Mechanism of *Agrobacterium* β-Glucosidase: Kinetic Studies†

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Received March 27, 1992; Revised Manuscript Received July 30, 1992

ABSTRACT: The β-glucosidase from *Agrobacterium faecalis* (previously *Alcaligenes faecalis*) has been subjected to a detailed kinetic investigation with a range of substrates to probe its specificity and mechanism. It has a relatively broad specificity for the substrate sugar moiety and exhibits a classical pH dependence for its kinetic parameters with three different substrates and an identical pH dependence for its inactivation by a mechanism-based inactivator, cyclophellitol. Measurement of k_{cat} and K_m values for a series of aryl glucoside substrates has allowed construction of a Bronsted plot, the concave-downward shape of which is consistent with the anticipated two-step mechanism involving a glucosyl–enzyme intermediate which is formed and hydrolyzed via oxocarbonium ion-like transition states. The slope of the leaving group-dependent portion of the Bronsted plot ($\beta_{lg} = -0.7$) indicates a large degree of bond cleavage at the transition state. Secondary deuterium kinetic isotope effects measured for five different aryl glucosides are also consistent with this mechanism and further suggest that the transition state for formation of the glucosyl–enzyme intermediate, probed with the slower substrates for which $k_H/k_D = 1.06$, is more S_N2-like than that for its hydrolysis (for which $k_H/k_D = 1.11$). Reasons for this difference are proposed, and values of K_i for several ground-state and transition-state analogue inhibitors are presented which support the concept of sp^2-hybridized transition states.

Structure/function studies on glycosidases to date have been hampered by difficulties either with the protein itself or with the substrates for it. Thus although a number of glycosidases have been studied in some detail either structurally or mechanistically, in no case have the two been satisfactorily combined [see the following for authoritative reviews: Legler (1990) and Sinnott (1990, 1987)]. Undoubtedly the best structural information available on a glycosidase is that on lysozyme (Imoto et al., 1972); however, detailed mechanistic studies on that enzyme have proved extremely difficult due to the complexity of the substrate and therefore the enormous amount of synthetic chemistry needed. These problems have also dogged efforts to probe its mechanism through site-directed mutagenesis (Malcolm et al., 1989; Anand et al., 1988). Details of the mechanism therefore remain obscure. Probably the most extensive mechanistic characterization has been performed on the (*lac z*) β-galactosidase from *Escherichia coli*, including studies of pH/activity dependence (Tenu et al., 1971), kinetic isotope effects (Sinnott & Souchard, 1973), linear free energy relationships (Sinnott & Withers, 1974), nucleophilic competition (Sinnott & Viratelle, 1973), alternate substrates and inhibitors (Lalegerie et al., 1982), affinity labels and suicide inactivators (Legler, 1990), and also site-directed mutagenesis (Cupples & Miller, 1988; Cupples et al., 1990). However, no significant progress has been made on the determination of its three-dimensional structure, despite its early crystallization (Wallenfels & Malhotra, 1961), presumably at least in part due to its very large size, a tetramer of monomer molecular weight 116 353. Other glycosidases which have been subjected to an almost comparable level of mechanistic investigation are the β-glucosidases from almond emulsin (Dale et al., 1985, 1986) and *Aspergillus wentii* (Legler et al., 1980). Unfortunately commercial sources of the almond enzyme contain at least two isoenzymes with different kinetic properties, and many of the published studies were performed with this mixture. Interpretations of such kinetic studies should therefore be made with caution; in addition, the enzyme is rather large (135 000) for structural studies. All work on the *A. wentii* enzyme has been performed on a single isoenzyme. However, the high molecular weight (170 000) and the heavy glycosylation of this enzyme, coupled with problems in its availability, make this an unlikely target for structural studies. A number of other glycosidases have been studied to varying extents, but the best-studied of these are polysaccharide-cleaving enzymes such as amylases and cellulases which suffer from many of the problems referred to with lysozyme. Structural information is available for several, but relatively little detailed mechanistic work has been done.

A clear need therefore exists for a study with a glycosidase of modest molecular weight and substrate requirements which is free of isoenzyme problems and available in a cloned and expressed form, thereby enabling both the production of large quantities of protein for structural studies and the application of techniques of mutagenesis. Such an enzyme is the β-glucosidase from *Agrobacterium faecalis* (previously typed as *Alcaligenes faecalis*). The complete purification of this enzyme from *Agrobacterium* has been described (Day & Withers, 1986), as has the cloning and expression in *E. coli* (Wakarchuk et al., 1986). In addition, several other communications on aspects of its mechanism have been published recently (Withers et al., 1987, 1988a,b, 1990). Characterization of the wild-type enzyme isolated from *Agrobacterium* revealed that it is dimeric, with a monomer molecular mass of 50 kDa. The substrate specificity of the wild-type enzyme was found to be relatively relaxed (Day & Withers, 1986), and the enzyme was shown to be capable of cleaving not only carbon–oxygen glycosidic linkages but also carbon–sulfur, carbon–nitrogen, and carbon–fluorine bonds. Subsequent sequence determination (Wakarchuk et al., 1986) has confirmed the monomer molecular mass of 50 kDa arising from 458 amino acids. This paper describes an extensive mechanistic study of the enzyme confirming that the wild-type and

† This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada.

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0006-2960/92/0431-9961$03.00/0

cloned enzymes have identical kinetic properties and providing insights into details of the mechanism. A subsequent paper describes investigations of the interactions of 2-substituted glycosides with the enzyme which have resulted in the identification of a key amino acid residue in the catalytic mechanism. Further insights into the role of that residue obtained through detailed analysis of mutants are described in the third paper in the series.

