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Solvent and α-secondary kinetic isotope effects on β-glucosidase ☆

Miaomiao Xie, Larry D. Byers *

Department of Chemistry, Tulane University, New Orleans, LA 70118, USA

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ABSTRACT

β-Glucosidase from sweet almond is a retaining, family 1, glycohydrolase. It is known that glycosylation of the enzyme by aryl glucosides occurs with little, if any, acid catalysis. For this reaction both the solvent and α-secondary kinetic isotope effects are 1.0. However, for the deglucosylation reaction (e.g., k_{cat} for 2,4-dinitrophenyl-β-*D*-glucopyranoside) there is a small solvent deuterium isotope effect of 1.50 (±0.06) and an α-secondary kinetic isotope effect of 1.12 (±0.03). For aryl glucosides, k_{cat}/K_M is very sensitive to the pK_a of the phenol leaving group [$\beta_{lg} \approx -1$; Dale et al., *Biochemistry* **25** (1986) 2522–2529]. With alkyl glucosides the β_{lg} is smaller (between −0.2 and −0.3) but still negative. This, coupled with the small solvent isotope effect on the pH-independent second-order rate constant for the glucosylation of the enzyme with 2,2,2-trifluoroethyl-β-glucoside [$^{D2O}(k_{cat}/K_M) = 1.23$ (±0.04)] suggests that there is more glycone–aglycone bond fission than aglycone oxygen protonation in the transition state for alkyl glycoside hydrolysis. The kinetics constants for the partitioning (between water and various alcohols) of the glucosyl-enzyme intermediate, coupled with the rate constants for the forward (hydrolysis) reaction provide an estimate of the stability of the glucosyl-enzyme intermediate. This is a relatively stable species with an energy about 2 to 4 kcal/mol higher than that of the ES complex. This article is part of a Special Issue entitled: Enzyme Transition States from Theory and Experiment.

1. Introduction

β-glucosidase (EC 3.2.1.21) from sweet almonds is a retaining glycohydrolase [1] and a member of the GH1 family of sequence-related glycosyl hydrolases [2]. The enzyme catalyzes the highly efficient hydrolysis of a variety of aryl and alkyl glycosides [3] *via* a double displacement mechanism (see Scheme 1).

The reaction utilizes two carboxylic (glutamic) acids with one of these functioning as a nucleophile leading to a glycosyl-enzyme intermediate (an acylal) and the other as a BrØnsted acid catalyst in the formation, and as a base catalyst in the breakdown, of this intermediate [4]. The mechanism of action of this enzyme has been extensively investigated and has revealed some intriguing features. Sweet almond β-glucosidase was one of the first enzymes examined for a secondary kinetic isotope effect. Dahlquist et al. [5] found an α-secondary deuterium KIE of $^{D}(k_{cat}/K_M) = 1.01$ for the enzyme-catalyzed hydrolysis of phenyl β-glucoside. This is significantly less than the value expected for a S_N1 ($D_N + A_N$) mechanism, or for that found for the acid-catalyzed hydrolysis ($^{D}k = 1.13$) of this substrate or for the lysozyme-catalyzed hydrolysis of its substrates [5]. This suggests a transition-state structure for the glycosylation of β-glucosidase occurs with very little oxocarbenium ion character. This is a fairly common feature for a variety of β-glucosidases [6–9] which often demonstrate larger secondary KIEs in the deglycosylation reaction than in the glycosylation reaction. In order to see if this is also the situation with the almond enzyme we examined the α-secondary deuterium KIE on k_{cat} for 2,4-dinitrophenyl β-glucoside hydrolysis, for which deglucosylation is the rate-limiting step [3].

The almond enzyme shows no evidence of protonation of the leaving group in the transition state for the hydrolysis of aryl glucosides. We found no solvent KIE [$^{D2O}(V/K) \approx 1.0$] on the pH-independent second-order rate constant for the enzyme-catalyzed hydrolysis of 4-nitrophenyl β-glucoside [3]. Rosenberg and Kirsch [10] found a large ^{18}O-kinetic isotope effect [$^{18}(V/K) = 1.038$] for the β-glucosidase-catalyzed hydrolysis of this substrate. Also, there is a large negative Brønsted coefficient ($\beta_{lg} \approx -1$) for the dependence of the enzyme glucosylation rate constant on the leaving group acidity (for phenols with $pK_a \geq 7$) [3]. While a transition state structure with substantial C–O cleavage and little protonation of the leaving group is plausible for glucosides with "good" leaving groups such as phenols with pK_as ≤10, it is difficult to imagine such a structure for the enzyme-catalyzed hydrolysis of unactivated substrates, such as methyl glucoside. Nevertheless, we found that there is no solvent kinetic isotope effect for the enzyme-catalyzed hydrolysis of methyl β-glucosidaside [11]. This was attributed to a fortuitous cancellation of a normal equilibrium isotope effect for the protonation of the enzyme-bound glucoside and an inverse kinetic isotope effect accompanying the cleavage of the

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* Corresponding author. Tel.: +1 504 861 7044.
E-mail address: byers@tulane.edu (L.D. Byers).

