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Scope and Mechanism of Carbohydrase Action: Stereospecific Hydration of D-Glucal Catalyzed by α- and β-Glucosidase†

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ABSTRACT: A unique demonstration is presented of the capacity of glycosidases to create anomeric configuration de novo. Purified *Candida tropicalis* α-glucosidase and sweet almond β-glucosidase have been found to attack the same substrate, D-glucal, and to convert this unusual glycosyl substrate (which lacks α or β anomeric configuration) to 2-deoxy-α- (or β-) D-glucose, respectively. The stereospecificity of the hydration reaction catalyzed by each enzyme in D_2O was revealed by the use of high-resolution (270 MHz) ^1H magnetic resonance spectroscopy. The α-glucosidase caused a specific axial protonation (deuteration) of D-glucal at C-2, and formation of 2-deoxy-α-D-[2(a)-^2H]glucose. The β-glucosidase catalyzed an oppositely directed axial protonation at C-2 and formation of 2-deoxy-β-D-[2(e)-^2H]glucose. These results are not accounted for by the generally accepted mechanisms of carbohydrase action derived from studies with glycosidically linked substrates alone. D-Glucal apparently binds to the enzymes with essentially the same overall orientation as the D-glucosyl moiety of glycosidically linked substrates (with the double bond of D-glucal lying essentially in the plane of the similarly bound D-glucosyl group). Thus, the α-glucosidase evidently protonates D-glucal from above the double bond and α-D-glucosidic substrates from below the glycosidic oxygen; β-glucosidase apparently protonates D-glucal from below the double bond and β-D-glucosides from above the glycosidic oxygen. A detailed mechanism is proposed for the hydration of D-glucal by each enzyme, involving an incipient glycosyl carbonium ion and assuming the presence at the active site of two carboxyl groups arranged to account for catalysis of glycosylations from glycosidically linked substrates. That D-glucal serves as a glycosyl substrate for these enzymes strongly supports the concept that glycosidases and glycosyltransferases are catalysts of glycosylation (i.e., glycosylases), since this concept does not make the usual assumption that carbohydrases are restricted to acting on substrates having a glycosidic bond and either α- or β-anomeric configuration.

The investigation of enzymatic glycosylation reactions that take place without glycosidic bond cleavage appears to hold considerable promise as an approach to gaining a more complete understanding of the catalytic actions of carbohydrases. Previous studies with glycosyl fluorides (Hehre et al., 1973, and cited references), for example, have yielded results that are incompatible with the long-held assumptions that glycoside hydrolases (EC 3.2) cause overwhelming hydrolysis of all their substrates, and that glycosyltransferases (EC 2.4) (transglycosylases, Hehre (1951)) require preexisting glycosidic bonds for their actions in synthesizing glycosidic linkages. These findings strongly support the thesis that a uniform chemical change, glycosyl-X + H-X′ $\rightleftarrows$ glycosyl-X′ + H-X, is effected in all reactions catalyzed by glycosidases (EC 3.2) and glycosyltransferases, including the formation and breakdown of glycosyl-enzyme intermediates. Unlike the hydrolase and transferase models, this concept carries no assumption restricting the nature of the glycosyl donor or the proton source that may take part in a reaction, and none restricting the mechanism(s) whereby the former becomes protonated and the latter glycosylated. Since currently accepted mechanisms of carbohydrase action assume stereospecific protonation of a glycosidic bridge atom, the study of reactions involving substrates without a glycosidic bond may be seen as potentially

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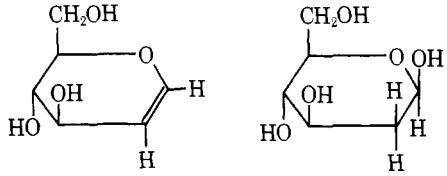
able to provide new insight into the catalytic mechanism of carbohydrases.

The interaction of glycals and glycosidases was first noted by Lee (1969) who observed that D-galactal is a powerful and highly specific inhibitor of β-galactosidases. Lehmann and Schröter (1972) and Wallenfels and Weil (1972) subsequently found D-galactal to be a substrate for *Escherichia coli* β-galactosidase, a finding confirmed by Wentworth and Wolfenden (1974). The first authors further reported that D-glucal (I) is specifically utilized (*e.g.*, hydrated to 2-deoxy-D-glucose (II))



I II

by sweet almond β-glucosidase, but that it is not attacked by *Saccharomyces* yeast α-glucosidase (maltase). Mechanisms were suggested for the binding and cleavage of D-galactal and D-glucal by β-galactosidase and β-glucosidase, respectively. However, no explanation was given as to why catalytically productive binding should occur only with β-glycosidases.

The present study had its origin in an interpretation of these results with glycals in terms of the above concept that the action of carbohydrases is to convert one glycosylic (not necessarily glycosidic) bond to another. The bond between C-1 and C-2 in glycals that is attacked by β-glycosidases was regarded by Hehre et al. (1973) as meeting the definition of a glycosylic bond, i.e., the union joining the anomeric carbon atom of the cyclical form of a sugar to the anomeric hydroxyl, or to any group replacing this hydroxyl. Thereby, a new view of the glycals as internal C-glycosyl compounds was obtained. D-Glucal (I), for example, was visualized as an internal 2-deoxy-D-glucosyl compound (Hehre, 1974).[1]

Reexamination of the capacity of α-glucosidase to attack D-glucal was undertaken despite Lehmann and Schröter's (1972) negative result with a yeast maltase, since it appeared likely that this failure might simply reflect the inability of a particular α-glucosidase to mobilize the 2-deoxy-D-glucosyl (in contrast to the D-glucosyl) moiety. Carbohydrases of a given type are known to differ in the stringency of their requirement as to the structure of the glycosyl moiety of substrates; *e.g.*, some β-glucosidases do and some do not hydrolyze β-D-xylosides, or β-D-galactosides, in addition to β-D-glucosides. The C-1 symmetry and, hence, lack of α or β configuration of D-glucal should present no greater barrier to attack of the double bond between C-1 and C-2 by appropriate α-glucosidases than by β-glucosidases. Indeed, as reported in abstract form (Hehre, 1974) and fully described below, the α-glucosidase from a species of *Candida* yeast has a high capacity to utilize D-glucal as a substrate.

