Carbohydrate Research, 198 (1990) 123–132
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

HYDROLYSIS OF β-D-GLUCOPYRANOSYL FLUORIDE TO α-D-GLUCOSE CATALYZED BY *Aspergillus niger* α-D-GLUCOSIDASE*

EDWARD J. HEHRE, HIROKAZU MATSUI†, AND CURTIS F. BREWER

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

(Received July 12th 1989; accepted for publication, September 12th, 1989)

ABSTRACT

Aspergillus niger α-D-glucosidase, crystallized and free of detectable activity for β-D-glucosides, catalyzes the slow hydrolysis of β-D-glucopyranosyl fluoride to form α-D-glucose. Maximal initial rates, V, for the hydrolysis of β-D-glucosyl fluoride, *p*-nitrophenyl α-D-glucopyranoside, and α-D-glucopyranosyl fluoride are 0.27, 0.75, and 78.5 μmol.min^{-1}.mg^{-1}, respectively, with corresponding V/K constants of 0.0068, 1.44, and 41.3. Independent lines of evidence make clear that the reaction stems from β-D-glucosyl fluoride and not from a contaminating trace of α-D-glucosyl fluoride, and is catalyzed by the α-D-glucosidase and not by an accompanying trace of β-D-glucosidase or glucoamylase. Maltotriose competitively inhibits the hydrolysis, and β-D-glucosyl fluoride in turn competitively inhibits the hydrolysis of *p*-nitrophenyl α-D-glucopyranoside, indicating that β-D-glucosyl fluoride is bound at the same site as known substrates for the α-glucosidase. Present findings provide new evidence that α-glucosidases are not restricted to α-D-glucosylic substrates or to reactions providing retention of configuration. They strongly support the concept that product configuration in glycosylase-catalyzed reactions is primarily determined by enzyme structures controlling the direction of approach of acceptor molecules to the reaction center rather than by the anomeric configuration of the substrate.

INTRODUCTION

Until recently, α-D-glucosidases [E.C. 3.2.20] were assumed to act only on α-D-glucosylic substrates and to catalyze reactions with water or other external cosubstrates that proceed with retention of configuration. However, α-glucosidases

*Supported by Research Grants GM-25478 (to E.J.H.) and CA-16054 (to C.F.B.) from the National Institutes of Health; Core Grant P30 CA-13330 from the National Institutes of Health; NMR Instrumentation Grants I-S10 RR02309 from the National Institutes of Health and DMB-8413723 from the National Science Foundation.

†Research Associate in Microbiology and Immunology, on leave from the Departmental of Agricultural Biochemistry, Hokkaido University, Sapporo, Japan.

0008-6215/90/$ 03.50

of various biological origins are now known to also catalyze hydration and glycosyl-transfer reactions with enolic glycosyl donors lacking α- or β-anomeric configuration, for instance D-glucal[1,2], D-*gluco*-heptenitol* [3,4] and D-*gluco*-octenitol* [5]. In every instance where the stereochemical outcome has been determined (twelve reactions involving four different α-D-glucosidases) the product configuration created *de novo* from the enolic substrate was found to be *alpha*. Thus the steric outcome of reactions of α-glucosidases with prochiral glycosyl donors consistently matches that of reactions with α-D-glucosylic substrates. Furhermore, substantial evidence of the same kind exists from studies[2,5,6] with various other glycosylases acting on enolic glycosyl donors.

The long-accepted view of glycosidase specificity (*e.g.*, ref. 7) is that there is an absolute dependence on the anomeric configuration of the glycosyl bond attacked; that glycosidases show the same specificity for glycosyl fluorides as for glycosides[8]. We have since demonstrated, however, that certain glycosylases which hydrolyze glycosidic substrates with inversion of configuration catalyze stereochemically complementary reactions with the α and β anomers of the appropriate glycosyl fluoride. Beta amylase reacts with both α- and β-maltosyl fluoride[9]; glucoamylase, glucodextranase, and trehalase utilize both anomers of D-glucosyl fluoride[10–12]; *Bacillus pumilus* β-D-xylosidase acts on both α- and β-D-xylosyl fluoride[13]. In each case, reactions catalyzed with the normally disfavored anomer are analogous to reversals of hydrolysis but they are, in fact, irreversible reactions lying beyond the scope of the principle of microscopic reversibility.

