Glucosidase-Catalyzed Hydrolysis of α-D-Glucopyranosyl Pyridinium Salts: Kinetic Evidence for Nucleophilic Involvement at the Glucosidation Transition State

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Abstract: Kinetic isotope effects (KIEs) on the yeast α-glucosidase-catalyzed hydrolysis of two α-D-glucopyranosyl pyridinium salts were measured at 25 °C and pH = 6.8. The measured KIEs on k_{cat} for the ^{2}H-2, ^{13}C-1, and ^{15}N-1′ labeled substrates α-D-glucopyranosyl pyridinium bromide (**1**) and α-D-glucopyranosyl isoquinolinium bromide (**2**) were, respectively, 1.115 ± 0.006 and 1.106 ± 0.009, 1.028 ± 0.006 and 1.027 ± 0.005, and 1.019 ± 0.007 and 0.985 ± 0.005. KIEs for the spontaneous hydrolysis of α-D-glucopyranosyl 4′-bromoisoquinolinium bromide (**3**) were measured at 80 °C and pH = 6.8. The measured KIEs on k_{hyd} for the ^{2}H-1, ^{2}H-2, ^{13}C-1, and ^{15}N-1′ labeled substrate α-D-glucopyranosyl 4′-bromoisoquinolinium bromide (**3**) were, respectively, 1.189 ± 0.009, 1.094 ± 0.010, 1.005 ± 0.002, and 1.015 ± 0.004. The KIEs obtained in this study of yeast α-glucosidase-catalyzed hydrolysis reactions are consistent with a transition state which involves a small degree of nucleophilic attack by an enzyme active site carboxylate group at the anomeric carbon center, whereas the corresponding transition state for the uncatalyzed reaction in water does not involve any nucleophilic assistance. In addition, the equilibrium isotope effects for deprotonation of the conjugate acid (K_a^{14}/K_a^{15}) of isoquinoline was measured to be 1.0216 ± 0.0005.

Introduction

Glycosidases are a large family of enzymes that catalyze hydrolysis of the glycosidic bond, a linkage that is found in carbohydrates. This enzyme family can be subdivided into two classes of enzymes based on the stereochemical outcome of their hydrolysis reactions: (1) retaining glycosidases which generate a product that retains the anomeric configuration of the substrate and (2) inverting glycosidases which result in a product with inverted anomeric configuration.[1] Catalytically-efficient glycosidases require that two acidic amino acid residues, Asp and/or Glu, be located in the active-site. Furthermore, maximal enzymatic activity occurs in a pH region in which one of these essential carboxylate groups is protonated, while the other is ionized.[1] Since the work herein involves a retaining α-glucosidase isolated from yeast, further mechanistic discussions will be confined to retaining glycosidases.

The generally accepted hydrolysis mechanism for retaining glycosidases was initially proposed by Koshland[2] and is shown in Scheme 1.[1] An alternative mechanism has been proposed in which, during acetal hydrolysis, initial cleavage occurs to the endocyclic carbon−oxygen bond.[3] However, this particular mechanism cannot account for the rapidity with which glucosidase-catalyzed hydrolysis occurs with both glycosyl fluorides and glycosyl pyridinium salts as substrates.[4] Since the time of Koshland's original proposal of the double displacement mechanism for retaining glycosidases,[2] a general consensus has emerged in support of the idea that the active-site carboxylic acid residue facilitates exocyclic C−O bond cleavage by simultaneous protonation of the leaving group oxygen atom (Scheme 1). The proposed general-acid catalyst function of the protonated acidic residue is substantiated experimentally by solvent deuterium[5] and leaving group oxygen-18[6] kinetic isotope effects (KIEs) on the enzyme-catalyzed glycosidic hydrolysis. The precise function of the other essential active-site residue is more controversial. Two possible functions for the carboxylate group include the following: (1) the anionic CO_2^- group acts as a nucleophile and thus assists in leaving group departure;[1] or (2) the anionic CO_2^- group acts electrostatically to stabilize the glycosyl oxocarbenium ion intermediate in the active-site.[7] The proposed electrostatic model which is shown in Scheme 2 requires that the active-site carboxylate remain sufficiently separated from the anomeric carbon at the reaction transition state (TS). The consequence of this model is that the reaction occurs by an S_N1 mechanism to generate an electrostatically-stabilized oxocarbenium ion intermediate.[7]

Recently, Richard *et al.* argued that during the, β-galactosidase-catalyzed hydrolysis of alkyl β-D-galactosides an oxocarbenium ion intermediate was probably formed at the active-site, as required by the electrostatic model.[8] This conclusion was based on the observed selectivity of the galactosyl-enzyme intermediate between reaction with methanol and water.

Whereas circumstantial support for the nucleophilic role of the carboxylate in catalysis comes from Withers and co-workers who have shown that a series of 2-deoxy-2-fluoro-β-D-glycoside derivatives react with the corresponding β-glycosidase to form a stable 2-deoxy-2-fluoro-α-D-glycosyl enzyme intermediate.[9] However, isolation of a covalently-bound reaction intermediate provides no information with respect to the presence or absence of nucleophilic catalysis at the glycosylation TS. That is,