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$$E + GlcOR \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E{\bullet}GlcOR \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} E\text{-}Glc \xrightarrow[H_2O]{k_3} E + GlcOH$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad \underset{ROH}{\nearrow}$$

Scheme 1. Double displacement mechanism of ß-glucosidase.

glycosidic bond. In order to confirm this hypothesis, and to get an idea of the extent of proton transfer to the anomeric oxygen in the transition state we decided to examine the hydrolysis reactions with more acidic (fluorinated) alkyl glucosides.

2. Materials and methods

2.1. Reagents

Almond β-glucosidase, specific activity ≈ 25 units/mg (salicin, 37°, pH 5.0), was obtained from Sigma-Aldrich Chemical Co., St. Louis, MO and further purified (to a specific activity ≈ 35 units/mg) by ion exchange chromatography on DEAE Sepharose (eluting with increasing NaCl concentrations at pH 6.0). SDS-PAGE (4–12% gradient cross-linked NuPage™ gel, Invitrogen Life Technologies) gave a single band corresponding to a molecular weight of ~65,000, consistent with this being the subunit of the homodimeric isozyme A [12]. Protein concentration was determined by absorbance at 278 nm based on a value of $E^{1\%} = 18.8$ [11,12]. D_2O (99.9%) was obtained from Cambridge Isotope Laboratories. 4-Nitrophenyl β-*D*-glucopyranoside (pNPG[1]), DCl, NaOD, buffers and other reagents were obtained from Sigma-Aldrich.

2.2. Synthesis

All compounds were characterized by ^{1}H-NMR (400 MHz, Varian Unity Inova 400 spectrometer).

2,4-Dinitrophenyl β-*D*-glucopyranoside (DNPG[1]) was prepared by the method of Koeners et al. [13] *via* the reaction of 2,3,4,6-tetra-*O*-acetyl-β-*D*-glucopyranose with 2,4-dinitrofluorobenzene. The 1-deutero analog of the tetraacetate was prepared by the method of Berven and Withers [14] *via* reduction of corresponding lactone with $NaBD_4$ (Sigma-Aldrich, 98 atom % D). ^{1}H-NMR analysis of deuterated substrate indicated an extent of isotopic incorporation greater than 95%. The 1-proteo compound was prepared in an identical manner, using $NaBH_4$.

Ethyl β-*D*-glucopyranoside was prepared by the method of Koenigs and Knorr from α-bromo-2,3,4,6-tetra-O-acetylglucose [15] with a flash column chromatography step (EtOAc/hexane 2:3) for the purification of the ethyl glucoside tetraacetate, followed by Zemplen deacetylation (NaOMe in MeOH), neutralization with Dowex 50W-X8)*, concentration under reduced pressure and purification by column chromatography (silica gel, CH_2Cl_2/CH_3OH, 5:1), yielding a solid which was crystallized from EtOAc/MeOH, mp 97–100°, lit. 98–100° [16].

2,2,2-Trifluoroethyl β-*D*-glucopyranoside was prepared by the method of Xue et al. [17]. Isopropyl β-*D*-glucopyranoside was prepared enzymatically (800 units of β-glucosidase in 10 ml of 90% 2-propanol containing 3 mmol glucose) as described by Lu et al. [18]. The resulting syrup was subjected to flash column chromatography (silica gel, CH_2Cl_2/MeOH, 5:1) and collected as a white solid. The 1,1,1,3,3,3-hexafluoro analog of isopropyl β-*D*-glucopyranoside was prepared by the method of Gueyrard et al. [19].

