Catalytic Versatility of *Bacillus pumilus* β-Xylosidase: Glycosyl Transfer and Hydrolysis Promoted with α- and β-D-Xylosyl Fluoride†

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Received October 28, 1986; Revised Manuscript Received February 5, 1987

ABSTRACT: *Bacillus pumilus* β-xylosidase, an enzyme considered restricted to hydrolyzing a narrow range of β-D-xylosidic substrates with inversion of configuration, was found to catalyze different stereochemical, essentially irreversible, glycosylation reactions with α- and β-D-xylopyranosyl fluoride. The enzyme promoted the hydrolysis of β-D-xylopyranosyl fluoride at a high rate, $V = 6.25$ μmol min^{-1} mg^{-1} at 0 °C, in a reaction that obeyed Michaelis–Menten kinetics. In contrast, its action upon α-D-xylopyranosyl fluoride was slow and characterized by an unusual relation between the rate of fluoride release and the substrate concentration, suggesting the possible need for two substrate molecules to be bound at the active center in order for reaction to occur. Moreover, ^{1}H NMR spectra of a digest of α-D-xylosyl fluoride showed the substrate to be specifically converted to α-D-xylose by the enzyme. The observed retention of configuration is not consistent with direct hydrolysis by this "inverting" enzyme but is strongly indicative of the occurrence of two successive inverting reactions: xylosyl transfer from α-D-xylosyl fluoride to form a β-D-xylosidic product, followed by hydrolysis of the latter to produce α-D-xylose. The transient intermediate product formed enzymically from α-D-xylosyl fluoride in the presence of [^{14}C]xylose was isolated and shown by its specific radioactivity and ^{1}H NMR spectrum as well as by methylation and enzymic analyses to be 4-*O*-β-D-xylopyranosyl-D-xylopyranose containing one [^{14}C]xylose residue. The results are related to our earlier findings with β-amylase, glycoamylase, glucodextranase, and trehalase, which also had appeared to be strictly limited to catalyzing the hydrolysis of glycosidic substrates with inversion but which also were found to have functionally flexible catalytic groups capable of catalyzing nonhydrolytic glycosylation reactions by mechanisms other than for hydrolysis.

The study of enzymic glycosylation reactions catalyzed without glycosidic bond cleavage has in recent years emerged as a powerful approach providing a much altered understanding of the catalytic and mechanistic capabilities of glycosylases. That is, through the use of glycosyl fluorides and enolic glycosyl substrates, various well-known glycoside hydrolases and glycosyltransferases have been found to have functionally flexible catalytic groups and the ability to promote glycosylation reactions with different substrates by different mechanisms (Hehre et al., 1977, 1979, 1980, 1982, 1986; Lehmann & Zieger, 1977; Kitahata et al., 1980; Schlesselmann et al., 1982; Lehmann & Schlesselmann, 1983; Tsumuraya et al., 1984a; Kanda et al., 1986; Kasumi et al., 1986). Studies with such truncated glycosyl substrates have, for example, revealed the catalytic versatility of four well-known "inverting glycoside hydrolases" (β-amylase, glucoamylase, glucodextranase, and trehalase), which were long considered restricted to hydrolyzing particular α-D-glycosidic substrates and the limited reversal of such hydrolysis. Each of these enzymes has been shown (Hehre et al., 1979, 1982; Kitahata et al., 1980; Kasumi et al., 1986) to catalyze sterically complementary hydrolytic and nonhydrolytic reactions, respectively, with the α- and β-anomers of an appropriate glycosyl fluoride.

The question of whether comparable catalytic flexibility might be demonstrated for an enzyme known to hydrolyze β- rather than α-glycosidic substrates with inversion has now been examined with the purified β-xylosidase of *Bacillus pumilus*. This extensively studied enzyme has been considered limited to hydrolyzing aryl β-D-xylopyranosides and lower xylosaccharides with absolute specificity for the β-D-xylopyranosyl configuration (Kersters-Hilderson et al., 1969, 1984; Claeyssens et al., 1975), to act with configurational inversion (Kersters-Hilderson et al., 1976, 1978), and to lack glycosyl transferring ability (Kersters-Hilderson et al., 1978, 1984; van Doorslaer et al., 1979).

We have recently reported (Kasumi et al., 1985) that *B. pumilus* β-xylosidase is able to utilize both α- and β-D-xylopyranosyl fluorides as substrates and to catalyze different reactions with each. This study was undertaken to elucidate the nature and possible mechanisms of the reactions catalyzed with these two substrates. The results are discussed with respect to the proposal (Hehre et al., 1979) that glycoside hydrolases and glycosyltransferases (i.e., glycosylases) in general may have catalytic groups that are functionally flexible beyond the requirements of the principle of microscopic reversibility and that an individual glycosylase may have different

† This study was supported by U.S. Public Health Service Research Grant GM-25478 (to E.J.H.) from the National Institute for General Medical Sciences. C.F.B. was supported by Research Grant CA-16054 and by Core Research Grant P30-CA-13330 from the National Cancer Institute, National Institutes of Health.

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0006-2960/87/0426-3010$01.50/0

reaction pathways with different substrates.

