Mechanism of *Agrobacterium* β-Glucosidase: Kinetic Analysis of the Role of Noncovalent Enzyme/Substrate Interactions†

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ABSTRACT: The role of noncovalent interactions in the catalytic mechanism of the *Agrobacterium faecalis* β-glucosidase was investigated by steady-state and pre-steady state kinetic analysis of the hydrolysis of a series of monosubstituted aryl glycosides, in which the hydroxyl groups on the glycone were substituted by hydrogen or fluorine. Contributions of each hydroxyl group to binding of these substrates at the ground state are relatively weak (interaction energies of 3.3 kJ/mol or smaller) but are much greater at the two transition states (glycosylation and deglycosylation). The strongest transition state interactions were at the 2 position (at least 18 and 22 kJ/mol for glycosylation and deglycosylation, respectively) with the interactions at the 3 and 6 positions contributing at least another 9 kJ/mol of binding energy at both transition states. The interaction at the 4 position is less crucial to transition state binding but important for stabilization of the glycosyl−enzyme intermediate. Comparison of observed rates with those for spontaneous hydrolysis of the same substrates provides evidence for oxocarbenium ion character at both transition states, that for deglycosylation apparently having the greater positive charge development at the anomeric center.

The *Agrobacterium faecalis* β-glucosidase (Abg)[1] has proven to be a good model system for investigation of the mechanism of action of "retaining" β-glycosidases, those which hydrolyze the glycosidic bond with net retention of anomeric configuration. Past studies of this enzyme (Day & Withers, 1986; Kempton & Withers, 1992; Street et al., 1992) established that its mechanism is similar to that proposed by Koshland (1953) for this class of enzyme in which hydrolysis proceeds via a two-step, double-displacement mechanism. During the first step (glycosylation) an enzymic nucleophile [demonstrated to be Glu358 in this case (Withers et al., 1990)] attacks the glycoside at C1, resulting in formation of a covalently linked α-glucosyl−enzyme intermediate, with general acid-catalyzed release of the aglycon. In a second step (deglycosylation), the anomeric center of the glycosyl−enzyme intermediate is attacked by water in a general-base-catalyzed process to yield the β-glycose. The transition states for both glycosylation and deglycosylation are believed to possess significant oxocarbenium ion character (Kempton & Withers, 1992).

One aspect of the mechanism of Abg which has not been investigated is the role in catalysis of noncovalent interactions between the enzyme and the substrate glycone, both in ground state and transition state binding. Such interactions are thought to be crucial to enzymatic catalysis (Jencks, 1975; Pauling, 1946; Wolfenden & Kati, 1991; Fersht, 1986). Since the substrates for glycosidases are polyhydroxylated, it seems probable that many of the interactions for such enzymes would involve hydrogen bonding. Indeed, such is clearly the case as seen in the many three-dimensional structures of these enzymes now published [see McCarter and Withers (1994) for a recent compilation]. Estimates of the magnitudes and polarities of these hydrogen bonds can be obtained under favorable circumstances through the use of modified substrates in which the individual hydroxyl groups on the glycon have been replaced by hydrogen or by fluorine. The basis of this approach is that replacement of a sugar hydroxyl group by a hydrogen (deoxy sugar) will remove all significant hydrogen bonding at that position, while replacement by fluorine will only allow hydrogen bonds in which the fluorine acts as an acceptor, and then only weakly. Analysis of changes in binding interactions (K_d, K_i, or in some cases K_m) allows estimation of the contributions to ground state interactions whereas analysis of changes in rates (k_{cat}/K_m or k_{cat}) provides insights into transition state interactions. This approach has been applied *inter alia* to the study of glycogen phosphorylase (Street et al., 1986, 1989), phosphoglucomutase (Percival & Withers, 1992), and *Escherichia coli* β-galactosidase (McCarter et al., 1992). However, in none of these cases has it been possible to look at binding interactions at *both* transition states, glycosylation and deglycosylation. This would require pre-steady-state kinetic analyses coupled with the use of substrates with excellent chromogenic leaving groups for which the deglycosylation step would likely be rate-limiting. This report describes such

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[1] Abbreviations: Abg, *Agrobacterium faecalis* β-glucosidase; DNP, 2,4-dinitrophenol; PNP, 4-nitrophenol; DNP2Fglucoside, 2,4-dinitrophenyl-2-deoxy-2-fluoro-β-D-glucoside; DNP3Fglucoside, 2,4-dinitrophenyl-3-deoxy-3-fluoro-β-D-glucoside; DNP4Fglucoside, 2,4-dinitrophenyl-4-deoxy-4-fluoro-β-D-glucoside; DNP6Fglucoside, 2,4-dinitrophenyl-6-deoxy-6-fluoro-β-D-glucoside; DNP3Dglucoside, 2,4-dinitrophenyl-3-deoxy-β-D-glucoside; DNP4Dglucoside, 2,4-dinitrophenyl-4-deoxy-β-D-glucoside; DNP6Dglucoside, 2,4-dinitrophenyl-6-deoxy-β-D-glucoside.

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a study in which we investigate the role of hydrogen bonding between the substrate glycon and Abg in both substrate binding and catalysis by detailed kinetic analysis of the hydrolysis of a series of deoxy- and deoxyfluoro-2′,4′-dinitrophenyl-β-D-glycopyranosides.

MATERIALS AND METHODS

Synthesis. Details of the syntheses of the DNPglycosides used in this study have been published elsewhere (Namchuk et al., 1995). PNPglucoside and PNPmannoside were obtained from Sigma Chemical Co. 2′-Chloro-4′-nitrophenyl-β-D-glucopyranoside was a generous gift from Dr. Marc Claeyssens, Universiteit Gent, Belgium. Melting points (mp) were determined on a Laboratory Devices Mel-Temp II melting point apparatus and are uncorrected. Proton (^{1}H) nuclear magnetic resonance (nmr) spectra were acquired on a 300 MHz Varian XL-300 or a 200 MHz Bruker AC-200. When possible, tetramethylsilane (TMS) was used an internal reference (δ = 0.00 ppm). Spectra obtained in D_2O are referenced externally to 2,2-dimethyl-2-silapentane-5-sulfonate (δ = 0.00 ppm). ^{19}F NMR spectra were acquired on a 200 MHz Bruker AC-200 instrument, with proton decoupling. Chemical shifts are quoted relative to $CFCl_3$ (δ = 0.00 ppm), the external reference used being trifluoroacetic acid (δ = 76.53 ppm).

