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LOCATION OF A PROTON-DONATING GROUP AT THE *re*-FACE OF A β-D-GALACTOSIDASE-BOUND, DIASTEREOTOPIC SUBSTRATE

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ABSTRACT

(*Z*)-3,7-Anhydro-1,2-dideoxy-2-deuterio-D-*galacto*-oct-2-enitol (**1**) was used as a diastereotopic probe, in order to elucidate the stereochemistry of protonation by β-D-galactosidase. Compound **1** can be converted by the enzyme into 1,2-dideoxy-2-deuterio-D-*galacto*-3-octulopyranose (**2**), which was submitted to periodate degradation. Propanoic acid derived from C-1, 2, and 3 of **2** has the (*S*) configuration, which proved the enzymic protonation of **1** to have taken place exclusively from the *re*-face.

INTRODUCTION

The action of β-D-galactosidase, like that of other glycoside-cleaving enzymes, may involve two catalytically functioning groups[1]. One, being acidic, is responsible for activation of the aglyconoxy group by protonation, the other, as an essential, basic group, for the stabilization of the remaining glycopyranosyl group. The models, adopted from the lysozyme system, consider two carboxylate groups, one in its protonated form, and the other as its conjugate base[2]. Carboxylate groups could, indeed, be either located and identified[3], or their presence at the active site indicated[4]. There is as yet no proof for the necessity of two different carboxylate groups acting from opposite sides of the pyranoid plane, as implicated in the lysozyme model. Bifunctional catalysis (the bilateral model) could well be executed by only one carboxylic group acting from only one side (the monolateral model).

The same group could be responsible for acid, as well as base, catalysis. As experimental evidence, obtained through stereospecific, enzymic protonation of glycals, has only been given for a carboxylate group "beneath" the pyranoid plane for β-glycosidases[2,5], and "above" the plane in the case of an α-glycosidase[2], all results so far can be explained with only one carboxylic acid group lying in the proper position (monolateral model; see Fig. 1).

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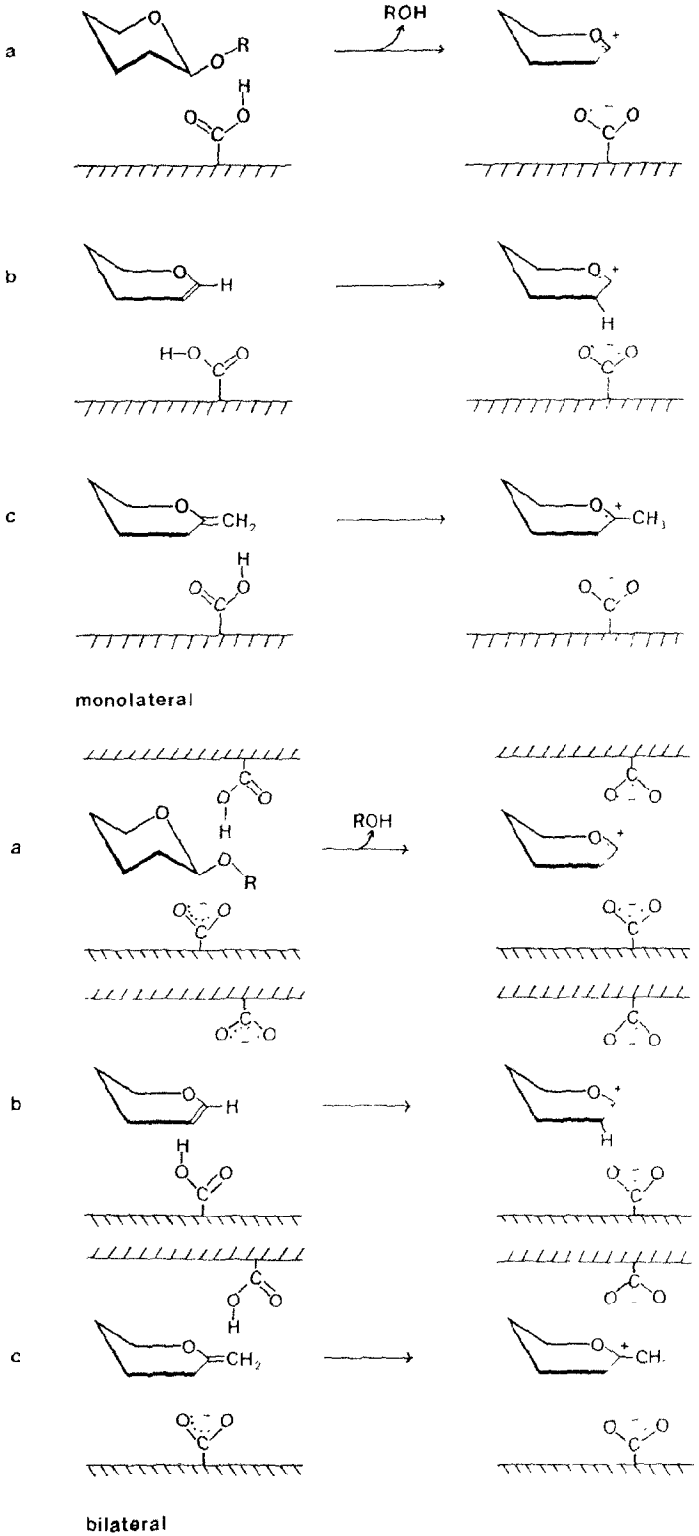
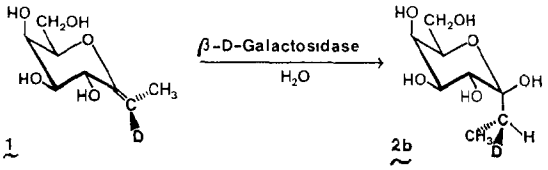


