# Inactivation of a β-Glucosidase through the Accumulation of a Stable 2-Deoxy-2-fluoro-α-D-glucopyranosyl–Enzyme Intermediate: A Detailed Investigation†

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ABSTRACT: The inactivation of glycosidases by 2-deoxy-2-fluoroglycosides has been shown previously to occur via the accumulation of a covalent 2-deoxy-2-fluoro-α-D-glucopyranosyl enzyme intermediate [Withers, S. G., & Street, I. P. (1988) *J. Am. Chem. Soc. 110*, 8551]. Further characterization of this process with *Agrobacterium* β-glucosidase is described, and the range of glycosides engaging in this behavior is examined. Inactivation is shown to be accompanied by the release of a stoichiometric "burst" of aglycon, thereby providing a new class of active site titration agents for glycosidases. The rate of inactivation is shown to be very strongly dependent on the leaving group ability of the aglycon, the slowest inactivator studied (*p*-nitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside) yielding only partial inactivation due to turnover of the intermediate becoming competitive with its formation. Such turnover of the intermediate is shown to be greatly accelerated by transglycosylation to a suitable glycoside bound in the aglycon site, resulting in the release of a disaccharide product which was isolated and characterized. The pH dependences of both the formation and the hydrolysis of the 2-deoxy-2-fluoroglycosyl–enzyme closely resemble those of each step for normal catalysis, indicating that the same catalytic groups are involved in both processes. A model system for the partial "steady-state" inactivation observed previously [Withers, S. G., Rupitz, K., & Street, I. P. (1988) *J. Biol. Chem. 263*, 7929] with certain other glycosidases was established by incubating the enzyme with an inactivator known to undergo relatively rapid transglycosylation in the presence of various concentrations of a suitable reactivator. The extent of steady-state inactivation observed in each case agreed closely with that predicted according to the kinetic scheme proposed.

Specific inhibitors of glycosidases are of interest in a number of quite different applications. These include mechanistic studies of the enzymes themselves (Lalegerie et al., 1982), study of glycoprotein processing (Elbein et al., 1984), and potential therapeutic uses such as the control of glucose release from the gut via specific inhibition of gut α-glucosidases (Truscheit et al., 1981). Several naturally occurring non-covalent inhibitors of glycosidases are known, such as acarbose (Schmidt et al., 1977) and nojirimycin (Schmidt et al., 1979). In addition, a number of active site-directed inhibitors of glycosidases have been described. These are generally glycosides containing such reactive functionalities as isothiocyanates, epoxides, and α-halocarbonyls. However, relatively few of the most selective mechanism-based inhibitors have been described, these essentially being the conduritol epoxides (Legler, 1968, 1990) including the naturally occurring cyclophellitol (Withers & Umezawa, 1991) and the glucosylmethyl triazenes (Marshall et al., 1981). This paper describes a novel approach to mechanism-based inhibition of glycosidases, involving the stabilization of a "normal" intermediate in the catalytic pathway.

As detailed in the previous paper (Kempton & Withers, 1992), the mechanism of "retaining" glycosidases involves a double-displacement reaction in which a glycosyl–enzyme intermediate is formed and hydrolyzed via oxocarbonium ion-like transition states. Enzymic catalysis is therefore effected through stabilization of these transition states, both by electrostatic stabilization of the developing positive charge and by provision of suitable hydrogen-bonding interactions to bind to the partially planar (most likely half chair) sugar moiety. One possible route to the inactivation of such enzymes would involve the formation of a very stable glycosyl–enzyme intermediate, but this necessitates slowing down the deglycosylation step [$k_{deglycosylation} = 150\ s^{-1}$ (Kempton & Withers, 1992)] considerably. Such an effect has been achieved to a limited extent previously by removing important transition-state interactions through modification of the 2-position of the sugar moiety. Thus, under favorable circumstances, the enzymic hydrolysis of certain 2-deoxyglycosides and glycals involves the accumulation of a 2-deoxyglycopyranosyl enzyme intermediate [see Legler (1990), Sinnott (1990), and Lalegerie et al. (1982) for reviews]. However, the lifetime of the intermediates so generated has been too short to permit their direct observation by physical techniques, though in one case (Roeser & Legler, 1981) the identity of the amino acid involved was determined through denaturation trapping of the intermediate and subsequent sequencing. Interactions at this 2-position would therefore appear to be of critical importance for glycosyl transferases (Roeser & Legler, 1981; Street et al., 1989; Wolfenden & Kati, 1991) presumably because this is the hydroxyl whose orientation changes the most in proceeding from the ground state to the transition state; thus interactions at this position are particularly effective in achieving specific transition-state stabilization.

Destabilization of the oxocarbonium ion-like transition state can also be achieved inductively through introduction of electron-withdrawing substituents in the sugar moiety, preferably at the 2-position such that the inductive destabilization of the positively charged transition state is maximized (Capon, 1969; Withers et al., 1986). A fluorine substituent is ideal

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for this task in the study of enzymes, not only because it is the most electronegative substituent possible but also because it is smaller than the hydroxyl it replaces and thus will not suffer steric problems and because it has the potential to act as an NMR "handle". In addition, the limited hydrogen-bonding possibilities for the fluorine will mean that some transition-state binding interactions will also likely be disrupted, leading to further rate reductions. Such a strategy might be expected to be particularly successful for β-glycosidases, for which it appears (Kempton & Withers, 1992) that the transition state for deglycosylation has more oxocarbonium ion character than that for glycosylation; thus the fluorine at C-2 would be expected to slow the breakdown of the intermediate more than it slows its formation. Further, the incorporation of aglycons of high leaving group ability should ensure that the glycosylation step remains faster than deglycosylation.

Under these conditions, it has indeed proved possible to generate effective mechanism-based inactivators of β-glycosidases, and preliminary reports on this topic have described their development for several types of enzymes (Withers et al., 1988). Brief accounts have also been published of their use to prove the intermediacy of a covalent α-D-glycopyranosyl–enzyme intermediate on a β-glucosidase (Withers & Street, 1988) and of the identification of the position of attachment in the cases of *Agrobacterium* β-glucosidase (Withers et al., 1990) and *Cellulomonas fimi* exoglucanase (Tull et al., 1991). This paper describes a detailed investigation of their mode of action and a demonstration of their relevance to the normal pathway of catalysis.

## MATERIALS AND METHODS

*Synthesis.* The syntheses of the inactivators 2-deoxy-2-fluoro-β-D-glucopyranosyl fluoride (2FβGlu-F),[1] 2-deoxy-2-fluoro-β-D-mannosyl fluoride (2FβMan-F), 2-deoxy-2-fluoro-β-D-galactopyranosyl fluoride (2FβGal-F), and 2′,4′-dinitrophenyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-fluoro-β-D-glucopyranoside (2,4-DNP2FGlu) have been described previously (Street et al., 1986; Withers et al., 1988; Berven et al., 1990). The synthesis of glucosyl benzene was performed according to Bonner (1963). The synthesis of the aryl 2-deoxy-2-fluoro-β-D-glucopyranosides was performed according to the following general procedure. 3,4,6-Tri-*O*-acetyl-2-deoxy-2-fluoro-α-D-glucopyranosyl bromide (Shelling et al., 1984) (1 mmol) dissolved in acetone (3 mL) was added to the phenol (2 mmol) suspended in 1 N NaOH (2 mL), and the mixture was stirred for 24 h at room temperature. The solvent was then evaporated in vacuo, the resulting aqueous slurry was diluted with water and extracted with dichloromethane (3 × 50 mL), and the combined organic extracts were washed twice with base (saturated $NaHCO_3/H_2O$), dried over $MgSO_4$, filtered, and then concentrated to a gum by evaporation. Most products crystallized immediately upon addition of ethanol, and all were purified and fully characterized by $^1H$ NMR, as well as by elemental analysis. Deacetylation of glycosides with leaving group $pK_a$ values >6 was effected by use of sodium methoxide in methanol (Sinnott & Souchard, 1973), while more reactive glycosides were deprotected using HCl in dry methanol according to Ballardie et al. (1973). Characterization data for the compounds so prepared are as follows.