p-Nitrophenyl-3,4,6-tri-O-acetyl-2-ammonium-2-deoxy-β-D-glucopyranoside Hydrochloride (**2**). The glucosyl bromide (**1**) (Wolfrom, 1961) (0.5 g, 1.1 mmol) was dissolved in 10 mL of acetone, treated with *p*-nitrophenol (0.8 g, 5.8 mmol) and 2 M NaOH (2 mL), and then stirred for 1.5 h at room temperature. The reaction mixture was diluted with chloroform, the organic layer washed with 2 M NaOH, and the solvent evaporated *in vacuo* to yield a crude oil which was dissolved in excess diethyl ether and filtered. The product was precipitated from the filtrate by bubbling HCl gas through the solution, filtered, and crystallized from ethanol. The compound was extremely hygroscopic (0.10 g, 20%). Mp >210 °C (dec). ^{1}H NMR (200 MHz, DMSO-d_6): δ 8.98 (broad s, ~1.5 H, NH_3), 8.30, 7.40 [d, 4 H, $J_{2',3'}$ 9 Hz, (aryl H)], 5.81 [d, 1 H, $J_{1,2}$ 8 Hz, H(1)], 5.40, 4.98 [t, 2 H, J 9.8 Hz, H(3)+H(4)], 3.90−4.15 [m, >3 H, H(2) + H(6) + water)], 3.90−3.68 [m, 1 H, H(5)], 2.10, 2.06, 2.01 (3 × s, 9 H, 3 × OAc). Anal. Calcd for $C_{18}H_{23}N_2O_{10}Cl$ + $0.5H_2O$: C, 45.82; H, 5.13; N, 5.94. Found: C, 46.04; H, 5.10; N, 5.86.

p-Nitrophenyl-2-ammonium-2-deoxy-β-D-glucopyranoside Hydrochloride (**3**). The acetylated glucoside (**2**) was deacetylated using the procedure of Ballardie et al. (1973) with the following modifications. The glucoside (150 mg, 0.33 mmol) was dissolved in 20 mL of dry methanol, cooled to 0 °C, and treated with dry acetyl chloride (0.3 mL, 4.2 mmol). The solution was stirred at room temperature for 48 h and then 4 h at 40 °C. The reaction mixture was evaporated to dryness *in vacuo* and the resultant oil triturated with dry diethyl ether and then again evaporated to dryness several times. The product solidified after several evaporations *in vacuo* with diethyl ether and drying *in vacuo* for several days. The compound was further purified on a silica gel column (7:2:1 ethyl acetate/ethanol/water) and isolated as a flaky white solid (73 mg, 68%). Mp >210 °C (dec); ^{1}H NMR (200 MHz, D_2O): δ 8.20−7.22 [m, 4 H, H(2′) + H(3′) + H(4′) + H(5′)], 5.57 [d, 1 H, $J_{1,2}$ 9.0 Hz, H(1)], 3.96−3.35 [m, 6 H, H(2) + H(3) + H(4) + H(5) + H(6)]. Anal. Calc. for $C_{12}H_{17}N_2O_6Cl$ + 0.6 CH_3CH_2OH: C, 45.51; H, 5.91; N, 8.15. Found: C, 45.20; H, 5.63; N, 7.72.

Phenyl-6-deoxy-6-fluoro-β-D-glucopyranoside (**4**). Phenyl-β-D-glucopyranoside (Kempton & Withers, 1992) (200 mg, 0.8 mmol) was dissolved in 5 mL of dry CH_2Cl_2, cooled to −23 °C, and then treated with DAST (0.72 mL, 4 mmol) using the procedure of Card and Reddy (1983). The solution was slowly warmed to room temperature, stirred for 45 min, again cooled to −23 °C, and quenched by addition of excess methanol. After evaporation of the solvent *in vacuo*, the resulting syrup was purified on a silica gel column (5% MeOH/EtOAc) and the product crystallized from ethyl acetate/petroleum ether and isolated as a white powder (70 mg, 40%). Mp 135−137 °C (lit. 143−145 °C); ^{1}H NMR (300 MHz, CD_3OD): δ 7.30 [m, 2 H, H(2′) + H(6′)], 7.14−6.99 [m, 3 H, H(3′) + H(4′) + H(5′)], 4.91 [d, 1 H, $J_{1,2}$ 7.5 Hz, H(1)], 4.69 [ddd, 1 H, $J_{6a,F}$ 48, $J_{6a,6b}$ 10, $J_{6a,5}$ 2 Hz, H(6a)], 4.65 [ddd, 1 H, $J_{6b,F}$ 48 Hz, $J_{6b,6a}$ 10 Hz, $J_{6b,5}$ 6 Hz, H(6b)], 3.73−3.40 [m, 4 H, H(2) + H(3) + H(4) + H(5)]. ^{19}F NMR (188.3 MHz, D_2O): δ −235.52. Anal. Calcd for $C_{12}H_{15}O_5F$ + 0.75 H_2O: C, 53.04; H, 6.12. Found: C, 52.78; H, 5.93.

p-Nitrophenyl-6-deoxy-6-fluoro-β-D-glucopyranoside (**5**). *p*-Nitrophenyl-β-D-glucopyranoside (400 mg, 1.3 mmol) was dissolved in 10 mL of dry CH_2Cl_2, cooled to −23 °C, and treated with DAST (0.8 mL, 1.3 mmol). The solution was slowly warmed to room temperature, stirred for 35 min, then worked up in the same manner as (**4**), and the product isolated as white crystals (35 mg, 9%). Mp 178−181 °C (lit. 186−189 °C); ^{1}H NMR (300 MHz, acetone-d_6): δ 8.23 + 7.27 [d, 4 H, $J_{2',3'}$ 8 Hz, aryl (H)], 5.27 [d, 1 H, $J_{1,2}$ 8 Hz, H(1)], 4.88−4.51 [m, 2 H, H(6)], 3.86 [dddd, 1 H, $J_{H,F}$ 25, $J_{5,4}$ 10, $J_{5,6a}$ 5, $J_{5,6b}$ 2 Hz, H(5)], 3.65−3.46 [m, 3 H, H(2) + H(3) + H(4)]. ^{19}F NMR (188.3 MHz, D_2O): δ −235.60. Anal. Calcd for $C_{12}H_{14}NO_7F$ + 0.5 H_2O: C, 46.15; H, 4.84; N, 4.49. Found: C, 45.83; H, 4.87; N, 4.18.

