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Hydrolysis of α- and β-D-glucosyl fluoride by individual glucosidases: new evidence for separately controlled "plastic" and "conserved" phases in glycosylase catalysis *

Hirokazu Matsui [1], Yoshimasa Tanaka [2], Curtis F. Brewer [3], John S. Blanchard [4] and Edward J. Hehre **

From the Departments of Microbiology and Immunology, Molecular Pharmacology, and Biochemistry, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461 (USA)

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ABSTRACT

α-Glucosidases from sugar beet seed and ungerminated rice catalyzed the hydrolysis of β-D-glucopyranosyl fluoride to form α-D-glucose. The reactions were slow, with $V/K = 11–15 \times 10^{-3}$ or ~1–2% of that for hydrolysis of *p*-nitrophenyl α-D-glucopyranoside, but were not due to any impurity in the substrate or to contaminating β-glucosidase or glucoamylase. Furthermore, almond β-glucosidase promoted hydrolysis of α-D-glucosyl fluoride to form β-D-glucose at an exceedingly low rate, $V/K = 4 \times 10^{-4}$. This weak reaction did not stem from any impurity in the substrate or to contamination with α-glucosidase or glucoamylase, but it was partly (~20%) attributable to a trace of accompanying trehalase. That all three glucosidases acted upon both α- and β-D-glucosyl fluoride, albeit at low efficiency with the disfavored anomer, reflects the previously demonstrated ability of each enzyme's catalytic groups to respond flexibly to substrates of different types. That the disfavored D-glucosyl fluoride in each case was converted into a product of the same configuration as from enitols or favored D-glucosyl substrates provides additional evidence for the two-step nature of the chemical mechanisms of glucosidases, in which the stereochemistry of water attack on the enzyme-stabilized oxocarbonium ion is strictly maintained, regardless of the initial anomeric configuration of the substrate.

INTRODUCTION

α and β-Glucosidases have long been considered to act only on α- (or only on β-) D-glucosylic substrates and to be "retaining enzymes". However, the frailty of

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[1] Research Associate in Microbiology and Immunology, on leave from the Department of Agricultural Chemistry, Hokkaido University, Sapporo, Japan.

[2] Visiting Scholar in Microbiology and Immunology, on leave from the doctoral studies program, Department of Agricultural Chemistry, Hokkaido University, Sapporo, Japan.

[3] Affiliated with the Department of Molecular Pharmacology.

[4] Affiliated with the Department of Biochemistry.

** Corresponding author at: Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, N.Y. 10461, USA.

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this traditional view has been made evident through studies of the stereochemical course of reactions catalyzed by various glucosidases with enolic glycosyl donors. In all such reactions for which the relevant data are available, the particular α- or β-glucosidase catalyst has been found to protonate the substrate [D-glucal[1,2] or (*Z*)-3,7-anhydro-1,2-dideoxy-D-*gluco*-oct-2-enitol ("D-*gluco*-octenitol")[3]] from a direction opposite that assumed for protonating its D-glucosidic substrates. At the same time, all reactions with D-glucal[1,2], 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol ("D-*gluco*-heptenitol")[4,5], or "D-*gluco*-octenitol[3] for which the steric outcome has been determined have yielded hydration and/or transfer products of the same anomeric configuration as formed by the particular glucosidase from D-glucosylic substrates. The conservation of specific steric outcome in reactions with enolic glycosyl donors (which have no α- or β-anomeric configuration to retain) reveals that each tested glucosidase has the means to create a specific configuration from substrates with a prochiral anomeric carbon. Comparable findings have been reported in studies of enitol utilization by a variety of glycosylases in addition to those cited here for glucosidases, leading us to propose[2,3,6] that glycosylase catalysis consists of two separate parts: a plastic phase in which functionally flexible catalytic groups provide for reactivity with substrates of different types, and a stereochemically conserved phase, separately controlled by protein structures that limit the direction of approach and orientation of water or other acceptors to the reaction center.

We recently reported independent evidence for this unconventional view[7], in that a crystalline α-glucosidase from *Aspergillus niger*, free of detectable activity for β-D-glucosides, was found to hydrolyze β-D-glucosyl fluoride (with low efficiency compared to good substrates) to form α-D-glucose. The importance of learning whether such behavior is demonstrable beyond this initial result with a single α-glucosidase preparation prompted the present study.

