Selective, Noncompetitive Assimilation of Bovine Testicular β-Galactosidase and Bovine Liver β-Glucuronidase by Generalized Gangliosidosis Fibroblasts

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ABSTRACT Bovine liver β-glucuronidase and testicular β-galactosidase were assimilated by generalized gangliosidosis fibroblasts at respective rates of 90 and 464 times the rate of assimilation of horseradish peroxidase. Assimilation of either of the two enzymes by the fibroblasts was saturable, suggesting the participation of receptor-mediated adsorptive endocytosis for internalization. The rate of assimilation of either enzyme was not affected by high levels of the other enzyme, suggesting that distinct receptors for each enzyme occur on the fibroblasts' cell surface. Furthermore, although assimilation of β-galactosidase was inhibited by mannose, methyl mannosides, mannosyl α1→2 mannose, and mannose-6-phosphate, these compounds did not detectably inhibit the assimilation of β-glucuronidase. These results suggest that testicular β-galactosidase was assimilated by the well-established phosphomannosyl recognition system. However, liver β-glucuronidase was assimilated by a distinct, noncompeting, and as yet undefined, recognition system.

INTRODUCTION

Specific lysosomal enzyme deficiencies have been shown to be responsible for several inherited storage disorders, including the mucopolysaccharidoses, lipidoses, and mucolipidoses (1–3). Identification of the defective enzyme systems, coupled with evidence that extracellular lysosomal hydrolases are assimilated by enzyme-deficient fibroblasts, has led to the suggestion that enzyme replacement therapy could be used to treat this class of disorders. Indeed, when a specific corrective enzyme is assimilated by cultured cells, the metabolic defect in these cells is corrected, i.e., materials that accumulate as a result of the enzyme deficiency are effectively catabolized (4–11). Although the results of clinical enzyme replacement therapy have been generally disappointing, they have stimulated attempts to elucidate the mechanism by which lysosomal enzymes, added externally to cells, find their way into the intracellular catabolic sites.

Investigation of the factors involved in the assimilation of enzymes has led to the suggestion that specific recognition markers contained on the lysosomal enzymes facilitate the assimilation of the enzymes by the cells. Neufeld et al. (12) have postulated that extracellular enzymes are selectively assimilated by a series of steps, including binding of the enzyme to the cell surface through carbohydrate residues, endocytosis, and, finally, fusion of the endocytotic vacuoles with the lysosomes. A number of protein-bound carbohydrate recognition markers and their corresponding cell-surface receptors have been described to date. Ashwell and coworkers (13) demonstrated that terminal β-galactosyl residues are required for the assimilation of serum glycoproteins by mammalian liver cells, whereas avian liver cells specifically recognize terminal β-*N*-acetylglucosaminyl residues (14). Bahl (15) has observed that mannosyl residues participate in the selective uptake of gonadotropin by rat testis cells, and Baynes and Wold (16) found that terminal α-mannosyl residues are required for the clearance of ribonuclease B from the circulation by rat liver. Many studies support the carbohydrate-directed uptake of lysosomal enzymes by human skin fibroblasts. Mannose phosphate residues of

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human platelet β-glucuronidase (17), a human α-iduronidase (18), bovine testicular β-galactosidase (19), and a variety of other human lysosomal enzymes (20) have been suggested as recognition markers for the selective uptake of the respective enzymes by fibroblasts. The uptake of rat liver β-glucuronidase has been suggested to proceed through participation of a recognition marker that does not contain mannose-6-phosphate (21).

This paper presents kinetics of the assimilation of bovine testicular β-galactosidase and bovine liver β-glucuronidase by generalized gangliosidosis skin fibroblasts. Both β-galactosidase (22) and β-glucuronidase (4) have been shown to be actively assimilated by skin fibroblasts; both enzymes act intracellularly to correct the accumulation of sulfate-containing compounds in cells deficient in the respective enzymes (4, 7); and after their assimilation, both β-glucuronidase (5) and β-galactosidase (22) have been demonstrated histochemically in the interior of the cells. It is therefore assumed that the assimilated enzymes enter the cells and reach a functional lysosomal destination in the enzyme-deficient cells. Evidence is presented below that supports the view that bovine β-galactosidase and β-glucuronidase are selectively assimilated by skin fibroblasts by distinct, noncompeting systems. Preliminary communications of this work have been presented (23, 24).