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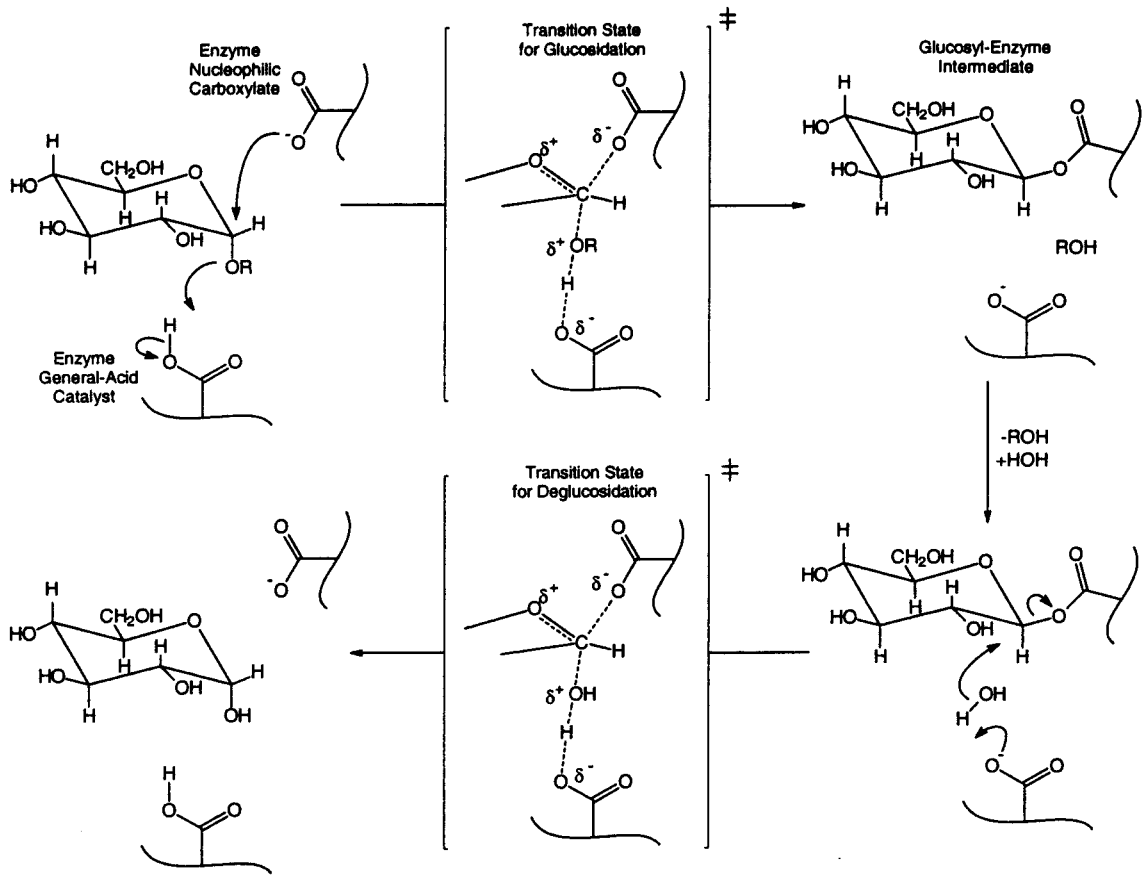
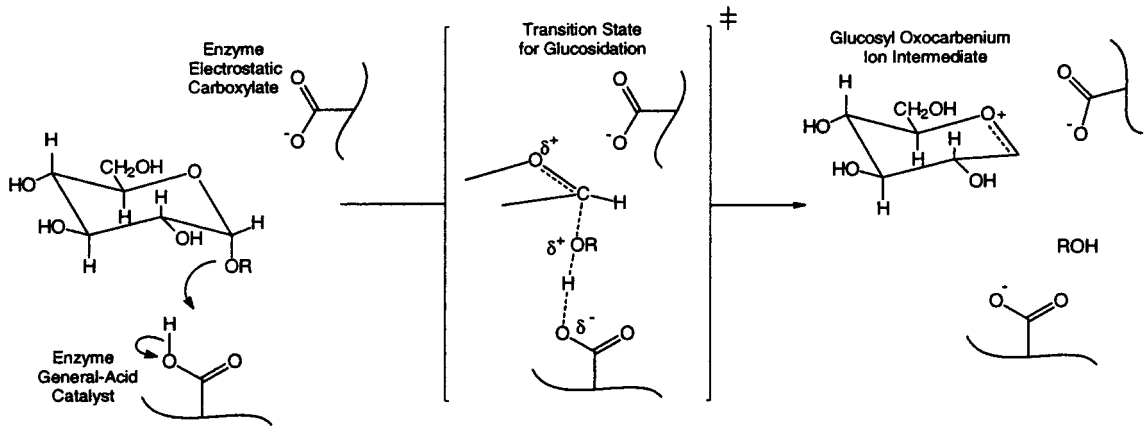
Scheme 1



Scheme 2



structural information of this kind cannot answer the question of whether bond formation occurred during or subsequent to the rate-limiting step. Data which pertain to this aspect of the mechanism must be kinetic in nature.

Measurement of a KIE allows the reaction TS to be probed, while affording the smallest possible perturbation to the system. The magnitude of an α-secondary deuterium KIE (α-SDKIE) is frequently used as a mechanistic indicator of the nucleophilic involvement in substitution reactions.[10,11] Knier and Jencks have shown that, although a methoxymethyl derivative reacts with added anions via a concerted mechanism (S_N2), the observed α-SDKIEs (k_H/k_D) vary from 1.18 (reaction with I^-) to 0.99 (reaction with F^-), a range that encompasses typical values for, respectively, S_N1 and S_N2 reactions.[12] Consequently, for substitutions taking place at acetal centers, the magnitude of an α-SDKIE is not a reliable probe for the occurrence of a weak, nucleophilic component at the reaction TS. Another, albeit smaller KIE that can possibly be used to distinguish between S_N1 (dissociative TS) and S_N2 (concerted bond formation and bond cleavage) reactions is the reaction center carbon KIE (k^{12}_C/k^{13}_C).[13] For example, a typical k^{12}_C/k^{13}_C value for an S_N1

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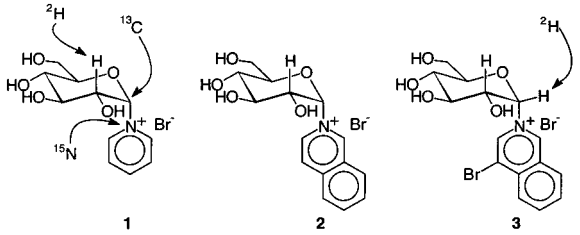
reaction is 1.00−1.01, whereas the corresponding value for an S_N2 reaction is generally in the range of 1.03−1.08.[13]

Anomeric carbon KIE measurements for α-D-glucopyranosyl fluoride hydrolysis reactions suggest that there is a distinct mechanistic difference between the reaction catalyzed by sugar beet seed α-glucosidase and the water-promoted reaction (*vide supra*). Tanaka *et al*. measured an anomeric carbon kinetic isotope effect (k^{12}_C/k^{14}_C) of 1.014 ± 0.009 on k_{cat}/K_M for the hydrolysis of α-D-glucopyranosyl fluoride catalyzed by the retaining sugar beet seed α-glucosidase at 25 °C.[14] This value is equivalent to a ^{13}C-KIE (k^{12}_C/k^{13}_C) of approximately 1.007 ± 0.004.[15] Analysis of the observed KIEs on k_{cat}/K_M for the α-glucosidase-catalyzed hydrolysis of this substrate, combined with reaction TS modeling work, lead Tanaka *et al*. to conclude that nucleophilic participation did not occur during rate-limiting C−F bond cleavage (S_N1).[14] In contrast, Banait and Jencks demonstrated that the reactions of α-D-glucopyranosyl fluoride in aqueous solution with added anions are bimolecular and thus proceed through a concerted mechanism (S_N2).[16] In a separate account Zhang *et al*. reported that the anomeric ^{13}C-KIE (k^{12}_C/k^{13}_C) for the reaction of α-D-glucopyranosyl fluoride with azide ion is 1.085 ± 0.008 and with water is 1.032 ± 0.003.[17] These results are in support of the conclusion of Banait and Jencks[16] that the substitution reactions of α-D-glucopyranosyl fluoride occur by a concerted mechanism (S_N2).

A second dichotomy exists for the hydrolysis of glucoside derivatives in aqueous solutions.[18] Namely, when the leaving group in the reaction is the anionic fluoride ion, glucoside hydrolysis occurs via an S_N2 mechanism,[16] whereas the neutral leaving groups methanol[19] and pyridine[18,19] cause glucosyl substitution reactions to proceed via an S_N1 mechanism. With respect to the reactions of glucopyranosyl derivatives in water, the apparent leaving group-based alternate substitution mechanism is a consequence of the borderline stability of the glucopyranosyl oxocarbenium ion, a species that reacts with the solvent with an estimated rate constant (k_{H_2O}) of $1-3 \times 10^{-12}$ s^{-1}.[16,18]

