) catalyzed by a fixed concentration of enzyme and k_{cat} = 156 s^{-1} for the latter reaction (Sinnott & Souchard, 1973), are listed in Table 1. The small change in k_{cat} for β-galactosidase-catalyzed hydrolysis of **1**-OC_6H_4-4-NO_2 from 156 s^{-1} at pH 7.0 to 120 s^{-1} determined here at pH 8.6 is in good agreement with the change calculated from the pH−rate

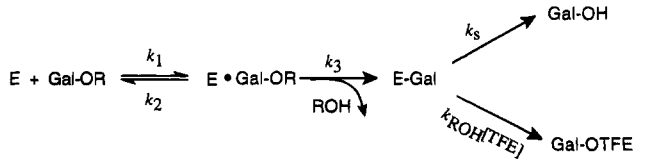
Table 1: Kinetic Parameters for β-Galactosidase-Catalyzed Cleavage of Alkyl β-D-Galactosides[a]

alkoxy leaving group	pK_a[b]	k_{cat} (s^{-1})	k_3[c] (s^{-1})	K_m (mM)	K_d[d] (mM)	k_{cat}/K_m ($=k_3/K_d$) (M^{-1} s^{-1})
CH_3CH_2O	16.0	7.5	7.5	5.5	5.5	1400
$-Mg^{2+}$ [e]		0.033	0.033	5.6		5.9
pH 7.0[f]		7.5		1.7		4400
$HOCH_2CH_2O$	15.1	110	130	7.3	8.5	1.5×10^4
$CH_3OCH_2CH_2O$	14.8	37	39	2.3	2.4	1.6×10^4
$ClCH_2CH_2O$	14.3	250	390	1.0	1.5	2.5×10^5
FCH_2CH_2O	14.2	55	60	3.3	3.5	1.7×10^4
Cl_2CHCH_2O	12.9	230	340	0.31	0.46	7.4×10^5
CF_3CH_2O	12.4	410	970	0.61	1.44	6.7×10^5
$-Mg^{2+}$ [e]		8.1	~8.1	0.62		1.3×10^4
pH 7.0[f]		510		0.32		1.6×10^6
OC_6H_4-4-NO_2	7.1	120	140	0.051	0.060	2.3×10^6
$-Mg^{2+}$ [e]		26				

[a] At 25 °C in 25 mM sodium pyrophosphate buffer (pH 8.6) containing 1.0 mM $MgCl_2$. [b] Jencks, W. P., Regenstein, J. (1976) in *Handbook of Biochemistry and Molecular Biology, Physical and Chemical Data*, 3rd ed. (Fasman, G. D., Ed.); Vol. 1, pp 305–351, CRC Press, Cleveland, OH. [c] Calculated from the experimental parameters k_{cat} and k_s = 750 s^{-1} using eq 2. [d] Calculated from the experimental parameters K_m and k_s = 750 s^{-1} using eq 3. [e] For reaction of the Mg^{2+}-free enzyme in the presence of 10 mM EDTA. [f] For reaction at pH 7.0 (25 mM sodium pyrophosphate).

equation for enzyme-catalyzed hydrolysis of **1**-OC_6H_4-4-NO_2 (Selwood & Sinnott, 1990). Values of k_{cat} for the cleavage of **1**-OR by the magnesium-free enzyme are also reported in Table 1. Essentially identical values of k_{cat} were observed for the cleavage of **1**-OCH_2CH_3 by a Mg^{2+}-free commercial preparation of β-galactosidase purchased from Sigma and a homogeneous enzyme preparation purified by a procedure similar to that of Brake et al. (1978). This shows that β-galactosidase, and not a contaminating enzyme, is responsible for the low levels of activity for cleavage of this relatively poor substrate.

Scheme 2



Values of the microscopic rate constant k_3 (s^{-1}) for cleavage of enzyme-bound **1**-OR, calculated using eq 2

$$k_3 = k_s k_{cat}/(k_s - k_{cat}) \quad (2)$$

derived for Scheme 2 and the experimental rate constants k_{cat} and k_s = 710 s^{-1} for reaction of the galactosylated enzyme at pH 8.6 (Selwood & Sinnott, 1990), are given in Table 1. In many cases $k_3 \approx k_{cat}$, and the largest difference between the experimental and derived rate constants is only 2.4-fold.

