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Hydrolysis of glycosylpyridinium ions by anomeric-configuration-inverting glycosidases

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ABSTRACT

The hydrolyses of five β-D-xylopyranosylpyridinium ions by the β-D-xylosidase of *Bacillus pumilus* proceed with k_{cat} values 10^8–10^9-fold larger than the rates of spontaneous hydrolysis of the same compounds. Log(k_{cat}) values correlate well with aglycon pK_a [$\beta_{lg}(V) = -0.52$, r = 0.99], whereas the correlation of log(k_{cat}/K_m) is poor [r = 0.77; $\beta_{lg}(V/K) = \sim -0.6$]. The (1→3)-β-D-glucanase of *Sporotrichum dimorphosporum* hydrolyses 4-bromo-2-(β-D-glucopyranosyl)isoquinolinium ion with a rate enhancement of 10^8. The amyloglucosidase II of *Aspergillus niger* hydrolyses three α-D-glucopyranosylpyridinium ions with rate enhancements of 10^5–10^8. The efficient hydrolysis of glycosylpyridinium ions by these three inverting glycosidases, the catalytic mechanism of which is unlikely to involve a nucleophile from the enzyme, makes it improbable that the hydrolysis of glycosylpyridinium ions by retaining glycosidases, discovered some years ago, is initiated by addition of a catalytic nucleophilic carboxylate group of the enzyme to the pyridinium ring.

INTRODUCTION

Some years ago, we discovered that β-D-galactopyranosylpyridinium ions were hydrolysed by the *lacZ* β-D-galactosidase of *Escherichia coli*[1]. k_{cat} Values for these substrates were 10^{10}–10^{12} fold greater than their rates of spontaneous hydrolysis, indicating that electrophilic or acidic catalysis, structurally impossible with these substrates, played no crucial part in the catalytic efficiency of the enzyme[2]. Similar observations were made with the *ebg* enzymes of *E. coli*[3,4], the β-D-glucosidase of *Aspergillus wentii*[5] and sweet almonds[6], yeast α-D-glucosidase[7], and the α-L-arabinofuranosidase III of *Monilinia fructigena*[8], as well as with *E. coli* and snail glucuronidase[9]. All of these enzymes yield the reducing sugar product in the same anomeric configuration as the substrate. They therefore probably work by a double-displacement mechanism involving, as intermediates, glycosylated aspartate

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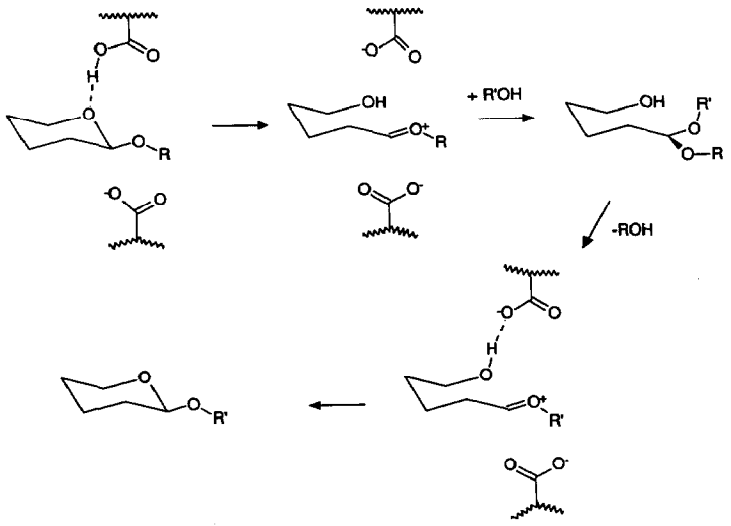
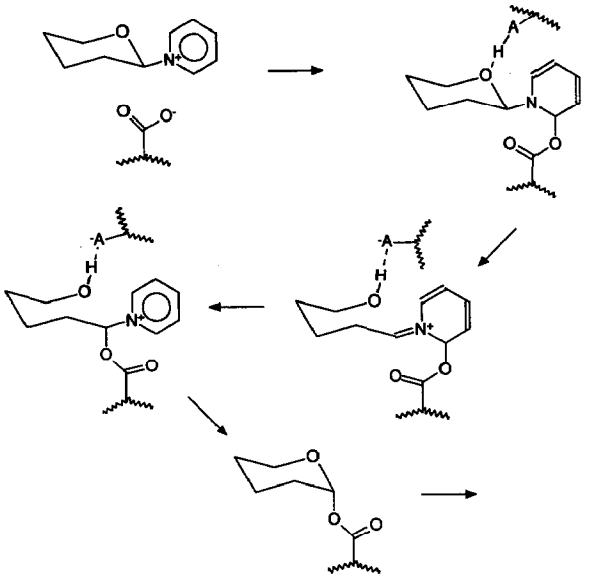


Scheme 1. Ring opening mechanism for c → c glycosidases according to Fleet[11] or Post and Karplus[12]. The full oxocarbonium ions drawn are not essential to this mechanism, and may be taken to represent oxocarbonium-ion-like transitions leading to and from covalent acylals.

or glutamate residues in which the sugar residue has the opposite anomeric configuration to the substrate[10].