The present study was initiated to probe the mechanism of hydrolysis for glycosidase-catalyzed reactions involving glycopyranosides that contain a neutral leaving group. Prior, pertinent reports in the literature include an observed anomeric ^{14}C-KIE on k_{cat}/K_M of 1.044 ± 0.004 ($k^{12}_C/k^{13}_C \approx 1.023$[15]) for the hydrolysis of the furanoside inosine catalyzed by the *Crithidia fasciculata* nucleoside hydrolase.[20] This KIE is consistent with a reaction TS in which a weak nucleophilic attack is occurring at the anomeric center in concert with departure of the protonated purine leaving group.[20] Similar conclusions were drawn from a study of the catalyzed hydrolysis of adenosine monophosphate (AMP) by AMP nucleosidase from *Azotobacter vinelandii*.[21,22] However, furanosides display a marked reactivity difference compared to the reactivity of pyranosides.[23] For example, the anomeric ^{13}C-KIEs on the specific-acid catalyzed hydrolysis of methyl α-D-glucopyranoside is $k^{12}_C/k^{13}_C = 1.007 \pm 0.001$, a value that is consistent with the unassisted departure of methanol from the protonated glucoside,[19] whereas contrasting anomeric ^{14}C-KIEs of 1.044 ± 0.003 ($k^{12}_C/k^{13}_C \approx 1.023$[15])[21] and 1.019 ± 0.004 ($k^{12}_C/k^{13}_C \approx 1.010$[15])[24] have been reported for the acid-catalyzed hydrolysis of AMP and the spontaneous hydrolysis of NAD^+, respectively; both of these hydrolysis reactions of furanosides involve neutral leaving groups.

The present report contains the details of kinetic isotope effect studies on k_{cat} for the yeast α-D-glucosidase catalyzed hydrolysis of both α-D-glucopyranosyl pyridinium (**1**) and isoquinolinium (**2**) bromides. Also included are the details of a KIE study on the spontaneous hydrolysis reactions of α-D-glucopyranosyl 4′-bromoisoquinolinium bromide (**3**). The position of the various isotopically-labeled atoms that were utilized in the glucosidase KIE study are identified in structure **1**. The additional isotopically-labeled position studied in the spontaneous hydrolysis reaction is shown in structure **3**. The ^{15}N equilibrium isotope effects on the protonation of isoquinoline is also included in this report.



Materials and Methods

Sodium dihydrogen phosphate and disodium hydrogen phosphate buffering reagents were of "Analar" grade and were used without further purification. Yeast α-glucosidase type III suspended in 3.2 M $(NH_4)_2SO_4$ solution (product number G-7256, lot 28F0437), bovine serum albumin (BSA; product number A-6003, lot 122H9318), and *p*-nitrophenyl-α-D-glucopyranoside were purchased from Sigma. Milli-Q water (18.2 MΩ cm^{-1}) was used for the kinetic experiments. NMR spectra were acquired using either a Bruker AMX-400 or a Bruker AMX-600 spectrometer. Electron impact (70 eV) mass spectra were obtained using a Hewlett Packard 5985 mass spectrometer. Melting points are reported as uncorrected values. Both (^{15}N)-ammonium chloride (99.9 atom %; lot no. OU2253) and (^{15}N)-pyridine (99.8 atom %; lot no. NS1035) were purchased from Isotec Inc. 1-(^{13}C)-Glucose (99 atom %; lot no. 061992) was purchased from Omicron Biochemical Inc. 2-(^{2}H)-Tetra-*O*-acetyl-α-D-glucopyranosyl bromide (>95 atom %) was synthesized by the route of Hosie and Sinnott.[25] All labeled and unlabeled α-D-glucopyranosyl pyridinium, isoquinolinium, and 4′-bromoisoquinolinium bromides were synthesized using a previously published procedure.[26]

(^{15}N)-Isoquinolinium Chloride. A solution of ^{15}N ammonium chloride (2.0 g; 36.7 mmol) and sodium acetate (3.28 g) in water (30 mL) was added to a solution containing homophthalaldehyde[27] (5.92 g; 40 mmol) in tetrahydrofuran (30 mL). The resulting mixture was stirred for 30 min at room temperature, the volume was reduced (30 mL) using an aspirator, and the pH of the solution was adjusted to 2. Following extraction of the acidic solution with ether (2 × 30 mL), the aqueous phase was adjusted to pH 8. Dichloromethane (3 × 50 mL) was utilized to extract the labeled isoquinoline. The organic layers were combined, dried ($MgSO_4$), filtered, and concentrated at aspirator pressure to give a brown oil. Kugelröhr distillation at aspirator pressure

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gave a colorless oil (4.2 g; 89%). Dry hydrogen chloride gas was passed into an ice-cold ether (50 mL) solution of the distilled isoquinoline to give the hydrochloride salt as a colorless solid (5.2 g; yield from $^{15}NH_4Cl$ 85%). The isotopic enrichment was judged to be greater than 99% by mass spectral analysis.

(^{15}N)-4-Bromoisoquinoline. A solution of (^{15}N)-isoquinolinium chloride (2.0 g; 12 mmol) and bromine (0.93 mL; 18 mmol) in nitrobenzene (3 mL) was heated to 180 °C for 5 h. After cooling, the reaction mixture was poured into an aqueous sodium bicarbonate solution and extracted with ethyl acetate. The organic layer was dried (Na_2SO_4), filtered, and evaporated to dryness. Purification of the residue by flash column chromatography (silica; 5% ethyl acetate in hexane) gave a colorless solid (2.20 g; 88%): mp 40−42 °C (lit.[28] 41−42 °C).

Enzyme Kinetics. The yeast α-glucosidase suspension in ammonium sulfate was placed in a Centricon-30 microfilter and washed three times with 50 mM phosphate buffer (2 mL, pH = 6.8) by three successive centrifugations (5000 rpm; 45 min). After the final centrifugation, the concentrated enzyme solution (0.5 mL) was stored at 5 °C. Enzyme-catalyzed glycoside hydrolysis kinetics were followed using a Cary-3E UV−vis spectrophotometer equipped with the Cary six cell Peltier constant temperature accessory.

***K*$_M$ and *V*$_{max}$ Measurements.** For all kinetic runs, a stock solution of the enzyme was made by diluting the α-glucosidase concentrate into 50 mM phosphate buffer, pH = 6.8, containing 1 mg/mL BSA. Reactions were initiated by the injection of the stock solution of α-glucosidase into a pre-equilibrated (30 min, 25 °C) 3 mL solution of 50 mM phosphate buffer (pH = 6.8; [BSA] = 1 mg/mL) containing the required substrate concentration. The change in absorbance at 265 or 340 nm was followed as a function of time for, respectively, the pyridinium and the isoquinolinium salts. V_{max} and K_M values were determined by nonlinear least squares fitting of v versus [S] data using the commercial program Enzfitter.