EXPERIMENTAL

General procedures.—Thin-layer chromatography (TLC) was carried out with plates of Silica Gel G (Analtech) developed with 5:1 EtOAc–EtOH. Optical absorbance was measured with a Gilford Stasar II or Model 120 spectrophotometer. D-Glucose was determined using a D-glucose oxidase–4-aminoantipyrine reagent ARII (Wako, Tokyo) in 2 M tris(hydroxymethyl)aminomethane hydrochloride buffer[2,8], pH 7.0.

Anomeric D-glucosyl fluorides.— D-Glucopyranosyl α- and β- fluorides, prepared by catalytic deacetylation of the analytically pure crystalline tetraacetates[7,9] were purified on columns of dry Silica Gel 60 developed with 5:1 EtOAc–EtOH. On TLC the α-D-glucosyl fluoride showed a single spot at R_f 0.58; the β anomer, a single spot at R_f 0.66. The ^{1}H NMR spectra (see Results) confirmed the assigned structures and showed no sign of the other anomer in either case.

Concentration of fluoride ion was measured with a specific fluoride-ion electrode and Orion Ionalyzer 910, standardized concurrently with NaF. Test and control solutions were examined after addition of 1.5–3 vol of 1 M NaOAc buffer (pH 5.2) containing 1 M NaCl.

β-Maltose monohydrate was a laboratory preparation, free from glucose and higher maltosaccharides. Amylopectin was prepared from potato starch by Schoch's[10] pentanol fractionation method; *p*-nitrophenyl α-D-glucopyranoside, *p*-nitrophenyl β-D-glucopyranoside, α,α-trehalose dihydrate, salicin, and cellobiose were crystalline reagents.

Fractionation and characterization of enzymes.—Columns (2.5 × 95 cm) of Sephadex G-100 (Pharmacia) were equilibrated with 0.05 M acetate buffer at pH 4.8, and developed with the same buffer. Columns (0.9 × 20 cm) of CM-Sepharose (Pharmacia) were equilibrated with 0.05 M acetate buffer of pH 6.2; gradient elution was carried out with buffer containing 0–0.8 M NaCl.

Protein concentrations were estimated from absorbance measurements at 280 nm, using $E^{1\%}_{1cm}$ values of 10.8 for rice α-glucosidase[11], 13.6 for sugar beet seed α-glucosidase[12], and 9.38 for almond β-glucosidase (present study), obtained with solutions made with weighed, lyophilized enzyme.

Activities of the α-glucosidase preparations with maltose, amylopectin and *p*-nitrophenyl (PNP) β-D-glucopyranoside were assayed[7] at 30°C and pH 4.8. For β-glucosidase preparations, assays with PNP β-D-glucoside were made by mixing 60 μL of enzyme with 120 μL of 0.05 M acetate buffer (pH 4.8) containing 0.01% Triton X-100, and 120 μL of 10 mM PNP β-D-glucoside. After incubation (30°C, 10 min) 1.5 mL of M carbonate buffer of pH 10.0 was added; finally, the optical density at 420 nm was measured against a *p*-nitrophenol standard. Rates of glucose formation from maltose, amylopectin, and α,α-trehalose were determined for incubated (30°C, 30 min) test mixtures containing 40 μL enzyme, 60 μL water, 200 μL of the pH 4.8 buffer, and 200 μL of 5 $mg.mL^{-1}$ substrate.

To gauge the ability of each glucosidase (before final purification) to promote fluoride release from either anomer of D-glucosyl fluoride, mixtures comprising 40 mM of α- (or β-) D-glucosyl fluoride and an appropriate concentration of enzyme in pH 4.8 acetate buffer were incubated (30°C, 12 min) concurrently with a control of the glucosyl fluoride in buffer alone. After dilution with 1 M NaCl–1M acetate buffer of pH 5.2, the fluoride-ion concentration in each mixture was measured with a specific electrode; the level in the test mixture, corrected for that in the buffer control (< 25% of the test value), allowed calculation of the enzymically catalyzed fluoride release rate.