Experimental Procedures

General Methods. Thin-layer chromatography (TLC) was carried out on 20 × 20 cm silica gel G plates (Analtech) developed with ether/petroleum ether, 4:1, for acylated compounds and ethyl acetate/ethanol, 5:1 (unless otherwise noted), for nonacylated compounds. Visualization was by the sulfuric acid–char method. Paper chromatograms (descending) were made with Whatman No. 1 paper and 1-butanol/ethanol/water, 13:8:4; staining was with silver nitrate. Free fluoride ion concentrations were measured with Orion microprocessor ionanalyzer Model 901 and combination electrode Model 96-09. Test solutions and sodium fluoride standards were examined after dilution with 3 volumes of TISAB [1 M sodium acetate buffer of pH 5.2, 1 M sodium chloride, 0.4% 1,4-cyclohexanebis(dinitrilotetraacetic acid) monohydrate]. Optical absorbance measurements were made with Gilford Stasar II and Model 120 spectrophotometers; optical rotations were made with a Rudolph & Sons Model 70 polarimeter and 2-dm tubes. Solvent removal was carried out in rotary vacuum evaporators at 30 °C unless otherwise noted. Crystallization was judged with a Bausch & Lomb polarizing microscope with red retardation plate. Melting points were determined with a Mel-Temp block and are uncorrected.

Fourier transform ^1H NMR spectra at 200 MHz were recorded in D_2O (99.8 atom % D, Stoehler Chemicals) with a Varian XL-200 spectrometer; chemical shifts (ppm) refer to 3-(trimethylsilyl)propanesulfonic acid sodium salt. ^1H and ^{19}F NMR spectra at 100 and 94 MHz, respectively, were recorded with a Jeol PFT-100 spectrometer operated in the pulse FT mode; experimental conditions, including reference materials, are described in the text.

Assays of Enzymic Activity. B. pumilus β-xylosidase was routinely assayed according to Kersters-Hilderson et al. (1982), with one unit promoting release of 1 μmol of *p*-nitrophenol/min from 4 mM *p*-nitrophenyl β-D-xylopyranoside in digests (25 °C) with 0.01 M phosphate buffer at pH 7.15 and 1 mM ethylenediaminetetraacetic acid (EDTA). *p*-Nitrophenyl (PNP) release was measured as increase in absorption at 400 nm, with a conversion factor of 10.718. Protein was determined by the method of Lowry et al. (1951) standardized with bovine serum albumin.

B. pumilus β-Xylosidase. Initial steps in preparing the enzyme (cultivation, harvesting, and disruption of the bacilli; streptomycin treatment and ammonium sulfate fractionation of cell-free extracts) followed Kersters-Hilderson et al. (1982) except that the lysozyme- and DNase-treated bacteria were ruptured in a French pressure cell rather than by sonication. The β-xylosidase at this stage (55–75% ammonium sulfate fraction, assaying 0.17 unit/mg of protein) was next subjected to affinity chromatography on CH-Sepharose 4B (Pharmacia) coupled to *p*-aminobenzyl 1-thio-β-D-xylopyranoside (Kersters-Hilderson et al., 1984). A 1.5 × 16.5 cm column, set up at 4 °C with the coupled gel already equilibrated with 0.01 M phosphate buffer of pH 7.15 plus 1 mM EDTA, was washed with the same buffer until the effluent showed negligible absorbance at 288 and 444 nm. Fractionation of the enzyme with this column and subsequent purification steps were carried out at 4 °C.

A sample of crude soluble enzyme (67 PNP β-xylosidase units; ca. 400 mg of protein) in 75% saturated ammonium sulfate was sedimented at 20000*g*, dissolved in 12 mL of the 0.01 M phosphate buffer (plus 1 mM EDTA) at pH 7.15, and applied to the affinity column. Loading was followed by washing with buffer until the effluent was colorless, then by desorption with 0.01 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer (pH 7.4) containing 30% ethylene glycol, 1 mM EDTA, and 5 mM *p*-nitrophenyl β-D-ribopyranoside. Fractions showing high activity for *p*-nitrophenyl β-D-xyloside were pooled; the recovered enzyme (23.5 units) had a specific activity of 2.2 units/mg of protein.

Final purification was achieved by hydrophobic chromatography (Kersters-Hilderson et al., 1984). β-Xylosidase (19.8 units, 2.2 units/mg), sedimented from 80% saturated ammonium sulfate, was dissolved in 3 mL of 0.01 M phosphate buffer of pH 7.15 containing 1 mM EDTA and 25 g/100 mL ammonium sulfate. This was applied to a 0.9 × 18 cm column of phenyl-Sepharose CL-4B (Pharmacia) that had been equilibrated with the same solution. Elution was effected with a 90-mL linear gradient of decreasing ammonium sulfate concentration (25 to 0%) in the phosphate buffer and increasing ethylene glycol concentration (0 to 50%) in 10 mM Tris-HCl buffer of pH 8.0 containing 1 mM EDTA. The pooled active enzyme (18 mL), diluted with 60 mL of 10 mM Tris/1 mM EDTA buffer of pH 8.0, was dialyzed vs. several 1-L changes of the latter buffer and concentrated by centrifugation in Amicon CF-25 ultrafiltration membrane cones, yield 16.9 units (5.1 units/mg of protein). The concentrate was treated with ammonium sulfate to 80% saturation and the purified enzyme recovered by ultracentrifugation, as needed.

α- and β-D-Xylopyranosyl Fluoride Tri-O-acetates. Crystalline β-D-xylose tetra-*O*-acetate, 25 g, was reacted with 70 mL of anhydrous HF at −15 °C (20 min) then at 20 °C (10 min). After the mixture was shaken with ice-cold chloroform and water, the organic phase was repeatedly washed, dried with sodium sulfate, and concentrated to a clear syrup. This was mixed with an equal volume of absolute ethanol followed by storage under vacuum over sodium hydroxide at 4 °C for 3 days, which led to a deposit of α-D-xylosyl fluoride triacetate crystals. The latter, separated from the mother liquor and recrystallized from ethanol, weighed 2.8 g and gave a single spot on TLC (R_f 0.95). A further 2.4 g of the α-anomer (and 1.55 g of pure β-D-xylosyl fluoride triacetate, R_f 0.90) was recovered from the mother liquor by fractionation on columns of dry silica gel 60 with ether/petroleum ether, 4:1, as solvent.