METHODS

Bovine liver β-glucuronidase (type B-10), mannose-6-phosphate, and methyl α-mannoside were obtained from the Sigma Chemical Co., St. Louis, Mo. Mannosyl α1 → 2 mannose, prepared by chemical synthesis, was a generous gift of Dr. Irwin Goldstein, University of Michigan. Methyl β-D-mannoside (22), and bovine testicular inhibitor glycoproteins and glycopeptides were prepared as described previously (25).

Bovine testicular β-galactosidase, purified by affinity chromatography, catalyzed the hydrolysis of 5,940 nmol *p*-nitrophenyl-β-D-galactopyranoside/min per mg protein, and was essentially homogeneous (26). 1 U of β-galactosidase was defined as that amount that catalyzed the hydrolysis of 1 nmol substrate/min at 37°C and pH 4.3.

A commercial preparation of bovine liver β-glucuronidase catalyzed the formation of 1,000 nmol of phenolphthalein/min per mg protein when phenolphthalein-β-D-glucuronic acid served as substrate. Because highly purified bovine liver β-glucuronidase has been reported to exhibit a specific activity of 10,500 nmol/min per mg protein (27), the commercial enzyme preparation was assumed to be ~10% pure. In the present studies, β-glucuronidase activity was routinely measured using *p*-nitrophenyl-β-glucuronic acid as substrate. Reaction mixtures contained: 0.5 μmol *p*-nitrophenyl-β-glucuronic acid; 25 μl citrate phosphate buffer, pH 5.0, prepared as described by McIlvaine (28); 0.05 μg bovine serum albumin; and 1–5 U of β-glucuronidase in a total of 0.05 ml. Reaction mixtures were incubated at 37°C and the reaction terminated by the addition of 0.5 ml of 0.5 M glycine-NaOH buffer, pH 10.0. The *p*-nitrophenol formed was measured at 400 nm using a molar extinction coefficient of 17,500. Control mixtures lacked either enzyme or substrate. 1 U of β-glucuronidase was defined as that amount of enzyme that formed 1 nmol nitrophenol/min. The commercial enzyme preparation exhibited a specific activity of 3,000 (nitrophenol) U/mg protein. Bovine liver β-glucuronidase was also prepared by affinity chromatography (29). After two affinity chromatography steps, the purified preparation catalyzed the hydrolysis of 10,000 nmol *p*-nitrophenyl-β-glucuronic acid/min per mg protein.

Horseradish peroxidase (3,170 U/mg protein, Reinheitszahl purity number = 3.0) was obtained from Worthington Biochemical Corp., Freehold, N. J. Activity of the peroxidase was determined by measuring the decomposition of hydrogen peroxide; *O*-dianisidine served as hydrogen donor (30). 1 U was defined as that amount of enzyme that decomposed 1 μmol of peroxide/min at 25°C.

Enzyme assimilation studies. Enzyme K assimilation values (18) were 11.0 U/ml for β-galactosidase and 400±10 U/ml for both commercial and affinity-prepared β-glucuronidase.

Type I generalized gangliosidosis fibroblasts, strains KD and WG 370, were obtained from the Repository for Mutant Human Cell Strains, Montreal Children's Hospital, Quebec, Canada. Fibroblasts were grown in Eagle's minimal essential medium supplemented with 10% fetal calf serum. Growth medium was replaced at 3-d intervals and cells were transferred upon reaching confluence. Before measuring enzyme assimilation, the cells were detached from the flasks by trypsinization and suspended in fresh medium and 2×10^5 cells plated into 35-mm plastic petri dishes. After growth of the fibroblasts for 3 d, the medium was replaced with 1.35 ml Eagle's minimal essential medium containing 10% calf serum, and 0.02 M Hepes-HCl buffer, pH 6.8. At timed intervals, 0.15 ml of 0.85% NaCl, containing the indicated enzymes (and, if appropriate, inhibitors of enzyme assimilation), was added. After incubation at 37°C for the specified time, the medium was removed and the fibroblast monolayer rinsed with saline. Growth medium (2 ml) was added, and the cells detached from the petri dish by scraping with a rubber policeman. The detached cells were chilled in an ice bath, thoroughly dispersed using a Pasteur pipette, and pelleted for 3 min at 700 *g*. The cell pellet was washed in cold saline by resuspension and centrifugation, and the cells were suspended in 0.1 ml of distilled water and disrupted by freezing and thawing three–four times in a dry ice-acetone mixture. Aliquots of the lysates were analyzed for enzyme activity by the procedures described above and for protein content (31). Using the same enzyme preparation and fibroblast strain, repeated determinations of enzyme uptake gave values that were reproducible to within 10%. Both β-glucuronidase and β-galactosidase were stable in the buffered culture medium for the duration of each experiment.

