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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.

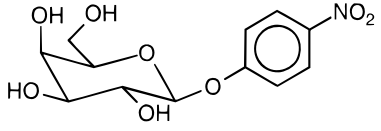
[d] Data from Ref. 2.

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

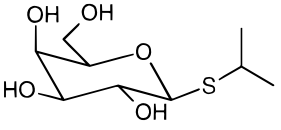
[f] For reactions of unlabeled Gal-OPNP in H_2O and D_2O (*2*). The values of the kinetic parameters used to calculate these isotope effects were determined at the pL maximum for the respective reactions.

(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.

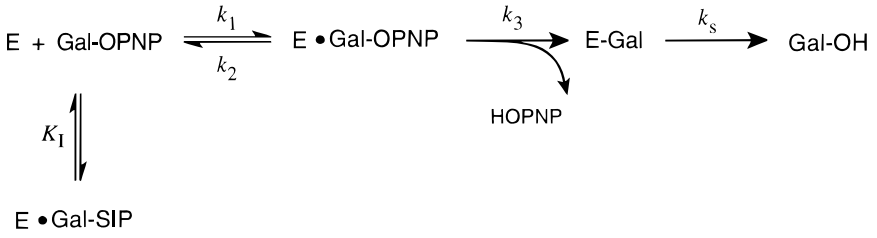
Enzyme assays. Enzyme assays were carried out at 25°C in 33 mM sodium phosphate at pH 7.0 containing 1 mM $MgCl_2$ and 130 mM NaCl; or in 33 mM sodium phosphate at pD 7.4 containing 1 mM $MgCl_2$ and 130 mM NaCl. The enzyme-catalyzed hydrolysis of 4-nitrophenyl β-D-galactopyranoside at pH 7.0 and at 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

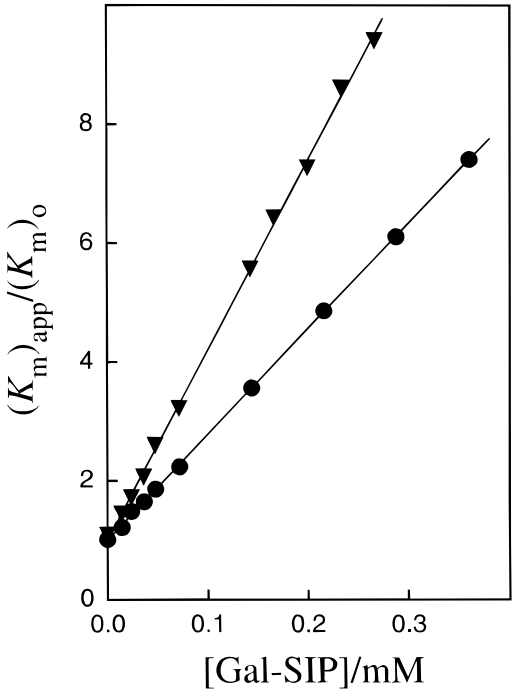


FIG. 1. The dependence of $(K_m)_{app}/(K_m)_o$ for the hydrolysis of Gal-OPNP catalyzed by β-galactosidase on the concentration of β-D-thiogalactopyranoside Gal-SIP in H_2O at pH 7.0 (33 mM sodium phosphate, ●) and in D_2O at pD 7.4 (33 mM sodium phosphate, ▼) at 25°C.

