Purification and mechanistic properties of an extracellular α -L-arabinofuranosidase from *Monilinia fructigena*

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1. The α -L-arabinofuranosidase isoenzyme designated AFIII [Laborda, Archer, Fielding & Byrde (1974) J. Gen. Microbiol. 81, 151–163] was purified by sequential isoelectric focusing, hydrophobic chromatography, gel filtration and chromatofocusing. 2. The enzyme is a monomer of M_r 40000. 3. On inactivation of the enzyme with ^3H -labelled 1- α -L-arabinofuranosylmethyl-3-*p*-nitrophenyltriazene, 0.64 mol of α -L-arabinofuranosylmethyl residues/mol of enzyme is estimated to become attached to protein. 5. Neither first-order nor second-order rate constants for hydrolyses of aryl α -L-arabinofuranosides are dependent upon leaving-group acidity [$\beta_{\text{lg}}(V) = -0.16 \pm 0.11$; $\beta_{\text{lg}}(V/K) = -0.11 \pm 0.07$; $n = 7$; $\Delta pK_a = 4.5$]. 6. Bond-breaking is nonetheless rate-limiting, as is shown by a value of $^{18}(V)$ of 1.030 ± 0.007 for the hydrolysis of *p*-nitrophenyl arabinoside. 7. Proton-donation to the leaving group is thus far advanced at the rate-limiting transition state for this enzyme. 8. Four α -L-arabinofuranosyl pyridinium salts are substrates, and an approximate $\beta_{\text{lg}}(V)$ value of -0.9 can be estimated. 9. The absolute rate enhancement with the 4-bromoisoquinolinium salt, 2.5×10^9 , is comparable with that observed with pyranosidases. 10. Ring-opening mechanisms can therefore be dismissed, even though they are known in the acid-catalysed hydrolysis of arabinofuranosides.

INTRODUCTION

Glycoside hydrolysis is one of the simplest chemical reactions catalysed by enzymes, yet aspects of the mechanisms of glycosidases remain little understood. For 'retaining' glycopyranosidases, which work by a double-displacement mechanism, the balance of evidence (reviewed by Sinnott, 1987) implies glycopyranosylation-like transition states leading to and from a covalent glycosylated carboxylate as the glycosyl-enzyme intermediate, with acidic catalysis being of variable importance, most of the catalytic power of the enzyme coming from non-covalent interactions, and non-covalent events being sometimes kinetically significant.

Even this type of glycosidase, however, continues to provoke novel mechanistic proposals: recently Fleet (1985) and Post & Karplus (1986) have suggested that certain pyranosidases might open the pyranose ring of their substrates and first break the endocyclic C-1–O-5 bond (see Scheme 1). There is no precedent for such a process in the non-enzymic hydrolysis of pyranosides (reviewed by Sinnott, 1984), although ring-opening can be observed in some circumstances during the non-enzymic hydrolysis of furanosides (Capon & Thacker, 1967; Lönnberg & Kulonpää, 1977; Lönnberg *et al.*, 1977; Lönnberg & Valtonen, 1978).

In fact the ring-opening mechanism cannot hold for any glycosidase for which the corresponding fluoride or pyridinium salt is a good substrate, since these aglycones, unlike an oxygen aglycone, cannot stabilize a positive charge in a way that would make the ring-opening mechanism chemically plausible (see Scheme 1). Furthermore, the observation of a negative β_{lg} value, or a direct leaving-group ^{18}O kinetic isotope

effect, would, were the ring-opening mechanism taking place, require the chemically implausible supplementary hypothesis that decomposition of some hemiacetal species had become rate-limiting. Such arguments can be used to dismiss the ring-opening mechanism for all glycopyranosidases for which these kinetic measurements have been made (Sinnott, 1987), but this still leaves ring-opening tenable as a mechanistic possibility supported by theoretical calculation.

