THE ROLE OF GLYCOSIDICALLY BOUND MANNOSE IN THE ASSIMILATION OF β-GALACTOSIDASE BY GENERALIZED GANGLIOSIDOSIS FIBROBLASTS

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Summary: Bovine testicular β-galactosidase is rapidly assimilated by generalized gangliosidosis skin fibroblasts. The enzyme contains equimolar amounts of mannose and glucosamine and strongly binds to concanavalin A-Sepharose. Pretreatment of β-galactosidase with a mannosidase preparation from Aspergillus niger reduced the rate of assimilation of the enzyme 97%. These data indicate that mannosyl residues play a role in assimilation of the enzyme. This conclusion is supported by observed inhibition of β-galactosidase assimilation by mannose, methyl α- and β-mannopyranosides, and mannose-containing testicular glycoproteins.

Skin fibroblasts from patients with generalized gangliosidosis are nearly devoid of β-galactosidase (1). When the fibroblasts are exposed to exogenous bovine testicular β-galactosidase, the enzyme: a) is rapidly taken up by the cells (400 times more rapidly than horseradish peroxidase), b) is observed in a perinuclear region of the cell by histochemical staining, and c) leads to correction of a moderate accumulation of sulfate-containing compounds that occurs in the fibroblasts (2,3). Hickman et al. (4) have suggested a means by which cells might assimilate extracellular hydrolases. This involves a series of steps including binding of the enzyme to the cell surface through carbohydrate residues, pinocytosis, and fusion of the pinocytotic vacuoles with the lysosomes. This hypothesis and the observed specific assimilation of serum glycoproteins containing β-galactosyl termini by liver cells (5) suggested

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the possibility that carbohydrate residues of β-galactosidase are involved in the assimilation of this enzyme by generalized gangliosidosis fibroblasts. The results described below support this hypothesis and further indicate that mannosyl residues of the enzyme are required for its efficient cellular assimilation.

Materials and Methods: Bovine testicular β-galactosidase was prepared as previously described (6) and was further subjected to gel filtration on Sephadex G-100 to remove galactose-containing compounds arising from the affinity support. The purified enzyme exhibited a specific activity of 5940 units/mg protein. A unit of all glycosidases described in this report is defined as that amount of enzyme which forms 1 nmole of nitrophenol/min from the appropriate p-nitrophenyl glycoside at 37° and pH 4.3.

Skin fibroblasts were obtained and grown as previously described (7). The rate of enzyme assimilation was measured after exposure of the cells to the enzyme for 3 h in Eagles minimal essential medium containing 10% calf serum and HEPES buffer (pH 6.8) at a final concentration of 0.02 M. Following incubation the cell monolayer was rinsed with saline, scraped from the petri dish, washed by centrifugation and lysed by 3 cycles of freezing and thawing. Aliquots of the cell extract were assayed for enzyme activity and protein (6). Potential inhibitors of enzyme assimilation were tested by mixing the compound with growth medium containing β-galactosidase prior to addition to the cell culture.

Jack bean α-mannosidase and bovine liver β-glucuronidase (Type B-10) were obtained from the Sigma Chemical Co., St. Louis, Mo., and concanavalin A-Sepharose from Pharmacia, Piscataway, N.J. Aspergillus niger α-mannosidase was prepared by the procedure of Matta and Bahl (8) and β-N-acetylglucosaminidase by the procedure of Bahl and Agrawal (9). Wheat germ agglutinin was prepared by affinity chromatography on chitin (10) and was coupled to cyanogen bromide-activated Sepharose as described by Cuatrecasas (11). Glucosamine was determined on an amino acid analyzer after hydrolysis in 4 N HCl; neutral sugars by gas-liquid chromatography of the corresponding alditol acetate derivatives after resin-catalyzed hydrolysis (12); and sialic acid by a thiobarbituric acid procedure after hydrolysis with 0.01 M H_2SO_4 (13). Methyl β-mannoside was prepared from methyl tetra-O-acetyl β-mannoside (kindly provided by Dr. I.J. Goldstein, The University of Michigan). The deacetylated compound was recrystallized as the isopropyl alcoholate (14). All sugars and sugar derivatives were recrystallized and were homogeneous when examined by gas-liquid chromatography.

