Glucocerebrosidase: Reconstitution from Macromolecular Components Depends on Acidic Phospholipids

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The reconstitution of glucocerebrosidase activity from its inactive macromolecular components depended on acidic membrane phospholipids.

Glucocerebrosidase was identified with acid β -glucosidase activity measured with the synthetic substrate 4-methylumbelliferyl β -glucoside (Ho, 1973). Both enzyme activities depended on the association of two inactive macromolecular components, factors P and C (Ho & O'Brien, 1971; Ho *et al.*, 1973a). Factor P was a soluble heat-stable glycoprotein fraction isolated from the spleen of a patient with adult Gaucher's disease. Factor C, from normal human spleen, was heat-labile and membrane-bound. The concentration of freely soluble factor P activity in the spleen of two patients with adult Gaucher's disease was 10–15 times normal, whereas factor C activity was barely detectable (Ho & O'Brien, 1971; Ho, 1973).

Factor C was activated, in the absence of factor P, by sodium taurocholate to give an enzyme with kinetic properties differing from that of the factor P-dependent enzyme (Ho, 1973). Factor C could be solubilized under a variety of conditions; although membrane-bound factor C reacted optimally with factor P, solubilized factor C was preferentially activated by taurocholate (Ho, 1973). The requirement for a third component was predicted (Ho, 1973) in the association of factors P and C.

In the present communication the identification of the third component is described. Factor C, partially purified in the soluble form, recombined with factor P to give active enzyme only in the presence of acidic phospholipid.

Experimental

Materials. Normal human spleens and spleens from patients with adult Gaucher's disease were provided by Dr. A. D. Patrick, Institute of Child Health, University of London, U.K.

Radioactive glucocerebroside, made by *N*-acylation of glucosylsphingosine with [14 C]stearic acid (Kopaczky & Radin, 1965), was a gift from Dr. Norman Radin, University of Michigan, Ann Arbor, Mich., U.S.A.

The following materials were from commercial sources as indicated: Triton X-100 (B grade) and sodium taurocholate (A grade) from Calbiochem,

Los Angeles, Calif., U.S.A.; 4-methylumbelliferyl β -D-glucopyranoside, 2,5-diphenyloxazole, 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene, phosphatidic acid (ex egg lecithin), phosphatidylserine (ex ox brain), phosphatidylinositol (ex yeast) and phosphatidylethanolamine (ex yeast) from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.; cholesterol, 1,4-dioxan and naphthalene from BDH Chemicals Ltd., Poole, Dorset, U.K.; phosphatidylcholine (egg yolk) from Sigma Chemical Co., St. Louis, Mo., U.S.A.; Sephadex G-150 from Pharmacia, Uppsala, Sweden; silica-gel-precoated plates from E. Merck A.-G., Darmstadt, Germany.

Methods. Factor C was solubilized and partially purified. The entire procedure was carried out at 4°C. A homogenate (15%, w/v) of normal human spleen in distilled water was centrifuged at 100000g for 45 min. The residue was resuspended in water and sedimented once again by centrifuging at 100000g for 45 min. The washed sediment from each g wet weight of spleen was suspended in 5 ml of 0.1% (v/v) Triton X-100 in 5 mM-sodium phosphate buffer, pH 7.0. The mixture was vortexed for 25 s at the end of each of two 5 min intervals while maintained at 4°C, and then centrifuged at 100000g for 45 min. The resultant clear supernatant (containing solubilized factor C activity) was applied directly to a column (2.5 cm $\times$ 45 cm) of Sephadex G-150 (Ho, 1973) equilibrated with 5 mM-sodium phosphate buffer, pH 7.0, containing 5 mM-NaCl. The column was eluted with the same buffer at a flow rate of 22.8 ml/h, and fractions (3.8 ml) were collected. Protein was monitored continuously by measuring the extinction at 280 nm. Each fraction was assayed for factor C activity by using both the synthetic and the natural substrates (Ho, 1973) as described below. Factor C activity was found exclusively in the protein peak at the void volume (V_0).

Factor P was isolated from the spleen of a patient with adult Gaucher's disease as previously described (Ho & O'Brien, 1971), but omitting the enzymic digestion steps. This preparation had a specific activity of 420 units/mg of protein.

Since purified factors P and C were each devoid of enzyme activity, it is possible to redefine the activity

of each directly as the glucocerebrosidase activity obtained when assayed in the presence of an excess of the other factor. Thus 1 unit of factor P activity gives 1 unit of enzyme activity (1 nmol/h) when assayed in the presence of an excess of factor C, and vice versa.

Total membrane lipid was prepared from the washed 100000g sediment from 1 g wet wt. of normal human spleen. This was suspended in 1 ml of water and mixed with 5 ml of chloroform-methanol (2:1, v/v). After centrifugation (120g, 2min), the upper phase was aspirated off, and the lower phase was dried in a desiccator connected to a vacuum pump. The solids were extracted with chloroform-methanol (2:1, v/v); insoluble precipitates were removed by filtration through glass-wool. The solution was dried down once more and redissolved in 1 ml of chloroform-methanol (2:1, v/v).