We therefore now present data on the catalytic mechanism of an α -L-arabinofuranosidase of *Monilinia fructigena*. Whereas ring-opening during non-enzymic hydrolyses of *O*-glycopyranosides lacks chemical precedent, ring-opening during acid catalysis of the hydrolysis of *O*-arabinofuranosides of electron-donating aglycones has been firmly established (Lönnberg & Kulonpää, 1977; Bennet *et al.*, 1985). If ring-opening can be dismissed in a system chemically biased towards it, then it can probably be dismissed in general as a mechanistic possibility for glycosidases.

Further reasons for investigating this enzyme include a dearth of mechanistic information about *O*-furanosidases, as compared with *O*-pyranosidases and *N*-furanosidases, especially NAD glycohydrolases (Yost & Anderson, 1983; Schuber *et al.*, 1979; Bull *et al.*, 1978, the importance of arabinofuranosidase in infection by the fungus that is responsible for the brown rot of apples (Byrde *et al.*, 1973), and the existence of preliminary protein-chemical and mechanistic information. Thus culture filtrates of the fungus were shown to contain two isoenzymes, a low- M_r form ($\overline{M}_r$ 40000) of pI 6.5 and optimum pH 5, and a high- M_r , possibly self-associating, form of pI 3.0 and optimum pH 4 (Laborda *et al.*, 1973). Both isoenzymes give products with retention of

Abbreviation used: AFIII, α -L-arabinofuranosidase isoenzyme III.

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Scheme 1.

configuration (Fielding *et al.*, 1981), but only the low- M_r form, designated AFIII (Laborda *et al.*, 1974), was subject to active-site-directed irreversible inhibition by the suicide inactivator 1- α -L-arabinofuranosylmethyl-3-*p*-nitrophenyltriazene (Marshall *et al.*, 1981).

MATERIALS AND METHODS

Materials

Proteins from the culture filtrate of *Monilinia fructigena* were concentrated and subjected to isoelectric focusing by Dr. R. J. W. Byrde and Dr. A. H. Fielding, Long Ashton Research Station, Bristol, as described by Laborda *et al.* (1973). The fractions containing AFIII from several runs, which were not required for those workers' immediate purposes, were pooled and kept frozen.

The chemical syntheses of aryl α -L-arabinofuranosides (Widdows, 1979; Kelly, 1985) and of α -L-arabinofuranosyl 4-bromoisoquinolinium and 3,5-dimethylpyridinium bromides (Sinnott & Wijesundera, 1985) are described elsewhere. The non-crystalline α -L-arabinofuranosylpyridinium and 4-methylpyridinium bromides were made similarly and gave correct ¹H-n.m.r. spectra.

α -L-Arabinofuranosylmethylamine was prepared as follows. We used the method of de las Heras & Fernández-Resa (1982) for the production of acylated glycosyl 1,2-*trans*-cyanides. Acetylation of L-arabinose by the method of Börjeson *et al.* (1963), followed by flash chromatography [silica gel, with ethyl acetate/chloroform (1:1, v/v) as eluent] yielded 1,2,3,4-tetra-*O*-acetyl- α -L-arabinofuranose as a chromatographically homogeneous syrup. Reaction of this material (27.4 g) with trimethylsilyl cyanide (25 ml) (Reetz & Chatziiofidis, 1982) in dry nitromethane (400 ml) for 25 min in the presence of boron trifluoride etherate (1.2 ml), followed by work-up as described by de las Heras &