o-Nitrophenyl-6-deoxy-6-fluoro-β-D-glucopyranoside (**6**). *o*-Nitrophenyl-β-D-glucopyranoside (Aldrich Chemical Co.) (70 mg, 0.23 mmol) was suspended in 5 mL of dry CH_2Cl_2, cooled to −23 °C, and reacted with DAST (0.090 mL, 0.74 mmol). The reaction was slowly warmed to room temperature, stirred for 35 min, cooled again to −23 °C, and quenched by addition of excess methanol. The solvent was evaporated *in vacuo* and the resulting oil purified by column chromatography (5% MeOH/EtOAc). The product was isolated as a clear oil (12 mg, 17%). ^{1}H NMR (200 MHz, acetone-d_6): δ 7.76 [d, 1 H, $J_{3',4'}$ 9 Hz, H(3′)], 7.56 [t, 1 H, $J_{4',3'}$ 9, $J_{4',5'}$ 9 Hz, H(4′)], 7.40 [d, 1 H, $J_{6',5'}$ 9 Hz, H(6′)], 7.14 [t, 1 H, $J_{5',4'}$ 9 Hz, $J_{5',6'}$ 9, H(5′)], 5.13 [d, 1 H, $J_{1,2}$ 7 Hz, H(1)], 4.56 [ddd, 1 H, $J_{6a,F}$ 47, $J_{6a,6b}$ 9, $J_{6a,5}$ 2 Hz, H(6a)], 4.53 [ddd, 1 H, $J_{6b,F}$ 47, $J_{6b,6a}$ 9, $J_{6b,5}$ 5 Hz, H(6b)], 3.80−3.55 [m, 1 H, H(5)], 3.52−3.30 [m, 3 H, H(2−4)] ^{19}F NMR (188.3 MHz, D_2O): δ −235.17.

2′-Chloro-4′-nitrophenyl-6-deoxy-6-fluoro-β-D-glucopyranoside (**7**). 2′-Chloro-4′-nitrophenyl-β-D-glucopyranoside (50 mg, 0.15 mmol) was suspended in 5 mL of dry CH_2Cl_2, cooled to −23 °C, and reacted with DAST (0.070 mL, 0.58 mmol). The reaction was worked up in the exact same manner as **6** to yield the product as a clear oil which was crystallized from EtOAc/petroleum ether (15 mg, 30%). Mp 172−175 °C; ^{1}H NMR (200 MHz, acetone-d_6): δ 8.32 [s, 1 H, H(6′)], 8.21 [d, 1 H, $J_{4',5'}$ 10 Hz, H(4′)], 7.59 [d, 1 H, $J_{5',4'}$ 10 Hz, H(5′)], 5.39 [d, 1 H, $J_{1,2}$ 7.8 Hz, H(1)], 4.71

[ddd, 1 H, $J_{6a,F}$ 48, $J_{6a,6b}$ 10, $J_{6a,5}$ 2 Hz, H(6a)], 4.63 [ddd, 1 H, $J_{6b,F}$ 47, $J_{6b,6a}$ 10, $J_{6a,5}$ 5 Hz, H(6b)], 3.99−3.75 [m, 1 H, H(5)], 3.70−3.44 [m, 3 H, H(2−4)]. ^{19}F NMR (188.3 MHz, D_2O): δ −235.64. Anal. Calcd for $C_{12}H_{14}NO_8ClF$ + 0.2 H_2O: C, 42.23; H, 4.00; N, 4.10. Found: C, 42.29; H, 4.29; N, 3.90.

Enzymology. All buffer chemicals used were obtained from Sigma Chemical Co. Abg used for these studies was obtained as described previously (Kempton & Withers, 1992). Enzyme concentrations were determined by their absorbance at 280 nm (ϵ = 2.184 mg mL^{-1} cm^{-1}). All absorbance measurements were made on a Phillips PU-8800 UV−visible spectrometer equipped with a circulating water bath. Extinction coefficients have been reported previously (Kempton & Withers, 1992). The buffer used for all kinetic studies was 50 mM sodium phosphate buffer, pH 6.8, containing 0.1% bovine serum albumin. Studies were performed at 37 °C unless otherwise stated.

Steady-State Kinetics. Steady-state rate constants were determined as described previously (Kempton & Withers, 1992). Concentrations of DNPglycosides were determined by measuring the absorbance of the intact substrate at 255 nm (ϵ = 10.7 cm^{-1} mM^{-1}) or by total hydrolysis of the glycoside and determination of the final concentration of phenol released using the extinction coefficients reported (Kempton & Withers, 1992). Rates were determined by initial rates analysis following the release of phenol with time which was found to be linear below 8% substrate depletion. Values for k_{cat} and K_m were determined by weighted fitting of the observed rate data to the Michaelis−Menten equation using GraFit (Leatherbarrow, 1990).

Pre-Steady-State Kinetics. Pre-steady-state kinetic data were acquired using an Applied Photophysics MV 17 microvolume stopped-flow spectrophotometer equipped with a Grant constant temperature bath. The data were accumulated using the stopped flow work station and fit using a robust implementation of the Marquart algorithm (Bevington, 1969). The stop volume was set at 100 μL and the reaction monitored by following the release of dinitrophenol at 360 nm (ϵ = 14.0 mM^{-1} cm^{-1}).

The concentration of enzyme used in each reaction was chosen such that a burst with a total absorbance change of ΔA_{360} = 0.03 was obtained in each case, which was sufficient to obtain reliable data under the conditions of the experiment. For the dinitrophenyl substrates, the reactions were carried out at 5 °C in 50 mM sodium phosphate buffer at pH 6.80. Rates were determined at five or more concentrations of substrate which were chosen to bracket K_d for that substrate whenever possible. The rate measurement was repeated four times at each concentration of substrate and the traces averaged. The rate of glycosylation at each substrate concentration (k_2) was determined by fitting the averaged trace to a first order equation or to an equation which fits a pre-steady-state followed by a steady state, as appropriate. Values of K_d and k_2 were determined by fitting the observed rate constants to the Michaelis−Menten equation by a weighted nonlinear regression, using the GraFit program (Leatherbarrow, 1990). Standard error values for these parameters were provided by the same program. Pre-steady-state analyses of the PNPglycosides were carried out in the same manner with the exception that the reaction was followed at 400 nm (ϵ = 7.18 mM^{-1} cm^{-1}) and at 37 °C.

HPLC Procedures for Product Characterization. Hydrolysis of the DNPglycosides was carried out as described for the steady-state analysis. All analyses were carried out on a Waters HPLC equipped with a UK6 manual injector and Waters 441 UV detector. Aliquots of reaction mixtures (30 μL) containing substrate and Abg were injected onto the HPLC equipped with an analytical Dextro-pak column (Waters) and eluted with 20% acetonitrile/water. Enzyme was removed from these solutions by a precolumn of Iatro beads (Iatron Laboratories). Products were detected by their A_{280} and identified by their retention time in comparison with standards, using the Baseline 710 operating system. The identity of the 3FDNPglucoside transglycosylation product was confirmed by liquid SIMS mass spectrometry of the per-*O*-acetylated product as follows. A sample of the transglycosylation product purified by HPLC was evaporated to dryness and the residue acetylated in acetic anhydride/pyridine (Behrend & Roth, 1904).

RESULTS

Abg has been shown previously (Kempton & Withers, 1992) to follow the kinetic scheme shown below.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E{-}S \xrightarrow{k_3} EP$$

The following kinetic parameters apply to this scheme.