We now report evidence for the ability of *A. niger* α-D-glucosidase, purified to homogeneity, to catalyze the hydrolysis of both α- and β-D-glucosyl fluoride with the formation of α-D-glucose in each case.

RESULTS

Figure 1 illustrates the results of fractionation of an 18-mg sample of crystalline *A. niger* α-glucosidase on Sephadex G-100. A single, symmetrical peak was obtained for protein content and for enzymic activities (glucose production) from maltose and amylopectin. The peak fractions 31–35, pooled for use in the present experiments, contained 12.54 mg protein, 585 units of activity for maltose (46.7 μmol maltose hydrolyzed.min^{-1}.mg^{-1}), 2.89 units of activity for amylopectin (0.23 μmol glucose released.min^{-1}.mg^{-1}), and no detectable activity for *p*-nitrophenyl α-D-glucopyranoside the last trace of which (3×10^{-3} unit) was eluted in later fractions.

The finally purified enzyme catalyzed the slow hydrolysis of β-D-glucosyl fluoride. A mixture comprising 40mM substrate and 0.54 mg/mL α-glucosidase in 0.4M acetate buffer of pH 4.8 (with 0.01% Triton X-100) was monitored for fluoride

*Abbreviations: D-*gluco*-heptenitol = 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol; D-*gluco*-octenitol = (*Z*)-3,7-anhydro-1,2-dideoxy-D-*gluco*-oct-2-enitol; ± = standard error of the mean.

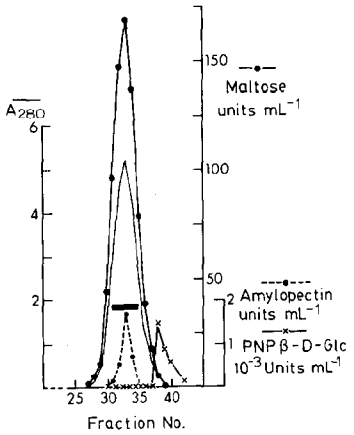

Fig. 1. Fractionation of crystalline *A. niger* α-D-glucosidase on Sephadex G-100. Fractions monitored for protein, by absorption at 280 nm ——, and for ability to hydrolyze maltose —●—, amylopectin --●--, and *p*-nitrophenyl β-D-glucoside —x—. The illustrated activities for amylopectin and *p*-nitrophenyl β-D-glucoside hydrolysis are enlarged 20- and 20 000-times, respectively, relative to that for maltose hydrolysis. Fractions 31–35 (solid bar) were pooled to provide the enzyme used in the present work.

release at 20-s intervals from 1–5 min at 24°. The increments in fluoride-ion meter readings between successive intervals were uniform and corresponded to an initial enzymic hydrolysis rate of 0.149 μmol.min^{-1}.mg^{-1} after correction for the appreciable rate of spontaneous hydrolysis (0.075 μmol.min^{-1}) of 40mM of β-D-glucosyl fluoride in buffer alone, measured under identical conditions. This activity of the α-glucosidase preparation for β-D-glucosyl fluoride, ~0.3% of that for maltose and 65% of that for amylopectin, cannot be attributed to contaminating β-glucosidase: entirely negative results were obtained with cellobiose and salicin as well as with *p*-nitrophenyl β-D-glucoside in assays capable of detecting <0.1% as much activity with these compounds as found with the β-D-glucosyl fluoride.

The possibility that reactivity with β-D-glucosyl fluoride might be due to contamination with glucoamylase (E.C. 3.2.1.3) was next examined, as the α-glucosidase preparation showed some activity (0.23 unit.mg^{-1}) for amylopectin. Glucoamylase not only is abundantly produced by the *A. niger* from which the β-glucosidase was isolated, but was shown earlier[10] to convert β-D-glucosyl fluoride into β-D-glucose by a two-step process. Purified *A. niger* glucoamylase with a specific activity for amylopectin of 16 μmol glucose liberated.min^{-1}.mg^{-1} (Matsui *et al.*, 1989) was examined and found to act upon 40mM β-D-glucosyl fluoride (at pH 4.8 and 24° at the rate of 0.36 μmol.min^{-1}.mg^{-1}. To account for the observed rate of β-D-glucosyl fluoride hydrolysis by the β-glucosidase, ~40% of the protein in the α-glucosidase preparation would have to be glucoamylase, but then amylopectin would be hydrolyzed 25 times faster than found with the α-glucosidase.

