Labeling and Identification of the Postulated Acid/Base Catalyst in the α-Glucosidase from *Saccharomyces cerevisiae* Using a Novel Bromoketone C-Glycoside†

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ABSTRACT: α-Glucosidase from *Saccharomyces cerevisiae* is a member of a sequence-related family of α-glycosidases (family 13) that includes digestive α-amylases and commercially important cyclodextrin glucanotransferases. These enzymes catalyze the hydrolysis of α-linked oligosaccharides by a two-step mechanism involving a glycosyl−enzyme intermediate. A novel bromoketone C-glycoside inactivator, 1′-bromo-3′-(α-D-mannopyranosyl)-2′-propanone, has been synthesized and used to label the putative acid/base catalyst (Glu-276) of yeast α-glucosidase. Electrospray ionization mass spectrometry was used to demonstrate stoichiometric labeling of the protein. The labeled residue was identified by comparative liquid chromatographic/mass spectrometric analysis of peptic digests of labeled and unlabeled enzyme samples, which confirmed the unique presence of two labeled peptides of *m*/*z* 745 and 694. Subsequent tandem mass spectrometric analysis in the daughter-ion scan mode showed the two peptides to have an overlapping sequence in which Glu-276 was the labeled residue. Together with active-site-directed protection against inactivation with deoxynojirimycin, these results prove that Glu-276 is located within the active site of yeast α-glucosidase and, thus, provide further evidence for this residue playing an important role in catalysis.

There has been widespread interest in glycosidases in recent years, largely due to their role in a multitude of biological systems and, as a result, their potential as therapeutic targets. In addition, enzymes such as cellulases and amylases are important for the industrial degradation of biomass. Structural information, especially with respect to the active site, is needed in order to understand and classify these enzymes. Furthermore, the identification of active-site residues provides the basis for engineering of new proteins with modified or improved function.

Glycosidases can be classified as "retaining" or "inverting", depending on the anomeric configuration of the initially formed product with respect to the substrate. Both types possess two catalytic carboxylic acid residues within the active site. Retaining glycosidases employ a "double-displacement mechanism" in which, in the first step, one residue provides general acid catalysis and the other provides nucleophilic catalysis to form a covalent glycosyl−enzyme intermediate (*1, 2*). In the second step, general base-catalyzed hydrolysis of this intermediate yields the product with overall retention of anomeric stereochemistry. Inverting glycosidases use a single-step mechanism in which general base catalysis facilitates direct attack by water at the anomeric center, with general acid-catalyzed departure of the aglycon. All steps proceed via transition states with substantial oxocarbenium ion character.

A wide variety of mechanism-based inhibitors and affinity labels have been developed in order to label and identify catalytically important residues (*3*−*5*). The catalytic nucleophile has been labeled and identified in many cases by the use of mechanism-based inhibitors such as the conduritol epoxides (*3, 6*), 2-deoxy-2-fluoroglycosides (*7*−*10*), and 5-fluoroglycosyl fluorides (*11, 12*). Identification of the labeled residues has been achieved by peptic digestion of the labeled enzymes and the location of the labeled peptide determined by means of mass spectrometry or by use of a radioactive label (*5, 13*).

Affinity labels have also been widely used, including glycosyl epoxides (*14, 15*), *N*-(bromoacetyl)glycosylamines (*16*−*18*), and glycosyl isothiocyanates (*19*). Notable successes were the selective labeling and identification of the acid/base catalytic residue in the β-(1,4)-glucanase (Cex) from *Cellulomonas fimi* with *N*-(bromoacetyl)-β-D-cellobiosylamine (*20*) and the identification of Glu-198 as the putative acid/base catalyst in the cyanogenic β-glucosidase from cassava with *N*-(bromoacetyl)-β-D-glucosylamine (*18*). Whereas many O-linked affinity labels have the drawback of being susceptible to enzyme-catalyzed hydrolysis, *N*-(bromoacetyl)glycosylamines have the advantage of being inert in this respect. Unfortunately, this class of compounds is currently only available in the β-configuration, and access to the α-configured analogues seems unlikely. While there are a few examples of α-configured affinity labels, such as

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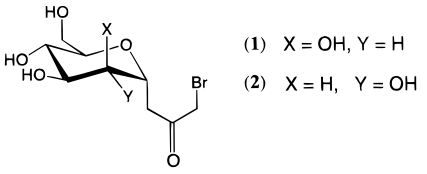


FIGURE 1: Target materials: α-C-mannoside **1** and α-C-glucoside **2**.

1,2-epoxy-3-(α-D-glucopyranosyl)propane, which was shown to be an effective irreversible inactivator of α-glucosyl transferases (*21*), the wide variety of affinity labels in the *β*-series has generally not been reproduced in the α-series.

Interesting enzyme targets for α-configured glycosyl-based affinity labels are α-mannosidases and α-glucosidases, particularly those from glycosidase familes 13 and 38 (*22*−*24*). Certain biological disorders are associated with family 38 mannosidases, such as mammalian Golgi α-mannosidase II, a current target for anticancer drugs, and lysosomal α-mannosidase, a deficiency of which is associated with the hereditary disease α-mannosidosis (*25*−*28*). Similarly, the α-glucosidases from family 13 include commercially important amylases and cyclodextrin glucanotransferases. The active-site nucleophile has been labeled and identified for both jack bean α-mannosidase (family 38) (*29*) and certain α-glucosidases and glycosyl transferases from family 13, including the *Saccharomyces cerevisiae* α-glucosidase (*12*, *30*, *31*), by trapping glycosyl−enzyme intermediates. No reagents for the reliable identification of the acid/base catalyst in these glycosidases have been described, however. Other inactivators, such as conduritol B epoxide (*32*), conduritol B aziridine (*33*), and an α-difluoroalkyl glucoside (*34*), which might play this role have been shown to cause time-dependent inactivation of yeast α-glucosidase, but the labeled residues were not identified.

