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Gaucher disease: Isolation and comparison of normal and mutant glucocerebrosidase from human spleen tissue

(copurification/immunotitration/heat-stable factor/structural mutation)

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ABSTRACT **Glucocerebrosidase was purified 26,000-fold from spleens from normal humans and from patients with Gaucher disease (Gaucher spleens). The specific activities of the purified normal and mutant enzymes with glucocerebroside as substrate were 8.5×10^5 and 5.4×10^4 nmol/mg of protein per hr, respectively. The ratio of enzymatic activities was constant throughout the isolation procedure. The two enzymes appeared to be similar by other parameters such as substrate affinity, heat lability, and pH optimum. Immunotitration with glucocerebrosidase antiserum showed equivalent quantities of crossreacting material in extracts of normal and Gaucher spleens. These data strongly suggest that the genetic basis of Gaucher disease is a structural mutation of glucocerebrosidase. The results of sodium dodecyl sulfate gel electrophoresis also indicate that there are differences between the normal and the Gaucher disease enzyme.**

The presence of residual glucocerebrosidase activity in tissue specimens from patients with Gaucher disease is well documented. The amount of this activity in tissues such as liver, spleen, cultured fibroblasts, and leukocytes has been reported to range from 5 to 40% of normal (1–4). An essential step for the elucidation of the molecular basis of this inherited lysosomal storage disorder was contingent on a systematic characterization of the residual enzyme. We have undertaken such a study in order to determine whether this metabolic disorder represents a regulatory or a structural mutation. Other investigators have compared properties of normal and Gaucher-disease glucocerebrosidase and have reported differences in K_m, heat lability, or pH activity profile (5, 6, ‡). Because those studies were carried out with crude enzyme extracts, the relevance of the observations is somewhat limited. We report here a systematic comparative characterization of glucocerebrosidase from normal and Gaucher-disease spleens during a 26,000-fold enrichment.

METHODS

Enzyme Assay. Glucocerebrosidase was routinely assayed by incubation with 59 nmol of D-[1-C^{14}]glucocerebroside (21,000 cpm) in 0.2 ml of 75 mM potassium phosphate buffer, pH 5.9/0.12% Cutscum/0.12% sodium taurocholate at 37°. The enzymatically cleaved [1-C^{14}]glucose was subsequently measured as described (7). One unit of enzymatic activity is defined as catalyzing the hydrolysis of 1 nmol of substrate/hr under these conditions. Protein was determined by the method of Lowry *et al.* (8) with human serum albumin as standard.

Enzyme Purification. Normal spleen tissue was collected as autopsy material from trauma victims. The Gaucher spleen was obtained after splenectomy from a 44-year-old woman with type I Gaucher disease. All tissue was stored at −40°.

The procedure outlined below essentially represents a modification of the method developed for the isolation of human placental glucocerebrosidase (9). All procedures were carried out at 4°. The tissue samples were thawed and cut into small pieces; 500-g portions were homogenized in a Waring Blendor with 1.5 liters of 25 mM phosphate buffer (pH 6.5). After centrifugation, the 10,000 × *g* pellet was homogenized with 1.5 liters of 25 mM sodium citrate buffer, pH 7.0/1% sodium cholate. The suspension was centrifuged and the 10,000 × *g* supernatant was adjusted to 30% $(NH_4)_2SO_4$. After another centrifugation, the 10,000 × *g* supernatant was adjusted to pH 5.35 with 1 M citric acid. The pellet was suspended in 600 ml of 25 mM citrate phosphate buffer, pH 5.0/5 mM EDTA/1 mM dithiothreitol. n-Butanol was slowly added with stirring to this suspension to a concentration of 20% followed by centrifugation at 10,000 × *g* for 30 min. The lower aqueous phase (625 ml) was collected and dialyzed against 7 liters of 50% ethylene glycol in 50 mM citrate phosphate buffer, pH 5.0/5 mM EDTA/1 mM dithiothreitol. Ethanol (340 ml) was slowly added to the dialyzed solution (410 ml) and, after centrifugation at 10,000 × *g* for 30 min, an additional 480 ml of ethanol was added to the supernatant. The pellet was collected after centrifugation at 10,000 × *g* for 30 min and suspended in 10 ml of 10% ethylene glycol in 100 mM citrate phosphate buffer, pH 5.0/5 mM EDTA/1 mM dithiothreitol. After standing for approximately 12 hr in an ice bath, the solution was centrifuged at 100,000 × *g* for 1 hr for final clarification. The supernatant was used in the subsequent chromatographic steps.