The rate found for the promotion of β-D-glucosyl fluoride hydrolysis by the α-glucosidase, though modest, indicated that the steric course of the reaction might

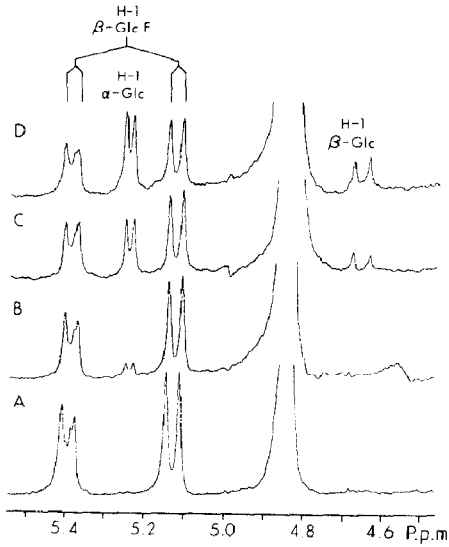

Fig. 2. ^{1}H-n.m.r. spectra recorded in buffered D_2O (PD 5.2) at 200 MHz; chemical shifts (p.p.m.) refer to sodium 4,4-dimethyl-4-silapentanesulfonate. A: 40mM β-D-glucosyl fluoride in buffered D_2O, pD 5.2, 21°, 4 min. B, C, D: 40mM β-D-glucosyl fluoride with 7.5 mg.mL^{-1} *A. niger* α-glucosidase in buffered D_2O, pD 5.2, incubated at 21° for 8, 45 and 84 min, respectively.

be determined by ^{1}H-n.m.r. spectroscopy. To this end, the enzyme was concentrated in a collodion-membrane apparatus; exhaustively dialyzed at 8° against 0.05M acetic acid-d_4/NaOD buffer of pD 5.2 to replace the enzyme's exchangeable ^{1}H atoms by ^{2}H atoms; and finally adjusted to 7.5 mg.mL^{-1} with the buffer. A test mixture was prepared by adding 0.60 mL (4.5 mg) of the dialyzed enzyme to 24 μmol of β-D-glycosyl fluoride freshly dried *in vacuo* from the stock solution in methanol; 0.60 mL of the pD 5.2 buffer was added to a duplicate 24-μmol sample of the substrate as a control.

The test mixture was monitored at 21° by 200-MHz ^{1}H-n.m.r. spectra recorded at intervals from 4 to 84 min; the β-D-glucosyl fluoride–buffer control was examined after 4 and 84 min. Figure 2 illustrates the downfield region of spectra of (A), the freshly dissolved substrate in buffer and (B–D) of the enzymic reaction-mixture after 8, 45, and 84 min at 21°. Spectrum A records the H-1 axial proton resonance of β-D-glucosyl fluoride, a doublet of doublets* centered at 5.23 p.p.m. with $J_{1,2}$ 6.9 Hz and $J_{1,F}$ 53.1 Hz; no signal referable to the anomeric proton of α- or β-D-glucose is present. Spectra B–D of the incubated enzymic digest show progressive diminutions of the substrate H-1 resonance with time. All show, in addition, a new doublet at 5.24 p.p.m., $J_{1,2}$ 3.8 Hz, attributable to the H-1

*The anomalous difference in the *J* splitting pattern, with different transition levels in the downfield (5.49 p.p.m.) resonance, has been noted previously. It is eliminated at a higher field and appears as the result of a frequency-dependent process (see ref. 10).

resonance of α-D-glucopyranose; this is clear at 8 min (spectrum B) and increases to the end of the test period. A second new resonance doublet, centered at 4.66 p.p.m. with $J_{1,2}$ 8 Hz, is barely evident at 8 min (spectrum B) but is well defined in spectra C and D after 45 and 84 min of incubation; this signal is due to the H-1 axial proton of β-D-glucopyranose. At each time-period examined, the 5.24 p.p.m. resonance is larger than that at 4.66 p.p.m., clearly demonstrating that α-D-glucose is the primary product of the hydrolysis of β-D-glucosyl fluoride by the α glucosidase.

