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Steric Course of the Hydration of D-*gluco*-Octenitol Catalyzed by α-Glucosidases and by Trehalase†

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ABSTRACT: Crystalline *Aspergillus niger* α-glucosidase and highly purified preparations of rice α-glucosidase II and *Trichoderma reesei* trehalase were found to catalyze the hydration of [2-^{2}H]-D-*gluco*-octenitol, i.e., (*Z*)-3,7-anhydro-1,2-dideoxy-[2-^{2}H]-D-*gluco*-oct-2-enitol, to yield 1,2-dideoxy-[2-^{2}H]-D-*gluco*-octulose. In each case, the stereochemistry of the reaction was elucidated by examining the newly formed centers of asymmetry at C-2 and C-3 of the hydration product. The C-1 to C-3 fragment of each isolated [2-^{2}H]-D-*gluco*-octulose product was recovered as [2-^{2}H]propionic acid and identified by its positive optical rotatory dispersion as the *S* isomer, showing that each enzyme had protonated the octenitol (at C-2) from above its *re* face. ^{1}H NMR spectra of enzyme/D-*gluco*-octenitol digests in D_2O showed that the α-anomer of [2-^{2}H]-D-*gluco*-octulose was exclusively produced by each α-glucosidase, whereas the β-anomer was formed by action of the trehalase. The trans hydration catalyzed by the α-glucosidases was found to be very strongly inhibited by the substrate; the cis hydration reaction catalyzed by the trehalase showed no such inhibition. Special importance is attached to the finding that in hydrating octenitol each enzyme creates a product of the same anomeric form as in hydrolyzing an α-D-glucosidic substrate. This result adds substantially to the growing evidence that individual glycosylases create the configuration of their reaction products by a means that is independent of donor substrate configuration, that is, by a means other than "retaining" or "inverting" substrate configuration.

In recent years much new insight into the catalytic capabilities of glycosidaes and glycosyltransferases has been obtained through the study of reactions catalyzed without glycosidic bond cleavage. Studies with glycosyl fluorides and enolic glycosyl donors have, for example, provided several converging lines of evidence for the ability of individual glycosylases to promote different stereochemical reactions. Enzymes that hydrolyze glycosidic substrates with configurational inversion have been found to hydrolyze the corresponding anomer of a glycosyl fluoride but also to catalyze stereocomplementary nonhydrolytic reactions with the other anomer (Hehre et al., 1979, 1982; Kitahata et al., 1980; Kasumi et al., 1986, 1987). Glycosidases and certain glucanases (i.e., β-amylase and cellulases) that catalyze glycal hydration have been shown to protonate that type of substrate from a direction opposite that generally assumed for their glycosidic substrates (Lehmann & Zieger, 1977; Hehre et al., 1977, 1986; Kanda et al., 1986). Other evidence for the functional versatility of glycosylases comes from the finding that an inverting exo-α-glucanase (glucodextranase) catalyzes the hydration of 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol (D-*gluco*-heptenitol) to form 1-deoxy-β-D-*gluco*-heptulopyranose (β-D-heptulose), while promoting reactions leading to α-D-heptulosyl transfer products (Hehre et al., 1980; Schlesselmann et al., 1982). Finally, a clear example of protonation by two diferent catalytic groups of β-galactosidase was obtained with the aid of a new type of enolic glycosyl donor, (*Z*)-3,7-anhydro-1,2-dideoxy-D-*galacto*-oct-2-enitol. Lehmann and Schlesselmann (1983) found β-galactosidase to promote hydration of this octenitol by protonating it from the top (*re*) face, whereas the same enzyme had been shown to protonate D-galactal from below its *si* face (Lehmann & Zieger, 1977).

In view of the significant information obtained about β-galactosidase through its reaction with D-*galacto*-octenitol, the D-*gluco* analogue was prepared (Brewer et al., 1984) in the

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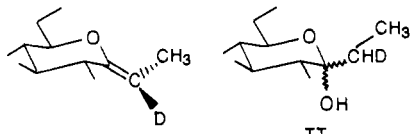
expectation that it might be advantageously used to investigate the catalytic functioning of enzymes that mobilize the D-glucosyl residue. Heretofore, D-glucal was the only substrate known to be converted by such enzymes to products from which the direction of substrate protonation (and full reaction stereochemistry) can be determined.

The present report deals with the steric course of hydration of D-*gluco*-octenitol and its 2-deuterio derivative (I), catalyzed by *Trichoderma reesei* trehalase and by α-glucosidases prepared from rice and *Aspergillus niger*. We envisioned that, by elucidating both the direction of protonation of this substrate and the configuration of the hydration product, 2-deuterio-D-*gluco*-octulose (II), new basic information might



I II

be gained regarding the catalytic abilities of α-glucosidases and of trehalase. For the latter enzyme, which only recently was shown to utilize substrates other than α,α-trehalose and its close analogues (Hehre et al., 1982; Kasumi et al., 1986), D-*gluco*-octenitol offered the first opportunity to examine catalytic behavior with a prochiral substrate.

For the study described below, 2-deuterio-D-*gluco*-octenitol (I) was synthesized and used for elucidating the direction of substrate protonation by the enzymes. The protonic form of D-*gluco*-octenitol was used for kinetic experiments and for determining the anomeric configuration of the hydration product (II). The results are compared with those of related reports dealing with the stereochemistry of reactions catalyzed by α-glucosidases, trehalase, and other glycosylases. In particular, the present findings are discussed with respect to their bearing on the factors involved in determining the steric course of a given reaction catalyzed by a glycosylase.