A candidate for the acid/base catalyst in family 13 α-glucosidases has been identified by sequence alignment and through site-directed mutagenesis of conserved acidic residues (*35*), as well as from the available three-dimensional structures. Indeed, the X-ray structures reveal two possible candidates, of which one is the more likely on the basis of the structures of enzyme/substrate and enzyme/inhibitor complexes (*36*, *37*). Although the roles of acidic residues have not been probed by mutagenesis with yeast α-glucosidase (*S. cerevisiae*), sequence alignment with other family members indicates the acid/base catalyst as being Glu-276. In the case of family 38 (α-mannosidases), there are several conserved acidic residues, but, as yet, there is no evidence to indicate which residue provides acid/base catalysis.

The α-C-mannoside (**1**) and α-C-glucoside (**2**)[1] (Figure 1) were chosen as synthetic targets in our attempt to develop novel α-configured affinity labels for α-mannosidases and α-glucosidases, respectively. These compounds were designed as C-linked glycosides since such compounds do not possess a labile glycosidic bond, which is prone to enzyme-catalyzed hydrolysis. In addition, the α-configuration at the pseudoanomeric position is easily accessible synthetically. The α-bromocarbonyl functionality, which proved successful in the *N*-(bromoacetyl)glycosylamines, has been employed here also, this time in the ketone form rather than the amide form. Here we report the synthesis of α-C-mannoside (**1**), attempts to synthesize the α-C-glucoside (**2**), and investigation into the ability of (**1**) to inactivate various α-glycosidases. Further, we report the use of **1** to specifically label and identify the postulated acid/base catalyst of yeast α-glucosidase.

[1] Abbreviations: C-glycoside, carbon glycoside; α-PNPG, 4-nitrophenyl α-D-glucopyranoside; α-PNPM, 4-nitrophenyl α-D-mannopyranoside; LC/MS, liquid chromatographic/mass spectrometric; ESMS, electrospray mass spectrometry; IPTG, isopropyl thio-*β*-D-glucopyranoside.

EXPERIMENTAL PROCEDURES

General Procedures. All buffer chemicals and other reagents were obtained from the Sigma/Aldrich Chemical Co. unless otherwise noted. Recombinant yeast α-glucosidase and pepsin (from porcine mucosa) were obtained from Boehringer Mannheim.

Synthetic reactions were monitored by TLC using Merck Kieselgel 60 F_{254} aluminum-backed sheets. Compounds were detected by charring with 10% ammonium molybdate in 2 M H_2SO_4 and heating. Flash chromatography under a positive pressure was performed with Merck Kieselgel 60 (230−400 mesh) using the specified eluants. ^{1}H NMR spectra were recorded on a Brüker WH-400 spectrometer at 400 MHz (chemical shifts quoted relative to $CDCl_3$ or to DSS when taken in D_2O). ^{13}C NMR spectra were recorded on a Varian XL-300 at 75 MHz or a Brüker AC-200 at 50 MHz and are proton-decoupled with $CDCl_3$ or acetone (30.5 ppm) as a reference.

Synthesis of 1′-Bromo-3′-(α-D-mannopyranosyl)-2′-propanone (**1**). *3′-(2,3,4,6-Tetra-O-acetyl-*α*-D-mannopyranosyl)-1′-propene (**3**).* α-D-Mannose per-*O*-acetate (20.8 g, 53.3 mmol) and allyltrimethylsilane (9.75 mL, 61.3 mmol) were dissolved in acetonitrile (250 mL) and cooled to 0 °C under nitrogen. To the stirred mixture were added boron trifluoride diethyl etherate (12.1 mL, 98 mmol) and trimethylsilyl trifluoromethanesulfonate (2 mL, 10.3 mmol) sequentially, dropwise. After being stirred for 1 h, the mixture was allowed to warm to room temperature, after which stirring was continued for a further 16 h. The solution was then poured into a mixture of saturated aqueous $NaHCO_3$ (300 mL) and diethyl ether (700 mL). After the mixture was shaken, the organic layer was isolated, washed further with $NaHCO_3$ (300 mL), water (300 mL), and brine (300 mL), and then dried ($MgSO_4$). The solvent was evaporated in vacuo and the residue purified on silica gel (diethyl ether/petroleum ether, 2:5; bp 35−60 °C) to give **3** (10.9 g, 55%, α:*β* 6:1, inseparable mixture) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.70−5.80 (1 H, m, H-2′), 5.00−5.30 (5 H, m, H-1a′, H-1b′, H-2, H-3, H-4), 4.29 (1 H, dd, $J_{6a,6b}$ = 12.0 Hz, $J_{6a,5}$ = 6.3 Hz, H-6a), 4.08 (1 H, m, H-6b), 4.01 (1 H, ddd, J = 9.1, 6.0, and 3.2 Hz, CH−O), 3.87 (1 H, ddd, J = 9.1, 6.4, 2.9 Hz, CH−O), 2.51 (1 H, m, H-3a′), 2.32−2.43 (1 H, m, H-3b′), 2.09, 2.06, 2.04, 2.01 (12 H, 4 × s, 4 × OAc). CIMS (NH_3): *m*/*z* (relative intensity) 313 $(M - OAc)^+$ (20), 373 $(M + H)^+$ (25), 390 $(M + NH_4)^+$ (100%).

