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Gaucher's Disease: Deficiency of 'Acid' β-Glucosidase and Reconstitution of Enzyme Activity *In Vitro*

(spleen/components of enzyme)

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ABSTRACT **The spleen from a patient with adult Gaucher's disease was shown to be deficient in a β-glucosidase (EC 3.2.1.21) isoenzyme that has optimal activity at pH 4.0–4.3, and is stimulated by 0.02% Triton X-100. A mixture of spleen homogenates from a control and from the patient contained β-glucosidase activity equivalent to 2–3 times the theoretical expected activity. The increase in enzyme activity occurred at pH 4.0–4.3; the magnitude of the increase was proportional to the amount of each homogenate added. Two factors, one called factor P from the patient's spleen, the other called factor C from the control spleen, were responsible for a reconstitution of β-glucosidase activity *in vitro*. Factor P is tentatively identified as an acid glycoprotein.**

Leucocytes (1) and skin fibroblasts (2) have a specific deficiency of an 'acid' β-glucosidase (EC 3.2.1.21), with a pH optimum of 4.0, in adult Gaucher's disease. In skin fibroblasts (2), the enzyme was markedly stimulated by Triton X-100 at pH 4.05. Subsequently, we began to characterize the enzyme from spleen and found an unexpected increase in β-glucosidase activity when spleen homogenates from the control and the patient were mixed. Two factors appear to be responsible for the reconstitution of β-glucosidase activity. We call them factor C from control spleen and factor P from the patient's spleen. These factors are isolated in crude and partially purified form, respectively. The mechanism of the reconstitution of β-glucosidase activity from these factors is investigated. Factor P is tentatively identified as an acid glycoprotein.

MATERIALS AND METHODS

The patient is a 12-year-old girl who has chronic noncerebral (adult) Gaucher's disease, who was splenectomized to ameliorate hemolytic anemia.

Spleens were obtained at autopsy (controls) or after splenectomy (patient) and stored at −20°C until use.

β-Glucosidase (β-D-glucoside glucohydrolase) was assayed (2) with 4-methylumbelliferyl-β-D-glucopyranoside (Koch Light, England) as substrate. 1 unit of enzyme activity represents 1 nmol of substrate cleaved per mg of protein per hr.

Gel filtration was performed on Sephadex G-50, prepared as recommended by the manufacturer (Pharmacia), in 5 mM sodium phosphate (pH 7.0)–5 mM NaCl. Standardization of the column was done with Blue Dextran (Pharmacia) (A_{620nm}); bovine-serum albumin fraction V (Sigma) (A_{280nm}); cytochrome *c* (Sigma) (A_{412nm}); and sucrose [orcinol reagent (3)].

Ultrafiltration was done as described (4).

Polyacrylamide gel electrophoresis was in a discontinuous Tris (pH 9.0)–Tris·glycine (pH 8.5) buffer recommended by the manufacturer (Canalco), with 5–7% acrylamide gels. Running time was 1.25 hr at 4°C, with a current of 2 mA per tube. Gels were then fixed in 10% Cl_3CCOOH and stained for proteins with Amido Black (0.05%) or Coomassie Blue (0.024%) in 10% acetic acid. Destaining was performed overnight in the same solvent.

Cellulose acetate strip electrophoresis was done on cellulose acetate strips (Buchler) in 0.04 M sodium phosphate buffer (pH 7.0). Running time was 1.25 hr at 300 V at 4°C. After electrophoresis, the strip was cut into 5-mm sections and assayed for β-glucosidase activity.

Partial Purification of Factor P. A 12% (w/v) homogenate of the patient's spleen in distilled water was centrifuged for 45 min at 100,000 × *g*. The supernatant was heated in 100°C for 3 min, adjusted to pH 4–5 with citrate–phosphate buffer (0.01 M, final concentration) and centrifuged again (1,000 × *g*, 15 min). The supernatant was concentrated to 20% of the original volume by ultrafiltration, adjusted to pH 7.0 with dibasic sodium phosphate (final concentration of phosphate not exceeding 0.02 M), and digested with 1 mg/ml of Pronase (B grade, Calbiochem) for 4 hr at 50°C. The mixture was precipitated with 9 volumes of ethanol at 0°C and redissolved in 0.01 M phosphate buffer (pH 7.0)–0.01 M NaCl. 5 μl of α-Amylase (Type 1-A from hog pancreas, in a suspension containing about 20,000 U/ml, Sigma) was added per milliliter and digestion proceeded for 2 hr at 37°C. The mixture was precipitated with 9 volumes of ethanol at 0°C, redissolved in 0.025 M citrate–phosphate buffer (pH 5.0), and digested with 0.5 mg/ml of hyaluronidase (Type IV from bovine testes, Sigma) for 3 hr at 37°C. Cl_3CCOOH (10%) was added slowly with constant mixing at 0°C to a final concentration of 2%. The supernatant was dialysed against distilled water for 16 hr at 4°C. Protein that precipitated during dialysis was discarded. The total recovery of factor P activity was about 85%. The overall increase in specific activity (units/mg of protein) was about 100-fold. 1 unit of factor P activity is defined as an increase of 1 unit of enzyme activity in the presence of the experimentally determined excess of factor C, prepared as described below.