KIE Measurements. The stability of a dilute enzyme solution (containing 1 mg/mL BSA) incubated at 25 °C was evaluated by testing the α-glucosidase activity with the substrate *p*-nitrophenyl-α-D-glucopyranoside at periodic intervals. The measured enzyme activity remained invariant within experimental error over a period of greater than 24 h. Therefore, at V_{max} conditions all KIEs were measured by the addition of a concentrated solution of substrate to a dilute, BSA-containing solution of the enzyme. A typical experimental protocol involved the addition of concentrated substrate solution (50 μL) to an equilibrated α-glucosidase solution (2.95 mL) contained in a 10 mm path-length cell, to give a final substrate concentration of between 7 and 15 times the K_M. For glucosidase-catalyzed hydrolysis of the isoquinolinium salt, the absorbance change was measured at 340 nm, whereas the pyridinium salt reaction was monitored at 270 nm for lower and 273 nm for higher concentrations of the glucoside. The order of the isotopically-labeled compounds assayed was varied (i.e., ^{1}H ^{2}H ^{2}H ^{1}H ^{1}H ^{2}H ^{2}H ^{1}H etc.). Consecutive pairs of kinetic runs were compared, with the quoted isotope effects representing the mean and standard deviation of at least seven such dyads. Bias on the part of the experimenter was eliminated as a possibility by performing a "double-blind" estimation for the k^{12}_C/k^{13}_C KIE on α-glucosidase-catalyzed hydrolysis of **2**. That is, the substrate samples were prepared by and their respective identities were only known to an independent third party. The carbon-13 labeled sample was observed to react more slowly in each of the eight dyads that were used to calculate the KIE and its associated standard error.[6]

Test for Possible Product Inhibition. In order to rule out the involvement of product inhibition, a solution containing both isoquinoline and glucose (50 μL; 6.0 mM) or pyridine and glucose (50 μL; 90 mM) was added to an equilibrated α-glucosidase solution (2.90 mL) in a 10 mm path-length cell, a concentrated solution of **2** (50 μL; 6.63 mM) was added, and the α-glucosidase-catalyzed hydrolysis reaction was monitored by recording the absorbance change at 340 nm.

KIE Measurements for the Spontaneous Hydrolysis of 3. Two separate solutions containing either labeled or unlabeled salt (~1.6 mg/100 mL) in 10 mM phosphate buffer (1:1 NaH_2PO_4:Na_2HPO_4; μ = 1.0, KCl) were prepared, and their respective absorbances at 238.5 nm (10 mm path-length cells) were adjusted to be approximately equal by the addition of buffer to the solution having the higher absorbance. Solutions (3 mL) of unlabeled and isotopically-labeled compounds were placed in an alternating fashion (for example, ^{1}H, ^{2}H, ^{1}H, ^{2}H, ^{1}H, and ^{2}H) into each of the six sample cells in the Peltier constant-temperature accessory of a Cary-3E UV−vis spectrophotometer, and the corresponding labeled or unlabeled isotopic species (^{2}H, ^{1}H, ^{2}H, ^{1}H, ^{2}H, and ^{1}H) were placed into the six reference cells. The experimental time course of absorbance at 238.5 nm was fit to eq 1 using a nonlinear least squares program, where ΔA which represents the absorbance change for complete hydrolysis of the unlabeled substrate was measured in a separate experiment. The remaining kinetic terms ΔB (the change in absorbance for the labeled substrate), Abs_∞ (the absorbance after complete reaction), k (the rate constant for hydrolysis of the unlabeled substrate), and c (the kinetic isotope effect) were treated as variables for the fit of the data to eq 1.

$$Abs(t) = \Delta A \exp(-kt) + \Delta B \exp(-kt/c) + Abs_\infty \qquad (1)$$

In all cases, $\Delta A \sim -\Delta B$ and k was within experimental error of that which was measured in a separate experiment. To reduce the systematic error caused by temperature gradients between the reference and sample compartments, the above series of experiments were repeated using the opposite arrangement of labeled and unlabeled substrates in the various UV−vis cells. The kinetic isotope effect was calculated from pairs of runs obtained for each position in the cell block and using eq 2 where k_L/k_H is the measured kinetic isotope effect and LS and LR denote the position of the unlabeled (light) isotopomer in, respectively, the sample or the reference compartment of the spectrometer.

$$KIE = \sqrt{\left(\frac{k_L}{k_H}\right)_{LS}\left(\frac{k_L}{k_H}\right)_{LR}} \qquad (2)$$

Equilibrium Isotope Effect on Isoquinoline Protonation. A solution containing a 2:1 molar ratio of ^{15}N:^{14}N isoquinolinium chlorides (90 mg) in 10% v/v CH_3CH_2OD:H_2O (10 mL) was prepared, and a portion of this solution (0.6 mL) was placed into 11 standard 0.5 mm NMR tubes. Various amounts of an NaOH solution in 10% v/v CH_3CH_2OH:H_2O were added into ten of the NMR tubes such that the fraction of protonated isoquinoline in the 11 NMR tubes varied from 0 to 100%. An inverse-gated proton decoupling pulse sequence was used to acquire the ^{13}C NMR spectra (150.9 MHz) from the samples which were maintained at a solution temperature of 25.0 ± 0.1 °C. The observed signals were referenced internally to the ^{13}C-methyl signal of the ethanol cosolvent.

Table 1. Observed Kinetic Parameters for the Yeast α-Glucosidase Catalyzed Hydrolysis of **1** and **2** in 50 mM Phosphate Buffer Solutions at pH = 6.8 and Temperature = 25 °C

	K_M (μM) for **1**	K_M (μM) for **2**	k_{cat} (**1**)/k_{cat} (**2**)[a]
this work[b]	97 ± 8	7.7 ± l.l[c]	2.6
ref 23[d]	66 ± 6	6.6 ± 0.6	3.25

[a] k_{cat} values were not determined in the present study, but the quoted values are the relative V_{max} values corrected for the different molar-absorption changes on hydrolysis. [b] [BSA] = 1 mg/mL. [c] A repeat determination gave a value of 7.0 ± 0.6, μM. [d] [BSA] = 0 mg/mL.

Results

In order to measure KIEs on k_{cat} for an enzyme-catalyzed reaction, the kinetic parameters K_M and V_{max} must be determined so that appropriate experimental conditions can be chosen. Table 1 presents the observed catalytic constants for the α-glucosidase-catalyzed hydrolysis of **1** and **2** in 50 mM phosphate buffer containing 1 mg/mL BSA at a pH of 6.8 and a temperature of 25 °C. In addition, the corresponding values reported in a study by Hosie and Sinnott are listed.[25]

KIE experiments were performed at substrate concentrations between 7 and 15 times the K_M value for the substrate. The observed KIEs obtained for the isotopic substitutions 2-^{2}H, 1-^{13}C, and 1′-^{15}N on the yeast α-glucosidase-catalyzed hydroly-

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Table 2. Observed Kinetic Isotope Effects on k_{cat} for the Yeast α-Glucosidase Catalyzed Hydrolysis of **1** and **2** in 50 mM Phosphate Buffer Solutions at pH = 6.8, [BSA] = 1 mg/mL, and Temperature = 25 °C[a]

compd	k_H/k_D	k_{12C}/k_{13C}	k_{14N}/k_{15N}
1	1.119 ± 0.022	$1.028_5 \pm 0.011$	1.018 ± 0.011
	1.115 ± 0.020	1.028 ± 0.010	1.020 ± 0.010
	$1.110_6 \pm 0.009_6$	$1.027_9 \pm 0.008_6$	
	$1.118_3 \pm 0.009_7$		
2	1.101 ± 0.019	1.026 ± 0.011	0.984 ± 0.012
	1.101 ± 0.015	$1.026_7 \pm 0.009_2$	0.978 ± 0.010
	1.102 ± 0.022	$1.026_2 \pm 0.009_5$	0.985 ± 0.022
	1.115 ± 0.015	1.031 ± 0.018[b]	$0.988_6 \pm 0.007_3$

[a] The quoted values are the mean and standard deviation from between seven and ten independent kinetic runs. [b] "Double-blind" measurement.