We herein describe the ability of the α-glucosidase from *Candida tropicalis* and the β-glucosidase from sweet almonds to catalyze the stereospecific hydration of D-glucal to give the α and β anomers of 2-deoxy-D-glucose, respectively. High-resolution (270 MHz) proton nuclear magnetic resonance (NMR)[2] was used to determine the nature of the hydration reaction in each case. The findings are discussed in relation to the currently accepted models for carbohydrase-catalyzed reactions.

Experimental Procedures

Highly purified α-glucosidase from *Candida tropicalis*, variety japonica (Sawai, 1958, 1960; Sawai and Hehre, 1962), was the generous gift of Dr. Teruo Sawai. The enzyme had been purified by a multistage procedure as previously reported (Sawai, 1967) and was free from β-glucosidase activity, from dextrinogenic action on starch, and from hydrolytic activity on sucrose. Crystalline β-glucosidase from sweet almonds was purchased from Boehringer Mannheim Corp., N.Y. It had a specific activity of 40 IU/mg as assayed with salicin.

D-Glucal (D-*arabo*hexal) was prepared from 3,4,6-tri-acetyl-D-glucal (Aldrich Chemical Co.) following the procedure of Shafizadeh and Stacey (1952). Crystallization from ethanol yielded the product, $[\alpha]^{26°}_D = -7.1°$ (*c* 1.0, in water) unchanged on standing (lit. $[\alpha]^{19°}_D = -7.2°$ (Bergmann and Schotte, 1921)). The product gave a single spot on paper chromatography and was completely converted to 2-deoxy-D-glucose by treatment with 2 N H_2SO_4 (4 °C, 24 h).

2-Deoxy-β-D-glucose (2-deoxy-β-D-*arabino*-hexopyranose) was the grade III crystalline product (lot 12C-5200) from Sigma Chemical Co. The sample had a mp of 148–150 °C (uncorrected) (lit. 148 °C (Bergmann et al., 1922)); $[\alpha]^{23°}_D = +26.5°$ at 2 min to +48.2° after 35 min (*c* 0.50, in water), $k_1 + k_2 = 0.06$ min^{-1}; $[\alpha]^{21°}_D = +17.6°$ at 2 min to +43.1° after 90 min (*c* 0.50, in deuterim oxide, 99.7 atom % D), $k_1 + k_2 = 0.011$ min^{-1} (lit. $[\alpha]^{17.5°}_D = +38.3°$ to +45.9° after 35 min (*c* 0.52, in water), Overend et al., 1949). All measurements of optical rotation were made in a Rudolph and Sons precision polarimeter (Model 70) and 2-dm tubes.

Spectrophotometric Assay of 2-Deoxy-D-glucose. A modification of the glucose oxidase–chromogen procedure for determining D-glucose was used to quantitatively determine 2-deoxy-D-glucose in the presence of D-glucal. McComb et al. (1957) had found 2-deoxy-D-glucose oxidized at ~12% of the rate for D-glucose. To a mixture of 0.20 mL of glucose oxidase (special glucostat reagent, Worthington Biochemicals), 1.8 mL of 0.15 M phosphate (pH 7.0) buffer, and 2.0 mL of chromogen was added 1.0 mL of the sample solution. Standard solutions containing 0.8, 1.6, 2.4, and 3.2 μmol/mL of 2-deoxy-D-glucose were run concurrently. After 10 min at 25 °C, the mixtures were acidified with two drops of 4 N HCl and the optical density was read at 400 nm in a Gilford Model 300 Microsample spectrometer. D-Glucal gave optical-density readings equivalent to 0.4% of those produced by 2-deoxy-D-glucose. With correction, the latter could be accurately determined even in the presence of a 50-fold excess of D-glucal.

Proton Magnetic Resonance Spectrometry. High-resolution ^{1}H NMR spectra in D_2O (99.7 atom % D, Merck, Sharpe and

[1] This view was developed as follows. In the classical synthesis of D-glucal from 2,3,4,6-tetra-*O*-acetyl-α-D-glucosyl bromide, the reductive removal of bromine from C-1 and of acetoxy from C-2 may be considered to convert the D-glucosyl to a 2-deoxy-D-glucosyl moiety. The loss of asymmetry and of anomeric configuration at C-1 that accompanies its sharing a further pair of electrons with C-2 would not be a reason to deny the continued existence of the 2-deoxy-D-glucosyl moiety, since a C-1 atom devoid of asymmetry and anomeric configuration is present also in glycosyl oxycarbonium ions. The enolic bond between C-1 and C-2 is, of course, much different from the single bond that joins a glycosyl group in glycosidic linkage. Nevertheless, it is possible to imagine that the stronger (σ bond) component of the enolic bond might correspond to the bond that normally forms part of the 2-deoxy-D-glucosyl ring structure, and that the weaker (π bond) component might correspond to the glycosylic bond.

[2] Abbreviations used are: NMR, nuclear magnetic resonance; FID, free induction decays.