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3}$$

$$K_m = \frac{k_1 + k_2}{k_1} \frac{k_3}{k_2 + k_3}$$

$$k_{cat}/K_m = \frac{k_1 k_2}{k_{-1} k_2}$$

For such a system, substrates with excellent leaving groups will likely have $k_2 > k_3$. In this situation, the k_{cat} value reflects k_3 and pre-steady-state analysis of the hydrolysis kinetics yields values for k_2 and K_d (where $K_d = k_{-1}/k_1$). The determination that k_3 is the rate-limiting step is best made by the observation of a burst phase in the pre-steady-state analysis by stopped flow kinetic studies. In addition, a strong indication that this is likely to be the case is usually obtained from the very low K_m values determined in the steady-state analysis since K_m values decrease progressively as the ratio k_3/k_2 drops.

Steady-State Kinetics. Michaelis−Menten parameters for hydrolysis of the aryl glycosides are presented in Tables 1 and 2. Values of k_{cat}/K_m for the modified glycosides are one to three orders of magnitude smaller than that for the parent substrate, DNPglucoside. Since k_{cat}/K_m is the second order rate constant for the first irreversible step in the reaction (in this case, glycosylation), it is apparent that replacement of the ring hydroxyl groups of the DNPglucoside decreases the rate of formation of the glycosyl−enzyme intermediate. A similar decrease in k_{cat} was also observed in most cases, with the interesting exception of those substrates missing an

Table 1: Steady-State Kinetic Parameters for Hydrolysis of DNPGlycosides by Abg at 37 °C

substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
DNPglucoside	0.0218 (0.0012)	130 (3)	5960
DNP6Dglucoside	0.0227 (0.004)	44.8 (0.25)	1974
DNP4Dglucoside	0.170 (0.01)	380 (11)	2260
DNP3Dglucoside	0.029 (0.003)	9.9 (0.42)	342
DNP6Fglucoside	0.0020 (0.0001)	8.68 (0.15)	4340
DNP4Fglucoside	0.020 (0.0016)	9.62 (0.27)	481
DNP3Fglucoside	0.0020 (0.0004)	1.12 (0.05)	560
DNP2F glucoside[a]		$\sim 2.0 \times 10^{-7}$	
DNPgalactoside	0.840 (0.10)	175 (8)	208
DNP4Fgalactoside	0.500 (0.034)	137 (4)	271
DNPalloside	0.0068 (0.0002)	0.100 (0.007)	14.7
DNPmannoside	0.106 (0.014)	0.126 (0.007)	1.19

[a] Data obtained from Street et al. (1992).

Table 2: Steady-State Kinetic Parameters for Hydrolysis of PNPGlycosides by Abg at 37 °C

substrate	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
PNPglucoside	0.082	141	1720
PNPmannoside[a]	0.020	0.10	5
PNP2Dglucoside	0.015	0.025	1.67
PNPgalactoside[b]	5.0	275	55
PNP2F glucoside[c]		$\sim 2.0 \times 10^{-7}$	
PNP2NH_2glucoside	0.056 (0.009)	6.5×10^{-4} (3.3×10^{-5})	1.16×10^{-2}

[a] Data obtained from Day and Withers (1986). [b] Data obtained from Kempton and Withers (1992). [c] Data obtained from Street et al. (1992).

Table 3: Steady-State Kinetic Parameters for the Hydrolysis of the Aryl 6-Deoxy-6-Fluoroglucosides by Abg at 37 °C[a]

6-deoxy-6-fluoro-β-D-glucoside	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m (sec^{-1} mM^{-1})
2,4-dinitrophenyl	0.0020 (0.0001)	8.52 (0.15)	4260
2-chloro-4-nitrophenyl	0.0035 (0.001)	12.5 (0.6)	3570
2-nitrophenyl	0.044 (0.0029)	19.5 (1.2)	443
4-nitrophenyl	0.359 (0.037)	49.5 (2.5)	138
phenyl	1.640 (0.380)	0.81 (0.06)	0.494

[a] Standard errors given in parentheses.

equatorial 4-substituent (DNPgalactoside, DNP4Fgalactoside, and DNP4Dglucoside). In fact, the k_{cat} value for the DNP4Dglucoside is significantly higher than that for DNPglucoside. Kinetic parameters for the hydrolysis of a series of aryl 6-deoxy-6-fluoro-β-glucopyranosides were also determined, and these are presented in Table 3.

Lineweaver–Burk plots of the kinetic data for all of the substrates tested were linear, with the exception of that for DNP3Fglucoside. In this case a biphasic plot was obtained as shown in Figure 1. A similar phenomenon had been observed previously in the hydrolysis of PNPxyloside (Kempton & Withers, 1992) and shown to be due to a change in reaction mechanism wherein reaction at high substrate concentrations involves transglycosylation to a second substrate molecule while simple hydrolysis occurs at the lower concentrations.

To determine if transglycosylation was affecting values for the steady-state rate constants, the mode of deglycosylation for several DNPglucosides was investigated by HPLC analysis of products of the Abg-catalyzed reactions. At a DNP3Fglucoside concentration of 0.055 mM, the release of

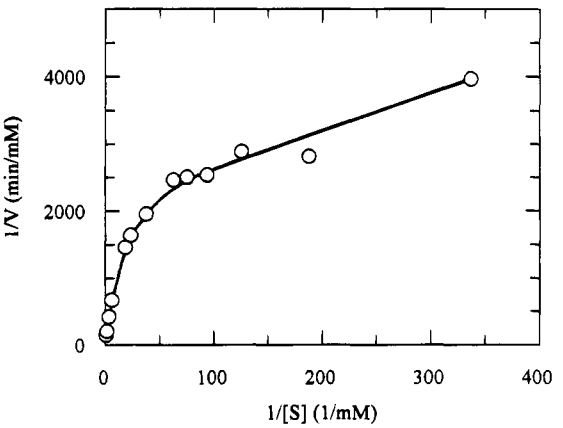

FIGURE 1: A Lineweaver–Burk analysis of the hydrolysis of DNP3Fglucoside by Abg.

Table 4: Pre-Steady-State Kinetic Parameters for Reaction of DNPGlycosides with Abg at 5 °C

substrate[a]	K_d (mM)	k_2 (sec^{-1})	k_2/K_d (mM^{-1} s^{-1})
DNPglucoside	0.65 (0.022)	1300 (21)	2000
DNP6Dglucoside	2.7 (0.34)	530 (35)	195
DNP3Dglucoside	0.43 (0.04)	33 (1.3)	77
DNP6Fglucoside	5.6 (2.0)	1800 (500)	320
DNP4F glucoside	2.7 (0.58)	90 (14)	34
DNP3F glucoside	0.31 (0.04)	81 (5)	266
DNP2F glucoside	0.34 (0.05)	0.355 (0.02)	1.06
DNPgalactoside[b]	2.74 (0.37)	22.1 (1.7)	8.1
DNPmannoside	2.65 (0.37)	1.04 (0.10)	0.392

[a] Neither DNP4Fgalactoside nor DNP4Dglucoside show a pre-steady-state "burst"; therefore, no pre-steady-state kinetic data were obtained. [b] K_d and k_2 were estimated from K_m and k_{cat} determined at 5 °C.

