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CELL-SPECIFIC DIFFERENCES IN MEMBRANE β-GLUCOSIDASE FROM NORMAL AND GAUCHER CELLS

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Summary

Two isozymes of membrane-bound β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) with activity towards 4-methylumbelliferyl-β-D-glucopyranoside have been identified in human cells. One of these isozymes was found to have a pH optimum of 5.0, a K_m of 0.4 mM and to be rapidly inactivated at pH 4.0 ("acid-labile"). The second isozyme had a pH optimum of 4.5, a K_m of 0.8 mM and was stable at pH 4.0 ("acid-stable"). Cultured long-term lymphoid lines and peripheral blood leukocytes contained both isozymes while cultured skin fibroblasts contained only the "acid-stable" form in detectable amounts. The specific activity of the "acid-stable" isozyme was severely reduced in cultured skin fibroblasts, cultured long-term lines and peripheral leukocytes from patients with Gaucher's disease. The specific activity of the "acid-labile" enzyme in the latter two cell types was apparently unaffected. The β-glucosidase activity in all three cell types examined was predominantly particulate but the enzyme could be solubilized with low concentrations of Triton X-100. The solubilized enzyme required sodium taurocholate (0.2%) for maximum activity. Solubilized β-glucosidase did not exhibit the cell-specific differences in pH optimum and K_m shown by the membrane-bound enzyme.

Introduction

Gaucher's disease is a sphingolipidosis with an autosomal recessive mode of inheritance. The major clinical manifestations of the disease, such as splenomegaly, hepatomegaly and bone lesions, apparently stem from the accumulation of glucocerebroside in cells of the reticuloendothelial system [1]. Brady and coworkers [2,3] demonstrated that this accumulation is due to the deficiency of a β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) (glucocerebrosidase) which catalyzes the hydrolytic cleavage of glucocerebroside to

ceramide and glucose. This finding was independently confirmed by Patrick [4] using both the natural substrate and an artificial substrate, *p*-nitrophenol-β-D-glucopyranoside. A second artificial substrate, 4-methylumbelliferyl-β-D-glucopyranoside, has since been used for the study of β-glucosidases and reduced activity towards this substrate has been demonstrated in liver and spleen [5], leukocytes [6,7] and cultured skin fibroblasts [8—10] from patients with Gaucher's disease.

In an earlier study [6] evidence was presented for the existence in peripheral leukocytes of two isozymes of β-glucosidase with differing pH optima. In cells from patients with Gaucher's disease, activity of the isozyme with the more acidic pH optimum appeared to be the more severely diminished. In this report we present results on some properties of β-glucosidase from peripheral leukocytes, cultured long-term lymphoid lines (derived from blood lymphocytes by transformation with phytohemagglutinin or Epstein-Barr virus) and cultured skin fibroblasts. The results provide evidence for the existence of two discrete isozymes and for the specific deficiency of one of these in patients with Gaucher's disease.

Materials and Methods

Cultured cells. Long-term lymphoid cell lines were established after stimulation of peripheral lymphocytes with phytohemagglutinin and subsequent incubation with Epstein-Barr virus, or after stimulation with phytohemagglutinin only [11,12]. The procedures used for culture and harvesting of these cells have been given in detail elsewhere [13]. Skin fibroblasts, obtained from a biopsy from the inner forearm, were cultured and harvested as previously described [13].

Peripheral leukocytes. Blood was drawn by venipuncture in a heparinized syringe. Leukocytes were prepared by sedimentation in 1% Dextran and preferential lysis of erythrocytes with 0.83% NH_4Cl. Cells were washed twice with 0.9% saline and stored as packed cells at −20° C. Cells were assayed after no more than a few days storage at this temperature.