Fernández-Resa (1982), treatment with decolorizing charcoal and flash chromatography, gave 2,3,4-tri-*O*-acetyl- α -L-arabinofuranosyl cyanide (7.9 g, 28% yield) as a syrup homogeneous on t.l.c. on silica-gel plates with light petroleum (b.p. 60–80 °C)/ethyl acetate (3:2, v/v) (R_F 0.27). ¹H n.m.r. (60 Mz) δ relative to tetramethylsilane (p.p.m.) ([²H]chloroform) 2.1 (9H, m, 3 $\times$ CH₃CO), 4.3 (3H, m, 4-*H* + 5a-*H* + 5b-*H*), 4.75 (1H, s, 2-*H*), 5.08 (1H, m, 3-*H*) and 5.25 (1H, s, 1-*H*). This material (7.9 g) was reduced with LiAlH₄ according to the procedure described by Coxon & Fletcher (1964) for the β -D-galactopyranosyl compound. The intensely deliquescent product (1.2 g, 30% yield) was obtained crystalline from dry methanol. It co-chromatographed (h.p.l.c., C₁₈ column, water as eluent) with the material produced previously (Marshall *et al.*, 1981) and was further characterized by ¹³C and ¹H n.m.r. (JEOL FX200 instrument) (200 MHz on ¹H) in ²H₂O, with chemical shifts given relative to sodium 3-(trimethylsilyl)propanesulphonate, and nuclei numbered according to the IUPAC name 1-amino-1-deoxy-2,5-anhydro-L-mannitol. ¹³C-n.m.r. peaks were observed at 83.2 (5-*C*), 82.6 (2-*C*), 78.0 and 76.8 (3-*C* and 4-*C*), 61.4 (6-*C*) and 42.4 p.p.m. (1-*C*). The observed ¹H-n.m.r. spectrum could be simulated by using the following chemical shifts (δ) and coupling constants: H-1a, 2.798 p.p.m.; H-1b, 2.860 p.p.m.; H-2, 3.892 p.p.m.; H-3, 3.971 p.p.m.; H-4, 4.0225 p.p.m.; H-5, 3.862 p.p.m.; H-6a, 3.761 p.p.m.; H-6b, 3.686 p.p.m.; J_{1a-1b} 13.79 Hz; J_{1a-2} 6.88 Hz; J_{1b-2} 3.47 Hz; J_{2-3} 6.28 Hz; J_{3-4} 6.10 Hz; J_{4-5} 6.19 Hz; J_{5-6a} 3.47 Hz; J_{5-6b} 5.91 Hz; J_{6a-6b} 12.26 Hz.

³H-labelled α -L-arabinofuranosylmethylamine was synthesized as follows. The Raney-nickel-catalysed exchange of the carbon-bound protons of carbohydrates (Koch & Stuart, 1977) was used to introduce ³H from ³H-labelled water, essentially as described by van Diggelen *et al.* (1981) for the β -D-galactopyranosyl compound. ¹H- and ¹³C-n.m.r. spectra of the material

exchanged with $^2\text{H}_2\text{O}$ indicated that complete exchange of H-1a, H-1b, H-6a and H-6b had taken place, H-3 and H-4 were partially exchanged, and H-2 and H-5 were not exchanged. Exchange of unlabelled α -L-arabinofuranosylmethylamine (50 mg) with ^3H -labelled water (1.25 ml; 5 Ci/ml) afforded 8 mg (16%) of a material of specific radioactivity 0.55 Ci/mg.

Methods

Assay of α -L-arabinofuranosidase activity. α -L-Arabinofuranosidase activity was assayed during enzyme isolation with 1 mM-*p*-nitrophenyl α -L-arabinofuranoside, in 0.1 M sodium acetate/acetic acid buffer, pH 5.0, at 30 °C. For assay of chromatography fractions, the reaction was stopped by the addition of NaHCO_3 and the absorbance was read at 400 nm; for accurate assay of pooled fractions the absorbance was measured continuously at 345 nm. The unit of activity is transformation of 1 $\mu\text{mol/min}$ under these conditions.

Determination of protein. Protein concentrations below 0.1 mg/ml were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, and between 0.1 and 1.0 mg/ml by the biuret method of Gornall *et al.* (1949). Protein purity was assessed by electrophoresis on slabs of polyacrylamide gel in the presence of SDS (Laemmli, 1970). A linear gradient of 10–20% acrylamide was used, and the gel was stained with Coomassie Blue. Ferritin (M_r 450 000), catalase (M_r 240 000), bovine serum albumin (M_r 68 000) and cytochrome *c* (M_r 12 500) were used as M_r standards.

Measurement of radioactivity. Radioactivity was measured in an LKB Rac Beta liquid-scintillation counter with an external tritium standard. Liquid samples (up to 1 ml) were suspended in liquid scintillant (Unisolve E; Koch-Light Ltd.) (10 ml), and kept at 4 °C overnight in the dark before their radioactivity was counted. Protein samples, whether precipitates or solutions, were digested with 30% (w/v) H_2O_2 for 1 h at 70 °C before their radioactivity was counted.

