Stereochemical Studies of D-Glucal Hydration by α-Glucosidases and Exo-α-glucanases: Indications of Plastic and Conserved Phases in Catalysis by Glycosylases†

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ABSTRACT: α-Glucosidases from *Aspergillus niger*, pig serum, ungerminated rice, buckwheat, and sugar beet seeds (but not from brewers' yeast or honeybee) were found to catalyze the hydration of D-glucal. Each reactive α-glucosidase, incubated with D-glucal in D_2O, was shown to protonate (deuteriate) this prochiral substrate from above its *re* face, i.e., from a direction opposite that assumed for protonating α-D-glucosidic substrates. At the same time, D-glucal hydration catalyzed by three of the α-glucosidases that acted rapidly enough in D_2O to determine product configuration was found to yield 2-deoxy-D-glucose of the same specific (α-) configuration as the D-glucose produced from α-D-glucosidic substrates. These findings substantially extend those reported earlier for the hydration of D-glucal by one (*Candida tropicalis*) α-glucosidase preparation. Together with other recent results, they suggest that the process of catalysis by α-glucosidases (and perhaps glycosylases in general) may comprise two separate and separately controlled parts, namely, a "plastic" phase concerned with substrate protonation and a substrate-unrelated "conserved" phase concerned with the creation of product configuration. In contrast to the α-glucosidases, three "inverting" exo-α-glucanases (*Arthrobacter globiformis* glucodextranase; *Rhizopus niveus* and *Paecilomyces varioti* glucoamylase) were found to protonate D-glucal from below its *si* face. Further, whereas the catalysis of D-glucal hydration by the α-glucosidases was intensively inhibited by excess substrate, that promoted by the exo-glucanases showed no detectable substrate inhibition.

Studies of enzymic glycosylation reactions catalyzed with nonglycosidic substrates (glycosyl fluorides, glycals, and related enolic glycosyl donors) have in recent years yielded abundant evidence for the ability of various individual glycosylases to catalyze different stereochemical reactions with different substrates (Lehmann & Zieger, 1977; Hehre et al., 1977, 1979, 1982, 1986; Kitahata et al., 1981; Schlesselmann et al., 1982; Lehmann & Schlesselmann, 1983; Tsumuraya et al., 1984; Kanda et al., 1986; Kasumi et al., 1986, 1987). Early studies, for example, showed several enzymes to protonate a glycal substrate from a direction opposite that assumed for protonating their glycosidic substrates (Hehre et al., 1977; Lehmann & Zieger, 1977). These and subsequent observations of the functional flexibility of a range of glycosylases provide an advance of broad significance, directly contradicting the common belief that an enzyme acts on all substrates by a single mechanism ("the" mechanism of the enzyme).

In addition to illustrating this functional versatility, reactions catalyzed with a glycal by one α-glucosidase of wide specificity as well as by several β-glycosidases have shown an unusual feature that would appear to provide the basis for achieving a further significant advance toward understanding catalysis by glycosylases: each of these enzymes was found to protonate an appropriate glycal from a direction opposite that assumed for protonating its glycosidic substrates, yet to convert the glycal to a product of the same configuration as produced from its glycosidic substrates (Hehre et al., 1977; Lehmann & Zieger, 1977; Kanda et al., 1986). In order to learn more about this dichotomy, further studies of reactions catalyzed with nonglycosidic substrates by enzymes active upon α-D-glucosidic substrates appeared especially important. Only one α-glucosidase (from *Candida tropicalis* yeast) has been reported to catalyze D-glucal hydration (Hehre et al., 1977); that from a second source (bakers' yeast) had been found by Lehmann and Schröter (1972) to be without apparent action on this compound. Only one "inverting" exo-α-glucanase (β-amylase) has so far been shown to act on a glycal (maltal), with no information yet available on the configuration of the reaction product (Hehre et al., 1986).

The availability of a series of highly purified α-glucosidases and inverting exo-α-glucanases of different biological origins gave the opportunity to gain further basic information about the catalytic capabilities of these types of enzymes. The present study examines the frequency with which α-glucosidases and exo-α-glucanases catalyze the hydration of D-glucal. With all enzymes found to promote the reaction, the direction of protonation of the substrate was investigated by conducting the reaction in deuterium oxide and determining through ^1H NMR spectroscopy the location of the deuteron at C-2 of the reaction product. With enzymes that catalyzed hydration rapidly enough, both protonation direction and product configuration were determined by following the reaction in D_2O in the NMR spectrometer (Hehre et al., 1977). For enzymes acting slowly on D-glucal, the protonation (deuteriation) direction was determined by ^1H NMR spectra of the isolated

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reaction product (Lehmann & Zieger, 1977; Hehre et al., 1979). The results of these examinations and of kinetic studies with the enzymes of both types are discussed in relation to other reports of reactions catalyzed by glycosylases with nonglycosidic substrates.

