Short Communication

Glucocerebrosidase: Reconstitution of Activity from Macromolecular Components

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Glucocerebrosidase activity was reconstituted *in vitro* from a soluble glycoprotein factor (P) and a particle-bound factor (C). The physiological significance of the system is discussed.

Acid β -glucosidase (EC 3.2.1.21), which is specifically deficient in adult Gaucher's disease (Beutler & Kuhl, 1970; Ho *et al.*, 1972), was reconstituted *in vitro* from two macromolecular factors, P and C (Ho & O'Brien, 1971). Factor P, a glycoprotein fraction isolated from the spleen of a patient with adult Gaucher's disease, was soluble, extremely heat-stable and devoid of β -glucosidase activity. Factor C, a crude particulate preparation from normal human spleen, was heat-labile and insoluble, and was associated with minimal amounts of β -glucosidase activity. When incubated together under appropriate conditions, the two factors appeared to associate to give a remarkable increase in β -glucosidase activity (up to 40–50 times that in the factor C preparation). Although factor P accumulated in adult Gaucher's disease (10–15 times normal), factor C activity was barely detectable in the patient's spleen (Ho & O'Brien, 1971).

Since Gaucher's disease is characterized by the accumulation of glucocerebroside, it was decided to test whether glucocerebrosidase could be reconstituted from factors P and C, and thus assess the physiological significance of the system in the catabolism of glucocerebroside.

Experimental

Materials. The following materials were from commercial sources as indicated: Triton X-100 and Pronase (B grade) from Calbiochem, Los Angeles, Calif., U.S.A.; 4-methylumbelliferyl β -D-glucopyranoside from Koch-Light Laboratories Ltd., Coln-

brook, Bucks., U.K.; silica-gel precoated plates from E. Merck A.-G., Darmstadt, Germany; α -amylase (type 1A from hog pancreas) and hyaluronidase (type IV from bovine testis) from Sigma Chemical Co., St. Louis, Mo., U.S.A.; [14 C]stearic acid from New England Nuclear Corp., Boston, Mass., U.S.A.

Methods. Factors P and C were prepared as previously described (Ho & O'Brien, 1971). A brief description is given below.

Factor P was isolated from the spleen obtained on splenectomy from a patient with adult Gaucher's disease. A 12% (w/v) homogenate of the spleen in distilled water was centrifuged (100000g for 45 min) to obtain a clear supernatant. This was heated for 3 min at 100°C, adjusted to pH 5 and centrifuged (1000g for 15 min). The supernatant was concentrated by vacuum dialysis, then treated in sequence with Pronase (1 mg/ml), α -amylase (100 units/ml) and hyaluronidase (0.5 mg/ml). Trichloroacetic acid was added in the cold to a final concentration of 2% (w/v), and the supernatant was dialysed against distilled water overnight. Protein precipitated during dialysis was centrifuged off. The overall purification was 100-fold.

Factor C was a crude particulate preparation from normal human spleen obtained at autopsy. A 12% (w/v) homogenate of the spleen in distilled water was centrifuged (100000g for 45 min) to obtain a particulate fraction. This was resuspended in distilled water, adjusted to pH 5 and centrifuged (1000g for 15 min). The residue was washed several times and resuspended in distilled water.

Assay of 4-methylumbelliferyl β -glucosidase (Ho *et al.*, 1972) was modified as follows. To 20 μ l of enzyme solution was added 20 μ l of 0.1 M-sodium acetate buffer, pH 4.5, containing 0.1% (v/v) of Triton X-100 and 4 mM-4-methylumbelliferyl β -D-glucopyranoside. This modification was made to allow both

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4-methylumbelliferyl β -glucosidase and glucocerebrosidase to be assayed in the same buffer system.

Glucocerebrosidase was assayed with glucocerebroside *N*-acylated with [14 C]stearic acid (Kopaczynk & Radin, 1965) and diluted with non-radioactive glucocerebroside from the spleen of a patient with Gaucher's disease to a specific radioactivity of 5000 c.p.m./nmol. A 6.8 nmol portion of glucocerebroside was transferred to a small test tube, evaporated to dryness in a stream of N_2 and re-dissolved in 20 μ l of 0.1 M-sodium acetate buffer, pH 4.5, containing 0.1% of Triton X-100. Enzyme was added, and the final volume of the incubation mixture was adjusted to 40 μ l. The test tube was sealed with Parafilm and incubated for 30 min or 60 min at 37°C. The reaction was stopped by the addition of 50 μ l of water followed by 0.5 ml of chloroform-methanol (2:1, v/v). The phases were clarified by centrifugation (120g for 2 min). The upper phase was aspirated off and the lower phase was washed once with a theoretical upper phase (Folch *et al.*, 1957). The washed lower phase was evaporated to dryness in a stream of N_2 . The solids

were extracted with chloroform-methanol (2:1, v/v) and applied in 13 mm streaks to silica-gel precoated plates. Ceramide (8 μ g) was applied as marker in some lanes. The plate was developed in chloroform-methanol-acetic acid (45:1:4, by vol.) for 15 min at room temperature and air-dried, and the marker was located by using an iodine chamber. The sections corresponding to ceramide and glucocerebroside were scraped off individually and transferred to a scintillation vial. This was mixed with 0.4 ml of water and 10 ml of 1,4-dioxan [containing 5 g of 2,5-diphenyloxazole (PPO) and 100 g of naphthalene/l] and its radioactivity was counted in a Beckman LS-250 scintillation counter. The counting efficiency was 90–95%. The recovery of radioactivity in ceramide and glucocerebroside was 100%. The non-enzymic (blank) hydrolysis was about 0.01 nmol/h.

