Lignocellulose degradation by *Phanerochaete chrysosporium*: purification and characterization of the main α -galactosidase

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The main α -galactosidase was purified to homogeneity, in 30% yield, from a solid culture of *Phanerochaete chrysosporium* on 1 part wheat bran/2 parts thermomechanical softwood pulp. It is a glycosylated tetramer of 50 kDa peptide chains, which gives the N-terminal sequence ADNGLAIPQMG(?W)NT(?W)NHFG(?W)DIS(?W)DTI. It is remarkably stable, with crude extracts losing no activity over 3 h at 80 °C, and the purified enzyme retaining its activity over several months at 4 °C. The kinetics of hydrolysis at 25 °C of various substrates by this retaining enzyme were measured, absolute parameters being obtained by active-site titration with 2',4',6'-trinitrophenyl 2-deoxy-2,2-difluoro- α -D-galactopyranoside. The variation of k_{cat}/K_m for 1-naphthyl- α -D-galactopyranoside with pH is bell-shaped, with $\text{p}K_1 = 1.9_1$ and $\text{p}K_2 = 5.5_4$. The $^{2D}(V/K)$ value for *p*-nitrophenyl- α -D-glucopyranoside is 1.031 ± 0.007 at the optimal pH of 3.75 and 1.114 ± 0.006 at pH 7.00, indicating masking of the intrinsic effect at optimal pH. There is no α -³H effect on binding galactose

[$^{2D}(K_i) = 0.994 \pm 0.013$]. The enzyme hydrolyses *p*-nitrophenyl β -L-arabinopyranoside ~ 510 times slower than the galactoside, but has no detectable activity on the α -D-glucopyranoside or α -D-mannopyranoside. Hydrolysis of α -galactosides with poor leaving groups is Michaelian, but that of substrates with good leaving groups exhibits pronounced apparent substrate inhibition, with K_{is} values similar to K_m values. We attribute this to the binding of the second substrate molecule to a β -galactopyranosyl-enzyme intermediate, forming an $E^*\beta\text{Gal}'\alpha\text{GalX}$ complex which turns over slowly, if at all. 1-Fluoro- α -D-galactopyranosyl fluoride, unlike α -D-galactopyranosyl fluoride, is a Michaelian substrate, indicating that the effect of 1-fluorine substitution is greater on the first than on the second step of the enzyme reaction.

Key words: family 27 glycohydrolase, mechanism, substrate inhibition.

INTRODUCTION

Phanerochaete chrysosporium is the most thoroughly studied organism capable of degrading all the components of lignocellulose, lignin, cellulose and hemicellulose. The degradation of lignin by this organism is well documented [1–3], and its cellulolytic system is receiving increasing attention [3–6]. However, although the organism's capacity to breakdown hemicellulose has been amply demonstrated, of the individual enzymes that must be responsible, only a 4-*O*-methyl α -glucuronidase [7] and β -xylosidase/glucosidase [8] have been isolated. Both these enzymes act on xylan-based hemicellulose. The main softwood hemicelluloses, though, are based on $\beta(1 \rightarrow 4)$ glucomannans decorated with $\alpha(1 \rightarrow 6)$ -linked galactopyranosyl residues. As a contribution to an eventual full understanding of how *P. chrysosporium* acts on its substrate, we now report the isolation and characterization of the main α -galactosidase.

A further motivation for investigating this enzyme is its potential application in pulp bleaching. Since the original suggestion that xylanases might act as bleach-boosters [9], they have been in use for nearly a decade in the pulp and paper industry [10]. A puzzle, though, is that xylanases can be used successfully with softwood pulp, yet xylan is a comparatively minor component of softwood hemicellulose. The main softwood hemicelluloses are (galacto)glucomannans, with covalent linkages to lignin through the galactose units [11]. The mannanase from *Trichoderma reesei* has been shown to aid pulp bleaching [12],

but not the enzyme from *Pseudomonas fluorescens* subsp. *cellulosa* [13]. Studies on the α -galactosidase of *T. reesei* [14] have recently been reported [15,16]. Three α -galactosidase genes from *T. reesei* have also been cloned by expression in *Saccharomyces cerevisiae* [17]. However, the *T. reesei* enzyme has limited stability, thus reducing its suitability for practical application.

This paper therefore addresses the basic protein chemistry and mechanistic characterization of the species responsible for cleaving α -galactosyl residues when *P. chrysosporium* grows on its natural substrate.

MATERIALS AND METHODS

Materials

General laboratory and inorganic chemicals were obtained from BDH (Poole, Dorset, U.K.) and were of reagent grade or higher. Organic chemicals were obtained from Sigma/Aldrich (Gillingham, Dorset, U.K.), as were the following substrates: *p*-nitrophenyl glycosides of α -D-glucopyranose, α -D-mannopyranose, α -D-xylopyranose and β -L-arabinopyranose, and *p*-nitrophenyl, 6-bromo-2-naphthyl, 1-naphthyl and *o*-nitrophenyl- α -D-galactopyranosides. Yeast extract was from Difco Laboratories (Detroit, MI, U.S.A.). Thermomechanical softwood pulp was from Rygene-Smith & Thommesen A/S (Ryken, Norway), production date 22 August–26 August 1994.

Abbreviations used: 1-Nap- α -D-Galp, 1-naphthyl- α -D-galactopyranoside; PNP, *p*-nitrophenyl.

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Synthetic substrates, inhibitors and inactivators

1-Fluoro-galactopyranosyl fluoride (**I**) (see Figure 1) was prepared as described by K et al. [18], and α -galactopyranosyl fluoride as described by Thiem and Wiesner [19]. A sample of galactotetrazole (**II**) [20] was a gift from Professor Dr. Andrea Vasella (ETH, Zürich, Switzerland) and was used as provided. *p*-Nitrophenyl 1-[^2H]- α -D-galactopyranoside was synthesised from 1-[^2H]-D-galactose (98% ^2H , from Cambridge Isotope Laboratories, Andover, MA, U.S.A.) by acetylation, fusion of the acetate with *p*-nitrophenol and anhydrous zinc chloride, as described by Montgomery et al. [21], and Zemplén deacetylation. The deuterated substrate was obtained after recrystallization from methanol/ether as a material identical {melting point (m.p.) 172–174 °C, cf. non-isotopic compound: lit. 173 °C [22]} with the unlabelled compound, but for the absence of a 1 H doublet at δ 5.74 ($J = 3.52$ Hz) in the ^1H NMR spectrum in $^2\text{H}_2\text{O}$.