The efficient hydrolysis of glycosylpyridinium salts by retaining glycosidases would appear to preclude mechanisms for the hydrolysis of *O*-glycosides involving opening of the sugar ring, as proposed by Fleet[11] for the general case and by Post and Karplus[12] for lysozyme (Scheme 1), since an analogous mechanism for hydrolysis of the pyridinium salts would require the generation of an electron-deficient centre next to the full positive charge of the pyridinium nitrogen atom. However, Franck[13] has attempted to reconcile ring opening mechanisms for e → e (retaining β) glycopyranosidases with their hydrolysis of glycosylpyridinium salts by suggesting that the nucleophilic aspartate or glutamate residue that eventually formed the glycosyl–enzyme intermediate first attacked the pyridine ring nucleophilically, the ring opened (in the normal mechanism of glycosylamine hydrolysis), and then the system underwent a 1,3- or 3,3-sigmatropic rearrangement (Scheme 2).

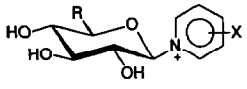
If glycosylpyridinium salts are substrates for retaining glycosidases because the carboxylate group forming the glycosyl–enzyme intermediate can add to the pyridinium ring, then glycosylpyridinium ions should not be substrates for glycosidases which do not have such a group. Configuration-inverting glycosidases fall into this class. These enzymes are generally considered to operate by a single displacement mechanism involving a nucleophilic water molecule, partly deprotonated by a base catalytic group, with proton donation to the leaving group from an acid catalytic group[10]. The X-ray crystal structures of the three inverting glycosidases that have so far been determined[14–16] show the acid catalytic group clearly. The base catalytic group is discernible in *Aspergillus* glucoamylase[16], and in the



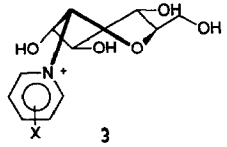
Scheme 2. Mechanism for hydrolysis of glucosylpyridinium salts by e → e glycosidases proposed by Franck[13]. The covalent glycosyl–enzyme intermediate is the same as that generated from *O*-glycosides.

CelD gene-product from *Clostridium thermocellum*[15], but not in the catalytic domain of the cellobiohydrolase II from *Trichoderma reesei*[14].

Inverting glycosidases, though, are commonly highly specific with respect to the leaving group, and fail to hydrolyse aryl glycosides, so that their failure to hydrolyse glycosylpyridinium salts would not be mechanistically informative. We now report that three inverting glycosidases, the β-D-xylosidase of *Bacillus pumilus*[17–19], a (1 → 3)-β-D-glucanase from *Sporotrichum dimorphosporum*[20,21], and *Aspergillus niger* amyloglucosidase II, which do hydrolyse aryl glycosides, also hydrolyse β-D-xylopyranosyl- (**1**), β-D-glucopyranosyl- (**2**), and α-D-glucopyranosyl- (**3**) -pyridinium bromide.



1 R = H
2 R = CH_2OH



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EXPERIMENTAL

Substrates.—β-D-Xylopyranosyl-[22], β-D-glucopyranosyl-[5], and α-D-glucopyranosyl-pyridinium bromide[7] are described elsewhere. *p*-Nitrophenyl β-D-glucopyranoside, *p*-nitrophenyl β-D-xylopyranoside, and laminarin were obtained from Sigma, St Louis, MO.

Enzymes.—Amyloglucosidase II of *Aspergillus niger* was the kind gift of Dr. M. Schülein, NOVO A/S, Bagsvaerd, Denmark: it was assumed to be 100% pure and 100% active as supplied. The molecular weight per active site was taken as 97 k[16].

The (1 → 3)-β-D-glucanase from the Basidiomycete ATCC 24562 (identified as *Sporotrichum dimorphosporum*) was isolated by a procedure based upon that of Peterson and Kirkwood[20] and described elsewhere[23]. It gave one band in SDS-PAGE electrophoresis (Pharmacia Phast system) and had a specific activity of 400 U/mg when assayed against laminarin[24]. For calculation of k_{cat} values, enzyme with the maximum literature specific activity of 570 U/mg[20] was assumed to be 100% pure and 100% active, and to have a molecular weight per active site of 51 k[21].