Experimental Procedures

General Procedures. Thin-layer chromatography (TLC) was carried out with 5 × 20 cm plates of silica gel G (Analtech) with ethyl acetate/2-propanol/water (50:18:9) as solvent unless otherwise noted. Spots were visualized by spraying with anisaldehyde/sulfuric acid and heating at 110 °C (Stahl & Kaltenbach, 1961). Preparative chromatograms were made by using 23 × 56 cm sheets of Whatman No. 1 paper prewashed with the developing solvent (1-butanol/ethanol/water, 13:8:4). Test samples (0.6 mL) were applied as a 20-cm band and chromatographed (descending) for 18 h. Reaction components were located with the aid of silver nitrate stained end guide strips. Product elutions were made with methanol. Solvent removal was carried out by the use of rotary vacuum evaporators operated at 30 °C.

2-Deoxy-D-glucose was determined in the presence or absence of D-glucal by using a glucose oxidase plus 4-aminoantipyrine reagent (Wako Pure Chemicals, Ltd., Tokyo) in tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer of pH 7.0 standardized with crystalline 2-deoxy-D-glucose, grade III (Sigma).

D-Glucal. Samples (5 mmol) of 3,4,6-tri-*O*-acetyl-D-glucal (Aldrich Chemical Co.) were deacetylated in 40 mL of freshly prepared 0.025 M sodium methoxide in dry methanol. Mixtures were held at 25 °C until reaction was complete (usually 5–6 h) as judged by TLC; then 3 g of silica gel 60 (Brinckmann) was added. The mixture was dried under vacuum and chromatographed on a 2.5 × 36 cm column of dry silica gel 60, with ethyl acetate/ethanol (5:1) as developer. Fractions containing abundant D-glucal (phenol/sulfuric acid method) were combined and concentrated under vacuum at 30 °C. The syrupy product was further dried from absolute ethanol and from benzene and then kept for 1–2 days in a vacuum desiccator over calcium sulfate; the usual yield was 0.45–0.50 g. The pure D-glucal (<0.05% 2-deoxy-D-glucose content) was recovered in the form of highly hygroscopic crystals that appeared as highly birefringent plates under the polarizing microscope. It was usually kept as a solution of known concentration in dry methanol at −20 °C, protected from moisture. As needed, aliquots were dried in test tubes in a rotary vacuum evaporator. For kinetic experiments, 300 or 400 mM solutions were prepared in water and filtered through Swinnex HA 0.45-nm membranes (Millipore Corp.).

Enzymes. The α-glucosidases examined, each purified to apparent homogeneity, included the major form (II) of the enzyme from ungerminated rice (Murata et al., 1979), assaying at ca. 38 μmol of maltose hydrolyzed/(min·mg) in 0.04 M acetate buffer of pH 4.0 and at 30 °C. Buckwheat α-glucosidase (Kanaya et al., 1976) assayed at ca. 60 μmol of maltose hydrolyzed/(min·mg) in 0.04 M acetate at pH 5.0 and 37 °C; sugar beet seed α-glucosidase (Chiba et al., 1978; Matsui et al., 1978), at ca. 20 maltose units/mg at pH 4.5 and 37 °C; pig serum neutral α-glucosidase (Hibi et al., 1976), at 11 μmol of maltose hydrolyzed/(min·mg) in McIlvaine buffer at pH 6.3 and 37 °C. In these assays, the substrate (maltose) concentration was 5.6 mM with the enzyme protein concentration derived from its specific extinction coefficient at 280 nm; glucose was measured by the glucose oxidase/Tris method. Honeybee α-glucosidase I (Takewaki et al., 1980) assayed at 19 μmol of phenyl α-D-glucoside hydrolyzed/(min·mg) at pH 6.7 and 33 °C. Each of these enzymes yielded α-D-glucose specifically upon hydrolyzing maltotriose or phenyl α-maltoside (Chiba et al., 1983).

Apsergillus niger α-glucosidases examined included the recently crystallized enzyme (Kita et al., 1984) which assayed at 39.7 μmol of maltose hydrolyzed/(min·mg) (with 5.6 mM maltose) in 0.04 M acetate buffer at pH 4.0 and 30 °C, and a highly purified preparation of the enzyme (kindly furnished by Dr. John Pazur) shown both to hydrolyze maltose and to convert it extensively to α-D-glucobioses (Pazur & Ando, 1961; Pazur et al., 1977).