2',4',6'-Trinitrophenyl 2-deoxy-2,2-difluoro-3,4,6-tri-*O*-acetyl- α -D-galactopyranoside was prepared by picrylation [23] of 2-deoxy-2,2-difluoro-3,4,6-tri-*O*-acetyl- α -D-galactopyranose [24] by Dr. Calvin J. Chany II, University of Illinois at Chicago. It was deacetylated at 4 °C in anhydrous methanol saturated with HCl. After 2 h the solution was concentrated *in vacuo* at less than 30 °C (water pump) and yielded a yellow solid which was purified by column chromatography on silica gel (eluent: diethyl ether) to yield 2',4',6'-trinitrophenyl 2-deoxy-2,2-difluoro- α -D-galactopyranoside (**III**) as a yellow powder, m.p. 120–123 °C (dec.). ^1H NMR ($^2\text{H}_2\text{O}$): 3.59 (2 H, d, $J = 6.2$, H-6), 3.89 (1 H, dd, $J_1 = 6.2$, $J_2 = 6.2$, H-5), 4.10 (1 H, m, H-4), 4.23 (1 H, ddd, $J_{\text{axial F},3} = 22.8$, $J_{\text{equatorial F},3} = 7.3$, $J_{3,4} = 3.8$, H-3), 5.84 (1 H, apparent d, $J_{1,\text{F}} = 0$, $J_{1,\text{F}} = 6.7$, H-1), 9.04 (2 H, s, aromatic H).

α -D-Galactopyranosylmethyl *p*-nitrophenyl triazene (**IV**) was synthesized as previously described [25], except that the diborane reduction of C-2,3,4,6-tetra-*O*-acetyl α -D-galactopyranosyl formamide was quenched with methanol saturated with HCl gas rather than aqueous HCl. The crystalline precipitate of α -D-galactopyranosylmethylamine hydrochloride {m.p. 214–217 °C (dec.), [lit. 213–217 °C (dec.)] [25]} gave a ^{13}C NMR spectrum in accord with that reported previously [25]. The amine was converted into the triazene by reaction with *p*-nitrobenzene diazonium tetrafluoroborate in buffered aqueous solution [26].

Buffer solutions

Buffer solutions were prepared at room temperature by addition of concentrated sodium hydroxide solution to the acid form of the buffer until the desired pH was reached, as indicated by a Mettler 350 pH/ion meter fitted with a Mettler Inlab 413 combination pH electrode; the electrode was calibrated with pH 4.00 and 7.00 standards. Citrate buffers were prepared from citric acid. Phosphate buffer, pH 7.00, was prepared from sodium dihydrogen orthophosphate, and low-pH phosphate buffer from H_3PO_4 .

Maintenance and harvest of *P. chrysosporium* spores

P. chrysosporium ME-446 was grown on malt extract agar in a 125 ml Erlenmeyer flask at 37 °C for 5 days to allow sporulation. Flasks were then sealed and stored at 4 °C until needed. Spores were harvested by addition of 10–20 ml of a sterile 0.05% Tween-80 solution, followed by suspension of the spores by stirring with a magnetic bar. Spore concentration was calculated from the absorbance of a suitably diluted aliquot of spore suspension, assuming $D_{650} = 1.0$ (path length 1.0 cm) $\equiv 5.0 \times 10^6$ spores/ml.

Figure 1 Structures of substrates and other ligands

Routine α -glycosidase assays

A stopped enzyme assay was used wherein the enzyme preparation was incubated with 4.0 mM *p*-nitrophenyl- α -D-glycoside in 50 mM sodium citrate, pH 5.0, (total volume 500 μl) at either 37 or 50 °C (water-bath temperature). After 5 min, the reaction was stopped by addition of an equal volume of 0.2 M sodium carbonate and absorbance (*p*-nitrophenolate $\lambda_{\text{max}} = 410$ nm, $\epsilon = 18500 \text{ M}^{-1} \cdot \text{cm}^{-1}$) was determined on a Cecil single-beam UV/visible spectrophotometer.

Protein assay

Total protein was determined by the method of Bradford [27,28] from a standard curve for BSA.

SDS/PAGE

SDS/PAGE was performed using the Bio-Rad Mini Protean II apparatus, employing the discontinuous buffer system of Laemmli [28–30]. Proteins were detected by staining either with Coomassie Brilliant Blue R-250 [28] or silver stain, using a protocol based on that of Morrissey [31].

Molecular masses of protein bands were determined from a direct fit to a logarithmic function of R_f values of molecular-mass standards (Pharmacia Low Molecular Weight Markers, Pharmacia, Uppsala, Sweden) run on the same gel.

Enzymic deglycosylation of purified α -galactosidase

Enzymic deglycosylation of purified α -galactosidase with *N*-glycosidase F [peptide- N^4 -(*N*-acetyl- β -glucosaminyl)-asparagine amidase, EC 3.5.1.52] was performed using the Boehringer-Mannheim *N*-glycosidase F deglycosylation kit (Boehringer-Mannheim UK, Lewes, East Sussex, U.K.) following the protocol outlined in the accompanying documentation. Samples were then examined directly by SDS/PAGE.

Protein sequence analysis

The protein sequence was kindly determined by Dr. R. Burke (UMIST Department of Biomolecular Sciences), by means of automated Edman degradation chemistry on a Perkin-Elmer 476A protein sequencer. Liquid samples of native protein were applied to glass fibre membranes pre-treated with Biobrene Plus (Perkin-Elmer, Foster City, CA, U.S.A.) and subjected to repetitive pulsed-liquid sequencing. Degradation products (phenylthiohydantoin-amino acid derivatives) were separated by reverse-phase HPLC and identified by comparison of retention time with phenylthiohydantoin-amino acid standards (Perkin-Elmer).

Purification

Culture harvest

Ten 2.0 litre Erlenmeyer flasks were charged with thermo-mechanical softwood pulp (32 g), wheat bran (16 g), distilled H₂O (100 ml), ammonium sulphate (1.46 g), potassium dihydrogen orthophosphate (0.73 g) and yeast extract (0.73 g). The flasks were autoclaved (121 °C, 15 min), inoculated with 1.0×10^8 spores/flask and incubated at 37 °C for 96 h. The contents of each flask were homogenized with 280 ml of 0.2 M sodium acetate, pH 5.0, containing 0.05 % Tween-80 and 100 µg/ml PMSF in a Waring blender at 4 °C (cold room). The homogenized material was centrifuged at $1 \times 10^4 g$ for 10 min and the supernatant was decanted and vacuum filtered through a Whatman GF/A glass fibre filter.

Ammonium sulphate precipitation

EDTA was added to the crude extract to a final concentration of 1.5 mM. Ammonium sulphate was then added in portions with stirring at 4 °C to 50 % saturation (at 4 °C). Stirring was continued for 1.5 h and then the precipitated protein was centrifuged down ($1 \times 10^4 g$ for 10 min). The supernatant was decanted off and additional portions of ammonium sulphate were added to the supernatant to bring the concentration to 80 % saturation. Stirring was continued for an additional 1.5 h and precipitated protein was pelleted by centrifugation ($1 \times 10^4 g$ for 10 min). The supernatant was decanted and the pellet was re-dissolved in 50 mM sodium phosphate, pH 7.0, containing 1.5 M ammonium sulphate (150 ml) followed by removal of undissolved material by centrifugation ($1 \times 10^4 g$ for 10 min).

