Synthesis of Glucosylceramide by Purified Calf Spleen β-Glucosidase

Srinivasa S. Raghavan, Richard A. Mumford and Julian N. Kanfer,
E.K. Shriver Center at the W.E. Fernald State School, Waltham,
MA 02154 and Neurology Research, Massachusetts General Hospital,
Boston, MA 02114.

Received March 19, 1974

Summary: Radioactive glucocerebroside was formed when purified calf spleen β-glucosidase was incubated in the presence of 4-methylumbelliferyl-β-D-glucoside and (^{14}C) labeled ceramide. The product was identified by cochromatography on thin layer plates and carrier dilution and crystallization to constant specific activity. The radioactive glucocerebroside was also a substrate for hydrolysis by this same β-glucosidase preparation employed for its synthesis resulting in the liberation of labeled ceramide. Neither methyl-β-D-glucoside nor free D-glucose were effective in producing radioactive glucocerebroside when incubated with labeled ceramide in this system.

Glucocerebrosidase present in various mammalian tissues (1-3) catalyzes the hydrolysis of the β-linkage present in glucosylceramide yielding glucose and ceramide as the products. This enzyme activity is markedly diminished in the inherited disorder, Gaucher disease (4,5) in which large amounts of glucosylceramide accumulate in the patients spleen (7-9). The enzyme has been partially purified from human spleen tissue (1), rat intestinal mucosa (2) and beef spleen (10), and highly purified from human placental tissue (11). The technique of affinity chromatography has been employed to obtain in a single step a highly purified β-glucosidase preparation from calf spleen that cleaves the β-glucosidic bond of 4-methylumbelliferone (4MU)-β-D-glucoside, glucocerebroside and glucosylsphingosine (12). This enzyme preparation has been found to catalyze a transglucosylation reaction, which results in glucosylceramide formation.

Methods

4MU-glucosides were obtained from Pierce Chemical Co., Rockford,

Ill. The synthesis of N-stearoyl-1-^{14}C sphingosine has been reported earlier (1), and the compound was repurified by column chromatography on silica gel (0.05-0.2 mm, extra pure, 70-325 mesh, E. Merck, cat. no. 7754). Labeled ceramide, 50 mg, was applied to a column (1.6 cm I.D.) containing 10 g silica gel in chloroform and was washed with 100 ml of the solvent. The column was then eluted with chloroform:methanol: acetic acid (90:2:8) and 1 ml portions were collected. Individual fractions were monitored both for radioactivity by scintillation counting and for the purity of labeled ceramide by thin layer chromatography (TLC) on Silica Gel G plates employing the same solvent system. The specific activity of the purified labeled ceramide was 684,500 cpm/μmole.

Assay conditions for transglucosylation

The incubation mixture contained 220 nmoles of labeled ceramide, 1 mg sodium taurocholate, 50 μmoles of citrate-phosphate buffer, pH 4.5, 1 μmole of 4MU-β-D-glucoside or 0.5 μmole of 4MU-α-D-glucoside and 42.6 units of β-glucosidase in a total volume of 0.4 ml. One unit of enzyme is defined as that amount required to hydrolyze 1 nmole of 4MU-β-D-glucoside/hr under these incubation conditions. Boiled enzyme controls were included and all samples were incubated for 3 hr. The reaction mixture was extracted with 20 volumes of chloroform:methanol (2:1) and subjected to the Folch partitioning procedure (13). Suitable aliquots of the lower phase were spotted on Silica Gel G plates (Analtech) along with authentic standards and developed with chloroform:methanol:water (65:25:4). Radioactive spots were detected by autoradiography and standards identified by exposure to iodine vapour.

Product identification

In order to isolate the labeled product formed after incubation of the enzyme with 4MU-β-D-glucoside and N-stearoyl-1-^{14}C sphingosine, the contents of 20 identical incubation tubes were pooled and the lipids subjected to preparative thin layer chromatography. The radioactive

band corresponding to standard glucocerebroside was scraped, transferred to a small column and eluted with chloroform:methanol:water (60:35:8). An aliquot containing approximately 12,000 cpm of this material was mixed with 50 mg authentic glucocerebroside and the mixture was crystallized from hot methanol to carry out carrier dilution studies.

Enzymatic hydrolysis of the labeled glucocerebroside

An aliquot of labeled glucocerebroside isolated by preparative TLC was incubated with the enzyme preparation which had been employed to catalyze its formation. The reaction mixture contained 36 nmoles of substrate, 1 mg sodium taurocholate, 50 μmoles of citrate-phosphate buffer, pH 4.5, and 40 units of β-glucosidase in a total volume of 0.3 ml. A boiled enzyme control was included and the samples were incubated for 3 hr. The lipids were extracted as described earlier, spotted on Silica Gel G plate along with standards and developed with chloroform:methanol:acetic acid (90:2:8) as the solvent system. The standards were located after exposure to iodine vapour and radioactive spots located by autoradiography.