Determination of Michaelis–Menten parameters. Michaelis–Menten parameters for all substrates except 4-methylumbelliferyl α -L-arabinofuranoside were determined by continuous monitoring of u.v. absorbance by a Unicam SP.1800 or SP.8-200 spectrophotometer fitted with thermostatically controlled cell-blocks: wavelengths and absorption-coefficient changes are given in Table 1. The hydrolysis of the fluorogenic substrate was followed continuously in a partly jacketted cell in a Perkin–Elmer 3000 spectrofluorimeter, with monitoring at 450 nm and excitation at 360 nm. Because of the quenching of aglycone fluorescence by the substrate, it was necessary to construct a response curve for 4-methylumbelliferone for each substrate concentration used. Michaelis–Menten parameters were calculated by linear least-squares treatment of plots of V against $V/[S]$.

Determination of ^{18}O kinetic isotope effect. The ^{18}O kinetic isotope effect for hydrolysis of *p*-nitrophenyl α -L-arabinofuranoside was measured at a substrate

concentration of 8 mM (10 K_m), with the labelled glycoside described by Bennet *et al.* (1985), and the procedure used by Hosie & Sinnott (1985) for the equivalent measurement on α -glucosidase, except that the reaction was monitored at a wavelength of 345 nm, the isosbestic point of *p*-nitrophenol and its anion. Successive comparisons of 13–17 pairs of rates of hydrolysis of labelled and unlabelled substrates, with recrystallization of the substrates between each set of measurements, gave isotope effects of 1.0535 ± 0.034 , 1.0312 ± 0.014 , 1.0395 ± 0.014 and 1.0299 ± 0.007 .

Isolation of enzyme. All chromatography was carried out in the cold-room.

Preliminary tests had shown that AFIII was precipitated at between 50% and 60% saturation of $(\text{NH}_4)_2\text{SO}_4$. Consequently, for the hydrophobic column the start buffer was 10 mM-sodium acetate/acetic acid buffer, pH 5.0, in 26.4% (w/v) $(\text{NH}_4)_2\text{SO}_4$. The combined AFIII fractions from the isoelectric-focusing columns (11.9 mg of protein, total activity 12.75 $\mu\text{mol/min}$) were dialysed against this solution, and applied to a 2.5 cm $\times$ 20 cm packed bed of phenyl-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) equilibrated with start buffer. The start buffer was then pumped through the column, at a rate of 25 ml/h, until a protein peak had come off, and then a linear gradient to a final buffer composition of 50% (v/v) ethylene glycol in 10 mM-sodium acetate/acetic acid buffer, pH 5.0, was applied, the total volume being 1 litre. The portions containing enzyme activity were concentrated and dialysed against 0.1 M-sodium acetate/acetic acid buffer, pH 5.0, to give 2.15 mg of protein and 8.67 units of activity (3.8-fold purification).

The enzyme solution was concentrated to 7 ml, and then applied to a 2.5 cm $\times$ 20 cm column of Sephadex G-100 (Pharmacia), equilibrated with 0.1 M-sodium acetate/acetic acid buffer, pH 5.0. The column was then eluted with this buffer at a rate of 4 ml/h, and 5 ml fractions were collected. Enzyme appeared in fractions 8–12, which on concentration gave 1.16 mg of protein and 6.4 units of activity (1.37-fold purification).

The M_r determination was carried out on the same column.

The enzyme from the Sephadex G-100 column was dialysed against 25 mM-imidazole/HCl buffer, pH 7.0, and applied to a 0.9 cm $\times$ 20 cm column of Polybuffer Exchanger PBE 94 (Pharmacia), equilibrated with the same buffer. A linear gradient of this buffer (200 ml) and Polybuffer 74 (Pharmacia) (25 ml, adjusted to pH 5.0 with conc. HCl, and made up to 200 ml) (200 ml) was applied. Fractions (3 ml) were collected at a flow rate of 34 ml/h. A range of overlapping peaks of both protein and activity, the patterns corresponding to one another, was obtained in fractions 80–138. Since no kinetic differences could be detected in the behaviour of enzyme from separate parts of the pattern of peaks, we attributed this detection of multiple molecular forms to differing degrees of glycosylation, and combined fractions to give, after concentration and dialysis, 0.14 mg of protein of specific activity 9.39 units/mg.