Crude Preparation of Factor C. Factor C was prepared by centrifugation of a 12% (w/v) homogenate of the control spleen in distilled water at 100,000 × *g* for 45 min. The residue obtained was resuspended in water and adjusted to pH 4–5

with citrate–phosphate buffer (0.01 M final concentration). The suspension was centrifuged 15 min at 1000 × *g* and the supernatant was discarded. The precipitate was washed once with distilled water and resuspended in distilled water. Factor C activity was assayed by measuring the increase in β-glucosidase activity produced in a mixture with an experimentally determined excess of purified factor P.

Thin layer chromatography was on plates coated with silica gel (Merck) with the solvent described by Gal (5). Hexoses and hexosamines were visualized with 2-aminobiphenyl reagent (6).

Total hexoses were determined on aliquots of purified factor P by the Winzler orcinol–sulfuric acid method (3).

Sialic acid content was determined on aliquots of purified factor P by the resorcinol method (7).

Hexosamine content was determined on a hydrolyzed sample of factor P (3 N HCl for 3 hr at 100°C) according to Rondle and Morgan (8) with the modification of Boas (9).

RESULTS AND DISCUSSION

Deficiency of acid β-glucosidase in spleen

The patient's spleen was 5- to 10-fold deficient in total β-glucosidase activity as compared to controls. The deficiency was most pronounced when activity was assayed between pH 4.0–4.3. At this pH, Triton X-100 (0.02%) stimulated β-glucosidase activity in control spleens about 40%, whereas no stimulation occurred in the patient's spleen. These results are in agreement with those obtained in skin fibroblasts (2). An acid β-glucosidase (optimum about pH 4.0, 100% stimulated by Triton) was isolated by gel filtration of supernatants from control spleens (Fig. 1, peak I of control). The very small amount of enzyme activity eluted at about the same position from the spleen supernatant prepared from the patient (Fig. 1) was quite different. It had optimal activity at pH 5.0 and was not stimulated by Triton X-100. Peak II from both types of spleen (Fig. 1) represents a 'neutral' β-glucosidase (pH optimum of 5.0–7.0) that was unaffected in Gaucher's disease.

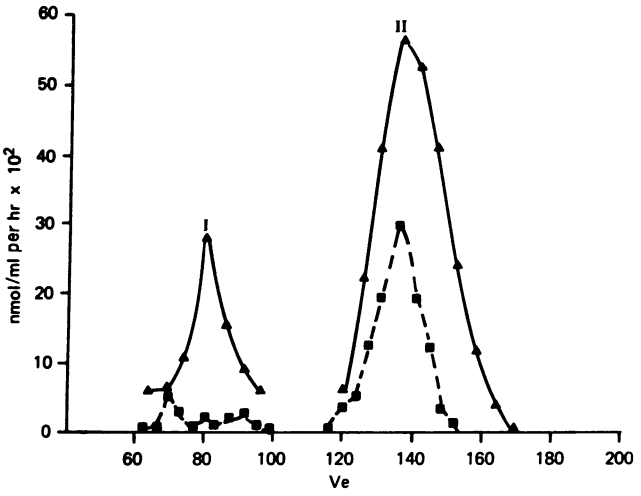

FIG. 1. Sephadex gel filtration of β-glucosidase. ▲——▲, control spleen; ■——■, patient's spleen. The 100,000 × *g* supernatant of a 14% homogenate of the spleen was applied to the column. β-Glucosidase activity in each 3-ml fraction was assayed at pH 5.0.