Pure tri-*O*-acetyl-α-D-xylopyranosyl fluoride had mp 84.5–86 °C and $[\alpha]^{23}_D$ +67.3° (*c* 1, chloroform) [lit. (Brauns, 1923) mp 87 °C and $[\alpha]_D$ +67.24°]. ^{19}F NMR and ^1H NMR spectra, recorded in $[^2H_6]$acetone at 94 and 100 MHz, respectively, agreed with those reported by Hall and Manville (1969). Anal. Calcd for $C_{11}H_{15}O_7F$: C, 47.48; H, 5.43; F, 6.83. Found: C, 47.72; H, 5.40; F, 6.75.

Pure microcrystalline tri-*O*-acetyl-β-D-xylosyl fluoride had $[\alpha]^{22}_D$ −58.2° (*c* 1.56, chloroform), [lit. (Lundt & Pedersen, 1966) $[\alpha]_D$ −56.4° (*c* 1.6, chloroform)]. ^{19}F NMR and ^1H NMR spectra recorded in $[^2H_6]$acetone at 94 and 100 MHz agreed with those of Hall and Manville (1969).

α-D-Xylopyranosyl Fluoride. Crystalline tri-*O*-acetyl-α-D-xylosyl fluoride (usually 1 mmol) was deacetylated with freshly prepared 0.03 M sodium methoxide in dry methanol (0 °C for 10 min, then at 25 °C for 1–1.5 h). Chromatography on a 1 × 35 cm column of dry silica gel 60, with ethyl acetate/ethanol, 5:1, as developer, gave pure α-D-xylosyl fluoride (ca 0.7 mmol) as highly birefringent crystals, with fluoride/D-xylose ratios of 0.95–0.98 and a free fluoride content of <0.3%. All preparations gave a single spot, R_f 0.69, on TLC (ethyl acetate/ethanol, 10:1) and were free from β-D-xylosyl fluoride (R_f 0.75). α-D-Xylosyl fluoride was kept as a solution in dry methanol, stored at −20 °C protected from

moisture. As needed, aliquots were dried under vacuum (30 °C) in small plastic tubes immediately before use. A ^{19}F NMR spectrum of α-D-xylosyl fluoride in 0.1 M [$^2H_4$]-acetate/D_2O buffer of pD 5.6, recorded at 5 °C at 94 MHz, showed a multiplet centered at −154.6 ppm upfield from trichlorofluoromethane in [$^2H_6$]acetone, with $J_{1,F}$ = 52.5 Hz and $J_{2,F}$ = 28.1 Hz. ^1H NMR spectra are described under Results.

β-D-Xylopyranosyl Fluoride. Crystalline β-D-xylopyranosyl fluoride triacetate (usually 350 μmol) was deacetylated at 0 °C (usually 1.5 h) in freshly prepared 0.03 M sodium methoxide in dry methanol and then chromatographed on a silica gel 60 column as described for the α-anomer. The amorphous product (200–250 μmol) was kept as a solution in dry methanol at −20 °C, protected from moisture. Preparations showed a fluoride/xylose ratio of 0.90–0.92 and a free fluoride concentration of 2–3% of the total fluorine. A ^{19}F NMR spectrum of β-D-xylosyl fluoride in 0.1 M [$^2H_4$]acetate/D_2O buffer of pD 5.6, recorded at 5 °C at 94 MHz, showed a chemical shift at −143.9 ppm upfield from trichlorofluoromethane in [$^2H_6$]acetone, with $J_{1,F}$ = 53.7 Hz and $J_{2,F}$ = 11.0 Hz. A ^1H NMR spectrum of a solution in D_2O buffered at pD 7.6, recorded at 200 MHz and 23 °C, showed the H-1 chemical shift centered at 5.19 ppm with $J_{1,2}$ = 5.5 Hz, $J_{1,F}$ = 51 Hz, and $J_{2,F}$ = 11.8 Hz. The $J_{2,F}$ coupling constants indicate that β-D-xylosyl fluoride in aqueous solution is mainly of the $^4C_1$ conformation.

[^{14}C]Xylose and Radioactivity Measurements. Uniformly labeled [^{14}C]-D-xylose, 30 μCi and 76 μCi/μmol (Amersham), was dissolved in methanol along with sufficient chromatographically pure xylose to make 20.0 mL of 25.0 mM [^{14}C]xylose, 0.060 μCi/μmol. A 1:25 dilution of the latter in water was used as a counting standard. Radioactivity measurements were made with a Beckman LS 230 scintillation counter. Samples (120 μL) were vigorously shaken with 4.5 mL of Aquasol (New England Nuclear) in plastic minivials. Counts per minute values, replicated to the 0.5% confidence level, were corrected for background; counting efficiency was 80% of theoretical.

Results

The ability of *B. pumilus* β-xylosidase to utilize both α- and β-D-xylopyranosyl fluoride as substrates was first discerned with samples of purified enzyme that had lost a great part of their activity in transit between laboratories. In the following experiments, purified enzyme preparations of high specific activity were used. All digest and control mixtures were incubated in 0.05 M phosphate buffer of pH 7.15 (or pD 7.65) containing 1 mM EDTA.

Kinetics of Reactions with α- and β-D-Xylosyl Fluoride. Test mixtures containing 3.3–10 mM β-D-xylosyl fluoride and 5 μg (0.014 unit)/mL β-xylosidase at pH 7.15 were incubated, along with substrate/buffer controls, in an ice–water bath for 15 min and then analyzed for fluoride content with a specific-ion electrode. A linear relation was found between $1/v$ and $1/S$, from which values of V = 6.25 μmol min^{-1} mg^{-1} (at 0 °C) and K_m = 1.8 mM β-D-xylosyl fluoride were calculated for an enzyme sample that hydrolyzed 4 mM *p*-nitrophenyl β-D-xyloside at a rate of 2.8 μmol min^{-1} mg^{-1} (at 25 °C). A direct comparison at 25 °C showed 4 mM β-D-xylosyl fluoride to be enzymically hydrolyzed ca. 12 times faster than 4 mM *p*-nitrophenyl β-D-xyloside.

