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Solvent Deuterium Isotope Effect on the Binding of β-D-Galactopyranosyl Derivatives to β-Galactosidase (*Escherichia coli, lac Z*)[1]

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A value of 1.8 has been determined for $(K_I)_{HOH}/(K_I)_{DOD}$, the ratio of the values of K_I for competitive inhibition of β-galactosidase by isopropyl β-D-thiogalactopyranoside in H_2O and D_2O. This is similar to the value of 1.7 for $(K_m)_{HOH}/(K_m)_{DOD}$, the ratio of the Michaelis constants determined for the β-galactosidase-catalyzed hydrolysis of 4-nitrophenyl β-D-galactopyranoside (Gal-OPNP) in H_2O and D_2O. The similarity of these solvent deuterium isotope effects suggests that the observed isotope effect on K_m corresponds, mainly, to the isotope effect on the dissociation constant K_d for Gal-OPNP. The implications of these results for the interpretation of the solvent deuterium isotope effects on k_{cat} and k_{cat}/K_m for β-galactosidase-catalyzed hydrolysis of Gal-OPNP is discussed.

INTRODUCTION

We would like to understand the mechanistic implications of the following kinetic isotope effects that have been reported in the literature for the β-galactosidase-catalyzed hydrolysis of 4-nitrophenyl β-D-galactopyranoside (Table 1) (*1–3*).

(1) The small and essentially identical α-deuterium kinetic isotope effects on k_{cat}/K_m and k_{cat} (Table 1) (*1,2*), which require that the changes in hybridization at the α-carbon of the substrate on proceeding to the rate-limiting transition states for k_{cat}/K_m and k_{cat} be essentially the same.

(2) The ^{18}O-leaving group isotope effects on k_{cat}/K_m and k_{cat}. Significant isotope effects are observed on each of these kinetic parameters, consistent with significant changes in bonding at oxygen on proceeding to the respective rate-determining transition states (*3*), but the difference in these isotope effects is consistent with somewhat greater changes in bonding at the rate-determining transition state for k_{cat} compared with that for k_{cat}/K_m.

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TABLE 1

Kinetic Isotope Effects on the Hydrolysis of 4-Nitrophenyl β-D-Galactopyranoside Catalyzed by β-Galactosidase

Isotope effect[a]	Kinetic parameter	
	k_{cat}/K	k_{cat}
α-Deuterium isotope effect[b]	1.07 ± 0.03[c]	1.04 ± 0.02[d]
^{18}O-Leaving group isotope effect[e]	1.014 ± 0.003	1.022 ± 0.002
Solvent deuterium isotope effect[f]	1	1.7

[a] The ratio of the kinetic parameters for reactions with the substrate Gal-OPNP, or the reaction medium, labeled with the light and heavy isotopes.

[b] At pH 7.0 for reactions of Gal-OPNP labeled with hydrogen and deuterium at the anomeric carbon.

[c] Data from Ref. 1.

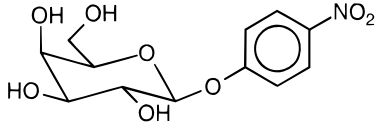
[d] Data from Ref. 2.

[e] At pH 7.0 for reactions of Gal-OPNP labeled with ^{16}O and ^{18}O at the leaving-group oxygen (*3*).

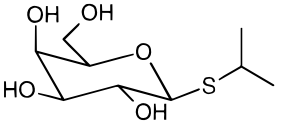
[f] For reactions of unlabeled Gal-OPNP in H_2O and D_2O (*2*). The values of the kinetic parameters used to calculate these isotope effects were determined at the pL maximum for the respective reactions.

(3) The contrasting large difference between the values of 1.0 and 1.7, respectively, for the solvent deuterium isotope effects on k_{cat}/K_m and k_{cat} (*2*). This, nominally, is consistent with very different degrees of cleavage of the bond to a solvent-derived hydron at the transition state for k_{cat} (significant bond cleavage) and k_{cat}/K_m (little bond cleavage). However, it is not easily reconciled with the much smaller differences observed for the α-deuterium isotope effects and the ^{18}O-leaving group isotope effects on k_{cat}/K_m and k_{cat} (Table 1).