*3′-(2,3,4,6-Tetra-O-acetyl-*α*-D-mannopyranosyl)-2′-propanone (**5**).* Palladium chloride (0.28 g, 1.58 mmol) and cuprous chloride (1.6 g, 16.2 mmol) were stirred in aqueous dimethylformamide (DMF/H_2O, 7:1; 10 mL) under an atmosphere of oxygen for 1 h. 3′-(2,3,4,6-Tetra-*O*-acetyl-

α-D-mannopyranosyl)-1′-propene (**3**) (6.04 g, 16.2 mmol) was then added as a solution in aqueous dimethylformamide, and the mixture was stirred vigorously for a further 24 h. The mixture was then poured into 2 M HCl (100 mL), which was extracted with diethyl ether (5 × 40 mL). The combined organic fractions were washed successively with saturated aqueous $NaHCO_3$ (100 mL), water (2 × 100 mL), and brine (100 mL) and then dried ($MgSO_4$). After filtration, the solvent was evaporated in vacuo and the residue purified on silica gel (ethyl acetate/petroleum ether, 1:1; bp 35−60 °C) to give starting material (3.09 g, 8.31 mmol) and **5** (703 mg, 1.81 mmol, 23%) as a colorless oil (yield based on recovered starting material). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.22 (1 H, dd, $J_{3,4}$ = 7.4 Hz, $J_{3,2}$ = 3.3 Hz, H-3), 5.05−5.10 (2 H, m, H-2 and H-4), 4.46 (1 H, dt, $J_{1,2}$ = 4.7 Hz, $J_{1,3a'}$ = 9.2 Hz, $J_{1,3b'}$ = 4.7 Hz, H-1), 4.38 (1 H, dd, $J_{6a,6b}$ = 12.0 Hz, $J_{6a,5}$ = 6.8 Hz, H-6a), 4.13 (1 H, dd, $J_{6b,5}$ = 3.8 Hz, H-6b), 3.92 (1 H, td, $J_{5,4}$ = 6.8 Hz, H-5), 2.82 (1 H, dd, $J_{3a',3b'}$ = 16.0 Hz, H-3a′), 2.61 (1 H, dd, H-3b′), 2.03−2.09 (15 H, 5 × s, 4 × OAc and H-1′). ^{13}C NMR-APT (50 MHz, $CDCl_3$): δ 204.1 (C-2′), 170.3, 169.8, 169.6, 169.2 (4 × OAc), 71.4, 69.2, 69.0, 67.9, 67.0 (5 × CH), 61.4 (C-6), 43.2 (C-3′), 29.9 (C-1′), 20.4 (4 × OAc). CIMS (NH_3): *m/z* (relative intensity) 329 $(M - OAc)^+$ (100), 389 $(M + H)^+$ (50), 406 $(M + NH_4)^+$ (20). HRMS $(M + H)^+$, calcd 389.1448, found 389.1458.

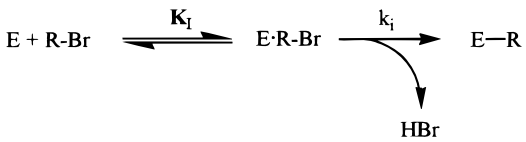
*3′-(α-D-Mannopyranosyl)-2′-propanone (**6**).* Ketone **5** (703 mg, 1.81 mmol) was dissolved in a methanol/water/triethylamine mixture (7:3:1) and allowed to stand for 2 h at room temperature. The solvent was evaporated in vacuo and the residue purified on silica gel (10% methanol/dichloromethane) to give **6** (342 mg, 1.56 mmol, 86%) as a colorless syrup. $[\alpha]_D$ +29° (*c* 0.61, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.44 (1 H, ddd, $J_{1,3a'}$ = 8.7 Hz, $J_{1,3b'}$ = 5.2 Hz, $J_{1,2}$ = 2.2 Hz, H-1), 3.88 (1 H, t, $J_{2,3}$ = 2.2 Hz, H-2), 3.79−3.83 (2 H, m, H-3, H-6a), 3.74 (1 H, dd, $J_{6a,6b}$ = 12.2 Hz, $J_{5,6b}$ = 6.0 Hz, H-6b), 3.67 (1 H, t, $J_{3,4}$ and $J_{4,5}$ = 9.0 Hz, H-4), 3.55 (1 H, ddd, $J_{5,6a}$ = 2.4 Hz, H-5), 3.10 (1 H, dd, $J_{3a',3b'}$ = 17.0 Hz, H-3a′), 2.88 (1 H, dd, H-3b′), 2.29 (3 H, s, Me). ^{13}C NMR (50 MHz, $CDCl_3$): δ 204.0 (C-2′), 75.0, 73.9, 70.1, 70.8, 67.5 (5 × CH), 61.3 (C-6), 42.5 (C-3′), 28.0 (C-1′). CIMS (NH_3): *m/z* (relative intensity) 203 $(M - OH)^+$ (100), 221 $(M + H)^+$ (80), 238 $(M + NH_4)^+$ (10%). HRMS $(M + H)^+$ calcd 221.1025; found 221.1025.

*1′-Bromo-3′-(α-D-mannopyranosyl)-2′-propanone (**1**).* Ketone **6** (330 mg, 1.1 mmol) was dissolved in methanol (1.5 mL) to which methanolic bromine (1.5 mL, 1.13 M in methanol) was added. The mixture was left to stand for 30 min at 40 °C in darkness. The solvent was then removed in vacuo and the residue purified twice on silica gel (column A, 10% methanol/chloroform; column B, ethyl acetate/methanol/water, 27:2:1) to give **1** (210 mg, 64%) as a colorless syrup. $[\alpha]_D$ +37° (*c* 1.95, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.45 (1 H, ddd, $J_{1,3a'}$ = 8.6 Hz, $J_{1,3b'}$ = 5.3 Hz, $J_{1,2}$ = 2.6 Hz, H-1), 4.28 (2 H, s, H-1′), 3.87 (1 H, t, $J_{2,3}$ = 2.6 Hz, H-2), 3.77−3.81 (2 H, m, H-3, H-6a), 3.72 (1 H, dd, $J_{6a,6b}$ = 12.2 Hz, $J_{5,6b}$ = 6.0 Hz, H-6b), 3.66 (1 H, t, $J_{3,4}$ and $J_{4,5}$ = 8.9 Hz, H-4), 3.55 (1 H, ddd, $J_{5,6a}$ = 2.6 Hz, H-5), 3.27 (1 H, dd, $J_{3a',3b'}$ = 17.3 Hz, H-3a′), 2.98 (1 H, dd, H-3b′). ^{13}C APT (75 MHz, D_2O): δ 202.9 (C-2′), 67.2, 70.6, 70.9, 67.7, 74.8 (5 × CH), 61.1 (C-6), 36.2, 39.1 (2 × CH_2). ESMS: *m/z* (relative intensity) 299.3 $(M + H)^+$ (100), 301.1 $(M + H)^+$ (100). Anal. Calcd for $C_9H_{15}O_6Br$: C, 36.14; H, 5.05. Found: C, 36.33; H, 5.26.