Glucosidases.—Rice α-glucosidase (Type V, Sigma) was found to catalyze hydrolysis of maltose and PNP β-D-glucoside at rates of 19.0 and 0.024 $\mu mol.min^{-1}.mg^{-1}$, respectively. For purification, 30.6 mg of the enzyme was fractionated on successive columns of Sephadex G-100, CM-Sepharose, and Sephadex G-100. In each case, individual fractions were assayed against maltose and PNP β-D-glucoside; those with the highest specific activity for maltose were

retained. The finally recovered enzyme (6.0) mg hydrolyzed maltose and PNP β-D-glucoside at rates of 37.2 and 0.00041 μmol.min^{-1}.mg^{-1}, respectively; amylopectin was hydrolyzed at 2.27 μmol glucose min^{-1}.mg^{-1}; salicin and cellobiose were not detectably catalytically processed.

α-Glucosidase from sugar beet seeds, homogeneous on ultracentrifugation and disc electrophoresis, was kindly provided by Professor Seiya Chiba (Hokkaido University). It was reported to hydrolyze maltose and soluble starch at similar rates to form D-glucose as sole product[12,13], and to yield α-D-glucose from phenyl α-maltoside[14]. A 15-mg sample was fractionated on two successive columns of Sephadex G-100 to remove a small trace of contaminating β-glucosidase. Properties of the beet α-glucosidase preparation (4.64 mg) recovered for use are described under Results.

Sweet almond β-glucosidase (Type 1, Sigma) was found to hydrolyze salicin at the rate of 17.2 μmol.min^{-1}.mg^{-1}; α,α-trehalose and maltose at rates of 0.0040 and 0.0023 μmol.min^{-1}.mg^{-1}, respectively. Rigorous tests showed the preparation to be without detectable activity for potato amylopectin or dextran T2000 (Pharmacia). To further purify the β-glucosidase, 51 mg was fractionated on Sephadex G-100 (four 1.5 × 45 cm columns). Fractions were assayed against salicin, α,α-trehalose, and maltose and those of highest activity for salicin were pooled. The recovered enzyme (9.3 mg) hydrolyzed PNP β-D-glucoside and salicin at rates of 62.5 and 25.7 μmol.min^{-1}.mg^{-1}, respectively; α,α-trehalose and maltose at rates of 0.0018 and 0.0031 μmol.min^{-1}.mg^{-1}, respectively. This preparation was used to examine the steric course of α-D-glucosyl fluoride hydrolysis by ^{1}H NMR spectroscopy, following which the enzyme was dialyzed and refractionated on Sephadex G-100. The resulting preparation, described in the Results section, was used to evaluate the possible contribution of trehalase to the preparation's activity with α-D-glucosyl fluoride.

^{1}H NMR spectroscopy.—Spectra were recorded in D_2O at pD 5.2 and 25°C using 200-, 400-, or 500-MHz Varian spectrometers. Buffered D_2O at pD 5.2 was prepared with 0.05 M acetate-d_4, NaOD, and 99.8 atom% D_2O. Chemical shifts (ppm) refer to sodium 4,4-dimethyl-4-silapentanesulfonate.

RESULTS

In initial tests, purified sugar beet seed α-glucosidase[12,13], kindly furnished by Professor S. Chiba, and commercial samples of rice α-glucosidase and almond β-glucosidase, were found to catalyze the hydrolysis of both α- and β-D-glucosyl fluoride when incubated with 40 mM of either substrate at 30°C and pH 4.8. In each case, the rate of fluoride release observed with the "wrong" anomer (corrected for hydrolysis by buffer) was low, ≤ 0.15 μmol.min^{-1}.mg^{-1}.

As a first step in investigating these slow reactions, each enzyme was further fractionated in order to improve its specific activity and to detect and remove traces of accompanying carbohydrases. Fig. 1 illustrates, for sugar beet seed

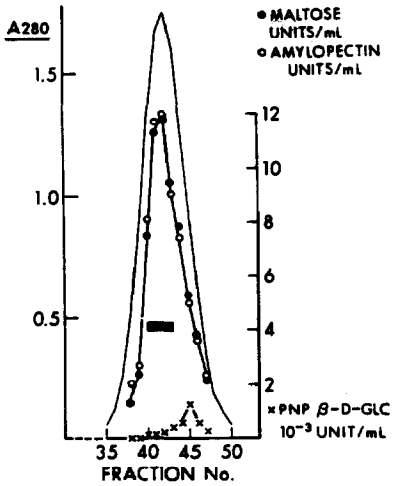


Fig. 1. Second of two successive fractionations of purified sugar beet seed α-glucosidase on Sephadex G-100. Individual fractions assayed for rates of glucose formation from maltose (•), potato amylopectin (○), and PNP β-D-glucoside (×). Shaded block indicates the fractions recovered for experimental use.