*4′-Nitrophenyl 2-Deoxy-2-fluoro-β-D-glucopyranoside* was recrystallized from ether to produce fine white needles (0.156 g, 0.514 mmol, 62%), mp 142–144 °C. $^1H$ NMR ($CD_3OD$): 8.20 [d, 2 H, $J$ = 9 Hz, H(3′), H(5′)], 7.10 [d, 2 H, $J$ = 9 Hz, H(2′), H(6′)], 5.35 [dd, 1 H, $J_{1,2}$ = 8, $J_{1,F}$ = 3 Hz, H(1)], 4.50–4.25 [m, 1 H, $J_{1,2}$ = 8, $J_{2,3}$ = 10, $J_{2,F}$ = 48 Hz, H(2)], 4.00 [dd, 1 H, $J_{2,3}$ = 9, $J_{3,4}$ = 2 Hz, H(3)], 3.65–3.45 [m, 4 H, H(4), H(5), H(6), H(6′)]. Anal. Calcd for $C_{12}H_{14}O_7NF$: C, 47.52; H, 4.68; N, 4.62%; Found: C, 47.66; H, 4.68; N, 4.61%.

*3′,4′-Dinitrophenyl 2-Deoxy-2-fluoro-β-D-glucopyranoside* was recrystallized from methanol/dichloromethane/acetone to produce yellowish needles (0.024 g, 0.069 mmol, 87%), mp 138–139 °C (dec). $^1H$ NMR ($CD_3OD$): 8.15 [d, 1 H, $J_{5',6'}$ = 11 Hz, H(5′)], 7.65 [d, 1 H, $J_{2',6'}$ = 2 Hz, H(2′)], 7.50 [dd, 1 H, $J_{5',6'}$ = 11, $J_{2',6'}$ = 2 Hz, H(6′)], 5.48 [dd, 1 H, $J_{1,2}$ = 8, $J_{1,F}$ = 3 Hz, H(1)], 4.50–4.25 [dt, 1 H, $J_{1,2}$ = 8, $J_{2,3}$ = 8, $J_{2,F}$ = 51 Hz, H(2)], 3.85 [dd, 1 H, $J_{2,3}$ = 8, $J_{3,4}$ = 2 Hz, H(3)], 3.60–3.30 [m, 4 H, H(4), H(5), H(6), H(6′)]. Anal. Calcd for $C_{12}H_{13}O_9N_2F + 0.5H_2O$; C, 40.34; H, 3.96; N, 7.84%; Found: C, 40.29; H, 4.19; N, 7.69%.

*2′,4′,6′-Trichlorophenyl 2-Deoxy-2-fluoro-β-D-glucopyranoside* was recrystallized from acetone/dichloromethane/hexane to produce off-white solid (0.045 g, 0.124 mmol, 60%), mp 165–167 °C (dec). $^1H$ NMR ($CD_3OD$): 7.40 [s, 2 H, H(3′), H(5′)], 5.30 [dd, 1 H, $J_{1,2}$ = 8, $J_{1,F}$ = 3 Hz, H(1)], 4.45–4.20 [dt, 1 H, $J_{1,2}$ = 8, $J_{2,3}$ = 8, $J_{2,F}$ = 49 Hz, H(2)], 3.85–3.30 [5 H, H(3), H(4), H(5), H(6), H(6′)]. Anal. Calcd for $C_{12}H_{12}O_5Cl_3F + 0.5H_2O$; C, 38.88; H, 3.63; Found: C, 38.88; H, 3.49.

*4′-Chloro-2′-nitrophenyl 2-Deoxy-2-fluoro-β-D-glucopyranoside* was recrystallized from ethyl acetate/hexane to produce yellowish crystals (0.038 g, 0.113 mmol, 81%), mp 122–124 °C. $^1H$ NMR: 7.85 [d, 1 H, $J_{3',5'}$ = 2 Hz, H(3′)], 7.60 [dd, 1 H, $J_{3',5'}$ = 2, $J_{5',6'}$ = 11 Hz, H(5′)], 7.45 [d, 1 H, $J_{5',6'}$ = 11 Hz, H(6′)], 5.40 [dd, 1 H, $J_{1,2}$ = 8, $J_{1,F}$ = 3 Hz, H(1)], 4.40–4.15 [dt, 1 H, $J_{1,2}$ = 8, $J_{2,3}$ = 8, $J_{2,F}$ = 49 Hz, H(2)], 3.95 [dd, 1 H, $J_{2,3}$ = 14, $J_{3,4}$ = 2 Hz, H(3)], 3.60–3.30 [4 H, H(4), H(5), H(6), H(6′)]. Anal. Calcd for $C_{12}H_{13}O_7NFCl + 0.5H_2O$; C, 41.56; H, 4.08; N, 4.04%; Found: C, 41.25; H, 4.41; N, 3.70%.

*2′,5′-Dinitrophenyl 2-Deoxy-2-fluoro-β-D-glucopyranoside* was recrystallized from acetone/dichloromethane to produce yellowish crystals (0.018 g, 0.052 mmol, 62%), mp 122–124 °C. $^1H$ NMR: 8.30 [d, 1 H, $J$ = 2 Hz, H(6′)], 8.00 [m, 2 H, H(3′), H(4′)], 5.57 (dd, 1 H, $J_{1,2}$ = 8, $J_{1,F}$ = 3 Hz, H(1)], 4.40–4.20 [dt, 1 H, $J_{1,2}$ = 8, $J_{2,3}$ = 8, $J_{2,F}$ = 49 Hz, H(2)], 3.95 [dd, 1 H, $J_{2,3}$ = 14, $J_{3,4}$ = 2 Hz, H(3)], 3.70 [dd, 1 H, $J_{1,2}$ = 8, $J_{2,3}$ = 14 Hz, H(2)], 3.80–3.40 [4 H, H(4), H(5), H(6), H(6′)]. Anal. Calcd for $C_{12}H_{13}O_9N_2F + 0.5H_2O + CH_2Cl_2$: C, 35.31; H, 3.65; N, 6.34%; Found: C, 35.35; H, 3.98; N, 6.31%.

*2′,4′-Dinitrophenyl 2-Deoxy-2-fluoro-β-D-glucopyranoside* was recrystallized from acetone/dichloromethane/hexane to produce yellowish needles (0.016 g, 0.046 mmol, 58%), mp 123–126 °C. $^1H$ NMR ($CD_3OD$): 8.75 [d, 1 H, $J_{3',5'}$ = 2 Hz, H(3′)], 8.50 [dd, 1 H, $J_{3',5'}$ = 2, $J_{5',6'}$ = 10 Hz, H(5′)], 7.65 [d, 1 H, $J_{5',6'}$ = 10 Hz, H(6′)], 4.45–4.20 [dd, 1 H, $J_{2,3}$ = 10, $J_{2,F}$ = 51 Hz, H(2)], 3.93 [dd, 1 H, $J_{2,3}$ = 10, $J_{3,4}$ = 3 Hz, H(3)], 3.82–3.60 [m, 3 H, H(4), H(5), H(6)], 3.50 [m, 1 H, H(6′)]. Anal. Calcd for $C_{12}H_{13}O_9N_2F + 2.5H_2O$:

[1] Abbreviations: PNPGlu, *p*-nitrophenyl β-D-glucopyranoside; PGlu, phenyl β-D-glucopyranoside; 2,4-DNPGlu, 2′,4′-dinitrophenyl β-D-glucopyranoside; 2,4-DNP2FGlu, 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside; 2,4-DNP2FGal, 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-D-galactopyranoside; PNP2FGlu, 4′-nitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside; PNP, 4-nitrophenol; DNP, 2,4-dinitrophenol; 2FβGlu-F, 2-deoxy-2-fluoro-β-D-glucopyranosyl fluoride; 2FβGal-F, 2-deoxy-2-fluoro-β-D-galactopyranosyl fluoride; 2FβMan-F, 2-deoxy-2-fluoro-β-D-mannopyranosyl fluoride.