Experimental Procedures

Substrates. Crystalline 4,5,6,8-tetra-*O*-acetyl-(*Z*)-3,7-anhydro-1,2-dideoxy-D-*gluco*-oct-2-enitol (octenitol) was prepared as recently reported (Brewer et al., 1984); crystalline 4,5,6,8-tetra-*O*-acetyl-(*Z*)-3,7-anhydro-1,2-dideoxy-[2-^{2}H]-D-*gluco*-oct-2-enitol was synthesized by a modified and improved procedure (Weiser et al., unpublished results). Octenitol and [2-^{2}H]octenitol were prepared by deacetylating the respective tetra-*O*-acetates in freshly prepared 0.02 M sodium methoxide in dry methanol; mixtures were held at 0 °C for 10 min, and then at 25 °C until thin-layer chromatography (TLC) showed reaction to be complete (usually 3 h). Stock solutions of each octenitol in dry methanol were kept at −20 °C, protected from moisture. Aliquots were dried under vacuum in test tubes just prior to use. ^{1}H NMR spectra of octenitol in D_2O show H-1 (d, 3 H) at 1.59 ppm and H-2 (q, 1 H) at 5.25 ppm. ^{1}H NMR spectra of [2-^{2}H]octenitol show H-1 to be a singlet at 1.59 ppm, indicating the presence of a deuteron at C-2; integration was consistent with H-1 (3 H).

Enzymes. α-Glucosidase II from ungerminated rice seed (noncommercial cultivar grown at Hokkaido University) was purified to apparent homogeneity as judged by ultracentrifugation and polyacrylamide disc electrophoresis (Takahashi et al., 1971; Murata et al., 1979). This enzyme has been shown to produce exclusively α-D-glucose from maltotriose and phenyl α-maltoside (Chiba et al., 1979, 1983). One sample, which catalyzed the hydrolysis of maltose at the rate of 35 μmol/(min·mg) at 30 °C, pH 4.0, and 5.6 mM maltose, was used in preparing the hydration product from digests of [2-^{2}H]-octenitol and was used also in kinetic experiments. Configuration of the hydration product was determined with a second batch of the enzyme (19.9 units/mg) as well as with a rice α-glucosidase made from commercial grain (Sigma) and further purified by fractionation on columns of Sephadex G-100 and CM-Sepharose (Pharmacia) to a specific activity of 32.3 units/mg. Each of the rice α-glucosidase samples was found to have negligible activity for *p*-nitrophenyl (PNP) β-D-glucoside or salicin (0.01% of the activity for maltose).

α-Glucosidase from *A. niger* was a crystalline sample prepared by Kita et al. (1984) and shown by these authors to be homogeneous on polyacrylamide disc electrophoresis and to produce exclusively α-D-glucose from maltotriose and phenyl α-maltoside (Kita et al., 1984). It hydrolyzed maltose at the rate of 39.7 μmol/(min·mg) (30 °C, pH 4.0, and 5.6 mM maltose); activity for PNP β-D-glucoside and salicin was 0.001% of that for maltose.

Trehalase from *T. reesei* QM 9414 (Vijayakumar et al., 1978), kindly supplied by Dr. E. T. Reese and further purified by fractionation with cold acetone, hydrolyzed 25 mM α,α-trehalose at the rate of 16 μmol/(min·mg) (30 °C, pH 4.4). Its activity for PNP β-D-glucoside was 0.11% of that for trehalose. The enzyme has been reported to hydrolyze trehalose with inversion of configuration of the mobilized glucose residue (Alabran et al., 1986).

Enzyme stocks were kept at 4 °C as suspensions in ammonium sulfate. For use, aliquots were brought to 90% saturation with ammonium sulfate and ultracentrifuged; the sedimented enzyme was dissolved in the required buffer.

Methods Used with Enzymic Digests and Digest Components. Thin-layer chromatography was carried out with silica gel G plates (Analtech) developed with ethyl acetate/ethanol 5:2. Spots were visualized by spraying with anisaldehyde–sulfuric acid and heating at 105 °C. (Stahl & Kaltenbach, 1961). Determinations of 1,2-dideoxy-D-*gluco*-octulose were made by a cuprimetric reduction procedure (Hehre et al., 1980) standardized with octulose prepared by acid hydration of the octenitol. Solvent removal was carried out with rotary vacuum evaporators operated at 30 °C unless otherwise noted.

Chromatographic separation of the hydration product from enzymic digests of 2-deuteriooctenitol was carried out with 1-butanol/ethanol/water 13:8:4 and 23 × 56 cm sheets of prewashed Whatman No. 1 paper, with 0.75 mL of digest usually applied per sheet. After descending development for 24 h, silver nitrate stained guide strips located the octulose product, which was then eluted in methanol from the unstained center panels. Eluates containing octulose produced by a particular enzyme were combined, clarified by centrifugation, and dried under vacuum. Each product was then dissolved in 2.5 mL of deuterium oxide, passed through a Swinnex-HA 0.45-μm Millipore filter, dried in a vacuum evaporator at 45 °C, and finally redissolved in deuterium oxide for examination by ^{13}C and ^{1}H NMR spectroscopy. On completion of these examinations, each octulose sample was redried in preparation for cleavage with recovery of the C-1 to C-3 fragment.