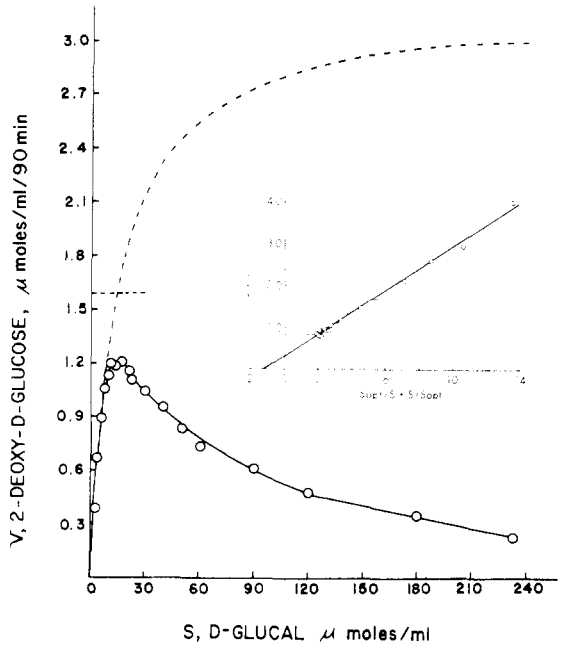


FIGURE 1: Initial rate of 2-deoxy-D-glucose formation, v, as a function of D-glucal concentration, S. Digests (pH 4.6) contained 10 μg/mL *C. tropicalis* α-glucosidase and differing concentrations of D-glucal; incubation, 90 min at 30 °C. The solid line is the theoretical curve based on the equations, $v = V_{max}S/(S + K_m + S^2/K_{SS})$, and the computed values of V_{max} = 3.17 μmol mL^{-1} (90 min)$^{-1}$; K_m = 14.2 mM; K_{SS} = 20.9 mM. The dashed line is the theoretical curve based on the Michaelis–Menten equation, $v = V_{max}S/(S + K_m)$, with intercept at $V_{max}/2$. Insert: fit of data to theoretical curve in plot of $1/v$ vs. $S/S_{opt} + S_{opt}/S$, using the computed value of S_{opt} = 17.2 mM.

Dohme, Ltd., Canada) were recorded in the Fourier transform mode. Preliminary data were obtained using a Jeol PFT-100 spectrometer interfaced with a Nicolet Series 1000 computer. Final experiments, recorded below, were carried out using a Brucker HX-270 spectrometer interfaced with a Nicolet 80 computer system. A Diablo disk system was used to store the NMR data before Fourier transformation. Suppression of the residual HOD resonance in the D_2O solutions was achieved by presaturating this signal at the water resonance position prior to signal accumulation.

For the study of D-glucal hydration by α- and β-glucosidase by NMR spectroscopy, D-glucal and the enzymes were extensively lyophilized and dialyzed, respectively, in D_2O to exchange their labile hydrogens for deuterium atoms. At time zero, sample solutions were prepared by adding 0.6 mL of the appropriate enzyme (0.3 mg of α-glucosidase; 3 mg of β-glucosidase) in pD 5.2 deuterium oxide to 12 μmol of deuterium-exchanged D-glucal. The solution was transferred to a 5-mm NMR tube, and ^{1}H NMR spectra were recorded once every 128 s for the first 30 min, then once every 38 min for the next 4 h. Each spectrum consisted of 64 free-induction decays (FID) using 2-s repetition times. At the end of four such sets of FID accumulations, the data were stored on a memory disk and later Fourier transformed. The sample temperature was 28 ± 1 °C.

Results

Hydration of D-Glucal by α-Glucosidase. The capacity of *C. tropicalis* α-glucosidase to catalyze the essentially complete hydration of D-glucal to 2-deoxy-D-glucose was shown as follows. Test and control mixtures, containing 40 mM D-glucal and 0.4 mg/mL active or heat inactivated enzyme at pH 4.6, were incubated at 4 °C for 24 h. Paper chromatography of the test digest revealed essentially complete conversion of the substrate to a single new spot migrating with the same R_f value as 2-deoxy-D-glucose. On analysis by the glucose oxidase method, the expected quantity of 2-deoxy-D-glucose was found. No hydration of D-glucal was detected in the control by either method.

The initial rate, v, of formation of 2-deoxy-D-glucose as a function of D-glucal concentration, S, in the presence of the α-glucosidase is shown in Figure 1. In this experiment, mixtures were prepared containing 2 to 233 μmol/mL D-glucal, 10 μg/mL α-glucosidase, and 16 μmol/mL acetate buffer (pH 4.6). After incubation (30 °C, 90 min), each mixture was analyzed for 2-deoxy-D-glucose content (with correction for residual D-glucal) by the glucose oxidase method. All digests showed less than 20% of the substrate hydrated. As shown in Figure 1, the rate passes through a maximum as the concentration of substrate is increased. The concentration providing the optimal rate, S_{opt}, was computed to be 17.2 mM by the iterative method of Marmasse (1963). This method also allows solution of the kinetic equation of Haldane (1930), $V_{max}/v = (1 + K_m/S) + S/K_{SS}$. Thereby, V_{max} was calculated to be 3.17 μmol mL^{-1} (90 min)$^{-1}$ (3.5 μmol min^{-1} (mg of α-glucosidase)$^{-1}$); K_m, 14.2 mM; K_{SS}, 20.9 mM. V_{opt}, the optimal rate (at S_{opt}), was calculated from the relationship, $V_{opt} = V_{max}/[1 + 2(K_m/K_{SS})^{1/2}]$, to be 1.20 μmol mL^{-1} (90 min)$^{-1}$. The close fit of the theoretical curve, based on the Haldane equation, to the experimental points is shown in Figure 1 (solid line). In addition, a close fit of the experimental points with a linear theoretical curve was achieved using the relationship, $1/v$ vs. $S/S_{opt} + S_{opt}/S$, derived by Friedenwald and Maengwyn-Davies (1954). These results represent solid evidence for a dead-end type of inhibition (cf. Cleland, 1970) involving complexes of an α-glucosidase molecule combined with two molecules of D-glucal.

In a limited comparison, *C. tropicalis* α-glucosidase was found to be a much more active catalyst of D-glucal hydration than sweet almond β-glucosidase. In tests using 18 mM substrate, for example, the rate of 2-deoxy-D-glucose formation was found to be 0.033 μmol min^{-1} (mg of purified β-glucosidase)$^{-1}$. The rate was 40-times higher, 1.34 μmol min^{-1} (mg of protein)$^{-1}$, with the *Candida* α-glucosidase. Under the same conditions, the β-glucosidase catalyzed the hydrolysis of phenyl and methyl β-D-glucopyranosides at rates of 1.31 and 0.29 μmol min^{-1} mg^{-1}, respectively; phenyl and methyl α-D-glucopyranosides were hydrolyzed by the α-glucosidase at rates of 1.00 and 0.61 μmol min^{-1} (mg of protein)$^{-1}$ respectively.