RESULTS AND DISCUSSION

Bovine testicular β-galactosidase, prepared as described above, was nearly homogeneous when examined by disc gel electrophoresis (Fig. 1) and, at pH 7.5, enzymatic activity corresponded to the major protein-staining band. A molecular weight of 68,000 was established by SDS gel electrophoresis (15) and gel filtration (6). These results indicate that the enzyme consists of a single polypeptide chain.

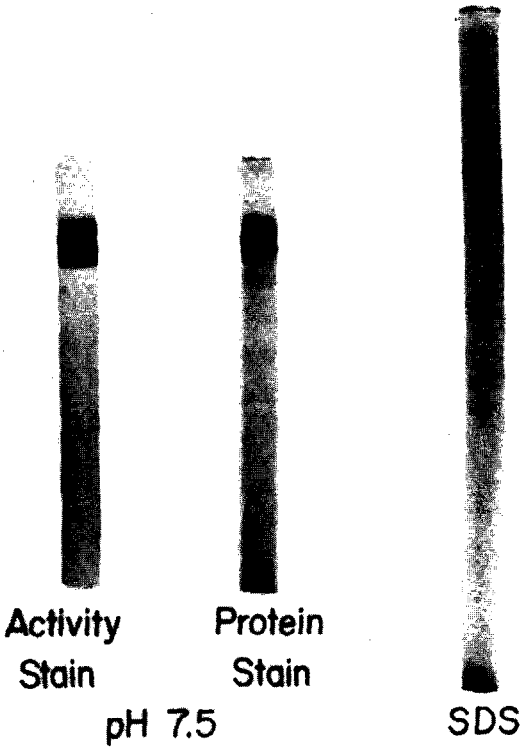

Fig. 1. Disc gel electrophoresis of bovine testicular β-galactosidase (80 units) after gel filtration as described in Methods. Electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed by the procedure of Weber and Osborn (15); in gels run in phosphate buffer at pH 7.5, protein was located with Amido-Swartz stain (6) and enzyme activity by incubation for 30 min in a solution containing 5-bromo-4-chloro-3-indolyl-β-galactoside (16).

As reported for a number of other lysosomal enzymes (17), bovine testicular β-galactosidase is a glycoprotein; analysis of the purified enzyme revealed the presence of mannose (0.16 μmole/mg protein) and glucosamine (0.16 μmole/mg protein). Other sugars commonly found in glycoproteins including galactose, fucose and sialic acid were not detected (<0.01 μmole/mg protein). The enzyme was not bound to wheat germ agglutinin-Sepharose under conditions described by Norden and O'Brien (18), suggesting the absence of terminal β-N-acetylglucosaminyl residues.

The rate of β-galactosidase assimilation by fibroblasts was a linear

TABLE I

Lectin Affinity and Assimilation of β-Galactosidase after Pretreatment with Glycosidases

Pretreatment[a]	Assimilation of treated β-galactosidase[b]	% β-galactosidase eluted from concanavalin A-Sepharose[c]	
	units/3 h / *mg cell protein*	ethylene glycol (50%)	CH_3 α-Man (0.75 M)
Bovine β-galactosidase (1000 units)	2.5	<1	100
Bovine β-galactosidase (1000 units) + jack bean α-mannosidase (1000 units)	2.4	<1	100
Bovine β-galactosidase (1000 units) + *A. niger* β-N-acetylglucosaminidase (1000 units)[d]	2.0	15	83
Bovine β-galactosidase (1000 units) + *A. niger* α-mannosidase (140 units)[e]	0.07	65	33
A. niger α-mannosidase (140 units)[e]	2.5[f]		

[a]Preincubations (1 ml) contained 0.25 ml McIlvaine citrate-phosphate buffer, pH 4.3; 0.02% NaN_3; and the indicated glycosidases. The mixtures were incubated for 72 h at 37° and dialyzed for an additional 24 h at 4° against 2 changes of normal saline.