^{1}H NMR spectra at 400 MHz were recorded in D_2O by using Varian VXR 400 and Jeol JNM GX 400 Fourier transform (FT) spectrometers. Chemical shifts (ppm) refer to 3-(trimethylsilyl)propanesulfonic acid sodium salt. ^{13}C and ^{1}H NMR spectra at 50 and 200 MHz, respectively, were recorded in D_2O with a Varian XL 200 spectrometer. ^{13}C chemical shifts (ppm) are reported relative to dioxane at 67.4 ppm. Deuterium oxide was 99.89 atom % D; buffers were prepared with acetic acid-d_4 and NaOD (Hewlett-Packard).

Cleavage and Recovery of [2-^2H]Propionic Acid from [2-^2H]Octulose. The octulose (0.72–0.81 mmol) isolated from individual [2-^{2}H]octenitol/enzyme digests was dissolved in water (60 mL), and solid sodium metaperiodate (5 mmol) was added under stirring at 5 °C in the dark. After 24 h all starting material had disappeared (TLC analysis). Material volatile at room temperature was then collected in a liquid nitrogen trap. Titration with 0.1 N NaOH gave 50% of the theoretically expected amounts of formic acid (C-4, C-5, C-6, C-7) and propionic acid (C-1, C-2, C-3). Apparently the initially formed propionate ester (C-1, C-2, C-3 and C-6, C-7, C-8) is only partly hydrolyzed under the slightly acidic conditions of periodate cleavage. The nonvolatile material from the foregoing procedure was dissolved in 1 N H_2SO_4 (20 mL) and the mixture heated for 35 min at 85–90 °C in a closed vessel. After cooling, the volatiles were again trapped in liquid nitrogen and titrated. The total yield of volatile acids was about 80%. After neutralization to pH 9.5–10, the aqueous solution of the sodium salts of formic and propionic acids from each [2-^{2}H]octulose was adjusted to ca. 4 mL. For the isolation of propionic acid, aliquots (200 μL) were acidified with an equal volume of 4 N H_2SO_4 and submitted to high-performance liquid chromatography (HPLC) fractionation (approximately 20 runs). The recovered propionic acid, well separated from formic acid by this procedure, was immediately neutralized by titration with 0.01 N NaOH solution and lyophilized. The sodium propionate derived from the different octuloses contained, according to ^{1}H NMR integration, more then 95% of the theoretically possible amount of deuterium at C-2. ^{1}H NMR (D_2O, 250 MHz) without internal standard showed 1.04 (dd, 3 H, H-1) and 2.15 ppm (m, 1 H, H-2, –C*H*D–); $J_{1,2}$ = 7.8 Hz, $J_{1,D}$ = 1.2 Hz, and $J_{2,D}$ = 2.2 Hz. Elemental analysis, however, indicated the presence of some extraneous inorganic material in the samples. Hence the free propionic acid was isolated by mixing each sample with an excess of crystalline phosphoric acid and distilling all volatile material under gradual warming to about 55 °C into a liquid N_2 cooled receiver. Since the condensation of some water along with propionic acid was unavoidable, the amounts of both were determined in each sample by mass spectroscopy, kindly carried out by Dr. Jürgen Wörth (University of Freiburg). No compound other than propionic acid and water was detected.

Octulose Reference Standard. A solution of octenitol (20 μmol) in 0.5 mL of 0.025 N sulfuric acid was kept at 100 °C for 15 min and then chilled, neutralized with cold 0.025 N NaOH, and finally diluted to 10.0 mL. The sample gave a single spot on TLC (R_f 0.5).

Results

In preliminary tests, highly purified α-glucosidases of several biological sources (*A. niger*, rice, *Candida tropicalis*, buckwheat, pig serum), as well as *T. reesei* trehalase, were found to catalyze the hydration of octenitol. In each case, digests (30 °C, 18 h) comprising 50 mM substrate and 10 mg/mL enzyme showed, by TLC and reducing sugar analysis, nearly complete conversion of the enitol (R_f 0.8) to a product behaving as 1,2-dideoxy-D-*gluco*-octulose (R_f 0.5); octenitol concurrently incubated in pH 4.4 buffer was not detectably hydrated. Results obtained with respect to the kinetics and stereochemistry of the octenitol hydration reaction catalyzed by three of the enzymes (rice α-glucosidase II, crystalline *A. niger* α-glucosidase, and *T. reesei* trehalase) are described below.

Kinetics. To examine the relation between the initial rate of hydration and the substrate concentration, a series of 80-μL digests was prepared containing octenitol in a 10-fold range of concentrations plus 0.2 mg/mL enzyme (or buffer alone). Digests with rice or *A. niger* α-glucosidase, buffered at pH 4.0, were incubated at 30 °C for 2 h; those with the *T. reesei* trehalase at pH 4.4 were kept at 30 °C for 40 min. Concentrations of 1,2-dideoxy-D-glucooctulose were determined for test and buffer control mixtures, according to a cuprimetric method (Hehre et al., 1980) with octulose standards; initial rates of enzymically catalyzed hydration [V = μmol/(min·mg)] were calculated after correction for control values.