α-glucosidase, the second of two fractionations on Sephadex G-100. A common peak of glucose-forming activity from maltose and amylopectin is found, plus a mostly separable minor peak (shown 1000-fold enlarged) of activity for PNP β-D-glucosde. The pool of α-glucosidase fractions recovered for use hydrolyzed maltose and amylopectin at rates of 8.46 and 8.42 μmol.min^{-1}.mg^{-1}, respectively; PNP β-D-glucoside at 0.00023 μmol.min^{-1}.mg^{-1}. ^{1}H NMR spectra of incubated enzyme–amylopectin and enzyme–α-glucosyl fluoride test-mixtures (not illustrated) showed the enzymic reaction product to be α-D-glucose, indicating the essential absence of glucoamylase in the sugar beet enzyme preparation.

The sample of rice α-glucosidase (Sigma), which initially had far too much accompanying β-glucosidase activity (0.024 unit.mg^{-1}) for the present use, was purified by successive fractionations on Sephadex G-100, CM-Sepharose, and Sephadex G-100 columns. The final preparation hydrolyzed maltose, amylopectin, and PNP β-D-glucosde at rates of 37.2, 2.27, and 0.00041 μmol.min^{-1}.mg^{-1}, respectively. Almond β-glucosidase (Sigma), further purified by fractionation on Sephadex G-100, hydrolyzed PNP β-glucoside and salicin at rates of 62.5 and 25.7 μmol.min^{-1}.mg^{-1}, respectively; maltose and α,α-trehalose at 0.0031 and 0.0018 μmol.min^{-1}.mg^{-1}, respectively.

^{1}H NMR. analysis of reactions promoted with the "wrong" D-glucosyl fluoride anomer.—To learn the stereochemical course of the reactions catalyzed by the purified α-glucosidases with β-D-glucosyl fluoride, and by the purified almond β-glucosidase with α-D-glucosyl fluoride, each enzyme preparation was exhaustively dialyzed against three changes of D_2O at pD 5.2 (during 48 h at 6°C) to replace exchangeable protons. Test and control mixtures in each case comprised 30 μmol of the desired D-glucosyl fluoride anomer, freshly dried under vacuum from

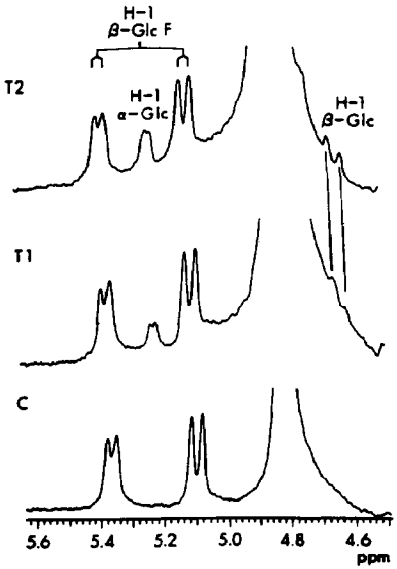


Fig. 2. Hydrolysis of β-D-glucosyl fluoride by purified rice α-glucosidase. Downfield sections of ^1H NMR spectra in D_2O, recorded at 200 MHz. T1, test mixture of 7.5 mg.mL^{-1} α-glucosidase plus 40 mM β-D-glucosyl fluoride in D_2O at pD 5.2, incubated at 22°C for 40 min; T2, same mixture incubated at 22°C for 75 min; C, control of 40 mM β-D-glucosyl fluoride in the pD 5.2 buffer, incubated at 22°C for 30 min.

stock solution in methanol, taken up in 0.75 mL of enzyme solution in D_2O buffered at pD 5.2 (or in 0.75 mL of the pD 5.2 buffer alone). Mixtures were immediately transferred to an NMR tube and examined at several periods of incubation.