C, 40.83; H, 4.46; N, 7.27%; Found: C, 40.75; H, 4.40; N, 6.90%.

*Enzymology.* *Agrobacterium* β-glucosidase was purified and assayed as described previously (Kempton & Withers, 1992), and protein concentration was determined using the absorbance value of $E_{280}^{0.1\%} = 2.20\ cm^{-1}$. Determination of the concentration of β-glucosidase active sites was also achieved by titrating samples of the enzyme with 2,4-DNP2FGlu as follows: sodium phosphate buffer (50 mM, pH 6.8) containing β-glucosidase was placed in a 1-cm path length glass cuvette, and the temperature was allowed to equilibrate to 37 °C in the spectrophotometer. The inactivator 2,4-DNP2FGlu (at twice the estimated enzyme concentration) was added and the optical density at 400 nm recorded. After corrections had been made for the added volume and the initial absorbance of the glucoside sample, the enzyme concentration was calculated using 11 300 $M^{-1}\ cm^{-1}$ as the molar extinction coefficient of the 2,4-dinitrophenolate anion at 37 °C and pH 6.8. A similar protocol was followed to assay fluoride released from 2FβGlu-F using a colorimetric dye-binding assay for fluoride except that in this case the enzyme was dialyzed into 10 mM HEPES buffer, pH 6.8, to avoid interference from the phosphate buffer. This assay (Megregian, 1978) is based on the ability of fluoride to destroy the orange zirconium eriochrome complex and is sensitive in the 0.5–1.0 μM range.

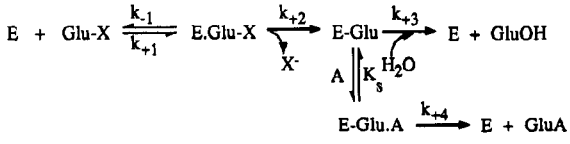
Time-dependent inactivation of β-glucosidase was monitored as described previously (Withers et al., 1987, 1988). The time-dependent release of phenolate from the aryl 2-deoxy-2-fluoroglucosides was monitored by placing the desired concentration of the inactivator in a 1-cm quartz cell in the thermostated cell block of a Pye Unicam PU8800 double-beam spectrophotometer in buffer containing BSA. After thermal equilibration, a sample of enzyme (at 37 °C) was injected into the cell, the contents were mixed rapidly, and the absorbance was directly monitored. Rates of release of 2,4-dinitrophenolate from 2,4-DNP2FGlu were determined using an Applied Photophysics stopped flow instrument, monitoring release of dinitrophenolate by the absorbance change at 400 nm. Release of phenolate was found to be pseudo-first-order in all cases except that of PNP2FGlu, in which case the earlier stages of the reaction were nonetheless approximately first order. Data were analyzed to extract inactivation parameters as described previously (Withers et al., 1987, 1988). The pH dependence of the inactivation reaction with 2FβGlu-F was determined by measuring the inactivation rates at a series of pH values within the pH-stability range of the enzyme (Kempton & Withers, 1992) using a fixed (0.13 mM) concentration of 2FβGlu-F in 50 mM sodium phosphate buffer containing 145 mM NaCl and 0.1% BSA at 37 °C. The partial "steady-state" inactivation by 2FβGlu-F in the presence of different concentrations of glucosyl benzene was monitored by incubating *Agrobacterium* β-glucosidase with 2FβGal-F (0.26 mM) in the presence of a range (0–19.1 mM) of concentrations of glucosyl benzene. Residual activity of the enzyme was monitored using PNPGal (3 mM) as substrate.

*Turnover of the Covalently Inactivated Enzyme.* Inactivated enzyme was loaded onto a column of Sephadex G-25 (19.5 cm × 1 cm), and the column was eluted with 50 mM sodium phosphate buffer, pH 6.8. The eluant was monitored at 280 nm, and fractions containing protein were collected and assayed for the presence of any residual inhibitor. The turnover of the isolated covalent complex was then monitored at 37 °C in 50 mM sodium phosphate buffer (pH 6.8) containing 1 mg $mL^{-1}$ BSA in the presence or absence of reactivating ligand. Periodically, samples were removed from the incubation mixture and assayed for β-glucosidase activity using 1.5 mM PNPGlu as described above. Reactivation of the 2-fluorogalactosyl–enzyme was found to be very significantly accelerated by PNPGlu during the actual assay, causing significant errors in the estimation of residual activity. Samples of this enzyme were therefore assayed with PNPGal (3.0 mM), since this is still an excellent substrate for the native enzyme (Day & Withers, 1986; Kempton & Withers, 1992) but is a very poor ligand for promoting reactivation. The activity of the fully reactivated enzyme sample was determined by a number of different methods: for rapid reactivation, the enzyme was incubated until a constant activity was attained over several assays. For experiments where reactivation was slow but large amounts of the inhibited enzyme were available, the end point activity was calculated from the ratio of the optical densities at 280 nm before and after gel permeation chromatography multiplied by the initial activity of the sample prior to inhibition. The pH dependence of the hydrolytic reactivation reaction was determined using the 2-deoxy-2-fluoro-galactosyl enzyme since it is the only species which turns over on an experimentally accessible time scale. Samples were incubated at the pH values indicated in 50 mM sodium phosphate buffer containing 145 mM NaCl and 0.1% BSA at 37 °C, and aliquots were taken at time intervals and assayed at pH 6.8 using PNPGal.

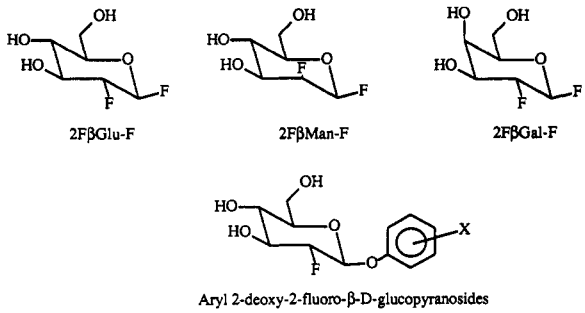
*$^{19}F$ NMR.* Spectra were recorded on a Bruker HXS-270 spectrometer operating at 254 MHz in the Fourier-transform mode with a 5-mm $^{19}F$ probe. A spectral width of 40 000 Hz was used, with a 90° pulse angle (15 μs). Experiments were performed using gated proton decoupling (decoupler on during acquisition) with a repetition delay of 2 s. Chemical shifts are quoted relative to $CFCl_3$ ($\delta = 0.00$ ppm) and were measured with reference to an internal sample of 6-deoxyfluoro-α-D-glucopyranosyl phosphate ($\delta = 237.48$ ppm). Exponential line broadening used prior to Fourier transformation was generally 20 Hz, and all line width data have been corrected for this. Protein samples were prepared by concentrating *Agrobacterium* β-glucosidase in a Centricon microconcentrator (30 000-Da cut-off) until the sample volume had been reduced to approximately 1 mL. The sample was then diluted 2-fold with deuterated buffer and reconcentrated to a final volume of less than 0.4 mL. Paramagnetic impurities were removed from the deuterated water by pretreatment with Chelex. The final enzyme concentration was determined using the dinitrophenolate burst method after dilution of a small aliquot (5–10 μL) into 0.5 mL of 50 mM sodium phosphate buffer, pH 6.8. Solutions of effectors (pH adjusted to 6.8) were added directly to make up the samples (final volume 0.4 mL). Data acquisition was performed at a probe temperature of 30 °C.