Preparation of cell extracts. Packed fibroblasts, lymphoid lines or leukocytes were suspended in three volumes of distilled water and subjected to five cycles of freezing and thawing. Lymphoid line and leukocyte extracts were then diluted with a further two volumes of distilled water. Fibroblast extracts were diluted with nine volumes of distilled water. Extracts were prepared immediately prior to assay for β-glucosidase. Membrane preparations were obtained by centrifugation of cell extracts at 20 000 rev./min (Sorvall SS-34 rotor) for 30 min at +4° C. The resulting pellet was washed once with distilled water by centrifugation as above.

β-Glucosidase assay. All assays were carried out with the artificial substrate 4-methylumbelliferyl-β-D-glucopyranoside (Koch-Light Labs. Ltd., Colnbrook, England). 1.2. mM solutions were made up in citrate/phosphate buffer, pH 4.0 or 5.1. The buffer was made by mixing 0.2 M disodium phosphate and 0.1 M citric acid to the required pH and diluting 4-fold prior to use [9]. Reaction mixtures contained 0.1 ml substrate and 0.02 ml sample. Reaction tubes were sealed and incubated for 20 min at 37° C after which time the reaction was

stopped with 4 ml 0.085 M glycine/carbonate buffer, pH 10.0. All assays were performed in triplicate. Fluorescence was measured with either a Turner model 111 fluorimeter or a Beckman model 772 ratio fluorimeter.

Using lymphoid line extracts the amount of substrate split increased linearly with time for at least 75 min at both pH 4.0 and pH 5.1. The amount of substrate split was also directly proportional to the amount of cell extract added over the range 8—160 μg protein per assay. Protein was determined by the method of Lowry et al. [14]. Fibroblast assays usually contained from 6 to 10 μg protein. Crude sodium taurocholate (British Drug Houses) and Triton X-100 (Sigma) were included in some assays as described in the text.

Patients. All patients presented initially with splenomegaly and anemia at ages ranging from 5 to 54 years. In all cases typical Gaucher cells were observed in bone marrow aspirates. None of these patients showed any signs of neurological involvement.

Results and Discussion

pH optimum and K_m

Fibroblast β-glucosidase was optimally active at pH 4.5. Activity was 80—90% maximum at pH 4.0 and 5.0 but fell rapidly at assay pH values above and below these values. The enzyme was essentially inactive at pH 3.0 or pH 7.0. In contrast, lymphoid line or peripheral leukocyte β-glucosidase was optimally active at pH 5.0. In all lymphoid line or leukocyte preparations the pH curve was asymmetrical and in most cases a distinct shoulder, or second optimum, occurred at pH 4.0. The prominence of this shoulder varied between individual cell lines or leukocyte preparations. As outlined below, β-glucosidase in human cells is predominantly membrane bound. It was found that washed membrane preparations from fibroblasts or lymphoid lines gave results identical to those obtained with complete cell extracts. The cell-specific variation in pH optimum is therefore not dependent on the presence of soluble cell components in the reaction mixture.

Incubation of lymphoid line or leukocyte extracts in 12.5 mM phosphate/citric acid buffer, pH 4.0, prior to assay, caused an alteration in the shape of the pH curve. While activity at pH 4.0 and below was unaffected, activity at higher pH values was considerably reduced. In a typical experiment preincubation at pH 4.0 (room temperature) caused a loss of activity ranging from 61% at pH 5.0 to 72% at pH 6.5. The reduction in activity was essentially complete after 5 min. Identical pretreatment of fibroblast membrane preparations at pH 4.0 resulted in only a slight fall in β-glucosidase activity (less than 10%) and no change in the shape of the pH curve.

In addition to these differences in pH optimum and stability at pH 4, fibroblast and lymphoid line β-glucosidase differed slightly in K_m for the 4-methylumbelliferyl substrate. K_m values for both cell types were measured at pH 5.1, using at least seven substrate concentrations covering the range 0.1—4.5 mM. Lineweaver-Burk plots were linear over this range. Duplicate determinations gave average K_m values of 0.81 mM for the fibroblast enzyme and 0.39 mM for the lymphoid line enzyme.