MATERIALS AND METHODS

Materials. All *o*-nitrophenyl, *p*-nitrophenyl, and phenyl glycosides, isopropyl β-D-thioglucopyranoside, 1-deoxynojirimycin, 1,6-anhydro-β-D-glucopyranose, gluconolactone, and all buffer chemicals were obtained from Sigma Chemical Co. Phenols were obtained from either Sigma Chemical Co., Aldrich Chemical Co., or Fluka. Gluconohydroximolactone and gluconophenylurethane were kind gifts of Dr. Andrea Vasella. β-D-Glucosylamine was synthesized from D-glucose as previously described (Cusack et al., 1973) as was glucosyl benzene (Bonner, 1963). 2′,4′-Dinitrophenyl β-D-glucopyranoside was synthesized from 2,3,4,6-tetra-*O*-acetyl D-glucopyranose and fluoro-2,4-dinitrobenzene according to published procedures (van Boom et al., 1980; Ballardie et al., 1973). Synthesis of all other protected aryl glucosides was achieved from 2,3,4,6-tetra-*O*-acetyl α-D-glucopyranosyl bromide and the sodium salt of the corresponding phenol using the aqueous acetone method (Conchie & Levvy, 1963). Deprotection of sensitive glycosides (phenol $pK_a < 6$) was achieved using HCl in dry methanol (Ballardie et al., 1973) while other glycosides were deprotected using sodium methoxide in methanol (Sinnott & Souchard, 1973) with the exception of acetylated 2′,4′,6′-trichlorophenyl β-D-glucopyranoside which was deprotected using AG1-X8 (OH^-) resin in dry methanol (Reed et al., 1981). All products were obtained in crystalline form and characterized by melting point, ^1H NMR, and elemental analysis. These analyses were consistent with the expected structure and literature data when available in each case. Melting points determined for the deprotected substrates are as follows: 4′-chlorophenyl β-D-glucopyranoside, 172–174 °C [lit. mp 173–175 °C] (Nath & Rydon, 1954); 4′-bromophenyl β-D-glucopyranoside, 175–176 °C; 4′-*tert*-butylphenyl β-D-glucopyranoside, 145–147 °C [lit. mp 145–146 °C] (Nath & Rydon, 1954); 4′-cyanophenyl β-D-glucopyranoside, 188–189 °C [lit. mp 193–194 °C] (Nath & Rydon, 1954); 3′-nitrophenyl β-D-glucopyranoside, 166–168 °C [lit. mp 166–168 °C] (Nath & Rydon, 1954); β-naphthyl β-D-glucopyranoside, 184–186 °C; 3′,5′-dichlorophenyl β-D-glucopyranoside, 215–217 °C; 2′,5′,6′-trichlorophenyl β-D-glucopyranoside, 190–194 °C; 2′,5′-dinitrophenyl β-D-glucopyranoside, 166–168 °C; 3′,4′-dinitrophenyl β-D-glucopyranoside, 152–153 °C; 4′-chloro-2′-nitrophenyl β-D-glucopyranoside, 159–160 °C.

Synthesis of deuterated aryl β-D-glucopyranosides was achieved by exactly the same route, starting from 1,2,3,4,6-penta-*O*-acetyl β-D-[1-^2H]glucopyranose which was itself synthesized according to the published procedure (Berven & Withers, 1986). Melting points of products obtained were identical to those of the corresponding protiated material in each case, and ^1H NMR spectra were identical except for the absence of the anomeric proton and related couplings. Careful integration of the region of the spectrum where the anomeric proton should resonate revealed no more than 2% contamination of the deuterated material with protiated compound.

Recombinant *Agrobacterium* β-glucosidase was purified from *E. coli* by a modification of the method utilized for isolation of the native enzyme from *Agrobacterium* (Day & Withers, 1986), as follows. Protein was expressed in *E. coli* JM101 from the lac promoter of pTZ18R. Cells were grown overnight in 2 mL of LB amp at 37 °C. One milliliter of a culture was used to inoculate 1 L of M56 salts (Gerhardt et al., 1981) supplemented with 0.6% (v/v) glycerol, 0.2% casein, and 100 μg/mL ampicillin. Cultures were grown for 6 h, transferred to a 110-L fermentor (L. H. Fermentation) containing 60 L of the same medium plus 0.1 mM IPTG, and grown until $2OD_{600}$. Cells were harvested by Sharples continuous centrifugation at 31000*g*, and the cell paste was stored at −20 °C. All subsequent manipulations were carried out at 4 °C except for the Pharmacia FPLC chromatography, which was performed at room temperature. Cell extracts were prepared by grinding with 2.5 times their weight in alumina powder (Schleif & Wensink, 1981). The extraction buffer was 25 mM sodium phosphate, pH 7.0, 10 mM 2-mercaptoethanol, and the alumina pelleted by centrifugation at 5000*g* for 10 min. Nucleic acids were removed from the crude extract by precipitation with 1.5% streptomycin sulfate (3 h at 4 °C) followed by centrifugation (20 min, 17000*g*). The clarified extract was loaded onto a column of DEAE Sephacel (48 cm × 2.6 cm) which had previously been equilibrated with 50 mM sodium phosphate buffer, pH 7.0. This column was eluted with a 2 × 1 L linear gradient of 0–1 M sodium chloride in the starting buffer. Fractions containing β-glucosidase were pooled and dialyzed overnight against 50 mM sodium phosphate buffer, pH 7.00, and then after filtration loaded onto a Q-Sepharose column (10.5 cm × 1.6 cm) and eluted with a 2 × 1 L linear gradient of 0–1 M NaCl in 50 mM sodium phosphate buffer, pH 7.0. Fractions containing β-glucosidase were pooled and concentrated using Amicon Centriprep 10 centrifuged ultrafiltration devices. After centrifugation (18000*g*, 10 min) to clarify, the concentrated protein (10–20 mg mL^{-1}) was loaded onto a Sephacryl S-200 high-resolution column (53 cm × 2.6 cm) equilibrated with 50 mM sodium phosphate buffer, pH 7.00. Fractions containing the highest specific activity protein were pooled and stored at 4 °C in the presence of 1 mM sodium azide. The final purification step produced β-glucosidase judged to be homogeneous by SDS–PAGE. Protein concentration was determined using the absorbance value of $E_{280}^{0.1\%} = 2.20$ cm^{-1}, a value obtained by measurement of the absorbance at 280 nm of an enzyme solution whose concentration had been accurately determined by quantitative amino acid analysis in the presence of a known concentration of norleucine.

Kinetic Studies. All kinetic studies were performed by following changes in UV/vis absorbance using matched quartz cells in a Pye-Unicam PU-8800 spectrophotometer equipped with a circulating water bath which maintained the cells at a temperature of 37 °C. Cells of 1-cm path length were employed for all experiments. The buffer employed for all kinetic experiments (apart from those involving pH variation) was 50 mM sodium phosphate buffer, pH 6.8, containing 0.1% BSA. Extinction coefficients for phenols and glycosides were determined by measuring the absorbances of carefully prepared stock solutions of each compound in the same buffer at 37 °C. Extinction coefficient differences for each glycoside/phenol combination at the wavelength of interest were confirmed by measuring the change in absorbance of a known concentration of each glycoside after the addition of a small volume of β-glucosidase. The wavelengths employed (nm) and molar extinction coefficient differences determined ($\Delta\epsilon$, M^{-1} cm^{-1}) at that wavelength for each glucoside were as follows: 2′,4′-dinitrophenyl, 400 nm, 10 910; 2′,5′-dinitro-

phenyl, 440 nm, 4288; 3′,4′-dinitrophenyl, 400 nm, 11 009; 4′-chloro-2′-nitrophenyl, 425 nm, 3546; 2′,4′,6′-trichlorophenyl, 312 nm, 2736; 4′-nitrophenyl, 400 nm, 7280; 2′-nitrophenyl, 400 nm, 2170; 3′,5′-dichlorophenyl, 280 nm, 732; 3′-nitrophenyl, 380 nm, 385; 4′-cyanophenyl, 270 nm, 3101; 4′-bromophenyl, 288 nm, 680; 4′-chlorophenyl, 278 nm, 580; β-naphthyl, 325 nm, 816; phenyl, 277 nm, 778; 4′-*tert*-butylphenyl, 272 nm, 725.