2.3. Kinetics

The reactions were monitored on a Hewlett-Packard model 8452A diode array spectrophotometer equipped with a circulating water bath (T = 25.0 ± 0.1 °C). Concentrated enzyme solutions (~10 mg/ml ≈ 150 μM subunits) were prepared in 0.01 M MES[1] buffer containing 0.01 *M* NaCl at pH 6.3, and the reactions were initiated by addition of enzyme. With pNPG[1] as substrate, product production was monitored spectrophotometrically by measuring the absorbance at 400 nm ($\varepsilon = 2.9 \times 10^3\ M^{-1}\ cm^{-1}$ at pH 6.3). With DNPG as substrate the reaction was monitored at 400 nm ($\varepsilon = 1.09 \times 10^4\ M^{-1}\ cm^{-1}$ for 2,4-dinitrophenolate) under first order conditions ($[S] \ll K_M \approx 0.8$ mM). When larger substrate concentrations were used (i.e., in the determination of the Michaelis–Menten parameters) higher wavelengths were used (e.g., at 480 nm, $\varepsilon = 760\ M^{-1}\ cm^{-1}$). These parameters were determined by monitoring the initial velocities at substrate concentrations ranging from ~0.3 to 9 mM. (Substrate concentrations were determined from the absorbance of dinitrophenolate following complete enzymatic hydrolysis of a sample from the stock substrate solutions.) The reactions were initiated by the addition of enzyme solution (final concentration ≈ 0.03 units/ml = 12 nM) and the initial velocities were fit to the Henri–Michaelis–Menten equation *via* non-linear regression. The α-secondary kinetic isotope effect on k_{cat} for DNPG hydrolysis was determined at pH 6.3 by measuring a series of (10–15) initial velocities with each substrate (the 1-deutero- and the 1-proteo-DNPG) at high ([S] ~12K_M) concentrations. The V_{max} values were calculated from these initial velocity measurements by multiplying them by the saturation correction factor ($1 + K_M/[S]$), which is V_{max}/v from the Henri–Michaelis–Menten equation.

For the reaction with alkyl glucosides, the reactions were monitored by the rate of glucose production by removal of an aliquot of the reaction mixture and then determination of the glucose concentration using the coupled enzyme assay of hexokinase (yeast) and glucose-6-phosphate dehydrogenase (*L. mesenteroides*), containing 10 mM ATP, 5 mM $MgSO_4$, 1 mM NAD^+, pH 7.5, and measuring the resulting absorbance at 340 nm due to the formation of NADH ($\varepsilon = 6.32 \times 10^3\ M^{-1}\ cm^{-1}$ [20]). The K_M values for these substrates were determined by measuring the inhibition of pNPG[1] hydrolysis under first-order conditions. For the reverse reactions (Glc + ROH → GlcOR), the rate constant for the alcoholysis of the glycosyl-enzyme was obtained by monitoring the product partitioning when *p*-nitrophenol is produced from pNPG in the presence of various concentrations of the alcohol. The product ratio ([Glc]/[GlcOR]) is equal to the rate-constant ratio ($k_{ROH}[ROH]/k_W[H_2O]$) where k_W is the rate constant for the reaction of water with the glucosyl-enzyme and k_{ROH} is the rate constant for the reaction of the alcohol with the glucosyl-enzyme. In determining the product ratio, the glucose concentration (measured as described above and corrected for the absorbance of pNPG and of *p*-nitrophenol/phenolate at 340 nm) and the *p*-nitrophenol/phenolate concentration (= [Glc] + [GlcOR]) were used to calculate the concentrations.

2.4. Solvent kinetic isotope effect

The V_{max}/K_M values for hydrolysis of 2,2,2-trifluoroethyl β-*D*-glucopyranoside were determined under pseudo zero-order conditions by adding enzyme (final concentration = 30 nM) to an appropriately buffered solution containing 10 mM substrate ($\ll K_M \approx 120$ mM at pH 5) and measuring the initial velocity (by removing aliquots and determining the glucose concentration at various times). The initial velocity was divided by the substrate concentration to yield V_{max}/K_M. The buffer solutions consisted of 0.01 M buffer [formate, pL(= pH/pD) 3.0–4.0; acetate, pL 3.5–5.8; MES[1], pL 5.5–7.0; PIPES[1], pL 6.1–7.6], prepared in deionized water (or D_2O) containing 0.01 M NaCl. pH measurements were made using a glass combination electrode (Accumet pH meter).

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pD values were estimated by adding 0.41 to the pH meter reading [21]. The data were fit to the following equation:

$$\log\ k_{obs} = \log\left[k^{lim}/\left(1 + 10^{(pK1-pL)} + 10^{(pL-pK2)}\right)\right] \quad (1)$$