Dissociation constants for release of **1**-OR from β-galactosidase (K_d), calculated from the experimental parameter K_m using eq 3, are given in Table 1. Equation 3 was derived

$$K_d = \frac{k_2}{k_1} = \frac{K_m(k_3 + k_s)}{k_s} \quad (3)$$

for Scheme 2 with the assumption that the binding of **1**-OR to β-galactosidase is reversible ($k_2 \gg k_3$). This assumption

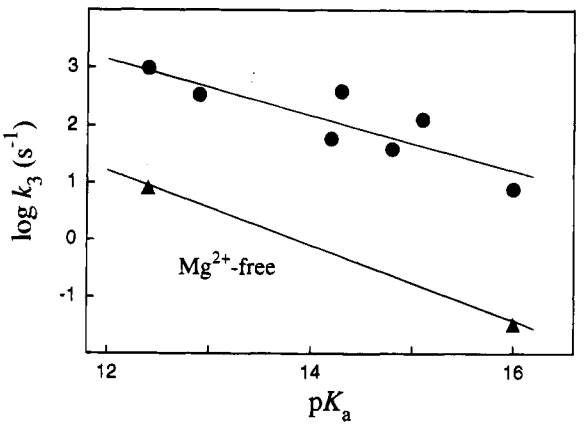


FIGURE 2: Brønsted correlation with slope $(\beta_{lg})_{k_3} = -0.49 \pm 0.13$ of values of log k_3 for the β-galactosidase-catalyzed hydrolysis of **1**-OR. Key: (●) reaction in the presence of 1.0 mM Mg^{2+}; (▲) reaction of Mg^{2+}-free enzyme in the presence of 10 mM EDTA.

is justified by the observation that the values of k_{cat}/K_m for hydrolysis of **1**-OR are well below the limiting value of 10^7–10^9 M^{-1} s^{-1} for a "diffusion-limited" reaction (Hammes & Schimmel, 1970) which would be observed if binding of the substrate to the enzyme were effectively irreversible ($k_3 > k_2$).

Second-order rate constants $k_{cat}/K_m = k_3/K_d$ for β-galactosidase-catalyzed hydrolysis of **1**-OR are given in Table 1.

DISCUSSION

Alkyl β-D-Galactopyranosides: Reactivity and Rate-Determining Step. The second-order rate constants k_{cat}/K_m ($=k_3/K_d$) for cleavage of **1**-OR (Table 1) are smaller than those for cleavage of **1**-OC_6H_4-4-NO_2. However, this is due partly or entirely to the tighter binding of **1**-OC_6H_4-4-NO_2, and in some cases enzyme-bound **1**-OR actually shows a significantly higher reactivity toward cleavage despite the much greater chemical reactivity of **1**-OC_6H_4-4-NO_2. For instance, similar values of k_{cat} are observed for cleavage of **1**-OC_6H_4-4-NO_2 and **1**-OCH_2CH_2OH in spite of the 8 unit difference in the pK_a of the 4-nitrophenol and 2-hydroxyethanol leaving groups, which greatly favors uncatalyzed cleavage of the former substrate in water. We conclude that aryl β-D-galactopyranosides are in fact relatively poor substrates for β-galactosidase, when the low basicity of the aryl oxide anion leaving group is taken into consideration, and that the enzyme provides a much larger acceleration of the hydrolysis of substrates with strongly basic alkoxide anion leaving groups. These data suggest that the enzyme provides much more effective electrophilic catalysis of glycoside hydrolysis when the leaving group is a strongly basic alkoxide ion.

The kinetic parameters for cleavage of **1**-OAr show poor Brønsted correlations with the pK_a of the phenol leaving group (Sinnott & Souchard, 1973). By contrast, the first-order rate constants k_3, and the second-order rate constants $k_{cat}/K_m = k_3/K_d$, for β-galactosidase-catalyzed cleavage of **1**-OR show fair logarithmic correlations with the pK_a of the alkyl alcohol leaving group (Figures 2 and 3, respectively), with slopes of $(\beta_{lg})_{k_3} = -0.49 \pm 0.13$ and $(\beta_{lg})_{k_{cat}/K_m} = -0.75 \pm 0.14$, respectively. These Brønsted correlations provide strong evidence that the rate of enzyme-catalyzed cleavage of **1**-OR is limited by cleavage of the glycosidic bond. It

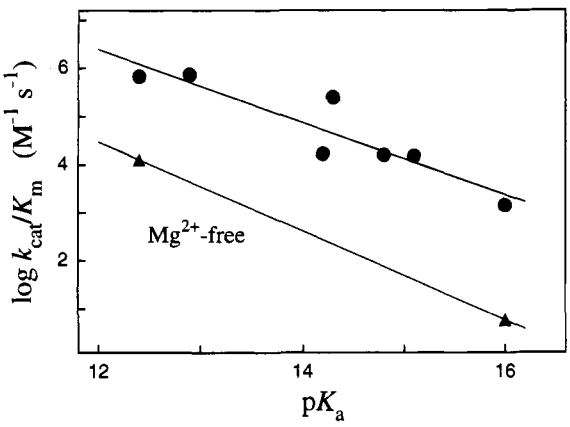


FIGURE 3: Brønsted correlation with slope $(\beta_{lg})_{k_{cat}/K_m} = -0.75 \pm 0.14$ of values of log k_{cat}/K_m for the β-galactosidase-catalyzed hydrolysis of **1**-OR. Key: (●) reaction in the presence of 1.0 mM Mg^{2+}; (▲) reaction of Mg^{2+}-free enzyme in the presence of 10 mM EDTA.