Results

A typical radioautogram is shown in Fig. 1 demonstrating that a radioactive spot (lane 1) corresponding to standard glucocerebroside (lane 4) was obtained when 4MU-β-D-glucoside was used as the glucose donor and N-stearoyl-1-^{14}C sphingosine as the acceptor. This was absent in the boiled enzyme control (lane 3) and only a trace was seen in tubes containing 4MU-α-D-glucoside as the potential glucose donor (lane 5). This small amount might be due to the presence of trace quantities of 4MU-β-D-glucoside as a contaminant in this commercial preparation of 4MU-α-D-glucoside. The boiled enzyme control containing 4MU-α-D-glucoside (lane 6) did not appear to contain any such radioactive product. The intense radioactive spot seen at the solvent front in all the samples is labeled ceramide, employed as the glucose acceptor in these reaction

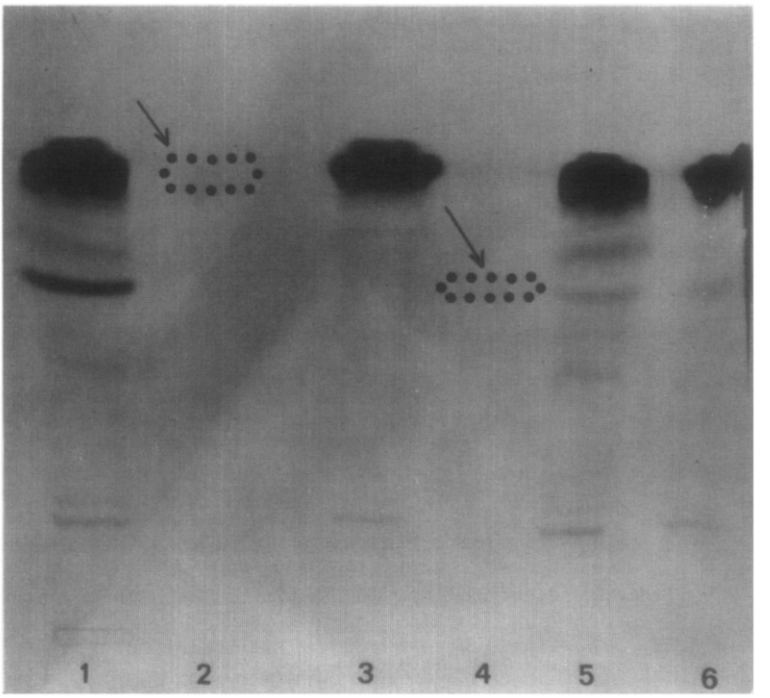

Fig. 1: Radioautogram obtained from thin layer chromatography of lipids extracted after incubating β-glucosidase with the following: Lane 1 - native enzyme with 4MU-β-D-glucoside and labeled ceramide; Lane 3 - boiled enzyme with 4MU-β-D-glucoside and labeled ceramide; Lane 5 - native enzyme with 4MU-α-D-glucoside and labeled ceramide; Lane 6 - boiled enzyme with 4MU-α-D-glucoside and labeled ceramide; Lane 2 - standard nonhydroxy fatty acid containing ceramide; Lane 4 - standard glucocerebroside. Details of incubation and TLC conditions are provided in the Methods section.

mixtures and this material cochromatogrammed with the ceramide standard (lane 2). The enzymic product which cochromatogrammed with authentic glucocerebroside was isolated by preparative thin layer chromatography and subjected to carrier dilution experiment by crystallization from boiling methanol. A constant specific activity of 116 cpm/mg was attained, which indicated that glucocerebroside is formed in these reaction mixtures.

The radioactive glucocerebroside synthesized was a substrate for hydrolysis by this same β-glucosidase enzyme preparation, resulting in the liberation of labeled ceramide as shown in Fig. 2. A radioactive