The sample was applied to a 5.0 × 60 cm Sephadex G-200 column equilibrated with 10% ethylene glycol in 100 mM citrate phosphate buffer, pH 5.0/5 mM EDTA/1 mM dithiothreitol. Peak enzyme fractions eluted from the column were added to a 0.9 × 30 cm decyl-agarose column (Miles Laboratories) equilibrated with the same 10% ethylene glycol buffer. The enzyme was eluted with 400 ml of a linear gradient of ethylene glycol ranging from 40 to 80% in 100 mM citrate phosphate buffer, pH 5.0/5 mM EDTA/1 mM dithiothreitol. Peak enzyme fractions were dialyzed against 20% ethylene glycol in the above buffer and subsequently added to a 0.9 × 30 cm column packed with octyl-Sepharose (Pharmacia) equilibrated with 20% ethylene glycol in 100 mM citrate phosphate buffer, pH 5.0/5 mM EDTA/1 mM dithiothreitol. The column was eluted with a linear gradient of 400 ml of ethylene glycol ranging from 40 to 90% in 100 mM citrate phosphate buffer, pH 5.0/5 mM EDTA/1 mM dithiothreitol.

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Abbreviation: HSF, heat-stable factor.

‡ Turner, B. M. & Hirschhorn, K. (1977) *Abstract, American Society of Human Genetics Meeting*, San Diego, CA, p. 109A.

Table 1. Glucocerebrosidase isolation from 500 g of normal human spleen tissue

Step or fraction	Activity* Total units	Activity* Units/ml	Protein, mg/ml	Specific activity, units/mg protein	Enrichment, fold	Recovery, %
Total tissue homogenate	2.2×10^6	1,000	41.0	24	1.0	100
Detergent homogenate of particulate fraction	1.3×10^6	760	25.0	30	1.2	60
10,000 × *g* supernatant of detergent homogenate	1.5×10^6	940	13.0	72	3.0	68
Butanol extract	7.8×10^5	1,900	2.0	850	39	35
Ethanol precipitate	3.5×10^5	18,000	31.0	5,800	240	16
Gel filtration	2.4×10^5	1,800	0.066	27,000	1,100	11
C_{10} hydrophobic chromatography	1.8×10^5	3,600	0.030	120,000	5,000	8
C_8 hydrophobic chromatography	1.5×10^5	3,900	0.0170	230,000	9,600	7
C_{10} hydrophobic chromatography	4.4×10^4	3,100	0.0052	620,000	26,000	2

* Enzyme assays were carried out with 296 μM glucocerebroside. At optimal substrate concentration, the specific activity of the final preparation is 890,000 units/mg of protein.

Peak enzyme fractions were pooled, dialyzed, and rechromatographed on a decyl-agarose column as before.

RESULTS AND DISCUSSION

Normal and mutant glucocerebrosidase activities copurified in similar fashion (Tables 1 and 2). At no step in the purification procedure was there a consistent difference between the two enzymes with respect to stability, percentage recovery, or resolution. Both activities filtered through G-200 in a molecular weight range of 200,000. The two enzymes were consistently eluted from hydrophobic columns with the ethylene glycol concentration 60–70%.

Sodium dodecyl sulfate gel electrophoresis of the purified enzyme preparations revealed somewhat divergent and heterogeneous protein profiles (Fig. 1). Although purified placental glucocerebrosidase (9) showed a single protein band with a molecular weight of 68,000, normal and Gaucher glucocerebrosidases from spleen each displayed a major protein band with a molecular weight of 66,000 and 57,000, respectively, as well as several minor bands in the range of 60,000–50,000. With the limited amount of protein material available at present, we can only speculate on the nature and significance of these divergent protein patterns. Heterogeneity due to differences in the amount of bound carbohydrate is not unusual for glycoprotein hydrolases such as these (10). If future studies show that essentially all of the proteins in these highly purified preparations represent glucocerebrosidase enzyme, the noted differences of the normal and Gaucher enzyme might shed additional light on the molecular basis of this metabolic disorder.

The ratio of specific activities of normal and Gaucher enzyme remained relatively constant during 26,000-fold enrichment of both activities. This finding implies that the glucocerebrosidase deficiency in this Gaucher disease patient does not result from a depressed synthesis of normal enzyme. The data favor the alternate mode of abnormal genetic expression—the formation of a structurally altered and catalytically deficient glucocerebrosidase protein—because the specific activity of the highly purified Gaucher enzyme was only 6% that of the equally purified normal enzyme. However, comparison of the relative specific activities of the two enzymes solely on a protein basis is tenuous at this time because the absolute purity of the final enzyme preparations can be questioned due to their heterogeneous protein profiles on electrophoresis. An additional basis for comparing the relative catalytic activities of the two enzymes was obtained by observing the similarity of the immunoprecipitation curves of the normal and Gaucher enzymes with anti-glucocerebrosidase serum (Fig. 2). If one assumes antigenic equivalency, the amount of protein associated with 16 units of Gaucher glucocerebrosidase was essentially equivalent to the protein representing 300 units of normal enzyme.