Rates of enzyme-catalyzed hydrolysis were determined by incubating the appropriate concentration of substrate in buffer containing BSA at 37 °C in a 1-cm cuvette located in the thermostated block of the spectrophotometer. Reaction was initiated by the addition of enzyme (in BSA-containing buffer) from a syringe, and the reaction was monitored at the appropriate wavelength. Rates were determined at 7–10 different substrate concentrations ranging from approximately 0.15 times the value of the K_m ultimately determined to 7 times its value. Values of K_m and k_{cat} were determined from these rates by means of a nonlinear regression analysis (Wilkinson, 1961). K_i values for inhibitors were determined by first estimating the approximate K_i value by using a "range-finding" assay at fixed (0.1 mM) PNPGlu[1] concentration and widely varied inhibitor concentration and plotting the data in a Dixon plot, for which the intersect with the horizontal line drawn at $1/V_{max}$ gives an approximate value of K_i. A full K_i determination was then carried out at a series of six different substrate concentrations bracketing the K_m value with each of five inhibitor concentrations bracketing the approximate K_i value. The nonlinear regression analysis program GraFit (Leatherbarrow, 1990) was used for fitting of all such data.

Isotope effects were determined by comparison of the initial rates of hydrolysis of high (10 times the K_m value) concentrations of protio and deuterio substrates determined spectrophotometrically. Quartz cells were filled with the appropriate concentration of diluted enzyme and incubated at 37 °C, reaction being initiated by the addition of a small volume (50–100 μL) of (thermally equilibrated) substrate. Rates of protio and deuterio substrate hydrolysis were determined in alternation until a total of 9 or 10 rates for each (protio and deuterio) substrate had been measured. Average rates for the protio and deuterio substrates were then calculated, and the rate was taken to give the isotope effect. Rate ratios were also determined at higher substrate concentrations (20 times K_m when possible) and found to be the same as those determined at the lower concentration in all cases, thus demonstrating that the effect measured is not a trivial consequence of the presence of inhibitory impurities in one sample.

The stability of the enzyme at different pH values was estimated by incubating samples of the enzyme in 50 mM sodium phosphate/145 mM sodium chloride buffer at a series of pH values, removing aliquots at different times and assaying under standard conditions. Subsequent determinations of k_{cat} and K_m were then only made at pH values at which the enzyme was stable (>95% activity retained) over a 5-min period. The pH dependence of the enzyme was investigated by determining k_{cat} and K_m values using stopped assays at a series of pH values between 5.5 and 8.8. These involved incubating the substrate (0.9 mL) in 50 mM sodium phosphate/145 mM sodium chloride at the desired pH value until the temperature had stabilized at 37 °C and then adding thermally equilibrated enzyme (0.01 mL) to start the reaction. After 5 min (when reaction had proceeded to less than 5–10% completion), the reaction was terminated by the addition of 1.2 M NaOH (0.025 mL), causing the pH to rise to approximately 11. The extent of reaction was determined by quantitating the phenolate released spectrophotometrically. In order to ensure that pH values had not changed significantly during reaction, the pH values of a series of mock reaction mixtures containing the highest substrate concentration employed at each starting pH value were monitored directly for the 5-min period. No significant changes were observed, even at the pH extremes. The pH dependence of the inactivation by cyclophellitol was determined by measuring the inactivation rate constants (k_i and K_i) exactly as described previously (Withers & Umezawa, 1991) at a series of pH values covering the same pH range as above. All assays were performed under standard conditions. Values of pK_a's were assigned by fitting to the appropriate equations using the program GraFit (Leatherbarrow, 1990).

Nucleophilic competition experiments were performed by measuring rates of reaction at different substrate concentrations in the presence and absence of 0.1 M DTT, thereby determining k_{cat} and K_m values. Since DTT itself significantly increases the rate of breakdown of substrates, it was necessary to perform careful controls for each reaction in which no enzyme was added and thereby correct the observed rate for this background. Enzyme concentrations were increased in experiments where the background hydrolysis was significant in order to minimize this source of error.

Table I: Michaelis–Menten Parameters for the Hydrolysis of Aryl Glucosides by *Agrobacterium* β-Glucosidase

phenyl glycoside substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
4-nitrophenyl D-glucoside	0.078	169	2170
4-nitrophenyl D-galactoside	5.0	275	55
4-nitrophenyl D-mannoside	0.02	0.12	6
4-nitrophenyl D-xyloside[a]	(i) 0.22	1.85	8.4
	(ii) 3.3	8.0	2.4
4-nitrophenyl D-fucoside	0.12	139	1157
4-nitrophenyl α-L-arabinoside	0.33	32.6	98.9
2-nitrophenyl D-galactoside	9.3	267	28.8
2-nitrophenyl D-xyloside[a]	(i) 0.025	1.04	42
	(ii) 2.4	2.36	1.0
phenyl D-galactoside	16	0.91	0.06

[a] Parameters indicated (i) are for the hydrolysis reaction and (ii) are for the transglycosylation reaction.

RESULTS

Substrate Specificity. Kinetic parameters for a range of substrates are presented in Table I. At the relatively low concentrations of substrate studied in most cases (up to approximately 8 times the K_m value), there was no evidence of transglycosylation through transfer to another molecule of substrate. However, the Lineweaver–Burk plots for two of the substrates studied, the two xylosides, revealed transglycosylation, resulting in rate enhancement over that expected, at higher substrate concentrations. An example of such a Lineweaver–Burk plot is shown in Figure 1, where biphasic behavior is clearly evident. Gas chromatographic analysis of silylated reaction mixtures at low (0.2 mM) and high (2 mM) *p*-nitrophenyl xyloside concentrations confirmed that simple hydrolysis was occurring at low substrate concentrations with production of *only p*-nitrophenol and xylose in equal amounts. At the higher concentrations of *p*-nitrophenyl xyloside (2 mM), the ratio of xylose to *p*-nitrophenol was much lower, and a new product was observed which was identified as a nitrophenyl

[1] Abbrevations: PNPGlu, *p*-nitrophenyl β-D-glucopyranoside; PGlu, phenyl β-D-glucopyranoside; 2,4-DNPGlu, 2′,4′-dinitrophenyl β-D-glucopyranoside.