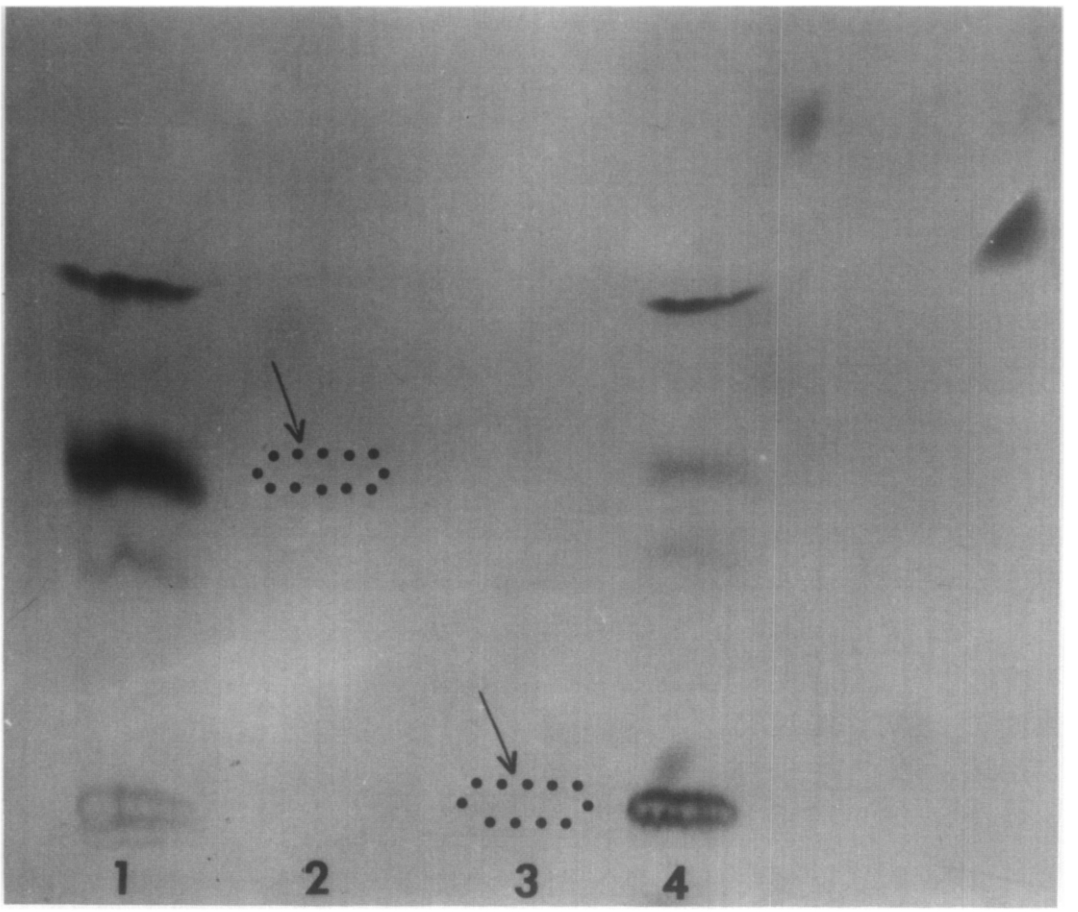

Fig. 2: Radioautogram obtained from thin layer chromatography of lipids extracted after incubating β-glucosidase with radioactive glucocerebroside synthesized by the same enzyme. Lane 1 - radioactive glucocerebroside plus native enzyme; Lane 2 - reference nonhydroxy fatty acid containing ceramide; Lane 3 - reference glucocerebroside; Lane 4 - radioactive glucocerebroside plus boiled enzyme. Details of incubation and TLC conditions are provided in the Methods section.

spot (lane 1) corresponding to standard ceramide (lane 2) was formed. In the boiled enzyme control (lane 4) the radioactivity remained at the origin which corresponds to the location of standard glucocerebroside (lane 3). The spot at the solvent front is a contaminant present in this substrate.

Radioactive glucocerebroside was obtained when the enzyme was in-

cubated with nonradioactive glucocerebroside as the glucose donor and labeled ceramide as the glucose acceptor. However, neither methyl-β-D-glucoside nor free D-glucose were effective in producing radioactive glucocerebroside when incubated with labeled ceramide in this system.

Discussion

The calf spleen β-glucosidase purified by affinity chromatography has been shown to hydrolyze 4MU-β-D-glucoside and glucocerebroside (12). The present experiments demonstrate that when either 4MU-β-D-glucoside or glucocerebroside is incubated in the presence of labeled ceramide, radioactive glucocerebroside is produced. This synthesis is presumably not due to a simple reversal of hydrolysis of glucocerebroside since free glucose, a product of this cleavage, is not effective in the reversal of this reaction. The 'transglucosylation' reaction may occur via a 'glucosylated enzyme', a presumed intermediate formed in the hydrolysis of both 4MU-β-D-glucoside and glucocerebroside as follows:

$$\text{4MU-β-D-glucoside} + \text{Enzyme} \rightleftharpoons \text{E-glucose} + \text{4MU} \quad (1)$$

$$\text{Glucocerebroside} + \text{Enzyme} \rightleftharpoons \text{E-glucose} + \text{Ceramide} \quad (2)$$

$$\text{E-glucose} + H_2O \longrightarrow \text{Glucose} + \text{Enzyme} \quad (3)$$

The E-glucose formed by the forward reaction of 1 may react with added ceramide to result in the reverse reaction of 2 and therefore produce glucocerebroside in these incubations. This observation is consistant with the postulate that the same enzyme protein catalyzes the hydrolysis of both 4MU-β-D-glucosidase and glucosylceramide.