Three exo-α-glucanases were studied. The glucodextranase from *Arthrobacter globiformis* which catalyzes endwise hydrolysis of dextrans with release of β-D-glucose (Ohya et al., 1978) was purified to apparent homogeneity by a multistep procedure involving ammonium sulfate precipitation of the enzyme from culture fluid, Sephadex G-100 fractionation, and DEAE-cellulose column chromatographic fractionation. The glucodextranase, which assayed at 21.94 μmol of glucose/(min·mg) (Nelson–Somogyi method) released from 0.25% dextran T2000 (Pharmacia) at pH 6.0 and 30 °C, gave a single protein band on polyacrylamide and sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (G. Okada, unpublished results). The glucoamylase of *Paecilomyces varioti*, purified to apparent homogeneity as judged by polyacrylamide electrophoresis, had a specific activity for soluble starch of ca. 50 μmol of glucose/(min·mg) at pH 4.0 and 37 °C (Takeda et al., 1985); the enzyme hydrolyzes maltooligosaccharides to form glucose of β-configuration. Twice-recrystallized glucoamylase from *Rhizopus niveus* (Seikagaku Kogyo Ltd., Tokyo), listed as producing 395 mg of glucose from soluble starch/[(30 min)·mg] at 40 °C, was found to yield 15.0 μmol of glucose/(min·mg) from soluble starch (Mallinckrodt) at pH 4.8 and 30 °C. The glucose produced is exclusively of β-configuration (Tsujisaka, 1960). Most enzymes were kept at 4 °C as suspensions in 90% saturated ammonium sulfate. Amounts as required were sedimented by high-speed configuration, dissolved in buffer of the desired composition, and dialyzed if necessary.

^1H NMR Spectra. Fourier transform proton NMR spectra were recorded in D_2O (99.8 atom % D) at 200 MHz with a Varian XL 200 spectrometer. Chemical shifts (ppm) refer to 3-(trimethylsilyl)propanesulfonic acid sodium salt. Buffered solutions of deuterium oxide of desired pD were prepared by using glacial acetic acid-d_4 (Thompson-Packard) and NaOD (KOR Isotopes); pD was assigned by adding 0.40 to pH meter readings.

Enzyme/D-Glucal Digests in D_2O, for Isolation of 2-Deoxy[2-^2H]-D-glucose Hydration Product. Enzyme (in 0.20–0.25 mL of D_2O) was dialyzed in 6-mm Spectrapor membrane tubing (A. H. Thomas) (6 °C, with stirring) for three successive 24-h periods, against changes of appropriately buffered deuterium oxide in a 15-mL cylinder. Following dialysis, the volume was adjusted to 0.60 mL with the buffered D_2O, and the enzyme was added to a sample of D-glucal that had been dried in vacuo (in a 12 × 75 mm test tube) from stock solution in methanol and finally dried from methyl alcohol-d_4. The tube with digest was corked and sealed, placed in a larger tube that was also sealed, and incubated at 30 °C. At intervals, samples were examined by TLC until sufficient 2-deoxy-D-glucose was judged to be present to permit isolation and characterization. The digest was then chromatographed, and the 2-deoxy-D-glucose product was eluted with methanol. The eluates were concentrated to ca. 1.5 mL and centrifuged. The clear supernatant fluid was dried, and the product taken

up in 2 mL of deuterium oxide; this solution was passed through a Swinnex HA 0.45-nm membrane filter. The 2-deoxy-D-glucose content was measured by the glucose oxidase method, and a suitable amount of product from each enzyme was analyzed by ^{1}H NMR spectroscopy.

Kinetic Experiments. Initial rates of enzymic hydration of D-glucal were measured at different substrate concentrations. Appropriate aliquots of an aqueous 300 or 400 mM D-glucal solution that had been filtered through a Swinnex HA 0.45-nM membrane were diluted with water to make 1.00 mL of substrate of the desired concentration. At 1-min intervals, 0.20 mL of enzyme in 0.2 M acetate buffer (or buffer alone) was added to each. Rice II and *A. niger* α-glucosidase additions were 0.20 mg/mL solutions in buffer of pH 4.0; buckwheat α-glucosidase and *P. varioti* glucoamylase were 0.60 mg/mL solutions in 0.2 M buffer of pH 4.8; *R. niveus* glucoamylase was 1.0 mg/mL in the pH 4.8 buffer; *A. globiformis* glucodextranase was in 0.2 M acetate of pH 5.7 plus 5 mM $CaCl_2$; each enzyme stock was filtered through a 0.45-nm Millipore membrane. Individual reaction mixtures were incubated at 30 °C for 30 (α-glucosidases) or 120 min (exo-α-glucanases) and then treated with 2.0 mL of 2 M Tris-HCl buffer of pH 7.0 and 0.40 mL of glucose oxidase reagent; after an additional 30-min incubation at 30 °C, optical density was measured at 505 nm against standards of 2-deoxy-D-glucose. Rates of enzymically catalyzed D-glucal hydration were calculated after correction for the 2-deoxy-D-glucose content of concurrently incubated substrate/buffer control mixtures.