Fig. 2 illustrates spectra recorded at 200 MHz for a mixture of rice α-glucosidase (7.5 mg/mL) and 40 mM β-D-glucosyl fluoride incubated at 22°C. A control spectrum (C) of substrate in buffer (22°C, 30 min) shows the H-1 (dd) resonance of the β-D-glucosyl fluoride centered at 5.24 ppm, plus a minute signal at 5.22 ppm due to H-1 of α-D-glucose. Spectrum T1, of the test digest after 40 min incubation, shows a prominent doublet at 5.22 ppm $J_{1,2}$ 8.4 Hz representing the H-1 of α-D-glucose, and a considerably smaller doublet at 4.63 ppm, $J_{1,2}$ 8.4 Hz, assignable to the H-1 of β-D-glucose. Both resonances are increased by 75 min (spectrum T2) at which time integration showed the α anomer to constitute 70% of the total glucose (twice the proportion expected for equilibrated D-glucose), and the substrate to be 22% hydrolyzed. The findings clearly show that α-D-glucose is the primary enzymic reaction product; also, that it arose from β-D-glucosyl fluoride and not from any trace impurity in the latter.

Fig. 3 illustrates spectra recorded at 400 MHz for the reaction catalyzed by the purified sugar beet α-glucosidase preparation (5.9 mg/mL) with 40 mM β-D-glucosyl fluoride. Spectrum C1, representing a substrate–buffer control held at 25°C for 20 min, show the substrate's H-1 resonance; just detectable H-1 signals of α-

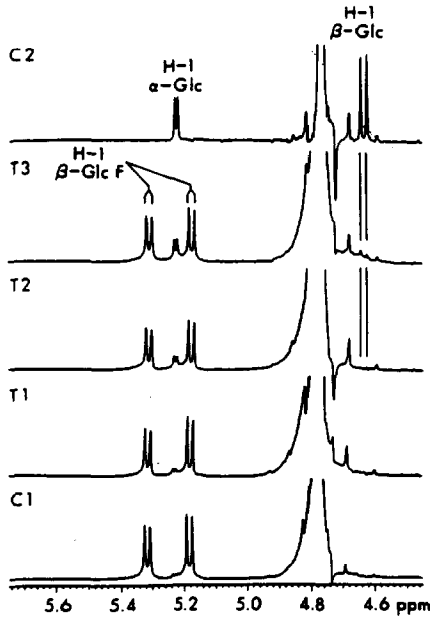


Fig. 3. Hydrolysis of β-D-glucosyl fluoride by sugar beet α-glucosidase. Downfield sections of ^{1}H NMR spectra in D_2O recorded at 400 MHz. T1–T3, test mixture of 5.9 mg.mL^{-1} of purified sugar beet seed α-glucosidase plus 40 mM β-D-glucosyl fluoride in D_2O at pD 5.2, incubated at 25°C for 7, 20, and 38 min, respectively; C1, control of 40 mM β-D-glucosyl fluoride in the pD 5.2 buffer incubated at 25°C for 20 min; C2, control of 10 mM anomerically equilibrated D-glucose in D_2O.

and β-D-glucose at 5.23 and 4.64 ppm, respectively, and no sign of the H-1 resonance of α-D-glucosyl fluoride (compare Fig. 4C). Spectra T1–T3 represent the α-glucosidase–β-D-glucosyl fluoride test-mixture incubated at 25°C for 7, 20, and 38 min, respectively. A clear doublet at 5.23 ppm ($J_{1,2}$ 3.3 Hz) of the H-1 of α-D-glucose is found at 7 min (T1). Its intensity, already greater than in the control at 20 min, increases progressively with time. In contrast, the H-1 resonance of β-D-glucose at 4.64 ppm is barely detectable in the 7 and 20 min spectra. Only by 38 min (spectrum T3) does the signal appear appreciably larger than in the β-D-glucosyl fluoride–buffer control (C1). Integration of the H-1 resonances in spectrum T3 indicates that ~15% of the β-D-glucosyl fluoride substrate was hydrolyzed by 38 min, with α-D-glucose accounting for ~80% of total D-glucose. Anomerically equilibrated D-glucose has ~36% α-D-glucose (spectrum C2). These data establish that α-D-glucose is the primary enzymic product and that it arises from β-D-glucosyl fluoride and not from a contaminant thereof.