Proton NMR Study of the Hydration of D-Glucal by α- and β-Glucosidases. In order to examine the detailed mechanism of hydration of D-glucal catalyzed by *Candida* α-glucosidase and sweet almond β-glucosidase, the changes brought about by each enzyme at the double-bond position of D-glucal were studied by use of high-resolution proton magnetic resonance spectroscopy.

The reference spectra used for resonance assignments were obtained with a solution of 2-deoxy-β-D-glucose in D_2O. The spectrum obtained 2 min after solution of the sugar (Figure 2A) includes peaks at 4.8, 2.25, and 1.5 ppm, which correspond to the resonances assigned by Hall and Manville (1968) to the C-1, C-2 equatorial, and C-2 axial protons of the β anomer. After anomeric equilibration (270 min), these peaks, as expected, are present in reduced size (Figure 2B), while new resonances at 5.25, 2.1, and 1.7 ppm appear which are attrib-

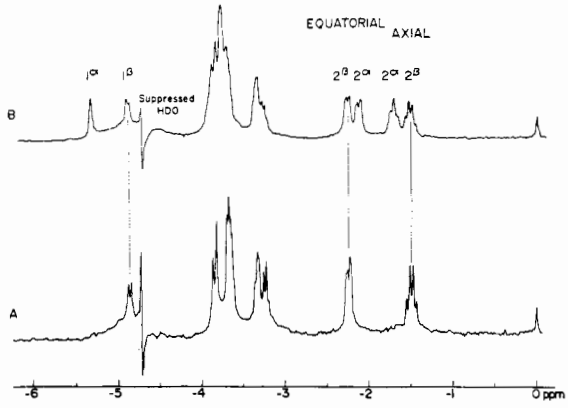


FIGURE 2: ^{1}H NMR spectra at 270 MHz of 2-deoxy-β-D-glucose in D_2O at 28 °C. (A) Two minutes after solution; (B) after 270 min.

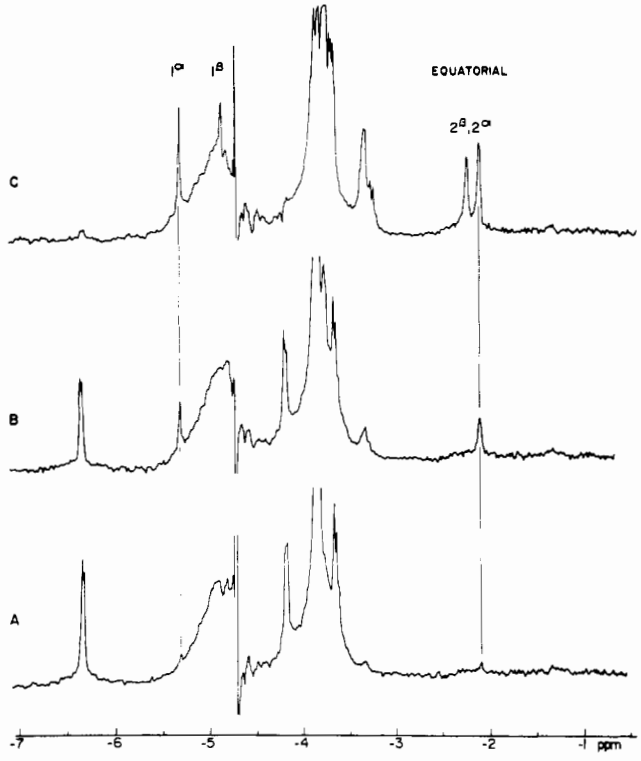


FIGURE 3: ^{1}H NMR spectra at 270 MHz of the 2-deoxy-D-glucose produced in a digest with 20 mM D-glucal and 0.5 mg/mL *Candida* α-glucosidase in D_2O (pD 5.2), incubated at 28 °C. (A) Eight minutes; (B) 30 min; (C) 200 min.

utable to the C-1, C-2 equatorial, and C-2 axial protons of the α anomer (Hall and Manville, 1968).[3]

The action of *Candida* α-glucosidase on D-glucal was examined by following the proton magnetic resonance spectrum of the 2-deoxy-D-glucose formed during incubation (28 °C) of the enzyme–substrate mixture in D_2O (pD 5.2). The results (Figure 3) show clearly that the enzymatically formed 2-deoxy-D-glucose is the α anomer. Moreover, the reaction product, which is detected at 8 min and is more abundant after 30 min of incubation, is identified at 2-deoxy-α-D-[2(a)-^{2}H]glucose by the resonances at 5.25 and 2.1 ppm, characteristic of the C-1 and C-2(e) protons of the α anomer, and by

[3] The τ values observed for C-1, C-2 equatorial (C-2(e)), and C-2 axial (C-2(a)) proton resonances, for both α and β anomers, averaged 0.54 ppm higher than the values reported by Hall and Manville (1968). The relative positions of all resonances were, however, identical.

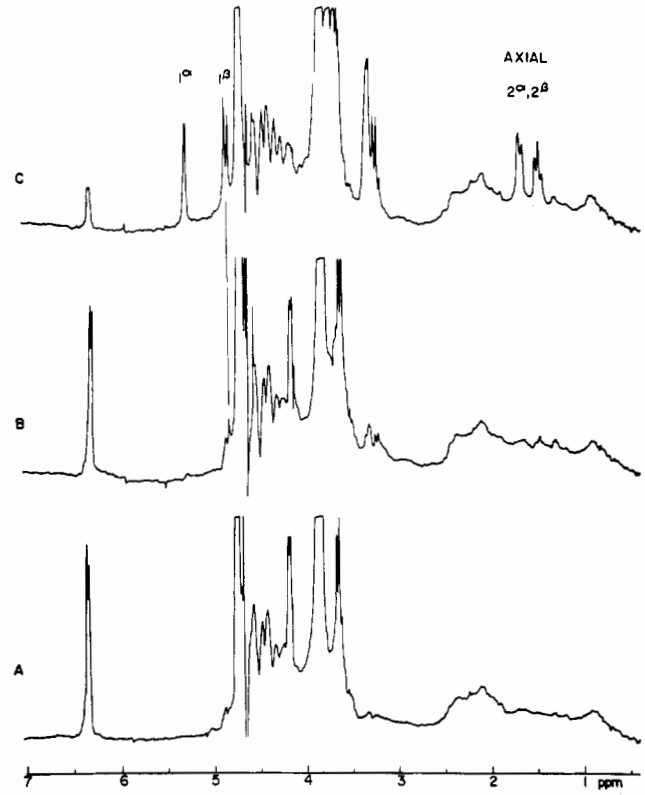


FIGURE 4: ^{1}H NMR spectra at 270 MHz of the 2-deoxy-D-glucose produced in a digest with 20 mM D-glucal and 5 mg/mL sweet almond β-glucosidase in D_2O (pD 5.2), incubated at 28 °C. (A) Eight minutes; (B) 30 min; (C) 180 min.