[b]Assimilation of treated β-galactosidase was tested at a level of 25 units/ml of growth medium.

[c]β-Galactosidase (100 units in 1 ml 0.05 M Tris-HCl, pH 7.5 containing 1 mg/ml bovine serum albumin, 0.15 M NaCl, and 0.02% NaN_3) was applied at 25° to a column containing 0.5 ml concanavalin A-Sepharose and the column washed with 8 ml of the buffer-salts mixture. The column was eluted sequentially with the indicated compounds dissolved in the same buffer-salts mixture (8 ml of each eluant).

[d]Contaminated with α-mannosidase (5 units).

[e]Contaminated with β-mannosidase (370 units) and β-N-acetylglucosaminidase (810 units).

[f]Pretreated α-mannosidase added to culture medium containing untreated β-galactosidase (25 units/ml).

function of the concentration of the enzyme in the medium at levels below 40 units/ml. In the presence of 25 units of β-galactosidase/ml, 2.5 ± 0.3 units of enzyme were assimilated/3 h/mg fibroblast protein; uptake of the enzyme was linear with time over this period. Above approximately 60 units of β-galactosidase/ml medium the rate of uptake was constant; approximately 5 units of the enzyme were assimilated/3 h/mg cell protein under these conditions.

Effect of Pretreatment of β-Galactosidase with Glycosidases. Preincubation of the enzyme at pH 4.3 in the absence of other glycosidases did not change the rate of its assimilation. Pretreatment of β-galactosidase with A. niger α-mannosidase resulted in up to 97% reduction in its rate of assimilation (Table I). Although the α-mannosidase preparation contained a substantial amount of β-N-acetylglucosaminidase, this latter activity was not primarily responsible for the lowered rate of assimilation since pretreatment of β-galactosidase with purified β-N-acetylglucosaminidase reduced the uptake only by 20% (Table I). Further, reduction in the rate of assimilation was not due to: a) the presence of inactive β-galactosidase because preincubation did not result in a detectable loss of β-galactosidase hydrolytic activity, or b) inhibition of assimilation by α-mannosidase because addition of α-mannosidase and β-galactosidase directly to cell cultures did not result in lowered rates of assimilation (Table I). The A. niger α-mannosidase preparation contained both α- and β-mannosidase; therefore, it is not certain which mannosidase destroyed the assimilability of the β-galactosidase. Indeed, a concerted action of the two mannosidases seems possible in view of the common presence of both α- and β-mannosyl residues in many glycoproteins. However, it appears likely that α-mannosyl residues are removed from β-galactosidase by the α-mannosidase preparation since binding of β-galactosidase to concanavalin A-Sepharose is markedly reduced. Untreated β-galactosidase was bound to concanavalin A-Sepharose and was not eluted with 50% ethylene glycol. After mannosidase treatment, 67% of the β-galactosidase was eluted with ethylene glycol (Table I).

Pretreatment of β-galactosidase with jack bean α-mannosidase did not alter

TABLE II

Inhibitors of β-Galactosidase Assimilation

Substance added to the growth medium	% Inhibition of β-galactosidase uptake[a]
Mannose (0.1 M)[b]	21
CH_3 α-Man (0.1 M)	46
CH_3 β-Man (0.1 M)	57
Testis glycoprotein(s) (17 μg/ml)	61
Galactose, N-acetylglucosamine, chitobiose, CH_3 α-Glc, CH_3 α-Gal, CH_3 α-GlcNAc, CH_3 β-Gal, or CH_3 β-Glc (0.1 M)	<5
Orosomucoid, ovalbumin, ribonuclease b (1 mg/ml)	<5
β-Glucuronidase (900 μg/ml, 1350 units/ml)[c]	<5

[a]Assimilation of β-galactosidase was tested at a level of 7 μg (40 units)/ml medium.