DNP during the reaction was accompanied by accumulation of a product identified as a fluorinated dissacharide by mass spectral analysis of the purified, acetylated product ($M + 1 = 724$ g/mol). When the same reaction was carried out at 0.002 mM substrate concentration, however, the transglycosylation product did not accumulate to an appreciable extent. Examination of the Abg-catalyzed reaction with DNPglucoside and 4FDNPglucoside indicated that, at saturating substrate concentration (0.4 mM), no transglycosylation was occurring with these substrates, thereby confirming that the linear Lineweaver–Burk plots seen for these, and presumably the other, substrates in the study reflected hydrolysis of the glycosyl–enzyme intermediate and not a transglycosylation process.

Pre-Steady-State Kinetics. A pre-steady-state burst was observed in most cases, indicating that deglycosylation is rate limiting for the majority of these substrates, as would be expected with the activated DNP leaving group. However, no pre-steady-state burst was observed for those substrates without an equatorial substituent at the 4 position, paralleling the anomalies observed with regard to increases in k_{cat} values. Pre-steady-state kinetic analysis of the hydrolysis of the parent DNPglucoside at 37 °C revealed that reactions were too fast for reliable analysis: thus all pre-steady-state experiments were carried out at 5 °C. Even at this lower temperature many of the reactions were very fast-resulting in considerable uncertainties in some of the higher values determined. Pre-steady-state kinetic parameters measured are shown in Table 4, and a representative

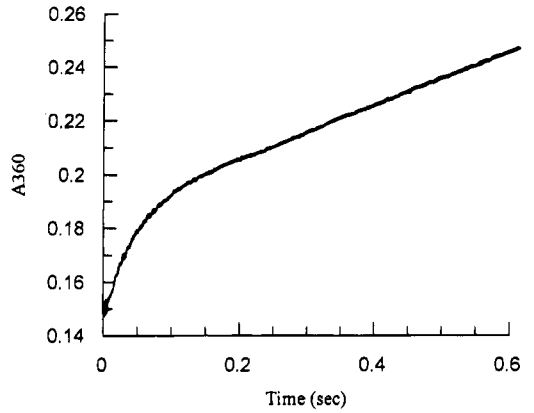

FIGURE 2: Pre-steady-state burst for DNP4Fglucoside with Abg at 5 °C. Concentration of DNP4Fglucoside = 1.1 mM.

Table 5: Inactivation Parameters for the 2-Position Substituted Glycosides with Abg at 37 °C

inactivator	K_i (mM)	k_i (s^{-1})	k_i/K_i (mM^{-1} s^{-1})
DNP2F glucoside	0.245 (0.02)	4.72 (0.22)	19.3
DNP2F mannoside	1.15 (0.7)	0.498 (0.012)	0.433
PNP2F glucoside[a]	1.670	2.83×10^{-4}	1.65×10^{-4}
DNP2Cl glucoside	8.3 (1.5)	0.285 (0.033)	0.0345

[a] Data obtained from Street et al. (1992).

burst is shown in Figure 2 for DNP4Fglucoside. Unfortunately the different temperatures studied make a comparison of the steady-state and pre-steady-state kinetic parameters for the DNPglycosides difficult. However, the slower glycosylation rates for the PNPglycosides allowed pre-steady-state analysis at 37 °C, and the following values were determined: PNPmannoside, $k_2 = 48$ s^{-1}, $K_d = 7.7$ mM, $k_2/K_d = 5.5$ mM^{-1} s^{-1}; PNP2Dglucoside, $k_2 = 19$ s^{-1}, $K_d = 4.4$ mM, $k_2/K_d = 4.2$ mM^{-1} s^{-1}; PNPglucoside, $k_2/K_d = 3300$ mM^{-1} s^{-1}. A reasonable agreement is seen between k_2/K_d values and k_{cat}/K_m values obtained from steady-state analyses, as would be expected since both parameters pertain to the same step in the reaction.

Inactivation Parameters. Kinetic parameters for the time-dependent inactivation of Abg by a series of 2,4-dinitrophenyl glycosides substituted at the 2-position are presented in Table 5. These represent glycosylation rate constants, k_2 for each compound since inactivation is a consequence of the accumulation of the glycosyl−enzyme intermediate in each case.

Calculation of $\Delta\Delta G^{\circ\ddagger}$ Values for the Steps in the β-Glucosidase Mechanism. Changes in the reaction parameters observed for Abg-catalyzed hydrolysis of the DNPglycosides upon substitution of the glycone hydroxyl groups can be related to changes in the equilibrium free energy (for dissociation constants) and activation free energy (for rate constants) using the expression given below. Here k_{par} and k_{sub} are the kinetic or dissociation constants for the parent (DNPglucoside) and substituted glycosides, respectively.

$$\Delta\Delta G^{\circ} = RT \ln(k_{par}/k_{sub})$$

These values of $\Delta\Delta G^{\circ\ddagger}$ for each hydroxyl group have been calculated for the initial binding (from K_d values at 5 °C), for the glycosylation step (from k_2 values at 5 °C and from k_{cat}/K_m values at 37 °C), and for the deglycosylation step k_3 (from k_{cat} values at 37 °C). They are presented in Table 6.

Table 6: Free Energy Changes at Each Step Resulting from Hydroxyl Substitution on DNP Glycosides

substitution	$\Delta\Delta G^0$, K_d (kJ mol^{-1})	$\Delta\Delta G^{\circ\ddagger}$, k_2 (kJ mol^{-1})	$\Delta\Delta G^{\circ\ddagger}$, k_3 (kJ mol^{-1})	$\Delta\Delta G^{\circ\ddagger}$, k_{cat}/K_m (kJ mol^{-1})
6-deoxy	3.3	2.1	2.7	2.9
4-deoxy				2.5
3-deoxy	−1.0	8.5	6.6	7.4
2-deoxy			~22[a]	
6-fluoro	5.0	−0.8	7.0	0.8
4-fluoro	3.3	6.2	6.7	6.5
3-fluoro	−1.7	6.4	12	6.1
2-fluoro	−1.5	19	52	15

[a] Value derived from a comparison of k_{cat} for this substrate with k_{cat} for PNPglucoside.

DISCUSSION

As is clear from the values of the rate constants and the free energy changes, substitution of the glycone hydroxyl groups has a significant effect on each of the two steps of Abg catalysis. Further, the magnitude of the effect varies with both the position of the substitution and the step in the reaction.