can shown by direct analysis that the chemical step for formation of the galactosyl–enzyme intermediate (k_3, Scheme 2) is slower than other steps that are potentially rate-determining for the enzyme-catalyzed reaction:

(1) The values of k_{cat} (s^{-1}, Table 1) for β-galactosidase-catalyzed cleavage of **1**-OR are at least 1.7-fold smaller than $k_s = 710$ s^{-1} (Scheme 2) for hydrolysis of the E-Gal reaction intermediate. Values of k_3 (Table 1) for the cleavage step can be readily calculated from the experimental data using eq 3.

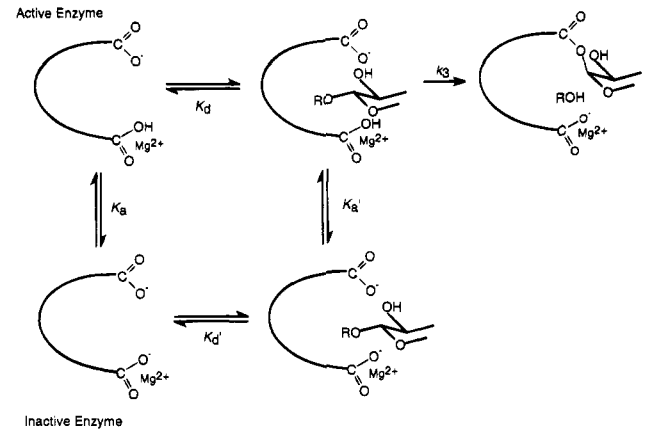
(2) Values of k_{cat}/K_m (M^{-1} s^{-1}) for cleavage of **1**-OR are not limited by the rate of substrate binding to the enzyme, because the largest value, $k_{cat}/K_m = 6.7 \times 10^5$ M^{-1} s^{-1} for reaction of **1**-OCH_2CF_3, is well below the limiting rate constants of $\sim 10^7$–10^9 M^{-1} s^{-1} expected for a "diffusion-limited" enzyme-catalyzed reaction (Hammes & Schimmel, 1970).

Binding Effects. The difference in $(\beta_{lg})_{k_3} = -0.49$ and $(\beta_{lg})_{k_{cat}/K_m} = -0.75$ for β-galactosidase catalysis of the cleavage of **1**-OR shows that there is a weak correlation between log K_d for substrate release and the pK_a of the alcohol leaving group with a slope of $(\beta_{lg})_{K_d} = 0.26 \pm 0.09$. There are at least two possible explanations for this correlation.

(1) $(\beta_{lg})_{K_d}$ may represent stabilization of an inactive, fully ionized, form of the enzyme by nonproductive electrostatic interactions between the enzyme and electron-withdrawing alkyl substituents at **1**-OR (Scheme 3).

These experiments were carried at pH 8.6, in the region where the pH–rate profiles for k_{cat}/K_m and k_{cat} for enzyme-catalyzed hydrolysis of **1**-OC_6H_4-4-NO_2 begin to show a downward break due to deprotonation of an essential acidic residue (Selwood & Sinnott, 1990).[1] This ionization will not affect the value of $(\beta_{lg})_{k_{cat}/K_m}$ for reaction of **1**-OR with the active form of free enzyme, because a decrease in the concentration of the active form of the enzyme will lead to the same proportional decrease in k_{cat}/K_m for cleavage of all

Scheme 3



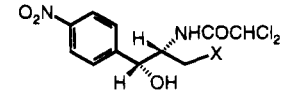
1-OR (Jencks, 1971). The observation that the ratios of k_{cat}/K_m for hydrolysis of **1**-OCH_2CH_3 and **1**-OCH_2CF_3 at pH 8.6 and 7.0 are identical, within experimental error, confirms that $(\beta_{lg})_{k_{cat}/K_m}$ is independent of pH.