Table 3. Calculated Kinetic Isotope Effects on k_{cat} for the Yeast α-Glucosidase Catalyzed Hydrolysis of **1** and **2** in 50 mM Phosphate Buffer Solutions Containing BSA (1 mg/mL) at a pH of 6.8 and a Temperature of 25 °C[a]

compd	k_H/k_D	k_{12C}/k_{13C}	k_{14N}/k_{15N}
1	1.115 ± 0.006[b]	1.028 ± 0.006	1.019 ± 0.007
2	1.106 ± 0.009	$1.026_8 \pm 0.005_4$	0.985 ± 0.005

[a] The quoted values are the weighted averages of the mean and standard deviation calculated from the data given in Table 2. [b] The value cited in ref 23 is $1.08_5 \pm 0.02$ (no added BSA).

Table 4. Observed Kinetic Isotope Effects on the Uncatalyzed Hydrolysis of **3** in 10 mM Phosphate Buffer Solutions and Temperature = 80 °C (μ = 1.0, KCl)[a]

k_{H1}/k_{D1}[b]	k_{H2}/k_{D2}[c]	k_{12C}/k_{13C}	k_{14N}/k_{15N}
1.1898	1.0841	1.0029	1.0195
1.1931	1.0947	1.0043	1.0126
1.1914	1.0899	1.0070	1.0160
1.1899	1.0888	1.0022	1.0164
1.1703	1.1106	1.0064	1.0085
1.1962			1.0182

[a] The quoted values are the mean and standard deviation from between seven and ten independent kinetic runs. [b] α-Secondary deuterium kinetic isotope effect. [c] β-Secondary deuterium kinetic isotope effect.

sis of **1** and **2** in 50 mM phosphate buffer ([BSA] = 1 mg/mL; T = 25 °C; pH = 6.8) are presented in Table 2. The best estimate for each KIE and its associated standard deviation was calculated using a standard weighted average method and the data listed in Table 2.[29] Values calculated in this manner are reported in Table 3.

An experimental protocol that is based on the UV−vis spectroscopic method of Rosenberg and Kirsch (see Experimental Section)[30] was utilized in order to measure KIEs on k_{hyd} for the spontaneous hydrolysis of **3**. The KIEs for the isotopic substitutions ^{2}H-1, ^{2}H-2, ^{13}C-1, and ^{15}N-1′ on the spontaneous hydrolysis of **3** at 80 °C are presented in Table 4, while the means and standard deviations of these values are given in Table 5. In addition the observed rate for hydrolysis of **3** at 80 °C (μ = 1, KCl) is $1.35 \pm 0.03 \times 10^{-4}$ s^{-1} (mean and standard deviation of four independent runs) a value that agrees with the rate of $1.73 \pm 0.04 \times 10^{-4}$ s^{-1} at 81.3 °C reported by Hosie *et al.*[26]

The ^{15}N equilibrium isotope effect (EIE) on the acidity constant K_a for isoquinoline-H$^+$ (Scheme 3) was determined so that the KIE data for the α-glucosidase-catalyzed C−N$^+$ bond cleavage reaction could be correlated with the EIE data for deprotonation of the corresponding H−N$^+$ bond. The EIE was

(29) Taylor, J. R. *An Introduction to Error Analysis*; University Science Books: Mill Valley, 1982; pp 147−150.

(30) Rosenberg, S.; Kirsch, J. F. *Anal. Chem.* **1979**, *51*, 1379−1383.

Table 5. Calculated Kinetic Isotope Effects on the Uncatalyzed Hydrolysis of **3** in 10 mM Phosphate Buffer Solutions at a pH of 6.8 and a Temperature of 80 °C (μ = 1.0, KCl)[a,b]

k_{H1}/k_{D1}	k_{H2}/k_{D2}	k_{12C}/k_{13C}	k_{14N}/k_{15N}
1.1885 ± 0.0092	1.0936 ± 0.0102	1.0046 ± 0.0021	1.0152 ± 0.0040

[a] The quoted values are the means and standard deviations (σ_{n-1}) calculated from the data given in Table 4. [b] Values corrected to 25 °C[41] for the ^{2}H-1, ^{2}H-2, ^{13}C-1, and ^{15}N-1′ substituted α-D-glucopyranosyl 4′-bromoisoquinolinium salt are, respectively, 1.227, 1.112, 1.005_4, and 1.018.

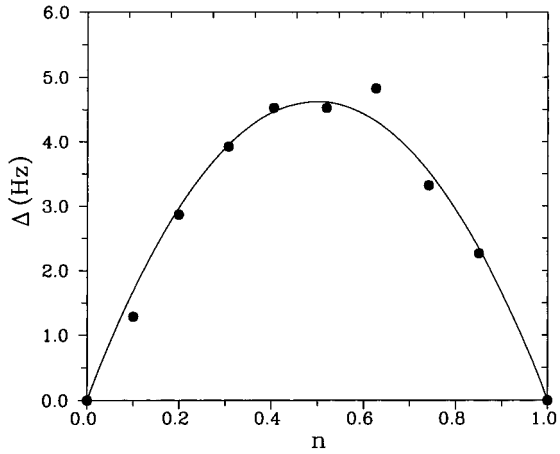
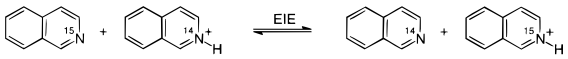


Figure 1. The observed difference (△) between the ^{13}C chemical shifts for C-6 of the ^{14}N and ^{15}N isotopomers of isoquinoline as a function of the fraction (n) of the ^{14}N-isotopomer protonated. The curved line is the calculated best nonlinear fit to eq 3.

Scheme 3



determined using an identical ^{13}C-NMR method to that employed by Rabenstein and Mariappan in the determination of the ^{15}N-EIE for the ionization of glycine (see Experimental Section).[31] The EIE (K_a^{14}/K_a^{15}) can be calculated according to eq 3 if no observable ^{14}N/^{15}N isotope effect occurs on the ^{13}C chemical shifts of either the neutral or the protonated base.[31]

$$\Delta = \frac{Rn\Delta^{14}}{Rn - n + 1} - n\Delta^{14} \qquad (3)$$

In eq 3, Δ is the observed chemical shift difference between the two isotopically labeled isoquinolines ($\delta^{15} - \delta^{14}$), $R = K_a^{14}/K_a^{15}$, $\Delta^{14} = \delta_p - \delta_d$ (where δ_p and δ_d are the observed chemical shifts for the protonated and deprotonated ^{14}N species, respectively), and n is the fractional concentration of the protonated form of the ^{14}N compound.[31] Chemical shift data obtained from the C-6 atom of isoquinoline was used to calculate an EIE of 1.0216 ± 0.0005, while the corresponding data for the C-8 atom gave $K_a^{14}/K_a^{15} = 1.0219 \pm 0.0005$. Figure 1 presents the chemical shift difference (C-6 carbon atom of isoquinoline) between the ^{15}N and the ^{14}N isotopomers as a function of n (fraction of ^{14}N protonated form). In addition, the calculated nonlinear least squares fit to eq 3 is plotted in Figure 1.