What has not been considered when interpreting the solvent deuterium isotope effects is the possibility that the rate-limiting transition states for k_{cat} and k_{cat}/K_m *are* similar with respect to extent of transfer of a solvent-derived hydron to the oxygen leaving group, but that the difference in these isotope effects reflects a solvent isotope effect on the dissociation constant for the substrate Gal-OPNP. We report here that the solvent deuterium isotope effect on the inhibition constant K_I for the competitive inhibitor isopropyl β-D-thiogalactopyranoside (Gal-SIP) is similar to the solvent deuterium isotope effect on K_m for Gal-OPNP. This result provides a precedent for a solvent deuterium isotope effect on the affinity of sugar derivatives for β-galactosidase, and it suggests that the difference in the solvent isotope effects on k_{cat} and k_{cat}/K_m reported in Table 1 is due largely to a solvent isotope effect on substrate binding. Therefore, the difference in the extent of cleavage of the bond to a solvent-derived hydron on proceeding to the respective rate-determining transition states for k_{cat} and



Gal-OPNP



Gal-SIP

k_{cat}/K_m is much smaller than that suggested by the difference in the observed solvent deuterium isotope effects.

MATERIALS AND METHODS

Materials. Reagent grade organic and inorganic chemicals were obtained from commercial sources and were used without further purification. Water was distilled and passed through a Milli-Q water purification system. 4-Nitrophenyl β-D-galactopyranoside (Gal-OPNP), isopropyl β-D-thiogalactopyranoside (Gal-SIP), and β-galactosidase (Grade VIII from *Escherichia coli.*) were purchased from Sigma. Deuterium oxide (99.9%) was purchased from Cambridge Isotope Laboratories. Solution pH was determined using an Orion Model 601A pH meter equipped with a Radiometer GK2321C combination electrode that was standardized at pH 7.00 and 10.00. Values of pD were obtained by adding 0.40 to the observed pH meter reading (*4*).

The concentration of isopropyl β-D-thiogalactopyranoside (Gal-SIP) in stock solutions in H_2O or D_2O was determined as the concentration of D-galactose that is formed upon quantitative hydrolysis of the sugar derivative. Gal-SIP was hydrolyzed for six hours at 100°C in a sealed vial that contained concentrated HCl or DCl. The solution was then neutralized with NaOH or NaOD and a measured aliquot added to a cuvette that contained 0.90 mM NAD^+ in sodium pyrophosphate buffer at pH 8.6. The concentration of D-galactose was determined from the change in absorbance at 340 nm observed upon addition of galactose dehydrogenase ($\Delta\varepsilon = 6200\ M^{-1}\ cm^{-1}$). There was good agreement (±5%) between the concentrations of Gal-SIP determined by this method and those calculated from the weighed mass of Gal-SIP in the stock solutions.

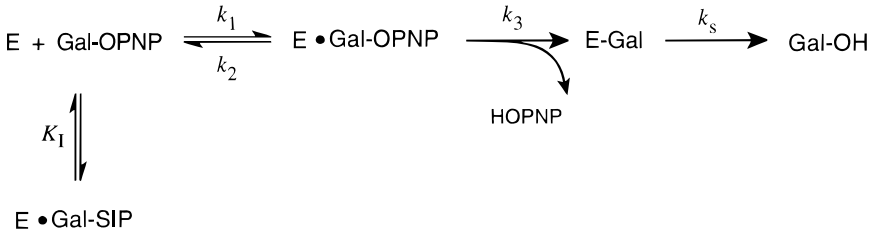
Enzyme assays. Enzyme assays were carried out at 25°C in 33 mM sodium phosphate at pH 7.0 containing 1 mM $MgCl_2$ and 130 mM NaCl; or in 33 mM sodium phosphate at pD 7.4 containing 1 mM $MgCl_2$ and 130 mM NaCl. The enzyme-catalyzed hydrolysis of 4-nitrophenyl β-D-galactopyranoside at pH 7.0 and at pD 7.4 was monitored by following the increase in absorbance at 405 nm. The initial velocities for the enzyme-catalyzed reaction were calculated using values of 8900 and 7200 $M^{-1}\ cm^{-1}$ for the difference in extinction coefficients ($\Delta\varepsilon_{405}$) of Gal-OPNP and 4-nitrophenol in H_2O and D_2O, respectively, that was determined for the complete hydrolysis of a known concentration of this substrate in the respective buffers (*5*).