*Preparation and Purification of the Transglycosylation Product.* The reaction mixture used to prepare a sample of the transglycosylation product contained the following: 0.73 mM β-glucosidase, 3.0 mM 2FβGlu-F, and 32 mM glucosyl benzene dissolved in 50% deuterated sodium phosphate buffer, pH 6.8. The sample was incubated at 30 °C, and the progress of the reaction was monitored periodically by $^{19}F$ NMR. Incubation was continued until all the 2FβGlu-F had been consumed and then the reaction mixture was transferred to a Centricon microconcentrator (30 000-Da cut-off) and diluted to 2 mL with sodium phosphate buffer. The volume was reduced to 0.5 mL by centrifugation (3000*g*), the fraction containing the low molecular weight components was removed, and the protein-containing fraction was diluted to 2 mL. This process was repeated four times, and then the low

Scheme I: Kinetic Mechanism of a Two-Step Hydrolase, Including Transglycosylation




Scheme II: Structures of Inactivators




molecular weight fractions were pooled and lyophilized. This sample was desalted and partially purified on a column of Bio-Gel P-2 (1.5 cm × 84 cm). Fractions containing fluorinated glycoside (as determined by $^{19}F$ NMR) were pooled, lyophilized, and acetylated in 0.2 mL of dry pyridine by slow addition of 0.1 mL of acetic anhydride. The reaction was left overnight at room temperature and then quenched by addition of 1 mL of methanol. After 2 h solvents were removed by evaporation at reduced pressure, and the residue was examined by thin-layer chromatography on silica gel (ethyl acetate/hexane 1:1). The chromatogram showed two components ($R_f$ 0.63 and 0.42), of which the major component ($R_f$ 0.63) comigrated with an authentic sample of 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl benzene. The remainder of the product mixture was chromatographed on a small column of silica gel (ethyl acetate/hexane 1:1) to give the pure minor component from the mixture. Characterization of the product was achieved by $^{19}F$ and $^{1}H$ NMR as described under Results.

## RESULTS

*Inactivation of Agrobacterium β-Glucosidase.* Assuming that only the chemical steps are kinetically important in the reaction mechanism of a "retaining" β-glucosidase, then the mechanism becomes that of a two-step hydrolase (Scheme I). In this scheme, E is the enzyme, GluX is the glycoside, X is the aglycon, E·GluX is the noncovalent Michaelis complex, E–Glu is the covalent glycosyl–enzyme intermediate, and GluOH is the glycose. The kinetic constants are $k_1$ and $k_{-1}$, the association and dissociation rate constants, $K_d$ is the resultant dissociation constant, and $k_2$ and $k_3$ are, respectively, the first-order rate constants for formation and hydrolysis of the glycosyl–enzyme. An alternative pathway of turnover via transglycosylation to ligand A can sometimes occur with a rate constant $k_4$ and a ligand dissociation constant $K_S$. In the absence of such transglycosylation, if hydrolysis of the glycosyl–enzyme (E–Glu) is extremely slow ($k_3 \ll k_2$), then inactivation occurs, and the inactivation parameters may be determined by monitoring the time-dependent loss of activity in the presence of different concentrations of the inactivators. Alternatively, the inactivation process may be monitored by following the release of the aglycon ($X^-$).

Both such approaches have been employed in monitoring the reaction of *Agrobacterium* β-glucosidase with a series of aryl 2-deoxy-2-fluoroglycosides. For the most rapid inactivator (2,4-DNP2FGlu), the inactivation was best monitored by following the release of dinitrophenolate spectrophotometrically, using a stopped-flow instrument. Reaction with the other inactivators was followed by monitoring the time-dependent loss of enzyme activity and/or by monitoring the release of phenolate in a double-beam spectrophotometer. In those cases where both approaches were applied to a single inhibitor, very similar results were obtained. In all cases but one (PNP2FGlu), pseudo-first-order reaction kinetics were observed, allowing simple fitting of the data. In the case of PNP2FGlu, the release of PNP followed a first-order time course initially but settled into a steady state of nitrophenolate release after several hours. A typical trace for such an experiment is shown in Figure 1. In this case, reaction was also followed by monitoring the time-dependent loss of enzyme activity, and inactivation was found to be incomplete, reaching a steady-state level of inactivation whose magnitude depended upon the concentration of inactivator employed. Results obtained from these analyses are shown in Table I along with the $pK_a$ values of the departing phenols. Results are plotted in the form of Hammett relationships in Figure 2.

*Stoichiometry of Inactivation: Active Site Titration.* Since inactivation by 2,4-DNP2FGlu involves the reaction of 1 equiv of inactivator with 1 equiv of enzyme, and should therefore result in the release of 1 equiv of dinitrophenolate with no significant turnover of the intermediate, these compounds have the potential to serve as excellent active site titration agents. The stoichiometry of the reaction was confirmed with 2,4-DNP2FGlu and 2Fβ Glu-F, in the first case by monitoring the dinitrophenolate released spectrophotometrically and in the second case by measuring the fluoride released using a colorimetric dye-binding assay, the enzyme concentration used

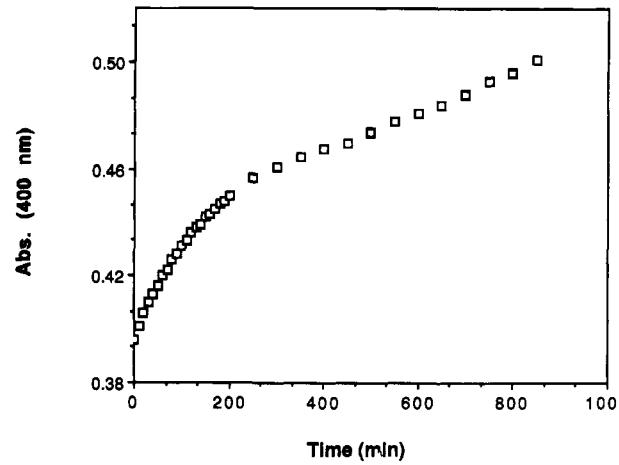



FIGURE 1: Release of PNP from PNP2FGlu on reaction with *Agrobacterium* β-glucosidase. *Agrobacterium* β-glucosidase (9.2 μM) was added to PNP2FGlu (1.28 mM) in a 1-cm cell.

Table I: Inactivation Parameters for the Reaction of β-Glucosidase with Aryl 2-Deoxy-2-fluoro-β-D-Glucopyranosides

| phenol substituent | $pK_a$ | $K_i$ (mM) | $k_i$ ($min^{-1}$) | $k_i/K_i$ ($mM^{-1}$ $min^{-1}$) |
|---|---|---|---|---|
| 2,4-dinitro | 3.96 | 0.245 | 123 | 502 |
| 2,5-dinitro | 5.15 | 0.035 | 5.0 | 145 |
| 3,4-dinitro | 5.36 | 0.718[a] | 0.652[a] | 0.908[a] |
| 4-chloro-2-nitro | 6.35 | 1.38[a] | 2.25[a] | 1.63[a] |
| | | 1.82[b] | 2.86[b] | 1.57[b] |
| 2,4,6-trichloro | 6.39 | 0.143[a] | 2.07[a] | 14.5[a] |
| | | 0.077[b] | 0.952[b] | 12.4[b] |
| 4-nitro | 7.16 | 1.67 | 0.0165 | 0.0099 |

[a] Determined using phenolate burst method. [b] Determined using time-dependent inactivation method.