Fig. 4 shows spectra at 500 MHz for a test mixture of almond β-glucosidase (8.0 mg/mL) and 40 mM α-D-glucosyl fluoride (spectra T1–T3). A control spectrum (C) of substrate in the pD 5.2 buffer, incubated for 30 min, shows the H-1 of α-D-glucosyl fluoride centered at 5.72 ppm with $J_{1,2}$ 2.8 Hz and $J_{1,F}$ 53.5 Hz. No sign of the H-1 of β-D-glucosyl fluoride (dd, centered at 5.25 ppm) or those of α- or β-D-glucose is evident. In the test mixture at 7 min (spectrum T1) a new clear

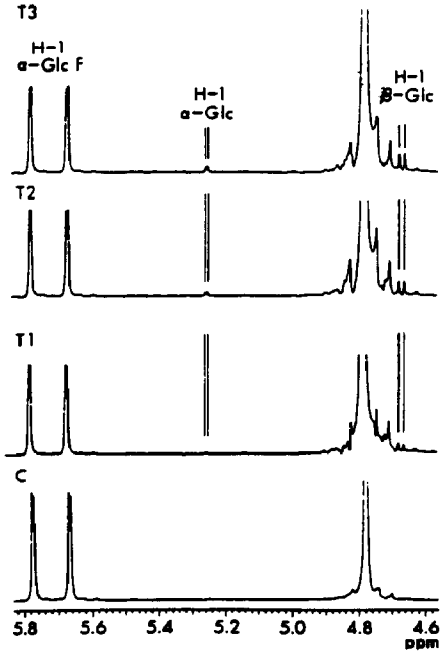


Fig. 4. Hydrolysis of α-D-glucosyl fluoride by almond β-glucosidase. Downfield sections of ^{1}H NMR spectra recorded in D_2O at 500 MHz. T1–T3, test mixture of 8.0 mg.mL^{-1} of purified almond β-glucosidase plus 40 mM α-D-glucosyl fluoride in D_2O buffered at pD 5.2, incubated at 25°C for 7, 28, and 41 min, respectively; C, control of 40 mM α-D-glucosyl fluoride in the pD 5.2 buffer, incubated at 25°C for 30 min.

doublet is present at 4.67 ppm ($J_{1,2}$ 8 Hz) representing H-1 of β-D-glucose, together with a barely detectable signal at 5.26 ppm referable to H-1 of α-D-glucose. Both doublets increase with time (spectra T2 and T3 at 28 and 41 min, respectively), with that of the β-D-glucose remaining dominant at each period. By integrating the H-1 resonances in each spectrum the β-anomer was estimated to comprise 85% of all D-glucose at 7 min, 77% at 28 min, and 69% at 41 min — in all cases above the 64% expected in anomerically equilibrated D-glucose (compare, Fig. 3, spectrum C2). Integration further showed that 12% of the α-D-glucosyl fluoride substrate had been hydrolyzed by 41 min.

Evaluation of the role of accompanying enzymes in the observed reactions.—The ^{1}H NMR findings show clearly that the rice and sugar beet seed α-glucosidase preparations hydrolyzed β-D-glucosyl fluoride to form α-D-glucose, and that the almond β-glucosidase preparation hydrolyzed α-D-glucosyl fluoride to form β-D-glucose. The minute β-glucosidase activity detected in the rice and sugar beet α-glucosidase preparations could only have accounted for some of the low proportion of β-D-glucose found in the digests with β-D-glucosyl fluoride. The considerable glucose-forming activity from amylopectin shown by the rice and, especially, sugar beet seed α-glucosidase raised the question of possible contamination by glucoamylase. ^{1}H NMR spectra (not illustrated) of mixtures of the purified rice

α-glucosidase (14 μg/mL) or sugar beet seed α-glucosidase (40 μg/mL) plus 40 mM α-D-glucosyl fluoride in D_2O at pD 5.2 showed a large H-1 resonance signal of α-D-glucose, and barely detectable H-1 resonance of β-D-glucose, after brief (7 min) incubation at 25°C. These results exclude any significant contamination of either α-glucosidase preparation by glucoamylase which, in any case, produces β-D-glucose from β-D-glucosyl fluoride[9]. Trace contamination could only have contributed to the small proportion of β-D-glucose found in the spectra of Figs. 2 and 3.