Several kinetic parameters of the two enzyme preparations were evaluated. The Michaelis–Menton constants, pH optima, and heat labilities of normal and Gaucher enzyme were similar (Table 3). The most striking differences were observed in the relative specific activities of the enzymes. With both natural and synthetic substrates, normal glucocerebrosidase was found to be approximately 16 times more active than the mutant enzyme.

Table 2. Glucocerebrosidase isolation from 500 g of Gaucher spleen tissue

Step or fraction	Activity* Total units	Activity* Units/ml	Protein, mg/ml	Specific activity, units/mg protein	Enrichment, fold	Recovery, %
Total tissue homogenate	117×10^3	53	39	1.4	1.0	100
Detergent homogenate of particulate fraction	71×10^3	36	21	1.7	1.2	61
10,000 × *g* supernatant of detergent homogenate	79×10^3	45	17	2.6	1.9	68
Butanol extract	64×10^3	148	2.3	64	46	55
Ethanol precipitate	42×10^3	2100	6.5	320	230	36
Gel filtration	25×10^3	160	0.120	1,300	930	21
C_{10} hydrophobic chromatography	16×10^3	290	0.043	6,700	4,800	14
C_8 hydrophobic chromatography	9.0×10^3	204	0.0095	21,000	15,000	8
C_{10} hydrophobic chromatography	2.3×10^3	160	0.0042	38,000	27,000	2

* Enzyme assays were carried out with 296 μM glucocerebroside. At optimal substrate concentration, the specific activity of the final preparation is 57,000 units/mg of protein.

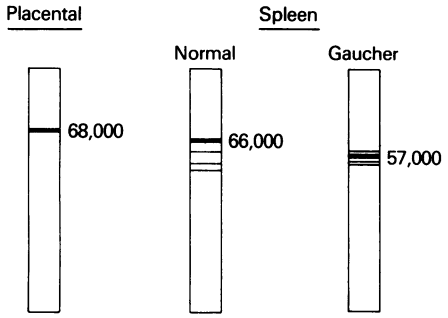

FIG. 1. Diagram of protein patterns of purified glucocerebrosidases in sodium dodecyl sulfate/polyacrylamide disc gel electrophoresis carried out according to Laemmli (13). Protein (40 μg) was treated with 1% sodium dodecyl sulfate and 10% 2-mercaptoethanol at 100° for 5 min prior to electrophoresis. The specific activities of the normal placental and spleen enzymes were 700,000 and 620,000 units/mg of protein, respectively. The specific activity of Gaucher glucocerebrosidase was 38,000 units/mg of protein. Standard marker proteins were used to calibrate the gels according to molecular weight. The thickness of the bands is indicative of the relative staining of the various protein bands with Coomassie blue.

Gaucher liver and spleen extracts have been shown to contain a heat-stable factor (HSF) that has the capacity to stimulate the activity of normal glucocerebrosidase (11, 12). Consequently, the influence of HSF on the stability and catalytic activity of the two enzymes was investigated. HSF was observed to extend the half-lives of both enzymes at 50° by a factor of 2–3. When an equal number of units of normal and Gaucher enzyme were assayed with increasing concentrations of HSF, maximal stimulation was reached at similar concentrations of the factor. On the other hand, the maximal stimulation achieved with normal enzyme was 5 times higher than that reached with Gaucher enzyme (Fig. 3). Consequently, HSF stimulation of catalytic activity appears to be considerably less efficient with the mutant enzyme. The potential physiological significance that can be derived from these *in vitro* observations remains unclear at present; however, previous studies have suggested that HSF probably does not normally play a significant role in

