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BRANCH SPECIFICITY OF β-D-GALACTOSIDASE FROM *Escherichia coli**

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ABSTRACT

The "branch specificities" of the β-D-galactosidases from *Escheichia coli*, jack bean, *Aspergillus niger*, and human liver were investigated with two branched oligosaccharide substrates, one which forms part of a complex-type biantennary *N*-linked glycan (compound **1**) and a structure having blood group I activity (compound **2**), respectively. Both substrates were available as radioactive compounds having a known distribution of ^{3}H and ^{14}C label in each of the terminal galactosyl groups, which allowed accurate estimation of the branch specificity of the enzymes from the ratio of ^{3}H and ^{14}C radioactivity in the galactose released by these hydrolases. It was found that the β-D-galactosidase from *E. coli* preferentially released the galactosyl group at the 1→3 branch of compound **1** and that at the 1→6 branch of compound **2**. By contrast, the other β-D-galactosidases investigated showed little or no branch specificity. These results suggest that the branch specificity of the β-D-galactosidase from *E. coli* has to be explained from a specific recognition of certain parts of the aglycon of the substrates by this enzyme rather than from a better accessibility of the galactose at one particular branch.

INTRODUCTION

Recent studies on the biosynthesis of branched glycans in *N*-glycosyl proteins and substances showing blood group I activity have demonstrated that several glycosyltransferases preferentially attach a sugar group to one particular branch of these glycans[1–5]. This feature, which has been defined as "branch specificity"[1], has been suggested as being responsible for the occurrence of glycans, the branches of which differ systematically in the composition and structure of their terminal portions[1,3–5]. Potentially, however, some such asymmetric glycans might also have evolved as a result of the release of sugars from one particular branch during processing of the materials in the cell, or by the action of certain, branch-specific glycosidases during isolation from their sources.

*Dedicated to Roger W. Jeanloz.

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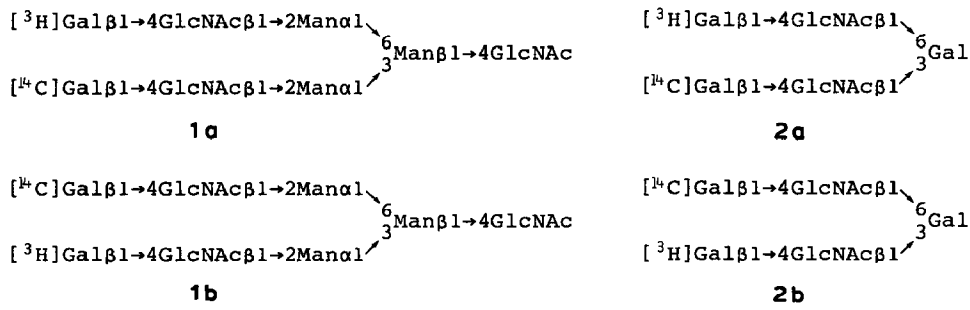

Fig. 1. Structures of compounds **1** and **2** synthesized *in vitro* by using UDP-Gal: 2-acetamido-2-deoxyglucoside β1→4-galactosyltransferase. Compounds **1** and **2** were used as substrates to estimate the branch specificity of β-D-galactosidases. Each compound consisted of a mixture of two isotopic isomers. For compound **1**, the ratio of **1a** to **1b** had been determined[3] to be 3.7:1 and the specific radioactivities of [^{3}H]Gal and [^{14}C]Gal were 1.4 and 0.63 Ci per mol, respectively. Compound **2** was present[1] in ratio of 20:1 (**2a** to **2b**) and the specific radioactivities of [^{3}H]Gal and [^{14}C]Gal in this oligosaccharide were 0.36 and 0.54 Ci per mol, respectively.

This study was undertaken in order to investigate the possible branch specificity of one particular class of glycosidases, the β-D-galactosidases. Two unique, branched substrates for these enzymes having a known distribution of ^{3}H and ^{14}C label in each of the terminal galactosyl groups were used. It was found that indeed one of the β-D-galactosidases tested showed a pronounced branch specificity with both substrates.