We now consider the effect of stabilization of the inactive form of the enzyme by polar interactions between electron-withdrawing groups at the alkoxy side chain of **1**-OR and carboxylate anions (Jencks, 1971). These interactions will not affect k_{cat} at low pH, where the major form of the enzyme is fully active. A change in the leaving group at **1**-OR from strongly to weakly basic may cause a decrease in pK_a' (Scheme 3) if the interactions between the electron-withdrawing substituents and the dianionic form of the enzyme are significant. If so, then the inactive form of the enzyme would accumulate at progressively lower pH as the basicity of the leaving group is decreased, and this would cause a change to more positive values of $(\beta_{lg})_{k_3}$ as the pH is increased to pK_a and above. The observation that the ratios of k_{cat} for hydrolysis of **1**-OCH_2CH_3 and **1**-OCH_2CF_3 are the same, within experimental error, at pH 8.6 and 7.0 shows that $(\beta_{lg})_{k_3}$ does not change significantly over this pH range, and suggests that pK_a' (Scheme 3) is independent of the alkoxy group at **1**-OR.

(2) The following observations suggest that the correlation between log K_d and the pK_a of the alkyl alcohol leaving group at **1**-OR [$(\beta_{lg})_{K_d} = 0.26 \pm 0.09$] reflects the tendency of halo substituents, which decrease the basicity of the alkoxy anion leaving group, to also increase the hydrophobicity of the substrate and its affinity for the active site.

(a) There is a fair logarithmic correlation (not shown, $r = 0.84$) of dissociation constants (Table 1) for monosubstituted ethyl β-galactopyranosides (HO, F, H, Cl) and the Hansch π_2 hydrophobicity parameter for substitution at an alkyl chain.[2]

(b) There is good evidence that halomethyl groups at chloramphenicol derivatives **2** are hydrophobic compared to the methyl and hydroxymethyl group, and there is a strong



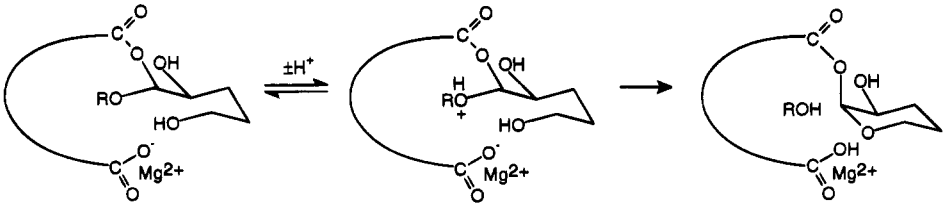
2 X = H, OH, F, Cl, Br, I

correlation between the hydrophobicities of **2** and their

[1] We wished to determine the kinetic parameters for galactosylation of β-galactosidase by alkyl β-D-galactopyranosides and for the reverse degalactosylation reaction by alkyl alcohols (Richard et al., 1995) under a single set of reaction conditions. Thus, these reactions were carried out at pH 8.6 because it was not practical to study the latter alkyl alcohol trapping reactions at pH 7.0.

[2] The π_2 parameters are taken from Table XIV of a review by Hansch and co-workers (Leo et al., 1971).

Scheme 4



affinity for chloramphenicol acetyl transferase (Cullis et al., 1991). A similar increase in the affinity of **1**-OR for β-galactosidase is expected upon addition of halogens to the alkoxy side chain, because the binding pocket of β-galactosidase is hydrophobic as evidenced by an enhanced affinity for aryl (K_m = 0.05−0.5 mM (Sinnott & Souchard, 1973)) compared with alkyl β-D-galactopyranosides (K_m = 0.5−5 mM, Table 1).

The binding interactions between β-galactosidase and the alkyl groups of **1**-OR may either be productive and expressed both at the Michaelis complex and the transition state for enzyme-catalyzed cleavage of **1**-OR, in which case they will lead to an increase in k_{cat}/K_m but will not affect k_3, or they may be nonproductive and expressed at the Michaelis complex but *not* at the transition state, in which case the requirement for loss of binding energy on moving from the Michaelis complex to transition state will cause k_3 to decrease but will not affect k_{cat}/K_m. The correlation between log K_d for reaction of **1**-OR and the pK_a of the alkyl leaving group (not shown) is very poor. Now, if the deviations of log K_d from this Brønsted correlation are caused mainly by nonproductive binding interactions, which are expressed only in the kinetic parameter k_3, then they should also lead to deviations from the Brønsted correlation for k_3 (Figure 2). However, productive interactions would be expressed in the kinetic parameter k_{cat}/K_m and would lead to deviations from this Brønsted correlation (Figure 3). In fact, significant deviations from both of these Brønsted correlations are observed, which suggests that the interactions between **1**-OR and β-galactosidase are both nonproductive and productive. We are unable to evaluate the effects of productive and nonproductive hydrophobic binding interactions on k_{cat}/K_m and k_3, respectively. Therefore, the difference in the values of $(\beta_{lg})_{k_3} = -0.49 \pm 0.13$ (Figure 2) and $(\beta_{lg})_{k_{cat}/K_m} = -0.75 \pm 0.14$ (Figure 3) represents the range of uncertainty in these electronic substituent effects.