A subsequent larger-scale isolation started from 580 mg of protein (295 units), and, after chromatofocusing, Sephadex G-100 chromatography and phenyl-Sepharose chromatography (in that order), gave 130 units of specific activity 7.6 units/mg.

Scheme 2.

Affinity labelling of AFIII. ^3H -labelled α -L-arabinofuranosylmethylamine (8 mg) was mixed with an equal weight of the unlabelled compound, and dissolved in water (1.0 ml) containing NaHCO_3 (8.4 mg, 1 equiv.). *p*-Nitrobenzenediazonium tetrafluoroborate (11.4 mg, 1 equiv.) was added, and the whole was stirred 30 min at 0°C . The mixture was filtered through glass-fibre filter papers, and washed with benzene (3×5 ml) and then with butan-1-ol (3×5 ml). The butanol extracts were back-extracted with water (5 ml), and then evaporated at ambient temperature (pressure approx. 65 Pa), to yield 1- α -L-arabinofuranosylmethyl-3-*p*-nitrophenyltriazene (2.5 mg, 8% yield), as assessed by u.v. absorbance (Sinnott & Smith, 1978).

AFIII (2.3 mg; 7.6 units/mg) in 10 mM-Tris/HCl buffer, pH 7.0, was incubated with triazene (1.0 mg) for 24 h at 30°C , by which time the enzyme activity was constant at 37% of the starting activity. To a sample (10 μl) was added aqueous 1% (w/v) bovine serum albumin solution (50 μl), and proteins were precipitated with aqueous 10% (v/v) trifluoroacetic acid (1 ml). The precipitate was filtered, washed well with trifluoroacetic acid and digested, and its radioactivity counted.

The bulk of the protein was then dialysed against inactivation buffer (3×1 litre), and loaded on to a $2.5 \text{ cm} \times 17 \text{ cm}$ column of Sephadex G-25 (Pharmacia), which was eluted with purified water at 5.2 ml/h, and 30 min fractions were collected. A peak of coincident radioactivity, protein and enzymic activity emerged over fractions 13–17, which were colourless, and a peak of absorbance and some radioactivity over fractions 23–28. The former is clearly labelled enzyme, the latter triazene decomposition products.

RESULTS AND DISCUSSION

Electrophoretograms of the purest samples of enzyme revealed the presence of a minor band of slightly lower M_r than the main band; although clearly detectable by eye, it produced less than 10% of the densitometer signal of the main band. Gel electrophoresis under denaturing conditions, in the presence of M_r standards, gave an M_r of between 34000 and 35000. Gel filtration gave an M_r of about 40000, in agreement with the results reported by Laborda *et al.* (1973). The enzyme is a monomer.

From the results of chromatofocusing, it is clear that the enzyme as isolated is a mixture of molecular forms. The relative activities towards 4-phenylphenyl and *p*-nitrophenyl α -L-arabinofuranosides of enzyme from four separate regions of the chromatofocusing column were the same within experimental error, and the K_m values of the *p*-nitrophenyl glycoside for the two most abundant fractions were identical. Since varying glycosylation patterns of extracellular fungal glycosidases, giving rise to enzyme microheterogeneity, are well known [see, e.g., Niku-Paavola *et al.* (1985) (*Trichoderma reesei* cellulase), Chu *et al.* (1985) (yeast invertase) and Moloney *et al.* (1985) (*Talaromyces emersonii* cellulase)], for the present purposes the enzyme can be treated as a single catalytic species.