3. Results

3.1. Secondary kinetic isotope effect

The α-deuterium secondary KIE for the hydrolysis of 2,4-DNPG was measured at pH 6.3 (0.01 M MES, 0.01 M NaCl, 25 °C). Estimates of the kinetic parameters, obtained under initial velocity conditions, for the enzymatic hydrolysis of [^{1}H]- and [^{2}H]-glucosides of 2,4-dinitrophenol are: $K^h_M = 0.79\ (\pm 0.08)$ mM, $K^d_M = 0.7\ (\pm 0.1)$ mM and $k^H_{cat} = 510\ (\pm 16)\ s^{-1}$, $k^D_{cat} = 450\ (\pm 23)\ s^{-1}$. Under pseudo first-order conditions, the measured rate constants, and thus the k_{cat}/K_M values, are identical [$6.46\ (\pm 0.09) \times 10^5\ M^{-1}\ s^{-1}$ for the protio substrate and $6.43\ (\pm 0.08) \times 10^5\ M^{-1}\ s^{-1}$ for the deuterio substrate] yielding a secondary KIE of $^D(k_{cat}/K_M) = 1.00$. This is not surprising since the second-order rate constant for this substrate is largely diffusion-controlled [3]. In order to obtain a more precise estimate of the isotope effect on k_{cat}, a series of initial velocity measurement were made with each substrate at high concentrations (9.2 mM ≈ 13.1 K_M for the deuterio-DNPG and 9.8 mM ≈ 12.4 K_M for the protio-DNPG). These initial velocities converted to V_{max} values by multiplying by the corresponding saturation correction factor, $(1 + K_M/[S]) = 1.076$ for [^{2}H]-substrate and 1.081 for the [^{1}H]-DNPG. The ratio of these maximal velocities yields a value for the α-secondary KIE of $^Dk_{cat} = 1.12\ (\pm 0.03)$.

3.2. Solvent kinetic isotope effects

3.2.1. On the deglucosylation step — dinitrophenyl glucoside hydrolysis

The value of k_{cat} for the hydrolysis of DNPG was found to vary by <3% between pH 5 and 6. When measured at the pH optimum (pH 5.2) this value (determined from 12 initial velocity measurements at [S] = 20 mM) is 990 (±28) s^{-1}. In D_2O (pD = 5.6) this value is 660 (±21) s^{-1}, corresponding to a solvent kinetic isotope effect of 1.50 (±0.06) on the deglucosylation reaction.

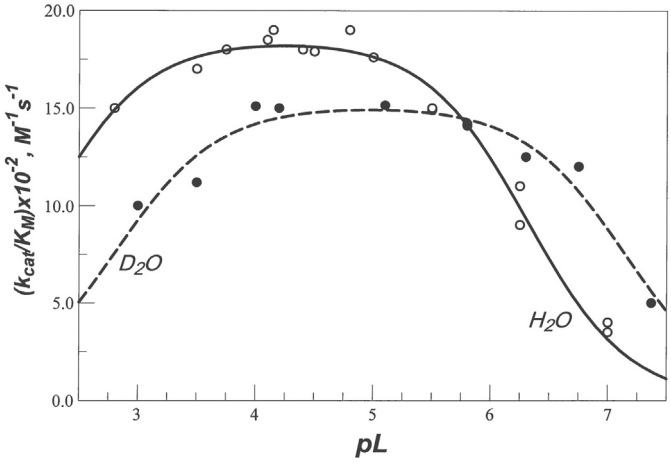

Fig. 1. Solvent isotope effect on the enzymatic hydrolysis of 2,2,2-trifluoroethyl β-*D*-glucopyranoside (25 °C). The lines are the fit to Eq. (1) with the following values: in H_2O (open circles); $(k_{cat}/K_M)^{lim} = 1.85(\pm 0.03) \times 10^3\ M^{-1}\ s^{-1}$, pK_{a1} ~2.2 (±0.2), $pK_{a2} = 6.31\ (\pm 0.05)$ and in D_2O (filled circles); $(k_{cat}/K_M)^{lim} = 1.51(\pm 0.05) \times 10^3\ M^{-1}\ s^{-1}$, pK_{a1} ~2.8 (±0.1), $pK_{a2} = 7.14\ (\pm 0.09)$, yielding a solvent isotope effect on the pH-independent second-order rate constant of $^{D2O}(k_{cat}/K_M)^{lim} = 1.23\ (\pm 0.04)$.

Table 1
Alkyl β-glucoside hydrolysis, pH 5.5.

ROH	pK_a	k_{cat}/K_M, $M^{-1}\ s^{-1}$	K_M, mM	k_{cat}, s^{-1}
CH_3CH_2OH	15.9	48 (±2)	230 (±12)	11 (±1)
CF_3CH_2OH	12.4	1620 (±58)	110 (±6)	178 (±11)
$(CH_3)_2CHOH$	17.1	130 (±8)	210 (±9)	27 (±2)
$(CF_3)_2CHOH$	9.3	8090 (±18)	79 (±4)	640 (±30)

3.2.2. On the glucosylation step — 2,2,2-trifluoroethyl β-D-glucopyranoside hydrolysis

Fig. 1 shows the pL (pH/pD) dependence of k_{cat}/K_M for the hydrolysis of TFEG[1] in H_2O and in D_2O. The data show typical increases in the apparent pK_as when the solvent is changed from H_2O to D_2O as well as a small, but significant, solvent kinetic isotope effect on the limiting second-order rate constant of $^{D2O}(k_{cat}/K_M) = 1.23\ (\pm 0.04)$.