A much different result was obtained with the α-anomer. In a typical experiment, digests comprising 8–167 mM α-D-xylosyl fluoride and 120 μg/mL of the 2.8 unit/mg enzyme were incubated at 30 °C (30 min) with substrate/buffer controls and then analyzed for fluoride content. Initial reaction rates, v = μmol min^{-1} mg^{-1}, plotted as a function of substrate concentration gave (Figure 1) an S-shaped curve that was upwardly concave below ca. 50 mM substrate. The rate was miniscule (1.8% of the routine assay value) with 8 mM α-D-xylosyl fluoride and 9 times faster with 40 mM substrate and with 120 mM α-D-xylosyl fluoride was 52% of the routine assay value. These results suggest that the reaction catalyzed with α-D-xylosyl fluoride is substrate activated.

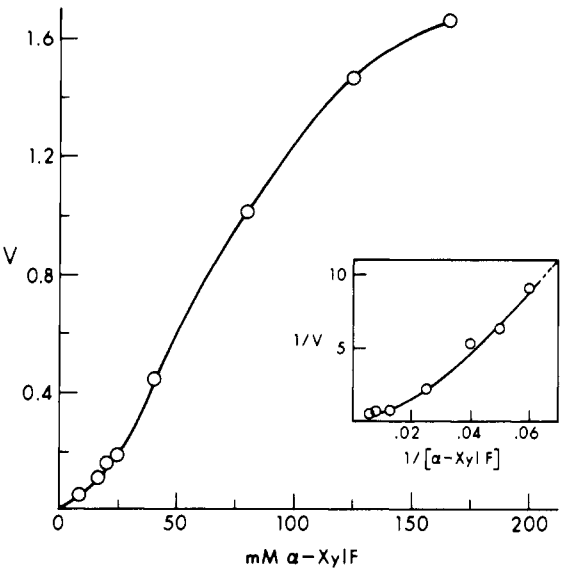


FIGURE 1: Kinetics of action of *B. pumilus* β-xylosidase upon α-D-xylopyranosyl fluoride at 30 °C and pH 7.15; v = μmol of enzymically released F^- min^{-1} mg^{-1}.

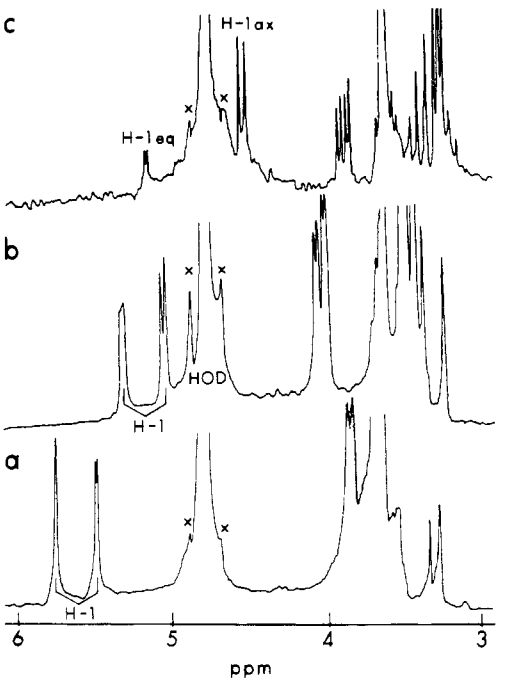


FIGURE 2: ^1H NMR reference spectra at 200 MHz recorded in deuterium oxide at 23 °C: (a) 50 mM α-D-xylopyranosyl fluoride; (b) 50 mM β-D-xylopyranosyl fluoride; (c) anomerically equilibrated D-xylose, showing the H-1 equatorial and H-1 axial resonances, respectively, of α- and β-D-xylose. × = spinning sideband.

Steric Course of the Reaction with α-D-Xylosyl Fluoride. To further evaluate the α-D-xylosyl fluoride reaction, a ^1H NMR study was made to learn the configuration of the xylose product. To this end, reference spectra of α- and β-D-xylosyl fluoride and of equilibrated D-xylose were recorded (Figure 2). It is evident that in α-D-xylosyl fluoride (spectrum 2a)

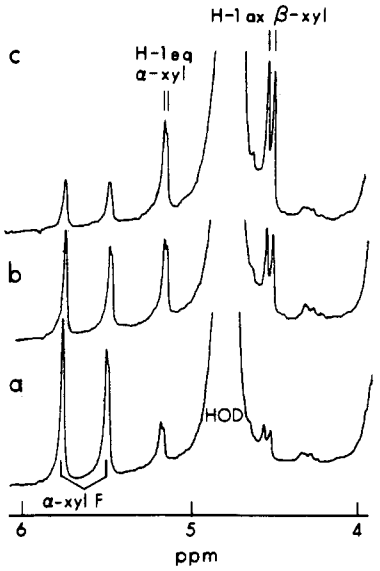


FIGURE 3: ^1H NMR spectra at 200 MHz of 50 mM α-D-xylopyranosyl fluoride/β-xylosidase digest conducted in 0.05 M [$^2H_3$]phosphate/D_2O buffer at pD 7.65 and 23 °C. The anomeric proton resonances of the substrate and of α- and β-D-xylose are shown after (a) 9.5-, (b) 20-, and (c) 55-min incubation.