TABLE I

SPECIFIC ACTIVITY OF β-GLUCOSIDASE IN CELLS FROM CONTROLS, OBLIGATE HETEROZYGOTES AND PATIENTS WITH GAUCHER'S DISEASE

Results are expressed in pmol/min per mg protein ± S.D. The range of values is shown in parentheses.

Cell type	Origin	No.	Specific activity	
			pH 4.0	pH 5.1
Lymphoid line	Controls	15	60.0 ± 36.0 (11.3—135.4)	98.6 ± 34.0 (48.3—160.3)
	Obligate heterozygotes	3	20.5 (11.4—33.5)	69.4 (57.2—79.5)
	Patient	1	25.8	96.0
Peripheral leukocytes	Controls	8	37.2 ± 6.1 (27.5—44.9)	83.7 ± 18.3 (65.2—112.8)
	Obligate heterozygotes	2	24.3, 26.0	105.1, 78.2
	Patients	4	7.0 ± 1.8 (5.1—8.9)	78.8 ± 11.8 (67.6—95.4)
Skin fibroblasts	Controls	20	1571 ± 302 (969—2065)	1128 ± 216 (744—1705)
	Obligate heterozygotes	5	799 ± 162 (561—982)	559 ± 112 (453—741)
	Patients	7	140 ± 30 (111—197)	183 ± 43 (121—252)

Specific activities in controls, heterozygotes and homozygotes for Gaucher's disease

The results of assays on long-term lymphoid lines, peripheral leukocytes and cultured skin fibroblasts from controls, obligate heterozygotes and patients with Gaucher's disease are summarized in Table I.

The specific activity of β-glucosidase in long-term lymphoid lines resembled that of the peripheral leukocyte enzyme. However, the two cell types differed in that the specific activity of the enzyme in lymphoid lines was far more variable than that of the leukocyte enzyme. Lines established from different donors varied by more than one order of magnitude.

In an attempt to establish the origins of this variation, nine of the cell lines were assayed on two or more occasions using either different preparations of the same line or different lines established from the same donor. The results of repeated assays were consistent with the original determinations ($P < 0.05$). The variation between cell lines would not therefore seem to be due either to irreproducibility of the assay system or to variations in culture conditions. Neither was the variability of β-glucosidase simply a reflection of amore general variation in lysosomal enzyme levels in lymphoid lines. No significant correlation existed between α-mannosidase, α-L-fucosidase and β-glucosidase activities in different cell lines.

As shown in Table I the specific activity of β-glucosidase in lymphoid line and leukocyte extracts from patients and heterozygotes was consistently below mean control values when assayed at pH 4.0. However, at pH 5.1 the specific activity of the patients' enzyme was, in all cases, within the control

range. In cultured skin fibroblasts from patients, activity was reduced at both pH values, in agreement with previous reports [8—10].

The pH curve of the "residual" β-glucosidase in leukocytes from two patients had a sharp optimum at pH 5.0 falling rapidly above or below this pH. Activity at pH 5.0 was reduced by at least 75% following incubation of the cell extract at pH 4.0 prior to assay. In contrast, β-glucosidase activity in cultured skin fibroblasts from patients was optimal over the range pH 4.5—5.0 and was unaffected by preincubation at pH 4.0.

Membrane association and effect of detergents

Despite the use of freeze-thawing procedure for cell disruption, 85—90% of the β-glucosidase activity in both cultured skin fibroblasts and long-term lymphoid lines was found to be associated with particulate material in the cell extract. Treatment of lymphoid line extracts with the detergent Triton X-100 led to a progressive reduction in β-glucosidase activity associated with the membrane fraction. The results of one experiment demonstrating this are shown in Fig. 1. Because of the biphasic pH curve in these cells, activities were measured at both pH 4.0 and pH 5.1. At the highest detergent concentration used (0.1%) about 20% of the initial activity at pH 5.1 remained associated with the membrane fraction, while at pH 4.0 membrane-bound activity was virtually absent. As shown in Fig. 1, a proportion of the activity released from the membrane fraction was recovered in the supernatant following Triton X-100 treatment. When washed membrane preparations, rather than complete cell extracts, were used for this experiment, recovery of activity in the supernatant fraction was considerably reduced.