Increase in β-glucosidase activity in mixed homogenates

When spleen homogenates from the patient and controls were mixed, there was an unexpected increase (2–3 times more than the theoretical activity expected) in β-glucosidase activity. The increase in activity occurred maximally at pH 4.0; little or no increase was evident at higher or lower pH values. The increase in β-glucosidase activity itself, when homogenates were mixed, and its dependence on pH were observed both in the presence and in the absence of Triton X-100.

The apparent restoration in β-glucosidase activity after mixing was dependent upon the concentration of both control- and patient-spleen homogenates, which suggested the possibility of an association reaction between at least two independent molecules in the two tissues. After the homogenates were mixed, a progressive increase was observed in the stimulation of activity by Triton X-100 at pH 4.05, indicating that the β-glucosidase activity that was restored by mixing of the homogenates was Triton-stimulated.

Nature of factor P

The nature of the factor in the patient's spleen that was responsible for the restoration of β-glucosidase activity (factor P) was investigated. This factor was readily soluble in water in the absence of detergent. It was not dialyzable, and Sephadex gel filtration indicated an apparent average molecular weight of 20,000. Since factor P appears to be a glycoprotein (see below), gel filtration data for molecular weight may be unreliable (10). Factor P was readily precipitated without loss of activity from cold acetone or ethanol. Extraction of a purified preparation of this factor in water with an equal volume of chloroform–methanol 2:1 at 0°C caused no loss of activity. However, factor P was destroyed irreversibly by overnight lyophilization. Nonetheless, it was remarkably thermostable, and was not destroyed by heating for 3 min at 100°C, nor was its activity destroyed after incubation with Pronase (1 mg/ml) for 10 hr at 50°C or with trypsin (0.2 mg/ml) for 4 hr at 37°C. After digestion with Pronase (1 mg/ml) for 4 days, about 20% of the activity of factor P was destroyed as compared to aliquots of the same sample that were incubated under identical conditions without Pronase.

Partially purified factor P was estimated to be at least 80% pure by staining after polyacrylamide gel electrophoresis. The major band in the preparation migrated just behind the marker dye, and possessed factor P activity as determined by sectioning the gel after electrophoresis and incubation of the sections directly with factor C. The purified preparation of factor P had a specific activity of 80 units (see *Methods*), an almost hundred-fold increase over that of the spleen homogenate from the patient.

Partially purified factor P contained significant amounts of galactose, mannose, and hexosamine after acid hydrolysis. Before hydrolysis, factor P gave a positive resorcinol reaction, indicative of the presence of sialic acid. Quantitative analysis gave a percent dry weight of hexosamine, hexose, and sialic acid of 5.5, 11.42, and 9.63%, respectively, which corresponds roughly to molar ratios of 1:2:1. Judging from a thin-layer chromatogram of hydrolysates of factor P, the amount of galactose was about equal to mannose. Determination of protein by the Lowry reaction (11) gave a value of 52.7% of the dry weight. Factor P is thus tentatively identified as an acid glycoprotein.

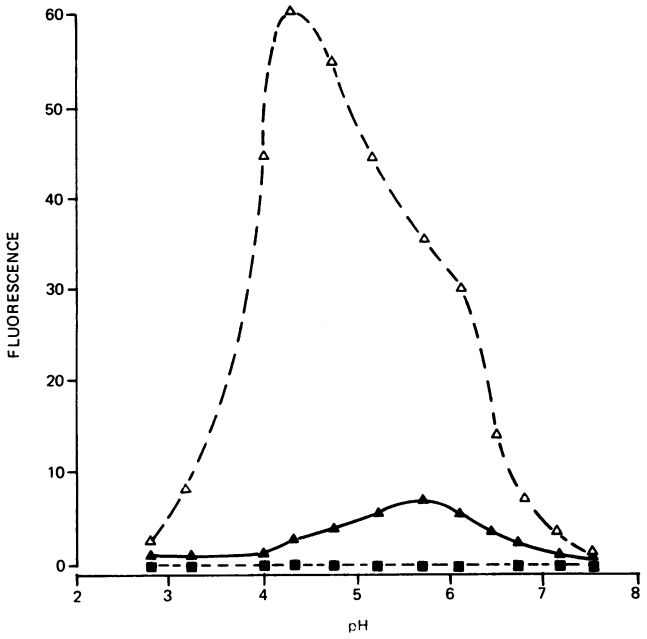

FIG. 2. Reconstitution of β-glucosidase. Activities at various pH values were determined after incubation for 30 min at 37°C in 0.02 M citrate–phosphate buffer (pH 4.05) with 0.02% Triton X-100. ■——■, factor P; ▲——▲, factor C; Δ——Δ, both factors.