Fig. 1. Monolateral and bilateral models for the enzymic conversion of glycosides (a), glycals (b), and heptenitols (c).

RESULTS AND DISCUSSION

*(Z)-3,7-Anhydro-1,2-dideoxy-2-deuterio-*D-galacto-*oct-2-enitol* (**1**) *as a diastereotopic probe.* — In glycosidase-catalyzed reactions, heptenitols "c" act as proton acceptors in the same way as glycosides "a" seem to. The proton, donated by the enzyme, is, in the former instance, stably fixed to a carbon atom, and remains with the pyranoid system, but, in the latter, it is attached to an oxygen atom, and is lost by exchange. Protonation could take place according to either the monolateral or the bilateral model. The two possibilities can be differentiated between with diastereotopic probes that yield diastereomeric product on enzymic conversion.

The synthesis of **1** has been described[6]. Its enzyme-catalyzed hydration should yield either 2-(*R*)- (**2a**) or 2-(*S*)-1,2-dideoxy-2-deuterio-D-*galacto*-3-octulopyranose (**2b**), depending on the direction of enzymic protonation. Because the enzymic protonation of D-galactal was proved to take place from below the pyranoid plane, protonation of **1**, also from below (*si*-face), yielding **2a**, would strengthen the feasibility of the monolateral model (not necessarily with only one functional group).



Protonation of **1** from above (*re*-face), yielding **2b**, would indicate at least two groups operating from opposite sides of the pyranoid plane (bilateral model).

Acid-catalyzed hydration of **1**. — Compound **1** is stable in protic solvents, but it can be hydrated in the presence of an acidic ion-exchange resin, and the homogeneous product has reducing properties. ^{1}H-N.m.r. investigations proved it to be the α anomer of **2**. No deuterium–proton exchange occurs at C-2 throughout the procedure.

Enzyme-catalyzed hydration of **1**. — Incubation of **1** with various concentrations of β-D-galactosidase in phosphate buffer, pH 6.8, also yields **2**. The conversion was conveniently monitored by t.l.c. For quantitative measurements (see Fig. 2), tritium-labelled substrate, [2-^{3}H]-**1**, having a specific radioactivity of 80 μCi/mmol, was used. Competitive inhibition of the reaction with isopropyl 1-thio-β-D-galactopyranoside (IPTG) indicated that **1** is hydrated at the site of D-galactoside cleavage. The hydration of **1** on a preparative scale was conducted with 1.1 mmol of the substrate. After the reaction was complete, the product was separated from the enzyme solution by dialysis, and the dialyzate was freeze-dried. Without further purification, this freeze-dried dialyzate was treated with sodium periodate solution. The major fragment from the periodate degradation, 2-propanoylglyceraldehyde, could not be detected;