The β-glucosidases from calf spleen [15] and human spleen [16] have been shown to require the bile salt sodium taurocholate, in partially purified form, for optimum activity. Lymphoid line β-glucosidase, solubilized with Triton

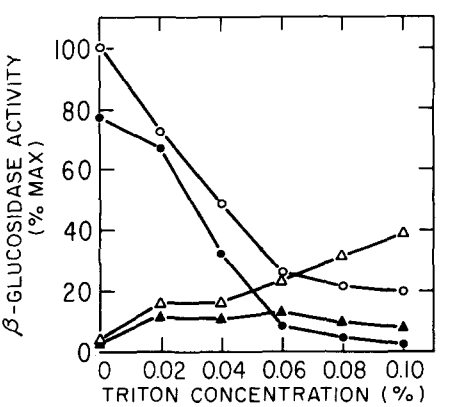

Fig. 1. β-Glucosidase activity (percent maximum) in supernatant (△,▲) and particulate (○,●) fractions following preincubation in the presence of varying concentrations of Triton X-100. Assays were carried out at pH 4.0 (closed symbols) and pH 5.1 (open symbols). Six 1-ml centrifuge tubes were set up, each containing 0.1 ml cell extract (one volume packed cells in three volumes water), 0.05 ml 30% glycerol, varying amounts of Triton X-100 and distilled water to a final volume of 0.3 ml. After 20 min at room temperature the tubes were centrifuged at 37 000 × *g* (4° C) for 30 min. Supernatants were removed and the pellets washed once in distilled water before being resuspended in 0.3 ml distilled water.

X-100, is also stimulated by taurocholate. In a typical experiment, increasing the taurocholate concentration of the reaction mixture from 0 to 0.2% (w/v) caused a progressive increase in β-glucosidase activity from 9 to 120 units. Increasing the taurocholate concentration above 0.2% caused no further increase in activity. The leukocyte and fibroblast enzymes showed a similar requirement for taurocholate following solubilization.

Solubilized β-glucosidases from different cell types had similar K_m values. These were 2.8 mM for the fibroblast enzyme and 4.5 mM for the lymphoid line enzyme. In both cases these values are considerably higher than those obtained with the membrane-bound enzyme (see above). Solubilization also resulted in a shift in pH optimum. When assayed in the presence of Triton X-100 (0.02%) and optimum concentration of taurocholate, lymphoid line, leukocyte and fibroblast β-glucosidases all gave symmetrical pH curves with a single optimum at pH 6.0. Under these assay conditions no cell-specific differences in the pH dependence of the enzyme were detectable.

β-Glucosidases from cultured skin fibroblasts and cultured long-term lymphoid lines differ in pH optimum, K_m, stability at pH 4 and in the extent of activity reduction in patients with Gaucher's disease. These observations can be accounted for by the presence of two distinct forms of β-glucosidase. These are, an "acid-stable" form, which has a pH optimum of 4.5 and is stable at pH 4.0, and an "acid-labile" form, which has a pH optimum of 5.0 and is inactivated at pH 4.0. We suggest that cultured skin fibroblasts contain predominantly the "acid-stable" enzyme, while long-term lymphoid lines and peripheral leukocytes contain both forms. It is of interest that membrane-bound β-glucosidase in human brain, liver, spleen and placenta has properties similar to those of the leukocyte and lymphoid line enzyme and consists of both "acid-stable" and "acid-labile" components (Turner, B.M., unpublished results). Among the tissues examined so far, cultured skin fibroblasts are unique in having only the "acid-stable" enzyme.