1-OCH_2CH_2OH shows a relatively low affinity for β-galactosidase, as measured by K_d, and a high reactivity when bound to β-galactosidase, as measured by k_3, which shows a positive deviation from the Brønsted correlation (Figure 2). These data are consistent with weak binding of the hydrophilic hydroxyethyl side chain in the Michaelis complex, and the development of productive binding interactions to the 2′-OH group (which is also present in the physiological leaving group glucose) on moving to the transition state which are "utilized" to stabilize this transition state (Jencks, 1975).

Reaction Mechanism: Endocyclic vs Exocyclic C−O Bond Cleavage. It has been suggested that the experimental work on β-galactosidase does not rigorously exclude enzymatic catalysis of endocyclic cleavage of the sugar (Franck, 1992) and, in rebuttal, forcefully argued that this mechanism is needlessly convoluted and lacks experimental support (Sinnott, 1993). The increasing kinetic parameters for enzyme-catalyzed cleavage of aryl β-D-galactopyranosides with decreasing basicity of the leaving group is inconsistent with endocyclic cleavage of these substrates, but it might be that cleavage of glycosides with strongly basic alkoxide anion leaving groups follows this pathway (Franck, 1992). This mechanism can now be excluded for reaction of these substrates by the observation of large negative values of β_{lg} for β-galactosidase-catalyzed cleavage of alkyl β-D-galactopyranosides **1**-OR (Figures 2 and 3). The negative Brønsted coefficients are consistent with the development of negative charge at the alkoxy oxygen on moving to the transition state for exocyclic cleavage of the glycosidic bond (see below). They are inconsistent with endocyclic bond cleavage (Franck, 1992), because formation of the cyclic galactosyl−enzyme intermediate would then require nucleophilic displacement of the alkoxy group from an acylal (Scheme 4). Such a reaction is without precedent in solution, where displacement of the carboxylate ion is favored by its weaker basicity compared to an alkoxide ion leaving group and by the more effective stabilization of a carbenium ion by an alkoxy compared to an acyl oxy group. The reaction is also extremely unlikely at an enzyme-bound intermediate and would require electrophilic activation of the alkoxy leaving group if it were to occur at all (Scheme 4). This would lead to a net buildup of positive charge at the alkoxy oxygen in the transition state and positive values of $(\beta_{lg})_{k_3}$ and $(\beta_{lg})_{k_{cat}/K_m}$.

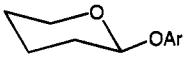
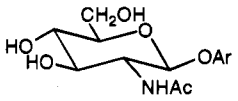
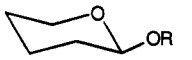
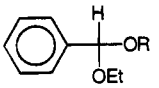
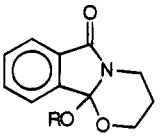
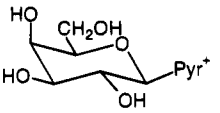
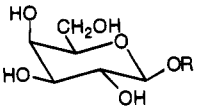
Interpretation of Structure−Reactivity Parameters. Structure−reactivity parameters β_{lg} for the nonenzymatic and β-galactosidase-catalyzed hydrolysis reactions of acetals are listed in Table 2. These data effectively exclude two mechanisms for the enzyme-catalyzed cleavage of the acetal substrate (Cordes & Bull, 1974).

(1) Unassisted cleavage of **1**-OR to form the galactosyl−enzyme intermediate and an alkoxide ion: This mechanism requires large negative values of $\beta_{lg} \leq -1.2$, such as have been observed for the spontaneous the cleavage of aryl acetals (Craze & Kirby, 1978; Piszkiewicz & Bruice, 1968) and galactosylpyridinium ions (Jones et al., 1977) (Table 2).

(2) Preequilibrium protonation of the leaving group, followed by cleavage of the galactopyranoside to form the galactosyl−enzyme intermediate and an alcohol leaving group: This mechanism requires small values of $\beta_{lg} \approx 0$, such as have been observed for the specific-acid-catalyzed cleavage of alkyl (Jensen et al., 1979) and aryl acetals (Jensen & Wuhrman, 1983).