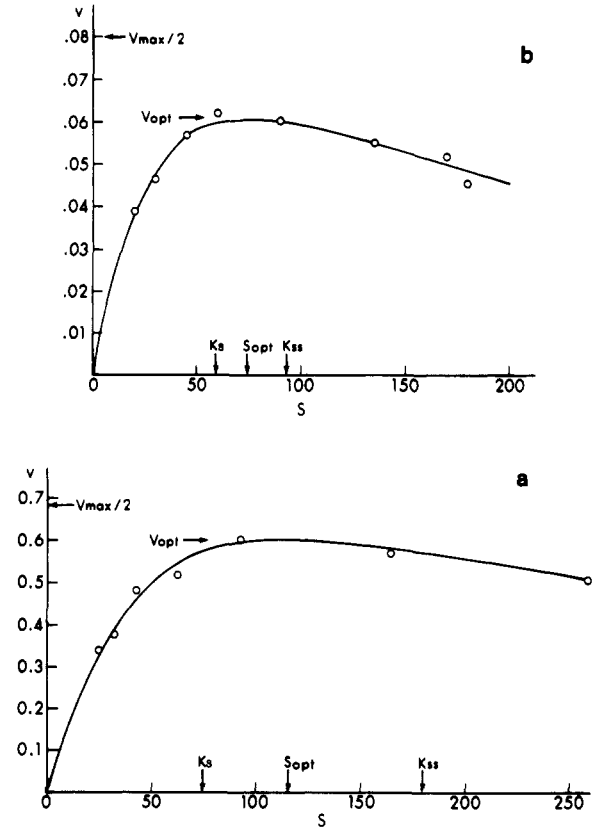


FIGURE 1: Plots of initial velocity, V = μmol/(min·mg), as a function of substrate concentration, S = mM octenitol: (a) rice α-glucosidase II; (b) crystalline *A. niger* α-glucosidase. Curves represent best fit of the data to the Haldane equation by application of the iterative method of Marmasse (1963).

As illustrated in Figure 1, octenitol hydration catalyzed by each of the α-glucosidases showed intense substrate inhibition. Plots of V versus S, when fitted to the Haldane equation by the iterative method of Marmasse (1963), produced values of K_s = 74, S_{opt} (concentration affording V_{opt}) = 115, and K_{ss} (apparent K_i of a second substrate molecule) = 179 mM octenitol, with V_{max} = 1.37 and V_{opt} (maximal observed value) = 0.60 μmol/(min·mg of rice α-glucosidase II) (Figure 1a). For crystalline *A. niger* α-glucosidase (Figure 1b), values of K_s = 59, S_{opt} = 74, and K_{ss} = 96 mM octenitol were obtained, with V_{max} = 0.16 and V_{opt} = 0.061 μmol/(min·mg). It is noteworthy that, with each α-glucosidase, especially that from *A. niger*, the maximally observed velocity, V_{opt}, was smaller than 0.5V_{max} and, also, that V_{opt} for the rice enzyme was about 10 times that for the *A. niger* enzyme. In contrast to the α-glucosidases, *T. reesei* trehalase catalyzed octenitol hydration without detectable substrate inhibition. In a series of digests with substrate concentrations between 8 and 240 mM, a linear relation was found between V^{-1} and S^{-1}, from which values of K_m = 11.7 mM octenitol and V_{max} = 0.41 μmol/(min·mg) were calculated.

Isolation and Characterization of [2-^2H]Octulose Formed Enzymically from [2-^2H]Octenitol. To further characterize

the product formed from octenitol by action of the different enzymes and, in particular, to allow investigation of the direction of protonation of the substrate by each enzyme, the hydration product was recovered from replicate 1.5–3.0-mL digests with [2-^{2}H]octenitol. In total, the digests comprised the following: rice α-glucosidase II (6 mg, 210 units) plus 1.2 mmol of [2-^{2}H]octenitol in 6 mL of 0.05 M acetate buffer of pH 4.0; crystalline *A. niger* α-glucosidase (18 mg, 720 units) plus 1.1 mmol of [2-^{2}H]octenitol in 5.4 mL of the pH 4.0 buffer; *T. reesei* trehalase (15 mg, 240 units) plus 1.2 mmol of [2-^{2}H]octenitol in 7.5 mL of 0.05 M acetate buffer of pH 4.4.

The digests were incubated at 30 °C until reducing sugar measurements and TLC examinations indicated the substrate to be nearly completely utilized (24, 32, and 50 h, respectively, with the rice, *A. niger*, and *T. reesei* enzymes). The hydration product in each case was recovered by paper chromatography (see Experimental Procedures).[1] Yields were as follows: rice α-glucosidase II product, 177 mg; *A. niger* α-glucosidase product, 160 mg; *T. reesei* trehalase product, 175 mg.

^{13}C NMR spectra of the three isolated (equilibrated) hydration products in D_2O were essentially superimposable. Each sample showed a proton-decoupled eight-carbon resonance spectrum that was consistent with [2-^{2}H]octulose of one anomeric form. Chemical shifts were as follows: C-1, 7.15 ppm (s); C-2, 30.62 ppm (t); C-3, 99.60 ppm (s); C-4 to C-7, 70.60 (s), 72.98 (s), 73.46 (s), and 74.60 ppm (s); C-8, 61.49 ppm (s). The triplet of the C-2 resonance with proton decoupling indicates the presence of a deuteron at C-2. ^{1}H NMR spectra in each instance showed H-1 as a doublet centered at 0.92 ppm with $J_{1,2}$ = 7.2 Hz, H-2 as a quartet centered at 1.73 ppm with $J_{1,2}$ = 7.2 Hz, and H-4 to H-8 as broad multiplets between 3 and 4 ppm. Integration showed H-1 to be 3 H and H-2 to be 1 H. The doublet at H-1 is consistent with the presence of one proton and one deuteron at C-2. The coupling patterns also indicate a 3:1 ratio of protons at C-1 and C-2, respectively. The presence of one doublet assignable to H-1 is consistent with one anomeric form of the product at equilibrium.