Further analysis of the series of spectra recorded with the digest incubated between 4 and 84 min, and of the substrate–buffer control at 21° for 84 min, was made by sectioning and weighing the H-1 resonance areas of the unutilized substrate and the α- and β-D-glucose products in 2-times enlarged copies of each spectrum. As illustrated in Fig. 3, 45% of the β-D-glucosyl fluoride in the mixture with enzyme was hydrolyzed by 84 min; <5% in the buffer control. Estimates of the proportion of D-glucose product present as the α anomer range from ~88% at 16 min to 75% at 84 min. Since, at equilibrium in D_2O, D-glucose comprises 36% α and 64% β anomer[19], it is evident that the D-glucose present after 84 min incubation (Fig. 2, spectrum D) was far from anomeric equilibrium, and that nonenzymic anomerization accounts for the low levels of β-D-glucose observed during incubation.

In a similar experiment, a mixture of 16mM α-D-glucosyl fluoride with 0.10 mg.mL^{-1} of dialyzed *A. niger* α-glucosidase in pD 5.3 buffer was monitored by ^{1}H-n.m.r. spectra at 21° and 200 MHz. The results (spectra not illustrated) showed α-D-glucose to be the primary reaction-product. A resonance doublet at 5.24 p.p.m., $J_{1,2}$ 3.8 Hz, appeared by 4 min and increased with time, while a small signal at 4.65 p.p.m., $J_{1,2}$ 7.7 Hz, attributable to H-1 of β-D-glucose, was first evident at

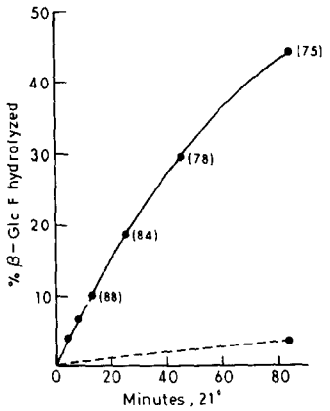

Fig. 3. Time course (% hydrolysis) for 40mM β-D-glucosyl fluoride in D_2O at pD 5.2 and 21°: ——, digest with 0.5 mg.mL^{-1} *A. niger* α-glucosidase; ----, substrate/buffer control. Bracketed figures show the % of the glucose product present as the α anomer. Data calculated from weighed sections, representing the H-1 resonances of β-D-glucosyl fluoride, α-D-glucose, and β-D-glucose, from 2-times enlarged copies of individual ^{1}H-n.m.r. spectra.

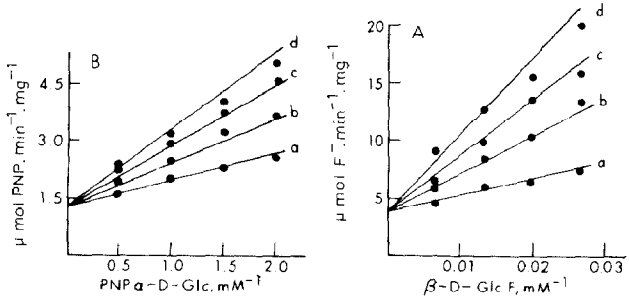

Fig. 4. A. Maltotriose inhibition of the hydrolysis of 37.5–150mM β-D-glucosyl fluoride by *A. niger* α-glucosidase: a, no inhibitor present; b–d, in presence of 0.5, 1.0, or 1.5mM maltotriose, respectively. B. β-D-glucosyl fluoride inhibition of the hydrolysis of 0.5–2.0mM *p*-nitrophenyl α-D-glucosidase by *A. niger* α-glucosidase: a, no inhibitor present; b–d in presence of 20, 40, or 60mM β-D-glucosyl fluoride, respectively. Data points are experimental. Lines drawn through the points are computed iterative fits to the equation $v = VA\{K(1 + I/K_{is}) + A\}$.

35 min. In view of the different steric course of the hydrolysis of β-D-glucosyl fluoride compared with the hydrolysis of α-D-glucosyl fluoride (or that of maltotriose and phenyl α-maltoside[18]), studies were made to determine whether β-D-glucosyl fluoride binds to the same active site as α-D-glucosyl substrates of the α-glucosidase.