Results

As was previously demonstrated for 4-methylumbelliferyl β -glucosidase (Ho *et al.*, 1972), a loss of specific activity of glucocerebrosidase occurred

Fig. 1. Reconstitution of glucocerebrosidase and 4-methylumbelliferyl β -glucosidase activities as a function of the concentrations of factors P and C

The concentration of one factor was held constant while that of the other factor was varied. Both enzyme activities were assayed as described in the text under 'Methods'; 4-methylumbelliferyl β -glucosidase activity has been decreased to one-half of the true value for better fit into the diagram. The concentration of each factor is expressed as μ g of protein used in each assay. ●, Glucocerebrosidase; ○, 4-methylumbelliferyl β -glucosidase. (a) Factor P varied, factor C constant at 64 μ g of protein; (b) factor C varied, factor P constant at 5 μ g of protein. Each point represents the average of two determinations, which agreed within 5%.

Table 1. *Reconstitution of glucocerebrosidase and 4-methylumbelliferyl β -glucosidase activities*

Each sample was assayed for both glucocerebrosidase and 4-methylumbelliferyl β -glucosidase activities as described in the text under 'Methods'. In the mixture of factors in this experiment, factor P was in excess. Enzyme activity is expressed as nmol of substrate cleaved/h per mg of protein. Each value represents the average of four determinations, which agreed within 5%.

Sample	Glucocerebrosidase activity (A)	4-Methylumbelliferyl β -glucosidase activity (B)	B/A ratio
Factor C	0.22	2.05	9.4
Factor P	0	0	0
Factors P+C	8.50	15.25	1.8

on dilution of the normal spleen homogenate. High-speed centrifugation (100000g for 45min) gave a clear supernatant and a particulate fraction each with about 10% of the total glucocerebrosidase activity that was in the original homogenate. A similar but less marked decrease in 4-methylumbelliferyl β -glucosidase activity was also observed (40% of the total activity in the supernatant, 10% in the particulate fraction). These results suggested that glucocerebrosidase activity, like part of the 4-methylumbelliferyl β -glucosidase activity, depended on the interaction between a soluble factor and an insoluble factor.

The reconstitution of enzyme activity was demonstrated by mixing factors P and C and assaying for both glucocerebrosidase and 4-methylumbelliferyl β -glucosidase activities. The amount of 'reconstituted' enzyme activity (activity in the mixture of factors P and C) varies as a function of the concentration of both factors. Where the concentration of one factor was held constant at a fairly high value, variation in the concentration of the other factor gave a typical sigmoid increase in reconstituted activity (Figs. 1a and 1b). This strongly suggested an association of the factors to produce enzyme activity. As in the reconstitution of acid β -glucosidase, preincubation of factor P with factor C in the absence of substrate led to the disappearance of factor P from the supernatant coinciding with the appearance of enzyme activity in the residue (Ho & O'Brien, 1971).

In the present buffer system used, maximal reconstitution of both glucocerebrosidase and 4-methylumbelliferyl β -glucosidase activities occurred at pH 4.5. The 4-methylumbelliferyl β -glucosidase/glucocerebrosidase activity ratio was markedly in favour of glucocerebrosidase in the reconstituted enzyme. One experiment is summarized to illustrate this point (Table 1). The 4-methylumbelliferyl β -glucosidase/glucocerebrosidase activity ratio in factor C was 9.4, whereas in the mixture of factors P and C it was decreased to 1.8. 4-Methylumbelliferyl β -glucosidase was stimulated 7.4-fold in the presence

of factor P, whereas glucocerebrosidase was stimulated 39-fold.

Discussion

The literature abounds with reports of disparity between enzyme activities measured with synthetic and natural substrates (see, e.g., Patrick, 1965; Kanfer *et al.*, 1972; Bowen & Radin, 1968), and no intelligent predictions on one that are based on the other could be made. Glycosidases have been extensively purified by using synthetic substrates for testing the activity and found to have no activity towards natural substrates (see, e.g., Jungawala & Robins, 1968). It therefore comes as a mild surprise that glucocerebrosidase and 4-methylumbelliferyl β -glucosidase activities could both be reconstituted from factor P and factor C. It may be noted, however, that the magnitude of reconstitution is much greater for glucocerebrosidase, indicating that in the absence of factor P a large part of the activity measured with the synthetic substrate is in fact not glucocerebrosidase activity.

The physiological significance of the factor P and factor C system in glucocerebroside catabolism is emphasized by the following: (a) glucocerebrosidase activity is preferentially reconstituted in a mixture of factors P and C; (b) the glucocerebrosidase activity attained is at least 70–80-fold higher than previously reported (Brady *et al.*, 1965) with a pH 7 assay procedure. The present high activity is perhaps the best indication that the enzyme system investigated is the more important physiologically in the breakdown of glucocerebroside. A defect in the system, such as a deficiency of factor C in adult Gaucher's disease (Ho & O'Brien, 1971), could explain the diminution in glucocerebrosidase activity first described independently by Brady *et al.* (1965) and Patrick (1965).

Of especial interest is the fact that factor P is the first substance ever described to have such a striking effect on the activity of a lysosomal glycosidase. Whether it may perhaps play a role in the activation

of lysosomes (about which so little is known) remains to be investigated.

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