Interpretation of these changes requires a consideration of the major factors underlying them, as discussed previously for similar studies with glycogen phosphorylase (Street et al., 1989) and β-galactosidase (McCarter et al., 1992). Changes in the kinetic parameters occasioned by substitution of the hydroxyl groups on the glycon result from a combination of two major effects, electronic and binding. Electronic effects have their origin in the considerable differences in electronegativities of these substituents (H, OH, F), and the different effects these will therefore have on the stability of a positively charged, oxocarbenium ion-like transition state: highly electronegative substituents will destabilize the transition state, slowing the reaction and *vice versa*. The magnitude of these effects will be dependent upon the distance of the substituent from the anomeric center. Binding effects arise from the noncovalent interactions which develop between the enzyme and substrate at the transition state for the step in question, thereby stabilizing the transition state and providing catalysis. Removal of individual interactions, most likely hydrogen bonding, will result in destabilization of the transition state, thus slowing the reaction. Unfortunately, it is not possible to separate these two effects completely and determine their individual contributions. However, as described below, a reasonable estimate of the relative importance of the two effects can be obtained, providing useful insights into catalysis. A third possible effect could arise from differences in conformation of the sugars or of rates of interconversion of conformers. These are probably, however, of minor significance for hexopyranosides since the C-5 hydroxymethyl group largely controls conformation, as has been shown previously for 2-deoxy-2-fluoro-β-D-mannosyl fluoride, a fluorosugar which might have been expected to be distorted. X-ray crystallographic analysis and two-dimensional NMR studies revealed no significant change in the ring conformation (Withers et al., 1986).

Electronic Effects. The electronic nature of the transition state can be probed by comparison of the enzymic process with the well characterized equivalent nonenzymic reaction, for which the mechanism is known (Poulter et al., 1981;

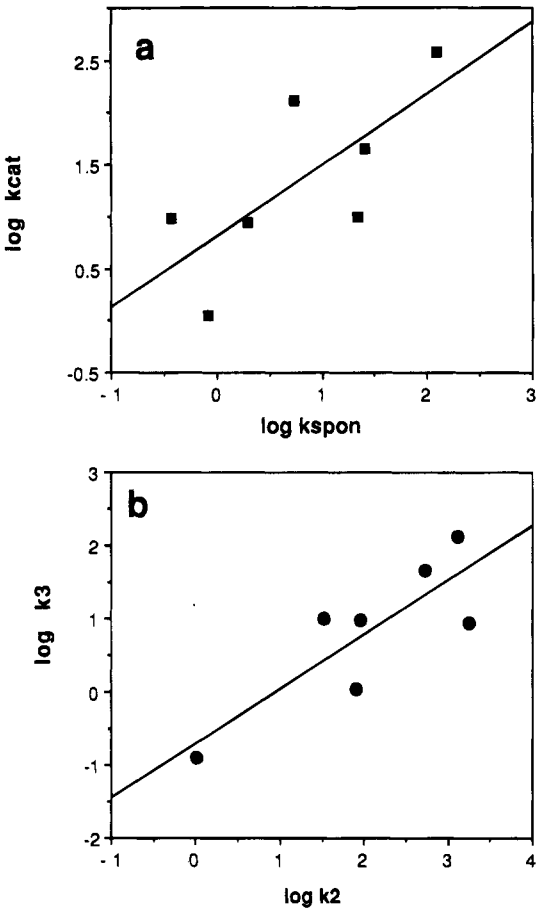

FIGURE 3: Linear free energy comparisons of the transition states for the enzymatic and spontaneous hydrolyses. (a) Plot of log k_{cat} versus log k_{spon}. (b) Plot of log k_3 versus log k_2.

Street et al., 1989; McCarter et al., 1992). In this study, the effect of the ring substituents on the electronic nature of the transition state was investigated by comparing the rate of enzyme-catalyzed hydrolysis of the DNPglycosides with their rates of spontaneous hydrolysis (Namchuk et al., 1995). A large body of evidence suggests that glycoside hydrolysis in solution proceeds via a transition state with substantial oxocarbenium ion character (Capon, 1969; Bennet & Sinnott, 1986). If the enzymic process proceeds via a similar transition state, a correlation should exist between the rates of spontaneous and enzymic hydrolysis for the same substrate. However, any such correlation will be masked by a background of scatter due to enzyme/substrate binding interactions not present in the spontaneous hydrolysis of the substrate. The plot of log k_{cat} versus log k_{spon} so derived shows such a correlation ($\rho = 0.73$), but only a very modest one. (Figure 3a). The considerable scatter in this plot is undoubtedly a consequence of the importance of noncovalent binding interactions in catalysis by this enzyme. Indeed, data for the DNP2Fglucoside were omitted since binding interactions at this position are particularly strong (*vide infra*), thereby distorting the plot. Nonetheless the correlation observed, albeit weak, does provide further evidence for an oxocarbenium ion-like transition state, consistent with predictions based upon secondary deuterium kinetic isotope effect studies (Kempton & Withers, 1992).

In a similar way, comparison of the rate constants for the glycosylation (k_2) and deglycosylation (k_3) steps should provide insights into the relative amounts of positive charge (oxocarbenium ion character) generated at the two transition states. Further, the scatter may well be reduced since it is likely that many of the binding interactions present in one step will also be important to some extent in the other. Such a free energy relationship is presented in Figure 3b. This plot of log k_3 versus log k_2 has a slope of 0.93 and a correlation coefficient of $\rho = 0.83$, the better correlation coefficient pointing to considerable similarity between the two transition states. Further, the slope of 0.93 might indicate (though the quality of the correlation and the different temperatures used leaves this in some doubt) that the deglycosylation transition state is more sensitive to substitution, and thus has more oxocarbenium ion character, than that for the glycosylation step. This conclusion is reinforced by inspection of the data in Table 6, where increases in $\Delta\Delta G^{\circ\ddagger}$ for the fluorosugars are much greater for the deglycosylation step than the glycosylation, whereas changes in $\Delta\Delta G^{\circ\ddagger}$ for the deoxysugar substrates are similar for the two steps. This would be consistent with similar previous suggestions based upon the larger secondary deuterium kinetic isotope effect for the deglycosylation step ($k_H/k_D = 1.10-1.12$) than the glycosylation step ($k_H/k_D = 1.05-1.07$) (Kempton & Withers, 1992).

Further support for this hypothesis is obtained from a comparison of rate constants for DNPmannoside, DNP2Fmannoside, and PNP2Dglucoside. All three compounds are missing an equatorial C-2 substituent: thus the same crucial binding interactions are absent in each case. However, DNPmannoside and PNP2Dglucoside have very similar deglycosylation rate constants, consistent with the removal of a large stabilising interaction worth 18 and 22 kJ mol^{-1}, respectively, while DNP2Fmannoside is turned over very much more slowly corresponding to a 41 kJ mol^{-1} increase in activation energy, suggesting large electronic effects in addition to the binding effects. By contrast glycosylation rate constants (from k_{cat}/K_m or k_i/K_i) for DNPmannoside and DNP2F mannoside are quite similar, corresponding to increases in the activation energy for glycosylation of 22−25 kJ mol^{-1} relative to the parent substrate and thereby suggesting that the electronic effect is much less important to glycosylation.