increasing concentrations of Gal-SIP in H_2O at pH 7.0 and in D_2O at pD 7.4 at 25°C, where $(K_m)_{app}$ is the value of the Michaelis constant determined at a given inhibitor concentration and $(K_m)_o$ is the Michaelis constant determined at [Gal-SIP] = 0 in H_2O or D_2O. The values of the pH and pD for these determinations are close to the pL maximum for k_{cat}/K_m determined for the β-galactosidase-catalyzed hydrolysis of Gal-OPNP. The data in Fig. 1 show a good fit to Eq. [2], which was derived for Scheme 1. The slope of the correlations for reaction in H_2O and D_2O, respectively, give the inhibition constants $(K_I)_{HOH} = 56 \pm 1$ mM and $(K_I)_{DOD} = 31 \pm 2$ mM for Gal-SIP. The parameter determined in H_2O is in fair agreement with the literature value of $K_I = 85$ mM for Gal-SIP (*6*). Combining these data for inhibition in H_2O and D_2O gives a value of $(K_I)_{HOH}/(K_I)_{DOD} = 1.8$ for the solvent deuterium isotope effect on K_I for Gal-SIP. The solvent deuterium isotope effect $(K_m)_{HOH}/(K_m)_{DOD} = 1.7$ for Gal-OPNP is in excellent agreement with the value that can be calculated from the difference in the solvent deuterium isotope effects on k_{cat} and k_{cat}/K_m (Table 1) (*2*).

$$(K_m)_{app}/(K_m)_o = 1 + \left(\frac{1}{K_I}\right)[\text{Gal - SIP}] \qquad [2]$$

DISCUSSION

A solvent isotope effect of $(K_I)_{HOH}/(K_I)_{DOD} = 1.8$ (Fig. 1 and Results) has been determined for inhibition of β-galactosidase by Gal-SIP at 25°C, where the inhibition constant K_I (M) is equal to the dissociation constant K_d for Gal-SIP (Scheme 1). This solvent deuterium isotope effect corresponds to a 0.35 kcal/mol *more favorable* binding of Gal-SIP to β-galactosidase in D_2O than in H_2O. By comparison, the binding of methyl α-D-mannopyranoside to the lectin concanavalin A at 25°C is 0.3 kcal/mol more favorable in D_2O than in H_2O (*7*). Solvent deuterium isotope effects of this type have been treated by considering the changes in solute-solvent interactions which occur on proceeding from the protein and free ligand to the protein-ligand complex (*7*,*8*). There is evidence that these changes are the result of differences in hydrogen-bond strength in H_2O and D_2O (*9*). However, there is no justification for discussing our limited data within the context of changes in solute–solvent interactions, which occur upon the binding of substrate or inhibitors to β-galactosidase.

There are two possible explanations for the difference in the values of 1.7 and ≈ 1 for the solvent deuterium isotope effects on k_{cat} and k_{cat}/K_m, respectively, for the β-galactosidase-catalyzed hydrolysis of Gal-OPNP (Table 1) (*2*). The isotope effect of $(K_m)_{HOH}/(K_m)_{DOD} = 1.7$ is equal to the isotope effect on the dissociation constant K_d for Gal-OPNP when $K_d \approx K_m$ for the enzyme-catalyzed reaction. Such effects on K_d are more likely to result from isotopic substitution at the aqueous reaction medium than from a single isotopic substitution at the substrate. Alternatively, there may be no significant solvent deuterium isotope effect on K_d. In this case, the conversion of the initial Michaelis complex to products must be much faster than the dissociation of substrate from this complex ($k_3 >> k_2$, Scheme 1) so that the observed isotope effect on K_m is equal to the isotope effect on the ratio k_3/k_1. The result is that the isotope effects on k_{cat} and K_m will cancel in k_{cat}/K_m provided there is no significant solvent isotope effect on the substrate binding step k_1.[3]

The following experimental observations provide strong support for the conclusion that the solvent deuterium isotope of 1.7 on K_m for β-galactosidase-catalyzed hydrolysis of Gal-OPNP is due mainly to a solvent isotope effect on the dissociation constant K_d for this substrate.

(1) There is no significant difference in the α-deuterium isotope effects on k_{cat}/K_m and k_{cat} for β-galactosidase-catalyzed hydrolysis of Gal-OPNP (Table 1), and no more than a small difference in the ^{18}O-leaving group isotope effect on these kinetic parameters (*3*). The observation that there is little difference in the transition states for k_{cat}/K_m and k_{cat} with respect to rehybridization of the α-carbon and bond cleavage to the 4-nitrophenoxy leaving group is difficult to reconcile with a large difference in these transition states with respect to proton transfer to the leaving group.