Affinity labelling of glycopyranosidases by the corresponding 1-glycosylmethyl-3-*p*-nitrophenyltriazenes arises by enzyme-catalysed generation of the glycosylmethanediazonium ion, which is captured by active-site nucleophiles or escapes from the enzyme active site (Sinnott, 1982). The incorporation of the L-arabinofuranosylmethyl residue into enzyme protein indicates that this pathway is also followed during inactivation of this furanosidase (Scheme 2). If it is assumed that pure completely active enzyme has a specific activity of 9.4 units/mg and an M_r of 40000, the acid-precipitation results indicate a stoichiometry of 0.64 mol of arabinofuranosylmethyl residues incorporated/mol of active site destroyed. That the radioactivity incorporated into acid precipitates was not a simple entrainment phenomenon was shown by Sephadex G-25 chromatography of labelled dialysed enzyme. The labelled enzyme still possessed 37% of the starting enzymic activity, and so it was possible to demonstrate the existence of coincident peaks of radioactivity, enzymic activity and absorbance at 280 nm at the exclusion limit, and also a separate peak of low- M_r slightly radioactive material. The labelled protein was colourless, whereas the low- M_r material was yellow, indicating that when the arabinofuranosylmethyl residue became attached to protein the *p*-nitroaniline chromophore was lost, in accord with the inactivation mechanism of Scheme 2.

The stoichiometry of incorporation of label calculated from the radioactivity of the excluded peak on the Sephadex G-25 column is somewhat lower (0.51) than

Table 1. Hydrolysis of α -L-arabinofuranosyl derivatives by AFIII

Leaving group	λ (nm)	$10^{-3}\Delta\epsilon$	K_m (mM)	k_{cat} (s^{-1})
0.1 M-Sodium acetate/acetic acid buffer, pH 5.0, at 30.0 °C				
3,4-Dinitrophenol	410	3.71	0.71 ± 0.08	25
3,5-Dinitrophenol	340	1.16	0.83 ± 0.04	34
<i>p</i> -Nitrophenol	345	2.47	0.82 ± 0.08	11.3
1-Naphthol	321	1.522	0.22 ± 0.01	5.8
4-Phenylphenol	250	-4.34	0.09 ± 0.01	1.8
Phenol	269	0.88	4.6 ± 0.6	29.3
4-Methylumbelliferone	(Fluorescence assay)		0.48 ± 0.02	7.0
50 mM-Sodium phosphate buffer, pH 7.0, at 25.0 °C				
4-Bromoisquinoline	344	-6.07	4.1	6.4
Pyridine	265	-2.58	3.7*	0.6
4-Methylpyridine	265	-2.48	0.7*	0.028
3,5-Dimethylpyridine	282	-3.76	11.7*	0.0086

* K_i value.

that calculated from the acid-precipitation experiment. A possible cause is loss of label from a glycosylmethylated carboxylate group. Affinity labelling of the *ebg* β -galactosidases of *Escherichia coli* with 1- β -D-galactopyranosylmethyl-3-*p*-nitrophenyltriazene results in partial attachment of the glycosylmethyl residue to a glutamate residue by a labile ester linkage (Fowler & Smith, 1983).

These stoichiometries are less than the correct value of 1.0 that should be obtained for completely pure and completely active enzyme. There are two possible causes: loss of label from the glycosylmethylated protein even during the acid-precipitation step, or denaturation of the enzyme during prolonged storage of the frozen isoelectric-focusing fractions containing crude AFIII (the purified enzyme loses activity completely on freeze-drying). There are precedents with this type of affinity label for both causes of low stoichiometries. Affinity labelling of commercial *lacZ* β -galactosidase of *E. coli* (Sinnott & Smith, 1978) gives a stoichiometry of 0.64, whereas freshly isolated enzyme gives a stoichiometry of 0.91, and the *ebg* β -galactosidases, even when freshly isolated, give stoichiometries around 0.5.

Michaelis-Menten parameters for seven aryl α -L-arabinofuranosides are given in Table 1. It is seen that the range of k_{cat} values is comparatively small, and that there is little discernible trend for k_{cat} to increase with aglycone acidity ($\beta_{lg} = -0.16 \pm 0.11$). Small k_{cat} values are associated with hydrophobic aglycones (4-methylumbelliferone, 1-naphthol, 4-phenylphenol), and the fact that substrates with these aglycones also have low K_m values suggests that non-productive binding through the hydrophobic moiety may be taking place. The range of k_{cat}/K_m values is thus smaller than the range of k_{cat} values, but even over this smaller range there is little evidence of systematic variation with acidity ($\beta_{lg} = -0.11 \pm 0.07$).