3.3. Alkyl glucoside hydrolysis

The cleavage of aryl glucosides catalyzed by almond β-glucosidase proceeds with little, if any, proton transfer [3,10]. The BrØnsted coefficient, based on k_{cat}/K_M for the hydrolysis of a series of 13 substituted phenyl glucosides is large and negative ($\beta_{lg} = -0.97$) [3]. In order to obtain a qualitative comparison for the enzyme-catalyzed hydrolysis of *alky* glucosides with that of *aryl* glucosides we compared the kinetics for the hydrolysis of ethyl and isopropyl glucosides with their fluorinated analogs. The results are shown in Table 1. It is clear that within each structurally analogous pair, the more acidic alcohol is the better leaving group. Furthermore, the sensitivity of either k_{cat}/K_M or k_{cat} to the aliphatic alcohol leaving group pK_a (β_{lg} ~ −0.3) is not nearly as large as it is for aryl leaving groups (with $pK_a \geq 7$).

3.4. Enzymatic synthesis of alkyl glucosides

The reactions illustrating the competition between transglucosylation, in the presence of added alcohols, and hydrolysis are summarized in Scheme 2:

The kinetics of product partitioning of pNPG (1 mM) between glucose and alkyl glucoside were determined in the presence of added alcohols at pH 5.5. Assuming that the reaction of the glucosyl-enzyme intermediate with water (i.e., formation of Glc) is first order in [H_2O] and its reaction with the accepting alcohol (i.e., formation of GlcOR) is first-order in [ROH] (*vide infra*) then the second-order rate constant ratio for these two reactions, k_{ROH}/k_W is readily obtained (e.g., see [22]) from the ratio of the initial velocities for *p*-nitrophenol production (v_{pNP}) and for glucose production (v_{glc}):

$$v_{pNP}/v_{glc} = 1 + k_{ROH}[ROH/k_W H_2O] \quad (2)$$

Fig. 2 shows the results obtained using 2-propanol and its hexafluoro analog. The results are linear at least up to the highest alcohol concentrations tested [ca. 20% v/v with $(CF_3)_2CHOH$ (=1.9 M) and 35% v/v with $(CH_3)_2CHOH$ (=4.6 M)]. Similar results were obtained with ethanol and 2,2,2-trifluoroethanol. This indicates that the reactions are, indeed, first-order in alcohol concentration and show no evidence of enzyme denaturation during the time course (several minutes) for which the reactions are followed. (This stability is not surprising. The 2-propyl glucoside was prepared by incubating the enzyme in 90% 2-propanol for 2 days at 50° [18].) The partitioning of the glucosyl-enzyme intermediate between the reaction with the alcohol and with water determined by this method is $k_{ROH}/k_W = 7.5$ for $(CF_3)_2CHOH$ and 4.0 for $(CH_3)_2CHOH$. With CF_3CH_2OH and CH_3CH_2OH the partitioning ratios are 9.2 and 5.9.

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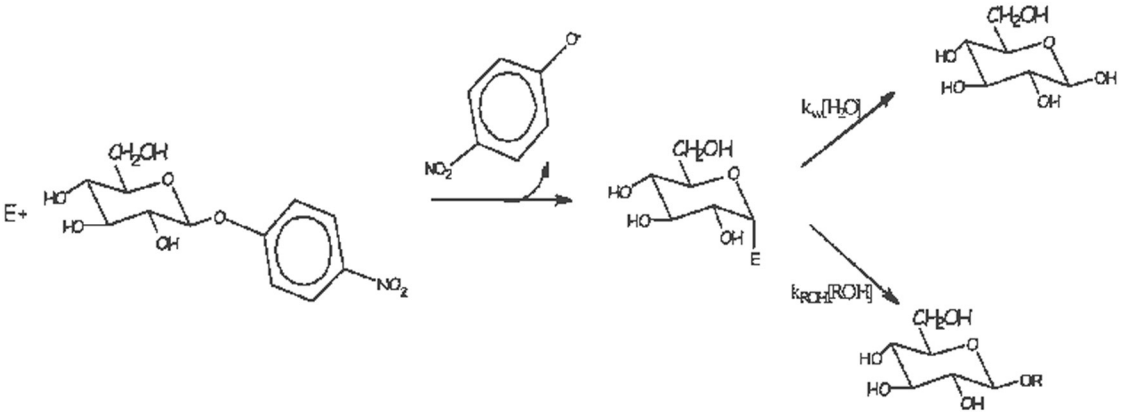

Scheme 2. Partitioning of intermediate.