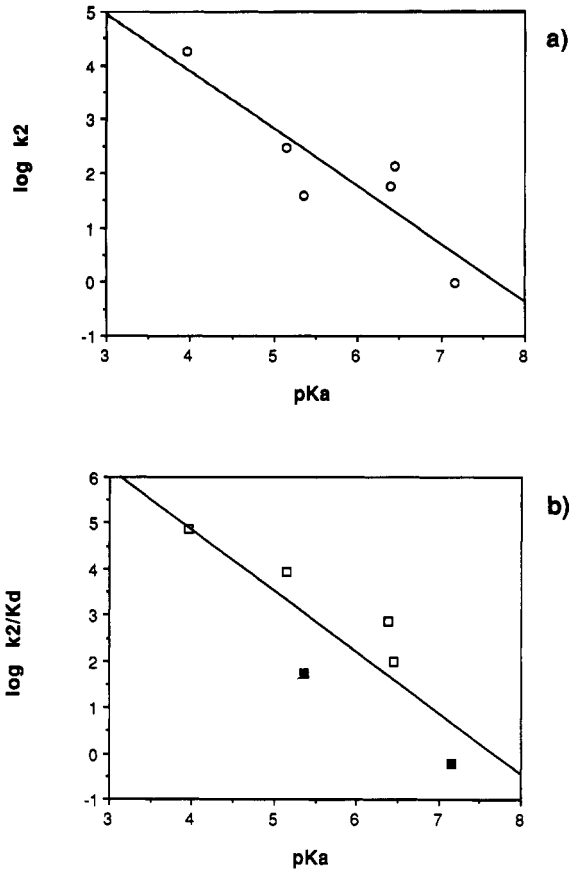



FIGURE 2: Dependence of inactivation rate on aglycon p$K_a$. (a) The logarithm of the inactivation rate constant $k_2$ for each aryl 2-deoxy-2-fluoroglucoside is plotted against the p$K_a$ of its aglycon; (b) log $k_2/K_d$ is plotted against the aglycon p$K_a$; (□) ortho-substituted compound; (■) non-ortho-substituted derivative.

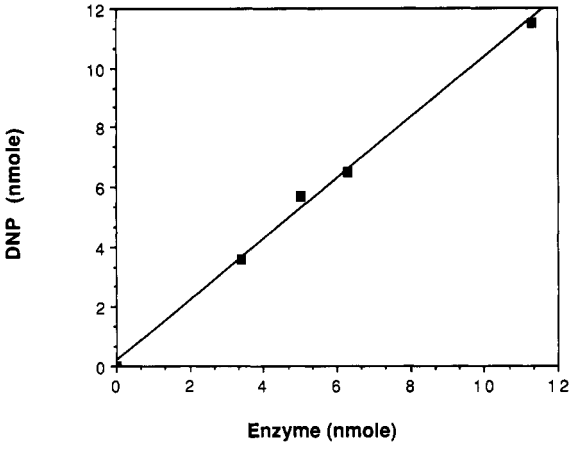



FIGURE 3: Measurement of the "burst" magnitude. Plot of nanomoles of DNP released (based upon absorbance readings at 400 nm) upon reaction of an excess of 2,4-DNP2FGlu with different amounts of enzyme.

being determined using the extinction coefficient of $E_{280}^{0.1\%}$ = 2.20 cm$^{-1}$ (Kempton & Withers, 1992). Results obtained for 2,4-DNP2FGlu are plotted in Figure 3. This experiment yielded a stoichiometry of 1.01 (±0.02) equiv of DNP released per equivalent of enzyme present. The results with the fluoride assay were less accurate but yielded a similar value of 0.93 (±0.05) equiv of fluoride released per equivalent of enzyme present.

*Reactivation of the 2-Deoxy-2-fluoro-*D*-glycopyranosyl–Enzyme Intermediate.* β-Glucosidase was inactivated with several different 2-deoxy-2-fluoro-D-glycosides and freed of excess inactivator, and the pseudo-first-order return of activity associated with hydrolytic turnover of the intermediate was monitored (Table II). Reactivation could in some cases be accelerated by the addition of alternate nucleophiles, and the most effective such ligands were generally found to be carbohydrates of the β-anomeric configuration, as shown in Table III. The kinetics of reactivation by transglycosylation to the hydrolytically stable glucosyl benzene were investigated for the 2-deoxy-2-fluoro-α-D-glucopyranosyl–, the 2-deoxy-2-fluoro-α-D-galactopyranosyl–, and the 2-deoxy-2-fluoro-α-D-mannopyranosyl–enzymes, and in all three cases the reactivation rate was found to be dependent upon the concentration of β-glucosyl benzene in a saturable fashion. Data were analyzed according to the following expression based upon Scheme I:

$$-\frac{d[E\text{–Glu}]}{dt} = \left(k_3 + \frac{k_4[A]}{K_s + [A]}\right)[E\text{–Glu}]$$

Thus when the reaction is carried out with a large excess of reactivating ligand A over enzyme, the reaction will become pseudo-first-order with respect to [E–Glu], and at saturating concentrations the observed first-order rate constant ($k_{obs}$) will be equal to ($k_3$ + $k_4$). Under these circumstances, the increase in activity (increase in free enzyme) with time can be related to the full activity of the sample by

$$[E] = [E_o](1 - \exp[-k_{obs}t])$$

Representative results are shown in Figure 4 for the reactivation of the 2-deoxy-2-fluoro-D-glucopyranosyl–enzyme, and values of the reactivation parameters $k_4$ and $K_S$ are presented in Table II.

*Identification of the Transglycosylation Product.* The identity of the 2-deoxy-2-fluoro-D-glucoside released from the enzyme as a consequence of reactivation by addition of glucosyl benzene was investigated by monitoring the reaction by $^{19}$F NMR and by isolation of the fluorinated product. $^{19}$F NMR investigation of a reaction mixture containing *Agrobacterium* β-glucosidase (0.6 mM), 2FβGlu-F (2.3 mM), and β-glucosyl benzene (35 mM) revealed the time-dependent appearance of

Table II: Rate Constants for Turnover of 2-Deoxy-2-fluoroglycosyl–Enzyme Intermediates

| enzyme intermediate | $k_3$ (hydrolysis) (min$^{-1}$) | $k_4$ (transglycosylation) (min$^{-1}$) | $K_S$ [glucosyl benzene] (mM) |
|---|---|---|---|
| 2-fluoroglucosyl– | $1 \times 10^{-5}$ | $(5.3 \pm 3.1) \times 10^{-3}$ | 59 ± 3.1 |
| 2-fluoromannosyl– | $1 \times 10^{-3}$ | $(6.4 \pm 14) \times 10^{-3}$ | 64 ± 14 |
| 2-fluorogalactosyl– | $5 \times 10^{-3}$ | $(355 \pm 4) \times 10^{-3}$ | 69 ± 9.5 |

Table III: Reactivation of 2-Deoxyfluoroglucosyl–Enzyme by Various Ligands[a]

| ligand | $k_{obs}$ (min$^{-1}$) | relative rate[b] |
|---|---|---|
| *p*-nitrophenyl β-glucoside | 0.027 | 1 |
| isopropyl β-thioglucoside | 0.0081 | 0.3 |
| salicin | 0.026 | 0.96 |
| cellobiose | 0.0006 | 0.02 |
| β-glucosyl benzene | 0.0013 | 0.05 |
| *p*-nitrophenyl α-glucoside | NR[c] | |
| *p*-nitrophenyl β-galactoside | NR | |
| glucose | NR | |
| methanol (1 M) | NR | |
| *p*-nitrophenol | NR | |

[a] All reactions were run at pH 6.8 and 37 °C in 50 mM sodium phosphate buffer containing BSA (1 mg mL$^{-1}$) and the ligand at a concentration of 20 mM, unless othewise stated. [b] Relative rate compared with PNPGlu. [c] NR, no reactivation detected over 120 min.