the absence of a C-2(a) (α) proton signal at 1.7 ppm. The spectrum obtained after incubation of the mixture for 200 min (Figure 3C) shows the C-1 and C-2(e) proton signals of both α and β anomers. It is to be noted that, although anomerization occurred at C-1, there is no epimerization at C-2 during the prolonged incubation. C-2(a) (α) and C-2(a) (β) proton resonances, at 1.7 and 1.5 ppm, would have been expected in the event of such a change, but neither is seen. This evidence of the configurational stability at C-2 provides added assurance that the α-glucosidase-catalyzed protonation (deuteration) of D-glucal is stereospecifically axial at the C-2 position.

The action of sweet almond β-glucosidase in hydrating D-glucal was similarly studied through proton magnetic resonance spectra of the 2-deoxy-D-glucose formed during incubation (at 28 °C) of an enzyme–D-glucal mixture in D_2O (pD 5.2). As shown in Figure 4, the results are opposite to those obtained with the α-glucosidase. The reaction product, though not seen at 8 min, is visualized after 30 min as a resonance doublet at 4.8 ppm marking the C-1 proton of 2-deoxy-β-D-glucose. A spectrum after 180 min (Figure 4C) shows the resonances of the C-1 and C-2(a) protons of both α and β anomers of 2-deoxy-D-[2(e)-^{2}H]glucose. The absence of signals corresponding to the C-2(e) (α) and C-2(e) (β) protons, at 2.25 and 2.1 ppm, confirms the stability of C-2 during anomerization of 2-deoxy-β-D-glucose, and shows that the β-glucosidase-catalyzed protonation (deuteration) of D-glucal is stereospecifically equatorial at the C-2 position.

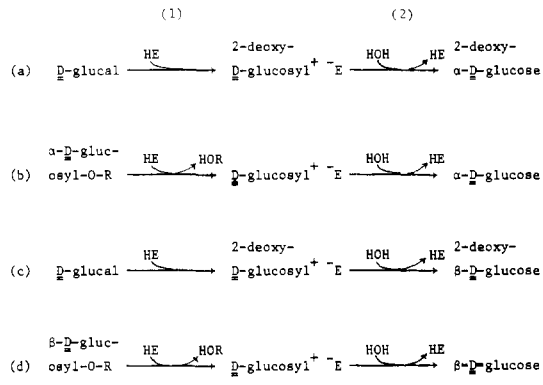
Discussion

The present study provides a unique demonstration of the capacity of glycosidases to create anomeric configuration de novo, in that an α- and a β-glucosidase have been shown to exert their different specificities on a common substrate (D-

glucal) that lacks an α- or β-anomeric configuration. This example of specific hydration of the same substrate and bond by an α- and by a β-glycosidase contradicts the long-standing belief that such enzymes attack only mutually exclusive substrates, and affords information on the basic requirements for carbohydrase action. That D-glucal serves as a specific glycosyl substrate for both of these "complementary" enzymes confirms in a striking way the concept (Hehre et al., 1973) that glycosidases and glycosyltransferases are glycosylases or catalysts of glycosylation. A key feature of this concept of carbohydrase action is its freedom from the usual assumption (evident for example in the designations α- and β-glucosidase) that carbohydrases are restricted to acting on substrates having a glycosidic bond and appropriate (α or β) anomeric configuration.

Scheme I illustrates the parallel way in which protonation

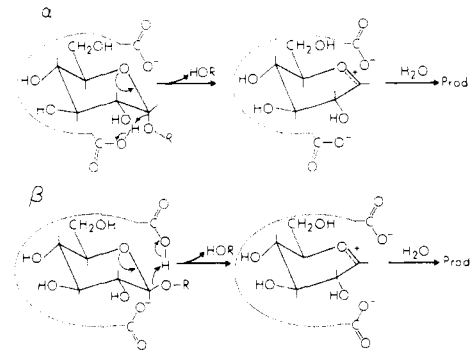
Scheme I



of a glycosyl donor and glycosylation of the protonating source (i.e., glycosyl acceptor) would account for (a) the newly recognized action of *Candida* α-glucosidase on D-glucal; (b) the actions of this enzyme on substrates of α-D-glucosyl-OR type; (c) reactions of sweet almond β-glucosidase with D-glucal first reported by Lehmann and Schröter (1972); and (d) the latter enzyme's actions on glycosidically linked substrates, and on β-D-glucosyl-F (Barnett et al., 1967). The first step (1) would involve productive substrate binding followed by specific protonation of D-glucal at C-2, or of D-glucosyl-OR substrates at the glycosidic oxygen. This catalytic step, in which an enzyme-derived proton effects the formation of a 2-deoxy-D-glucosyl (or D-glucosyl) cation from the substrate, is pictured as leading to the formation of a transient glycosyl carbonium ion-enzyme complex in each case. A second step (2) would effect breakdown of this complex by a directed attack of water (or other acceptor) at C-1, creating a product of α configuration (α-glucosidase) or β configuration (β-glucosidase), plus a reprotonated enzyme. Mechanistic studies of the acid-catalyzed hydration of vinyl ethers (Salomaa et al., 1966) provide a good reason for believing that an incipient glycosyl cation, lacking C-1 asymmetry, would be formed upon protonation of C-2 in the enzymatic reactions with D-glucal, and this is the basis for suggesting that end-product configuration, in the reactions with glycosidic substrates as well, is created anew and is not transmitted directly from substrate, as might be inferred from the custom of describing reactions catalyzed by these or other glycosidases as proceeding with retention (or inversion) of substrate configuration.