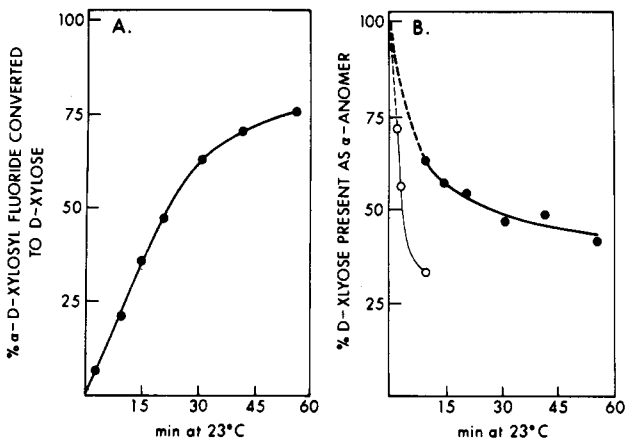


FIGURE 4: (A) Time course of β-xylosidase-catalyzed conversion of α-D-xylosyl fluoride to D-xylose, on the basis of measurements (see footnote 1) of the relative areas of the H-1 resonances of the substrate and of α- and β-D-xylose in individual ^1H NMR spectra at different times of incubation at 23 °C. (B) (●) Estimated percent of D-xylose in the enzymic digest that is present in α-anomeric form, on the basis of measurements (see footnote 1) of the relative areas of the H-1 resonances of α- and β-D-xylose in the individual spectra recorded during incubation at 23 °C; (○) similarly derived estimates of the percent α-D-xylose remaining with time (at 23 °C) in a solution of 15 mM α-D-xylose in the 0.05 M [$^2H_3$]phosphate/D_2O buffer of pD 7.65 used for the enzymic digest.

the H-1 resonance is a doublet of doublets centered at 5.65 ppm; whereas in β-D-xylosyl fluoride (spectrum 2b) this resonance is centered upfield, at 5.19 ppm. Spectrum 2c of anomerically equilibrated xylose shows the H-1 doublet of the β-anomer, at 4.54 ppm, to be approximately twice the size of the H-1 doublet of the α-anomer, at 5.16 ppm.

To determine the configuration of the xylose formed when β-xylosidase acts upon α-D-xylosyl fluoride, 5.1 unit/mg enzyme was exhaustively dialyzed against 0.05 M [$^2H_3$]phosphate/D_2O buffer of pD 7.65 containing 1 mM EDTA. The dialyzed enzyme (0.60 mL, 10.6 units) was added to 30 μmol of α-D-xylosyl fluoride freshly dried from methyl [^2H]alcohol, and the reaction was followed in the NMR spectrometer. Spectra recorded after 9.5, 20, and 55 min at 23 °C (Figure 3) show a progressive diminution of the H-1 resonance of the substrate as well as the early presence and increase with time of the anomeric resonances of α- and β-D-xylose. At 9.5 min (spectrum 3a), the 5.16 ppm H-1 doublet of α-D-xylose appears somewhat larger than the 4.54 ppm H-1 doublet of the β-anomer; by 20 min (spectrum 3b), these resonances appear about equal in area; after 55 min (spectrum 3c), when most of the substrate has been utilized, the H-1 resonance of β-D-xylose has become larger than that of the α-anomer. Even at this late stage of the reaction, however, the proportion of β- to α-D-xylose in the digest remains below that expected at anomeric equilibrium (compare Figure 2c).

Further information was obtained on the relative proportions of substrate and of each xylose anomer present at a given time by sectioning and weighing the H-1 resonance areas of a particular spectrum.[1] Figure 4A illustrates that the conversion of the 50 mM α-D-xylosyl fluoride to xylose occurred at a uniform rate during the first 15 min at 23 °C and was about 75% complete by 55 min. Figure 4B (solid points) indicates that the percentage of xylose present as the α-anomer fell continuously but remained well above the 33–35% expected at anomeric equilibrium. The increasing percentage of β-D-xylose with time is the result of anomerization. Estimates[1] made from ^1H NMR spectra of a control of pure α-D-xylose in the same pD 7.65 buffer (Figure 4B, open points) show that anomerization reached a near equilibrium value in 10 min at 23 °C. In sum, although the data do not rule out the possibility that a small proportion of the observed β-D-xylose is produced enzymically, they do show that *B. pumilus* β-xylosidase catalyzes the conversion of α-D-xylosyl fluoride to form α-D-xylose, with most or all of the β-anomer arising nonenzymically.

Xylosyl Transfer Catalyzed with α-D-Xylosyl Fluoride. To examine whether the conversion might involve a transfer reaction from α-D-xylosyl fluoride to form a β-xylosidic product, followed by enzymic hydrolysis of the latter to yield the observed α-D-xylose, efforts were made to detect the intermediate transfer product. In one trial, a digest comprising 60 mM α-D-xylosyl fluoride and 0.4 mL of 0.8 unit/mL β-xylosidase was incubated (30 °C, 30 min) along with substrate/buffer and xylose/enzyme controls. Each mixture was chromatographed as an 18-cm band on paper, with markers of xylose and xylobiose. Stained guide strips in each case showed xylose (scant in the α-D-xylosyl fluoride/buffer control), but neither xylobiose nor other product. Material eluted from the xylose region behaved as pure xylose on rechromatography; however, following treatment with 0.025 M sulfuric acid (100 °C, 10 min), the "xylose" recovered from the enzymic digest (though not that from either control) yielded a compound that migrated as xylobiose (R_{Xyl} 0.34) and that was hydrolyzed to xylose by the β-xylosidase. These findings indicate that the acid-labile product found comigrating with xylose was formed by β-xylosidase action upon α-D-xylosyl fluoride. Its features are consistent with those expected for a transfer product (xylobiosyl fluoride) that would be hydrolyzed by the enzyme to form α-D-xylose.