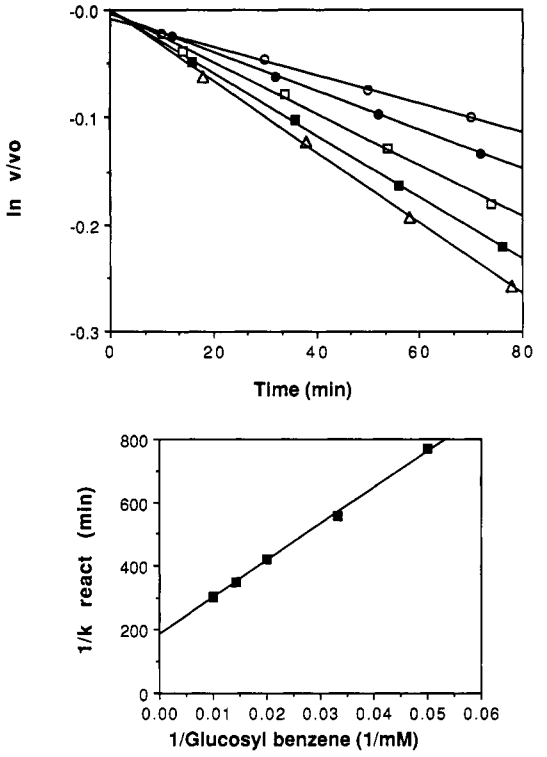



FIGURE 4: Reactivation of the 2-deoxy-2-fluoroglucopyranosyl–enzyme by glucosyl benzene. (a) Semilogarithmic plot of activity vs time at the following concentrations of glucosyl benzene: (○) 20 mM; (●) 30 mM; (□) 50 mM; (■) 70 mM; (△) 100 mM. (b) Double-reciprocal replot of first-order rate constants from panel a vs ligand concentration.

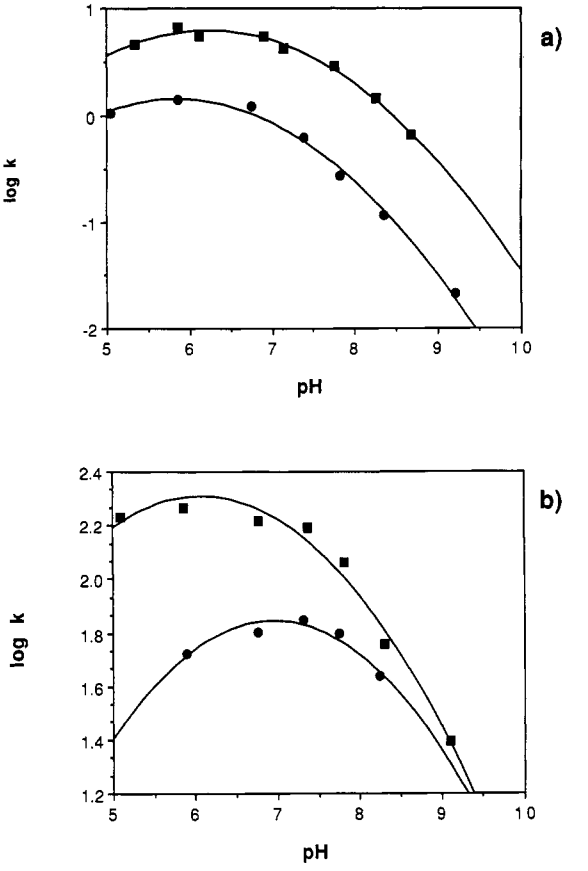



FIGURE 5: pH dependence of the inactivation and reactivation reactions. (a) Plot of log $k_2$ vs pH for 2Fβ Glu-F (●) and $k_{cat}$ for PGlu hydrolysis vs pH (■). (b) Plot of the hydrolytic reactivation rate constant $k_4$ for the 2-deoxy-2-fluorogalactosyl–enzyme vs pH (●) and the $k_{cat}$ for hydrolysis of 2,4-DNPGlu vs pH (■). Lines drawn through the points simply serve to illustrate the general shape of the plots.

a new resonance at $\delta$ = 199 ppm in addition to the resonances at $\delta$ 121 (due to fluoride released) and 197 ppm (due to the 2-deoxy-2-fluoro-α-D-glucopyranosyl–enzyme intermediate). Purification of this fluorinated product was accomplished by gel exclusion chromatography followed by acetylation of the product mixture and flash chromatography on a small column of silica gel. A single UV-active fluorinated product (approximately 20 μg) was obtained which had the following $^{19}F$ and $^1H$ NMR parameters. $^{19}F$ NMR (254 MHz, $CDCl_3$): $\delta$ 199.3 ppm (dd, $J_{gem}$ = 50, $J_{vic}$ = 14 Hz). $^1H$ NMR (400 MHz, $CDCl_3$): $\delta$ 7.28 (5 H), 5.37 (t, 1 H, $J$ = 9.4 Hz), 5.29 (dt, 1 H, $J$ = 8.9, 14.6 Hz), 5.05 (t, 1 H, $J$ = 9.6 Hz), 5.01 (t, 1 H, $J$ = 9.7 Hz), 4.65 (dd, 1 H, $J$ = 3.1, 7.8 Hz), 4.46 (AB multiplet, 2 H, $J$ = 12.5, 4.3, 1.0 Hz), 4.39 (d, 1 H, $J$ = 9.8 Hz), 4.16 (AB multiplet, 2 H, $J$ = 12.3, 2.0, 4.2 Hz), 4.00 (t, 1 H, $J$ = 9.6 Hz), 3.81 (m, 1 H), 3.71 (m, 1 H), 2.09 (s, 3 H), 2.07 (s, 3 H), 2.05 (s, 3 H), 2.05 (s, 3 H), 2.00 (s, 3 H), 1.79 (s, 3 H). These data confirm the product as a β-linked disaccharide of 2-deoxy-2-fluoro-D-glucose and glucosyl benzene but do not prove the linkage position other than to exclude a β-(1–6) linkage. The (1–4) assignment is therefore based upon the previous observations of principally (1–4)-linked products of transglycosylation by this enzyme (Kempton & Withers, 1992) and upon the fact that galactosides (with the opposite C-4 stereochemistry) are far less efficient reactivating ligands than glucosides (Table III). In any case, precise assignment of the structure does not affect the conclusions of this study.

A similar $^{19}F$ NMR experiment was performed using 2Fβ Man-F (1.5 mM) as the inactivator and glucosyl benzene (100 mM) as reactivator. Resonances observed initially were those at $\delta$ 121.0 ppm due to fluoride released and $\delta$ 201.0 ppm due to the covalent 2-deoxy-2-fluoro-α-D-mannopyranosyl–enzyme intermediate as well as resonances at $\delta$ 149.5 and 224.4 ppm due to excess inactivator. In this case, the new resonance which appeared had a chemical shift of 221.2 ppm, a value which is diagnostic of a 2-deoxy-2-fluoro-β-D-mannoside and approximately 20 ppm upfield of that from 2-deoxy-2-fluoro-α-mannosides (Phillips & Wray, 1971; Adamson et al., 1970; Withers & Street, 1988), thereby confirming the β-linkage of the products formed in this way.