To define a possible catalytic mechanism for the specific hydrations of D-glucal catalyzed by *Candida* α-glucosidase and sweet almond β-glucosidase, use has been made of information available on the catalytic elements of glycosidases and on the way in which they may function in reactions with glycosidically linked substrates. Previous workers have obtained considerable evidence for the presence of carboxyl groups in the active sites of several glycosidases, and have postulated possible catalytic mechanisms for these enzymes based on the disposition of the carboxyl groups observed in the active site of hen egg-white lysozyme (Phillips, 1967). The catalytic mechanism of lysozyme is generally believed to involve cooperative interaction between these two carboxyl groups such that one acts as a general acid which protonates the glycosidic oxygen atom of the substrate while the other, as a carboxylate anion, acts as a charge-stabilizing group or a nucleophilic-specific base. Such a mechanism is shown (in part) in Scheme II for an α- and a β-glucosidase acting on substrates of α- or

Scheme II

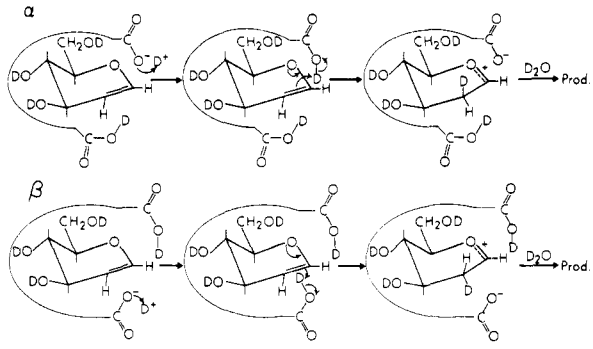


β-D-glucosyl-OR type, respectively. For the α-glucosidase, one carboxyl group is pictured as existing in a protonated state and located below the glycosidic oxygen atom; the second is proposed to exist as a carboxylate anion located above the D-glucosyl ring. For β-glucosidase, the reverse relationship is assumed, with the protonated carboxyl located above the glycosidic oxygen atom and the carboxylate ion below the D-glucosyl ring.

With respect to the hydration of D-glucal catalyzed by *Candida* α-glucosidase and by sweet almond β-glucosidase, the results of NMR analyses of reactions carried out in D_2O provide direct evidence for the formation of the α and β anomers of 2-deoxy-D-glucose, respectively. Further, the α-glucosidase reaction is found to involve stereospecific protonation (deuteration) of the double bond from above C-1 and C-2, leading to the incorporation of deuterium exclusively at the axial position of C-2 in the product, 2-deoxy-α-D-glucose. In the β-glucosidase reaction, the deuterium atom is found at C-2 exclusively in the equatorial position in the product, 2-deoxy-β-D-glucose, indicating protonation (deuteration) of the double bond from below C-1 and C-2.

We assume from a comparison of molecular models of D-glucal and a glycosidic substrate such as methyl α-D-glycopyranoside that D-glucal can bind to the donor site of α- or β-glucosidase in essentially the same manner as D-glucosylic groups of glycosidically linked substrates of appropriate configuration, i.e., via hydrogen bonding through the C-3, C-4, and C-6 hydroxyl groups. We envision, therefore, that when D-glucal binds to *Candida tropicalis* α-glucosidase, its overall orientation to the catalytic groups of the enzyme (presumed to be the two carboxyl groups discussed above) would be essentially the same as that of an α-D-glucosyl moiety of a glycosidically linked substrate (Schemes II and III). The double bond of D-glucal would lie essentially in the plane (ring oxygen,

Scheme III



C-2, C-3, and C-5 atoms) of a similarly bound α-D-glucosyl group, and proximate to the "active site" carboxylate anion of the protein which is assumed to be above the plane of the saccharide ring. Since double bonds are highly hydrophobic groups, one might expect that this carboxylate group would experience an increase in its effective pK_a and undergo protonation, thus becoming a potential general acid donor. Since the double bonds of vinyl ethers undergo facile general acid-catalyzed reactions (Salomaa et al., 1966), the double bond of D-glucal (a cyclic vinyl ether) would be expected to undergo protonation by this carboxyl group. This would lead to protonation (deuteration) of the double bond from above the plane of the substrate and concomitant collapse to an incipient 2-deoxy-D-glycosyl carbonium ion (Scheme III). Subsequent stereospecific hydrolysis of the glycosyl cation–enzyme complex would give the α-anomeric product, 2-deoxy-α-D-[2(a)-^{2}H]glucose. Since no deuterium was detected at the C-2 equatorial position in the product, the other "active-site" carboxyl group which is presumed to effect cleavage of the glycosidic bond of α-glycosidic substrates (Scheme II) apparently is not close enough to the double bond of D-glucal to protonate this bond.

In the case of D-glucal binding to β-glucosidase from sweet almonds, we envision the possibility that an opposite arrangement of the carboxyl and carboxylate groups in the enzyme's active site from that of α-glucosidase can account for the stereospecific hydration of the unsaturated sugar derivative. As shown in Scheme III, when D-glucal binds to β-glucosidase its orientation with respect to the catalytic groups of the enzyme is also expected to be essentially the same as that of a β-D-glucosyl moiety of a glycosidically linked substrate. The double bond of D-glucal would be proximate to the active-site carboxylate ion of the enzyme which is assumed to be below the plane of the double bond. The rise in the effective pK_a of this carboxyl group due to the proximity of the double bond of D-glucal would provide a source of protonation (deuteration) of the unsaturated substrate from below the plane of the double bond, thus leading to the formation of an incipient 2-deoxy-D-glycosyl carbonium ion as shown in Scheme III. This intermediate would be stereospecifically solvated to give the observed product, 2-deoxy-β-D-[2(e)-^{2}H]glucose. Since no deuterium was detected at the C-2 axial position of the product, the second active-site carboxyl group which is presumed to protonate the oxygen atom of β-glucosidic substrates (Scheme II) apparently is also not close enough to the double bond of D-glucal to protonate this bond.