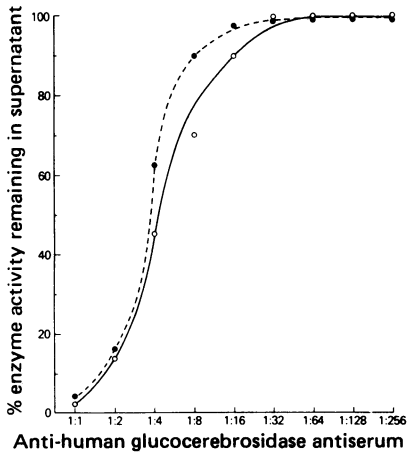

FIG. 2. Immunoprecipitation of human spleen glucocerebrosidase. Purified human placental glucocerebrosidase (specific activity, 1×10^6 units/mg of protein) was mixed with complete Freund's adjuvant and injected subcutaneously into a rabbit at a dose of 100 μg of protein per kg. A booster dose of the same enzyme and adjuvant mixture was given 3 weeks later. Blood was drawn and serum was prepared at 2-week intervals after the booster dose. Enzyme at the ethanol-precipitated stage (Tables 1 and 2) was used in this study. Enzyme protein (50 μg) from normal (●--●; 300 units) and Gaucher (○--○; 16 units) spleens was incubated in 0.30 ml of 15 mm NaCl/10 mM phosphate buffer, pH 7.0, containing 0–0.10 ml of either pre- or postimmunized rabbit serum. After incubation in an ice bath for 12 hr, the cloudy suspensions were centrifuged at 49,000 × *g* for 1 hr and the supernatant was assayed for remaining glucocerebrosidase activity.

Table 3. Kinetic properties of purified human spleen glucocerebrosidase

Parameter	Enzyme	
	Normal	Gaucher
K_m, M × 10^{-5}		
Glucocerebroside	10.9 ± 0.9	12.6 ± 0.6
4-Methylumbelliferyl-β-D-glucopyranoside	89 ± 6	178 ± 45
Maximum velocity, nmol/mg protein/hr		
Glucocerebroside	8.5×10^5	5.4×10^4
4-Methylumbelliferyl-β-D-glucopyranoside	4.2×10^5	3.1×10^4
pH optimum with glucocerebroside	5.0 – 6.0	5.0 – 6.0
Heat inactivation at 50° ($t_{1/2}$, min)		
pH 5.0	90	87
pH 6.0*	11 (31)	14 (30)
pH 7.0*	0.7 (1.8)	0.90 (1.8)

Assays with glucocerebroside were carried out as described in *Methods*. When the fluorogenic substrate was used, assays were carried out in 25 mM citrate phosphate buffer, pH 5.0/0.12% sodium taurocholate. The pH activity profiles with natural substrate were determined under normal assay conditions except that the buffer consisted of 40 mM citrate phosphate in the pH range 3–8. For the heat lability studies, aliquots of purified enzyme were incubated at 50° in 0.175 ml of 50 mM citrate phosphate buffer of the indicated pH containing 0.25 mg of Cutscum. At specified time intervals, tubes were transferred to an ice bath and adjusted to pH 6.0. Aliquots were subsequently assayed with the natural substrate in the usual manner. Heat stable factor (HSF) was prepared by heating a 40% homogenate of Gaucher spleen in H_2O at 100° for 3 min (11). The clear supernatant obtained after centrifugation at 49,000 × *g* for 30 min contained 9.5 mg of protein per ml and served as the source of HSF for all the studies; when used in the heat inactivation studies, 25 μl of the solution was included in the initial incubation at 50°. K_m data shown as mean ± SD.

* Heat-stable factor (HSF) was added to the incubations.

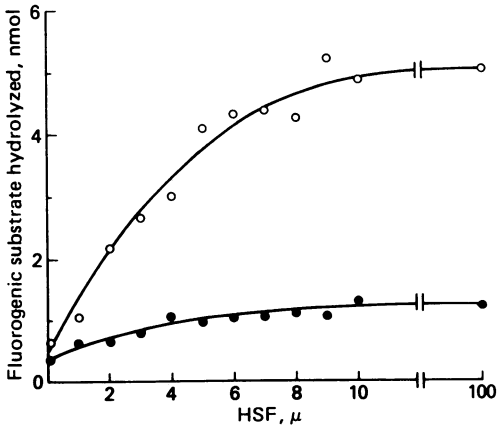

FIG. 3. Activation of normal and Gaucher glucocerebrosidase with HSF. HSF was prepared as described in Table 3. The assays consisted of varying aliquots of HSF (9.5 mg of protein per ml) in 0.15 ml of 25 mM citrate phosphate buffer (pH 5.0) containing 1.2 mM 4-methyl-umbelliferyl-β-D-glucopyranoside and 0.01% Triton X-100. Equal activities (28 units) of purified normal and Gaucher glucocerebrosidase were incubated in these solutions for 1½ hr at 37°. The reactions were stopped by the addition of base, and the fluorescence was determined as described (14). ○, Normal enzyme; ●, Gaucher enzyme.

the metabolism of glucocerebroside (12, 14). It now appears as if the significance of HSF in influencing the catabolism of glucocerebroside in Gaucher tissues might also have to be questioned.

In conclusion, the present report strongly suggests that the underlying molecular mechanism of type I Gaucher disease involves a structurally altered and catalytically deficient glucocerebrosidase enzyme.

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