Hydrophobic-interaction chromatography

Resolubilized active fractions from ammonium sulphate precipitation were applied to a Phenyl-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) column (2.5 cm $\times$ 20 cm) equilibrated with 50 mM sodium phosphate, pH 7.0, containing 1.5 M ammonium sulphate at room temperature. Elution was achieved by a 1.0 litre linear gradient of ammonium sulphate from 1.5 to 0 M in 50 mM sodium phosphate buffer, pH 7.0. Fractions containing α -galactosidase activity were pooled, stored at 4 °C and assayed for total protein and α -galactosidase activity.

The phosphate buffer system used for this step can be replaced with 50 mM sodium acetate, pH 5.0: this has no effect on the purification at this stage but avoids exposure of the enzyme to alkaline-to-neutral pH values, at which it is less stable. All the protein isolated for steady-state kinetics studies was produced using the phosphate buffer system, whereas subsequent purifications utilized the acetate buffer system.

Purification by ultrafiltration and dialysis to low pH

After 15-fold concentration on an Amicon PM-10 ultrafiltration membrane (Millipore, Watford, Hertfordshire, U.K.), pooled fractions from hydrophobic-interaction chromatography were dialysed at 4 °C against 20 mM sodium citrate, pH 3.0, and centrifuged at $1 \times 10^3 g$ for 10 min to remove precipitated protein. The supernatant was stored at 4 °C and assayed for total protein and α -galactosidase activity.

Cation-exchange chromatography

The enzyme solution was applied to a Mono-S cation-exchange column (Pharmacia, Uppsala, Sweden) equilibrated with 20 mM sodium citrate, pH 3.0, and eluted with a 50 ml linear gradient of 0–0.5 M sodium chloride. Fractions containing α -galactosidase activity were pooled, stored at 4 °C and assayed for α -galactosidase activity and total protein.

Gel-filtration chromatography

The pooled fraction from cation-exchange chromatography was concentrated before loading on a Pharmacia HR 10/30 Superose 12 column. Initial concentration (approx. 10-fold) utilized an Amicon PM-10 ultrafiltration membrane, followed by further concentration with an Amicon Microcon YM-10 apparatus, yielding 65-fold concentration overall. The gel-filtration column was run in 20 mM sodium acetate buffer, pH 5.0, containing 50 mM sodium chloride; 80 $\times$ 0.25 ml fractions were collected at a flow rate of 0.1 ml/min. In subsequent purifications, gel-filtration chromatography was performed on a Pharmacia HR 10/30 Superose 6 column, which gave better resolution of the α -galactosidase from contaminating proteins. All of the α -galactosidase used for kinetic studies resulted from purification on Superose 12, whereas data used in α -galactosidase native molecular mass calculations were obtained from Superose 6 chromatograms. Blue dextran, sweet potato β -amylase, yeast alcohol dehydrogenase, BSA, bovine erythrocyte carbonic anhydrase, horse heart cytochrome *c* and bovine lung apoprotein (all from Sigma) were used as native molecular-mass standards.

Anion-exchange chromatography

Pooled fractions from gel-filtration chromatography were loaded onto a Pharmacia Mono-Q HR 5/5 anion-exchange column equilibrated with 20 mM sodium acetate, pH 5.0. α -Galactosidase activity was eluted by a 23 ml linear gradient of sodium chloride from 0 to 0.5 M. Again, fractions containing α -galactosidase activity were checked for purity by SDS/PAGE, using silver-stain visualization, before pooling. Pooled fractions were assayed for total protein and α -galactosidase activity and were determined to be greater than 95 % pure by SDS/PAGE (visualization with Coomassie Brilliant Blue R-250).

Kinetic methods

Unless stated otherwise, all kinetic data were obtained at 25 °C. A Cary 1 UV/visible spectrophotometer controlled by an IBM compatible personal computer running Cary operating software, or a Perkin-Elmer Lambda 18 UV/visible controlled by an IBM compatible personal computer running Perkin-Elmer UV WinLab[™] for Windows[™] operating software, were used to monitor reactions involving chromophoric changes. The reaction temperature was maintained to ± 0.1 °C via water circulation through the cell block on the Cary spectrophotometer and via a Peltier-effect thermostated cell block on the Perkin-Elmer machine. Absorbance was followed continuously and initial rates

were determined by linear least-squares treatment of the time course for the first 10 % of the reaction. First-order rate constants were determined from time courses lasting at least 3 half-lives, with > 100 data points per run.

The hydrolyses of *o*- and *p*-nitrophenyl galactosides were followed at the isosbestic point of the ionization of the aglycone: 373.6 nm for *o*-nitrophenol and 347.3 nm for *p*-nitrophenol [32]. Molar absorption coefficients were determined in triplicate by hydrolysing the substrate to completion with enzyme: ϵ values ($M^{-1}\cdot cm^{-1}$) for the substrates were 479 ± 3 and 2865 ± 10 for the *o*- and *p*-nitrophenyl glycosides and 2266 ± 6 and 5399 ± 13 for the *o*- and *p*-nitrophenols respectively. The hydrolyses of 1-naphthyl and 6-bromo-2-naphthyl galactosides were followed at the wavelengths of greatest change in molar absorption coefficient, 322 and 326.6 nm respectively ($\Delta\epsilon$ 1770 and $1197 M^{-1}\cdot cm^{-1}$ respectively).

The kinetics of fluoride ion liberation were followed essentially as previously described [33].

α -Deuterium kinetic isotope effect measurements on the hydrolysis of *p*-nitrophenyl galactoside

The effect on k_{cat}/K_m was measured under first-order conditions ($[S] \ll K_m$) by directly fitting an exponential curve to experimental data. Eight separate measurements were made for each isotopomer in the order heavy-light-light-heavy, etc. The isotope effect was calculated by comparing the sequential light-heavy values pairwise and calculating the mean of the eight individual kinetic isotope effect values. The effect on k_{cat} was determined similarly, except that zero-order rates (over < 1 % reaction) at a substrate concentration of 3.80 mM replaced first-order rate constants.

pH values for pH rate profile data were measured immediately after kinetic runs with a Mettler 350 pH/ion meter and combination pH electrode with an integrated temperature probe calibrated with pH 4.0 and 7.0 standards.