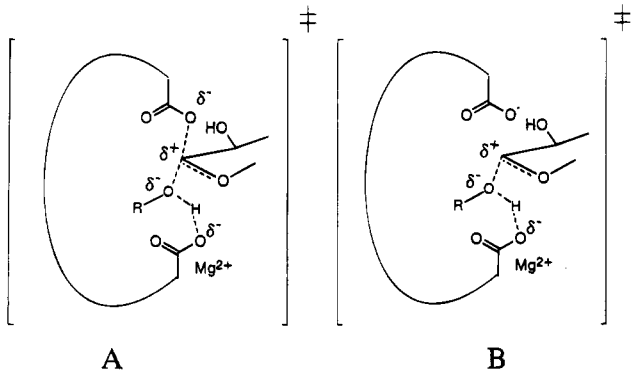
The values of β_{lg} for the β-galactosidase-catalyzed hydrolysis of **1**-OR lie between those observed for spontaneous cleavage reactions of acetals, in which there is no proton transfer to the leaving group, and the specific-acid-catalyzed cleavage reactions, in which there is full proton transfer to the leaving group. These data suggest that an electrophilic species at the enzyme catalyst functions to *partially* neutralize

Table 2: Values of β_{lg} for Spontaneous and Acid-Catalyzed Cleavage of Acetals

substrate	ref	leaving group	catalyst	β_{lg}
	*a*	substituted phenols	none	−1.28
	*b*	substituted phenols	none hydrogen ion	−1.23 0.05
	*c*	alkyl alcohols	hydrogen ion	−0.07
	*d*	alkyl alcohols	hydrogen ion cacodylic acid	0.31 −0.10
	*e*	alkyl alcohols	hydrogen ion acetic acid	0.24 −0.23
	*f*	substituted pyridines	none	−1.26
	*g*	alkyl alcohols	β-galactosidase	$(\beta_{lg})_{k_3} = -0.49$, $(\beta_{lg})_{k_{cat}/K_m} = -0.75$

[a] Calculated from the Hammett reaction constant $\rho^- = 2.7$ for the spontaneous hydrolysis reaction (Craze & Kirby, 1978) and $\rho^- = 2.11$ for the reference ionization reaction of substituted phenols (Jaffé, 1953). [b] Calculated from the Hammett reaction constants of $\rho^- = 2.6$ and $\rho^- = -0.11$ for the spontaneous and specific-acid-catalyzed hydrolysis reactions, respectively (Piszkiewicz & Bruice, 1968), and $\rho^- = 2.11$ for the reference ionization reaction of substituted phenols (Jaffé, 1953). [c] Jensen and Wuhrman (1983). [d] Jensen et al. (1979). [e] Gravitz and Jencks (1974). [f] Jones et al. (1977). [g] This work.

Chart 1



negative charge that develops at the leaving group as the reaction progresses to the transition state for glycoside cleavage.

An unambiguous interpretation of the Brønsted parameters determined in this work is not possible at the present time. Rather, these data will be discussed briefly within the context of several unresolved questions about the mechanism of action of β-galactosidase.

(1) Does the electrophilically assisted expulsion of the leaving group from **1**-OR proceed by a concerted A_ND_N (S_N2) (Guthrie & Jencks, 1989) mechanism with nucleophilic assistance by Glu-537 (Chart 1, transition state A) or by a stepwise $D_N + A_N$ (S_N1) mechanism, through an oxocarbenium ion reaction intermediate which is captured by Glu-537 in a subsequent step (Chart 1, transition state B)?

The stepwise mechanism is preferred here. The *solvolysis* reactions of glycosyl derivatives in water are thought to proceed by a stepwise mechanism (Banait & Jencks, 1991; Bennet & Sinnott, 1986) through glycosylpyranosyl oxocarbenium ion intermediates with lifetimes of $\sim 10^{-12}$ s (Amyes & Jencks, 1989). The transition state for hydrolysis of the acylal intermediate of β-galactosidase-catalyzed cleavage of glycosides ($k_s = 1300\ s^{-1}$) is stabilized relative to the reactant by more than 10 kcal/mol, compared with the transition state for hydrolysis of acylals in water ($k_{solv} = 4 \times 10^{-5}\ s^{-1}$ for spontaneous hydrolysis of β-D-glucopyranosyl benzoate at 78 °C) (Brown & Bruice, 1973). It is likely (but not proven) that at least part of this rate acceleration is due to specific stabilization of the oxocarbenium-like transition state (Sinnott & Souchard, 1973), which would also be expressed at an enzyme-bound oxocarbenium ion. Such stabilization of the intermediate of a stepwise reaction is not expected to cause a change to a concerted mechanism. Rather, changes from stepwise to concerted reaction mechanisms are observed upon *destabilization* of reaction intermediates, because the concerted mechanism provides an alternate pathway which avoids formation of highly unstable species (Richard & Jencks, 1984; Gandler & Jencks, 1982). The large azide ion selectivity of $k_{az}/k_s = 10^4\ M^{-1}$ for trapping of the galactosyl−enzyme intermediate of the E461G mutant form of β-galactosidase is consistent with formation of a relatively stable and highly selective oxocarbenium ion intermediate, and it is likely that an intermediate of equal or greater

stability is formed on the catalytic pathway for the native enzyme.[3]

(2) Is the electrophilic group that interacts with the leaving group in the reaction transition state a Mg^{2+} ion or an acidic residue at the enzyme or both?