Results

Highly purified preparations of α-glucosidase from five of seven different biological origins were found to catalyze the hydration of D-glucal as judged by TLC examinations (against 2-deoxy-D-glucose) of reaction mixtures (30 °C, 30 min) comprising 5 mg/mL enzyme and 30 mM D-glucal. Crystalline α-glucosidase of *A. niger* and purified preparations of rice II and buckwheat α-glucosidases were found to have caused nearly complete hydration of the substrate. α-Glucosidases from sugar beet seed and pig serum, as well as a highly purified *A. niger* preparation primarily studied as a glucosyltransferase and generously furnished by Dr. John Pazur, catalyzed hydration of ca. 15%, 10%, and 20% of the D-glucal, respectively, under these test conditions. Honeybee and brewers' yeast II α-glucosidase produced no detectable 2-deoxy-D-glucose (<1% D-glucal hydration) under the same conditions.

Measurements (by the glucose oxidase method) of the rate of 2-deoxy-D-glucose formation in digests (30 °C, 20 min) of 30 mM D-glucal with the α-glucosidases of *A. niger* and rice II (at pH 4.4) and of buckwheat (at pH 5.0) showed that each enzyme catalyzed the hydration at ca. 0.2 μmol/(min·mg). This rate indicated the possibility of determining the full stereochemistry of the reaction (both the direction of enzymic protonation of the D-glucal and the anomeric configuration of the 2-deoxy-D-glucose product) in these three cases, by conducting the reaction in buffered deuterium oxide and following its progress by ^{1}H NMR spectra (Hehre et al., 1977; Kanda et al., 1986).

^{1}H NMR Study of D-Glucal Hydration Catalyzed by A. niger, Rice II, and Buckwheat α-Glucosidases. Crystalline *A. niger* α-glucosidase was dialyzed against three changes of deuterium oxide buffered at pD 4.5 with 0.05 M deuterioacetate during a 24-h period in order to replace its exchangeable ^{1}H atoms by ^{2}H atoms. The dialyzed enzyme, 4.8 mg in 0.60 mL, was added to 60 μmol of D-glucal freshly dried from solution in methanol, and the mixture was immediately transferred to a 5-mm NMR tube and placed in the spectrometer.

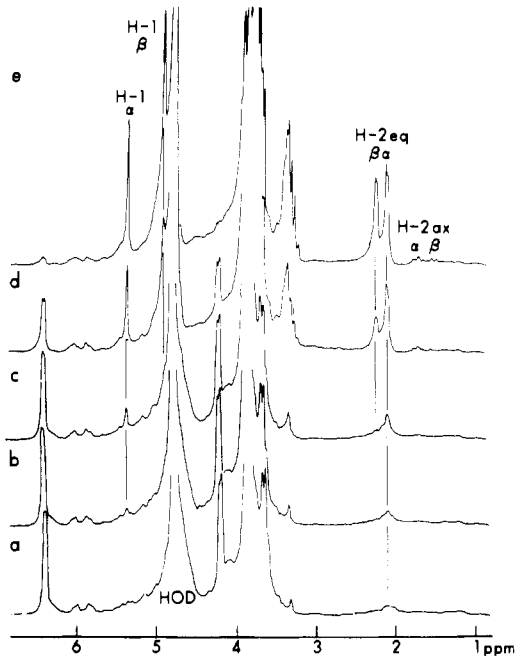

FIGURE 1: Fourier transform ^{1}H NMR spectra recorded at 200 MHz for a digest of 100 mM D-glucal by 6 mg/mL of crystalline *A. niger* α-glucosidase in deuterium oxide buffered at pD 4.5: spectra a, b, c, d, and e recorded after 3, 8, 25, 90, and 210 min, respectively, at 20 °C. Chemical shifts (ppm) refer to 3-(trimethylsilyl)propanesulfonic acid sodium salt.

Parts a, b, c, d, and e of Figure 1 illustrate spectra recorded at 200 mHz after 3-, 8-, 25-, 90-, and 210-min incubation, respectively, at 20 °C. Resonance assignments for the anomeric proton of D-glucal, and for the C-1, C-2 axial, and C-2 equatorial protons of α- and β-2-deoxy-D-glucose, are as previously reported (Hehre et al., 1977).