Direction of Enzymic Protonation in [2-^{2}H]Octenitol Hydration. The above [2-^{2}H]octulose samples, isolated from digests of [2-^{2}H]octenitol by the α-glucosidases and trehalase, were used in the following way to determine the direction of enzymic protonation of the enolic substrate. Each [2-^{2}H]-octulose was subjected to cleavage by periodate, following which the C-1 to C-3 fragment of the sugar was recovered as [2-^{2}H]propionic acid by HPLC and further purified by lyophilization (see Experimental Procedures). Polarimetric measurements were made with solutions (in 0.1 N HCl) of the [2-^{2}H]propionic acid samples, and specific optical rotations were calculated at five different wavelengths between 291 and 589 nm, through the kindness of Dr. Klaus Noack (Hoffmann-La Roche, Basel). As illustrated in Figure 2, the [2-^{2}H]propionic acid derived from the product formed from [2-^{2}H]octenitol by the *A. niger* α-glucosidase (A), rice α-glucosidase II (B), and *T. reesei* trehalase (C) in each case shows a positive optical rotatory dispersion (ORD) curve with positive specific optical rotations at all wavelengths examined. The observed rotations (average and standard deviation of 120–170 measurements per value) were comparable to those (Figure 2D) reported in the literature for (*S*)-[2-^{2}H]propionic acid.

[1] Transfer product formation from D-*gluco*-octenitol proved to be insignificant in contrast to the substantial production of such products from D-*gluco*-heptenitol by rice and *C. tropicalis* α-glucosidases and by glucodextranase (Schlesselmann et al., 1982).

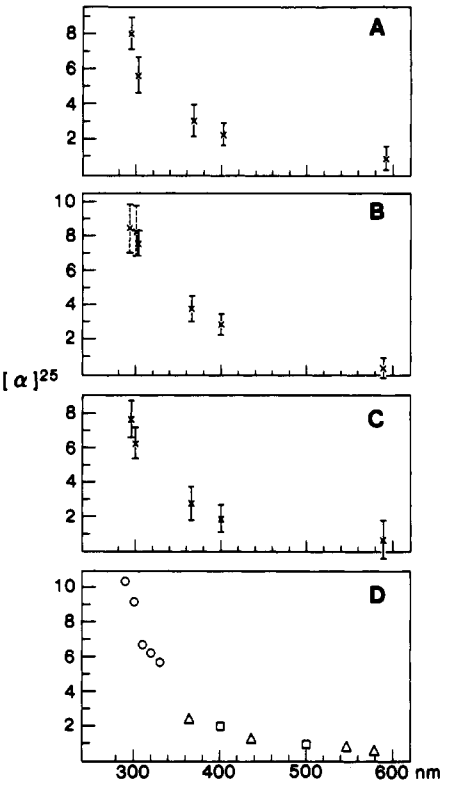


FIGURE 2: (A–C) Specific optical rotations, at various wavelengths, of [2-^{2}H]propionic acid derived from the product of 1,2-dideoxy-[2-^{2}H]-D-*gluco*-octenitol hydration, catalyzed respectively by *A. niger* α-glucosidase, rice α-glucosidase II, and *T. reesei* trehalase; each point (×) represents the average (with mean variation) of 120–170 polarimetric readings at concentrations of 2.15 g/L (A), 2.58 or 1.29 g/L (B), or 1.81 g/L (C) [2-^{2}H]propionic acid. (D) Specific optical rotations of (*S*)-[2-^{2}H]propionic acid reported by Retey et al. (1966) (△), Armarego et al. (1976) (□), and Adlersberg et al. (1977) (○).

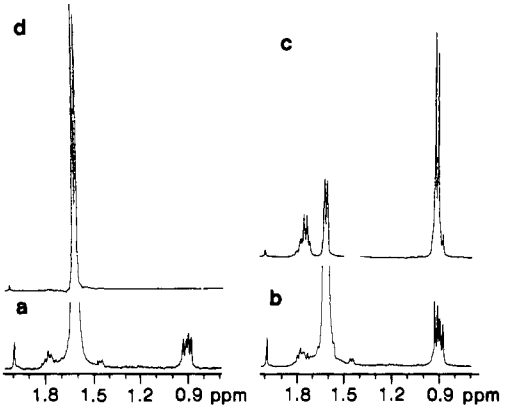


FIGURE 3: ^{1}H NMR spectra (upfield region) recorded at 400 MHz: (a, b, and c) digest containing 100 mM octenitol and 5 mg (80 units)/mL *T. reesei* trehalase in deuterium oxide buffered at pD 4.9, incubated at 20 °C for 65 min, 110 min, and 24 h, respectively; (d) control mixture of 100 mM octenitol in pD 4.5 buffer, incubated at 20 °C for 140 min.