Proton NMR studies of enzymic hydrolysis of α -D-galactopyranosides

1H NMR spectra were collected at ambient probe temperature (292 ± 1 K) with a Bruker AM spectrometer operating at 400.1 MHz, in the Chemistry Department of the University of Illinois, Chicago, IL, U.S.A. All reactions were performed in 2H_2O buffered with 50 mM sodium formate, p 2H 3.75, using high-quality 5 mm NMR tubes containing 600 μl total volume. Buffer exchange of enzyme was performed by concentration on Amicon Centricon concentrators (washed with 2H_2O to remove glycerol) followed by dilution to the original volume with 50 mM sodium formate, p 2H 3.75, three times. 1-Naphthyl- α -D-galactopyranoside (1-Nap- α -D-Galp; 2.7 mM) was hydrolysed with 0.022 $\mu g/\mu l$ enzyme. α -D-Galactopyranosyl fluoride (3.7 mM) was hydrolysed with 0.065 $\mu g/\mu l$ enzyme.

Attempted detection of transglycosylation products by HPLC

HPLC analyses were carried out using a Gilson HPLC system consisting of two model 306 pumps, a model 811C mixer, a model 805 manometric module and a model 118 UV/visible detector controlled by Gilson 712 HPLC Controller Software, v1.20. Samples were loaded onto a Spherisorb ODS1 reversed-phase C_{18} column (250 mm $\times$ 4.6 mm) and eluted with a gradient of solvent B from 20 to 55 % at a flow rate of 1 ml/min (solvent A: water, solvent B: acetonitrile. Both solvents were HPLC grade).

Sufficient enzyme was added to hydrolyse a 10 mM solution of *p*-nitrophenyl galactopyranoside in 50 mM sodium formate buffer, pH 3.75, within either approx. 120 or 240 min at room temperature. Aliquots were injected before addition of the enzyme, at 5 min after enzyme addition, and every 15 min for the first 1 h thereafter. Injections were made less frequently toward the end of the reaction. Elution standards included the *p*-nitrophenyl glycosides of α -galactopyranose, β -glucopyranose and β -cellobiose. The glucoside and galactoside eluted together, but there was baseline separation of the glucoside and the cellobioside.

Enzyme inactivation experiments

Triazene (IV) was tested as an inactivator by incubating enzyme with the triazene in 35 mM sodium phosphate, pH 7.0, or 20 mM sodium phosphate, pH 6.5, at 25 °C. Aliquots were removed at suitable intervals and assayed for remaining α -galactosidase activity against 1-Nap- α -D-Galp in 50 mM sodium formate, pH 3.75. In experiments to determine if the decomposition of the triazene was enzyme catalysed, the decrease in the absorbance of a solution of enzyme and triazene in 50 mM sodium phosphate, pH 7.0, was followed at 362 nm.

Initial experiments on inactivation of the enzyme by the picrate (III) were performed by incubating enzyme with the picrate (concentrations ranging from stoichiometric to 4 mM) in 50 mM sodium formate, pH 3.75, at 25 °C. Aliquots were removed at suitable time intervals and assayed for remaining α -galactosidase activity against 1-Nap- α -D-Galp in 50 mM sodium formate, pH 3.75. The enzyme-inactivation solution was diluted 1:300 upon addition to the assay mixture. The ability of 1-naphthyl- α -galactoside to reactivate inactivated enzyme by acting as a transglycosylation acceptor was measured by incubating the enzyme with 2.0 or 4.0 mM substrate and 57.1 μM picrate (III) and monitoring the absorbance change at 322.0 nm. Similar measurements were made with 1.8 or 3.6 mM *p*-nitrophenyl β -L-arabinopyranoside and 6.7 μM picrate (III) and monitoring the absorbance change at 347.3 nm. The ability of methyl- β -D-galactopyranoside to affect reactivation of inactivated enzyme was measured by incubating the enzyme with various concentrations of the methyl galactoside (from 29.5 μM to 84.5 mM) and 67 μM picrate (III) and monitoring picrate evolution at 400 nm. 'Burst' kinetics for α -galactosidase inactivation with picrate (III) were measured by incubating the enzyme with 6.9 or 10.8 mM D-galactose and 61.5 μM picrate (III) and monitoring the evolution of picrate at 400 nm ($\epsilon = 1.155 \times 10^4 M^{-1}\cdot cm^{-1}$) [34].

Mathematical treatment of data

All kinetic parameters were determined by direct fit to experimental data using Fig. P for Windows (Biosoft, Cambridge, U.K.) run on various IBM-compatible personal computers. Derivation and manipulation of rate equations was performed using *Mathematica* [35] v3.0 for Windows 95; eqns. (5)–(9) in particular were so derived.

RESULTS AND DISCUSSION

Protein isolation

The enzyme was purified to electrophoretic homogeneity by ammonium sulphate precipitation, hydrophobic-interaction chromatography, ultrafiltration and dialysis to low pH, cation-exchange chromatography, gel-filtration chromatography and

anion-exchange chromatography. Table 1 is the purification Table from a typical isolation. Aspects of the isolation deserving comment are the following.

(i) The enzyme isolated is not the only enzyme with α -galactosidase activity: peaks containing minor amounts (approx. 15 %) of such activity were obtained from both hydrophobic and cation-exchange chromatographies. However, the ratios of glycosidase activities obtained from the two peaks observed in hydrophobic chromatography (Table 2) suggest, but do not prove, that the minor α -galactosidase activities might be a side activity of (an) other enzyme(s).

(ii) Whereas SDS/PAGE analysis of protein obtained in the first purification indicated electrophoretic homogeneity, in the second and some later sequences of culture and purification, two closely spaced bands, not visible in gels of the first purification, were observed (Figure 2). This electrophoretic inhomogeneity was demonstrated to arise from differential glycosylation of a common peptide by treatment of material from both the first and second purifications with *N*-glycosidase F. In both cases the treated enzyme gave rise to a single band with an apparent molecular mass of 50 kDa (Figure 2), as compared with a molecular mass of 60 kDa for the untreated enzymes. The enzyme is thus heavily, and to some extent differentially, *N*-glycosylated. However, all kinetic measurements were made with enzyme from the first isolation with a common glycosylation pattern.

(iii) The stability of the enzyme (no loss in activity of crude or partially purified extracts over several months at 4 °C at pH 4 or 5; no loss of activity of crude extracts at 80 °C for several hours) was exploited in an acid precipitation and ultrafiltration step (Table 1). However, heat treatment of crude extracts, in the manner used for the isolation of the *Pycnoporus cinnabarinus* α -galactosidase [36] was considered inadvisable, since the enzyme so treated, while still active, failed to bind to the cation-exchange column (results not shown), suggesting possible deamidation reactions.

Table 1 Purification of the main α -galactosidase from *P. chrysosporium*

*Indicates activity was divided into more than one fraction, the reported values being those for the fraction with highest activity.