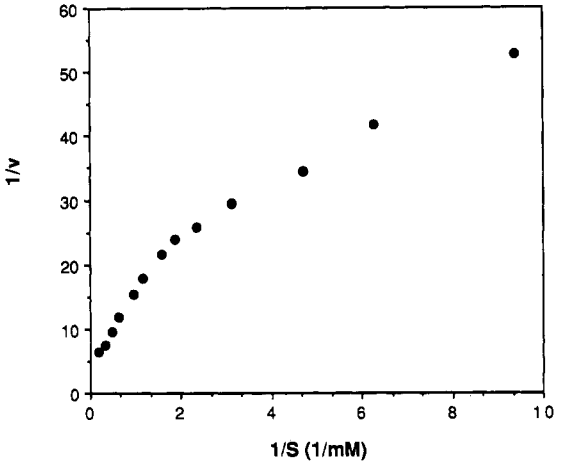


FIGURE 1: Lineweaver–Burk plot for the hydrolysis of *p*-nitrophenyl xyloside.

Table II: Inhibition Constants Determined for *Agrobacterium* β-Glucosidase

inhibitor	K_i (mM)
glucosyl benzene	3.4
isopropyl β-D-thioglucoside	4.0
glucosylamine	0.4
gluconolactone	0.0014
gluconophenylurethane	0.0012
gluconohydroximolactone	0.03
1-deoxynojirimycin	0.05
1,6-anhydro-β-glucopyranose	190
tris(hydroxymethyl)aminomethane	0.5

xylobioside by GCMS. Values of k_{cat} and K_m for both the hydrolytic reaction and the transglycosylation reaction were estimated by drawing straight lines through the two linear regions of the Lineweaver–Burk plot. Such an approach will give a reasonable estimate of the two values when, as in these cases, the K_m values are quite different.

Inhibitor Studies. Dissociation constants (K_i values) for a series of inhibitors of β-glucosidase are shown in Table II. This list includes inhibitors which mimic both the ground state and the transition state of the reaction catalyzed. All inhibitors were found to be directly competitive with substrate.

Substrate Reactivity. The consequences on kinetic parameters of change in the phenolate structure are shown in Table III, where the k_{cat} and K_m values for a series of aryl glucoside substrates of differing phenol leaving group ability (as measured by the pK_a of the phenol) are presented. These results are plotted in the form of Bronsted relationships in Figure 2: these are in many ways more like a Hammett plot, since they relate reaction rate and leaving group ability, but are called Bronsted plots since they fit the Bronsted catalysis equation. Secondary deuterium kinetic isotope effects on five different substrates have also been determined, and these are presented in Table IV, along with the pK_a values of the leaving phenols associated with each substrate.

pH Dependence. Values of k_{cat} and K_m at a series of pH values between 5.1 and 9.0 were determined for two different substrates, PNPGlu and PGlu. Values of k_{cat} only were determined for a third substrate, 2,4-DNPGlu, by measuring rates at a constant, high concentration of substrate (12 times its K_m value). Determination of K_m values at the two extremes of pH was carried out to ensure that the measured rate was not reduced beneath k_{cat} as a consequence of large increases in K_m values. These results are plotted in logarithmic form in Figure 3a,b. Values of k_{cat} for each substrate depend upon

Table III: Michaelis–Menten Parameters for the Hydrolysis of Aryl Glucosides by *Agrobacterium* β-Glucosidase

phenol substituent	pK_a[a]	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
2,4-dinitro	3.96	87.9	0.031	2800
2,5-dinitro	5.15	120	0.045	2700
3,4-dinitro	5.36	185	0.033	5600
2,4,6-trichloro	6.39	240	0.0092	26000
4-chloro-2-nitro	6.45	144	0.013	11000
4-nitro	7.18	169	0.078	2200
2-nitro	7.22	111	0.033	3400
3,5-dichloro	8.19	159	0.13	1200
3-nitro	8.39	108	0.19	570
4-cyano	8.49	129	0.21	600
4-bromo	9.34	28.8	0.56	52
4-chloro	9.38	29.6	0.64	46
2-naphthyl	9.51	25.3	0.16	160
H	9.99	5.44	2.12	2.6
4-*tert*-butyl	10.37	5.13	0.069	74

[a] Phenol pK_a values were taken from Barlin and Perrin (1966), Kortum et al. (1961), Robinson et al. (1960), and Ba-Saif and Williams (1988).

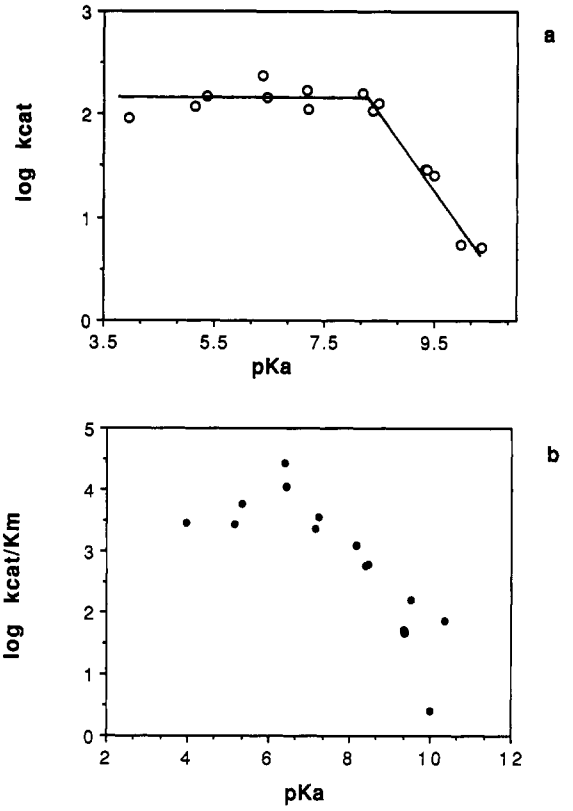


FIGURE 2: Bronsted plots relating rates of *Agrobacterium* β-glucosidase-catalyzed hydrolysis of aryl glucosides with the leaving group ability of the phenol. (a) Plot of log k_{cat} vs pK_a of the aglycon phenol; (b) plot of log k_{cat}/K_m vs pK_a of the aglycon phenol.