The "acid-stable" enzyme is deficient in lymphoid lines, leukocytes and cultured skin fibroblasts from patients with Gaucher's disease while the specific activity of the "acid-labile" enzyme is apparently unaltered. Our results show that the β-glucosidase activity found in leukocytes from patients consists primarily of the "acid-labile" enzyme. In contrast, the β-glucosidase found in skin fibroblasts from patients is stable at pH 4.0 and recent results (Turner, B.M., Beratis, N.G. and Hirschhorn, K., in preparation) suggest that this enzyme represents a genetically altered form of the "acid-stable" β-glucosidase found in normal cells.

The relationship between the two forms of membrane β-glucosidase remains to be established. We have shown that detachment of the enzyme from the membrane with Triton X-100 and assay in the presence of taurocholate, abolishes the cell-specific differences in pH optimum shown by the membrane-bound enzyme. This suggests that either the different properties of the "acid-stable" and "acid-labile" enzymes are dependent on attachment to membrane components, or that, of the two forms, only one is solubilized in an active state. In view of our finding that leukocytes from patients with Gaucher's disease contain predominantly the "acid-labile" form of the enzyme it was of interest to compare the effects of Triton-taurocholate treatment on the β-gluco-

TABLE II

THE EFFECT OF TRITON X-100 AND SODIUM TAUROCHOLATE ON LEUKOCYTE β-GLUCOSIDASE

Untreated samples were assayed in the normal way in the absence of Triton X-100 and Taurocholate.. Treated samples were preincubated for 20 min at room temperature in the presence of Triton X-100 (0.06%) and sodium taurocholate (0.48%). A small aliquot was removed (extract) and the remainder separated into supernatant and membranes by centrifugation for 15 min at 4°C and 10 000 × *g*. All treated samples were assayed in the presence of 0.02% Triton X-100 and 0.17% taurocholate and at pH 6.0. Results are expressed in pmol/min (total activity)

	Untreated activity		Treated activity		
	pH 4.0	pH 5.1	Extract	Membranes	Supernatant
Control	17.5	29.5	114.3	16.9	119.6
Patient	4.9	40.6	38.5	20.6	25.8

sidase activity of Gaucher and control leukocytes. The results of a representative experiment are summarized in Table II. While the total activity of the "acid-labile" enzyme (assayed at pH 5.1) was in fact greater in the patient's cells than in the control, activity of the solubilized enzyme from the patient's cells was only 22% of the control value. Conclusions about the properties of the normal enzyme based on experiments with deficient cells should obviously be regarded as tentative. However, these results indicate that at least in Gaucher cells, the "acid-labile" enzyme is not released from the membrane in an active state. The results shown in Table II probably account for the observation of Klibansky et al. [17] that β-glucosidase in the leukocytes of a patient with Gaucher's disease was deficient over the entire pH range of the enzyme when assayed in the presence of detergents.

Some work has been carried out on the interaction of human β-glucosidase (primarily derived from spleen) with cytoplasmic factors. A "heat-stable factor" from human spleen, probably a glycoprotein, activates human β-glucosidase [18,19] and increases its stability at low pH [20]. Acidic phospholipids enhance the activating effect of this "heat-stable factor" [21]. Most of these experiments were done in the presence of detergents and therefore caution is necessary in postulating an influence of soluble components on the native, membrane-bound enzyme. We have carried out a series of mixing experiments (in the absence detergents) between supernatant and membrane fractions from lymphoid lines and fibroblasts and have detected no significant alterations in the properties of the membrane-bound enzymes. However, despite these negative findings, the possibility remains that the "acid-stable" and "acid-labile" forms of β-glucosidase are the result of in vivo modification of the enzyme by soluble factors. Further studies on the relationship of the two forms are required and long-term lymphoid lines would seem to be a suitable source of enzyme for such investigation.

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