4. Discussion

4.1. Secondary α-deuterium KIE

The mechanism of action of retaining glycohydrolases, such as almond β-glucosidase, is a double-displacement reaction involving a covalent glycosyl-enzyme intermediate (Scheme 1). Consistent with this is the biphasic nature of the BrØnsted plot obtained when the log k_{cat} for hydrolysis of phenyl glucosides is plotted against the pK_a of the substituted phenol leaving groups (where the slope, β_{lg}, changes from −1 to 0 as the pK_a of the leaving group decreases below ~8) [3]. Thus, for aryl glucosides with good leaving groups, such as 2,4-DNP-Glc (leaving group $pK_a = 4.1$), the rate-limiting step is deglucosylation (i.e., $k_{cat} = k_3$). For this substrate, k_{cat} ~990 s^{-1} at pH 5.5 and 510 s^{-1} at pH 6.3. When k_{cat} was measured with both the protio and deuterio substrates, the α-deuterium secondary KIE was found to be 1.12 (±0.03), suggesting substantial sp^2- character of the anomeric carbon in the transition state for the deglucosylation reaction. This is in contrast to the enzyme-catalyzed hydrolysis of less reactive substrates (for which $k_3 \gg k_2$), such as phenyl glucoside (leaving group $pK_a = 10$), for which Dahlquist et al. [5] measured an α-deuterium secondary KIE of 1.01. It is known that there is substantial glycosidic bond cleavage in the transition state for the glycosylation reaction (k_2): 1) Rosenberg and Kirsch [10] found a large ^{18}O kinetic isotope effect on k_{cat}/K_M for the hydrolysis of pNPG suggesting nearly complete (89 ± 14%) C–O cleavage in the glucosylation (k_2) transition state. 2) The BrØnsted coefficient for this step is large and negative [3]. Indeed, this value ($\beta_{lg} = -1$) compared with the equilibrium β value for complete glycosidic bond cleavage (yielding the unprotonated phenoxide, ArO^-), determined by Richard et al. to be −1.56 for β-galactoside hydrolysis [22] and assuming that the β_{eq} for aryl glucoside hydrolysis is similar to this, suggests a transition state where bond cleavage is ~64% (100% β_{lg}/β_{eq}) complete. This data indicates that the lack of a secondary kinetic isotope for phenyl glucoside hydrolysis [5] is not the result of an early transition state with little, if any, bond cleavage. It is consistent with an almost S_N2-like transition state for the glucosylation reaction. There is a considerable body of evidence for an S_N2 (A_ND_N) type mechanism in the glycosylation step for many retaining glycohydrolases. For example, Berti's group [6], using natural abundance ^{13}C-NMR, has measured a large ^{13}C-KIE for almond β-glucosidase-catalyzed hydrolysis of methyl glucoside, indicative of an A_ND_N mechanism.

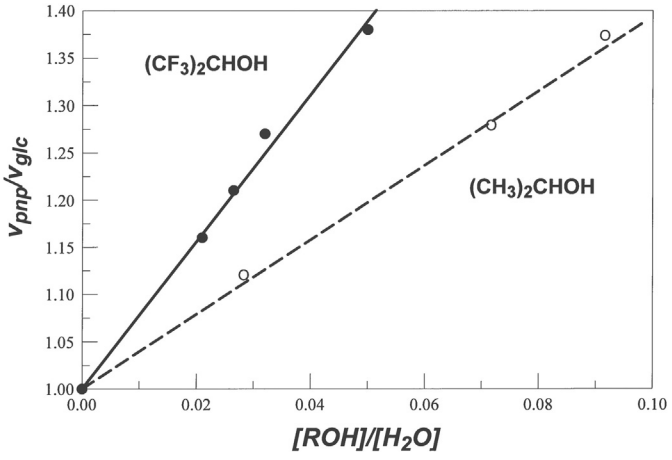

Fig. 2. Effect of increasing concentration of alcohol on the ratio of initial velocities of formation of *p*-nitrophenoxide and glucose (1 mM pNPG, 9 μg/ml β-glucosidase, .01 *M* MES, .01 *M* NaCl, pH 5.5, 25°).