Inhibition studies. — Initial rates of hydrolysis by the *A. niger* α-glucosidase were measured for three sets of test and control mixtures (all at pH 4.8 and 24° for 12 min) comprising 37.5–150mM β-D-glucosyl fluoride in the absence or presence of 0.5–1.5mM maltotriose as inhibitor; 5–20mM α-D-glucosyl fluoride alone or with 0.5–1.5mM maltotriose; 0.5–2mM *p*-nitrophenyl α-D-glucoside alone or in the presence of 20–60mM β-D-glucosyl fluoride as inhibitor. In all cases a linear competitive inhibition was observed. This is illustrated in Fig. 4 for maltotriose inhibition of β-D-glycosyl fluoride hydrolysis, and for β-D-glucosyl fluoride inhibition of *p*-nitrophenyl α-D-glucoside hydrolysis by the α-glucosidase.

When the data were fitted to the equation, $v = VA/\{K(1 + I/K_{is}) + A\}$ using Cleland's[20] program, the result showed α-D-glucosyl fluoride hydrolysis (V = 78.5 ±2 μmol.min^{-1}.mg^{-1}, K = 1.9 ±0.3mM) and β-D-glucosyl fluoride hydrolysis (V = 0.27 ±0.015 μmol.min^{-1}.mg^{-1}, K = 41.0 ±6.3mM) to be competitively inhibited by maltotriose at similar levels (K is 0.51 ±0.08mM and 0.43 ±0.05mM, respectively). Moreover, the hydrolysis of *p*-nitrophenyl α-D-glucoside (V = 0.75 ±0.02 μmol.min^{-1}.mg^{-1}, K = 0.52 ±0.06mM) was competitively inhibited by β-D-glucosyl fluoride (K_{is} = 30.1 ±3.4mM) even though *p*-nitrophenyl α-D-glucoside is a far better substrate than β-D-glucosyl fluoride (relative V/K ~200). These findings strongly support the view that β-D-glucosyl fluoride is bound by the α-glucosidase in the same manner as its α-D-glycosylic substrates.

DISCUSSION

Aspergillus niger α-glucosidase, purified to homogeneity and crystallized[18,21], was found to promote the hydrolysis of β-D-glucosyl fluoride at a low rate — ~0.3% of that of maltose or α-D-glucosyl fluoride. The observed hydrolysis of up to 45% of the β-D-glucosyl fluoride clearly rules out accompanying traces of α-D-glucosyl fluoride as a basis for the reaction. Several independent lines of evidence demonstrate that contamination by β-D-glucosidase or glucoamylase is not responsible for the reaction with β-D-glucosyl fluoride; for example, n.m.r. spectra showed the reaction product to be α-D-glucose whereas β-glucosidase[22] and glucoamylase[10] produce β-D-glucose from β-D-glucosyl fluoride. Further indications that the hydrolysis is catalyzed by α-glucosidase itself are that maltotriose, a favored substrate for the *A. niger* α-glucosidase[18,21] competitively inhibited the reaction with β-D-glucosyl fluoride, and that β-D-glucosyl fluoride competitively inhibited the reaction with *p*-nitrophenyl α-D-glucoside.

The initial rate of hydrolysis of 40mM β-D-glucosyl fluoride by 7.5 $mg.mL^{-1}$ enzyme at pD 5.2 and 21° was estimated (Fig 3) to be sixteen times that of the buffer-catalyzed reaction under the same conditions. The small enhancement of the hydrolysis rate by the enzyme, coupled with the stereospecificity of the reaction, would suggest that the mechanism may involve binding of β-D-glucosyl fluoride to α-glucosidase in the same productive orientation as α-D-glucosylic substrates, providing for suitable substrate alignment with a water molecule directed by protein to the reaction center from the *si* face. Reactant alignment could perhaps reduce entropy sufficiently to account for the modest (16-fold) increase in hydrolysis rate observed relative to that in aqueous buffer. Glucosyl fluoride hydrolysis has been considered[23] to represent an intramolecular elimination of fluoride involving a transition state with oxocarbonium-ion character, and evidence was found through α-secondary tritium, kinetic isotope-effect studies[24] that certain exo α-glucanases which catalyze α-D-glucosyl fluoride hydrolysis to β-D-glucose promote an SN1-type reaction by stabilizing the development of such a transition state. However, it is not known whether similar stabilization or an alternative SN2-type mechanism is involved in the hydrolysis of β-D-glucosyl fluoride by *A. niger* α-glucosidase.