β-D-Xylosidase was isolated from *Bacillus pumilus* 12 (the strain described by Simpson[25,26], kindly supplied by Dr. M. Claeyssens, Rijksuniversiteit te Gent), essentially according to literature procedures[17], but taking advantage of modern technology. The DEAE chromatography was carried out on a Waters 650 protein isolation system, using a DEAE-5PW column and 0.1 M Tris-HCl buffer, pH 8.0, 1 mM in EDTA and dithiothreitol, with a linear gradient (0–0.3M) of NaCl, and the gel permeation chromatography was carried out using Sephacryl 300, rather than Sephadex. The enzyme had a specific activity of 2.5 U/mg and gave one band on SDS-PAGE. It was dialysed against dithiothreitol (1.0 mM) before use. Enzyme with the maximal literature specific activity of 5.5 U/mg[27] was assumed to be 100% pure and 100% active, and to have a molecular weight per active site of 60 k[28].

Kinetic methods.—Rates of hydrolysis of glycosylpyridinium ions were followed with a Perkin–Elmer Lambda 6 spectrophotometer having a cell block fitted with a Peltier-effect-based temperature controller. Leaving groups, wavelengths (nm), and extinction coefficient changes (M^{-1} cm^{-1}) were, for the β-D-xylopyranosylpyridinium salts: 3-bromopyridine, 235, −271; 4-bromoisoquinoline, 345, −3480; nicotinamide, 247, −161; pyridine, 276, −238; and isoquinoline, 300, 896; and for the α-D-glucopyranosylpyridinium salts: 3-bromopyridine, 248, 509; pyridine, 247, 370; and 4-methylpyridine, 240, −789. Extinction coefficients at the same wavelength were assumed to be the same for β-D-xylopyranosyl- and β-D-glucopyranosyl-pyridinium ions. At least 10 initial rates were measured, and k_{cat} and K_m were calculated using the non-linear least-squares program *Kaleidagraph* (Synergy Software, Reading, PA).

The (1 → 3)-β-D-glucanase-catalysed hydrolysis of laminarin and its inhibition by 4-bromo-2-(β-D-glucopyranosyl)isoquinolinium bromide was followed by reducing sugar assay[24]: after 1 min, reactions were stopped by addition of alkaline copper reagent.

The inhibition of the β-D-xylosidase-catalysed hydrolysis of *p*-nitrophenyl β-D-xylopyranoside by 3-carbamoyl-1-(β-D-xylopyranosyl)pyridinium bromide was measured as described earlier[29].

RESULTS

In Table I are given kinetic parameters for the hydrolysis of xylopyranosylpyridinium salts by β-D-xylosidase of *B. pumilus*. In a previous study[27], we had measured the K_i values of some of these compounds, and had also been able to show qualitatively that the 3-carbamoylpyridinium salt was a slow substrate. With more enzyme available, we are now able to quantitate the catalysis, the similarity of the K_m for the 4-bromoisoquinolinium ion as a substrate and its K_i as an inhibitor providing some confidence that the hydrolyses are not being carried out by a trace contaminant (of, for example, a retaining xylosidase) in the enzyme preparation. With some substrates, saturation of the enzyme required substrate concentrations with very high initial absorbances, and so K_i values were taken as K_m values.

The correlation of $\log(k_{cat}/K_m)$ with leaving group pK_a is only fair ($r = -0.77$), but it is better for $\log k_{cat}$ ($r = -0.99$; $\beta_{lg} = -0.52$). This suggests that adventitious effects of the substituents on the pyridine affect binding, and that the degree of C–N bond cleavage at the single chemical transition state is rather less than that at the first chemical transition state of retaining β-D-glycosidases, for which β_{lg} values of -0.9 are commonly observed[1,3,5,6].

Comparison of k_{cat} values with first-order rate constants for the spontaneous hydrolyses of xylosylpyridinium salts[22] indicate that the enzyme accelerates C–N cleavage by a factor of 10^8 (for the more acidic leaving groups) to 10^9 (for the less acidic leaving groups).

The UV spectrum of a sample of the 4-bromo-2-xylosylisoquinolinium bromide completely hydrolysed by the *B. pumilus* xylosidase was identical to that of 4-bromoisoquinoline (λ_{max} 324, 312, 287, and 220 nm), and reducing sugar analysis[24] indicated that 80–85% of reducing sugar had been formed. This confirms that the expected products of the reaction, xylose and 4-bromoisoquinoline, are in fact formed.