*pH Dependence of Inactivation and Reactivation.* The pH dependence of the inactivation reaction for 2Fβ Glu-F is shown in Figure 5a, along with the pH profile for hydrolysis of the substrate PGlu (Kempton & Withers, 1992) for comparative purposes. The inactivation appears to depend upon two ionizations ($pK_1$ = 4.8, $pK_2$ = 7.2) which are quite close to those found for turnover of PGlu ($pK_1$ = 4.8, $pK_2$ = 7.6). The pH dependence of the reactivation reaction for the 2-deoxy-2-fluorogalactopyranosyl–enzyme is shown in Figure 5b, along with that for the substrate PNPGlu. Reactivation appears to be dependent upon two ionizations ($pK_1$ = 5.5, $pK_2$ = 8.4), the second of which is quite similar to that found for turnover of PNPGlu ($pK_2$ = 8.0). However, data on the reactivation reaction cover only a relatively narrow pH range since the galactosyl–enzyme was unstable at the pH extremes over the extended time course necessary for such a study; thus p$K$ values (particularly that of $pK_1$) may be affected considerably in this case.

*Modeling of "Steady-State" Partial Inactivation.* Incubation of *Agrobacterium* β-glucosidase with a fixed concentration of 2Fβ Gal-F in the presence of a series of concentrations of glucosyl benzene is found to result in the partial inactivation

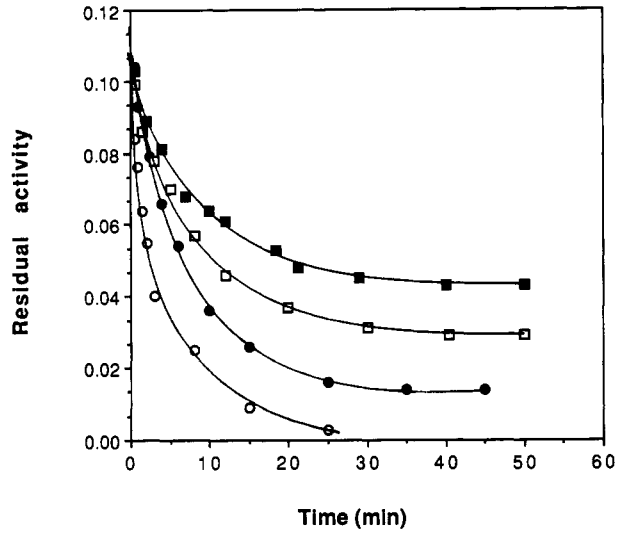



FIGURE 6: Partial steady-state inactivation by 2F$\beta$Gal-F at different reactivator concentrations. *Agrobacterium* $\beta$-glucosidase was incubated with 2F$\beta$Gal-F (0.26 mM) in the presence of glucosyl benzene at the following concentrations: (○) 0 mM; (●) 3.83 mM; (□) 7.66 mM; (■) 11.49 mM.

of the enzyme to an extent which depends inversely upon the concentration of glucosyl benzene present. This partial inactivation is due to the glucosyl benzene acting both as a competitive inhibitor, effectively slowing the rate of intermediate formation, and as a reactivating ligand, thereby increasing the rate of breakdown of the intermediate and decreasing the extent of accumulation of the intermediate, and results are shown in Figure 6. A similar experiment (not shown) in which the concentration of glucosyl benzene was kept constant (19 mM) and the concentration of 2F$\beta$Gal-F was varied yielded similar results. The kinetic model for this steady-state partial inhibition is identical to that in Scheme I except that the ligand A also binds as a competitive inhibitor to the free enzyme with a dissociation constant $K_i$. An expression describing the fraction of the enzyme accumulated in the glycosyl–enzyme form was derived:

$$\frac{[\text{E–Glu}]}{[\text{E}_0]} \equiv \frac{\dfrac{k_2K_S[\text{GluX}]}{K_dk_4[\text{A}]}}{1 + \dfrac{[\text{A}]}{K_i} + \dfrac{[\text{GluX}]}{K_d} + \dfrac{k_2K_S[\text{GluX}]}{K_dk_4[\text{A}]} + \dfrac{k_2[\text{GluX}]}{k_4K_d}}$$

where $K_d = (k_{-1}/k_{+1})$. Use of this expression allowed calculation of the expected levels of steady-state inactivation of the enzyme, and the values calculated using known values of the various rate and equilibrium constants (shown in the legend), along with those actually determined experimentally, are presented in Table IV.

Table IV: Partial Steady-State Inactivation by 2F$\beta$Gal-F in the Presence of Glucosyl Benzene. A Comparison of Experimentally Determined and Calculated Inactivation Levels

| 2F$\beta$Gal-F (mM) | glucosyl benzene (mM) | [E-Glu]/[E$_0$]$^a$ observed | [E-Glu]/[E$_0$]$^b$ calculated |
|---|---|---|---|
| 0.26 | 3.83 | 0.86 | 0.88 |
| 0.26 | 7.66 | 0.72 | 0.73 |
| 0.26 | 11.49 | 0.59 | 0.58 |
| 0.26 | 0 | 1.0 | 1.0 |
| 0.26 | 19.1 | 0.42 | 0.41 |
| 0.52 | 19.1 | 0.57 | 0.55 |
| 1.03 | 19.1 | 0.70 | 0.72 |

$^a$ Calculated from (initial activity − steady-state activity)/initial activity. $^b$ Calculated using the following kinetic constants: $K_d$ = 2.58 mM, $K_i$ = 3.4 mM, $K_S$ = 69 mM, $k_2$ = 3.6 min$^{-1}$, and $k_4$ = 0.355 min$^{-1}$ (Withers et al., 1988; Kempton & Withers, 1992).

## DISCUSSION

The results obtained here are completely consistent with the proposal that inactivation occurs via the accumulation of a relatively stable 2-deoxy-2-fluoro-$\alpha$-D-glycopyranosyl–enzyme intermediate which is nonetheless catalytically competent since it can undergo turnover, particularly in the presence of a reactivating ligand. The inactivation clearly occurs with a 1:1 stoichiometry, as shown by the active site titrations with 2F$\beta$Glu-F and particularly with 2,4-DNP2FGlu, and results in the formation of a covalent $\alpha$-D-glucopyranosyl–enzyme intermediate, as was shown by the previous $^{19}$F NMR experiments (Withers & Street, 1988). The nucleophilic amino acid involved in formation of this intermediate has been identified as glutamic acid residue 358 by labeling of the enzyme with [$^3$H]2,4-DNP2FGlu and subsequent sequencing of the $^3$H-labeled peptide purified by HPLC from peptic digests of this labeled protein (Withers et al., 1990). Competitive ligands protect against inactivation (Withers et al., 1987, 1988), indicating that this inactivation is occurring at the active site, and the similarity of the pH dependence of the inactivation reaction to that for enzymic hydrolysis of phenyl $\beta$-D-glucopyranoside, a substrate whose rate-limiting step is known to be the formation of the glycosyl–enzyme (Kempton & Withers, 1992), provides supportive evidence that the same catalytic machinery is being used in the two steps.

The design of these inactivators is based upon a perceived need for a very good leaving group on the 2-deoxy-2-fluoroglucoside in order to ensure that formation of the glycosyl–enzyme is faster than its decomposition. The need for this has been probed by study of the rates of inactivation with different aryl 2-deoxy-2-fluoro-$\beta$-D-glucopyranosides. The inactivation rate was indeed found to be strongly dependent upon the leaving group ability of the phenol, inactivation rates dropping almost four orders of magnitude on decreasing the leaving group ability from that of 2,4-dinitrophenolate (p$K_a$ = 4) to that of *p*-nitrophenolate (p$K_a$ = 7.2). Further, for the slowest inactivator studied, PNP2FGlu, the inactivation did not proceed to completion, reaching levels of inactivation which depended upon the concentration of inactivator employed. This is the expected outcome if the rates of glycosylation and deglycosylation (probably via transglycosylation to further PNP2FGlu) are approaching equivalence. This phenomenon of partial steady-state inactivation, which had also been seen previously (Withers et al., 1988) in the inactivation of several other glycosidases with their corresponding 2-deoxy-2-fluoroglycosyl fluorides, was therefore modeled using an inactivator with a relatively high transglycosylation rate, 2,4-DNP2FGal, and using glucosyl benzene as the reactivating ligand. Results obtained are entirely consistent with predictions based upon the appropriate kinetic model and support the mechanism previously proposed to explain this partial inactivation phenomenon (Withers et al., 1988). They also indicate that 2-deoxy-2-fluoroglycosides with leaving groups substantially poorer than *p*-nitrophenol would not serve as useful mechanism-based inactivators for the *Agrobacterium* $\beta$-glucosidase.