Binding Effects. Electronic considerations alone would predict that the rates of enzyme-catalyzed hydrolysis of the deoxy glycosides should all be greater than that of the parent DNPglucoside, as was seen in the nonenzymatic case (Namchuk et al., 1995). Since this is not observed, it is clear that noncovalent enzyme/substrate interactions with the glycon hydroxyl groups must play a major role in catalysis. Assuming these interactions take the form of hydrogen bonds, replacement of a glycon hydroxyl with hydrogen will result in deletion of any enzyme/substrate hydrogen bonding which normally takes place at the substituted position. Thus, $\Delta\Delta G^{\circ}$ or $\Delta\Delta G^{\circ\ddagger}$ values calculated for each of the steps in the Abg-catalyzed hydrolysis of the deoxyglycosides will provide a minimum estimate (Street et al., 1986, 1989) of the magnitude of the interaction at the substituted position. These are minimum estimates since the rate decrease may be mitigated by favorable electronic effects. An estimate of the polarity of the hydrogen bond at the substituted position may also prove possible since fluorine is arguably capable of accepting a hydrogen bond but cannot donate one (Street & Withers, 1986). Thus if the enzyme donates a hydrogen bond at the

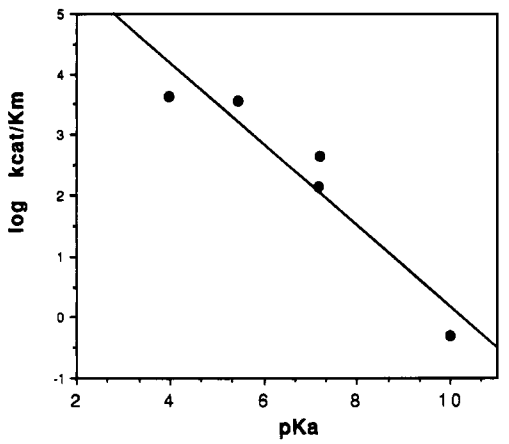

FIGURE 4: Brønsted plot for enzymatic hydrolysis of a series of aryl 6-deoxy-6-fluoro β-glucosides by Abg. A plot of log (k_{cat}/K_m) for the enzyme-catalyzed hydrolysis versus pK_a of the aglycon phenol.

substituted position, the deoxyfluoroglycoside may be able to regain some of the binding energy lost to the analogous deoxyglycoside, increasing the rate of hydrolysis for the deoxyfluoroglucoside in comparison to the analogous deoxy substrate, in opposition to electronic effects. If, however, the deoxyfluoro compound has a lower rate of hydrolysis than the analogous deoxyglycoside, no conclusion as to the polarity of the interaction at that position is possible since the rate reduction could arise from electronic or binding effects.

Inspection of the data in Table 6 reveals that, with the exception of the 6 position, contributions of individual hydroxyl groups to ground state binding interactions are much smaller than at the transition state. This is completely consistent with expectations based upon the notion that enzymes catalyze reactions by selective binding to, and stabilization of, their transition states. Estimates of the contributions of interactions at the glycosylation transition state derived from k_2 and k_{cat}/K_m are gratifyingly similar given the different temperatures employed and the fact that the preceding ground state species are different for the two parameters (the Michaelis complex for k_2 and free enzyme for k_{cat}/K_m). This suggests that the *differences* measured primarily reflect differences in transition state energies.

Interactions at the 3 and 4 positions appear to play little role in *ground state* substrate binding, and even to be slightly destabilizing, whereas effects at the transition states are more significant. Interactions at the 6-position, by contrast, provide substantial stabilization of the ground state, and only an equivalent stabilization at the transition state thus decreasing the efficiency of the reaction, as revealed in k_{cat}/K_m values. In order to probe this further, the series of 6-fluoro-substituted aryl glucosides was synthesised and subjected to kinetic analysis. The excellent ($\rho = 0.95$) Brønsted relationship obtained by plotting log (k_{cat}/K_m) vs pK_a (Figure 4) has a β_{lg} value of 0.7, identical to that obtained previously for the parent aryl glucosides (Kempton & Withers, 1992). This indicates that the transition states for the two processes are very similar, at least in regard to the negative charge accumulation on the departing oxygen atom.

Interactions at the 2 position are by far the most important, contributing at least 18 and 22 kJ mol^{-1} binding energy, respectively, to stabilization of the *transition states* for glycosylation and deglycosylation. The interactions at the 3 and 6 positions fulfill a similar function though they are much smaller in magnitude (7 and 3 kJ mol^{-1} at the glycosylation and deglycosylation steps, respectively). Previous studies of hydrogen bonds in protein−ligand interactions have shown that interactions between two neutral partners contrubute up to 6.3 kJ mol^{-1} while hydrogen bonds with a charged partner contribute approximately 12.5 kJ mol^{-1} (Fersht et al., 1986; Street et al., 1986, 1989). On the basis of these estimates, a hydrogen bond between the 2 position hydroxyl and at least one charged group on the protein seems likely, thereby implicating the 2-hydroxyl as the donor. One residue which must be charged, and must be close by is Glu358, the catalytic nucleophile. A hydrogen bond formed between the 2-hydroxyl and this residue could be optimized at the transition state, as the two groups approach each other while the glycosyl−enzyme bond is formed, but decrease in strength as the intermediate forms and the charge on the carboxylate oxygen is lost.

The weaker interactions at the 3 and 6 positions are more consistent with hydrogen bonds to neutral partners on the enzyme. Estimates of their polarities are possible following the approach described earlier since values of k_{cat}/K_m are larger for the 3- and 6-deoxyfluoro glucosides than for the analogous deoxy glycosides, suggesting that the enzyme donates a hydrogen bond at these two positions.

Interactions at the 4 Position. Kinetic parameters for substrates missing an equatorial hydroxyl group at the 4 position (DNPgalactoside, DNP4F galactoside, and DNP4Dglucoside) differ markedly from those of the other glycosides tested. First, no pre-steady-state burst was observed for these compounds, suggesting that glycosylation is rate limiting. Further, k_{cat} values for these compounds are greater than that for DNPglu, indicating that the deglycosylation rate is increased by removal of hydrogen bonding interactions at this position.