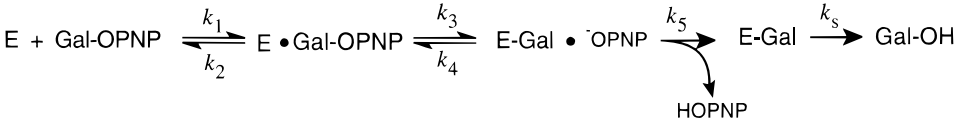
(2) A value of 1.8 was determined for $(K_I)_{HOH}/(K_I)_{DOD}$ for competitive inhibition of β-galactosidase by Gal-SIP in H_2O and D_2O, where the inhibition constant K_I is equal to the dissociation constant K_d for Gal-SIP. This is similar to $(K_m)_{HOH}/(K_m)_{DOD}$ = (30 μM)/(18 μM) = 1.7 (Results) determined for enzyme-catalyzed cleavage of Gal-OPNP at the same values of pL, which suggests that this is the solvent isotope effect on the dissociation constant for Gal-OPNP.

(3) A second explanation for any difference in the isotope effects on k_{cat} and k_{cat}/K_m is that the former kinetic parameter is limited by the rate constant k_3 for chemical bond cleavage, but that the latter parameter is limited by k_1 for formation of the Michaelis complex by diffusional encounter between enzyme and substrate (*2*).[3] In this case, the value of 5.6×10^6 M^{-1} s^{-1} for k_{cat}/K_m for β-galactosidase catalyzed cleavage of Gal-OPNP (*1*) would be essentially equal to k_1. However, this would be a rather small value for a rate constant for formation of a Michaelis complex (*10*). Furthermore, if the value of k_{cat}/K_m for β-galactosidase-catalyzed hydrolysis of Gal-OPNP were limited by k_1 for formation of the Michaelis complex, then the value of $K_m = 28$ $\mu M = k_3/k_1$ for this substrate (*1*) would be larger than the dissociation constant $K_d = k_2/k_1$ ($k_3 > k_2$ for rate-determining k_1); and, the value for K_m would be expected to decrease to that for K_d as the rate-determining step for k_{cat}/K_m changes from k_1 to k_3 for cleavage of less reactive aryl β-D-galactopyranosides. By contrast, the values of K_m increase to 35 and 43 μM, respectively, for enzyme-catalyzed hydrolysis of 4-cyanophenyl β-D-galactopyranoside ($k_{cat}/K_m = 1.7 \times 10^6$ M^{-1} s^{-1}) and 4-bromophenyl β-D-galactopyranoside ($k_{cat}/K_m = 7 \times 10^5$ M^{-1} s^{-1}), which are less reactive than Gal-OPNP ($k_{cat}/K_m = 5.6 \times 10^6$ M^{-1} s^{-1}) toward cleavage catalyzed by β-galactosidase (*1*).

In conclusion, the simplest explanation for the difference in the solvent deuterium isotope effects on k_{cat} and k_{cat}/K_m for β-galactosidase-catalyzed hydrolysis of Gal-OPNP (Table 1) is that this reflects, mainly, the solvent deuterium isotope effect on the dissociation constant for this substrate, which is similar to that determined here for the dissociation of Gal-SIP.