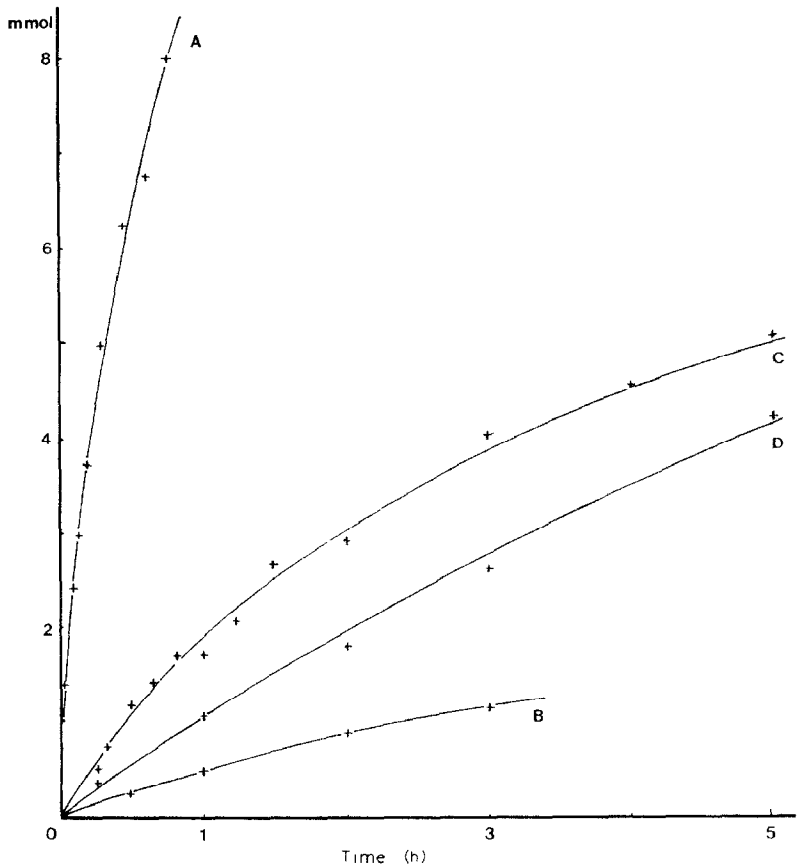


Fig. 2. Formation of (*S*)-1,2-dideoxy-α-D-*galacto*-[2-^{3}H]2-octulopyranose during enzymic hydration of (*Z*)-3,7-anhydro-1,2-dideoxy-D-*galacto*-[2-^{3}H]oct-2-enitol[6] ([2-^{3}H]**1**) in sodium phosphate buffer (50mM, pH 6.8) containing magnesium chloride (1mM) at 30°. {Key: A, [2-^{3}H]-**1** (50mM), β-D-galactosidase (870 U/mL); B, [2-^{3}H]-**1** (50mM), β-D-galactosidase (870 U/mL), and IPTG (57.2mM); C, [2-^{3}H]-**1** (39.3mM), β-D-galactosidase (154 U/mL); and D, [2-^{3}H]-**1** (39.3mM), β-D-galactosidase (154 U/mL), and IPTG (3.23mM).}

it was obviously hydrolyzed under the acidic conditions into propanoic acid and glyceraldehyde, the latter undergoing further degradation. After the reaction was complete, the fragments, namely, formaldehyde, formic acid, and propanoic acid, were separated from inorganic material by freeze-drying, and the volatile part, containing the fragment 2-deuteriopropanoic acid, was isolated as the sodium salt by extraction and crystallization. Measurement of the optical rotatory dispersion curve (see Fig. 3) proved that it had the (*S*) configuration, thereby also establishing the configuration of **2b**. A parallel experiment with **2**, obtained by acid-catalyzed hydration, yielded 2-deuteriopropanoate, not optically active.