Periodate oxidation [5 mM sodium metaperiodate in 10 mM sodium phosphate buffer (pH 7.0) for 15 min at room temperature] destroyed over 60% of the factor P activity, suggesting that the carbohydrate portion of the molecule is essential for biologic activity.

Nature of factor C

The factor present in control spleens (factor C) was only partially soluble after homogenization in water or dilute buffer. When the homogenate was centrifuged (100,000 × *g*, 45 min), 80–90% of the β-glucosidase activity remained in the supernatant, whereas nearly all of factor C was sedimented. Triton X-100 (0.1%) released most of the factor from the sediment, but the resultant preparation was much less potent. This result suggests that factor C is normally bound to lipoprotein membrane fragments, and that forces tending to disrupt this binding also inactivate factor C. Triton-solubilized factor C was retained by an ultrafiltration membrane (UM2, Amicon); this factor was eluted at about the same position as peak I β-glucosidase after gel filtration. Factor C was completely destroyed after 3 min at 100°C.

The crude preparation of factor C had a β-glucosidase activity equivalent to 2% of the total activity in the original homogenate. The small amount of β-glucosidase activity associated with it had a pH optimum of 5.0–6.5 (see Fig. 2). The relationship between this residual β-glucosidase activity and factor C is unknown. However, this preparation was highly potent, and its activity of factor C remained stable for at least one week at 4°C.

Assay for factors P and C activity

By means of serial dilutions of concentrated preparations of factors C and P, it was possible to arrive at concentrations of one factor that were 'saturating' (in excess) for a certain concentration of the other factor. This provided a crude basis for the assay of the activity of each factor. Thus, the activity of factor P is defined in terms of the increase in the units of β-glucosidase activity produced when it is assayed in the presence of an excess of factor C, and vice versa.

Reconstitution of 'acid' β-glucosidase activity *in vitro*

Purified factor P, having no β-glucosidase activity, was mixed with a crude preparation of factor C, which had very slight β-glucosidase activity. The mixture was incubated at 37°C for 30 min in 0.02 M citrate–phosphate buffer (pH

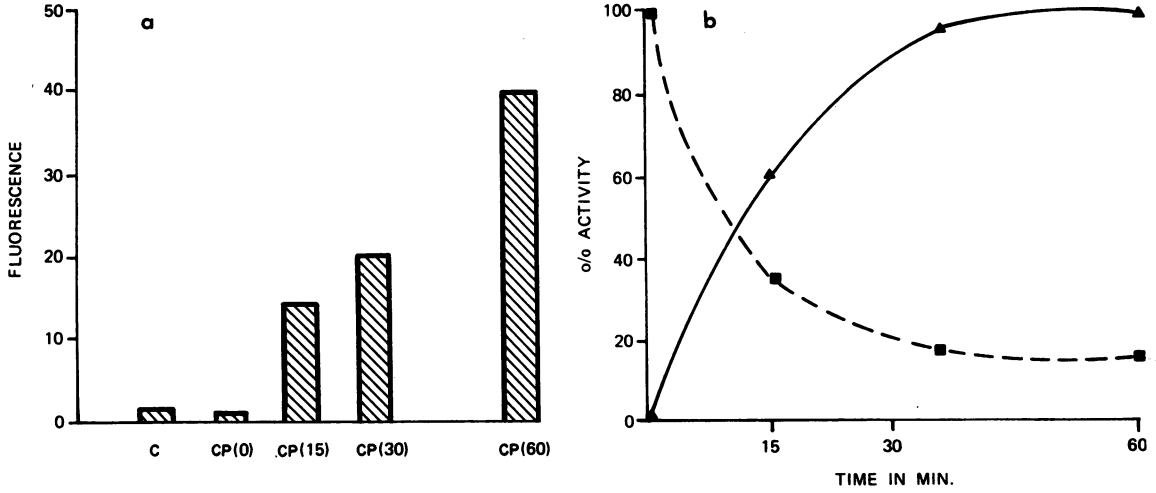

FIG. 3. (*a*) Generation of β-glucosidase measured as a function of the time of incubation at 37°C as in Fig 2. At the end of the incubation, the mixture was electrophoresed on cellulose acetate, sectioned, and assayed for β-glucosidase activity. The fluorescence represents the sum of activity in sections ±1 cm of the origin. C=factor C, CP = mixture of factors C and P; times of incubation (in min) in parentheses.