In a second trial, 75 mM α-D-xylosyl fluoride and 120 mM xylose were incubated (30 °C, 40 min) with β-xylosidase (0.8 unit/mL) in pH 7.15 buffer, along with controls of the substrates plus buffer and of xylose plus enzyme. Chromatograms

[1] From the weights of the H-1 resonance areas (sectioned from enlarged photographs of spectra) of α-D-xylosyl fluoride, α-D-xylose, and β-D-xylose in the same spectrum, the percent substrate converted to D-xylose was estimated as [(α-Xyl + β-Xyl) × 100]/[α-Xyl-F + α-Xyl + β-Xyl]. Similarly, the percent D-xylose product present as α-D-xylose was estimated as (α-Xyl × 100)/(α-Xyl + β-Xyl).

Table I: Products Separated from a Digest of 60 mM α-D-Xylosyl Fluoride plus 100 mM [^{14}C]Xylose, 0.060 μCi/μmol

substrates incubated (30 °C, 40 min) with	separated product (R_{Xyl})	analysis of 120-μL aliquots				from 3.5-mL digest (0.70-mL control)		
		f^a	cpm^b	μCi^c	total xylose (μmol)	μCi	total xylose (μmol)	specific radioactivity of xylose (μCi/μmol)
β-xylosidase	0.34	1/15	10340	0.0059	0.200	0.0885	3.00	0.0295
β-xylosidase	1.0	1/1800	18470	0.0105	0.192	18.9	346	0.0546
buffer	0.34	1/3	1050	0.0006		0.0018		
buffer	1.0	1/300	23570	0.0134		4.0		

[a] Fraction of separated product represented by a 120-μL aliquot. [b] All samples were counted concurrently along with aliquots of a 0.060 μCi/mL [^{14}C]xylose standard. [c] Calculated with respect to the 12630 cpm value determined with the 0.0072-μCi standard.

showed that only the α-D-xylosyl fluoride/enzyme digest contained a product migrating as xylobiose. Failure to detect this R_{Xyl} 0.34 material in the controls indicates that it was neither a contaminant of the xylose used in the digest, nor produced enzymically from xylose, not formed by interaction of the substrates in the absence of the enzyme.

A deeper understanding of the reaction catalyzed in the presence of xylose was achieved by analyses of a digest (3.5 mL) consisting of 60 mM α-D-xylosyl fluoride, 100 mM [^{14}C]xylose (0.060 μCi/μmol), and β-xylosidase (0.8 unit/mL) in buffer of pH 7.15. This was incubated (30 °C, 40 min) along with a control (0.7 mL) of the substrates at the same concentrations in buffer alone. The digest was then chromatographed on five sheets of Whatman No. 1 paper (the control on a single sheet) each with xylose and xylobiose end markers. Material in the R_{Xyl} 1.0 and R_{Xyl} 0.34 regions of digest and control chromatograms were quantitatively eluted with methanol, dried, and redissolved in known volumes of water; solutions of the products from the digest were passed through Swinnex 0.45-μm membrane filters.

Table I summarizes the results of analyses of aliquots of the products for radioactivity (by scintillation counting, concurrently with a [^{14}C]xylose standard) and for total xylose (phenol–sulfuric acid method). From the enzymic digest, the product migrating as xylobiose (R_{Xyl} 0.34) totaled 3.0 μmol (as xylose) and showed a specific radioactivity of 0.0295 μCi/μmol, i.e., half that of the [^{14}C]xylose originally added to the digest. Assuming the R_{Xyl} 0.34 product to be a disaccharide, this finding indicates that one xylose residue was derived from α-D-xylosyl fluoride and the other from the [^{14}C]xylose in the digest. The radioactivity recovered as R_{Xyl} 0.34 product, relative to total available radioactivity, was 0.47% for the enzymic digest and 0.045% for the substrate/buffer control. As the latter doubtlessly represents xylobiose with 0.06 rather than 0.03 μCi/μmol xylose, it is evident that more than 95% of the product from the digest was formed by enzymic action, presumably by xylosyl transfer from α-D-xylosyl fluoride to [^{14}C]xylose.

The xylose (346 μmol) recovered from the enzymic digest showed a 9% lower specific radioactivity than the original [^{14}C]xylose, indicating that 31 μmol of the recovered xylose is unlabeled and derived from the α-D-xylosyl fluoride. This amount, approximately 20 times the 1.5 μmol of unlabeled xylose recovered as R_{Xyl} 0.34 product, is consistent with what would be expected from the hydrolysis of a susceptible intermediate transfer product.

The R_{Xyl} 0.34 product recovered from the enzymic digest was characterized as follows. A solution in deuterium oxide gave a ^{1}H NMR spectrum (Figure 5a) which, apart from a few extraneous resonances due to impurities from the paper used in isolation, matched the spectrum (spectrum 5b) of a solution of equilibrated 4-*O*-β-D-xylopyranosyl-D-xylopyranose standard. The presence of a β-xylosidic linkage was indicated