The secondary KIE ($^Dk_{cat} = 1.12$) that we find with the reactive substrate, DNPG, (i.e., the KIE on k_3, the *deglucosylation* step) is of typical magnitude compared to other reactions involving formation of the glucopyranosyl cation (e.g., $^Dk = 1.13$ for the acid-catalyzed hydrolysis of phenyl β-glucoside [23]) but it is lower than that ($^Dk_{cat} = 1.25$) observed for the hydrolysis of 2,4-dintrophenyl β-galactoside catalyzed by β-galactosidase [24]. But even with β-galactosidase, which shows a substantially larger KIE on the deglycosylation reaction (k_3 in Scheme 1) than is seen here, or with other glycohydrolases [7,9,25] the isotope effect on the glycosylation reaction k_2 is unity [24]. Indeed, for most of the glycohydrolases examined (i.e., all of which we are aware) the secondary α-deuterium KIE on k_3 is significantly larger than that on k_2 (≈1.00) [7,9,24,25] suggesting an S_N2-like (A_ND_N) transition state for the glycosylation reaction and an S_N1-like ($D_N + A_N$) transition state for the deglycosylation reaction. Our results with the almond β-glucosidase are consistent with this.

4.2. Alkyl glucoside hydrolysis

For the reaction of aryl glucosides with the enzyme (k_{cat}/K_M), the more acidic the leaving group (up to $pK_a \approx 7$), the faster the reaction, corresponding to a $\beta_{lg} \approx -1$ [3]. Here we did not examine enough alkyl glucosides to obtain a reliable value of β_{lg}. However, if we look at the two pairs of structurally similar substrates shown in Table 1, we see that k_{cat} ($= k_2$) increases 16-fold when trifluoroethyl glucoside replaces ethyl glucoside. Fluorination of the of the leaving group decreases its pK_a by 3.5 units. This corresponds to a $\beta_{lg} \sim -0.3$. With the isopropyl and hexafluoroisopropyl glucosides it is also apparent that the more acidic alcohol is more reactive. This gives an apparent "β" value of $(\beta_{lg})_{k2} \sim -0.2$. Of course, neither of these "β" values are quantitatively reliable, being based on only two points, and the apparent difference between them is not statistically reliable. What is clear, however, is that

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the more *acidic* the alkyl alcohol leaving group the more reactive the glucoside will be. A similar phenomenon was observed by Richard et al. [26] who, working with β-galactosidase, clearly demonstrated increasing reactivity with galactosides of more acidic alkyl alcohols [$(\beta_{lg})_{k2} = -0.5(\pm 0.1)$, determined with a set of 7 structurally homogeneous galactosides].

4.3. Alkyl glucoside synthesis

This is simply the reverse of the glycosylation step and provides a direct measure of k_{-2} (Scheme 1). The partitioning of the glucosyl-enzyme intermediate (between reaction with ROH and H_2O) provides a measure of the relative values of the rate constants for the reverse step (k_{-2}) and the forward step (k_3). The observation that the partitioning ratios ($k_{ROH}/k_W = k_{-2}/k_3$) are greater with the more acidic (fluorinated) alcohols than the corresponding alkyl alcohols is consistent with base catalysis of addition of the alcohol to the intermediate. The "β_{nuc}" value based on the two pairs of alcohols examined is ~ −0.05 ("β_{nuc}" = −0.04 for hexafluoro-isopropanol/isopropanol and −0.06 for trifluoroethanol/ethanol) and suggests a small negative charge on the alcohol nucleophile in the transition state. This is analogous to the situation with β-galatosidase discussed by Richard et al. [22]. The partitioning ratio also allows us to construct a reaction coordinate diagram (Fig. 3) and reveals the relative stability of the glucosyl-enzyme intermediate. This intermediate (generated from ethyl glucoside) is higher in energy than the enzyme–substrate complex by 3.7 kcal/mol. Calculations based on the data (k_2/k_{-2}) obtained with the other glucosides (trifluoroethyl-, isopropyl- and hexafluoroisopropyl-) yield similar values ($\Delta\Delta G^{o\prime} = 2$ to 3 kcal/mol) for the energy difference between the covalent glucosylated enzyme and the non-covalent ES complex.