Enzyme Kinetics. All studies on yeast α-glucosidase were performed at 37 °C in 50 mM sodium phosphate buffer, pH 6.8, containing 0.1% bovine serum albumin. A continuous spectrophotometric assay based on the hydrolysis of α-PNPG was used to monitor enzyme activity by measurement of the rate of 4-nitrophenolate release (λ = 400 nm, ϵ = 7.28 × 10^3 M^{-1} cm^{-1} in the buffer above) using a UNICAM 8700 UV−visible spectrophotometer equipped with a circulating water bath.

The inactivation of α-glucosidase by **1** was monitored by incubation of the enzyme (∼0.04 mg/mL, 0.6 μM) under the above conditions in the presence of various concentrations of **1** at 37 °C. Residual enzyme activity was determined at the appropriate time intervals by addition of a 10-μL aliquot of the inactivation mixture to a solution of α-PNPG (2 mM, 750 μL, K_m = 0.18 mM) in the above buffer and measurement of 4-nitrophenolate release over a period of 1 min. Pseudo-first-order inactivation rate constants at each inactivator concentration (k_{obs}) were determined by fitting each curve to a first-order equation using the program GraFit (Leatherbarrow, R. J. *GraFit Version 3.0*; Erithacus Software Ltd., Staines, U.K., 1990). The value of k_i/K_I, assuming inactivation according to the kinetic model shown in Scheme 1, was determined from the slope of a plot of k_{obs} against inactivator concentration.

Scheme 1



Labeling and Proteolysis. Labeling of yeast α-glucosidase was achieved by incubating the enzyme (1 mg/mL [15 μM] × 30 μL) with **1** (2.5 μL × 52.8 mM) for 30 min in 50 mM sodium phosphate buffer (pH 6.8). This sample was then used directly for mass spectrometric analysis. When the sample was prepared for proteolytic digestion purposes, the inactivation was repeated using a more concentrated enzyme sample (6 mg/mL, 90 μM [50 μL] incubated with inactivator **1**, 52.8 mM [4 μL]). After incubating for 30 min, the sample was dialyzed against 50 mM sodium phosphate buffer (pH 6.8) using a centrifugal ultrafilter with a molecular weight cutoff of 30 000 (Millipore) by dilution to 500 μL, concentration to 50 μL, and then dilution with fresh buffer to 500 μL. This was repeated four more times, the last wash using pH 1.8 buffer (50 mM sodium phosphate acidified with 10% HCl). To the enzyme was then added 100 μL of buffer (pH 1.8) and 50 μL of pepsin (0.4 mg/mL pepsin in pH 1.8 buffer), and the mixture was incubated at room temperature for 45 min. LC/MS analysis of the proteolytic digest confirmed that the enzyme was completely digested.

Electrospray Mass Spectrometry. The analyses of the protein and peptide samples were carried out using a Sciex API-300 mass spectrometer interfaced with a Michrom UMA HPLC system (Michrom Bioresources, Inc., Auburn, CA). Inactivated yeast α-glucosidase (10−20 μg, labeled or unlabeled) was introduced into the mass spectrometer through a microbore PLRP column (1 mm × 50 mm) and eluted

with a gradient of 20−100% solvent B at a flow rate of 50 μL/min over 15 min (solvent A, 0.06% trifluoroacetic acid, 2% acetonitrile in water; solvent B, 0.05% trifluoroacetic acid, 90% acetonitrile in water). The MS was scanned over a range of 400−2300 Da, with a step size of 0.5 Da and a dwell time of 1 ms.

LC/MS analysis of the proteolytic digests was performed by loading a 10-μL sample of the pepsin digest (1.5 mg/mL) onto a C18 column (Reliasil, 1 mm × 150 mm), which was eluted at a flow rate of 50 μL/min with a gradient of 0−60% B over 45 min. In the single-quadrupole (normal LC/MS) mode, the MS conditions were as follows: the mass analyzer was scanned over the range of 300−2400 Da, with a step size of 0.5 Da, a dwell time of 1.5 ms, an ion source voltage (ISV) of 4.8 kV, and an orifice energy (OR) of 50V.

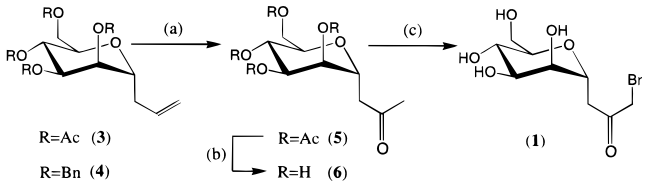
Methyl Esterification of Partially Purified Peptides. Partially purified peptide, labeled or unlabeled, was mixed with a freshly prepared solution of 2 M methanolic HCl, and the mixture was incubated at room temperature for 20 min. The excess reagent was removed by concentration under vacuum (SVC 100 Speed Vac), and the product was dissolved in 10% AcOH/MeOH. The sample was analyzed directly by flow injection into the mass spectrometer.

Aminolysis. Partially purified labeled peptide (10 μL) was incubated with concentrated ammonium hydroxide (5 μL) for 20 min, after which the sample was acidified with 25% aqueous acetic acid. The mixture was then subjected to MS analysis in the LC/MS mode.