Whatever its mechanism, this stereospecific reaction with a small D-glucosyl compound of the "wrong" configuration has a substantial meaning. The *A. niger* enzyme has been found to promote the hydration of D-glucal[2] and of D-*gluco*-octenitol[5] showing that it, in common with other α-glucosidases, is not limited to acting on substrates of α-anomeric configuration. The use of β-D-glucosyl fluoride further widens the range of structures susceptible to α-glucosidase attack, this time adding a compound of the explicitly "forbidden" β configuration. Its utilization (when β-D-glucosides are excluded) would suggest that the small (-F) equatorial substituent at C-1 of β-D-glucosyl fluoride allows productive binding, whereas the bulky, equatorially oriented aglycons of β-D-glucosides may clash and prevent proper binding.

Finally, the demonstration that β-D-glucosyl fluoride hydrolysis by *A. niger* α-glucosidase results in the specific formation of α-D-glucose provides strong new support for the concept that product (rather than substrate) configuration is the most constant feature of catalysis by α-glucosidases and by glycosylases in general. This concept was recently proposed[2,5,6] on the basis of findings on the steric outcome of enzymic reactions with enolic glycosyl donors. In each of more than twenty different reactions, catalyzed with such substrates by α-glucosidases and various other glycosylases, product configuration has been observed to match that of the products of comparable reactions with the enzyme's glycosidic substrates. Present findings provide the first demonstration of this phenomenon with a chiral substrate, with α-D-glucose formed from β- as well as from α-D-glucosyl fluoride just as α-products are formed by the enzyme from D-glucal and D-*gluco*-octenitol[2,5]. The uniform steric outcome of these reactions involving substrates with differently oriented glycosylic bonds (and which proceed with inversion, retention, or *de novo* creation of configuration, respectively) presents a much different view of α-glucosidase specificity than the traditional assumption of absolute restriction to substrates of α-anomeric configuration (namely with an axially oriented glucosylic bond) and to catalysis of reactions with retention of configuration. Enzyme structures which channel or limit the approach of water or other external co-substrate to the reaction center to a specific (*si*) orientation would appear to be the primary determinants of the steric outcome of reactions catalyzed by these enzymes.

EXPERIMENTAL PROCEDURES

General procedures. — Thin-layer chromatography (t.l.c.) was carried out with 5 × 20 cm plates of Silica Gel G (Analtech) developed with 3:2 Et_2O–petroleum ether for acetylated compounds and 5:1 EtOAc–EtOH for deacetylated compounds. Solvent removal was effected in rotary vacuum evaporators operated at 30°. Optical absorbance measurements were made with Gilford Stasar II and Model 120 spectrophotometers. D-Glucose determinations were made by using a D-glucose oxidase plus 4-aminoantipyrine reagent AR II (Wako, Tokyo) in 2M tris-(hydroxymethyl)aminomethane hydrochloride buffer, pH 7.0, in tests controlled with D-glucose standards.

Fluoride ion concentration was measured with a specific-ion electrode and an Orion Ionalyzer 901 standardized with sodium fluoride. Unless noted otherwise, test and control solutions were examined immediately after the addition of 1.5 volumes of TISAB [M sodium acetate buffer (pH 5.2), M sodium chloride, and 0.4% 1,4-cyclohexanebis(dinitrilotetraacetic acid) monohydrate].

Crystalline *p*-nitrophenyl α- and β-D-glucopyranoside, maltotriose, salicin, and cellobiose were purchased from Sigma; amylopectin was prepared from potato starch by the pentanol fractionation procedure of Schoch[14]. Pure crystalline α-D-glucosyl fluoride was prepared and characterized as reported earlier[10].

Fourier-transform proton n.m.r. spectra were recorded at 200 MHz in D_2O (99.8 atom% D, Cambridge Isotope Laboratories) with a Varian XL-200 spectrometer; chemical shifts (p.p.m.) refer to sodium 4,4-dimethyl-4-silapentanesulfonate.