EXPERIMENTAL

Materials. — The two bi[^{14}C,^{3}H]galactosylated oligosaccharide substrates used in this study and shown in Fig. 1 were synthesized as described previously from the corresponding agalacto compounds by use of bovine colostrum UDP-Gal*: 2-acetamido-2-deoxyglucoside β1→4-galactosyltransferase[1,3]. The agalacto form of compound **1** was obtained by digestion with *Escherichia coli* β-galactosidase of Galβ1→4GlcNAcβ1→2Manα1→3(Galβ1→4GlcNAcβ1→2Manα1→6)Manβ1→4GlcNAc, which was a kind gift of Dr. G. Strecker, Université de Lille, Villeneuve d'Ascq, France. GlcNAcβ1→3(GlcNAcβ1→6)Gal was kindly donated by Dr. A. Veyrières, Université Paris-Sud, Orsay, France. β-D-Galactosidase from human liver was kindly supplied by Dr. D. H. Joziasse from our department. β-D-Galactosidases from *Escherichi coli, Aspergillus niger*, and jack beans were purchased from Sigma, St. Louis, MO.

Enzymology. — In order to determine the branch specificity of the β-D-galactosidases, digestions of the double-labeled compounds **1** and **2** were carried out in a volume of 50 μL containing oligosaccharide substrate, 5 nmol in all instances, and

*The abbreviations used are: Gal, galactose; GlcNAc, *N*-acetylglucosamine; Man, mannose; Glc, glucose. All sugars are of the D configuration. L.c., high-pressure liquid chromatography.

in addition either sodium phosphate pH 7.3, 10 μmol; $MgCl_2$, 1 μmol, and β-D-galactosidase *(E. coli)* 0.32 unit; or sodium acetate pH 4.0, 5 μmol and β-D-galactosidase (jack bean), 0.03 unit; or sodium citrate pH 3.6, 5 μmol, and β-D-galactosidase *(A. niger)*, 0.05 unit; or sodium citrate–phosphate buffer pH 4.5, 5 μmol; NaCl, 3 μmol, and β-galactosidase (human liver), 0.001 unit. Incubations were conducted at 37° for various periods of time (10–120 min) and the release of [^{14}C]- and [^{3}H]-galactose was estimated by analyzing the incubation mixture on columns (1.5 × 50 cm or 1.6 × 200 cm) of Bio-Gel P-4 (200–400 mesh) in 0.05M ammonium acetate pH 5.2, which separates the oligosaccharide substrate from the liberated galactose. The branch specificities of the enzymes were calculated from the results obtained with incubations in which 15–25% of the total galactose was released, by using the following equation:

$$x = (a.b - 1)(a - b)^{-1} \qquad (I),$$

where x is the preference whereby galactose at the 1→6 branch relative to that at the 1→3 branch of the oligosaccharide substrate is released, a is the molar ratio of [^{3}H]Gal to [^{14}C]Gal of the galactose at the 1→6 branch in the substrate, and b that ratio of the galactose released by action of β-D-galactosidase. The ^{3}H and ^{14}C radioactivities were assayed by liquid-scintillation counting, using a double-labeling calculation method based on external-standard channel ratios.

RESULTS

The double-labeled compounds **1** and **2** were each obtained as a mixture of two isotopic isomers, which are forms of the compounds differing only in the radioactivity isotope whereby each of the terminal galactose residues are labeled (Fig. 1). The ratios of the isotopic isomers were determined by a method based on acetolysis and l.c., as described previously[1,3] and are given in the legend to Fig. 1.

Typical examples of the analysis of β-D-galactosidase digests on columns of Bio-Gel P-4 are shown for compound **1** in Fig. 2 and compound **2** in Fig. 3. In both instances β-D-galactosidase from *E. coli* had been used and the differences in ratios of [^{3}H]Gal to [^{14}C]Gal of the substrates and the galactose released indicate a preference of this enzyme for one of the branches of either substrate. In the case of compound **2**, a separation of the substrate and the monogalactosylated product was obtained on a long column of Bio-Gel (Fig. 3). The ratio of [^{3}H]Gal to [^{14}C]Gal in the latter material appeared to be the reciprocal of that of the peak of galactose released, illustrating the validity of our method for estimating the branch specificity. With the other β-D-galactosidases, little or no difference between the ratio of [^{3}H]Gal to [^{14}C]Gal in the substrate and that in the galactose released was observed (Bio-Gel profiles not shown), indicating that these enzymes have no pronounced branch specificity. Table I expresses quantitatively the branch specificities of the β-D-galactosidases tested as the ratio whereby the enzyme prefers to release the galactose at the 1→6 branch to that at the 1→3 branch.