Spectrum a (3 min) is essentially that of D-glucal, with an H-1 resonance at 6.4 ppm. This resonance is seen to diminish during incubation and is just visible in spectrum e, indicating nearly complete substrate utilization after 210 min at 20 °C. Spectrum b (8-min incubation) shows new signals centered at 5.35 and 2.1 ppm, representing the H-1 and H-2 equatorial resonances, respectively, of α-2-deoxy[2(a)-^{2}H]-D-glucose. Both resonances are seen to increase substantially with time (spectra c–e). After 25-min incubation (spectrum c), the first small signals are detected at 4.9 ppm (as a slight shoulder on the downfield limb of the HOD peak) and at 2.25 ppm. These represent the H-1 and H-2 equatorial resonances, respectively, of β-2-deoxy[2(a)-^{2}H]-D-glucose; they increase with time but approach the size of the H-1 and H-2 equatorial resonances of the α-anomer only after 210-min incubation when the D-glucal has been nearly completely utilized. The very small multiplets observed at 1.7 and 1.5 ppm after 90 and 210 min (spectra d and e) are assignable to the H-2 axial resonance of α- and β-2-deoxy[2(e)-^{2}H]-D-glucose. The minute amount of product protonated at the axial position at C-2 could reflect incomplete replacement of the enzyme's exchangeable protons during dialysis. All of the data are consistent with the specific conversion of D-glucal to 2-deoxy[2(a)-^{2}H]-α-D-glucose by the *A. niger* α-glucosidase, followed by nonenzymic anomerization of the product to form the later observed 2-deoxy[2(a)-^{2}H]-β-D-glucose component.

Similar ^{1}H NMR studies were made of the hydration of 100 mM D-glucal catalyzed by dialyzed rice α-glucosidase II (5.75 mg/mL in D_2O at pD 4.5) and by dialyzed buckwheat α-

Table I: 2-Deoxy[2-^{2}H]-D-glucose Samples Isolated from Digests (in D_2O) of D-Glucal with α-Glucosidases and with Exo-α-glucanases: Position of the Deuterium Atom at C-2

enzyme/D-glucal digests (0.60 mL) incubated at 30 °C					isolated 2-deoxy[2-^{2}H]-D-glucose	
enzyme kind and source	mg/mL	D-glucal (mM)	pD	30 °C incubation time (h)	yield[a] (μmol)	position of ^{2}H[b]
α-glucosidases						
rice seed	1.0	100	4.5	18[c]	43.0	axial at C-2
sugar beet seed	2.3	100	4.5	24	48.0	axial at C-2
pig serum	2.65	100	6.2	48[c]	51.2	axial at C-2
A. niger[d]	2.9	100	4.5	48	17.3	axial at C-2
exo-α-glucanases[e]						
A. globiformis	14.5	200	5.95	48[c]	32.6	equatorial at C-2
P. varioti	5.7	150	4.8	72	10.4	equatorial at C-2
R. niveus	29.7	150	5.2	44	26.5	equatorial at C-2

[a] Concurrently incubated D-glucal/buffer control digests contained <0.1 μmol of 2-deoxy-D-glucose (glucose oxidase method). [b] Determined by ^{1}H NMR spectra of solutions in D_2O. [c] A concentration of <1.2 M ^{1}H was found in a duplicate incubated D-glucal/enzyme digest; following addition of a known weight of 3-(trimethylsilyl)propanesulfonic acid sodium salt, an integrated ^{1}H NMR spectrum was used to measure the intensity of the HOD signal relative to that of the methyl group protons of the standard. [d] Sample studied by Pazur et al. (1977) as a glucosyltransferase. [e] Catalyze release of β-D-glucose from specific α-glucans.

glucosidase (7.0 mg/mL in D_2O at pD 5.0). The results in each case (not illustrated) were entirely comparable to those shown in Figure 1 for the *A. niger* enzyme. Spectra recorded within 10 min of incubation at 20 °C showed the formation of resonances at 5.3 and 2.1 ppm assignable to the H-1 and H-2 equatorial protons of the α-anomer of 2-deoxy-D-glucose. Late in incubation, resonances due the H-1 and H-2 equatorial protons of the β-anomer of 2-deoxy-D-glucose were present, but only traces of H-2 axial resonances of the equilibrated sugar were present. In sum, each of the three highly reactive α-glucosidases was observed to catalyze the same trans hydration of D-glucal, in which the substrate was protonated by enzyme from above the *re* face, to form 2-dexoy[2(a)-^{2}H]-α-D-glucose.