(*b*) Disappearance of factor P from the supernatant. A mixture of factors was incubated for various periods of time as in (*a*). The supernatant was assayed for factor P activity with excess factor C. The sediment was assayed for β-glucosidase activity. ■—■, factor P disappearance from supernatant; 100% represents the activity of the starting factor P sample. ▲—▲, appearance of β-glucosidase in the sediment; 100% represents the activity at 60 min.

4.05) containing 0.02% Triton X-100. Aliquots of the factor preparations were also incubated separately under identical conditions. At the end of the incubation, the activity of the β-glucosidase in each sample was examined at various pH values (Fig. 2). As can be seen, the β-glucosidase activity that was reconstituted in the mixture of factors had a pH optimum of 4.0–4.3. The β-glucosidase activity in the mixture measured at this pH was 40–50 times that of factor C.

No reconstitution occurred when factor P was incubated with a preparation from the patient's spleen that was made in the same way as factor C was prepared from control spleens. This demonstrated the absence of 'factor C activity' in the patient's spleen.

Mechanism of reconstitution of 'acid' β-glucosidase activity

Did the factors associate to form active enzyme molecules, or did one factor convert the other from an inactive to an active enzymic form?

To explore these possibilities, purified factor P was mixed with factor C, and the mixture was incubated for different times at 37°C in 0.02 M citrate–phosphate buffer (pH 4.05) containing 0.02% Triton X-100. At the end of each incubation period, aliquots of the mixture were applied to cellulose-acetate strips and electrophoresed. The strips were then blotted dry and sectioned. β-Glucosidase activity migrates within ±1 cm of the origin and factor P migrates rapidly toward the anode, widely separated from β-glucosidase activity. A time-dependent generation of β-glucosidase activity was seen (Fig. 3*a*), the longer the two factors were incubated together at 37°C, the greater was the resultant β-glucosidase activity. In the sample containing both factors mixed and kept at 0°C before electrophoresis [PC(0), Fig. 3*a*], β-glucosidase activity was not increased.

This experiment suggested that a time- and heat-dependent reaction between factors C and P took place in order to generate β-glucosidase activity. No permanent association of the factors to produce active enzyme molecules took place at 0°C.

To determine the nature of the reaction between the factors, the fate of factor P was followed after it was mixed with factor C in buffer and incubated at 37°C. Under the conditions used for reconstitution of β-glucosidase activity, the reconstituted enzyme sedimented completely after centrifugation of the mixture at 1,000 × *g* for 15 min, but factor P remained in the supernatant. If factor P is incorporated in some form into the enzyme its disappearance from the supernatant should be evident. This turned out to be the case. β-Glucosidase activity in the sediment was highest between 36–60 min. The disappearance of factor P from the supernatant coincided quantitatively and temporally with the appearance of β-glucosidase activity in the sediment (Fig. 3*b*).

About 25% of the factor P activity calculated to be present in the sediment with the reconstituted β-glucosidase activity could be solubilized by incubation at 37°C for 30–60 min with 0.1% Triton X-100. The recovery of factor P coincided with the amount of β-glucosidase activity that was lost from the sediment (about 30%). Incubation of the sediment with water or dilute buffers alone did not release factor P activity into the supernatant for up to 2 hr of incubation at 37°C and, concomitantly, little or no β-glucosidase activity was lost from the sediment.

The physiologic significance of the enzymic reconstitution reaction

Does the reconstitution of β-glucosidase activity observed *in vitro* have any significance *in vivo?* First of all, we attempted to ascertain whether factor P is a constituent of normal human spleen.

Factor P activity was present in a spleen preparation from a control prepared identically as was factor P from the patient except that the enzymic digestion steps were omitted. The preparation from the control spleen reacted with a factor C preparation in a manner identical to that of factor P from the diseased patient's spleen.

The activity of factor P in control spleens is about 6–10% of that in the patient's spleen. Electrophoretic and other properties must be examined before factor P from the two sources can be said to be identical.

One interpretation of our results is that adult Gaucher's disease may involve the deficiency of factor C, which is thermolabile and particle-bound, and which is involved in the formation of 'acid' β-glucosidase in association with another protein, factor P. The presence of factor P in the control spleen may indicate that a reaction between the two factors occurs under normal physiological conditions. It will be necessary to determine whether the restored β-glucosidase, which possesses activity for synthetic substrates, is also active against glucocerebroside, the glycolipid that accumulates in Gaucher's disease.

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