The hydrolysis of β-D-glucopyranosylpyridinium bromide by the (1 → 3)-β-D-glucanase of *S. dimorphosporum* yields 4-bromoisoquinoline and reducing sugar

TABLE I

Michaelis–Menten parameters for hydrolysis of β-D-xylopyranosylpyridinium ions by *B. pumilus* β-D-xylosidase in 10 mM sodium phosphate buffer 1 mM in EDTA, at pH 7.2 and 25.0°C

Leaving group	k_{cat}/K_m ($M^{-1}s^{-1}$)	$10^{-3}k_{cat}$(s^{-1})	K_m(mM)	K_i(mM)
3-Bromopyridine	37	67	1.73	
4-Bromoisoquinoline	277	58	0.28	0.36 [a]
Nicotinamide	5.4			7.9
Pyridine	1.47			6.76 [a]
Isoquinoiline	8.8			1.23 [a]

[a] Ref 29.

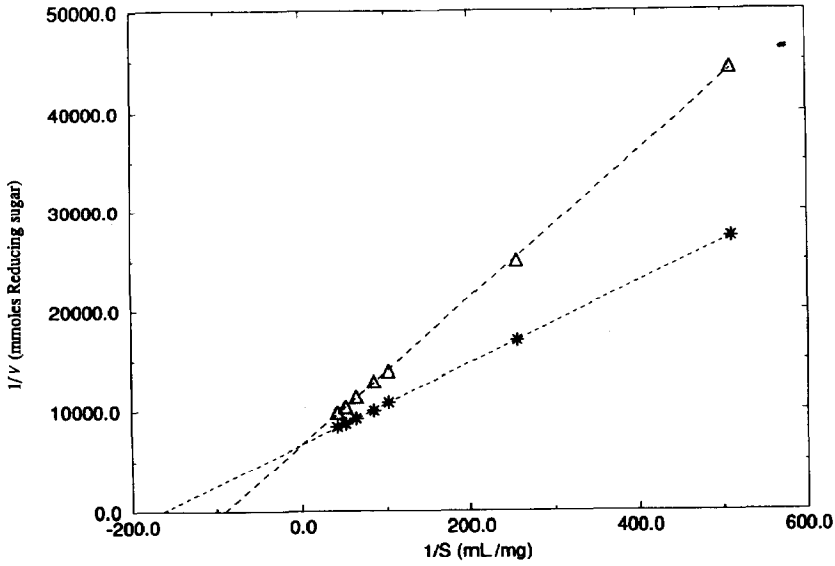

Fig. 1. Inhibition of the *S. dimorphosporum* hydrolysis of laminarin, catalysed by *S. dimorphosporum* (1 → 3)-β-D-glucanase (0.2 U/mL), by 4-bromo-2-(β-D-glucopyranosyl)isoquinolinium bromide: *, no inhibitor; Δ, 0.1 mM inhibitor. The K_m for laminarin is 5.9×10^{-3} mg/mL. The ordinate is the concentration of reducing sugar liberated per minute.

(80%), with $k_{cat} = 0.34\ s^{-1}$ and $K_m = 0.16$ mM at 25.0°C, in 50 mM sodium phosphate buffer, pH 6.0. Fig. 1 shows the inhibition of the hydrolysis of laminarin by this compound: a K_i value of 0.14 mM is obtained, indicating that transformation of the 4-bromo-2-(β-D-glucopyranosyl)isoquinolinium ion is indeed occurring in the active site of the (1 → 3)-β-D-glucanase, and not in that of a minor contaminating enzyme.

It was not possible to observe saturation of the (1 → 3)-β-D-glucanase with 3-carbamoyl-1-(β-D-glucopyranosyl)pyridinium ion, but a value of k_{cat}/K_m of 31 $M^{-1}s^{-1}$ can be estimated.

Extrapolation of data for the spontaneous hydrolysis of the 4-bromo-2-(β-D-glucopyranosyl)-isoquinolinium compound[22] to 25.0°C reveals that the (1 → 3)-β-D-glucanase brings about a rate enhancement of 10^9 on C–N cleavage.