With regard to the finding (Fig. 4) that α-D-glucosyl fluoride was converted to β-D-glucose by the β-glucosidase preparation, the latter's minute level of activity with maltose presents no problem since α-glucosidases produce α-D-glucose from α-D-glucosyl fluoride as shown by others[15] and noted here. On the other hand, special attention was given to the question of the degree to which the reaction observed in Fig. 4 might be due to the trace of trehalase found to accompany the β-glucosidase. Repeated assays had shown the activity of this enzyme preparation for α,α-trehalose to be extremely low, 0.0018 $\mu mol.min^{-1}.mg^{-1}$. However, as reported in earlier studies[16–18], trehalase hydrolyzes 20–40 mM α-D-glucosyl fluoride very rapidly (2 to 2.5 times faster than α,α-trehalose) to form β-D-glucose.

To examine this question, the β-glucosidase used in the ^1H NMR experiment of Fig. 4 was dialyzed free of reaction components and rechromatographed on Sephadex G-100. Fractions with the highest activity for PNP β-D-glucoside relative to α,α-trehalose were pooled and concentrated to give a solution assaying 42.8 $unit.mL^{-1}$ for PNP β-D-glucoside and 0.0012 $unit.mL^{-1}$ for trehalose. A later-eluted fraction showing a far lower ratio of β-glucosidase/trehalase activities (2.0 $unit.mL^{-1}$ with PNP β-D-glucoside and 0.0012 $unit.mL^{-1}$ with α,α-trehalose) was also recovered. A comparison was made of the two preparations, which had equivalent trehalase activity but much different β-glucosidase activities, by incubating each (60 μL) with 2.4 μmol of α-D-glucosyl fluoride for 4 h at 30°C, along with a substrate–buffer control at pH 4.8. The fluoride-ion concentration of each mixture, determined with a specific ion electrode, showed that the α-D-glucosyl fluoride was 8.8% hydrolyzed by the enzyme of high β-glucosidase content (corrected for hydrolysis in buffer); 1.8% hydrolyzed by the enzyme of low β-glucosidase content. The trehalase impurity thus accounts for ~ 20% of the observed activity of the purified almond β-glucosidase preparation with α-D-glucosyl fluoride. β-Glucosidase itself, however, is clearly responsible for most of the reaction catalyzed with this substrate.

Kinetic parameters.—Table I lists V and K values determined for initial stage reactions catalyzed by the three enzymes with each D-glucosyl fluoride anomer and the appropriate PNP D-glucoside. The rice and sugar beet seed α-glucosidases were generally similar in reactivity. With these two enzymes, α-D-glucosyl fluoride was by far the best substrate, with V/K values 25 to 100 times those for PNP α-D-glucoside, and 2700 to 5600 times those for β-D-glucosyl fluoride, a poor substrate with a much higher steady-state K_m value.

TABLE I

Reactivity of glucosidases with disfavored vs. preferred substrates

Glucosidase	Substrate [a]	V (μmol/min.mg)	K (mM)
Rice, α-	β-D-glucosyl F	0.83 ± 0.05	79.3 ± 7.2
	PNP α-D-glucoside	1.35 ± 0.04	2.35 ± 0.21
	α-D-glucosyl F	66.5 ± 1.25	1.13 ± 0.04
Beet, α-	β-D-glucosyl F	0.40 ± 0.03	27.3 ± 5.1
	PNP α-D-glucoside	4.07 ± 0.12	2.67 ± 0.16
	α-D-glucosyl F	95.8 ± 2.0	2.44 ± 0.23
Almond, β-	α-D-glucosyl F	0.15 ± 0.03	365.0 ± 91.0
	PNP β-D-glucoside	56.6 ± 1.5	4.6 ± 0.3
	β-D-glucosyl F	199.0 ± 15.0	28.3 ± 5.1

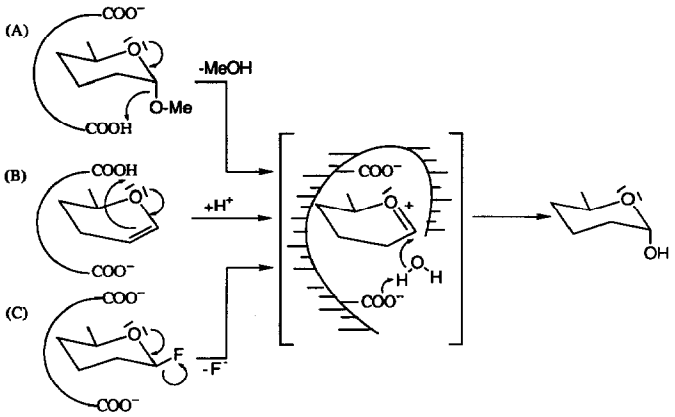
[a] PNP = *p*-nitrophenyl; pyranose forms throughout.