TABLE I

BRANCH SPECIFICITY OF β-D-GALACTOSIDASES[a]

Compound	*β-Galactosidase from*			
	E. coli	*Jack bean*	A. niger	*Human liver*
	Preference ratio			
1	1:5	1:1	1:1	1:1
2	8:1	2:1	1:1	1:1

[a]Branch specificity is expressed as the preference whereby the galactose residue at the 1→6 branch relative to that at the 1→3 branch of the oligosaccharide substrate is released.

DISCUSSION

The specificity of exoglycosidases, enzymes that hydrolyze the linkage between a terminal non-reducing sugar and an aglycon, is well documented[6,7]. These enzymes are in general highly specific for the sugar to be released as well as its anomeric configuration[6,7]. Exoglycosidases also frequently show specificity towards the position to which the sugar to be released is linked[6,7]. Thus β-D-galactosidases from different sources are specific for β-(1→4)-linked galactosyl residues to varying extents. The enzyme isolated from the culture fluid of *Diplococcus pneumoniae*[8] cleaves exclusively Galβ1→4GlcNAc but not Galβ1→3GlcNAc and Galβ1→6GlcNAc[9]. Similarly, β-D-galactosidase from jack

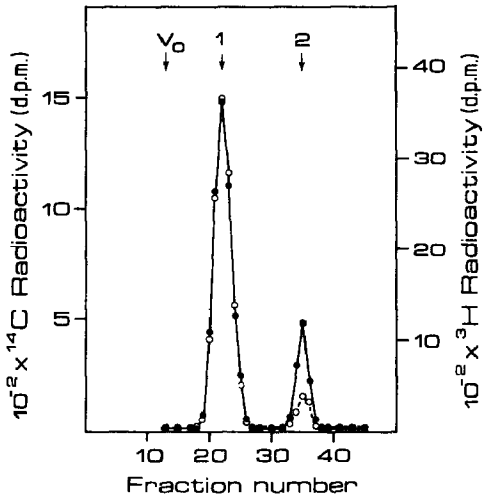

Fig. 2. Analysis of a β-D-galactosidase *(E. coli)* digest of compound **1** on Bio-Gel P-4. Incubation was conducted for 20 min and the mixture was then applied to a column (1.5 × 50 cm) of Bio-Gel P-4 (200–400 mesh) equilibrated and eluted with 0.05M ammonium acetate pH 5.2 at a flow rate of 15 mL per h. Fractions of 4 mL were collected and assayed for ^{14}C (●) and ^{3}H (○) radioactivity by liquid-scintillation counting. The arrows indicate the elution volume of (*1*) compound **1** and (*2*) galactose.