Factor C activity was assayed with both 4-methylumbelliferyl β -glucoside and glucocerebrosidase in 0.05 M-sodium acetate buffer, pH 4.5, containing 0.05% (v/v) Triton X-100 (Ho *et al.*, 1973a) in the presence of (a) 0.1% (w/v) sodium taurocholate (Ho, 1973) and (b) excess of factor P (Ho *et al.*, 1973a; Ho, 1973) supplemented with total membrane lipid equivalent to that extracted from 5 mg wet wt. of normal human spleen. Assay procedures for both the synthetic and the natural substrates have been described in detail (Ho *et al.*, 1973a; Ho, 1973).

The effect of membrane lipids on purified, solubilized factor C was examined as follows. Portions of the lipid in chloroform-methanol (2:1, v/v) were transferred to the test tubes and evaporated to dryness by exposure to air. The lipid was then redissolved in the appropriate assay buffer.

Results

The solubilization and purification of factor C is summarized in Table 1. The results for the natural

substrate only are given; those obtained with the synthetic substrate are analogous. More than 80% solubilization of factor C was achieved from the 100000g sediment. The final preparation remained stable at -20°C for at least 2 weeks. Previous preparations by a different solubilization procedure were extremely unstable (Ho, 1973). The novel procedure is mild and the higher concentration of Triton X-100 probably solubilized factor C together with small amounts of stabilizing membrane lipids.

The effect of membrane lipids on partially purified solubilized factor C is given in Table 2. For clarity the results for the synthetic substrate have been omitted, since they were closely analogous to those for the natural substrate. The acidic phospholipids stimulated factor P-dependent glucocerebrosidase activity between 16- and 25-fold. The order of effectiveness was phosphatidic acid > phosphatidylserine > phosphatidylinositol. The taurocholate-dependent enzyme activity was little affected. Phosphatidylcholine, phosphatidylethanolamine and cholesterol were not effective and did not prevent the stimulation of factor P-dependent enzyme activity by phosphatidic acid.

The acidic phospholipids acted antagonistically to taurocholate: the latter inhibited (by 40–50%) the stimulation of factor P-dependent enzyme activity by the acidic phospholipids. This was predictable from the previous observation that factor P and taurocholate maintained alternative conformations of the enzyme (Ho, 1973).

Discussion

Glucocerebrosidase is tightly membrane-bound under normal physiological conditions (Ho, 1973). The membrane-bound factor P-dependent enzyme was characterized by high affinity for glucocerebrosidase (low K_m) compared with an alternative form of the enzyme which was soluble and dependent on

Table 1. Solubilization and purification of factor C

Factor C was assayed as glucocerebrosidase activity. Taurocholate-dependent factor C activity was assayed in the presence of 0.1% (w/v) sodium taurocholate included in normal buffer [0.05 M-sodium acetate buffer, pH 4.5, containing 0.05% (v/v) Triton X-100]. Factor P-dependent activity was assayed in the presence of excess of factor P in the normal buffer system supplemented with total membrane lipid (see under 'Methods'). Specific activity is expressed as nmol/h per mg of protein. Protein was determined by the method of Lowry *et al.* (1951). Each value is an average of two determinations which agreed within 5%.

Fraction	Taurocholate-dependent activity			Factor P-dependent activity		
	Sp. activity	Total activity	%	Sp. activity	Total activity	%
100000g sediment	20.1	720	100	78.3	2819	100
Triton-soluble supernatant	100.2	601	84	381.8	2288	81
Triton-insoluble residue	4.1	124	17	16.0	490	17
cephadex G-150 peak	122.1	464	65	488.2	1855	66

Table 2. *Effect of membrane lipids on purified solubilized factor C*

Purified factor C (Sephadex G-150 peak) was assayed for glucocerebrosidase activity in the presence of taurocholate or of excess of factor P (see under 'Methods'). Controls were assayed in the normal buffer system without taurocholate or factor P. Lipids (or lipid mixtures) were added to the assay mixture at concentrations indicated in parentheses (the acidic phospholipids were added at optimum concentration). Glucocerebrosidase activity is expressed as nmol of substrate cleaved/h per mg of protein. Each value is an average of two determinations which agreed within 10%.

Lipid added	Glucocerebrosidase activity		
	Control	+ Taurocholate	+ Factor P
—	0	20.0	21.2
Phosphatidic acid (0.12 mM)	25.0	39.1	499.4
Phosphatidylserine (0.12 mM)	19.1	29.8	452.2
Phosphatidylinositol (0.12 mM)	18.7	27.5	324.8
Total membrane lipid*	21.0	20.2	488.2
Phosphatidylcholine (0.12 mM)	0	18.8	22.2
Phosphatidylethanolamine (0.12 mM)	0	18.6	15.5
Cholesterol (0.12 mM)	0	18.5	19.5
Phosphatidic acid (0.12 mM)+phosphatidylcholine (0.12 mM)	24.0	28.8	592.5
Phosphatidic acid (0.12 mM)+phosphatidylethanolamine (0.12 mM)	24.1	28.5	580.6
Phosphatidic acid (0.12 mM)+cholesterol (0.12 mM)	23.3	27.2	490.1

* Total membrane lipid added was equivalent to that extracted with chloroform-methanol (2:1, v/v) from the total membrane fraction (100000 g sediment) from 5 mg wet wt. of normal spleen.

taurocholate for activity. From this and other studies, it was suggested that membrane binding of enzymes may be associated with enzyme activation (Ho, 1973; Ho *et al.*, 1973b). The involvement of acidic membrane phospholipids in the association of factors P and C to give active enzyme is consistent with the hypothesis, and offers yet another refinement in the modulation of metabolic control through this multiple-component enzyme system.

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