Direction of Protonation of D-*Glucal by Other α-Glucosidases and by Exo-α-glucanases.* As previously noted, α-glucosidase preparations from sugar beet seeds, pig serum, and *A. niger* [considered a glucosyltransferse by Pazur et al. (1977)] catalyzed D-glucal hydration rather slowly. Similar behavior was observed with several exo-α-glucanases that hydrolyze specific α-glucans to form D-glucose of inverted (β-) configuration: namely, crystalline *R. niveus* glucoamylase, *P. varioti* glucoamylase, and *A. globiformis* glucodextranase. One mechanistically critical parameter for the reaction catalyzed by each of these slowly acting enzymes (the direction of protonation of the D-glucal substrate) could, nevertheless, be determined. Each enzyme was exhaustively dialyzed against appropriately buffered deuterium oxide under conditions shown capable of reducing the proton concentration to less than 1.2 M (see Table I, footnote *c*). Enzyme/D-glucal digests in detuerium oxide were incubated at 30 °C until TLC and reducing sugar examinations showed the presence of sufficient 2-deoxy-D-glucose to be characterized by ^{1}H NMR spectra. The digest was then chromatographed and the eluted hydration product, cleared of impurities from the paper, was dissolved in D_2O and examined by ^{1}H NMR spectra at 200 MHz. The digest conditions, product yield, and NMR results (location of the deuteron at C-2 in the product) are listed for each enzyme in Table I.

Figure 2 illustrates ^{1}H NMR spectra (upfield resonances) of four of the isolated (fully anomerized) 2-deoxy[2-^{2}H]-D-glucose products in Table I. Those products obtained with rice α-glucosidase II (spectrum a of Figure 2) and pig serum neutral α-glucosidase (spectrum b of Figure 2) show prominent resonances centered at 2.1 and 2.25 ppm, assignable to the equatorial H-2 proton of α- and β-2-deoxy-D-glucose, along with just-detectable multiplet signals at 1.7 and 1.5 ppm

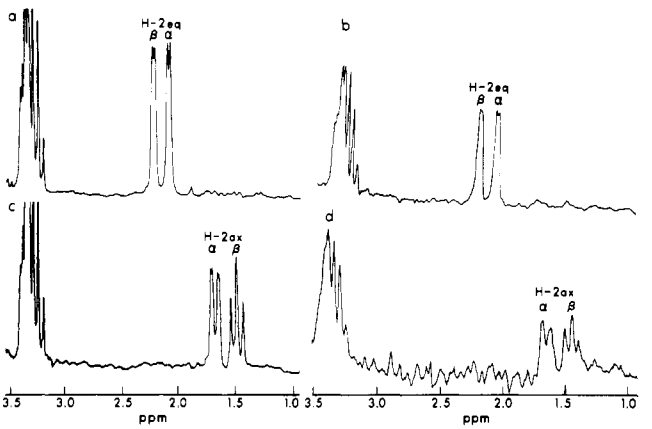

FIGURE 2: ^{1}H NMR spectra recorded in deuterium oxide at 200 MHz of anomerically equilibrated 2-deoxy-D-glucose isolated from digests of D-glucal with (a) rice α-glucosidase II, (b) pig serum neutral α-glucosidase, (c) *A. globiformis* glucodextranase, and (d) *P. varioti* glucoamylase.

referable to the axial H-2 protons of the sugar. Comparable spectra (not illustrated) were obtained with the products separated from digests with sugar beet seed α-glucosidase and the *A. niger* α-glucosidase (glucosyltransferase) furnished by Dr. Pazur. In contrast, the products obtained with *A. globiformis* glucodextranase (spectrum c of Figure 2) and *P. varioti* glucoamylase (spectrum d of Figure 2) show prominent resonances centered at 1.7 and 1.5 ppm, assignable to the axial H-2 proton of α- and β-2-deoxy-D-glucose, with barely detectable equatorial H-2 resonances at 2.1 and 2.25 ppm. A similar pattern (not illustrated) was found for the product formed by *R. niveus* glucoamylase. Thus, each of the α-glucosidases catalyzed the hydration of D-glucal to form specifically 2-deoxy[2(a)-^{2}H]-D-glucose, whereas each of the inverting exo-α-glucanases hydrated the same substrate to form specifically 2-deoxy[2(e)-^{2}H]-D-glucose. Enzymes of the first type protonated (deuteriated) D-glucal from above its *re* face, whereas those of the second type protonated the same substrate from below from *si* face.

Kinetic Studies. Examination was made of the relation between the initial rate of enzymic hydration of D-glucal and the concentration of the substrate. Test and control mixtures comprising 5–180 mM D-glucal and 40 μg/mL rice II (or crystalline *A. niger* α-glucosidase) in 0.04 M acetate buffer of pH 4.0 (or buffer alone) were incubated (30 °C, 30 min) and then assayed for 2-deoxy-D-glucose content with glucose oxidase at pH 7.0. Initial rates of D-glucal hydration by each