β-D-Glucosyl fluoride. — Tetra-*O*-acetyl-β-D-glucopyranosyl fluoride was prepared (Helferich and Gootz[15]) by treating tetra-*O*-acetyl-α-D-glucopyranosyl bromide in acetonitrile with silver(I) fluoride (Ventron Corp., Danvers, MA). The crude product obtained after filtration and solvent evaporation was freed of impurities by chromatography on columns of Silica Gel 60 (Brinkmann) developed with 4:1 Et_2O–petroleum ether (b.p. 37–57°). Fractions showing a single spot on t.l.c. were combined and recrystallized from Et_2O. The pure compound co-migrated with an authentic sample of β-D-glucosyl fluoride tetraacetate[10], and showed no trace of the readily distinguishable α anomer. A ^{1}H-n.m.r. spectrum recorded in $CDCl_3$ at 400 MHz was consistent with the assigned structure.

Anal. Calc. for $C_{14}H_{19}FO_9$ (350.30): C, 48.00; H, 5.47; F, 5.42. Found: C, 48.08; H, 5.53; F, 5.43.

Pure, amorphous β-D-glucosyl fluoride was prepared by deacetylating 0.3 g of the tetraacetate in 2 mL of 0.03M NaOMe in dry MeOH (25°, 3 H), followed by chromatography on a column of Silica Gel 60 developed with 4:1 EtOAc–EtOH. Fractions migrating as a single spot on t.l.c. were pooled, dried under vacuum, dissolved in MeOH and stored at −20°. For use, aliquots of the stock solution were dried under vacuum in small plastic test-tubes. Analyses showed the free fluoride content to be 0.4% and the molar ratio of fluoride to glucose following mild acid hydrolysis to be 0.97. A partial ^{1}H-n.m.r. spectrum is presented under Results.

α-D-Glucosidase. — Initial observations of the ability of *A. niger* α-D-glucosidase to attack β-D-glucosyl fluoride were made with a highly purified, preparation of the enzyme[16,17] kindly furnished by Dr. John Pazur. Present experiments were carried out with a crystalline sample of *A. niger* α-glucosidase prepared by Kita *et al.*[18] and shown by these authors to be homogeneous on polyacrylamide disc electrophoresis and to cleave exclusively the α anomer of D-glucose from maltotriose and phenyl α-maltoside. It hydrolyzed 5.6mM maltose at the rate of 45.3 μmol.min^{-1}.mg^{-1} at 30° and pH 4.8; activity for *p*-nitrophenyl β-D-glucoside and salicin was ≤0.001% of that for maltose. Before use, the enzyme (18 mg) was subjected to final fractionation on a 1.5 × 45 cm column of Sephadex G-100 (Pharmacia), developed with 0.05M acetate buffer of pH 4.8, in order to obtain an enzyme from which the last trace of β-glucosidase activity was removed. Fractions of 0.93 mL.10.5 min were collected and individually assayed for protein content and for hydrolytic activity for maltose, *p*-nitrophenyl β-D-glucoside, and amylopectin; one unit of activity represents the amount of enzyme required to effect the release of 1 μmol of glucose.min at 30° and pH 4.8. Protein concentration was determined[18] from the absorbance at 280 nm, $E^{1\%} = 14.6$ cm^{-1}. Assays for glucose formation from maltose and potato amylopectin were made by mixing 10 μL of a 1:100 dilution of enzyme fraction with 90 μL of water, 200 μL of 0.05M acetate

buffer of pH 4.8, and 200 μL of 5 mg/mL substrate; after incubation at 30° (5 min for maltose digests, 30 min for amylopectin digests) D-glucose was determined by the glucose oxidase procedure (activity in the case of maltose hydrolysis was based on one-half of the released glucose). β-Glucosidase activity was assayed by mixing 10 μL of undiluted enzyme fraction with 30 μL of water, 80 μL of the pH 4.8 buffer, and 80 μL of 3 mg.mL *p*-nitrophenyl β-D-glucoside; after 30 min at 30°, 1.0 mL of 0.5M carbonate buffer of pH 10 was added and the absorbance measured at 420 nm in comparison with standards of *p*-nitrophenol. Activities of the enzyme used for the present work are described under Results.