RESULTS

The assimilation of β-galactosidase and β-glucuronidase by generalized gangliosidosis fibroblasts was determined with increasing times of exposure of cells to exogenously added enzymes. The results, shown in Fig. 1, indicate that the cellular levels of these enzymes increased in a linear fashion for a period of at least 12 h. The rate of assimilation of each enzyme was routinely measured after 3 h exposure of the fibroblasts to growth medium containing enzyme, and was corrected for the endogenous levels of the respective enzymes. The short incubation time allowed sufficient enzyme assimilation to permit accurate measurements of cell-asso-

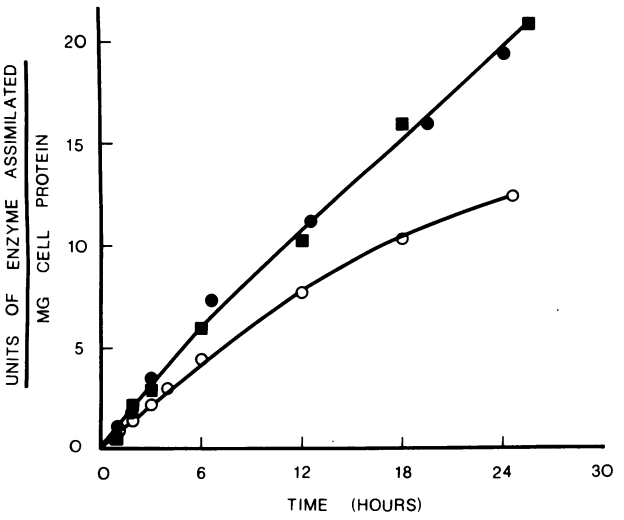

FIGURE 1 Assimilation of bovine testicular β-galactosidase and bovine liver β-glucuronidase by generalized gangliosidosis fibroblasts. Fibroblasts were exposed to β-galactosidase at a concentration of 20 U/ml medium, (○); to a commercial preparation of bovine liver β-glucuronidase at a concentration of 75 U/ml medium, (●); or to an affinity chromatography preparation of β-glucuronidase at a concentration of 100 U/ml medium, (■). Cells were harvested and washed at the indicated periods of time, and the cell-associated enzyme determined as described in Methods. Control cells were harvested immediately after adding enzyme-containing medium to the cells.

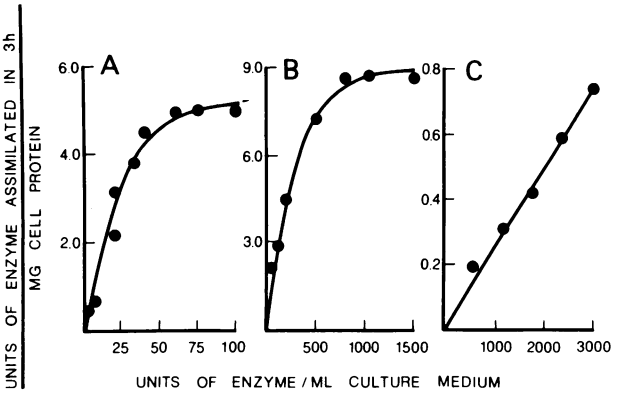

FIGURE 2 Effect of enzyme concentration on the rate of enzyme assimilation by generalized gangliosidosis fibroblasts. Fibroblasts were exposed for 3 h to medium containing the indicated concentrations of enzymes. The cells were harvested and cell-associated enzymes determined as described in Methods. Values obtained from control cultures not exposed to enzymes were subtracted. (A) bovine testicular β-galactosidase; (B) a commercial preparation of bovine liver β-glucuronidase; (C) horseradish peroxidase.

ciated enzyme activity and, at the same time, minimized errors attributable to growth of the fibroblasts, cellular catabolism of the assimilated enzymes, and inadvertent microbial contamination of the cultures during the assay procedure.