RESULTS AND DISCUSSION

The synthetic strategy toward the bromoketone **1** was based on acid-catalyzed bromination of the α-C-mannoside ketone **6**, which was obtained from the corresponding allyl-C-mannoside **3** as shown in Scheme 2. The allyl-C-mannoside is readily available as an inseparable 6:1 mixture of α- and β-stereoisomers from per-*O*-acetylated D-mannose (*38*). Oxidation to give the ketone **5** was achieved in one step using a Wacker oxidation. Deprotection and bromination of **6** gave the desired material (**1**). Although the Wacker procedure was low yielding, it was more convenient than the alternative multistep route which proceeded via epoxidation of **4**, reduction to the secondary alcohol and then oxidation to give, after deprotection, the ketone **6**.

Scheme 2



(a) (**3**), $CuCl_2$, $PdCl_2$, O_2, DMF/H_2O-7:1, 23%. (b) MeOH, H_2O, Et_3N, 7:3:1, 86%. (c) (**6**), MeOH, Br_2, 64%.

Synthesis of the α-C-glucoside analogue of **6** was attempted, using the readily available, benzylated gluco-analogue of **6** (*39*−*41*). Unfortunately, on removal of the benzyl protecting groups, cyclization occurs between the C-2 hydroxyl group and the ketone functionality, to produce a cyclic hemiacetal. Further, given the facility of this cyclization, it is probable that, even if **2** could be made, it would rapidly undergo cyclization via attack of the C-2 hydroxyl on the bromoketone. It was, therefore, not possible to synthesize the α-C-glucoside **2**.

Disappointingly, no time-dependent inactivation of either jack bean α-mannosidase or rat Golgi α-mannosidase II was observed upon incubation with the α-C-mannoside **1**. Presumably, this is because the reactive α-bromoketone is not bound directly adjacent to the acid catalytic side chain, but rather is bound in some other orientation. This is a common problem with affinity labels. This problem might be solved by use of glycosidic carbon chains of different lengths. It might also prove to be the case that, with other α-mannosidases, the reactive functionality may be appropriately positioned. Alternatively, a higher pH may be used, under which conditions the active-site group may be more reactive.

Fortunately, however, there was reason to believe that **1** might function as an inactivator *of* α-glucosidases. This expectation was based on an earlier report that yeast α-glucosidase has similar binding affinity for α-mannosides and α-glucosides. Ermert et al. demonstrated that α-PNPG is a substrate for yeast α-glucosidase (K_m = 0.18 mM) and that α-PNPM, although a 10^5 times slower substrate, binds very well, with K_m = 0.08 mM (*42*). Although K_m values are not reliable measures of true affinity, the low value measured indicates that mannosides should at least bind with reasonable affinity.

α-C-Mannoside **1** was found to cause rapid, time-dependent, and irreversible inactivation of α-glucosidase according to pseudo-first-order kinetics, as shown in Figure 2a. Residual enzyme activity, at several different concentrations of **1**, was measured over time, allowing pseudo-first-order rate constants for inactivation at each inactivator concentration to be determined by direct fit to a first-order expression (Figure 2a). A plot of the these observed pseudo-first-order rate constants versus inactivator concentration was linear (Figure 2b), allowing a second-order rate constant, k_i/K_I = 0.03 min^{-1} mM^{-1} (±0.002), to be obtained from the slope of this plot, assuming inactivation according to the kinetic model shown in Scheme 1. Higher concentration of inactivator could not be studied, in an attempt to see saturation kinetic behavior, since inactivation was too rapid under the conditions employed. Active-site-directed protection against inactivation was demonstrated by the use of 58 μM deoxynojirimycin, a known competitive inhibitor of α-glucosidase (K_i = 13 μM) (*12*), the first-order rate constant for inactivation at 0.98 mM **1** being reduced from 0.0497 to 0.0136 min^{-1} (Figure 2c).

Analysis of the nonlabeled enzyme by ESMS showed one major species with molecular mass 67 943 (±6) Da (Figure 3a). Similar analysis of the inactivated enzyme revealed a single major component with molecular mass 68 159 (±6) Da, thus indicating a mass of increase of 216 Da (Figure 3b). This indicates stoichiometric labeling of the protein, since the mass increase corresponds well, within experimental error, to the 219-Da mass increase expected due to labeling with one molecule of **1**. Stoichiometrically labeled enzyme was incubated with 4 mM **1** for a further hour; however, ESMS analysis of an aliquot showed no further labeling, indicating that the bromoketone does not react indiscriminately. Thus, despite the fact that **1** binds only weakly to the active site initially, as evidenced by the absence of saturating kinetics at concentrations up to 25 mM, the mass

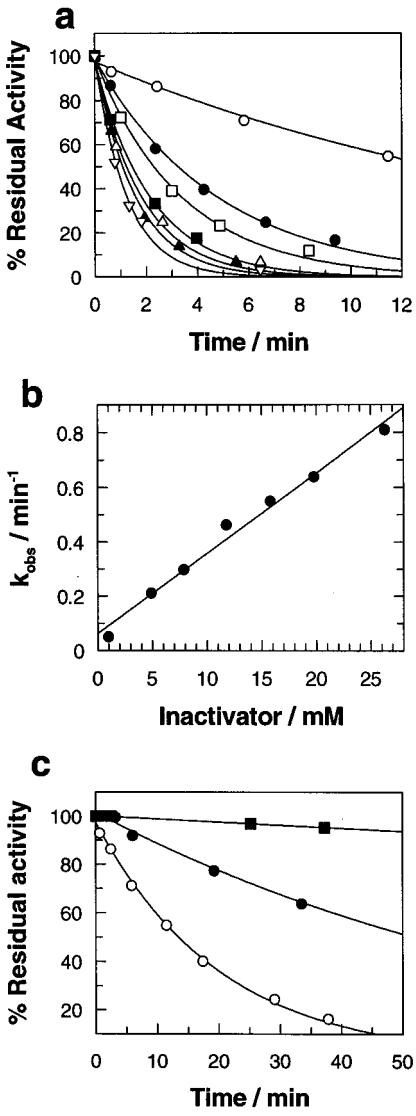


FIGURE 2: Inactivation of yeast α-glucosidase by **1**. (a) Plot of residual activity versus time at the following inactivator concentrations: (○), 0.98; (●), 4.9; (□), 7.9; (■), 11.8; (△), 15.8; (▲), 19.8; and (▽), 26.3 mM (activity ∝ rate of α-PNPG hydrolysis). (b) Plot of pseudo-first-order rate constants from (a) versus inactivator concentration. (c) Plot of residual activity versus time in the presence of (■) no inactivator; (●) 0.98 mM **1** and 58 μM deoxynojirimycin; and (○) 0.98 mM **1**.