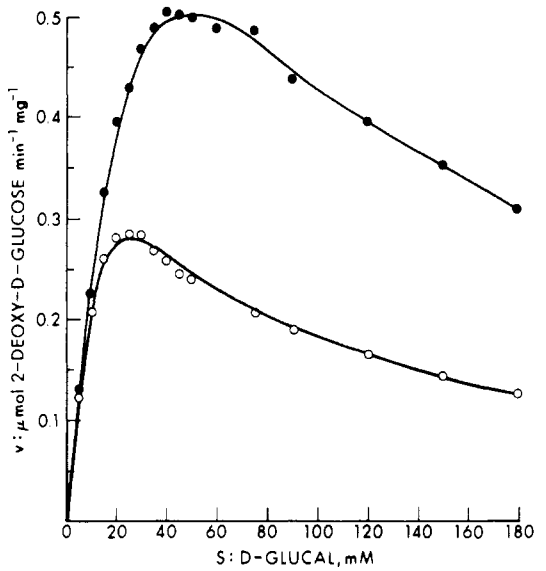

FIGURE 3: Initial rates of D-glucal hydration catalyzed by (●) rice α-glucosidase II and (○) crystalline *A. niger* α-glucosidase, plotted as a function of substrate concentration. Drawn curves express the equation $V_{max} = S/(S + K_s + S^2/K_{ss})$, with values for V_{max}, K_s, and K_{ss} computed from the experimental data for each enzyme.

enzyme were calculated after correction for the 2-deoxy-D-glucose content of substrate/buffer controls. The extent of 2-deoxy-D-glucose formation in all reactions was between 0.1% and 3.0% of available substrate.

Figure 3, which plots the speed of D-glucal hydration by rice II and *A. niger* enzymes versus substrate concentration, shows that the reaction in each case is intensively inhibited by excess substrate. A similar relation (not shown) also was found for the hydration catalyzed by buckwheat α-glucosidase. Kinetic parameters, calculated by an iterative least-squares method (Marmasse, 1963) for the hydration catalyzed by rice II and *A. niger* α-glucosidases, respectively, were V_{max} (assuming no inhibition), 1.82 and 0.65 μmol/(min·mg); V_{opt} (observed maximum), 0.51 and 0.28 μmol/(min·mg); K_s, 67.1 and 18.4 mM; S_{opt} (concentration affording V_{opt}), 51.9 and 27.8 mM; and K_{ss} (apparent K_i of a second molecule of substrate), 40.1 and 42.1 mM. In contrast to these findings with the α-glucosidases, no inhibition by excess D-glucal was detected in hydrations catalyzed by the exo-α-glucanases examined. *P. varioti* and *R. niveus* glucoamylase preparations showed a linear relationship between $1/V$ and $1/S$, affording, respectively, V_{max}, 0.021 and 0.044 μmol/(min·mg), and K_m, 150 mM and 600 mM D-glucal. *A. globiformis* glucodextranase produced an S-shaped V versus S curve and an upwardly concave $1/V$ versus $1/S$ curve, suggestive of substrate activation. The D-glucal hydration rates measured in all reactions with the exo-α-glucanases were for the initial stage (0.08–0.28% substrate conversion).

Discussion

The earlier reported ability (Hehre et al., 1977) of a broadly specific *Candida* yeast α-glucosidase to utilize D-glucal has been found to be shared by acidophilic α-glucosidases of various other origins—though not by that of the honeybee. For the first time, a neutral α-glucosidase (from pig serum) also was shown to catalyze D-glucal hydration; however, that from brewers' yeast was without apparent action as had been found (Lehmann & Schröter, 1972) with bakers' yeast α-glucosidase. Why the latter and the honeybee enzyme have little or no activity with D-glucal is not clear, but it would appear possible that the 2-OH group of a glucopyranosyl residue may be essential for productive binding of substrate by these particular α-glucosidases. Each of three inverting exo-α-glucanases examined was found to utilize D-glucal. All did so at sluggish rates as might be expected for enzymes that hydrolyze α-D-glucosides and lower oligosaccharides slowly compared with α-glucans.

Present findings of chief interest are those concerned with the stereochemistry of the newly demonstrated D-glucal hydration reactions. To begin with, those findings provide new distinctions between the α-glucosidases and exo-α-glucanases examined. Thus, all five reactive α-glucosidases (from *A. niger*, rice, buckwheat, sugar beet seeds, and pig serum) were shown to specifically protonate (deuteriate) D-glucal from above its *re* face, as found for the *Candida* enzyme (Hehre et al., 1977). This direction is different from that generally accepted for protonating the glycosidic oxygen atom of an α-D-glucosidic substrate, indicating that each of the α-glucosidases has functionally flexible catalytic groups. In contrast, the glucoamylase of *R. niveus* and of *P. varioti* and the glucodextranase of *A. globiformis* were all found to protonate D-glucal from below its *si* face, i.e., in the manner generally accepted for protonating α-D-glucosidic substrates. This behavior provides no evidence for the functional flexibility of the catalytic groups of these enzymes but also is not evidence for the lack of such flexibility. The catalytic versatility of both *Rhizopus* glucoamylase and *Arthrobacter* glucodextranase has been shown by their ability to catalyze stereocomplementary hydrolytic and nonhydrolytic reactions with α- and β-D-glucosyl fluoride (Kitahata et al., 1981). Each promotes both the hydrolysis of α-D-glucosyl fluoride to form β-D-glucose and the synthesis of α-D-glucosyl transfer products from β-D-glucosyl fluoride in reactions that are not reversals of each other. Also apparent is that the protonation of a glycal from the *si* face is not necessarily linked to the ability of an enzyme to act on α-glucans in exo fashion with inversion of configuration. β-Amylase, an inverting exo-α-glucanase generally similar to glucoamylase and glucodextranase, has been found to protonate maltal from above its *re* face (Hehre et al., 1986); and glycogen phosphorylase (configuration retentive in its reactions with α-D-glucosyl phosphate and maltosidic substrates), to protonate D-glucal from below its *si* face (Klein et al., 1982).