Interactions at the 4-position are best evaluated by consideration of the data for DNP4Dglucoside since hydrogen bonding will be eliminated and no repulsive interactions introduced. The k_{cat}/K_m value is reduced only 2-fold compared to DNPglucoside, indicating that the deglycosylation transition state is destabilized by only 2.5 kJ mol^{-1} by removal of this interaction. Much greater decreases (15−30-fold) are seen for DNPgalactoside and DNP4Fgalactoside suggesting the presence of additional repulsive steric interactions due to the axial substituent. By contrast, the deglycosylation step, k_3, for DNP4Dglucoside is actually accelerated at least 3-fold by removal of this substituent since k_{cat} increases to 380 s^{-1}. Given that k_2 is rate-limiting in this case, the true increase is likely much greater. Further evidence comes from studies using the 2-deoxy-2-fluoro-β-D-glycosyl fluorides, which have previously been shown to inactivate Abg by formation of a stable 2-deoxy-2-fluoroglycosyl−enzyme intermediate (Withers et al., 1988; Street et al., 1992). The value of k_i/K_i for inactivation of the enzyme was 18 times smaller for the 2-deoxy-2-fluorogalactosyl fluoride in comparison with the analogous glucoside, again showing that alteration of the interaction at the 4 position has a deleterious effect on the rate of glycosylation. However, the rate of hydrolysis of the galactosyl enzyme intermediate is 500 times *faster* than that of the glucosyl−enzyme intermediate (Street et al., 1992), showing the

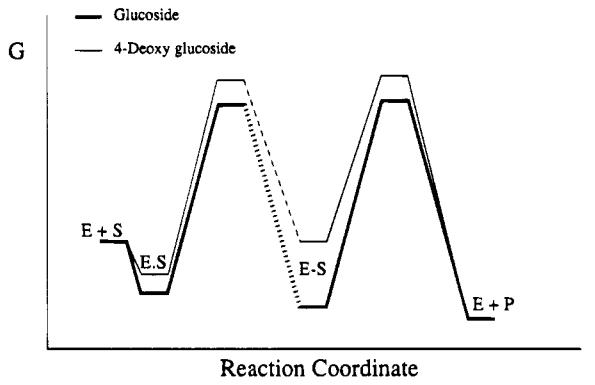

FIGURE 5: Generalized reaction coordinate diagram illustrating the hydrolysis of DNPglucoside and DNP4Dglucoside.

retarding effect of the equatorial substituent on the deglycosylation step.

In order to understand this unusual effect, it is important to consider not only the transition states but also the preceding ground states. It seems unlikely that a relatively remote substituent would differentially affect the two transition states to this degree, especially given that we have shown them to be quite similar. More probable is that the 4-hydroxyl provides an important interaction which stabilizes the intermediate more so than the two transition states or the Michaelis complex. As shown in Figure 5, the effect of this additional interaction would be to slow k_3 without affecting k_2. In the absence of structural information it is not possible to confidently assign a molecular basis to this; presumably, it is simply that a hydrogen bond at this position is maximised in the covalent intermediate.

It is noteworthy that the only 4-substituted DNPglucoside which does not follow this pattern is DNP4Fglucoside which has a low k_{cat} value and shows a burst. This difference in behavior cannot be a consequence of inductive effects since the DNP4Fgalactoside does not show the same behavior. Rather, it strongly suggests that the fluorine engages in productive hydrogen bonding at this position, thereby assigning the polarity of some of the hydrogen bonding at that position.

CONCLUSIONS

Hydrogen bonds between Abg and the substrate glycone play a crucial role in effecting catalysis. Initial substrate recognition involves a hydrogen bond at the 6 position and probably other interactions not addressed by the present study. Evidence from this and other studies of the enzyme suggest that the subsequent bond cleavage steps in the mechanism, glycosylation and deglycosylation, both pass through transition states with substantial oxocarbenium ion character. Hydrogen bonds at the 2, 3, and 6 positions aid in stabilizing the transition states for both steps with the interaction at the 2 position being by far the most important. Interestingly, the hydrogen bond at the 4 position appears to accelerate glycosylation but decelerate deglycosylation, most likely by maximization of its strength at the glycosyl–enzyme intermediate, rather than at the transition state. Increasing the lifetime of the intermediate in this way would allow the aglycon to diffuse from the active site and water to diffuse in and attack the intermediate, while still using the binding energy available in the aglycon site to catalyze the glycosylation step. This could be most important for the enzyme's natural substrates (cellobiose, cellotriose, etc.), for which binding interactions with the aglycon are essential to catalysis.

It is interesting to compare the data obtained in this study with that derived from the study of other retaining β-glycosidases. Although the importance of interactions at the other positions varies considerably, a common feature of all such enzymes examined to date is the importance of the interaction at the 2 position in stabilizing the transition state (Table 7). Some very strong interactions are involved, in the range of those which have been characterized elsewhere as short, low barrier hydrogen bonds (Cleland & Kreevoy, 1994; Frey et al., 1994), and it is of interest to speculate upon their origin. One source could be the change in geometry of the substrate, especially the orientation of the hydroxyl at C2, between the ground state $^4C_1$ chair and the transition state ^4H half chair, the hydrogen bond being optimized at the transition state geometry. In addition to changes in geometry, there is presumably an increase in acidity of the 2-hydroxyl at the positively charged transition state. This would result in a closer matching of the pK_a values of the 2-hydroxyl group and its protein partner, likely Glu358, thereby increasing the strength of the interaction, as required for such hydrogen bonds. Whether this interaction is a more general phenomenon is in question. However it is noteworthy that interactions at the 2-position in α-glycosidases and α-glycosyl transferases appear to be less important, values of 5.2 and 1.9 kJ mol^{-1} having been measured for glucoamylase (Sierks & Svensson, 1992) and glycogen phosphorylase (Becker et al., 1994). What is not clear is whether this is due to the fact that these are α-cleaving enzymes or whether it is due to other differences such as the fact that glucoamylase is an inverting enzyme or that glycogen phosphorylase is not a hydrolase. Clearly more data are required before any conclusions pertaining to differences in mechanisms of α- and β-glycosidases are possible, and work in this laboratory is currently underway which will aid in addressing this question.

Table 7: Comparison of Changes in Activation Free Energy Calculated from k_{cat}/K_m Using Analogously Substituted Deoxyglycoside Substrates[a]

modified position	*Agrobacterium* β-glucosidase	*E. coli (Lac-Z)* β-galactosidase[b]	*A. wentii* β-glucosidase[e]	A. oryzae β-glucosidase[c]	mammalian lactase[d]
2-deoxy	17.9	>34	32.1	43–45	*f*
3-deoxy	7.4			34.8	*f*
4-deoxy	2.5	15.5	21	29.1	5.2
6-deoxy	2.9	18.0		16	−4.8

[a] Activation free energies in kJ mol^{-1}. [b] Data from McCarter et al. (1992). [c] Data from Mega and Matsushima (1983). [d] Data from Rivera-Sagredo et al. (1992). [e] Data from Roeser and Legler (1981). [f] Rates were too low to be measured, indicating a large loss of binding energy upon deletion of the interactions at the 2 and 3 positions.

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