Purification step	Specific activity ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)	Fold purification	Yield of α -galactosidase activity (%)
Crude extract	0.808	1.00	100
(NH_4) ₂ SO ₄ precipitation	1.62	2.00	64.6*
Hydrophobic-interaction chromatography	4.10	5.07	54.3*
Ultrafiltration + dialysis to low pH	9.65	11.9	49.6
Cation-exchange chromatography	49.0	60.6	43.4*
Gel-filtration chromatography	130	161	29.7
Anion-exchange chromatography	137	170	23.1

Table 2 Enzyme activity against 4-nitrophenyl glycosides (nmol/min in 50 μl at 37 °C, in 50 mM sodium citrate, pH 5.0)

	PNP- α -D-Galp	PNP- α -D-Glcp	PNP- α -D-Manp	PNP- β -L-Arap
Peak 1 (255 ml)	27.3	1.84	0	2.43
Peak 2 (225 ml)	4.95	3.50	0	0

Figure 2 SDS/PAGE gel of purified α -galactosidase treated with *N*-glycosidase F

Lane, left to right: 1, molecular-mass markers (97.4, 66.2, 39.2, 26.6, 21.5, 14.4 kDa, top to bottom); 2, second purification, *N*-glycosidase F treated; 3, second purification; 4, first purification, *N*-glycosidase F treated; 5, first purification; 6, molecular-mass markers. The band visible at 35.5 kDa in lanes 2 and 4 is *N*-glycosidase-F.

Protein characterization

The enzyme consists of subunits of 60 kDa molecular mass, at least 10 kDa of which is carbohydrate. Gel filtration on Superose 6 with molecular-mass standards indicates a molecular mass of 250 kDa, indicating that the enzyme is a tetramer of identical subunits. The N-terminus, unusually for a *P. chrysosporium* extracellular hydrolase, is unblocked, permitting the N-terminal sequence to be determined: two sequencing runs gave ADNGLAITPQMG?NT?NHFG and ADNGLAITPQMG?NT?NHFG?DIS?DTULQ.

A BLASTP [37] search of the Non-redundant Peptide Sequence Database at the National Center for Biotechnology Information via the World Wide Web Interface (<http://www.ncbi.nlm.nih.gov/BLAST/>) using the confirmed 20 N-terminal amino acid sequence (ADNGLAITPQMG?NT?NHFG) returned several matches to known α -galactosidases or α -galactosidase precursors. From sequence similarity, it became apparent that the undetermined residues in the 20 amino acid sequence correlated with two tryptophan residues which were conserved in all 14 sequences matched with $P(N) < 1$, P is the 'expect' probability and is the number of equally good alignments that would be expected in searching a random sequence database with the query. Tryptophan identification with the Perkin-Elmer Applied Biosystems system is difficult, as diphenylurea, a by-product of Edman sequencing chemistry, and the tryptophan-phenylthiohydantoin derivative co-elute under the chromatography conditions used. It is highly likely, therefore, that these undetermined residues are in fact tryptophan residues. A second BLASTP search on the sequence, in which residues 13 and 16 were substituted with tryptophans, produced 26 matches, the very strong matches all being to family 27 α -galactosidases, and no strong matches to any other glycosyl hydrolases. Therefore the *P. chrysosporium* enzyme in all probability belongs to family 27 [38,39]; gene isolation is currently in progress.

Stereochemistry

Figure 3 displays the anomeric region of the ^1H NMR spectrum of 1-Nap- α -D-Galp during the course of its hydrolysis by *P. chrysosporium* α -galactosidase. The initial build-up of α -galacto-

Figure 3 Stereochemistry of the hydrolysis of 1-naphthyl α -galactoside determined by ^1H NMR

See Materials and methods section for reaction conditions. The anomeric resonances of 1-naphthyl galactoside, α -D-galactopyranose and β -D-galactopyranose are centred (relative to HO^2H at δ 4.70 ppm) on δ 5.82 ppm ($^1J_{\text{H1-H2}}$ 3.52 Hz), δ 5.12 ppm ($^1J_{\text{H1-H2}}$ 3.52 Hz) and δ 4.44 ppm ($^1J_{\text{H1-H2}}$ 8.21 Hz) respectively.

pyranose, and its slow mutarotation to β -galactopyranose, indicating a retentive, double-displacement mechanism, is clearly apparent (the signals corresponding to the α - and β -anomers are identifiable both from the chemical shift and the vicinal coupling constant, which is higher for the *trans*-diaxial disposition of the protons of the β -anomer). A similar experiment was performed with α -galactopyranosyl fluoride, in the course of which no self-condensation products (see below) could be detected.

Active-site titration and absolute k_{cat} values: inactivation by picrate (III)

The concentration of enzyme active sites was estimated using a reagent based on a principle established by Withers et al. [40]. That principle is based upon the generation of fluoroglycosyl-enzyme intermediates which turn over slowly, if at all, with concomitant generation of one equivalent of aglycone. Electron-

Figure 4 Liberation of picrate from inactivator (III) in the presence of enzyme

For details see Materials and methods section. The burst corresponds to 2,2-difluoro-galactosylation, the steady-state rate to spontaneous hydrolysis of the reagent

withdrawing substituents on the glycone destabilize the oxocarbenium-ion-like transition states leading to and from the glycosyl-enzyme intermediate, but a good leaving group makes the intermediate kinetically accessible. In the case of retaining α -glycosidases, F-for-H substitution at position 5, or F-for-H and F-for-OH substitution at position 2 is required, rather than the single F-for-OH substitution at position 2 that suffices for β -glycosidases [34].

Enzyme inactivation was so rapid with picrate (III), even as inactivator concentration approached that of the enzyme, that determination of the kinetics of loss of catalytic activity was not possible. However, when the evolution of picrate was monitored spectrophotometrically in the presence of 10.8 mM ($30 \times K_i$) D-galactose, a competitive inhibitor, a burst followed by a slow apparent steady-state rate was observed when the enzyme was incubated with 61.5 μ M [picrate (III)] (Figure 4 displays a typical trace). The 'steady-state' release of picrate represents the spontaneous hydrolysis of the reagent (probably by aryl-oxygen fission), rather than slow turnover of the 2-deoxy-2,2-difluoro-galactosyl enzyme intermediate, since the same rate is observed in the absence of enzyme.

In order to determine burst size, absorbance was fitted to eqn. (1).

$$\text{Absorbance} = a + b(1 - e^{-kt}) + ct \quad (1)$$

where a is the absorbance at $t = 0$, b is the burst size, and c is the 'steady-state' turnover rate. Three such determinations indicated an enzyme stock solution concentration of $7.54 \pm 0.53 \mu\text{M}$ (S.E.M.); this is in line with preliminary, less accurate, experiments at lower enzyme concentrations (the value obtained by Bradford protein assay, using a value of 60 kDa for the subunit molecular mass, was 5.88 μM). This active-site titration was standardized on the same day with data for hydrolysis of 1-naphthyl galactoside obtained under first-order conditions ($[S] \ll K_m$); the k_{cat}/K_m for this substrate is $5.47 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$. Enzyme activity was routinely checked against this substrate under first-order conditions and so all kinetic data are absolute.