Both postulated hydration mechanisms for D-glucal by α- and β-glucosidase in Scheme III require a carboxylate anion of both enzymes to become the source of protonation (deuteration) of the substrate. Although unusual, these mechanisms can account for the observed stereospecific trans addition of water across the double bond of D-glucal, with formation of the isomeric hemiacetal products, catalyzed by the two proteins. However, regardless of the proposed details of the mechanism of hydration of D-glucal by α- and β-glucosidase, the observation remains that these reactions catalyzed by the two proteins would seem to require protonation of the unsaturated sugar derivative from a source other than that associated with cleavage of the glycosidic linkage of glycosidic substrates. The incipient carbonium ion formed in the two reactions would thereafter resemble similar possible intermediates in reactions of the enzymes with D-glucosidic substrates.

This description of a possible catalytic mechanism for sweet almond β-glucosidase on D-glucal differs substantially from those proposed by others for the actions of this enzyme on D-glucal and of *Escherichia coli* β-galactosidase on D-galactal. Lehmann and Schröter (1972) assumed that binding to enzyme causes the double bond of a glycal to become strongly polarized; that such "activated" glycal undergoes spontaneous protonation at C-2, becoming a carbonium–oxonium ion; and, finally, that saturation occurs through a nucleophile. Wallenfels and Weil (1972) likewise picture the double bond of D-galactal as polarized prior to the catalytic action of β-galactosidase. We believe it is unlikely that the binding of D-glucal to the enzyme would suffice to effect polarization of its double bond, or that such prior polarization is needed for the conversion of this substrate to an incipient glycosyl cation. Indeed, spontaneous protonation of the double bond would fail to account for the stereospecificity of protonation at C-2 that occurs in the hydrations of D-glucal by α- and β-glucosidase and, presumably, in the reactions with D-galactal catalyzed by β-galactosidase.

Wentworth and Wolfenden (1974) have concluded that the hydration of D-galactal by *E. coli* β-galactosidase involves formation of a covalent 2-deoxy-D-galactosyl-enzyme intermediate. These authors found very slow rates of D-galactal binding (in inhibiting the hydrolysis of *o*-nitrophenyl β-D-galactoside) and of D-galactal release (as 2-deoxy-D-galactose), indicative of the possibility that a 2-deoxy-D-galactosyl-enzyme intermediate may be formed. Efforts to demonstrate such an intermediate by a sensitive isotopic method failed, but it is conceivable that a covalent adduct could arise by collapse of a 2-deoxy-D-galactosyl carbonium ion transiently stabilized by an anionic group located below the ring.

Several authors have investigated the catalytic mechanism of β-D-glucoside hydrolysis by sweet almond β-glucosidase. Legler (1968, 1975) has demonstrated that the inhibitor conduritol B epoxide reacts to form a covalent ester linkage with this enzyme. The results suggest that reactive carboxyl group(s) are present in the active site of the enzyme and are involved in bond formation with conduritol B. These groups may, therefore, participate in the catalytic action of the enzyme. A mechanism for hydrolysis of β-D-glucosides was proposed involving either carbonium ion formation and stabilization, or concerted general acid catalysis and nucleophilic attack by the enzyme to form a covalent glucosyl-ester intermediate. Evidence for the latter mechanism is found in the observation of Nath and Rydon (1954) that the influence of substituents on the V_{max} of hydrolysis of aryl β-D-glucosides by β-glucosidase was similar to that of their alkaline cleavage which proceeds by nucleophilic displacement. Dahlqvist et al. (1969) measured the secondary isotope effect on the rate of phenyl β-D-[^{2}H]glucoside hydrolysis by sweet almond β-glucosidase, and concluded that this effect, also, was similar to that of the base-catalyzed cleavage of the substrate. Other

evidence for a covalent intermediate includes Fink and Good's (1974) observation of an "aglycone burst" reaction between *p*-nitrophenyl β-D-glucoside and almond β-glucosidase in solutions of 50% aqueous Me_2SO at temperatures below −15 °C. This suggests the existence of a glycosyl-enzyme intermediate whose breakdown, at least at low temperatures, is rate limiting. Legler (1975), however, has failed to observe "burst" reactions with a series of *p*-nitrophenyl and 4-methylumbelliferyl β-D-glucoside substrates at 35 °C with this enzyme. On the basis of these results he has questioned the glucosyl-ester intermediate mechanism for the enzyme. The present results suggest that formation of a covalent intermediate by β-glucosidase would have to follow the formation of a glycosyl cation when D-glucal is the substrate (Scheme III) and probably, also, when β-D-glucosides are the substrates (Scheme II). A mechanism of double displacement at C-1 for β-glucosidase cannot account for the catalytic scope of the enzyme or for its capacity to create anomeric configuration de novo.

Nothing is known of the structure of the active site of *Candida tropicalis* α-glucosidase. The proposal of a glycosyl cation-mediated mechanism for this enzyme rests on its observed action on D-glucal, and is of necessity speculative with respect to the nature and arrangement of catalytic groups. The α-glucosidase from another species of yeast, *Saccharomyces oviformis*, has been reported to act by a double-displacement mechanism (Lai et al., 1974), based on recovery of a covalent glucosyl-enzyme compound from short-term reaction mixtures of the enzyme with methyl α-D-[^{14}C]glucoside. A test of the ability of this α-glucosidase to attack D-glucal might provide new insight on the mechanism of its action.