The Hammett relationships obtained for these aryl glycosides are disappointingly poor given the fact that a single chemical step is clearly rate-limiting for all compounds. Reasons for this poor fit are not clear but are probably not related to errors introduced by the use of several different techniques in extracting the parameters since quite reasonable correspondence between vaues was observed for the two

compounds which were investigated by both techniques. The scatter may be due to the presence of ortho substituents on some of the compounds, since such ortho substituents have been shown previously (Nath & Rydon, 1954) to perturb the kinetic parameters in comparable enzyme systems. Such effects were probably not apparent in the plot of log $k_{cat}$ vs $pK_a$ for the parent glycosides (Kempton & Withers, 1992) because the deglycosylation step was rate limiting for all the ortho-substituted compounds, obscuring such effects, though they do show up as scatter in the plot of log $k_{cat}/K_m$ vs $pK_a$. If the data for ortho-substituted compounds are correlated separately, then slopes of around $\beta_{lg} = -1.0$ are obtained in all cases whereas when the data are correlated with a single line, slopes of $\beta_{lg} = -0.9$ and $-1.2$ are obtained for the plots of log $k_i$ and log $k_i/K_i$, respectively. These slopes are all similar and large, indicating a substantial amount of charge separation at the transition state. Indeed, these are significantly larger than the slope for the parent glycosides ($\beta_{lg} = -0.7$), indicating that cleavage of the glycosidic bond is more complete, despite the presence of the inductively destabilizing fluorine substituent. Since it is unlikely that the transition state for the deoxyfluoro sugars has more oxocarbonium ion character, it must involve considerably more nucleophilic association of the active site carboxylate than occurs in the case of the parent substrates, possibly assisted by the more electronegative nature of the anomeric center.

The intermediate trapped in this way is therefore equivalent to that formed during normal catalysis, the only difference being its extremely slow formation and turnover rates, occasioned by the presence of the fluorine at C-2. The glycoside-promoted turnover of this intermediate via formation of a β-linked disaccharide product provides good evidence for the fact that this is a normal intermediate in catalysis, while the observation of saturation kinetics for the binding of the reactivating ligand, with similar reactivator $K_d$ values being measured for a series of different 2-deoxy-2-fluoroglycosyl–enzymes, shows that a single second sugar-binding site is involved for all species of inactivated enzyme, this presumably being the same site as that normally occupied by the second (reducing) sugar moiety of the natural substrate cellobiose. This increase in turnover rate by transglycosylation is a well-precedented phenomenon in glycosidase catalysis (Sinnott, 1990) and indeed forms the basis for the use of such enzymes in the synthesis of glycosides. The increased rate presumably derives from the binding energy between the enzyme and this second sugar which is used to stabilize the transition state for glycosyl transfer. Indeed, this is essentially the microscopic reverse of the first step in enzymic cleavage of the disaccharide cellobiose, a reaction which the enzyme performs with high efficiency, at a rate comparable to that found with glycosides containing much better leaving groups such as *p*-nitrophenolate. This substrate "activation" can only be explained if specific enzyme–substrate interactions at the "aglycon" site are stabilizing the transition state and lowering the activation barrier.

The general similarity of the pH dependence of the reactivation process (the 2-deoxy-2-fluorogalactosyl–enzyme was used in these studies because of its higher rates of hydrolytic reactivation) to that for hydrolysis of 2,4-dinitrophenyl glucoside (a substrate for which deglycosylation is the rate-limiting step) provides further evidence that the trapped intermediate is the same as that formed during normal catalysis. Unfortunately, the relative instability of the 2-deoxy-2-fluorogalactosyl–enzyme over the long time spans required for such experiments means that the data cover a narrower pH range than is desirable, and in fact the rates provided for the pH extremes may themselves be somewhat affected by this instability. Nonetheless, a pH dependence broadly similar to that found for the normal deglycosylation process appears to be observed.

One other interesting observation which further suggests that the species trapped is similar to the normal intermediate in catalysis is the fact that the 2-deoxy-2-fluoroglucosyl–enzyme is enormously stabilized toward proteolytic digestion compared to the free enzyme. Indeed, this frustrated early efforts to proteolytically cleave the labeled enzyme in efforts to identify the nucleophile (Withers et al., 1990). Whereas the free enzyme is rapidly cleaved by trypsin or chymotrypsin even without prior denaturation, the 2-deoxy-2-fluoroglucosyl–enzyme suffers no observable proteolysis by trypsin or chymotrypsin (1:50) even after prior exposure to very strongly denaturing conditions (8 M urea at 50 °C or 40% acetonitrile). This suggests an enormous stabilization of the protein structure due to the presence of the covalently linked 2-deoxy-2-fluoroglucose moiety. This large stabilization, which is likely a reflection of the stabilization afforded to the normal intermediate by binding interactions with the sugar moiety, is undergoing further investigation. In addition, it suggested this species as a likely candidate for crystallization, and indeed crystals of this complex have been obtained and are currently undergoing evaluation for X-ray crystallographic analysis.

The presence of the fluorine substituent at C-2 therefore slows the glycosylation and deglycosylation steps enormously. A measure of the degree to which the deglycosylation step is slowed upon replacement of the hydroxyl at C-2 by the fluorine is obtained by comparing the rate of the deglycosylation step for normal substrates ($k_{degly} = 150\ s^{-1}$) (Kempton & Withers, 1992) with that for spontaneous turnover of the 2-deoxy-2-fluoroglucosyl–enzyme intermediate ($2 \times 10^{-7}\ s^{-1}$), giving a value for the rate reduction of $7.5 \times 10^8$-fold. The rate reduction for the glycosylation step is more difficult to estimate since the rate of the glycosylation step for 2,4-DNPGlu is unknown and in any case is probably kinetically inaccessible since diffusive events are presumably slower. However, an *estimate* of this rate can be obtained by extrapolation of the leaving group-dependent part of the Hammett plot obtained for the parent substrates (Kempton & Withers, 1992), giving a predicted rate of $k_{gly} = 7 \times 10^8\ s^{-1}$ for 2,4-DNPGlu. Combining this with the measured rate of 2-deoxy-2-fluoroglucosyl–enzyme formation for 2,4-DNP2FGlu of $k_i = 1.8 \times 10^4\ s^{-1}$ yields a rate reduction of $3.9 \times 10^4$-fold. Similar comparison of data for PNPGlu and PNP2FGlu yields a rate ratio of $3.2 \times 10^6$. There is a significant difference between these two rate ratios which has its origin in the different slopes of the Hammett plots for the glucosides and the 2-deoxy-2-fluoroglucosides. The rate reductions on both steps are therefore enormous, reflecting both the importance of the interactions at this position to stabilization of the transition state and the inductive destabilization of the oxonium ion-like transition state afforded by the electronegative fluorine. Further, the fact that the deglycosylation transition state is destabilized more by the presence of the fluorine than the glycosylation transition state suggests either that interactions at this position are more important for the second step and/or that the second transition state has substantially more oxonium ion character than the first. This latter conclusion is particularly attractive in light of the data provided in the preceding paper (Kempton & Withers, 1992) concerning the relative charge developments at the two transition states.

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