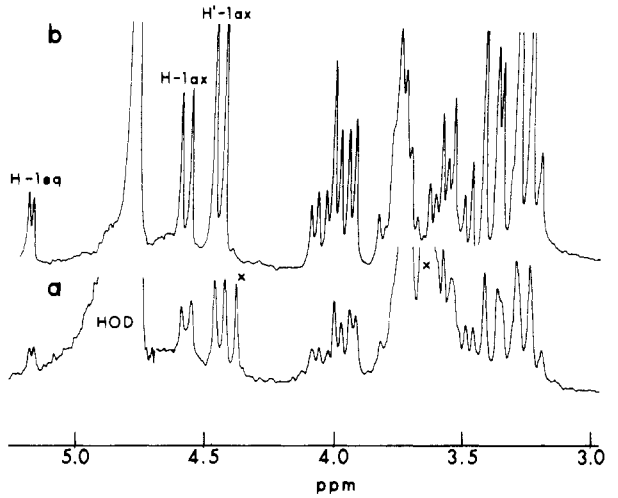


FIGURE 5: ^{1}H NMR spectra recorded in deuterium oxide at 200 MHz and 22 °C: (a) enzymically formed R_{Xyl} 0.34 product, 0.6 mg/mL; (b) anomerically equilibrated 4-*O*-β-D-xylopyranosyl-D-xylose standard, 3.6 mg/mL. H-1 eq and H-1 ax represent, respectively, the anomeric proton resonances of the reducing D-xylose unit of α- and β-xylobiose; the larger doublet, H′-1 (4.44 ppm, $J_{1,2}$ = 7.8 Hz), is the anomeric proton resonance of the β-D-xylopyranosyl residue of xylobiose; × = resonances attributable to impurities from paper used in isolating the compound.

by the large H-1 axial resonance doublet centered at 4.44 ppm ($J_{1,2}$ = 7.8 Hz), a point independently confirmed by the finding that the enzymic product was hydrolyzed to xylose by the β-xylosidase. In addition, both the enzymic R_{Xyl} 0.34 product (150 μg) and authentic xylobiose were methylated (Hakomori, 1964) and, following hydrolysis of the purified methyl derivatives of each by trifluoroacetic acid, were analyzed as the component alditol acetates by gas–liquid chromatography (Tsumuraya et al., 1984b). The alditol acetates from the enzymic product gave two major peaks having retention times of 0.67 and 1.46 relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (6.46 min). These values coincided with those found with the derivatives of authentic 4-*O*-β-D-xylopyranosyl-D-xylopyranose, indicating the enzymic product to be 4- and/or 2-*O*-D-xylopyranosyl-D-xylopyranose (2-linked and 4-linked xylopyranose are not distinguishable by this analysis).

Discussion

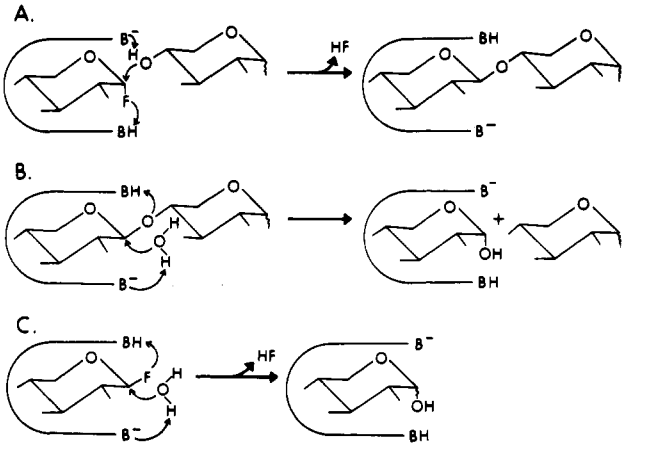
New insight has been obtained into the catalytic capabilities of the β-xylosidase of *B. pumilus* through its reactions with α- and β-D-xylopyranosyl fluoride. With α-D-xylosyl fluoride, the relation found between the initial rate of fluoride release and the substrate concentration suggested the possible need for two substrate molecules to be bound at the enzyme's active center in order for reaction to occur. The possibility that the upwardly concave v/S curve repeatedly observed between 2 and 50 mM α-D-xylosyl fluoride might simply indicate that

truly initial rates were not measured at low substrate concentrations (Allison & Purich, 1979) appears remote. All rates determined with substrate below 25 mM were for digests with <3% substrate utilization in an essentially irreversible reaction (F^- ion released), which, at 50 mM substrate, proceeds at zero order beyond 30% utilization (Figure 4A). Since *B. pumilus* β-xylosidase hydrolyzes β-D-xylosides with configurational inversion (Kersters-Hilderson et al., 1976), the overall retention observed with α-D-xylosyl fluoride suggests the occurrence of two successive inverting reactions: a xylosyl transfer between two substrate molecules to form a β-D-xylosidic product followed by its hydrolysis to yield α-D-xylose. Evidence for such a transfer product was obtained in enzymic digests with α-D-xylosyl fluoride as sole substrate. A related transfer product, isolated from a digest containing α-D-xylosyl fluoride and [^{14}C]xylose, was found to be indistinguishable from authentic 4-*O*-β-D-xylopyranosyl-D-xylopyranose. Specific radioactivity measurements showed that one of its residues had been derived from the [^{14}C]xylose and other from the α-D-xylosyl fluoride acting as donor substrate. Since xylobiose is a rapidly hydrolyzed substrate for β-xylosidase (Kersters-Hilderson et al., 1982), the xylobiose formed by the action of the enzyme upon α-D-xylosyl fluoride plus [^{14}C]xylose would not be expected to accumulate. The small amount of labeled transfer product recovered from the digest represents but a small fraction (<5%) of the total synthesized; most of which was then hydrolyzed.