Investigations into the site of attachment of the label are in progress.

Figure 5 pH- k_{cat}/K_m profile for the enzyme catalysed hydrolysis of 1-naphthyl galactoside

Results were obtained in 250 mM buffers (cation sodium) at 25 °C as follows: ■, ◆, phosphate; ▲, formate; ●, citrate (open circles were omitted from curve fit, see text).

pH-dependence of enzyme activity

Whereas substrates with poor leaving groups (naphthol, 6-bromonaphthol) exhibited well-behaved Michaelian behaviour, *o*- and *p*-nitrophenyl galactosides exhibited pronounced substrate inhibition (see below). Therefore the pH behaviour was investigated using 1-naphthyl galactoside. We confined the investigations to k_{cat}/K_m , since this is the steady-state parameter most likely to be interpretable in terms of ionizations of catalytic groups in the free enzyme. Moreover, as we were dealing with a very stable enzyme, k_{cat}/K_m was determined under first-order conditions with $[S]_0 \ll K_m$ (and also $\ll K_i$ for the product galactose), where eqn. (2) applies

$$[S] = [S]_0 \cdot \exp \left[\left(-\frac{[E]_t k_{\text{cat}}}{k_m} \right) \cdot t \right] \quad (2)$$

First-order rate constants were obtained by direct fit of absorbance to an exponential for at least three half-lives; the derived total change in absorbance corresponding to complete reaction was constant within 5%, confirming that enzyme denaturation during the course of the experiment did not contribute to the derived first-order constant. The pH-rate profile is given in Figure 5.

Over the pH range 2.4–4.5 (see Figure 5) there is a discrepancy between the rates in citrate buffer (open circles), and those in phosphate (solid squares) or formate buffers (solid triangles). That this was a consequence of inhibition by neutral citric acid was established by showing that doubling the buffer concentration (to 100 mM) had $< 10\%$ effect on k_{cat}/K_m measured in citrate or acetate buffers at pH 4.75, phosphate at pH 2.45 or formate at pH 3.75, whereas the value in citrate at pH 2.30 fell 30%. Since the $\text{p}K_a$ values of citrate are 3.1, 4.75 and 5.4, the absence of inhibition in citrate buffer, pH 4.75, means that neither the mono-anion nor the di-anion is inhibitory. Therefore the inhibition by citrate at pH 2.30 is due to neutral citric acid.

The similarity of k_{cat}/K_m values measured in different buffers at the same pH, and the absence of a buffer concentration effect, except for acid citrate, indicate that ionic strength effects can be

Figure 6 Enzyme-catalysed hydrolysis of PNP- α -D-Gal β in 50 mM sodium formate, pH 3.75

The fit is to the expression for substrate inhibition (eqn. 4).

neglected. Eqn (3) was therefore fitted to the data represented by the filled circles in Figure 5.

$$\log(k_{\text{cat}}/K_m) = \log[(k_{\text{cat}}/K_m)_{\text{max}}] - \log\left(1 + \frac{10^{-\text{pH}}}{K_{a_1}} + \frac{K_{a_2}}{10^{-\text{pH}}} + \frac{K_{a_2}}{K_{a_1}}\right) \quad (3)$$

The derived K_a values were $1.234 \times 10^{-2} \pm 1.387 \times 10^{-3}$ and $2.863 \times 10^{-6} \pm 1.591 \times 10^{-7}$ (corresponding to $\text{p}K_{a_1} = 1.91 \pm 0.05$ and $\text{p}K_{a_2} = 5.54 \pm 0.02$), i.e. the pH-dependence of the second-order rate constant is accurately described by a classical bell-shape.

Kinetics of action on galactosyl derivatives at the pH optimum

Of the substrates examined, *o*- and *p*-nitrophenyl galactosides and α -D-galactosyl fluoride exhibited pronounced substrate inhibition, whereas 1-naphthyl and 6-bromo-2-naphthyl galactosides and 1-fluoro galactosyl fluoride were hydrolysed according to Michaelian kinetics. Data for the hydrolysis of *p*-nitrophenyl galactoside are displayed in Figure 6. The data were fitted to the expression for simple substrate inhibition:

$$\frac{v_o}{[E]_t} = \frac{k_{\text{cat}}[S]}{K_m + [S] + \frac{[S]^2}{K_{\text{is}}}} \quad (4)$$

and gave the fit displayed in Figure 6, with values of r^2 of 0.986 for *p*-nitrophenyl and 0.988 for *o*-nitrophenyl galactosides. In the case of the α -galactosidase from *T. reesei*, apparent substrate inhibition in the hydrolysis of *p*-nitrophenyl galactoside had been shown to be associated with the formation of transglycosylation products, largely Gal β (1 $\rightarrow$ 6)Gal β PNP [16], where PNP is *p*-nitrophenyl. A second molecule of substrate was binding to the glycosyl-enzyme intermediate, yet the transfer of the α -galactosyl residue from the glycosyl-enzyme to a second molecule of substrate occurred more slowly than to water. However, we could find neither kinetic nor product-analytical evidence for such a process with the *P. chrysosporium* enzyme acting on nitrophenyl galactosides.

Scheme 1

Minimum kinetic scheme for substrate transglycosylation by a double-displacement glycosidase

Scheme 1 shows the minimal kinetic scheme for substrate transglycosylation by a double-displacement glycosyl hydrolase. This gives rise to rate equations of the form of eqn. (5), in which the parameters are given in terms of individual rate constants in eqns. (6)–(9).

$$\frac{v_o}{[E]_t} = \frac{k_{\text{cat}}[S] + k_{\text{cat}_2}[S]^2}{K_m + [S] + \frac{[S]^2}{K_{m_2}}} \quad (5)$$

$$k_{\text{cat}} = \frac{(k_1 k_2 k_3 (k_5 + k_{-4}))}{k_1 k_2 k_5 + k_1 k_3 k_5 + k_2 k_4 k_5 + k_4 k_5 k_{-1} + k_1 k_2 k_{-4} + k_1 k_3 k_{-4}} \quad (6)$$

$$k_{\text{cat}_2} = \frac{k_1 k_2 k_4 k_5}{k_1 k_2 k_5 + k_1 k_3 k_5 + k_2 k_4 k_5 + k_4 k_5 k_{-1} + k_1 k_2 k_{-4} + k_1 k_3 k_{-4}} \quad (7)$$

$$K_m = \frac{k_3 (k_2 + k_{-1}) (k_5 + k_{-4})}{k_1 k_2 k_5 + k_1 k_3 k_5 + k_2 k_4 k_5 + k_4 k_5 k_{-1} + k_1 k_2 k_{-4} + k_1 k_3 k_{-4}} \quad (8)$$

$$K_{m_2} = \frac{k_1 k_2 k_5 + k_1 k_3 k_5 + k_2 k_4 k_5 + k_4 k_5 k_{-1} + k_1 k_2 k_{-4} + k_1 k_3 k_{-4}}{k_1 k_4 (k_2 + k_5)} \quad (9)$$

Fitting of the rate-concentration data for *o*- and *p*-nitrophenyl galactosides to eqn. (5) rather than eqn. (4) resulted in unchanged values of r^2 and values of k_{cat_2} equal to zero. At the same time, no transglycosylation products could be detected when the enzymic hydrolysis of a 10 mM solution of *p*-nitrophenyl galactoside was followed to completion by HPLC.