[b]Values in parentheses indicate final concentration in the growth medium.

[c]β-Glucuronidase was rapidly assimilated by the fibroblasts with a maximal rate of uptake (in the presence or absence of β-galactosidase) of 8.4 ± 0.3 units/3 h/mg cell protein. The system was saturated with β-glucuronidase at concentrations above 500 units/ml growth medium.

either the assimilation of the enzyme by the fibroblasts or the binding of the enzyme to concanavalin A-Sepharose (Table I). The reason for the failure of jack bean α-mannosidase to release mannose from β-galactosidase is not readily apparent. Jack bean α-mannosidase is reported to remove terminal α-mannosyl residues from glycoproteins (19). The possibility cannot be eliminated that the effectiveness of the A. niger α-mannosidase preparation is due to the release of mannose by a sequential action of two or more enzymes in this preparation.

Inhibitors of β-Galactosidase Assimilation. Recrystallized, homogeneous mannose, when added to the β-galactosidase-containing medium at a level of 0.1 M, consistently reduced the rate of assimilation of β-galactosidase by about 21%

(Table II). A greater inhibition by mannose was found when commercial preparations were used without recrystallization (2). Methyl α- or β-Man at 0.1 M final concentration inhibited assimilation of β-galactosidase approximately 50% over that observed in control flasks. The inhibition of β-galactosidase assimilation by mannose and CH_3-mannosides was judged to be specific based on the failure of a number of other monosaccharides, glycosides, and glycoproteins to inhibit the assimilation of β-galactosidase (Table II) and by the failure of mannose or CH_3-mannosides to inhibit the uptake of bovine liver β-glucuronidase by fibroblasts (data not shown). The results of inhibition studies support the hypothesis that β-galactosidase binds specifically to mannosyl-binding receptors on the cell surface.

Crude testicular β-galactosidase was not readily taken up by fibroblasts. Prior to purification by affinity chromatography, assimilation of the enzyme was not detectable. This phenomenon proved to be due to the presence of inhibitors which passed through the β-galactosidase affinity support. The inhibitor(s) were further purified by adsorption to concanavalin A-Sepharose and elution with CH_3 α-Man as described by Norden and O'Brien (18). At levels of 17 μg inhibitor protein/ml medium and 7 μg β-galactosidase/ml medium, a 61% inhibition of β-galactosidase assimilation was observed (Table II). Analysis of the inhibitor preparation for neutral sugars gave 0.25 μmole mannose and 0.03 μmole galactose/mg protein. Viewed either from the standpoint of mannose content or protein, the preparation is a very effective inhibitor of galactosidase assimilation.

It is of interest to note that β-glucuronidase, an enzyme readily assimilated by fibroblasts (20), did not inhibit the assimilation of β-galactosidase even when β-glucuronidase was present at saturating levels (Table II). This observation together with the above described absence of inhibition of β-glucuronidase assimilation by mannosides suggests that the cellular mechanisms for the uptake of the two enzymes are different. However, the observed saturability of the assimilation of β-glucuronidase suggests that,

like β-galactosidase, β-glucuronidase is assimilated by a selective system. It remains to be determined whether the system involving β-glucuronidase is similar to that reported for the assimilation of serum glycoproteins containing terminal galactosyl residues by liver cells (5) or whether there are other, as yet undefined, recognition sites for glycoprotein assimilation. The availability of purified lysosomal enzymes and of fibroblasts lacking these enzymes (derived from glycosphingolipidosis, mucolipidosis and mucopolysaccharidosis patients) provides other systems for continuing studies on the multiplicity and mechanisms of glycoprotein assimilation.

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