These findings do not suffice to show the absolute specificity of the direction of protonation of the substrate by the enzymes, but they do show that, with each of the enzymes, the overall direction of protonation was from above the *re* face of the substrate.

Anomeric Configuration of the Octulose Produced Enzymically from Octenitol. In order to elucidate the full stereochemistry of the octenitol hydration reaction catalyzed by the rice and *A. niger* α-glucosidases and *T. reesei* trehalase, the configuration of the octulose produced by each enzyme was investigated. Digests of octenitol were set up in buffered

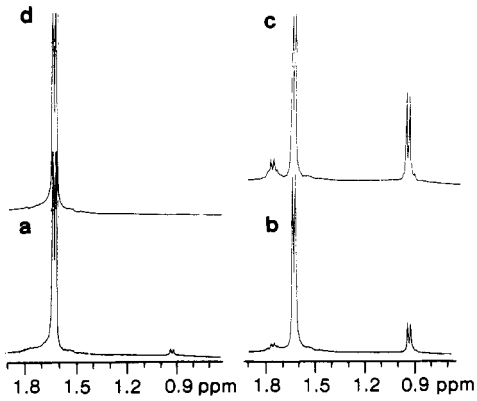


FIGURE 4: ^{1}H NMR spectra (upfield region) recorded at 400 MHz: (a, b, and c) digest containing 75 mM octenitol and 14.1 mg (280 units)/mL rice α-glucosidase II in deuterium oxide buffered at pD 4.5, incubated at 25 °C for 3, 28, and 70 min, respectively; (d) digest containing 75 mM octenitol and 0.05 mg (0.45 unit)/mL sweet almond β-glucosidase (Sigma), incubated at 25 °C for 40 min.

deuterium oxide and the reactions monitored by ^{1}H NMR spectra at 400 MHz.

T. reesei trehalase, exhaustively dialyzed against 0.05 M acetate-d_4/D_2O buffer of pD 4.9, was added to octenitol (freshly dried from solution in methanol-d_4) to give a digest comprising 100 mM octenitol and 5 mg (80 units)/mL trehalase. As illustrated in Figure 3, spectra recorded after 65-min, 110-min, and 24-h incubation at 20 °C show two H-] doublets, centered at 0.88 and 0.92 ppm. At 65 min (Figure 3a), the upfield doublet, assigned to the methyl protons of an axially oriented ethyl group with one ^{2}H at C-2, appears somewhat larger than the lower field (0.92 ppm) doublet. By 110 min (Figure 3b) both resonances have increased, especially that at 0.92 ppm.

After 24 h (Figure 3c) when most of the octenitol has been hydrated, the octulose H-1 resonance is seen to comprise predominantly the 0.92 ppm doublet, which occludes the downfield limb of the smaller doublet present at 0.88 ppm. The increasing proportion of the α-anomer of octulose after 1 h is evidence of anomerization at C-3, and the observed final preponderance of the α-anomer agrees with the results obtained with ^{13}C NMR spectra of the isolated [2-^{2}H]octulose sample. The integrated intensities and coupling constants of the H-1 and H-2 (1.73 ppm) octulose resonances in spectrum c of Figure 3 indicate the presence of one proton and one deuteron at C-2 of the product. The results are consistent with the production of the β-anomer of 1,2-dideoxy-[2-^{2}H]-D-*gluco*-octulose by trehalase action. Nonenzymatic β- to α-anomerization evidently accounts for much if not all of the observed α-anomer. However, the data do not suffice to show that production of the β-anomer by *T. reesei* trehalase is quantitatively specific.

By contrast, the octulose formed by action of the α-glucosidases on octenitol was found to be the α-anomer. In Figure 4, spectra a–c were recorded with a digest of 75 mM octenitol and 14.1 mg (280 units)/mL exhaustively dialyzed rice α-glucosidase II in 0.05 M acetate-d_4/D_2O buffer of pD 4.5. As shown in spectra a of Figure 4, a doublet centered at 0.93 ppm is present after 3-min incubation at 25 °C. This doublet, assignable to the three protons of C-1 in the equatorial ethyl group of α-octulose, increases rapidly with time. At 28 and 70 min (spectra b and c) it is found associated with the H-2 quartet, at 1.75 ppm, of [2-^{2}H]-α-octulose and with a very small doublet at 0.88 ppm of the C-1 protons in the axial ethyl group of β-octulose. This trace of late-appearing β-octulose is presumably the result of slight anomerization of the enzymically formed α-octulose. An origin related to the miniscule level of β-glucosidase activity (0.017 PNP β-D-glucoside unit/mL) present in the digest cannot be excluded but appears unlikely. Spectrum d of Figure 4, recorded for a control digest of 75 mM octenitol with 0.45 PNP β-D-glucoside unit/mL sweet almond β-glucosidase (Sigma), shows no β-octulose H-1 resonance after 40-min incubation at 25 °C.

^{1}H NMR spectra of a digest of 75 mM octenitol, with 15.2 mg (720 units)/mL α-glucosidase from rice of a different variety, showed a pattern of resonance development comparable in every detail to the pattern seen in Figure 4a–c. ^{1}H NMR spectra at 400 MHz (not illustrated) were recorded for a digest of 75 mM octenitol plus 19.9 mg (720 units)/mL crystalline *A. niger* α-glucosidase buffered at pD 4.5. A barely detectable H-1 doublet for α-octulose, at 0.92 ppm, was found after 3-min incubation at 25 °C. The intensity of the doublet increased throughout the period of observation (90 min) although not nearly so rapidly as with the rice α-glucosidase II digest under the same conditions. At 90 min, the spectrum showed the octulose H-2 resonance quartet centered at 1.75 ppm but no sign of the H-1 at 0.88 ppm assigned to the β-anomer of octulose.