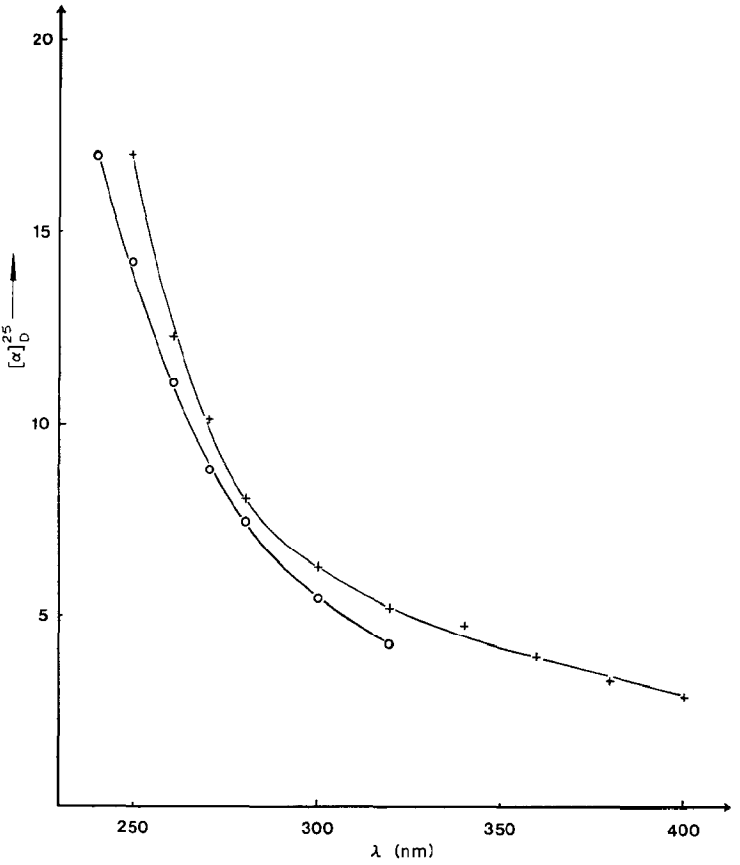


Fig. 3. O.r.d. curves of sodium (*S*)-2-deuteriopropanoate. [x-x-x, obtained from periodate decomposition of **2a** by enzymic hydration of **1** (*c* 0.8, H_2O); o-o-o, see ref. 7 (*c* 1.4, H_2O).]

CONCLUSION

Our results show that protonation of **1** by the β-D-galactosidase from *E. coli* occurs exclusively at the *re*-face of the double bond, *i.e.*, from "above" the pyranoid plane. Together with earlier results[5] on the stereoselective hydration of D-galactal, this proves the location of two catalytically active groups. The potentially proton-donating groups on the active site of the enzyme are situated at opposite sides of a plane given by the pyranoid ring of a bound substrate, which is in agreement with the "lysozyme model".

EXPERIMENTAL

General methods. — Melting points are uncorrected. Optical rotations were measured with a Perkin–Elmer 141 polarimeter, and optical rotatory dispersion with a Cary 60 spectropolarimeter. T.l.c. was performed on silica gel (Merck), using

5:1 (v/v) ethyl acetate–methanol as the solvent. Detection was effected by charring with sulfuric acid. Radioactive compounds were located with a Linear Analyser (Berthold). N.m.r. (internal Me_4Si, and sodium 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoate) data were obtained with a Bruker WM 250 (250 MHz) and a Varian EM 390 (90 MHz) spectrometer.

Enzyme-catalyzed hydration of **1**. — Compound **1** (200 mg) was incubated for 8 h at 30° in a solution of β-D-galactosidase from *E. coli* (1600 U) and phosphate buffer, pH 6.8 (50.0mM; 4 mL) containing 1mM magnesium chloride. When the reaction was complete (t.l.c., R_F 0.29–0.16), the products were separated by dialysis (4 times) at 4° with 10.0mM phosphate buffer, pH 6.8 (100 mL) containing 0.02M magnesium chloride. The combined dialyzates were freeze-dried, and the lyophilizate was used in the next step without purification. For characterization, a solution of a portion (50 mg) of the lyophilizate in methanol was passed through a column (0.5 × 5 cm) of silica gel. Colorless, syrupy **2b** (45 mg) was obtained; ^{1}H-n.m.r. data (D_2O, 250 MHz): δ 0.96 (d, 3 H, H-1), 1.78 (q, 1 H, H-2), 3.67 (d, 1 H, H-4), 3.66–3.79 (m, 2 H, H-8,8′), 3.86 (dd, 1 H, H-5), 3.99 (dd, 1 H, H-6), 4.03 (ddd, 1 H, H-7); $J_{1,2}$ 7.5, $J_{4,5}$ 10, $J_{5,6}$ 3.5, $J_{6,7}$ 1, $J_{7,8}$ 6.5, and $J_{7,8'}$ 6.5 Hz.