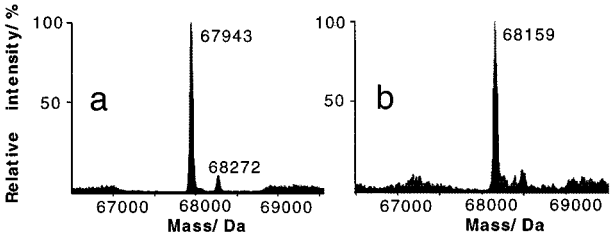


FIGURE 3: Electrospray mass spectrometry of yeast α-glucosidase. (a) Reconstructed mass spectrum of unlabeled enzyme; (b) reconstructed mass spectrum of inactivated, labeled enzyme.

spectrometric results and the active-site-directed protection afforded by deoxynojirimycin strongly suggest that **1** labels a single residue within the active site.

The identity of the labeled residue was determined by LC/MS analysis of a peptic digest of the labeled enzyme. Incubation of the enzyme for 30 min in the presence of 4 mM **1**, resulted in the loss of all enzyme activity. Prior to digestion of the enzyme, the excess inactivator was first removed from the sample by dialysis to avoid nonspecific reaction of the bromoketone with peptides. After 1 h at room temperature, LC/MS analysis of the intact labeled, dialyzed enzyme confirmed that there had been no observable loss of the label. Peptic digestion of the labeled enzyme gave a complex mixture of peptides, which was separated by microbore HPLC using the electrospray mass spectrometer as a detector in the LC/MS mode (Figure 4a). The labeled peptides were located within this chromatogram by comparison of the peptides present within digests of labeled and unlabeled enzyme, using the known mass difference between the labeled and unlabeled peptides as confirmation of the peptides of interest. All the peptides present in the labeled sample were also present in the unlabeled sample at comparable retention times, with the exception of two doubly charged peptides, *m/z* 745 and 694, which were observed only in the labeled sample. Figure 4b shows the mass spectra of peptides from the labeled enzyme digest, eluting with retention times between 25 and 27.5 min. The two doubly charged peptides, *m/z* 745 and 694, are clearly unique to the labeled enzyme digest and are absent in the digest from the unlabeled enzyme (Figure 4c). A small peak at *m/z* 747 exists in the unlabeled enzyme digest (Figure 4c) but is unrelated to the labeled peptide, *m/z* 745, present in the labeled enzyme digest. The doubly charged, unlabeled, counterparts, *m/z* 635.5 and 585 ($\Delta m/z$ = 109.5), were observed in the unlabeled enzyme digest. The former can be seen in Figure 4c, and the latter, which has a substantially different retention time (~21 min), was clearly seen in that region of the chromatogram. These signals correspond to unlabeled peptides of mass 1269 and 1168 Da, respectively, which increase by a mass of 219 Da when labeled with the inactivator **1**. Candidates for this peptide were identified by searching the amino acid sequence of the enzyme for all possible peptides with mass 1269 ± 2 Da and 1168 ± 2 Da. Twenty-three possible peptides were identified in each case.

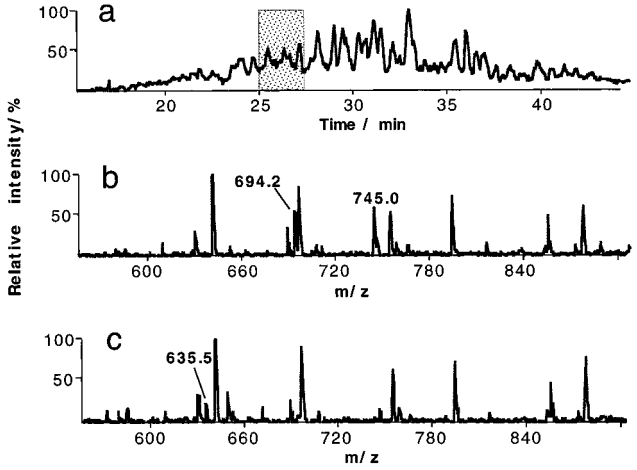


FIGURE 4: LC/MS analysis of labeled yeast α-glucosidase peptic digest. (a) Elution profile of labeled enzyme digest. (b) Mass spectrum of peptides with retention time 25−27.5 min, obtained from labeled enzyme digest. (c) Mass spectrum of peptides with retention time 25−27.5 min, obtained from unlabeled enzyme digest.

The identities of the labeled peptides were determined by tandem MS analysis as follows. The labeled peptide of *m/z*

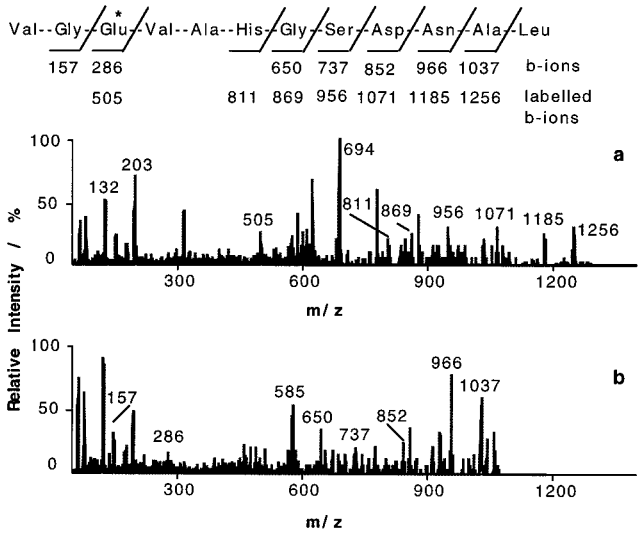


FIGURE 5: Tandem MS/MS daughter ion spectrum of (a) the C-mannoside-labeled peptide (*m*/*z* 694 in the doubly charged state) and (b) the corresponding unlabeled peptide (*m*/*z* 585 in the doubly charged state).