The hydrolyses of α-D-glucopyranosylpyridinium ions by the amyloglucosidase II of *A. niger* were slower: for the 3-bromopyridine, pyridine, and 4-methylpyridine leaving groups at 25.0°C in 50 mM sodium acetate buffer, pH 5.0, k_{cat} values were 0.045, 0.030, and 0.009 s^{-1}, respectively, and K_m values were 0.69, 1.79, and 0.88 mM. Fig. 2 shows the inhibition of the hydrolysis of *p*-nitrophenyl α-D-glucopyranoside by the 3-bromopyridinium salt, from which a K_i value of 0.58 mM can be derived, indicating that the hydrolysis of the pyridinium salts is being brought about at the active site of amyloglucosidase. Comparison of k_{cat} values with the rates of spontaneous hydrolysis of the same compounds[22] indicates that the rate

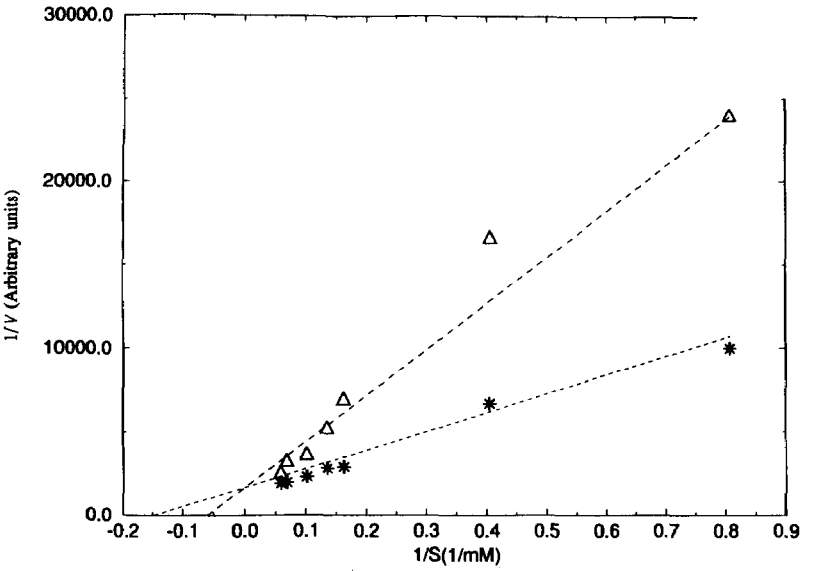

Fig. 2. Inhibition of the hydrolysis of *p*-nitrophenyl α-D-glucopyranoside, catalysed by *A. niger* glucoamylase II (0.32 U/mL), by 3-bromo-1-(α-D-glucopyranosyl)pyridinium bromide: *, no inhibitor; Δ, 0.1 mM inhibitor. The K_m for *p*-nitrophenyl α-D-glucopyranoside is 5.9×10^{-3} mg/mL.

enhancement is 10^5 for the 3-bromopyridinium compound, increasing to 10^8 for the more basic leaving groups.

DISCUSSION

It is clear that inverting β-glycosidases hydrolyse glycosylpyridinium ions in much the same way as retaining β-glycosidases. With 4-bromoisoquinoline as a leaving group, the rate enhancements of 10^8 for the β-D-xylosidase of *B. pumilus* and 10^9 for the (1 → 3)-β-D-glucanase of *S. dimorphosporum* can be compared with enhancements of 10^{10} for *lacZ* β-D-galactosidase of *E. coli*[2], and 10^9 for the *ebg* enzyme of the same organism[3] and the β-D-glucosidase A_3 of *Aspergillus wentii*[5]. Mechanisms for hydrolysis of the pyridinium salts which require the addition of a nucleophilic carboxylate to the pyridine ring are thereby rendered highly unlikely.

Whilst the amyloglucosidase undoubtedly hydrolyses α-D-glucosylpyridinium ions, the characteristics of the hydrolysis are puzzling. The k_{cat} values appear substantially independent of aglycon acidity, and so the absolute rate enhancements are very dependent on leaving group acidity, varying from low (10^5) for the 3-bromo derivative to 10^8 for the unsubstituted pyridinium compound. This pattern of behaviour may be related to the sugar ring of these compounds being in the $^1S_3$ conformation[22], whereas the enzyme probably transforms substrates in the $^4C_1$ conformation. If k_{cat} represented, not bond cleavage, but a conversion of the

enzyme-bound substrate into the $^4C_1$ conformation, then both the low efficiency with the better substrates and the absence of a dependence on aglycon acidity would become explicable. Whilst this system merits further investigation, for the present purposes the rate enhancement of 10^8 for the more basic leaving groups indicates that (inverting) amyloglucosidase is cleaving the C–N bond of α-D-glucopyranosylpyridinium ions with much the same efficiency as (retaining) α-D-glucosidase[7].

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