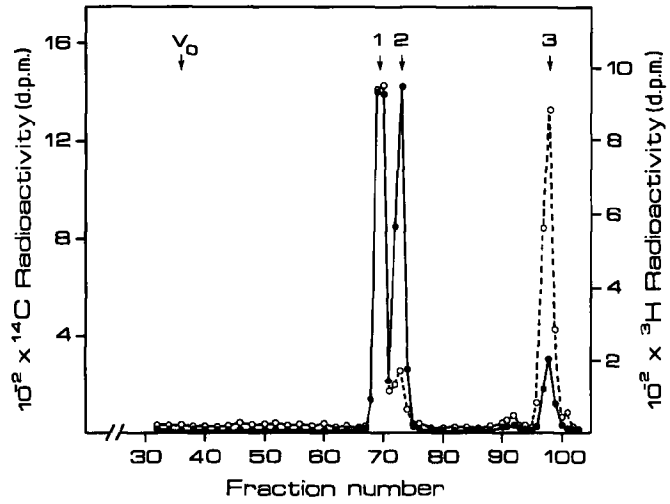

Fig. 3. Analysis of a β-D-galactosidase *(E. coli)* digest of compound **2** on Bio-Gel P-4. Digestion of compound **2** was conducted for 90 min. The resulting mixture was analyzed on a column (1.6 × 200 cm) of Bio-Gel P-4 (200–400 mesh) equilibrated and eluted in 0.05M ammonium acetate pH 5.2 at 37°. The flow rate was 14 mL per h and fractions of 4 mL were collected. Aliquots of the fractions were assayed for ^{14}C (●) and ^{3}H (○) radioactivity. The elution volumes of (*1*) compound **2**, (*2*) the mixture of the two isomeric monogalactosylated products, and (*3*) galactose are indicated.

beans releases β-(1→4)-linked galactose groups at a much higher rate than β-(1→3)-linked residues[7]. Bovine testicular β-D-galactosidase, however, acts equally well on Galβ1→3 and Galβ1→4 linked to GlcNAc, but shows low activity with Galβ1→6GlcNAc[10]. By contrast, *Escherichia coli* β-D-galactosidase is capable of cleaving both Galβ1→4Glc and Galβ1→6Glc[11].

This study demonstrates that β-D-galactosidase from *E. coli* also shows a high degree of branch specificity towards the two branched oligosaccharide substrates shown in Fig. 1; a structure that forms part of a complex-type biantennary *N*-linked glycan and a structure having blood group I activity, respectively. Studies on the conformation of biantennary *N*-linked glycans involving X-ray diffraction analysis[12,13], potential-energy hard-sphere exo-anomeric calculations[14,15] and n.m.r. spectroscopy[14–16] have indicated that the two branches of these glycans have different orientations in space. The 1→3 branch appears to take a fixed position with respect to the Manβ1→4GlcNAcβ1→4-GlcNAc core-portion of these glycans, which results in the exposure of the sugars of this branch, including the galactosyl group[12–14,16]. The 1→6 branch on the other hand has greater flexibility with respect to the core because of an additional freedom of rotation about the C-5–C-6 bond of the β-mannose-residue. This branch may occur in two interconvertible orientations: the one in which it is folded back onto the core is energetically favored[14,15]. In this conformation, the galactosyl group of the 1→6 branch would be less exposed and its access by enzymes might consequently be sterically hindered. Thus the preferential release by the β-D-galactosidase from *E. coli* of the galactosyl group at

the 1→3 branch in compound **1** could be seen as the result of better accessibility of this group in the favored conformation. However, if this were the explanation for branch specificity it is difficult to understand why the other β-D-galactosidases (Table I) do not show a similar preference for the galactose at the 1→3 branch.

With compound **2**, the β-D-galactosidase from *E. coli* shows a pronounced specificity for the galactosyl group at the 1→6 branch, whereas the other β-D-galactosidases show no or very little preference for this group (Table I). Also this result seems to indicate that the branch specificity of the enzyme from *E. coli* should not be explained from a difference in accessibility of the terminal galactoses at the two branches. Rather it appears that this specificity is an inherent property of the bacterial β-D-galactosidase.

The active center of the β-D-galactosidase from *E. coli* has been reported to consist of two subsites: the galactose site and the glucose site[17]. This glucose subsite appears to be specific for sugars in the pyranose form having the same structure as D-glucose at the 6-hydroxymethyl end[18]. The extent to which the glucose subsite recognises larger aglycons has not, however, been investigated. Our results in fact suggest that this subsite might interact in a specific manner with portions of the aglycon beyond the sugar to which the terminal galactose is attached, for instance the GlcNAcβ1→2Manα1→3Man and GlcNAcβ1→6Gal parts of compounds **1** and **2**, respectively.