694 was first partially purified using the conditions used for the profile shown in Figure 4a. This partially purified mixture was then introduced into the mass spectrometer by flow injection, and the peptide of *m*/*z* 694 was selected in the first quadrupole. Collision-activated dissociation of this ion at increased collision gas energy in the second quadrupole produced a series of daughter ions, which were analyzed in the third quadrupole. These daughter-ion scans revealed specific fragments consistent with a peptide sequence of VGEVAHGSDNAL, as shown in Figure 5a. The peak at *m*/*z* 694 corresponds to the intact, doubly charged, labeled peptide, and, although the intact, singly charged peptide was not observed, singly charged fragments of this peptide were seen. Peaks at *m*/*z* 1256 (b11), 1185 (b10), 1071 (b9), 956 (b8), and 869 (b7) correspond to peptides resulting from loss of amino acids from the C-terminus, i.e., all five peptides in the series: VGEVAHGSDNA (1256) − VGEVAHG (869). Furthermore, the mass of all these peptides is 219 Da above the value for the unlabeled peptide, thus confirming that the label is present on these peptide fragments. Peptide fragments of *m*/*z* 505 (b3) and 157 (b2) were also observed and correspond to VGE (labeled) and VG (unlabeled). Clearly, since the daughter ions b11−b7 still retain the label, Ser-281 and Asp-282 cannot be labeled. More directly, since the daughter ion b3 also retains the label, Glu-276 *must* be the labeled residue. Several other fragments resulting from loss of residues from the N-terminus were also observed (y-series). The peptide fragments *m*/*z* 132, 203, and 883 were prominent peaks which correspond to L, AL, and VAHGSDNAL, all of which are unlabeled and, therefore, provide further evidence that the glutamic acid is the only labeled residue.

As a double check of this sequence, the corresponding unlabeled peptide was partially purified from the control enzyme digest and subjected to the same tandem MS/MS analysis (Figure 5b). A fragmentation pattern similar to that of the labeled peptide was seen, but with the peptides showing *m*/*z* values consistent with the absence of the label (*m*/*z* 219), as shown in the scheme at the top of Figure 5. This sequence is identical to that surrounding Glu-276 in the full protein sequence, deduced from the nucleotide sequence (sp P07265).

Further evidence to prove that Glu-276 is, indeed, the labeled residue was the alkaline lability of the linkage between the peptide and the label, consistent with an ester but not with an ether or amine linkage. The labeled peptide was isolated as described above and reacted with aqueous ammonia. Subsequent LC/MS analysis of this sample revealed the complete loss of the *m*/*z* 694 peak and the appearance of a new peak of *m*/*z* 585. This is consistent only with loss of the label from Glu-276 since bonds to the serine, and particularly the histidine, would be stable in the presence of ammonia. Furthermore, treatment of a sample of the labeled peptide with acidic methanol and subsequent MS analysis revealed the loss of the peak in this doubly charged peptide of *m*/*z* 694 and the appearance of a new peak of *m*/*z* 708. The increase of *m*/*z* 14 in this doubly charged peptide suggests the formation of two methyl esters. One must be at the carboxyl terminus and the other at a side chain carboxyl. Since there are two possible side-chain carboxyls, one must already be esterified.

The labeled *m*/*z* 745 peptide corresponding to the unlabeled peptide of mass 1269 Da was also partially purified and then subjected to tandem MS analysis. The fragments observed were compatible only with the sequence MRV-GEVAHGSDN, in which the label was attached to glutamic acid. A very similar fragmentation pattern was observed from the unlabeled peptide but with several fragments having *m*/*z* values consistent only with the absence of the label. The sequence of this peptide clearly overlaps almost completely with that of the 1168-Da peptide. Together with methyl esterification and aminolysis experiments, analogous to those described above, the label was determined to be bound to Glu-276.

CONCLUSIONS

The labeling studies conducted confirm that the residue labeled by this new bromoketone affinity label is Glu-276. The stoichiometry of the labeling and the protection against inactivation provided by the competitive inhibitor deoxynojirimycin indicate that the inactivation is, indeed, active-site-directed. The residue labeled, Glu-276, is completely conserved within enzymes of glycosidase family 13 and corresponds to Glu-233 of porcine pancreatic α-amylase and Glu-257 of *Bacillus circulans* 251 cyclodextrin glucanotransferase. Indeed, three-dimensional studies of α-amylases and cyclodextrin glucanotransferases, particularly in complexes with substrates or inhibitors, have implicated this residue as the acid catalyst. These results strongly support that conclusion.

Despite its apparently weak noncovalent binding to the active site, the α-bromoketone functionality is clearly efficient at selectively alkylating the acid/base catalyst. Since there has been a dearth of reagents suitable for this task with glycosidases, this class of compounds should find more widespread applications. Second-generation compounds will be designed to have improved noncovalent binding affinity to the active site. One improvement for glycosidases which preferentially bind glycosides with an equatorial substituent at C-2 would be to circumvent the problem of intramolecular reaction by use of 2-deoxy or 2-deoxy-2-fluoro C-glycosides

rather than the epimeric manno sugar. Crystal structures of family 13 enzymes with such compounds bound to the active site would provide more insight into the structural relationship between conserved acidic residues within the active site and further clarify their mechanistic roles.