Mechanistic implications. The results reported here provide evidence against the proposal that there is a large difference in the degree of cleavage of the bond to a solvent-derived

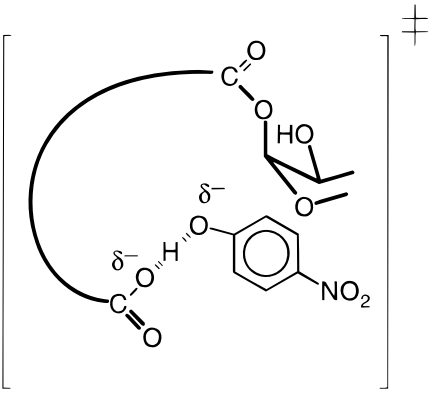
[3] The value of 1300 s^{-1} for k_s for addition of solvent to the galactosyl-enzyme intermediate (E-Gal) of β-galactosidase catalyzed hydrolysis of Gal-OPNP is much larger than the value of 156 s^{-1} for k_{cat} for this substrate (*1*). This requires that conversion of the Michaelis complex to the β-D-galactopyranosylated enzyme (k_3, Scheme 1) be rate-determining for k_{cat}.



SCHEME 2

hydron at the transition state for k_{cat} and k_{cat}/K_m (*2*), and suggest that the rate-determining transition states for these two processes with respect to proton transfer from solvent are similar. The *small* difference in the ^{18}O-leaving group effects on these kinetic parameters (Table 1) (*3*) may reflect the partly rate-determining nature of substrate binding on k_{cat}/K_m or a *"subtle methodological artifact"* resulting from the different experimental protocols for determination of the isotope effects on these kinetic parameters (*3*).

Selwood and Sinnott have proposed that the *β*-galactosidase-catalyzed hydrolysis of Gal-OPNP proceeds by stepwise cleavage of the bond to the 4-nitrophenoxy leaving group to form a galactosylated-enzyme-4-nitrophenolate complex (k_3, Scheme 2), followed by protonation of the 4-nitrophenolate ion (k_5, Scheme 2) by Glu-461 (*11*), which is proposed to be rate-limiting for k_{cat} (*2*). The rate-limiting transition state for k_5 (see **2**) can account for the following experimental observations: (a) The normal solvent deuterium isotope effect on k_{cat}, since proton transfer to the leaving group is rate determining. (b) The small α-deuterium isotope effect on k_{cat} for hydrolysis of Gal-OPNP (Table 1), since the hybridization at the α-carbon of the E-Gal intermediate is similar to that at substrate. (c) The ^{18}O-leaving group effect on k_{cat}, which would reflect the significant changes in the bonding to the leaving group on proceeding from Gal-OPNP to the transition state **2**.[4] Selwood and Sinnott have proposed that proton transfer in the putative rate-limiting step (k_5, Scheme 2) is to a Mg^{2+}-



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[4] We view a proposed concerted bimolecular displacement mechanism for *β*-galactosidase-catalyzed hydrolysis of Gal-OPNP as unlikely (*3*). There is good evidence that the reactions of nucleophiles with the *β*-D-galactopyranosylated form of the E461G mutant enzyme proceeds through a glycosyl cation intermediate, which is strongly stabilized by interactions with the enzyme (*15*). Such interactions increase the depth of the energy well for a reaction intermediate and will strongly favor stepwise reactions, which proceed through the intermediate, relative to concerted reactions which avoid formation of the oxocarbenium ion intermediate (*16,17*).

4-nitrophenolate chelate (*2*). However, this is not required by the experimental results, and it is possible that the 4-nitrophenolate ion is stabilized by interactions with hydrogen bond donors at the enzyme active site.

These kinetic isotope effect studies do not provide a unique description of the rate-determining transition state for β-galactosidase-catalyzed hydrolysis of Gal-OPNP, because it is not clear whether the small α-deuterium isotope effects on k_{cat} and k_{cat}/K_m rigorously exclude a mechanism in which cleavage of the C-OPNP bond is concerted with proton transfer to the leaving group. This concerted reaction mechanism is strongly favored for catalysis of cleavage of the physiological substrate lactose and other alkyl β-D-galactopyranosides (*5,12*) because of the large thermodynamic driving force for proton transfer from the acidic catalyst to the strongly basic alkoxide ion leaving group of the spontaneous reaction (*13,14*). However, the kinetic isotope effects on the β-galactosidase-catalyzed hydrolysis of these substrates have not been determined.

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