Relative to the activity of the two α-glucosidases with β-D-glucosyl fluoride (V/K 0.011, 0.015), the hydrolysis of α-D-glucosyl fluoride by the almond β-glucosidase (V/K 0.00042) is an exceeding weak reaction with a rate constant four orders of magnitude smaller than for the hydrolysis of β-D-glucosyl fluoride and PNP β-D-glucoside.

DISCUSSION

The ability of rice and sugar beet seed α-glucosidases and almond β-glucosidase to act on both α- and β-D-glucosyl fluoride, slowly hydrolyzing the "wrong" anomer with inversion, extends our initial finding that crystalline *Aspergillus niger* α-glucosidase catalyzes the conversion of β-D-glucosyl fluoride to form α-D-glucose[7]. The ability of each enzyme not only to act (albeit with low efficiency) on a substrate of the wrong configuration, but to yield a product of the same configuration as from "normal" substrates, strongly supports a model of glycosylase catalysis[2,3,6,19] distinct from the conventional paradigm and the associated generalization that α- and β-glycosidases are absolutely dependent on substrates of α- (or of β-) anomeric configuration.

A model is shown in Scheme 1 which includes the mechanistic features required to account for the stereochemical course of reactions catalyzed by these α-glucosidases with substrates of three different anomeric types: (A) methyl α-D-glucopyranoside, to represent α-D-glucosylic substrates; (B) D-glucal, representing prochiral (enolic) substrates; (C) β-D-glucosyl fluoride, the only presently known substrate of β configuration for α-glucosidases. We show these α-glucosidases possessing two carboxyl groups at the active site as in the case of hen's egg lysozyme, although the model does not depend on the chemical identity of the acidic and basic groups. Further, we suggest that substrates A–C bind with essentially the same orientation relative to these groups.



Scheme 1.

In the initial chemical event we envisage one of the lone pairs on the pyranose ring oxygen atom assisting in the cleavage of the C-1–O or C-1–F[20] bonds or in the stereospecific protonation[1–3] of C-2. Each of these events leads to a comparable oxocarbonium ion intermediate or transition state whose formation may be electrostatically stabilized by the presence of the negatively charged carboxylates. The catalytic flexibility of glucosidase function is illustrated in this variety of reactions with substrates of different configuration at C-1. A second common catalytic step follows in which the oxocarbonium ion is attacked by an incoming water molecule. This step, in contrast to the first, occurs with a conserved stereochemistry to yield a product of α configuration in all cases. We propose that this occurs by the protein molecule providing for the exclusive orientation of the water molecule and an appropriately positioned base such as the ionized carboxyl shown in Scheme 1.

The finding that almond β-glucosidase yields a β-anomeric product from α-D-glucosyl fluoride, as it does from β-D-glucosidic and enolic substrates[1,2], parallels the situation found for the α-glucosidases. More broadly, the present findings with glucosidases extend the evidence obtained with a variety of glycosylases[6,19,21] indicating that product configuration is the most strictly controlled feature in glycosylase catalysis. This provides a means of classifying glycosylases by stereochemical behavior * without implying a determinative relationship between the anomeric configuration of substrates and products. A subdivision into enzymes yielding hydrolytic or transfer products exclusively of α-D configuration, those yielding products exclusively of β-D configuration, and those yielding α-D products with certain acceptors and β-D products with others, would be in full accord with presently available information.

* The characterization of glycosylases as "retaining" or "inverting" enzymes[22] has practical usefulness in the context of reactions catalyzed with glycosidic substrates. However, these labels lack full generality since enzymes of both kinds also catalyze reactions having a different stereochemistry than the terms indicate.

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