Periodate degradation of **2b**. — A solution of crude **2b** (180 mg) in water (60 mL) was treated with sodium periodate (1.07 g), and kept in the dark for 24 h, the volatile products lyophilized at 0.01 Torr, and the lyophilizate made neutral with aqueous sodium hydroxide solution (0.1M), and freeze-dried. The lyophilizate, containing sodium 2-deuteriopropanoate and sodium formate, was extracted with boiling ethanol (40 mL), and the extract evaporated to dryness under diminished pressure. The residue was re-extracted with hot ethanol (20 mL), and the extract evaporated to dryness *in vacuo*, this procedure being repeated with 10 and 5 mL of boiling ethanol. Finally, the residue was dissolved in the minimum volume of ethanol, and treated with acetone (30 mL). The colorless crystals that formed slowly at 0° proved to be sodium (*S*)-2-deuteriopropanoate; yield, 24 mg (23.5%); ^{1}H-n.m.r. data (D_2O, 90 MHz): δ 1.15 (d, 3 H, CH_3) and 2.17 (m, 1 H, H-2).

Anal. Calc. for $C_3H_4DNaO_2$: C, 37.12; H (+D), 6.22. Found: C, 36.73; H (+D, as H), 5.61.

(R,S)-*1,2-Dideoxy-2-deuterio*-α-D-galacto-*2-octulopyranose* (**2a**, **2b**). — (*Z*)-3,7-Anhydro-1,2-dideoxy-2-deuterio-D-*galacto*-oct-2-enitol (**1**) (190.2 mg) was treated with water (15 mL) containing Amberlite IR-120 (H^+) resin for 2 h at 60°. After filtration, the filtrate was evaporated to dryness under diminished pressure, yielding a colorless foam consisting of a mixture of **2a** and **2b** (189 mg, 91%); $[\alpha]_{578}^{22}$ +58.9° (*c* 1.0, H_2O); ^{1}H-n.m.r. data (D_2O, 250 MHz): δ 0.96 (d, 3 H, H-1), 1.78 (q, 1 H, H-2), 3.67 (d, 1 H, H-4), 3.66–3.79 (m, 2 H, H-8,8′), 3.86 (dd, 1 H, H-5), 3.99 (dd, 1 H, H-6), 4.03 (ddd, 1 H, H-7); $J_{1,2}$ 7.5, $J_{4,5}$ 10, $J_{5,6}$ 3.5, $J_{6,7}$ 1, $J_{7,8}$ 6.5, and $J_{7,8'}$ 6.5 Hz.

For further characterization, the mixture of **2a** and **2b** was acetylated.

(R,S)-*4,5,6,7,8-Penta*-O-*acetyl-1,2-dideoxy-2-deuterio*-keto-D-galacto-*2-octulopyranose* (**3a**,**3b**). — The acetylation of a mixture (50 mg) of **2a** and **2b** in 1:1 (v/v)

acetic anhydride–pyridine (2 mL) was catalyzed by (4-dimethylamino)pyridine (5 mg). After 15 h, the mixture was processed as usual, and the dark-brown residue purified on a column (7 × 0.8 cm) of silica gel using 1 : 1 (v/v) ethyl acetate–petroleum ether (b.p. 60–70°) as the solvent. A mixture of the crystalline acetates **3a** and **3b** (56 mg, 55.7%) was obtained from ether–petroleum ether (b.p. 60–70°); m.p. 99°, $[\alpha]_{578}^{22}$ −12.8° (*c* 0.5, $CHCl_3$); ^{1}H-n.m.r. data ($CDCl_3$, 250 MHz): δ 0.98 (d, 3 H, H-1), 2.03, 2.04, 2.10, 2.13, 2.18 (5 s, 15 H, 5 OAc), 2.40–2.50 (m, 1 H, H-2), 3.88 (dd, 1 H, H-8), 4.28 (dd, 1 H, H-8′), 5.10 (d, 1 H, H-4), 5.32 (ddd, 1 H, H-7), 5.47 (dd, 1 H, H-5), 5.51 (dd, 1 H, H-6); $J_{1,2}$ 7, $J_{4,5}$ 1.9, $J_{5,6}$ 9.5, $J_{6,7}$ 2, $J_{7,8}$ 7.1, $J_{7,8'}$ 5.5, and $J_{8,8'}$ 12 Hz.

Anal. Calc. for $C_{18}H_{25}DO_{11}$: C, 51.55; H (+D) 6.49. Found: C, 51.80; H (+D, as H), 6.43.

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