Both the binding of a second molecule of substrate to the galactosyl-enzyme complex, to yield an inert $E'\beta\text{Gal}:\alpha\text{Gal}$ -PNP complex, and formation of an inert $E'(\alpha\text{Gal-PNP})_2$ complex, yield a rate law of the form of eqn. (4), but the meaning of K_{is} differs. In classical substrate inhibition K_{is} is the dissociation constant of the second substrate molecule from the non-productive ES_2 complex, whereas in the present case it is equal to $K_d(k_2 + k_3)/k_2$, where K_d is the dissociation constant of the second molecule of substrate from the galactosyl-enzyme, and k_2 and k_3 are the rates of formation and hydrolysis of this galactosyl enzyme.

We favour the formation of an inert $E'\beta\text{Gal}:\alpha\text{Gal}$ -PNP complex, since the occurrence of substrate inhibition only with substrates with good leaving groups is readily explained: with these substrates most of the enzyme that has bound the first substrate molecule will be present as the galactosyl-enzyme rather than the $E'\alpha\text{GalX}$ complex.

In the case of the fluoride, however, the $E'\beta\text{Gal}:\alpha\text{Gal}$ -F complex appears to be not completely inert. Figure 7 shows the fits to eqns. (4) and (5): the fit to transglycosylation is clearly

Figure 7 Concentration-dependence of the rate of liberation of fluoride ion from α -galactosyl fluoride

The fits are to the rate laws for simple substrate inhibition (eqn. 4, which gives the bottom curve at high $[S]$) and transglycosylation (eqn. 5, which gives the top curve at high $[S]$).

better (r^2 values of 0.982 and 0.992). In both cases, though, the errors associated with kinetic parameters (Table 3) are high due to the sparseness of data points at low $[S]$, an inevitable consequence of the comparative insensitivity of the fluoride ion electrode (detection limit around $1 \mu\text{M}$). Despite kinetic evidence favouring some transglycosylation, it was not detected by the NMR experiment used to determine the stereochemistry of action of the enzyme. This was carried out at 3.7 mM substrate, at which concentration, if the production of fluoride from the $\text{E}^*\beta\text{Gal}'\alpha\text{GalF}$ complex were due to transglycosylation to $\alpha\text{-D-Galp-(1} \rightarrow 6\text{)-}\alpha\text{-D-Galp-F}$, most of the product should have been disaccharide fluoride (Figure 7). However, we could detect only the anomeric resonances of α - and β -galactose and galactosyl fluoride. Overlap of the anomeric resonance of the transfer product with that of α -galactopyranose is unlikely, since both galactose anomeric resonances were clearly distinguishable from the anomeric resonances of raffinose [$\alpha\text{-D-Galp-(1} \rightarrow 6\text{)-}\alpha\text{-D-Glcp-}\beta\text{-D-Fruf}$]. It seems therefore likely that the turnover of the $\text{E}^*\beta\text{Gal}'\alpha\text{GalF}$ complex arises from slow hydrolysis of the βGal -enzyme linkage, but at a much lower rate than in the simple $\text{E}^*\beta\text{Gal}$ complex. Although the trend to more pronounced substrate inhibition for substrates with better leaving groups is

unambiguous, the k_{cat} values for 'good' substrates are not the same as they should be for clean rate-determining degalactosylation. The observation of substrate inhibition, though, will be a complex function of k_2 , k_3 and K_{is} , and, provided qualitatively significant quantities of the total bound enzyme are present as the galactosyl enzyme, substrate inhibition will be observed. The derived k_{cat} and K_{m} data for α -galactosyl fluoride are critically dependent, moreover, on data for substrate concentrations at the limit of resolution of our instrumentation.

Substrate specificity

The enzyme did not tolerate changes of glycone, no hydrolysis of the p -nitrophenyl α -glycosides of xylose, mannose or glucose being detectable at 3 mM concentrations (Figure 1). A weak activity on p -nitrophenyl β -L-arabinopyranoside was measured, but it was not possible to measure K_{m} at accessible substrate concentrations: the $k_{\text{cat}}/K_{\text{m}}$ value was some 10^3 lower than that of the corresponding galactoside (Table 3). We estimate an upper limit on $k_{\text{cat}}/K_{\text{m}}$ for the xyloside, mannoside and glucoside of around $5 \text{ M}^{-1} \cdot \text{s}^{-1}$.

Reversible inhibition

Preliminary experiments indicated that, surprisingly, galactose was a powerful inhibitor. Because of the problems of substrate inhibition, we measured only the competitive component of the inhibition, under conditions of $[S] \ll K_{\text{m}}$. We used first-order rather than initial-rate conditions, with 1-Nap- α -D-Galp as a substrate: direct fitting of the data to $k_{\text{obs}} = k_{\text{max}}/(1 + [I]/K_i)$ indicated the competitive K_i of D-galactose to be $0.357 \pm 0.016 \text{ mM}$. Product inhibition to this degree is rare with glycosidases, and the speculation that it might be related to the comparatively high proportion of aldehyde form of the sugar present at equilibrium [41] was readily tested. If the enzyme were binding this form exceptionally tightly, then there should be an equilibrium α - ^2H isotope effect on the proportion of straight-chain sugar present and hence on the apparent K_i . However, we could detect no such effect, and therefore conclude that the ring form of the sugar is being bound.

Galactotetrazole (**II**) has been shown to be a highly effective inhibitor of β -galactosidases, inhibiting with K_i values in the submicromolar range [20]. Tight binding is considered to arise both because of geometrical resemblance to the oxocarbenium-ion-like transition state and from in-plane protonation of the nitrogen attached to C-1 by the acid catalytic group. In fact, the

Table 3 Steady-state kinetic parameters of substrates and ligands

*Separated values determined in an experiment with the majority of the points at high $[S]$; $k_{\text{cat}}/K_{\text{m}}$ determined under first-order conditions.