In the absence of any information on which steps in the enzyme turnover sequence are reflected in the first-order and second-order rate constants for these substrates, no conclusions can be reached as to the chemical nature of any enzymic transition state from these data. We accordingly measured the ^{18}O kinetic isotope on the first-order rate constant for hydrolysis of *p*-nitro-

phenyl α -L-[1- ^{18}O]arabinofuranoside. The result, $^{18}(V) = 1.030 \pm 0.007$, indicates that cleavage of the exocyclic C-O bond is rate-limiting.

The magnitude of the effect will be affected by three factors: the degree to which the C-O bond is broken, the degree to which a proton is donated to the leaving O atom at the transition state, and the degree to which other processes are partially rate-limiting. The equilibrium ^{18}O isotope effect for conversion of *p*-nitrophenyl β -D-galactopyranoside into the galactopyranosyl cation and *p*-nitrophenolate ion has been calculated as 1.0425 (Rosenberg & Kirsch, 1981a); combined with the measured equilibrium isotope effect of 1.0181 on the acid dissociation constant of *p*-nitrophenol (Rosenberg & Kirsch, 1981a), this gives an estimated equilibrium isotope effect of 1.0244 for conversion of a nitrophenyl glycoside into the phenol and the glycosyl cation. The measured ^{18}O kinetic isotope effect for AFIII of 1.030 ± 0.007 is thus compatible with some process other than breaking of the exocyclic C-O bond being partly rate-determining only if the intrinsic effect on the bond-breaking step is close to the equilibrium effect of 1.0425 for bond-breaking without proton transfer. However, then the β_{lg} value would be close to -1.0, since the exocyclic bond would be broken but the exocyclic O atom would be unprotonated, as is shown by work on the β -glucosidase A of sweet almonds. Rosenberg & Kirsch (1981b) report a value of $^{18}(V/K)$ of 1.0377 ± 0.0061 for hydrolysis of *p*-nitrophenyl β -D-glucopyranoside, and Dale *et al.* (1986) report a β_{lg} (V/K) of -1.0 for the hydrolysis of aryl glucosides.

The observed isotope effect for AFIII-catalysed hydrolysis of *p*-nitrophenyl arabinoside is similar to that observed for the acid-catalysed hydrolysis of the same compound (1.023 ± 0.003 at 80 °C) (Bennet *et al.*, 1985), and, at this temperature, for the acid-catalysed hydrolysis of *p*-nitrophenyl β -D-glucopyranoside (Bennet *et al.*, 1987). However, at 50 °C, the latter effect increases to 1.0355 ± 0.0015 (Rosenberg & Kirsch, 1981b) as the mode of catalysis by H_3O^+ changes from specific acid to general acid (Bennet *et al.*, 1987; Rosenberg & Kirsch, 1981b).

The ^{18}O kinetic isotope effect for AFIII-catalysed hydrolysis of *p*-nitrophenyl arabinoside, then, establishes

that the rate-determining transition state C–O cleavage is almost complete. It is therefore possible to interpret the structure–reactivity parameters for aryl arabinoside hydrolysis in terms of a true β_{1g} of around -0.1 on the first bond-breaking step, on which ‘chemical noise’ arising from adventitious interaction of aryl substituents with the protein of the enzyme active site has been superimposed. This β_{1g} value indicates a degree of proton donation at the transition state comparable with that occurring during non-enzymic hydrolysis with H_3O^+ as the catalyst. The β -glucosidase A_3 of *Aspergillus wentii*, another extracellular fungal glycosidase, exhibits a similar pattern of behaviour (Legler *et al.*, 1981).