Discussion

Yeast α-Glucosidase Kinetic Parameters. In a 1985 study, Hosie and Sinnott reported that the rate-limiting step (k_{cat}) for the baker's yeast α-glucosidase catalyzed hydrolysis of α-D-

(31) Rabenstein, D. L.; Mariappan. S. V. S. *J. Org. Chem.* **1993**, *58*, 4487−4489.

glucopyranosyl pyridinium salts involves C−N bond cleavage.[25] Similar experimental conditions to those utilized by Hosie and Sinnott were employed in the present heavy-atom and secondary deuterium KIE study to ensure that C−N bond cleavage was the rate-limiting step in these reactions. In order to rule out the possibility that product release is partially rate limiting during the α-glucosidase-catalyzed hydrolysis of **1** and **2**, the reaction rate was monitored in the presence and absence of the products of hydrolysis of **1** and **2**. In these experiments the concentration of the reaction products was equivalent to 15 × K_M for the substrate from which they would have originated.[32] The α-glucosidase catalyzed hydrolysis rate of **2** was slowed by approximately 2.5% and 3.5% in the presence of glucose and either isoquinoline or pyridine, respectively. Consequently, the rate of product dissociation from the enzyme is greater than the corresponding rate of substrate dissociation. Therefore, product release for the enzyme is rapid compared to glucosylation of the enyzme, and isotope effects measured on k_{cat} are not modulated by this step.

The catalytic constants (Table 1) measured in the presence of BSA (1 mg/mL) for the baker's yeast α-glucosidase catalyzed hydrolysis of both substrates **1** and **2** are similar to those reported by Hosie and Sinnott,[25] who measured these parameters in the absence of BSA (Table 1). Therefore, under the conditions utilized in the present study, the α-glucosidase hydrolytic activity is similar to that previously reported in the literature.[25] In order to determine a KIE on the rate constant k_{cat} for an enzyme-catalyzed reaction, it is necessary to perform separate rate measurements for highly enriched isotopic substrates.[33,34] The major source of systematic error in the repeated determinations of individual rate measurements (V_{max}) for highly enriched isotopic substrates is variable enzyme concentration. In the present study, these systematic errors were minimized by use of an experimental protocol that called for the addition of a concentrated substrate solution to a dilute enzyme solution. Control reactions verified that the catalytic activity of the dilute baker's yeast α-glucosidase solutions containing BSA was invariant for the time scale of these experiments (see Experimental Section).

When interpreting an observed KIE it is important to remember that the magnitude of the effect depends on both the ground state and the transition state for the reaction being studied. In the case of the kinetic parameter k_{cat}, the reaction of interest is the first-order conversion of the enzyme−substrate (Michaelis) complex into the hydrolyzed products via the rate-limiting TS. For k_{cat}, the ground-state is an enzyme−substrate complex of unknown structure that is capable of many noncovalent interactions. Hence, computer modeling of the observed KIEs on k_{cat} using valence force-field programs such as BEBOVIB would require that more assumptions be made than are necessary for the modeling of the corresponding kinetic parameter k_{cat}/K_M, for which the ground-state is free substrate.

KIE Measurements for the Spontaneous Hydrolysis of 3. Measurement of KIEs on the spontaneous hydrolysis of either **1** or **2** was not practical since the estimated half-time for hydrolysis of **1** at 80 °C is approximately 33 h.[26] Therefore, in order to compare the KIEs measured for both the α-glucosidase-catalyzed and the spontaneous hydrolysis of α-D-glucopyranosides containing a neutral pyridinium leaving group, measurements were made for the isotopomers ^{2}H-1, ^{2}H-2, ^{13}C-1, and ^{15}N-1′ KIEs for the spontaneous hydrolysis of **3** at 80 °C.

Kinetic Isotope Effects: Anomeric Carbon. In 1980, it was stated by Melander and Saunders that "The clearest generalization one can draw about substitution reactions is that carbon isotope effects are substantially lower in S_N1 than in S_N2 reactions.".[13] A comparison of published k^{12}_C/k^{13}_C data for S_N1 reactions that generate carbenium ions with the analogous KIEs for glycosyl fluoride reactions where a weak nucleophilic interaction is present at the TS indicates that this statement must be qualified for dissociative reactions that are either S_N1 or S_N2 with a weak nucleophilic component. A general trend in observed carbon-KIEs for S_N1 reactions is that the more stable a carbenium ion intermediate, the smaller the measured KIE. For example, the equilibrium isotope effect (K^{12}_C/K^{13}_C) for the formation of the trityl cation by ionization of triphenylmethyl chloride in SO_2 at 0 °C is 0.9833 ± 0.0032,[35] whereas solvolysis of 1-phenylethyl bromide (via the 1-phenylethyl carbenium ion) in both methanol (25 °C) and ethanol (45 °C) gives observed k^{12}_C/k^{13}_C values of 1.0065.[36] Furthermore, within a series of substituted 1-phenylethyl bromides a correlation exists between the observed k^{12}_C/k^{13}_C value for methanolysis (0 °C)[37] and the estimated lifetime of the resultant carbenium ion intermediate (R^+) that forms in 50:50 v/v TFE:H_2O solutions.[38] Specifically, for the 4-methyl, the unsubstituted, and the 4-bromo substituted 1-phenylethyl bromides the reported ^{13}C KIEs[37] are 0.9995, 1.0050, and 1.0127, respectively, while the corresponding lifetimes in 50:50 v/v TFE:H_2O[38] are 2.5 × 10^{-10} s, 1 × 10^{-11} s, and 5 × 10^{-12} s.[39]

In addition, the anomeric carbon-KIE for the spontaneous reaction of 3 is 1.005 (80 °C), and this value is similar to the reported value of 1.007 for the specific-acid catalyzed hydrolysis of methyl α-D-glucopyranoside at 80 °C.[19] Both of these ^{13}C-KIE values are consistent with the analysis that glucopyranosides that contain neutral leaving groups hydrolyze by $D_N + A_N$ mechanisms,[18,19] via an oxocarbenium ion that has an estimated lifetime of 1−3 × 10^{-12} s^{-1}.[16,18]

In contrast, Zhang *et al*. reported that the anomeric ^{13}C-KIE (k^{12}_C/k^{13}_C) for the reaction of α-D-glucopyranosyl fluoride with azide ion is 1.085 and with water is 1.032.[17] Zhang *et al*. analyzed the KIEs for the hydrolysis of α-D-glucopyranosyl fluoride using the bond-order method of Sims and Lewis[14,40] and concluded that the reaction is concerted. Furthermore, these authors argued that the transition state for α-D-glucopyranosyl fluoride reaction with water is comprised of both nearly complete C−F bond cleavage and a small degree of C−O bond making. Although the charge difference at the anomeric center during the hydrolysis reactions of **3** and α-D-glucopyranosyl fluoride must be small, both TSs are highly dissociative, their respective ^{13}C-KIEs are markedly different where the reaction containing a small nucleophilic component displays the larger KIE.

Furthermore, the reaction of α-D-glucopyranosyl fluoride with azide ion, a reaction that manifests a Swain−Scott sensitivity parameter (s) of 0.18,[16] exhibits a substantial ^{13}C-KIE of 1.085 despite the large charge that must be present at the TS on the

(32) During the measurement of KIEs for the α-glucosidase-catalyzed reactions less than 10% of the substrate hydrolyzed during the measurements, so that the concentration of products in each kinetic run was at least 10-fold lower than that used in these experiments.

(33) Reference 10, pp 298−303.

(34) Recently, a new technique was introduced to measure KIEs on k_{cat}: Xue, H.; Wu, X.; Huskey, W. P. *J. Am. Chem. Soc.* **1996**, *118*, 5804−5805.

(35) Kresge, A. J.; Lichtin, N. N.; Rao, K. N.; Weston, R. E. *J. Am. Chem. Soc.* **1965**, *87*, 437−445.