The reaction catalyzed with β-D-xylopyranosyl fluoride, in contrast, appears to be a direct hydrolysis. Moreover, this substrate was found to be utilized at least 10 times faster than *p*-nitrophenyl β-D-xyloside and to have a K_m (1.8 mM) comparable to values reported for the latter substrate (Kersters-Hilderson et al., 1982; Marshall & Sinott, 1983). Further insight into the reactivity of β-D-xylosyl fluoride with β-xylosidase is gained from the following observations. Present NMR spectra confirm Hall and Manville's (1969) finding that tri-*O*-acetyl-β-D-xylopyranosyl fluoride in acetone exists mainly in the $^1C_4$ conformation but show that β-D-xylopyranosyl fluoride in aqueous solution is mostly though not entirely the $^4C_1$ conformer.[2] We assume that the productive substrate is the $^4C_1$ form as with the enzyme's other donor substrates, including α-D-xylopyranosyl fluoride, thus that its K_m would be somewhat lower than the 1.8 mM observed in the presence of some $^1C_4$ conformer. Moreover, since α-D-xylopyranosylpyridinium salts and other compounds with a ring constrained to the $^1C_4$ form were found by Marshall and Sinnott (1983) to be competitive inhibitors of hydrolysis by *B. pumilus* β-xylosidase, it is most probable that the $^1C_4$ form of β-D-xylopyranosyl fluoride would bind to the enzyme in such a way as to reduce the effective enzyme concentration. The true rate of hydrolysis of β-D-xylopyransoyl fluoride may therefore be higher than observed.

The mechanisms proposed for the reactions catalyzed by the β-xylosidase with α- and β-D-xylopyranosyl fluoride and β-D-xylopyranosides are illustrated in Scheme I. Little is known with certainty about the enzyme's catalytic groups. Kersters-Hilderson et al. (1969) initially suggested the participation of two dissociable groups (possibly an imidazole and a sulfhydryl group) in the catalytic event. More recently, a protonated sulfhydryl group located above the *re* face of a β-D-xylosidic substrate was proposed as the sole essential catalytic element (Kersters-Hilderson et al., 1984). To account

Scheme I



for the present findings, we assume the presence of a pair of catalytic groups, on opposite sides of the reaction center, that function in a coordinated way as a general acid and a general base. Scheme I depicts the mechanism postulated for the reaction catalyzed with α-D-xylopyranosyl fluoride in the presence of D-xylose and leading to the formation of 4-*O*-β-D-xylopyranosyl-D-xylose. The fluorine atom would be displaced by the 4-OH group of the acceptor, to produce the xylobiose, in a reaction involving general acid catalysis by a group located below the *si* face plus general base catalysis by a group above the *re* face. Scheme IB illustrates the mechanism proposed for the hydrolysis of xylobiose [cf. Marshall and Sinnott (1983)] on the basis that α-D-xylose is the first product (Kersters-Hilderson et al., 1976). In catalyzing this reaction, the functional roles of the catalytic groups are reversed relative to their roles in reaction A of Scheme I. The group above the *re* face, now acting as a general acid, would protonate the glycosidic oxygen atom, while that below the *si* face would act as a general base to assist the attack of water. The same mechanism is proposed for the hydrolysis of β-D-xylopyranosyl fluoride (Scheme IC), assuming the reaction product to be α-D-xylose as shown for the hydrolysis of aryl β-D-xylopyranosides by Kersters-Hilderson et al. (1976). As reaction A of Scheme I is not the reverse of reaction B or C, the opposing roles of the catalytic groups required to effect reaction A vs. either reaction B or C fall beyond requirements of the principle of microscopic reversibility.

The ability of *B. pumilus* β-xylosidase to catalyze transfer reactions with α-D-xylosyl fluoride as well as hydrolysis of β-D-xylosyl fluoride mirrors the earlier reported abilities of β-amylase, glucoamylase, glucodextranase, and trehalase (Hehre et al., 1979, 1982; Kitahata et al., 1980; Kasumi et al., 1986) to hydrolyze the α-anomer of an appropriate glycosyl fluoride and promote nonhydrolytic glycosylation reactions with the β-anomer. Marshall and Sinnott's (1983) conclusion that α-D-xylosyl fluoride is not a substrate for *B. pumilus* β-xylosidase was based on negative results of tests with enzyme and substrate in very low concentration. Substrate activation is significant in the reaction of β-xylosidase with α-D-xylosyl fluoride, as it is in the reactions promoted by inverting exo-α-glucanases with β-glycosyl fluorides (Hehre et al., 1979; Kitahata et al., 1980). The catalysts of xylosyl transfer by the β-xylosidase with α-D-xylosyl fluoride as donor opens the possibility of examining Marshall & Sinnott's (1983) interesting hypothesis that the reducing xylose residue of xylobiose binds in the normally disfavored $^1C_4$ conformation, by comparing the ability of xylopyranosides constrained in $^4C_1$ or $^1C_4$ conformation to serve as cosubstrates with α-D-xylosyl fluoride as donor.

[2] Kothe et al. (1979) found, by X-ray crystallography, that tri-*O*-acetyl-β-D-xylopyranosyl fluoride has the $^4C_1$ conformation in the crystalline state.

Present findings, finally, add to the considerable experimental support that has emerged for the proposal (Hehre et al., 1979) that most if not all glycosylases may possess functionally flexible catalytic groups and thus the ability to act on different substrates by different catalytic mechanisms. Glycosylases appear not to be alone in this respect. Evidence has been reported for the plasticity of the reaction mechanism involved in the deacylation of acyl-α-chymotrypsin, with the transition state occurring earlier in transesterification than in hydrolysis (Mishra & Klapper, 1986), in the hydrolytic cleavage of minimal vs. larger aryl groups from α-chymotrypsin (Stein et al., 1983), and in reactions catalyzed with different substrates by certain dehydrogenases (Scharschmidt et al., 1984; Hermes et al., 1984). However, the evidence appears particularly striking in the case of glycosylases where stereochemically different reactions have by now been found to be catalyzed by a considerable number of enzymes.

ACKNOWLEDGMENTS

We thank Dr. P. M. Silverman for kind assistance in rupturing *B. pumilus* cells with the French pressure cell and Dr. R. L. Whistler and Dr. E. T. Reese for samples of xylobiose.

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