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REFERENCES

1. McCarter, J., and Withers, S. G. (1994) *Curr. Opin. Struct. Biol. 4*, 885−897.
2. Sinnott, M. L. (1990) *Chem. Rev. 90*, 1171−1202.
3. Legler, G. (1990) Adv. Carbohydr. Chem. Biochem. 48, 319−385.
4. Mooser, G. (1992) in *The Enzymes* (Sigman, D. S., Ed.), 3rd ed., Vol. 20, pp 187−233, Academic Press Inc., New York.
5. Withers, S. G., and Aebersold, R. (1995) *Protein Sci. 4*, 361−372.
6. Febbrais, F., Barone, S. D., D′Auria, S., Rossi, M., and Nucci, R. (1997) *Biochemistry 36*, 3086−3075.
7. Gebler, J. C., Aebersold, R., and Withers, S. G. (1992) *J. Biol. Chem. 267*, 11126−11130.
8. Mackenzie, L. F., Brooke, G. B., Cutfield, J. F., Sullivan, P. A., and Withers, S. G. (1997) *J. Biol. Chem. 272*, 3161−3167.
9. Withers, S. G., Rupitz, K., and Street, I. P. (1988) *J. Biol. Chem. 263*, 7929−7931.
10. Withers, S. G., Warren, R. A. J., Street, I. P., Rupitz, K., Kempton, J. B., and Aebersold, R. (1990) *J. Am. Chem. Soc. 112*, 5887−5889.
11. McCarter, J., and Withers, S. G. (1996) *J. Am. Chem. Soc. 118*, 241−242.
12. McCarter, J., and Withers, S. G. (1996) *J. Biol. Chem. 271*, 6889−6894.
13. Tull, D., Miao, S., Withers, S. G., and Aebersold, R. (1994) *Anal. Biochem. 224*, 509−514.
14. Legler, G., and Bause, E. (1973) *Carbohydr. Res. 28*, 45−52.
15. Hoj, P. B., Condron, R., Traeger, J. C., McAuliffe, J. C., and Stone, B. (1992) *J. Biol. Chem. 267*, 25059−25066.
16. Naider, F., Bohak, Z., and Yariv, J. (1972) *Biochemistry 11*, 3202−3206.
17. Black, S., Laszlo, K., Tull, D., and Withers, S. G. (1993) *Carbohydr. Res. 250*, 195−202.
18. Keresztessy, Z., Kiss, L., and Hughes, M. A. (1994) *Arch. Biochem. Biophys. 314*, 142−152.
19. Schulman, M. L., Shiyan, S. D., and Khorlin, A. Y. (1976) *Biochim. Biophys. Acta 445*, 169−181.
20. Tull, T., Burgoyne, D. L., Chow, D. T., Withers, S. G., and Aebersold, R. (1996) *Anal. Biochem. 234*, 119−125.
21. Bombard, S., Maillet, M., and Capmau, M.-L. (1995) *Carbohydr. Res. 275*, 433−440.
22. Henrissat, B., and Bairoch, A. (1991) *Biochem. J. 280*, 309−316.
23. Henrissat, B., and Bairoch, A. (1993) *Biochem. J.* 293, 781−788.
24. Henrissat, B., and Bairoch, A. (1996) *Biochem. J. 316*, 695−696.
25. Neufeld, E. F. (1991) *Annu. Rev. Biochem. 60*, 257−280.
26. Daniel, P. F., Winchester, B., and Warren, C. D. (1994) *Glycobiology 4*, 551−556.
27. Bennet, J. K., Dembure, P. P., and Elsas, L. J. (1995) *Am. J. Med. Genet. 55*, 21−26.
28. Goss, P. E., Baker, M. A., Carver, J. P., and Dennis, J. W. (1995) *Clin. Cancer Res. 1*, 935−944.
29. Howard, S., He, S., and Withers, S. G. (1998) *J. Biol. Chem. 273*, 2067−2072.
30. Mooser, G., Hefta, S. A., Paxton, R. J., Shively, J. E., and Lee, T. D. (1991) *J. Biol. Chem. 266*, 8916−8922.
31. Mosi, R., He, S., Uitdehaag, J., Dijkstra, B. W., and Withers, S. G. (1997) *Biochemistry 36*, 9927−9934.
32. Winterhalter, R. (1976) Thesis (Diplom Biology), University of Cologne.
33. Caron, G., and Withers, S. G. (1989) *Biochem. Biophys. Res. Commun. 163*, 495−499.
34. Halazy, S., Danzin, C., Ehrhard, A., and Gerhart, F. (1989) *J. Am. Chem. Soc. 111*, 3484.
35. Svensson, B. (1994) *Plant Mol. Biol. 280*, 309−316.
36. Qian, M., Haser, R., Buisson, G., Duee, E., and Payan, F. (1994) *Biochemistry 33*, 6284−6294.
37. Knegtel, R. M. A., Strokopytov, B., Penninga, D., Faber, O. G., Rozeboom, H. J., Kalk, K. H., Dijkhuizen, L., and Dijkstra, B. W. (1995) *J. Biol. Chem. 49*, 29256−29264.
38. Nicolaou, K. C., Hwang, C.-K., and Duggan, M. E. (1989) *J. Am. Chem. Soc. 111*, 6682−6690.
39. Allevi, P., Anastasia, M., Ciuffreda, P., Fiecchi, A., and Scala, A. (1987) *J. Chem. Soc., Chem. Commun.*, 1245−1246.
40. Allevi, P., Anastasia, M., Ciuffreda, P., Fiecchi, A., and Scala, A. (1987) *J. Chem. Soc., Chem. Commun.*, 101−102.
41. Postema, M. H. D. (1992) *Tetrahedron 48*, 8545−8599.
42. Ermert, P., Vasella, A., Weber, M., Rupitz, K., and Withers, S. G. (1993) *Carbohydr. Res. 250*, 113−128.

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