Discussion

Crystalline *A. niger* α-glucosidase and highly purified preparations of rice α-glucosidase II and *T. reesei* trehalase were shown to catalyze the hydration of (*Z*)-1,2-dideoxy-[2-^{2}H]-D-*gluco*-oct-2-enitol (I), yielding 1,2-dideoxy-[2(*S*)-^{2}H]-D-*gluco*-3-octulopyranose (II). The specific *S* orientation of the ^{2}H atom at C-2 in each octulose product was determined by examining the latter's C-1 to C-3 fragment which was excised and recovered as pure [2-^{2}H]propionic acid. Each sample of the acid gave a positive optical rotatory dispersion curve, with specific rotations approximating the reported values for (*S*)-[2-^{2}H]propionic acid. All three enzymes thus protonated the octenitol predominantly from the top (*re*) face. With regard to configuration of the hydration product, the 1,2-dideoxy-D-*gluco*-octulose formed by the action of the *A. niger* and rice α-glucosidases proved to be exclusively the α-anomer; the *T. reesei* trehalase was found to give the β-octulose (the data do not show whether it produces only the β-anomer). Configuration was determined by ^{1}H NMR spectroscopy, with resonances of the H-1 (methyl group) protons centered at 0.92 and 0.88 ppm assigned, respectively, to the α- and β-anomers of 1,2-dideoxy-D-*gluco*-octulose. H-1 resonances at 0.92 ppm were preponderant in spectra of anomerically equilibrated 1,2-dideoxy-D-*gluco*-octulose which doubtless is the thermodynamically favored α-anomer (bulky substituents favor the equatorial position) as found for such close analogues as 1-deoxy-α-D-*gluco*-heptulose (Hehre et al., 1980) and D-*gluco*-α-L-*glycero*-3-octulopyranose (Westerlund, 1984). In sum, both α-glucosidases are found to catalyze a trans hydration of 1,2-dideoxy-D-*gluco*-octenitol, whereas the trehalase catalyzes an overall cis hydration of this substrate. Both types of hydration possibly proceed by way of a transient octulosyl carbonium ion/enzyme intermediate, with product configuration determined in a final step (Figure 5).

That the α-glucosidases and trehalase protonate octenitol from a direction opposite that generally assumed for their α-glucosidic substrates is indicative of the functional flexibility of their catalytic groups. This behavior parallels that observed with various α-glucosidases (including the present *A. niger* and rice preparations) in catalyzing D-glucal hydration; all were found to protonate the D-glucal from above its *re* face (Hehre et al., 1977; Chiba et al., 1988). No such comparison can be made with *T. reesei* trehalase as it does not appreciably utilize

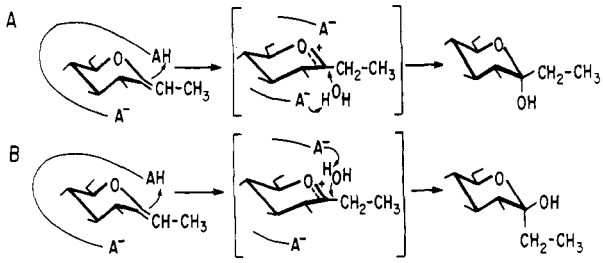


FIGURE 5: (A) Trans hydration of D-*gluco*-octenitol catalyzed by *A. niger* and rice α-glucosidase, with substrate protonation from the *re* face (ionization of the catalytic groups opposite that assumed for protonating α-D-glucosidic substrates). An enzyme-directed (general-base-catalyzed) attack of water from below the reaction center is assumed, yielding the observed α-D-*gluco*-octulose. (B) Cis hydration of D-*gluco*-octenitol catalyzed by *T. reesei* trehalase, with substrate protonated from the *re* face, and with enzyme-directed water attack from above the reaction center to yield β-D-*gluco*-octulose.

D-glucal[2] even though the catalytic flexibility of this and other trehalase preparations is evident from their ability to promote different stereochemical reactions with α- and β-D-glucosyl fluoride (Hehre et al., 1980; Kasumi et al., 1986). The behavior of the α-glucosidases in protonating D-*gluco*-octenitol and D-glucal from the same direction contrasts directly, however, with that of β-galactosidase, which was found by Lehmann and Schlesselmann (1983) to protonate D-*galacto*-octenitol from a direction opposite that observed with D-galactal (Lehmann & Zieger, 1977), thereby providing direct evidence for the operation of two different protonating groups in the enzyme. The dissimilar functioning of the α-glucosidases and β-galactosidase in acting on comparable enolic substrates represents a newly recognized manifestation of catalytic flexibility. It is not clear why α-glucosidases should protonate both octenitol and glucal oppositely from α-D-glucosides, or why β-galactosidase should protonate galactal (but not octenitol) oppositely from β-D-galactosides. However, the demonstrated occurrence of their dissimilar action patterns is significant in that it raises the basic question of what factors (apart from the proximity of a catalytic group to a glycosylic bond) are involved in determining the direction of protonation of a particular substrate by a given glycosylase.