Branch specificity of glycosidases has been reported in two other cases. *N*-Acetyl-β-glucosaminidase isolated from *Octopus vulgaris* has been shown to release from an ovalbumin glycopeptide the GlcNAc residue β-(1→4)-linked to the α-(1→3)-linked mannose of the core[19]. The β1→4 linkage whereby the bisecting GlcNAc residue (GlcNAcβ1→4Manβ) is attached to the glycopeptide was, however, completely resistant to hydrolysis by the enzyme. Recently, a specific α-D-mannosidase has been described in *Saccharomyces cerevisiae*[20]. This enzyme, which is most probably involved in the processing of oligosaccharides during biosynthesis of mannoproteins, specifically releases only one of the three terminal, α-(1→2)-linked mannosyl groups from a $Man_9GlcNAc$ oligosaccharide. Branch specificity of glycosidases, however, does not generally appear to be a property of these enzymes. Therefore the occurrence of asymmetric, branched glycan structures on mature glycoconjugates seems to be the result of the branch specificity of glycosyltransferases[1-5] rather than that of glycosidases.

REFERENCES

1 W. M. BLANKEN, G. J. M. HOOGHWINKEL, AND D. H. VAN DEN EIJNDEN, *Eur. J. Biochem.*, 127 (1982) 547–552.
2 M. R. PAQUET, S. NARASIMHAN, H. SCHACHTER, AND M. A. MOSCARELLO, *J. Biol. Chem.*, 259 (1984) 4716–4721.
3 W. M. BLANKEN, A. VAN VLIET, AND D. H. VAN DEN EIJNDEN, *J. Biol. Chem.*, 259 (1984) 15131–15135.
4 D. H. JOZIASSE, W. E. C. M. SCHIPHORST, D. H. VAN DEN EIJNDEN, J. A. VAN KUIK, H. VAN HALBEEK, AND J. F. G. VLIEGENTHART, *J. Biol. Chem.*, 260 (1985) 714–719.

5 D. H. VAN DEN EIJNDEN, D. H. JOZIASSE, W. M. BLANKEN, A. H. L. KOENDERMAN, W. E. C. M. SCHIPHORST, AND P. L. KOPPEN, in E. A. DAVIDSON, J. C. WILLIAMS, AND N. M. DIFERRANTE (Eds.), *Proc. VIIIth Int. Symp. Glycoconjugates,* Praeger, New York, 1985, pp. 285–286.
6 A. KOBATA, *Anal. Biochem.,* 100 (1979) 1–14.
7 Y.-T. LI AND S.-C. LI, *Methods Enzymol.,* 28 (1972) 702–713.
8 R. C. HUGHES AND R. W. JEANLOZ, *Biochemistry,* 3 (1964) 1535–1543.
9 J. C. PAULSON, J.-P. PRIEELS, L. R. GLASGOW, AND R. L. HILL, *J. Biol. Chem.,* 253 (1978) 5617–5624.
10 J. J. DISTLER AND G. W. JOURDIAN, *J. Biol. Chem.,* 248 (1973) 6772–6780.
11 R. E. HUBER, G. KURZ, AND K. WALLENFELS, *Biochemistry,* 15 (1976) 1994–2001.
12 V. WARIN, F. BAERT, R. FOURET, G. STRECKER, G. SPIK, B. FOURNET, AND J. MONTREUIL, *Carbohydr. Res.,* 76 (1979) 11–22.
13 J. DEISENHOFER, *Biochemistry,* 20 (1981) 2361–2370.
14 K. BOCK, J. ARNARP, AND J. LÖNNGREN, *Eur. J. Biochem.,* 129 (1982) 171–178.
15 J.-R. BRISSON AND J. P. CARVER, *Biochemistry,* 22 (1983) 3680–3686.
16 J.-R. BRISSON AND J. P. CARVER, *Biochemistry,* 22 (1983) 3671–3680.
17 P. J. DESCHAVANNE, O. M. VIRATELLE, AND J. M. YON, *J. Biol. Chem.,* 253 (1978) 833–837.
18 R. E. HUBER, M. T. GAUNT, AND K. L. HURLBURT, *Arch. Biochem. Biophys.,* 234 (1984) 151–160.
19 C. CECCARINI, A. D'ANIELLO, M. G. CACACE, AND P. H. ATKINSON, *Eur. J. Biochem.,* 132 (1983) 469–476.
20 S. JELINEK-KELLY, T. AKIYAMA, B. SAUNIER, J. S. TKACZ, AND A. HERSCOVICS, *J. Biol. Chem.,* 260 (1985) 2253–2257.