Although *Candida* α-glucosidase binds and utilizes D-glucal as a substrate, its catalytic action (hydration) is strongly inhibited by excess substrate. Kinetic analysis shows that this is dead-end inhibition, due to the formation of complexes of enzyme with two D-glucal molecules whose apparent equilibrium constants (K_m = 14.2 mM; K_{SS} = 20.9 mM) have an exceptionally close relationship, K_{SS}/K_m = 1.48 (*cf.* Bray and White, 1966). Indeed, no instance of substrate inhibition of a reaction with water, as extreme as that illustrated in Figure 1, has been previously reported for any carbohydrase as far as the authors are aware. A likely place where a second D-glucal molecule might bind with high affinity to an enzyme already associated with substrate, and also cause inhibition, might be at the acceptor site. *Candida tropicalis* α-glucosidase is known to accommodate an unusually wide variety of organic acceptors, including various sugars, alditols, and alcohols (Sawai, 1958, 1960; Sawai and Hehre, 1962). The presence of a D-glucal molecule at the acceptor site might interfere sterically with the formation and/or release of the sugar product, and with regeneration of active reprotonated enzyme. Whether the hydration of D-glucal by sweet almond β-glucosidase, or of D-galactal by β-galactosidase, may be inhibited in a similar way by excess substrate is not known. Several authors have proposed mechanisms whereby D-galactal causes the inhibition of the hydrolysis of β-D-galactosides by β-galactosidase. However, in no instance has the possibility been suggested that the inhibition might involve D-galactal binding to other than the donor substrate site or to other than free enzyme.

Present results, finally, confirm that D-glucal behaves as an internal 2-deoxy-D-glucosyl compound, and its enolic bond as a glycosylic bond. The participation of D-glucal as a glycosyl substrate in two sterically complementary reactions of hydration (glycosylations of water) catalyzed by carbohydrases shows that the "hidden" glycosyl group of this compound is fully functional even though it is linked to a carbon atom (C-2), lacks an asymmetric (anomeric) carbon atom, and is not bound glycosidically.

Note Added in Proof

Since completion of this work, we have learned (J. Lehmann, personal communication) that the action of β-galactosidase on a mixture of D-[2-^{2}H]galactal and glycerol in H_2O leads to the formation of glyceryl 2-deoxy-β-D-[2(a)-^{2}H]galactoside. This result, showing that β-galactosidase stereospecifically protonates D-galactal at C-2 from below the plane of the ring, is in complete accord with our findings.

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Activities of Lectins and Their Immobilized Derivatives in Detergent Solutions. Implications on the Use of Lectin Affinity Chromatography for the Purification of Membrane Glycoproteins†

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ABSTRACT: The effects of several commonly used detergents on the saccharide-binding activities of lectins were investigated using lectin-mediated agglutination of formalin-fixed erythrocytes and affinity chromatography of glycoproteins on columns of lectins immobilized on polyacrylic hydrazide-Sepharose. In the hemagglutination assays, *Ricinus communis I* (RCA_I) and *II* (RCA_{II}), concanavalin A (Con A), and the agglutinins from peanut (PNA), soybean (SBA), wheat germ (WGA), and *Limulus polyphemus* (LPA) were tested with several concentrations of zwitterionic, cationic, anionic, and nonionic detergents. It was found that increasing detergent concentrations eventually affected hemagglutination titers in both test and control samples, and the highest detergent concentrations not affecting lectin hemagglutinating activities were determined. The effects of detergents on specific binding of [^{3}H]fetuin and asialo[^{3}H]fetuin to and elution from columns of immobilized lectins were less severe when compared with lectins in solution, suggesting that the lectins are stabilized by covalent attachment to agarose beads. Nonionic detergents did not affect the binding efficiency of the immobilized lectins tested at concentrations used for membrane solubilization while cationic and zwitterionic detergents caused significant inhibition of Con A- and SBA-Sepharose activities. In sodium deoxycholate (>1%) only RCA_I-Sepharose retained its activity, whereas the activities of the other lectins were reduced dramatically. Low concentrations of sodium dodecyl sulfate (0.05%) inhibited only the activity of immobilized SBA, but at higher concentration (0.1%) and prolonged periods of incubation (16 h, 23 °C) most of the lectins were inactivated. These data are compared with previous reports on the use of detergents in lectin affinity chromatography, and the conditions for the optimal use of detergents are detailed.

The study of cell surface membrane glycoproteins has been facilitated by the use of lectins which bind saccharides in a highly specific manner (Sharon and Lis, 1972; Nicolson, 1974). Thus, the number and distribution of surface glycoproteins have been studied by employing radioactive, fluorescent, or electron-dense lectin derivatives (Nicolson, 1974; Sharon and Lis, 1975). For the study of the chemical nature of membrane glycoproteins, it is desirable to obtain them in a pure form. This is a complicated endeavor, because in the cell membranes that have been well characterized, glycoproteins are classified as integral membrane components (Singer and Nicolson, 1972); that is, they are presumed to be stabilized by hydrophobic forces and are not easily solubilized and separated from membrane lipids into aqueous, low ionic strength, neutral solutions. The use of chaotropic agents or detergents (for review, see: Helenius and Simmons, 1975), such as sodium dodecyl sulfate (Akedo et al., 1972; Susz et al., 1973; Gombos et al., 1974), Triton X-100 (Cuatrecasas, 1972; Findlay, 1974; Schmidt-Ullrich et al., 1975), and sodium deoxycholate (Allan and Crumpton, 1971; Allan et al., 1972; Hayman and Crumpton, 1972; Gurd and Mahler, 1974; Berzins and Bolmberg, 1975; Nachbar et al., 1976; Pitlick, 1976), enables effective solubilization of cell membrane components by breaking hydrophobic and ionic bonds. Once solubilized and "stabilized" in solution, integral membrane glycoproteins can be purified by conventional methods or by affinity chromatography on immobilized lectins. The latter technique has been applied to the fractionation and purification of glycoproteins from membranes of lymphocytes (Allan et al., 1972; Hayman and Crumpton, 1972), leukemic cells (Jansons and Burger, 1973), erythrocytes (Adair and Kornfeld, 1974; Findlay, 1974), brain cells (Susz et al., 1973; Gombos et al., 1974; Gurd and Mahler, 1974; Zanetta et al., 1975; Pitlick, 1976), platelets (Nachman et al., 1973), and viruses (Hayman et al., 1973).

Since lectins are made of subunits which, in many cases, are held together by noncovalent forces, detergents and chaotropic agents used for the solubilization of membrane components may dissociate the native lectin molecules and/or change their active conformation. In spite of the increasing use of lectin affinity chromatography for the isolation of membrane glycoproteins in detergent solutions, no detailed study has been undertaken to investigate the effects of detergents on the activities of lectins.

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