It is possible to correlate the picture of a transition state with the catalytic proton almost completely transferred to the leaving group with the acidity of the medium in which this extracellular enzyme normally acts. Work on the (non-enzymic) general-acid-catalysed hydrolysis of benzaldehyde aryl methyl and aryl ethyl acetals has shown that transition-state structure alters with the acid strength of the catalysing acid (Capon & Nimmo, 1975; Buffet & Lamaty, 1976). In the case of aryl ethyl (substituted methylsalicyl) acetals of benzaldehyde, the β_{1g} value changes from $+0.42$ for catalysis by H_3O^+ to -0.44 for catalysis by acetic acid (Buffet & Lamaty, 1976). These chemical precedents lead one to expect that the β_{1g} value obtained for the first chemical step of a glycosidase will vary according to the strength of the acid-catalytic group. The operation of natural selection will ensure that the enzyme uses as a general acid catalyst the strongest acid that will remain protonated in the medium in which the enzyme naturally acts. Therefore, the more acidic this natural medium, the more positive the β_{1g} value. The medium into which *M. fructigena* normally excretes AFIII (rotting fruit) is highly acidic.

A negative or near-zero β_{1g} value, like a direct ^{18}O kinetic isotope effect, is of course incompatible with a ring-opening mechanism with opening of the ring being rate-determining: such a process would be governed by a strongly positive β_{1g} value (Lönnerberg *et al.*, 1977). The leaving group of the natural substrate of the enzyme (another sugar residue) is less electronegative than the aryl glycosides used in the present study. It is therefore likely that, were the ring-opening mechanism to apply, the change of substrate from the natural one where, presumably, all steps would be of comparable kinetic importance (Albery & Knowles, 1976; Nambiar *et al.*, 1983) to an aryl glycoside would make ring-opening, rather than decomposition of some hemiacetal species, rate-limiting.

Table 1 also gives Michaelis–Menten parameters for the hydrolysis of four α -L-arabinofuranosylpyridinium salts. The difference in absorption coefficient between quaternary salt and protonated pyridine is too small for the reaction to be monitored conveniently, so the data pertain to a pH at which all four aglycones are completely deprotonated (7.0); moreover, to facilitate comparison with data on non-enzymic hydrolysis, the temperature of the measurements was $25^\circ C$.

It is clear, however, that the behaviour of the enzyme towards these substrates is similar to that of ‘retaining’ pyranosidases towards the corresponding pyranosylpyridinium ions. Thus, from the limited data available, it is possible to calculate a β_{1g} (V) value of -0.95 ± 0.23 for AFIII, similar to those observed with *lacZ*

β -galactosidase (-0.93 ± 0.03 ; Sinnott & Withers, 1974), *ebg* β -galactosidase (-0.8 ± 0.1 ; Burton & Sinnott, 1983), β -glucosidase A_3 (-0.96 ± 0.19 ; Legler *et al.*, 1981) and yeast α -glucosidase (-0.61 ± 0.06 ; Hosie & Sinnott, 1985). Likewise, the k_{cat} value for the bromoisouquinolinium ion is some 2.5×10^9 times greater than its rate of neutral, spontaneous hydrolysis (Sinnott & Wijesundera, 1985). This is an absolute rate enhancement similar to that brought about by pyranosidases (Legler *et al.*, 1981; Burton & Sinnott, 1983; Jones *et al.*, 1977).

The hydrolysis of α -L-arabinofuranosylpyridinium ions with high efficiency by AFIII enables the ring-opening mechanism to be unquestionably ruled out for this enzyme (Scheme 1).

CONCLUSIONS

A retaining furanosidase has been shown to resemble a number of retaining pyranosidases in three particulars: (1) suicide inactivation of the enzyme by the corresponding 1-glycosylmethyl-3-*p*-nitrophenyltriazene, with concomitant covalent attachment of the glycosylmethyl residue to the protein; (2) hydrolysis of aryl glycosides with a near-zero β_{1g} value, shown to apply to the chemical step by an ^{18}O kinetic isotope effect measurement; (3) hydrolysis of glycosylpyridinium ions with high efficiency.

The ring-opening mechanism can be dismissed for this enzyme, even though there is chemical precedent for it in the hydrolysis of alkyl arabinofuranosides. Proposals that the catalytic mechanism of pyranosidases can involve ring-opening, made in the absence of any chemical precedent for such a process (Fleet, 1985; Post & Karplus, 1986), therefore need not be considered further.

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