Table IV: Secondary Deuterium Kinetic Isotope Effects Measured with *Agrobacterium* β-Glucosidase

substrate	pK_a	k_H/k_D
2′,4′-dinitrophenyl glucoside	3.96	1.10 ± 0.02
4′-nitrophenyl glucoside	7.16	1.12 ± 0.02
3′-nitrophenyl glucoside	8.39	1.07 ± 0.02
4′-bromophenyl glucoside	9.34	1.06 ± 0.02
β-naphthyl glucoside	9.51	1.05 ± 0.02

two ionizations as follows: 2,4-DNPGlu (pK_1 = 3.6, pK_2 = 8.1); PNPGlu (pK_1 = 3.9, pK_2 = 8.0); PGlu (pK_1 = 4.8, pK_2 = 7.6). Values of k_{cat}/K_m likewise depend upon two ionizations as follows: PNPGlu (pK_1 = 5.0, pK_2 = 7.2); PGlu (pK_1 = 4.9, pK_2 = 7.0). The values of pK_1 are quite unreliable, but pK_2

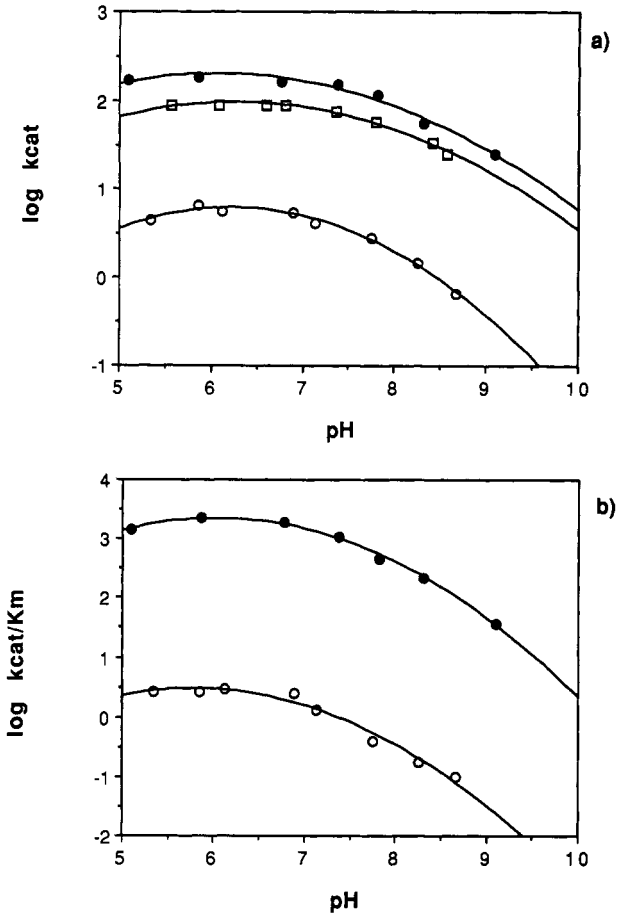


FIGURE 3: pH dependence of the hydrolysis of aryl glucosides by *Agrobacterium* β-glucosidase. (a) Plots of log k_{cat} vs pH for (●) PNPGlu, (○) PGlu, and (□) 2,4-DNPGlu; (b) plots of log k_{cat}/K_m vs pH for (●) PNPGlu and (○) PGlu. Lines drawn through points are simply intended to illustrate the shapes of the plots.

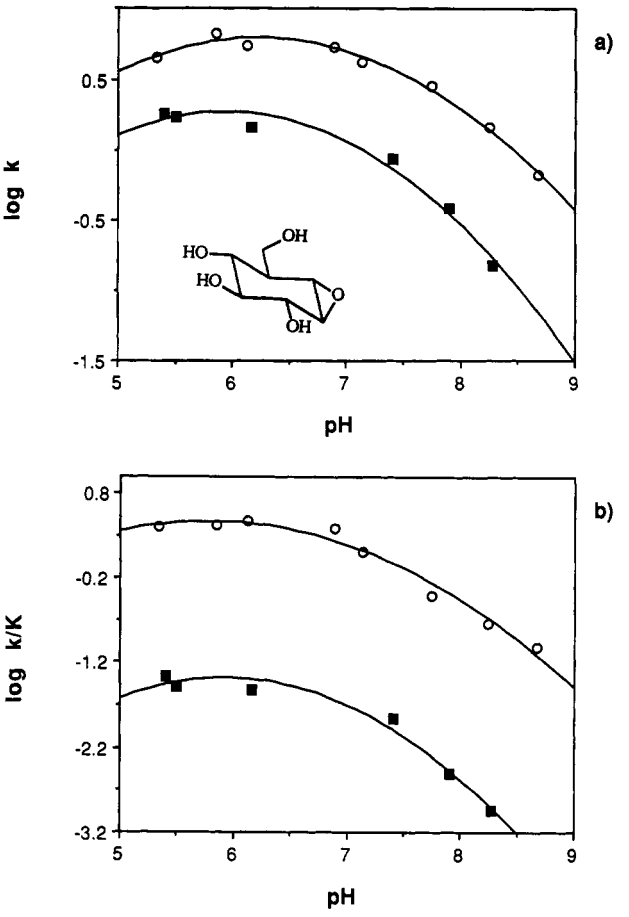


FIGURE 4: pH dependence of the inactivation of *Agrobacterium* β-glucosidase by cyclophellitol. (a) Plots of (■) log k_i vs pH for cyclophellitol and (○) log k_{cat} vs pH for PGlu; (b) plots of (■) log k_i/K_i vs pH for cyclophellitol and (○) log k_{cat}/K_m vs pH for PGlu. Lines drawn through points are simply intended to illustrate the shapes of the plots.

values should be reasonably accurate. In addition, the pH dependence of the inactivation of the enzyme by the mechanism-based inactivator cyclophellitol was investigated over the same pH range by measuring the inactivation rate constant, k_i, and the equilibrium binding constant, K_i, at each pH value. These results are presented in Figure 4 along with data for the substrate PNPGlu for comparison purposes. Inactivation by cyclophellitol appears to depend on two ionizations as follows: k_i (pK_1 = 4.4, pK_2 = 7.2); k_i/K_i (pK_1 = 4.5, pK_2 = 6.9).