4.4. Solvent isotope effect on trifluoroethyl-β-glucoside hydrolysis

The smaller sensitivity, on the leaving group pK_a, of the rate constant (k_2) for the glucosylation of the enzyme with alkyl glucosides than with aryl glucosides could reflect a transition state structure with a smaller extent of glycosidic bond cleavage and/or a larger degree of proton transfer from the enzyme to the oxygen (of the incipient alcohol product). The hydrolysis of trifluoroethyl glucoside (TFEG[1]) shows a solvent kinetic isotope effect [$^{D2O}(k_{cat}/K_M)^{lim} = 1.23\ (\pm 0.04)$], unlike the reaction of phenyl glucosides ($^{D2O}(k_{cat}/K_M)^{lim} \approx 1.0$ [3]). Although this a small isotope effect compared with those typically seen in general acid catalyzed reactions or with other hydrolytic enzymes, it *is* significant, particularly in view of the fact that no solvent isotope effect was detected on the pH-independent second-order rate constant for the β-glucosidase-catalyzed hydrolysis of methyl glucoside [$^{D2O}(k_{cat}/K_M)^{lim} = 1.05(\pm 0.08)$] [11]. Since no solvent isotope effect is seen on the binding of substrates (e.g., methyl or phenyl glucoside) it was concluded that the lack of a solvent isotope effect on the hydrolysis of methyl glucoside was a fortuitous cancellation of isotope effects (i.e., a normal isotope effect on a pre-equilibrium protonation of the bound substrate followed by an inverse isotope effect on the cleavage of the protonated glucosidic bond). Because the trifluoroethyl oxygen in TFEG is even less basic than that in methyl glucoside (the pK_a of trifluoroethanol is 3.5 units lower than that of ethanol), a pre-equilibrium protonation step is not very likely in the reaction. In order to avoid such an unstable intermediate (the enzyme-bound protonated glucoside) a concerted proton transfer from the acidic group (Glu) on the enzyme coupled to the glycosidic bond cleavage is expected, according to the principles of Jencks' libido rule [28]. A normal (albeit small) solvent isotope effect is consistent with this. While we are unable to quantitate the progression along the reaction coordinate where the transition state occurs, we can at least eliminate the two extremes. If protonation occurs before any glycosidic bond is cleaved in the transition state we would expect a larger solvent isotope effect and a $\beta_{lg} > 0$. If the transition state occurred when the cleavage of the protonated glycosidic bond was nearly complete we would expect a β_{lg} value near −0.56 (β_{eq} for glycoside hydrolysis [22]) and a solvent isotope effect <1 [due to the higher pK_a (12.4) of the alcohol than of the enzymic acid group ($pK_{a2} \approx 6.3$)]. Qualitatively, our results suggest a transition state structure where there is more glycosidic bond cleavage than protonation. Because of the stability of the glucosyl-enzyme intermediate (only about 2–4 kcal higher energy than the Michaelis complex, see Fig. 3), a late transition state (i.e., one resembling the product) for the glycosylation step is unlikely. It is, however, clear that leaving group protonation is more strongly coupled to C–O cleavage in alkyl glucoside hydrolysis than in aryl glucoside hydrolysis.

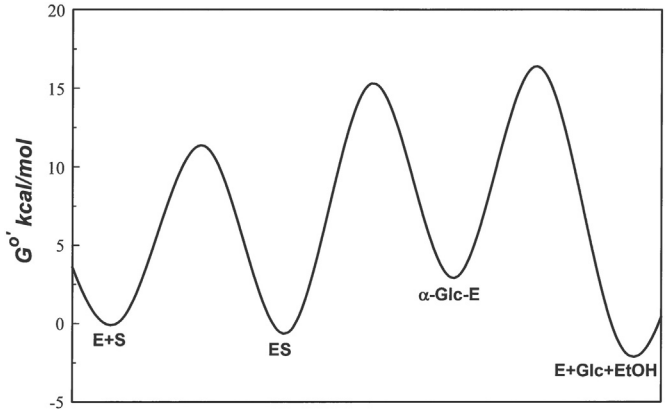

Fig. 3. Reaction coordinate diagram for the hydrolysis of ethyl β-*D*-glucopyranoside (pH 5.5, 25 °C, 55.6 M H_2O). The first step is assumed to be diffusion-controlled ($k_1 \approx 10^6\ M^{-1}\ s^{-1}$ for aryl glucosides [3]). The equilibrium constant for the overall reaction, $K_{eq}' \approx 15$ M, is from the value of Szekeres and Tettamanti [27]. The K_M ($= K_S$) for ethyl glucoside is 0.23 M, corresponding to a binding energy of −0.9 kcal/mol. For this substrate, $k_{cat} = k_2 = 11\ s^{-1}$ corresponding to an activation energy of 16 kcal/mol from the ES (15.1 kcal/mol from E + S). The value of k_{-2} ($= 6000\ s^{-1}$) corresponds to an activation energy of 12.3 kcal/mol from the glucosyl-enzyme intermediate. Thus, the energy of this covalent intermediate is 2.8 kcal/mol higher than that of the free enzyme + substrate and 3.7 kcal/mol higher than that of the enzyme–substrate complex ($k_2/k_{-2} = 1.8 \times 10^{-3}$).

Abbreviations

Glc	*D*-glucose
HEPES	N-(2-hydroxyethyl)-piperazine-N′-(2-ethansulfonate)
MES	2-(N-morpholino)ethane-sulfonate
DNPG	2,4-dinitrophenyl β-*D*-glucopyranoside
PIPES	piperazine-N,N′-bis(2-ethansulfonate)
pNPG	*p*-nitrophenyl β-*D*-glucopyranoside
TFEG	2,2,2-trifluoroethyl β-*D*-glucopyranoside

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