The present α-glucosidases and exo-α-glucanases also differed from each other with respect to the effect of substrate concentration on initial rates of D-glucal hydration. Reactions promoted by three representative α-glucosidases were all strongly inhibited by excess substrate. Analysis showed the data (Figure 3) to be consistent with an expression for inhibition of catalysis due to a dead-end combination of substrate with an inappropriate enzyme form (Cleland, 1970). However, perhaps the slowed 2-deoxy-D-glucose formation with excess substrate may be due to the increasing occurrence of a competing transfer reaction rather than to the diminutions of catalytic activity ("inhibition of the enzyme"). In any event, no inhibition of D-glucal hydration by excess substrate was detected with any of the present inverting exo-α-glucanase; similarly, none had been found for the maltal hydration reaction catalyzed by β-amylase (Hehre et al., 1986).

Special significance attaches to the stereochemical findings obtained for the D-glucal hydration reactions catalyzed by the present α-glucosidases. The observed consistent difference in protonating D-glucal versus α-D-glucosides extends the already substantial evidence, from studies with nonglycosidic substrates, for the ability of various individual glycosylases to catalyze different stereochemical reactions with different

substrates (Hehre et al., 1977, 1982, 1986; Lehmann & Zieger, 1977; Kitahata et al., 1981; Schlesselmann et al., 1982; Lehmann & Schlesselmann, 1983; Tsumuraya et al., 1984; Kasumi et al., 1986, 1987; Kanda et al., 1986). However, it is the information contained in the fully determined steric course of D-glucal hydration by the *A. niger*, rice II, and buckwheat α-glucosidases that, together with the results of related studies, provides fresh insight into the process of catalysis by α-glucosidases and, possibly, into catalysis by glycosylases in general. All three α-glucosidases were found to protonate D-glucal differently than proposed for α-D-glucosides, yet to convert the glucal to a product of the same (α-) anomeric configuration as that produced (Kita et al., 1984; Chiba et al., 1983) from maltotriose and phenyl α-maltoside. Similar characteristics had been observed earlier for reactions catalyzed by *C. tropicalis* α-glucosidase and sweet almond β-glucosidase with D-glucal (Hehre et al., 1977), by *Escherichia coli* β-galactosidase with D-galactal (Lehmann & Zieger, 1977), and by cellulases from *Irpex lacteus* and *A. niger* acting on cellobial (Kanda et al., 1986). Moreover, two of the presently examined α-glucosidase preparations (from *A. niger* and from rice) were recently found (Weiser et al., 1988) to protonate 1,2-dideoxy-D-*gluco*-octenitol from above the *re* face (opposite the direction assumed for α-D-glucosides) while catalyzing its hydration to yield 1,2-dideoxy-D-*gluco*-octulose of α-configuration.

Present findings with the *A. niger*, rice II, and buckwheat α-glucosidases thus provide a significant part of a body of evidence which reveals that the process of catalysis by glycosylases (in particular α-glucosidases) comprises two separate and separately controlled aspects. One is a substrate-dependent "plastic" part, whereby enzymic protonation of some substrates may differ from that of others; the other is a substrate-unrelated "conserved" aspect, concerned with the creation of product configuration. In our earlier study (Hehre et al., 1977) an enzyme-directed approach of acceptor substrates to the reaction center was assumed to account for the formation of products of α-configuration from D-glucal and α-D-glucosides by *Candida* α-glucosidase. At that time (1977), too few reactions with prochiral glycosyl donors had been examined to know whether this relation might hold generally. By now, however, some twenty reactions catalyzed by glycosylases with prochiral substrates have (without exception) yielded products with configuration matching that of comparable products formed by glycosidic substrates by the same enzyme. Present results strongly support the concept that an enzyme-specified approach of an acceptor to the reaction center is the sole or major determinant of product configuration in glycosylation reactions.

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