Substrate	k_{cat} (s^{-1})	K_{m} or K_i (mM)	K_{is} (mM)	$k_{\text{cat}2}$ ($\text{mM}^{-1} \cdot \text{s}^{-1}$)	$K_{\text{m}2}$ (mM)	$k_{\text{cat}}/K_{\text{m}}$ ($10^{-5} \text{ M}^{-1} \cdot \text{s}^{-1}$)
D-Galactose	—	0.357 ± 0.016	—	—	—	—
Tetrazole (II)	—	7.25 ± 0.43	—	—	—	—
p -Nitrophenyl α -L-arabinopyranoside	—	—	—	—	—	0.0263 ± 0.0009
1-Naphthyl α -galactoside	$171^* \pm 3$	$0.221^* \pm 0.017$	—	—	—	5.47^*
6-Bromo-2-naphthyl α -galactoside	77 ± 4	0.162 ± 0.023	—	—	—	4.7 ± 0.7
p -Nitrophenyl α -galactoside	272 ± 21	0.198 ± 0.023	0.254 ± 0.028	—	—	13.7 ± 1.9
o -Nitrophenyl α -galactoside	514 ± 33	0.30 ± 0.03	0.70 ± 0.07	—	—	17 ± 2
1-Fluorogalactosyl fluoride	5.6 ± 0.1	9.9 ± 0.4	—	—	—	0.0566 ± 0.0025
α -Galactosyl fluoride	87 ± 12	0.029 ± 0.013	0.36 ± 0.08	—	—	30 ± 14
	or	or	or	or	or	or
	140 ± 20	0.074 ± 0.018	—	46 ± 15	0.135 ± 0.030	19 ± 5

competitive inhibition constant (obtained under first-order conditions, as with galactose) is only 7.25 ± 0.43 mM. This weak binding could arise from clashes of the nitrogen atoms attached to N(5) with the enzyme protein and/or the inability of the acid catalytic group of an α -glycosidase to protonate N(1).

α - ^2H kinetic isotope effects

At the pH optimum of the enzyme, pH 3.75, an $^{2\text{D}}(V/K)$ effect of 1.031 ± 0.007 and an $^{2\text{D}}(V)$ effect of 1.093 ± 0.007 were measured for *p*-nitrophenyl α -D-galactopyranoside. The low value of $^{2\text{D}}(V/K)$ at pH 3.75 suggests that the isotopically silent steps are partly rate-determining at the pH optimum. This is commonly observed, and it is also commonly observed that isotope effects become close to intrinsic away from the pH optimum, as chemical steps are generally more pH-sensitive than non-chemical ones. Indeed, a value of $^{2\text{D}}(V/K)$ of 1.114 ± 0.006 was measured at pH 7.00; this is probably close to intrinsic, since the rate at this pH is only around a thirtieth of maximal.

The question then arises as to whether the effect is masked at optimal pH because the first irreversible step of the ES complex does not involve bond cleavage, or whether it is masked because the substrate is 'sticky', i.e. most encounters between enzyme and substrate result in the substrate being transformed (external commitment to catalysis = 1), so that k_{cat}/K_m represents the bimolecular diffusion of enzyme and substrate. As the value of k_{cat}/K_m (Table 3) is low but preceded [42] for diffusion-controlled glycosidase-substrate combination, *p*-nitrophenyl galactoside is probably a 'sticky' substrate.

If 1-naphthyl galactoside is likewise 'sticky', k_{cat}/K_m will represent, at least partly, diffusion-controlled enzyme-substrate combination. The $\text{p}K_a$ values derived from its variation with pH will then be kinetic $\text{p}K_a$ values arising from a pH-independent diffusional process and a bell-shaped dependence of the chemical step. The two limbs of the profile for the chemical step, were this kinetically accessible, would then be closer together than in the observed profile for k_{cat}/K_m . However, the facts that even the *p*-nitrophenyl glycoside is not fully sticky, as shown by the partial expression of the isotope effect even at the pH-optimum, and that the value of k_{cat}/K_m for 1-naphthyl galactoside is half that for *p*-nitrophenyl galactopyranoside, even though the two substrates have almost identical molecular masses (and hence similar diffusion coefficients), suggest that the commitments for the naphthyl substrate are low. Consequently the ionizations derived from Figure 5 will be close to those governing the ionizations of catalytic groups in the free enzyme.

The intrinsic value for $^{2\text{D}}(V/K)$ of 1.114 ± 0.006 is in the middle of the range observed for the first step of a retaining glycosidase, whereas the value $^{2\text{D}}(V)$ effect of 1.093 ± 0.007 is preceded for the hydrolysis of a glycosyl-enzyme intermediate [43], and both indicate a substantial degree of charge development on the glycone in the chemical transition states.

Effect of 1-fluorine substitution

We applied a mechanistic probe of higher resolution than an α - ^2H kinetic isotope effect to measure the degree of charge development at the first chemical transition state. The probe is the ratio of the second-order rate constants (k_{cat}/K_m values) for hydrolysis of a glycosyl fluoride and the almost isosteric 1-fluoro glycosyl fluoride [18,33]. Expressed logarithmically [i.e. as a free-energy difference $\Delta\Delta G^\ddagger = RT \ln [(k_{\text{cat}}/K_m)_F/(k_{\text{cat}}/K_m)_{F_2}]$] the ratio corresponds to an (unscaled) Brønsted slope calculated on two points. On the basis of the normal assumptions about linear free-energy relationships, then, numerically it should be proportional to the change in charge between ground state and

transition state. Although the difficulty of making measurements at fluoride concentration close to K_m means that the errors on the ratio itself are large, this translates into a comparatively small error on $\Delta\Delta G^\ddagger$, the value of which is 21 ± 2 kJ/mol. When compared with other measured values [18,33], this represents one of the larger effects so far measured and, if k_{cat}/K_m for the monofluoride represents diffusion-controlled binding, will be a lower limit.

Notably, 1-fluoro-galactosyl fluoride does not show substrate inhibition. If the only substrates showing substrate inhibition are those for which hydrolysis, rather than formation, of the glycosyl-enzyme is rate limiting, then the contrasting behaviours of the two fluorinated substrates is highly informative. Substitution of fluorine at C-1 changes the rate-determining step from degalactosylation to galactosylation. This means that the effect of the second fluorine is greater on the first step than on the second, and, by our assumptions that the effect is exerted by change of electronic demand, that the change in charge on the sugar between ES complex and the first transition state is greater than the change in charge on the sugar between the galactosyl-enzyme intermediate and the second transition state. However, microscopic reversibility suggests that this difference arises from differential effects on ground states rather than transition states. Certainly, microscopic reversibility requires the first and second transition states to be identical for virtual reactions (such as enzyme-catalysed washout of ^{18}O exchange from [1- ^{18}O]galactose), and it is reasonable that, at least for substrates with leaving groups that are electronically similar to water and which make no specific interactions with the protein, the first and second transition states will be very similar. Rather, the effect of fluorine is likely to be a ground-state effect, in which 1-fluoro substitution destabilizes the galactosyl-enzyme. If the ester bond in the glycosyl-enzyme is already polarized in some way towards the oxocarbenium ion, so that the sugar residue already carries a small positive charge, the effect of electron withdrawal by fluorine in the substrate can be readily understood.

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