Nucleophilic Competition. The values of k_{cat} determined for 2,4-DNPGlu and PGlu at three different concentrations of DTT are shown in Figure 5. A clear increase with increasing nucleophile concentration is seen for 2,4-DNPGlu, but no increase is observed for PGlu. Attempts to use methanol as the nucleophile were unsuccesful with this enzyme, since it appears to be particularly solvent sensitive. DTT appears to be a sufficiently better nucleophile than methanol such that lower concentrations could be employed and still give reasonable enhancements without affecting the protein structure.

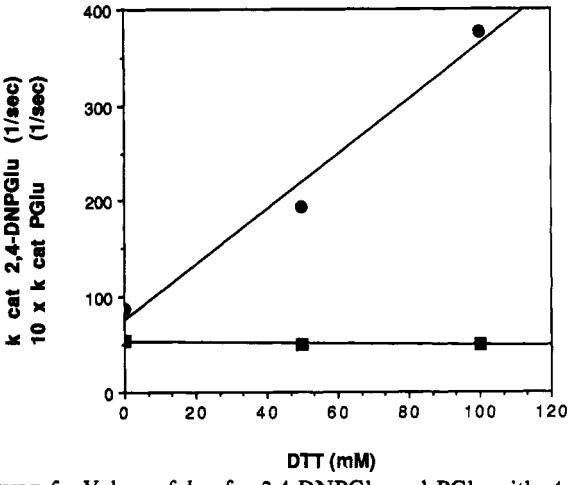
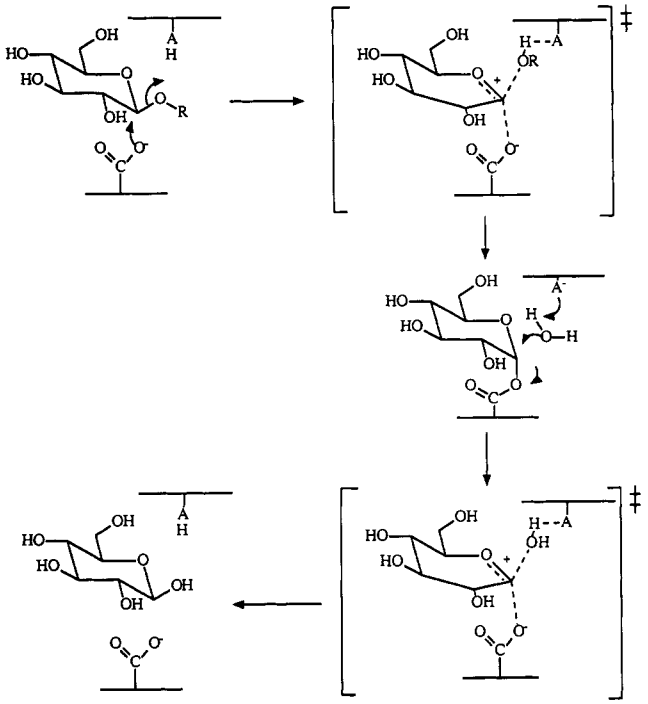


FIGURE 5: Values of k_{cat} for 2,4-DNPGlu and PGlu with *Agrobacterium* β-glucosidase at different DTT concentrations. (●) 2,4-DNPGlu; (■) PGlu. Note that the scale has been enlarged 10-fold for PGlu.

DISCUSSION

Comparison of the Recombinant Enzyme with the Wild Type. Inspection of the kinetic parameters for the recombinant enzyme in Table I and comparison with those determined for the wild-type enzyme isolated from *Agrobacterium* (Day & Withers, 1986) reveals no significant differences. The K_m values are very similar and k_{cat} values [based upon an A^{280} (1 mg/mL) of 2.20 mL/(mg·cm)] are of the same relative magnitude as was found for the native enzyme. In addition, an identical amino acid composition has been demonstrated (Wakarchuk et al., 1986), and no differences in mobility during SDS–PAGE electrophoresis could be detected. These observations all indicate that the recombinant enzyme is identical to the wild-type enzyme isolated from *Agrobacterium*.

Scheme I: Proposed Mechanism of Action of *Agrobacterium* β-Glucosidase



Substrate Specificity. Consideration of the previous data (Day & Withers, 1986) and that in Tables I and II confirms and strengthens the previous assertion that the enzyme has a very broad specificity. It is quite nonspecific with regard to the aglycone moiety, cleaving a variety of different linkages to a number of different aglycons. It is also unusually broad in its requirements for the glycone moiety since it can accommodate epimers at C-4 and C-2 with only a 40- and 400-fold drop in efficiency (based upon relative k_{cat}/K_m values), respectively. Its specificity for the 6-position substituent is relatively lax since it hydrolyzes xylosides (completely missing the C-6 hydroxymethyl) with only a 250-fold drop in efficiency and in fact hydrolyzes D-fucosides (galactosides missing the hydroxyl group at C-6) some 20-fold *more* efficiently than the corresponding galactoside. Whether or not this effect is also found in the glucoside series remains to be seen, but it is noteworthy that *p*-nitrophenyl fucoside is 2.6-fold better as a substrate for almond β-glucosidase than PNPGlu and 46-fold better than *p*-nitrophenyl galactoside (Dale et al, 1985). The ability of glucosidases to handle both galactosides and xylosides is not uncommon since, for example, the almond emulsin enzyme has a similar specificity (Dale et al., 1985). However, the ability to also handle mannosides is unusual.

Proposed Mechanism of Action. The generally accepted [though not universally so; see, for example, Post and Karplus (1986)] mechanism of action of glycosidases which cleave the glycosidic linkage with overall retention of configuration ("retaining" glycosidases) is essentially that originally proposed by Koshland (1953) (see Scheme I). This is a double-displacement mechanism involving an initial binding of the substrate to the enzyme followed by a general acid-catalyzed attack of an enzymic nucleophile upon the anomeric center to form a glycosyl–enzyme intermediate. This intermediate is then hydrolyzed by general base-catalyzed attack of water upon the anomeric center, forming the β-glycose product and returning the enzyme to its original protonation state. Both the formation and the hydrolysis of the glycosyl–enzyme intermediate likely proceed through transition states with substantial oxocarbonium ion character. There has been considerable discussion regarding the structure of this intermediate. One school of thought, based initially upon mechanistic deductions drawn from model-building studies with lysozyme, has suggested that it has an ion-pair structure involving a glycosyl oxocarbonium ion/enzymic carboxylate pair. The other school prefers a covalent structure which is formed and hydrolyzed via oxocarbonium ion-like transition states. The data in this and subsequent papers are strongly in support of the latter viewpoint and the mechanism shown in Scheme I reflects this view. The ring-opening mechanism (Post & Karplus, 1986) is not seriously considered here for the reasons reviewed recently (Sinnott, 1990) which render this mechanism untenable.