There is evidence that Mg^{2+} participates in electrophilic catalysis of hydrolysis of acetals of galactose by direct chelation to the leaving group (Sinnott et al., 1978; Sinnott, 1990); however, Glu-461 is also essential for catalysis (Cupples et al., 1990) and may act as a general-acid catalyst to protonate the leaving group. The X-ray crystal structure of β-galactosidase (Jacobsen et al., 1994) shows that an enzyme-bound Mg^{2+} lies in close proximity to Glu-461, but it does not establish the catalytic roles of these moieties.

There is insufficient data to assign without ambiguity the residue or cofactor that plays the primary role in electrophilic catalysis at the leaving group. However, there are several observations that are most easily explained by a direct role for Glu-461 in general acid−base catalysis, and a secondary role for Mg^{2+} which has not yet been clearly defined.

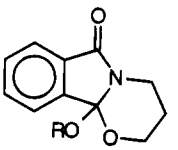
(a) Site-directed mutagenesis to replace Glu-461 by Gly-461 has a much larger effect on the catalytic activity than does removal of Mg^{2+}. For example, the Gly-461 for Glu-461 substitution leads to a 900-fold reduction in k_{cat} for β-galactosidase-catalyzed hydrolysis of **1**-OC_6H_4-4-NO_2 (Cupples et al., 1990), but removal of Mg^{2+} results in only a 5-fold reduction in this kinetic parameter (Table 1).

(b) Removal of Mg^{2+} leads to relatively small changes in $(\beta_{lg})_{k_3}$ and $(\beta_{lg})_{k_{cat}/K_m}$, from −0.49 to ∼−0.66 (Figure 2) and −0.75 to ∼−0.93 (Figure 3), respectively. This result is not easily rationalized by direct chelation of Mg^{2+} to the developing alkoxide ion in the reaction transition state, because removal of the chelated dication would be expected to result in a larger change in the "effective" negative charge (Hupe & Jencks, 1977) at the alkoxy oxygen than the ∼0.2 unit increases in negative charge observed here. For example, the addition of a proton to the transition state for the spontaneous cleavage of glycosides causes a ∼1.0 unit decrease in effective negative charge at the oxygen of the leaving group (Table 2). The magnitude of the charge−dipole interaction between Mg^{2+} and alkyl substituents is expected to show a $1/r^3$ dependence on the radius of separation of the two centers (Hine, 1975), so that the relatively small effect of Mg^{2+} on β_{lg} for cleavage of **1**-OR is consistent with a significantly larger separation between the enzyme-bound alkyl substituents and Mg^{2+} than between these substituents and a proton covalently bound to the alkyl oxygen in the specific-acid-catalyzed reaction.

The release of Mg^{2+} leads to the uptake of at least one proton by β-galactosidase, presumably by protonation of an amino acid side chain which functions as a ligand for the metal ion (Tenu et al., 1972). The small effect of removal of Mg^{2+} on β_{lg} might therefore reflect neutralization of developing negative charge at the leaving group by proton transfer from a side chain that is protonated upon release of Mg^{2+}.

The values of $(\beta_{lg})_{k_3} \approx -0.66$ (Figure 2) and $(\beta_{lg})_{k_{cat}/K_m} \approx -0.93$ (Figure 3) for reaction in the absence of Mg^{2+} are larger (more positive) than $\beta_{lg} = -1.2$ for the spontaneous cleavage of aryl pyranosides (Craze & Kirby, 1978; Piszkiewicz & Bruice, 1968). This is consistent with partial neutralization of developing negative charge at the leaving group by an interaction with a catalytic residue at the enzyme. Insertion of a Mg^{2+} close to Glu-461 (Chart 1, B) would cause an increase in the acidity of this group and a shift in the position of the transition state toward greater proton transfer to the leaving group, which would be reflected in a change to a more positive value of $(\beta_{lg})_{k_3}$ (Jensen et al., 1979; Capon & Nimmo, 1975; Ta-Shma & Jencks, 1986; Jencks & Jencks, 1977), as is observed here.

(3) If an acidic residue at the enzyme is acting to provide general-acid catalysis of the cleavage of **1**-OR, then (a) why is such catalysis observed for the enzymatic reaction, but not for the hydrolysis of glycosides in water (Fife, 1975) and (b) why are the values of β_{lg} for the β-galactosidase-catalyzed reaction significantly smaller (more negative) than $\beta_{lg} = -0.10$ for the cleavage of substituted benzaldehyde mixed acetals catalyzed by cacodylic acid (Jensen et al., 1979), or $\beta_{lg} = -0.23$ for cleavage of **3** catalyzed by acetic acid (Gravitz & Jencks, 1974) (Table 2)?