ACKNOWLEDGMENT

We thank Dr. John H. Pazur for samples of purified *A. niger* α-glucosidase (glucosyltransferase) used in preliminary experiments; Dr. John S. Blanchard for his kind help in analyzing the inhibition data.

REFERENCES

1 E. J. HEHRE, D. S. GENGHOF, H. STERNLICHT, AND C. F. BREWER, *Biochemistry*, 16 (1977) 1780–1787.
2 S. CHIBA, C. F. BREWER, G. OKADA, H. MATSUI, AND E. J. HEHRE, *Biochemistry*, 27 (1988) 1564–1569.
3 E. J. HEHRE, C. F. BREWER, T. UCHIYAMA, P. SCHLESSELMANN, AND J. LEHMANN, *Biochemsitry*, 19 (1980) 3557–3564.
4 P. SCHLESSELMANN, H. FRITZ, J. LEHMANN, T. UCHIYAMA, C. F. BREWER, AND E. J. HEHRE, *Biochemistry*, 21 (1982) 6606–6614.
5 W. WEISER, J. LEHMANN, S. CHIBA, H. MATSUI, C. F. BREWER, E. J. HEHRE, *Biochemistry*, 27 (1988) 2294–2300.
6 E. J. HEHRE, *J. Jpn. Soc. Starch. Sci.*, 36 (1989) 197–205.
7 H. M. FLOWERS AND N. SHARON, *Adv. Enzymol.*, 48 (1978) 29–95.
8 J. E. G. BARNETT, W. T. S. JARVIS, AND K. A. MUNDAY, *Biochem. J.*, 105 (1967) 669–672.
9 E. J. HEHRE, C. F. BREWER, AND D. S. GENGHOF, *J. Biol. Chem.*, 254 (1979) 5942–5950.
10 S. KITAHATA, C. F. BREWER, D. S. GENGHOF, T. SAWAI, AND E. J. HEHRE, *J. Biol. Chem.*, 256 (1981) 6017–6026
11 E. J. HEHRE, T. SAWAI, C. F. BREWER, M. NAKANO, AND T. KANDA, *Biochemsitry*, 21 (1982) 3090–3097.
12 T. KASUMI, C. F. BREWER, E. T. REESE, AND E. J. HEHRE, *Carbohydr. Res.*, 146 (1986) 39–49.
13 T. KASUMI, Y. TSUMURAYA, C. F. BREWER, H. KERSTERS-HILDERSON, M. CLAEYSSENS, AND E. J. HEHRE, *Biochemistry*, 26 (1987) 3010–3016.
14 T. J. SCHOCH, *Adv. Carbohydr. Chem.*, 1 (1945) 247–277.
15 B. HELFERICH AND R. GOOTZ, *Ber. Dtsch. Chem. Ges.*, 62 (1929) 2505–2507.
16 J. H. PAZUR AND T. ANDO, *Anch. Biochem. Biophys.*, 93 (1961) 43–49.
17 J. H. PAZUR, A. CEPURE, S. OKADA, AND S. FORSBERG, *Carbohydr. Res.*, 58 (1977) 193–202.
18 A. KITA, H. MATSUI, S. CHIBA, R. OHYA, AND T. SAKAI, *Abstr. Annu. Meet., Nogei Kagaku (Agr. Chem. Soc. Jpn)*, Tokyo, April, 1984, p. 545.
19 R. U. LEMIEUX AND J. D. STEVENS, *Can. J. Chem.*, 44 (1966) 249–262.
20 W. W. CLELAND, *Meth. Enzymol.*, 63 (1979) 103–117.
21 S. CHIBA, in *Handbook of Amylases and Related Enzymes* (Amylase Research Society of Japan, Eds.), Pergamon, Tokyo, (1988) pp. 112–116.
22 J. E. G. BARNETT, *Biochem. J.*, 123 (1971) 607–611.
23 C. WALSH, *Adv. Enzymol.*, 55 (1983) 197–289.
24 H. MATSUI, J. S. BLANCHARD, C. F. BREWER, AND E. J. HEHRE, *J. Biol. Chem.*, 264 (1989) 8714–8716.