Findings on the configuration of the product formed from octenitol by action of the α-glucosidases and the trehalase are significant for a different reason. Each enzyme not only creates product configuration de novo in hydrating this prochiral substrate but also controls the hydration reaction by some means so that the same configuration is formed (α by the α-glucosidases, β by the trehalase) as from the hydrolysis of an α-D-glucosidic substrate. These observations extend the findings of related studies to the point where the accumulated evidence is indeed substantial that enzymic reactions with prochiral (enolic) glycosyl donors consistently yield products whose configuration matches that of comparable products formed by the same enzyme acting on chiral (glycosidic or glycosyl) substrates. Related studies have shown that products of α configuration are formed from D-glucal by α-glucosidases, including the present *A. niger* and rice preparations (Hehre et al., 1977; Chiba et al., 1988), and by glycogen phosphorylases (Klein et al., 1982; Klein & Helmreich, 1985), and likewise from D-*gluco*-heptenitol by α-glucosidases (Hehre et al., 1980; Schlesselmann et al., 1982) and glycogen phosphorylases (Klein & Helmreich, 1985; Klein et al., 1986). Reactions catalyzed by β-galactosidase with D-galactal, D-*galacto*-heptenitol, and D-*galacto*-octenitol have been found to lead to products of the same (β-) anomeric form as reactions catalyzed with β-D-galactosides (Lehmann & Zieger, 1977; Brockhaus & Lehmann, 1977; Schlesselmann, 1981). Likewise, β-glucosidase has been found to act on D-glucal and D-*gluco*-heptenitol to form hydration products of β form (Hehre et al., 1977, 1980), and cellulases have been found to act on cellobial as well as on cellooligosaccharides to form products of β configuration (Kanda et al., 1986). The formation of β-D-*gluco*-octulose from octenitol by *T. reesei* trehalase [which hydrolyzes α,α-trehalose with release of the mobilized glucosyl residue as β-D-glucose (Alabran et al., 1983)] has a parallel in our earlier finding that *Arthrobacter globiformis* glucodextranase, which hydrolyzes dextran to form β-D-glucose (Sawai et al., 1976; Ohya et al., 1978), catalyzes D-*gluco*-heptenitol hydration to form β-D-*gluco*-heptulose.

Taken together, present findings and the above-cited observations show that product configuration in enzymic glycosylation reactions is not mechanistically tied to that of the donor substrate as the terms "retention" and "inversion" imply. The findings with prochiral substrates strongly suggest that product configuration is determined by inherent structural features of the enzyme that channel and direct the approach of water and other acceptor molecules to the reaction center.

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[2] The apparent inability of trehalase to catalyze D-glucal hydration, indicated by the negative results of tests under different conditions, is puzzling in view of its activity with D-*gluco*-octenitol. The absence of an OH group at C-2 or the different ring conformation of D-glucal might appear responsible, but neither factor can explain the observed further failure of trehalase to utilize D-*gluco*-heptenitol as a substrate.

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Stereochemistry and Accessibility of Prosthetic Groups in Flavoproteins†

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ABSTRACT: Using 8-demethyl-8-hydroxy-5-deaza-5-carba analogues of the appropriate flavin nucleotides, we determined the stereochemistry of interaction between coenzyme and substrate for several flavoproteins. The enzymes were D-amino acid oxidase, L-lactate oxidase, and D-lactate dehydrogenase, all three of which interact with pyruvate, as well as cyclohexanone monooxygenase and 2-methyl-3-hydroxypyridine-5-carboxylic acid oxygenase, which were both probed with nicotinamide nucleotides. L-Lactate oxidase and D-lactate dehydrogenase used the *si* face of the modified flavin ring while the other three enzymes showed *re*-side specificity. This selection of flavoenzymes includes FAD- and FMN-dependent enzymes, enzymes that follow a carbanion mechanism, and others that have hydride transfer as an integral part of their reaction pathway.

8-Demethyl-8-hydroxy-5-deaza-5-carbariboflavin (Ashton & Brown, 1980) is a flavin analogue whose specific features make it well suited for probing stereospecificities of flavin coenzymes in enzymic reactions. Its shape and charge usually do not interfere with binding at active sites of enzymes; it can be transformed to the FMN and FAD levels, respectively, by the well-characterized riboflavin kinase/FAD synthetase from *Brevibacterium ammoniagenes* (Spencer et al., 1976; Manstein & Pai, 1986); it is stable against reoxidation by air; it does not show the rapid two-electron disproportionation found in mixtures of oxidized and reduced 5-deazaflavins[1] (Spencer et al., 1976).

In the previous paper of this series (Manstein et al., 1986) we described a method for determining the stereochemistry

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[1] Abbreviations: MHPC-oxygenase, 2-methyl-3-hydroxypyridine-5-carboxylic acid oxygenase; $AcPyAD^+$, oxidized 3-acetylpyridine adenine dinucleotide; $AcPyADP^+$, oxidized 3-acetylpyridine adenine dinucleotide phosphate; 5-deazaflavin, 5-deaza-5-carbaisoalloxazine; 8-OH-5-deazaflavin, 8-demethyl-8-hydroxy-5-deaza-5-carbaisoalloxazine; [5-^{3}H]-8-OH-5-deaza$FADH_2$, reduced 8-OH-5-deazaFAD with one tritium label at carbon 5; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; MES, 2-(*N*-morpholino)ethanesulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography.

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