$$v = \frac{V_{max}[S]}{(K_m)_{app} + [S]} \qquad [1]$$

Values of $(K_m)_{app}$ and V_{max} for the β-galactosidase-catalyzed hydrolysis of Gal-OPNP in the presence of increasing fixed concentrations of the competitive inhibitor Gal-SIP were determined from the nonlinear least squares fit to Eq. [1] of the initial velocities determined for 10–12 different concentrations of Gal-OPNP using SigmaPlot from Jandel Scientific. The standard deviation for the kinetic parameters obtained from this fitting procedure is better than ±5%.

RESULTS

Values of $k_{cat} = 160\ s^{-1}$ and $K_m = 30\ \mu M$ were determined in H_2O at pH 7.0 and values of $k_{cat} = 89\ s^{-1}$ and $K_m = 18\ \mu M$ were determined in D_2O at pD 7.4 for β-galactosidase-



SCHEME 1

catalyzed cleavage of Gal-OPNP. These kinetic parameters and the derived solvent deuterium isotope effects of $(k_{cat})_{HOH}/(k_{cat})_{DOD} = 1.8$ and $(k_{cat}/K_m)_{HOH}/(k_{cat}/K_m)_{DOD} = 1.1$ are in good agreement with earlier literature values (*1,2*). Isopropyl β-D-thiogalactopyranoside (Gal-SIP) behaves as a classic competitive inhibitor of β-galactosidase (Scheme 1) and causes an increase in the value of the apparent Michaelis constant $(K_m)_{app}$ for enzyme-catalyzed hydrolysis of Gal-OPNP, but no change in the value of V_{max} for the reaction catalyzed by a constant concentration of enzyme.

Figure 1 shows the effect of increasing concentrations of Gal-SIP on $(K_m)_{app}/(K_m)_o$ determined for β-galactosidase-catalyzed hydrolysis of Gal-OPNP in the presence of

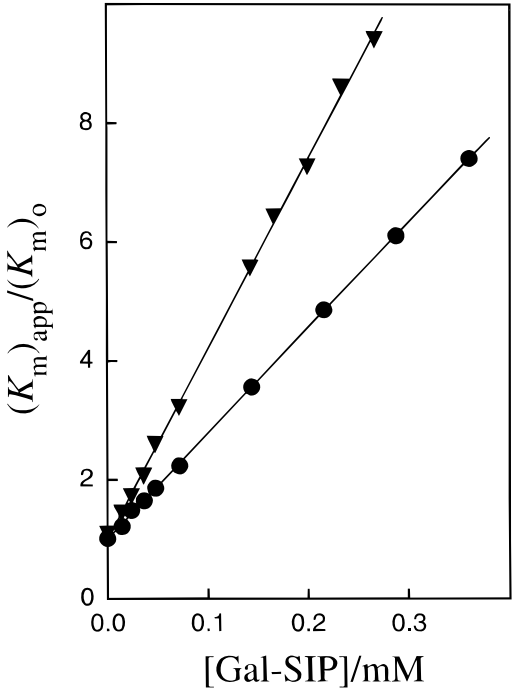


FIG. 1. The dependence of $(K_m)_{app}/(K_m)_o$ for the hydrolysis of Gal-OPNP catalyzed by β-galactosidase on the concentration of β-D-thiogalactopyranoside Gal-SIP in H_2O at pH 7.0 (33 mM sodium phosphate, ●) and in D_2O at pD 7.4 (33 mM sodium phosphate, ▼) at 25°C.