Evidence for a Two-Step Mechanism Involving Oxocarbonium Ion-Like Transition States: (a) Structure/Reactivity Studies. The finding of a biphasic Bronsted relationship (Figure 2a) with a concave-downward shape provides excellent evidence for a two-step mechanism, one step being glycosidic bond cleavage (formation of the glucosyl–enzyme intermediate). Substrates with relatively good leaving groups ($pK_a < 8$) show no significant dependence of their reactivity on phenol leaving group ability; thus for these substrates the initial bond-cleavage step is unlikely to be rate-limiting, and indeed the deglycosylation step was shown to be rate limiting for these substrates by the nucleophilic competition experiments performed with DTT as the nucleophile. Large increases in k_{cat} were observed for 2,4-DNPGlu as the DTT concentration was increased, but no increase was observed in the k_{cat} value for PGlu, a substrate for which glycosylation was shown to be rate-limiting (Figure 5). Kinetic isotope effect data (vide infra) also support this notion. Substrates with relatively poor leaving groups ($pK_a > 8$) show a significant dependence of k_{cat} on leaving group ability as shown by the line of slope $\beta_{lg} = -0.7$ (corresponding to a Hammett ρ value of 1.6) confirming that initial bond cleavage is rate limiting. This reaction constant is a little larger than that found for the hydrolysis of a series of aryl galactosides by Mg^{2+}-free *E. coli* (*lac z*) β-galactosidase ($\beta_{lg} = -0.5$) (Sinnott et al., 1978) but is identical to that ($\beta_{lg} = -0.7$) found for purified sweet almond β-glucosidase (Sinnott, 1990). Such a value indicates a fairly large degree of bond cleavage at the transition state, though the exact extent cannot be determined due to the unknown amount of proton donation.

A similar biphasic relationship, but much less well defined, is obtained if the logarithm of k_{cat}/K_m is plotted against phenol pK_a (Figure 2b). In this case it is much more difficult to fit a defined line to the plot with any degree of confidence; thus it is not possible to derive a meaningful β_{lg}. The fact that a biphasic relationship is observed in this case cannot be due to a change to rate-limiting deglycosylation at higher reactivities and is most likely due to the association of enzyme and substrate becoming rate-limiting with the more reactive substrates. The second-order rate constants (k_{cat}/K_m) for these substrates are certainly well within the range for which such association has been shown to be rate limiting in other enzyme systems (Gibson et al., 1990), including the almond β-glucosidase (Dale et al., 1986).

(b) Kinetic Isotope Effects. Secondary deuterium kinetic isotope effects provide insights into changes in hybridization at the substituted site in proceeding from the ground state to the rate-limiting transition state of the reaction. Although they may not be used to specifically distinguish between S_N1 and S_N2 mechanisms, they are useful in assessing the amount of carbonium ion character at the transition state (Knier &

Jencks, 1980). The isotope effects measured (Table IV) appear to fall into two groups, consistent with a two-step mechanism. Substrates for which the glycosylation step is rate limiting (aglycon $pK_a > 8$) show a relatively small, but nonetheless very significant, isotope effect of $k_H/k_D = 1.06$, whereas substrates for which deglycosylation is rate determining (aglycone $pK_a < 8$) show a larger isotope effect around $k_H/k_D = 1.11$. Secondary deuterium kinetic isotope effects upon the deglycosylation step of several other glycosidases have been measured previously and range from $k_H/k_D = 1.2$–1.25 for *E. coli* (*lac z*) β-galactosidase (Sinnott, 1978) to $k_H/k_D = 1.11$ for the β-glucosidase from *Stachyobotrys atra* (van Doorslaer et al., 1984) and $k_H/k_D = 1.09$ for the β-glucosidase from *Botrydiplodia theobromae* (Umezerike, 1988). The value observed here falls well within this range and provides strong evidence that the intermediate in this case is indeed covalent, since were the intermediate to be an ion pair, then inverse secondary deuterium kinetic isotope effects would have been expected.

The 6% isotope effect measured with the less reactive substrates is also consistent with glycosylation being the rate-limiting step in this case. Values of isotope effects measured previously for aryl glycosides in systems for which bond cleavage has been shown by other criteria to be rate determining range from 1.0 for *E. coli* (*lac z*) β-galactosidase to around 1.10 for several other systems (Dahlquist et al., 1969; Legler et al., 1980; van Doorslaer et al., 1984). These isotope effects suggest a relatively small amount of oxocarbonium ion character at the transition state, even though, at least in the case of *Agrobacterium* β-glucosidase, the Bronsted relationship indicated a large degree of bond cleavage at the first transition state with these substrates. This is consistent with the relatively late transition state for these poorer substrates and thus a large amount of preassociation of the enzymic nucleophile at the transition state.

Inhibitor Studies. The inhibition constants listed in Table II provide a measure of the affinity of the enzyme for the ground-state substrate structure and provide substantial support for the transition-state structure proposed earlier. Simple ground-state analogues such as glucosyl benzene and isopropyl β-D-thioglucopyranoside bind to the enzyme with millimolar affinities. Inhibitors which bear some resemblance to the proposed transition states, that is, which bear some positive charge or assume a conformation mimicking the half-chair, bind much more tightly with affinities down to the low micromolar range. The best of these inhibitors are the classical transition-state analogue gluconolactone, which has been shown to inhibit essentially all β-glucosidases (Legler, 1990) binding with an affinity considerably greater than that shown for the free sugar, and gluconophenylurethane (Beer & Vasella, 1986), which has also been shown to inhibit the almond β-glucosidase very effectively. Both of these bind with K_i values close to 1 μM, around 5000 times tighter than glucose binds (Day & Withers, 1986). The binding of glucosylamine is intermediate in strength, suggesting that the presence of the positive charge confers some additional affinity, but not to the extent seen with the β-glucosidase from *A. wentii* or the β-galactosidase from *E. coli*. Similarly relatively modest affinity is seen for 1-deoxynojirimycin, a compound that is an extremely potent inhibitor of some glycosidases. This presumably reflects slightly different relative placements of the positively charged nitrogen on the inhibitors and the negative charge with which it interacts in the different enzyme systems. The quite significant inhibition exhibited by Tris is consistent with that seen for a number of other glycosidases and is presumably another reflection of the general affinity of these enzymes for hydroxylated amines (Sinnott, 1990). The very weak binding of 1,6-anhydro-β-D-glucopyranose presumably reflects a poor affinity for sugars in a boat conformation, as was seen also with *E. coli* (*lac z*) β-galactosidase (Case et al., 1973).

pH Dependence. Examination of the plots in Figure 3 reveals that essentially the same pH dependence is seen for each of a series of three different substrates, one of which has the glycosylation step rate limiting and two of which appear to have deglycosylation as their rate-limiting step. This appears to be true for both ionizations in the enzyme/substrate complex (k_{cat} vs pH plots) and in the free enzyme (k_{cat}/K_m vs pH plots). All reactions depend upon the apparent presence of a protonated group of pK_a 7.6–8.1 whose deprotonation results in loss of activity. It also appears that the deprotonation of a group of $pK_a < 5$ results in loss of activity, but unfortunately the relatively rapid irreversible inactivation of the enzyme at pH values less than this precludes accurate determination of this pK_a value.