3

Studies of the nonenzymatic cleavage of acetals show that the observation of general-acid catalysis of cleavage of acetals is favored by the following (Fife, 1975): (i) stabilization of an oxocarbenium ion reaction intermediate relative to substrate; (ii) a decrease in the pK_a of the leaving group. Once general-acid catalysis *is* observed, then further increases in the stability of the oxocarbenium ion reaction intermediate are accompanied by a shift to more negative values of β_{lg} (Capon & Nimmo, 1975; Jensen et al., 1979; Ta-Shma & Jencks, 1986). The failure to observe general-acid catalysis of the cleavage of glycosides in water is best rationalized by a consideration of the effect of the conjugate base of the acid catalyst on the reverse reaction for addition of an alcohol to a highly unstable glycosyl oxocarbenium ion intermediate (Jencks, 1976). If there is no significant barrier to the addition of alkyl alcohols to a glycosyl oxocarbenium ion (Young & Jencks, 1977; Amyes & Jencks, 1989), then the barrier cannot be reduced by partial proton transfer to a base catalyst, so that catalysis of the reaction of the oxocarbenium ion and, by microscopic reversibility, catalysis of its formation from alkyl glycosides will be negligible. A barrier may be created by structural changes which decrease the reactivity of the oxocarbenium ion or the nucleophile. Once this barrier appears, it may by lowered by partial deprotonation of the nucleophile in a general-base-catalyzed reaction. Therefore, interactions between the enzyme and the substrate that increase the barrier to the addition of nucleophiles to the bound oxocarbenium ion intermediate, such as specific

[3] The ratio $k_{az}/k_s = 9 \times 10^3$ M^{-1} was calculated from a value of $k_s = 0.8$ s^{-1} for the E461G mutant (Cupples et al., 1990) and $k_{az} = 7500$ M^{-1} s^{-1}. The value of k_{az} 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 2) for reactions of 2-nitrophenyl β-D-galactopyranoside with the E461G mutant enzyme. This assumption is justified by the observation of a 17-fold increase in the velocity of hydrolysis of this substrate in the presence of increasing concentrations of azide ion (Huber & Chivers, 1993).

stabilization of this intermediate, will favor the observation of general-acid catalysis. Large negative values of β_{lg} are favored by strong stabilization of the oxocarbenium intermediate or by use of a weakly acidic catalytic acid residue (Capon & Nimmo, 1975; Jensen et al., 1979; Ta-Shma & Jencks, 1986).

(4) What is the change in effective charge at the alkyl alcohol leaving group on proceeding from **1**-OR in solution to the transition state for β-galactosidase-catalyzed hydrolysis of **1**-OR?

The structure−reactivity parameter β_{lg} provides a measure of the change in "effective charge" at the leaving group on moving from neutral reactants to the reaction transition state (Hupe & Jencks, 1977). The values of β_{lg} for β-galactosidase-catalyzed hydrolysis of **1**-OR determined in this work could be used to assign a formal transition state charge to the leaving group oxygen, if the "effective charges" at the alkoxy oxygen of the acetal **1**-OR and the alcohol **H**-OR are assumed to be equal. We have avoided this assumption and will show in the following paper that the hydrolysis reactions of **1**-OR behave as though the effective positive charge at the leaving group oxygen of **1**-OR is significantly larger than that at the oxygen of **H**-OR (Richard et al., 1995).

CONCLUSIONS

β-Galactosidase is an efficient catalyst of the cleavage of the glycosidic bonds to the strongly basic alkoxide leaving groups of alkyl β-galactopyranosides **1**-OR. Bond cleavage is partly or fully rate-determining for all of these enzyme-catalyzed hydrolysis reactions, and the kinetic parameters k_{cat} and k_{cat}/K_m increase relatively sharply with decreasing pK_a of the alkyl alcohol leaving group. Enzyme-catalyzed cleavage of **1**-OR does not follow an uncatalyzed or a specific-acid-catalyzed reaction pathway, as evidenced by Brønsted coefficients $(\beta_{lg})_{k_3}$ and $(\beta_{lg})_{k_{cat}/K_m}$ for the enzyme-catalyzed reaction that are different from those observed for these pathways in water (Table 2). The Brønsted correlations are consistent with concerted general-acid catalysis of leaving group expulsion by the enzyme but are insufficient to fully define the structure of the transition state for the enzyme-catalyzed reaction. This will require further experiments to determine the extent of cleavage of the C−O glycosidic bond in the transition state (*e.g.*, secondary α-deuterium isotope effects) and the extent of proton transfer to the leaving group (*e.g.*, solvent deuterium isotope effects).

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