(36) Stothers, J. B.; Bourns, A. N. *Can. J. Chem.* **1960**, *38*, 923−935.

(37) Bron, J.; Stothers, J. B. *Can. J. Chem.* **1969**, *47*, 2506−2509.

(38) Richard, J. P.; Rothenberg, M. E.; Jencks, W. P. *J. Am. Chem. Soc.* **1984**, *106*, 1361−1372.

(39) The lifetime of the 4-bromo derivative was not estimated, but the given lifetime is for the 3-methoxy compound which has a similar σ value, 3-methoxy (σ = 0.12), 4-bromo (σ$^+$ = 0.15). For a compilation of σ values see ref 11, pp 152−153.

(40) Sims, L. B.; Lewis, D. E. In *Isotopes in Organic Chemistry*; Buncel, E., Lee, C. C., Eds.; Elsevier: Amsterdam, 1984; Vol. 6, pp 161−259.

anomeric carbon. Therefore, it can be concluded that the magnitude of the anomeric ^{13}C-KIE is a sensitive probe for the occurrence of a nucleophilic component but is insensitive to small changes in charge, at the TS for pyranoside substitution reactions.

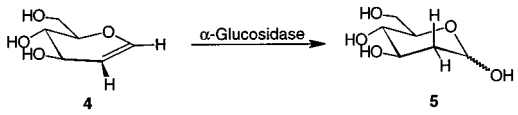
The measured anomeric carbon ^{13}C-KIEs for the α-glucosidase-catalyzed hydrolysis of both **1** and **2** (Table 3) are of a similar magnitude to the values reported for hydrolysis of α-D-glucopyranosyl fluoride in aqueous solution.[17] In addition, the similarity of these KIE values for the α-glucosidase-catalyzed hydrolysis reactions to the reported ^{13}C-KIE values on k_{cat}/K_M for the nucleoside hydrolase *(Crithidia fasciculata)* catalyzed hydrolysis of inosine (*vide supra*)[20] suggests that the α-glucosidase-catalyzed hydrolyses reported herein proceed via S_N2 mechanisms, albeit with a small degree of nucleophilic participation at the reaction TS. In addition, the measured KIEs for α-glucosidase catalyzed hydrolysis of both **1** and **2** are significantly larger ($>2\sigma$) than the corrected value obtained for the sugar beet seed α-glucosidase catalyzed hydrolysis of α-D-glucopyranosyl fluoride ($k^{12}_C/k^{13}_C \approx 1.007 \pm 0.004$).[15] These data indicate that at the TS for baker's yeast α-glucosidase-catalyzed hydrolysis of α-D-glucopyranosyl pyridinium salts, cleavage of the glucosidic C−N bond occurs with nucleophilic assistance from the active-site carboxylate group.

Kinetic Isotope Elects: α-Secondary Deuterium. The measured α-SDKIE value of 1.189 for the spontaneous hydrolysis of α-D-glucopyranosyl 4′-bromoisoquinolinium bromide (**3**) is significantly larger than the value of 1.137[19] reported for the specific acid-catalyzed hydrolysis of methyl α-D-glucopyranoside, despite previously stated conclusions that both of these compounds react via a dissociative transition state that has no nucleophilic component.[18,19,26] However, these two α-SDKIE values are on either side of the value of 1.154 (corrected to 80 °C[41]) for the reaction of α-D-glucopyranosyl fluoride with azide ion, a reaction that is characterized by a concerted S_N2 reaction, for example, inversion of configuration at the anomeric carbon. The similarity of these three α-SDKIEs serves to emphasize the previous conclusion that, for dissociative type reactions, α-secondary deuterium KIEs cannot be used to distinguish between unimolecular and bimolecular transition states.

Kinetic Isotope Effects: *β*-Secondary Deuterium. The measured values for the β-SDKIEs on the baker's yeast enzyme-catalyzed hydrolysis of both α-D-glucopyranosyl pyridinium and isoquinolinium bromides (**1** and **2**) and for the spontaneous hydrolysis of **3** are large and normal (i.e., $k_H/k_D > 1$, Table 3).

Although, these measured values on k_{cat} for the α-glucosidase-catalyzed hydrolysis reactions of **1** and **2** and the uncatalyzed hydrolysis of **3** are indistinguishable, they are larger than the corresponding values reported for (1) the specific-acid catalyzed hydrolysis of methyl α-D-glucopyranoside ($k_H/k_D = 1.087$[19,41]); (2) the weak nucleophilic reaction of water with α-D-glucopyranosyl fluoride ($k_H/k_D = 1.080$[17]); and (3) the sugar beet α-glucosidase-catalyzed hydrolysis of α-D-glucopyranosyl fluoride ($k_H/k_D \approx 1.01$,[15] corrected from the observed k_H/k_T value of 1.013 ± 0.003[14]). Since the principal cause of a β-SDKIE is hyperconjugative overlap of the bonding electrons of a C−H/D bond into the p-type orbital of a neighboring nascent carbenium ion, the observed magnitude is dependent on both the overlap-geometry and the charge development on the reaction center at the transition state.[10] A maximal, β-SDKIE will be observed for a "late" transition state when overlap of the C−H bond and the p-orbital is optimized, that is, when the C−H bond and the p-orbital are coplanar.[10] Conversely, a minimal value will be observed when either little charge has developed at the transition state or when the C−H bond and the p-orbital are orthogonal.[10] The similarity of the two β-SDKIE values for the acid-catalyzed hydrolysis of methyl α-glucopyranoside and the uncatalyzed hydrolysis of α-glucopyranosyl fluoride (1.087 and 1.080, respectively) suggests that similar to α-SDKIE measurements, β-SDKIEs cannot be used to distinguish between unimolecular and bimolecular transition states where the charges on the reaction center are similar. In contrast, when compared to the baker's yeast α-glucosidase-catalyzed reaction, the minuscule β-SDKIE value for the beet seed α-glucosidase-catalyzed reaction indicates that the two enzyme-catalyzed reactions must have different dihedral angles between the C−H bond and the p-orbital of the anomeric carbenium ions, resulting in different glucosyl ring conformations for the two TSs. Circumstantial support for the presence of different glucosyl ring conformations at the TSs for yeast versus beet seed α-glucosidase-catalyzed hydrolysis reactions is provided by the different reactivity of these two enzymes toward glucal hydration (Scheme 4).[42]

Scheme 4



The substrate glucal (**4**) is conformationally restricted by the presence of a 1,2-endocyclic double bond which limits the number of accessible glucosyl ring conformations. This substrate is catalytically hydrated by the sugar beet seed α-glucosidase, an enzyme which is proposed to hydrolyze glucosides via a flattened $^4C_1$ conformation,[14] whereas the yeast α-glucosidase which is proposed to hydrolyze glucosides via a boat conformation[25] is catalytically inactive toward glucal hydration.[42]