Detailed interpretation of pH dependence data is a very risky pastime (Knowles, 1976; Cleland, 1977), and, in this case, as also previously for β-glucosidase (Dale et al., 1986) and β-galactosidase (Tenu et al., 1971; Withers et al., 1978; Li et al., 1989), somewhat confusing and unsatisfying. The pH dependence of the deglycosylation step (2,4-DNPGlu and PNPGlu) might have been expected to reflect only the presence of a suitably deprotonated general base catalyst; thus the ionization of $pK_a < 5$ would be assigned to this group. The mechanistic basis of the apparent ionization of approximately pK_a 8 resulting in loss of activity upon deprotonation is not immediately apparent, though it does appear that the same value of $pK_2 = 8.0$ is found for these two substrates and that this value is higher than that ($pK_2 = 7.6$) found for PGlu, a substrate for which glycosylation is rate limiting. The glycosylation step would be expected to require the operation of both general acid catalysis and nucleophilic catalysis; thus the ionization of pK_a 8 might be ascribed to the loss of the proton from the acid catalytic group. However, since the principle of microscopic reversibility requires that this group be the same as that which acts as the general base catalyst during deglycosylation, this assignment requires that the group in question change its pK_a at least 3 pH units (8 to <5) from one step to the other. While not impossible, this seems unlikely. The ionization of $pK_a < 5$ in this case might be ascribed to the nucleophile, known to be the carboxylate side chain of glutamic acid 358 (Withers et al., 1990). Alternatively, these ionizations may not reflect changes in protonation state of discrete mechanistically important residues but rather be the consequence of other more remote ionizations elsewhere in the molecule which in some way affect the catalytic activity. It is therefore not a trivial task to interpret and understand these pH profiles. Nonetheless, they provide a useful way of characterizing the enzyme and can serve a very valuable function in understanding the consequences of mutations (see the third paper of this series) and in characterizing mechanism-based inactivators. An example of the latter is provided in Figure 4, which shows the pH dependence of the inactivation of *Agrobacterium* β-glucosidase by the mechanism-based inactivator cyclophellitol (Withers & Umezawa, 1991). This naturally occurring inactivator, an epoxide based upon a hydroxylated cyclohexane, is sufficiently similar in structure to a β-glucoside to be bound at the active site. It is then presumably protonated in the usual fashion, generating a reactive protonated epoxide which is rapidly attacked by a

nearby nucleophile, most likely the nucleophilic carboxylate Glu 358 (Withers et al., 1990), forming a stable ester derivative at the active site and thereby inactivating the enzyme. The pH dependence of the inactivation process is essentially identical to that for hydrolysis of substrates, as is illustrated for PGlu, a substrate for which the mechanistically analogous glycosylation step is rate determining. This provides some reassurance that the inactivation process is recruiting, and thereby inactivating, the normal catalytic machinery.

Conclusions. These results coupled with previous findings (Withers & Street, 1988; Withers et al., 1990) provide very strong support for the catalytic mechanism shown in Scheme I along with more detailed insights into the structures of the two transition states and the intermediate species. In particular, the isotope effects suggest that the second transition state has more oxocarbonium ion character than the first, at least with the substrates studied. A similar situation obtains for Mg^{2+}-free *E. coli* (*lac z*) β-galactosidase (Sinnott et al., 1978), where the isotope effect on the glycosylation step (k_H/k_D = 1.09) is considerably smaller than that on the deglycosylation step (k_H/k_D = 1.21). Values for the holoenzyme (Sinnott & Souchard, 1973; Rosenberg & Kirsch, 1981a,b) are even more pronounced (k_H/k_D = 1.04 and 1.25, respectively). It therefore seems likely that for β-glycosidases the glycosylation step has substantial S_N2 character, while the deglycosylation step is more S_N1-like. Unfortunately reliable data on the two steps in isolation do not seem to be available for any other single β-glycosidase.

It is of interest to speculate upon possible reasons for this apparent difference in transition-state structure, reflecting a greater degree of nucleophilic preassociation by the enzymic carboxylate (Glu 358 in the case of *Agrobacterium* β-glucosidase) at the first transition state (at least for the slower substrates) than by water at the second transition state. One relatively trivial explanation for this finding could be that the isotope effects for the first transition state have only been measured with relatively poor substrates containing leaving groups of low nucleofugacity; thus the reaction will inherently tend to be more associative in nature (S_N2-like), while the leaving group for the second step is the enzymic carboxylate, a relatively good leaving group, thus tending to make the reaction more dissociative. A second possible explanation is that since the nucleophile for the first step is anionic; it would likely preassociate more effectively with the developing positive charge at the anomeric center than the neutral water nucleophile. Such behavior has been demonstrated recently for nonenzymic solvolysis of acetals (Banait & Jencks, 1991) in the presence of anionic nucleophiles. A third possibility is that there is inherently more oxocarbonium ion character in the transition states for departure of an axial than an equatorial leaving group, as has also been seen in the acid-catalyzed hydrolysis of glycosides (Bennet & Sinnott, 1986; Sinnott & Jencks, 1980). This may have stereoelectronic origins (Deslongchamps, 1983) since the lone pair which is antiperiplanar to the axial leaving group could provide some assistance, making the reaction more dissociative. Departure of an equatorial leaving group would not be assisted and therefore would require more preassociation from the enzymic nucleophile. It may therefore be that the apparent stereoelectronic barrier to enzymic cleavage of the equatorial glycosidic bond is overcome not by substrate conformational changes (Deslongchamps, 1983) but by the enzyme forcefully preassociating its nucleophile, thereby changing the stereoelectronic demands to those of an "in-line" mechanism. This is the topic of further investigation.

ACKNOWLEDGMENT

We thank R. A. J. Warren and D. Trimbur for providing the recombinant *E. coli* cell paste, A. Vasella for providing the gluconohydroximolactone and phenylurethane inhibitors, and K. Rupitz and T. Andrews for technical assistance.

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