Rate of enzyme uptake as a function of enzyme concentration in the medium. The rate of assimilation of β-galactosidase and β-glucuronidase as a function of concentration of enzyme in the medium is shown in Fig. 2. Assimilation of β-galactosidase was directly proportional to the concentration of enzyme in the medium below 40 U/ml medium; β-glucuronidase assimilation was proportional to its concentration in the medium below 500 U/ml medium.

The rate of cellular assimilation of the lysosomal enzymes approached maximal rates above 60 U β-galactosidase and 750 U β-glucuronidase/ml medium (Fig. 2A and B). Saturability has been suggested as a criteria for demonstration of adsorptive endocytosis mediated by cell receptors (11, 12, 19, 20). Saturation of the uptake of horseradish peroxidase by the generalized gangliosidosis skin fibroblasts was not approached even at concentrations as high as 1 mg/ml (Fig. 2C). Inability to saturate the assimilation of peroxidase is evidence for its assimilation by nonadsorptive fluid endocytosis; the uptake of this enzyme has previously been used as a measure of fluid endocytosis in fibroblasts (5, 18, 22, 32). On a molar basis, the highest concentration of peroxidase tested was in excess of 100 times the concentration required to give maximal assimilation rates for β-galactosidase and β-glucuronidase.

Additional evidence for the presence of cell surface receptors for β-glucuronidase and β-galactosidase was obtained from a study of the selectivity of the assimilation processes. The rate of assimilation of β-galactosidase and β-glucuronidase measured at limiting concentrations was compared with that of peroxidase (Table I). Bovine β-galactosidase and β-glucuronidase were assimilated at respective rates of 464 and 90 times those observed for peroxidase. Thus, kinetic measurements strongly support the hypothesis that both bovine β-galactosidase and β-glucuronidase are assimilated by selective receptor-mediated adsorptive endocytosis.

Lack of competition in the assimilation of β-galactosidase and β-glucuronidase. To determine whether each enzyme was bound to the same or to distinct receptor sites, assimilation of the two enzymes was measured simultaneously in the same fibroblast culture. The results of these studies are presented in Table II. The rate of assimilation of β-galactosidase present at limiting concentrations was not altered in the presence of saturating levels of β-glucuronidase, and, conversely, saturating levels of β-galactosidase did not change the rate of assimilation of β-glucuronidase present at limiting concentrations. These results suggest that the rate-limiting step for the assimilation of each enzyme is different. A plausible explanation is that the two enzymes bind at specific but distinct receptor sites on the cell surface. This hypothesis is further supported by the results of studies concerned

TABLE I

Relative Rate of Assimilation of Bovine β-Galactosidase, Bovine β-Glucuronidase, and Horseradish Peroxidase at Limiting Enzyme Concentrations

Enzyme added	Concentration	Assimilation	Assimilation of added enzyme in 3 h/mg cell protein*	% Assimilation glycosidase / % Assimilation peroxidase
	U/ml	*U/3 h / mg cell protein*	%	
β-Galactosidase	30	3.5	11.6	464
β-Glucuronidase	200	4.5	2.25	90
Peroxidase	3,000	0.74	0.025	

* To compare the rate of assimilation of different enzymes, the percentage of added enzyme that would theoretically be removed from 1 ml medium over a 3-h period by cells containing 1 mg protein was calculated.

with the effect of inhibitors on the assimilation of each enzyme.

Inhibition of the assimilation of bovine β-glucuronidase and β-galactosidase. Mannose-6-phosphate and, to a lesser extent, other mannose-containing compounds were previously shown to inhibit the assimilation of bovine testicular β-galactosidase by fibroblasts (19, 22, 24, 25). The observed lack of competition between bovine testicular β-galactosidase and liver β-glucuronidase prompted a study of the ability of mannose-containing compounds to inhibit the assimilation of β-glucuronidase. A variety of mannose-containing carbohydrates that inhibited the assimilation of β-galactosidase did not inhibit the assimilation of β-glucuronidase. At a concentration of 0.1 M, mannose and methyl α- and β-mannosides inhibited β-galactosidase assimilation 21–57%, but inhibited the assimilation of β-glucuronidase < 5% (Table III). Synthetic mannopyranosyl α1 → 2 mannose strongly inhibited β-galactosidase assimilation (42% inhibition at a concentration of 2.0 mM) but did not detectably inhibit the assimilation of β-glucuronidase at the same concentration. Mannose-6-phosphate has been reported to inhibit the assimilation of human platelet β-glucuronidase (17) and bovine β-galactosidase (25), but does not detectably inhibit the assimilation of bovine liver β-glucuronidase (Table III). These results suggest that the assimilation of bovine liver β-glucuronidase proceeds by a process distinct from that observed for human platelet β-glucuronidase. A number of monosaccharides, disaccharides, and glycosides (shown in the footnotes to Table III) failed to inhibit the assimilation