Kinetic Isotope Effects: Leaving Group Nitrogen. The observed $k^{14}_N/k^{15}_N = 1.019$ for the baker's yeast α-glucosidase-catalyzed hydrolysis of α-D-glucopyranosyl pyridinium bromide (**1**) is of a similar magnitude to the EIE ($K_a^{14}/K_a^{15} = 1.0211 \pm 0.0004$[43]) for the dissociation constant of the conjugate acid of pyridine in water. This nitrogen-15 KIE value and the measured value for the ^{15}N-KIE of 1.018 (corrected to 25 °C[41]) on the spontaneous hydrolysis of α-D-glucopyranosyl 4′-bromoisoquinolinium bromide (**3**) are consistent with extensive C−N bond cleavage at the respective transition states. However, the observed $k^{14}_N/k^{15}_N = 0.985 \pm 0.005$ for the hydrolysis of α-D-glucopyranosyl isoquinolinium bromide is markedly different than the corresponding value for the pyridinium salt (**1**). The EIE for the ionization constant of isoquinolinium was measured in order to detect possible perturbation of the nitrogen atom by the fused aromatic ring (Scheme 3). Within experimental error, the evaluated EIE ($K_a^{14}/K_a^{15} = 1.0216 \pm 0.0005$) is identical to that obtained for pyridinium deprotonation,[43] a result that is not surprising considering the similarity of the two ionization constants [pK_a (pyridine-H$^+$) = 5.20, pK_a (isoquinoline-H$^+$) = 5.38[44]]. At a pH of 6.8, less than 4% of the isoquinoline in solution is protonated, a result that indicates that the observed greater rate of change in absorbance for the ^{15}N-isotopomer of **2** cannot be attributed to the formation of different percentages of protonated products. Instead, yeast α-glucosidase converts the ^{15}N-**2** enzyme−substrate complex to products faster than it

(41) Corrected assuming that $T \times \ln(k_H/k_D) =$ constant.

(42) Chiba, S.; Brewer, C. F.; Okada, G.; Matsui, H.; Hehre, E. J. *Biochemistry* **1988**, *27*, 1564−1569.
(43) Kurz, J. L.; Pantano, J. E.; Wright, D. R.; Nasr, M. M. *J. Phys. Chem.* **1986**, *90*, 5360−5363.
(44) Perrin, D. D. *Dissociation Constants of Organic Bases in Aqueous Solution*; Butterworths: London, 1965.

does the ^{14}N-**2** E−S complex. It has been proposed that a critical feature of the catalytic mechanism of glycosidases in the hydrolysis of pyranosides is distortion of the ground-state $^4C_1$ conformation toward a conformation that allows significant orbital interaction of the endocyclic oxygen atom and the forming p-type orbital of the oxocarbenium ion-like transition state.[7] Crystallographic evidence for this ring distortion has been published for a substrate analog that is bound to the active-site of the β-glycosidase enzyme lysozyme.[7] Accordingly, any conformational change that occurs on binding of the substrate to the enzyme to give the E−S complex will be expressed in the kinetic parameter k_{cat} (*vide supra*). In comparison to the binding of normal polysaccharide substrates which contain a flexible C−O−C linkage, the rigid C−N glycosidic linkage of glucopyranosyl isoquinolinium ions provides for greatly reduced degrees of freedom when binding to an enzyme active site.

A common feature of the active site of glycosidases and the binding region of sugar-binding proteins is the presence of several tyrosine and tryptophan residues,[1c] aromatic amino acid residues that can bind to the hydrophobic faces (above and below the ring) of carbohydrates. Similarly, aromatic glycoside substrates such as α-D-glucopyranosyl isoquinolinium ion can form strong intermolecular $\pi-\pi$ stacking interactions between the isoquinoline ring and the aromatic amino acid residues present in the active site of the glucosidase. Consequently, these $\pi-\pi$ stacking interactions in the Michaelis complex between the enzyme and the more rigid α-D-glucopyranosyl isoquinolinium ion substrate may lead to partial deformation of the isoquinolinium ring, in addition to effecting a pyranosyl ring conformational change. Distortion of the isoquinolinium ring would serve to reduce the overlap of the nitrogen p-orbital within the aromatic system which would, in turn, weaken the bonding interactions at the nitrogen atom. Subsequent cleavage of the glycosidic C−N bond would remove the aromatic distortion and thus give an inverse ^{15}N-KIE. Although speculative at this stage, the inverse ^{15}N-KIE observed for the α-glucosidase-catalyzed hydrolysis of **2** provides circumstantial evidence for substrate distortion at the stage of the initial Michaelis complex.

Transition State Differences, The Effect of Leaving Group. The substitution mechanism for α-D-glucopyranosyl fluoride changes from a concerted (S_N2) reaction in aqueous solution under nonenzymatic conditions,[16,17] to a dissociative (S_N1) reaction for the sugar beet α-glucosidase catalyzed hydrolysis.[14] For the enzyme-catalyzed reaction, the general-acid catalyzed assistance of fluoride departure provided by the active-site carboxylic acid must itself be sufficient for catalytic activity, with no additional nucleophilic assistance required from the active-site carboxylate during the carbon−fluorine bond cleavage. The equivalent general-acid catalyzed cleavage of α-D-glucopyranosyl fluoride by phosphate buffers has been observed in aqueous solution.[45] In contrast, the spontaneous hydrolysis of α-D-glucopyranosyl pyridinium salts proceeds through a dissociative mechanism (S_N1),[18,19,26] while yeast α-glucosidase-catalyzed hydrolysis of α-D-glucopyranosyl pyridinium salts occurs with nucleophilic assistance provided for C−N bond cleavage at the TS. The rate acceleration for enzyme-catalyzed hydrolysis of the C−N bond in glycopyranosyl pyridinium salts generally lies between 10^9 and 10^{12} (k_{cat}/k_{uncat}).[4] The enzyme-induced rate acceleration is in part accounted for by a degree of nucleophilic participation in conjunction with electrostatic stabilization by the active site carboxylate group at the reaction transition state, interactions that are absent for the analogous reaction that occurs in aqueous solution.

(45) Banait, N. S.; Jencks, W. P. *J. Am. Chem. Soc.* **1991**, *113*, 7958−7963.

Conclusions

The particular mechanism that occurs for the enzyme-catalyzed cleavage of glycosidic bonds is dependent on the nature of the leaving group. Yeast α-glucosidase catalysis of C−N bond cleavage for α-D-glucopyranosyl pyridinium salts is assisted by an enzyme active site nucleophile. In comparison, the sugar beet seed α-glucosidase-catalyzed C−F bond cleavage of α-D-glucopyranosyl fluoride requires no nucleophilic assistance during the C−F bond cleavage event. For the class of glycosidase enzymes studied in this report, the natural carbohydrate substrates are hydrolyzed by a mechanism that involves general-acid catalysis (protonation is occurring at the rate-limiting step for C−O bond cleavage). Therefore, the leaving group in glycosidase-catalyzed hydrolysis of these natural substrates is neither anionic (F^-) nor neutral (pyridine) but is instead a partially deprotonated alcohol. The results from this paper clearly indicate that the degree of nucleophilic assistance provided by the enzyme for hydrolysis of one substrate cannot necessarily be assessed by extrapolation from hydrolysis results obtained from another substrate. Specifically, the natural substrate of a glycosidase will yield a leaving group that at the reaction TS carries a partial negative charge. This type of leaving group is in contrast to a neutral leaving group which is generated during the hydrolysis of glucopyranosyl pyridinium salts or the anionic leaving group that develops during the hydrolysis of glucopyranosyl fluoride hydrolysis. In all cases the nucleophilic assistance provided by the enzyme will be contingent on the nature of the particular leaving group involved in the reaction.

In summary, the intimate mechanistic details of glycosidase catalyzed hydrolysis reactions depends on the nature and charge of the leaving group.

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Supporting Information Available: Experimental details (3 pages). See any current masthead page for ordering and Internet access instructions.

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