TABLE II

Simultaneous Assimilation of β-Galactosidase and β-Glucuronidase by Generalized Gangliosidosis Fibroblasts

Enzyme added	Concentration	Assimilation of: β-Galactosidase	Assimilation of: β-Glucuronidase
	U/ml medium	*U/3 h/mg cell protein*	
Experiment I			
β-Galactosidase	32	2.2	—
β-Galactosidase	32	2.3	4.3
β-Glucuronidase (commercial)	900		
Experiment II			
β-Glucuronidase (commercial)	76	—	1.7
β-Glucuronidase (commercial)	76	4.5	2.0
β-Galactosidase	150		
Experiment III			
β-Glucuronidase (affinity)	62	—	1.2
β-Glucuronidase (affinity)	62	3.6	1.3
β-Galactosidase	130		

TABLE III
Inhibition of Bovine β-Galactosidase and β-Glucuronidase Assimilation

Substance added*	Concentration	Assimilation of: β-Galactosidase	Assimilation of: β-Glucuronidase
	mM	*% Inhibition‡*	
Mannose	100	21	<5
Methyl α-mannoside	100	46	<5
Methyl β-mannoside	100	57	<5
Mannosyl α1 → 2 mannose	1.0	42	<5
Mannose-6-phosphate	0.5	70	<5

* At a final concentration of 100 mM the following gave <5% inhibition: galactose, glucose, lactose, *N*-acetylglucosamine, *N*-acetyllactosamine, methyl α- and β-galactosides, and methyl α- and β-glucosides.
‡ % Inhibition = 100

$$\times \frac{\text{(control assimilation)} - \text{(assimilation with inhibitor)}}{\text{(control assimilation)}}.$$

of either bovine testicular β-galactosidase or liver β-glucuronidase.

DISCUSSION

Bovine testicular β-galactosidase and liver β-glucuronidase are rapidly removed from the medium by generalized gangliosidosis fibroblasts. The rate of assimilation of these enzymes is too rapid to be explained solely on the basis of fluid endocytosis. Furthermore, both enzymes exhibit saturation kinetics suggesting that assimilation of β-galactosidase and β-glucuronidase proceeds via selective receptor-mediated processes. However, because neither enzyme inhibits the assimilation of the other, the presence of separate receptors for the two enzymes is suggested. This hypothesis is further substantiated by the results of inhibition studies with the two enzymes. The assimilation of β-galactosidase was inhibited by mannose-6-phosphate and other mannose-containing compounds, whereas these compounds did not detectably inhibit the assimilation of β-glucuronidase.

Glycosidically bound mannose phosphate residues participate in a recognition marker for the selective assimilation of bovine testicular β-galactosidase (19, 25) as well as for the assimilation of human platelet β-glucuronidase (17), human urinary α-L-iduronidase (12), and a variety of other lysosomal enzymes (20). Bovine liver β-glucuronidase is a glycoprotein that also contains high levels of mannose (27), but the assimilation of this type of β-glucuronidase by fibroblasts has not yet been linked to specific carbohydrate residues. Indirect evidence suggests that carbohydrate residues of β-glucuronidase participate in the recognition marker of bovine liver β-glucuronidase. For example, preliminary experiments indicate that crude testicular glycopeptides (25) inhibit β-glucuronidase assimilation, and that brief treatment of the enzyme with periodate abolishes enzyme uptake (unpublished observations). On the other hand, treatment of the enzyme with a variety of glycosidases to remove specific carbohydrate residues has not yet resulted in significant changes in the rate of assimilation. It should be noted that Strawser and Touster (33) have recently reported that rat liver β-glucuronidase binds to a microsomal protein via a peptide sequence that does not contain carbohydrates. Conceivably, a periodate-sensitive peptide sequence could participate in the assimilation of bovine liver β-glucuronidase. For these reasons, postulation of a carbohydrate containing a recognition marker on bovine liver β-glucuronidase may be premature and should await chemical characterization of the recognition marker region of the enzyme.

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