There are several reports in the literature of transglycosylation reactions being catalyzed by glycosidases. Both lysozyme (14) and purified N-acetyl-β-D-glucosaminidase from Aspergillus oryzae (15,16) catalyze the transfer of N-acetyl-β-D-glucosamine to suitable acceptors. Transgalactosylation catalyzed by E. coli β-galactosidase has been extensively studied and kinetic data support the formation of a galactosyl enzyme as the common intermediate in the hydrolysis of various β-

galactosides (17-21). Wallenfels and Fisher (22) have observed trans-galactosylation reaction catalyzed by the β-galactosidase from calf intestinal mucosa. Recently, Distler and Jourdian (23) demonstrated that β-galactosidase purified from bovine testes catalyzed a trans-galactosylation reaction with a high degree of acceptor specificity.

Gatt has reported (3) that the hydrolysis of glucocerebroside by β-glucosidase from ox brain is not reversible. This reverse reaction was examined by incubating ceramide and ^{14}C-glucose with the enzyme and the results of these experiments provided no evidence for the formation of labeled glucocerebroside. The enzyme did not catalyze an isotopic exchange reaction between ^{14}C-glucose and nonradioactive glucocerebroside. In the present experiments with β-glucosidase from calf spleen, the synthesis of glucocerebroside could be demonstrated by incubating 4MU-β-D-glucoside and ceramide. Neither free glucose nor methyl-β-D-glucoside were substrates for this synthesis.

Acknowledgements: Supported by grants from the USPHS #HD05515, HD04147 and NS10330 and Maternal and Child Health Project 906.

References

1. Brady, R.O., Kanfer, J.N. and Shapiro, D., J. Biol. Chem. 240, 39 (1965).
2. Brady, R.O., Gal, A.E., Kanfer, J.N. and Bradley, R.M., J. Biol. Chem. 240, 3766 (1965).
3. Gatt, S., Biochem. J. 101, 687 (1966).
4. Brady, R.O., Kanfer, J.N., Bradley, R.M. and Shapiro, D., J. Clin. Invest. 45, 1112 (1966).
5. Patrick, A.D., Biochem. J. 97, 176 (1965).
6. Rosenberg, A. and Chargaff, E., J. Biol. Chem. 233, 1323 (1958).
7. Suomi, E.D. and Agranoff, B.W., J. Lipid Res. 6, 211 (1965).
8. Kennaway, N.G. and Woolf, L.I., J. Lipid Res. 9, 755 (1968).
9. Kuske, T.T. and Rosenberg, A., J. Lab. Clin. Med. 80, 523 (1972).
10. Weinreb, N.J. and Brady, R.O., Methods Enzymol. 28, 830 (1972).
11. Pentchev, P.G., Brady, R.O., Hibbert, S.R., Gal, A.E. and Shapiro, D., J. Biol. Chem. 248, 5256 (1973).

12. Kanfer, J.N., Mumford, R.A., Raghavan, S.S. and Byrd, J., Anal. Biochem. (in press).
13. Folch, J., Lees, M. and Sloane-Stanley, G.H., J. Biol. Chem. 226, 497 (1957).
14. Banerjee, S.K., Kregar, I., Turk, V. and Rupley, J.A., J. Biol. Chem. 248, 4786 (1973).
15. Mega, T., Ikenaka, T. and Matsushima, Y., J. Biochem. 72, 1197 (1972).
16. Mega, T., Ikenaka, T. and Matsushima, Y., J. Biochem. 72, 1391 (1972).
17. Wallenfels, K. and Malhotra, O.M., Adv. Carbohydr. Chem. 16, 239 (1961).
18. Viratelle, O., Tenu, J.P., Garnier, J. and Yow, Y., Biochem. Biophys. Res. Commun. 37, 1036 (1969).
19. Tenu, J.P., Viratelle, O.M., Garnier, J. and Yow, Y., Eur. J. Biochem. 20, 363 (1971).
20. Stokes, T.M. and Wilson, I.B., Biochemistry 11, 1061 (1972).
21. Van der Groen, G., Wouters-Leysen, J., Yde, M. and De Bruyne, C.K., Eur. J. Biochem. 38, 122 (1973).
22. Wallenfels, K. and Fischer, J., Hoppe-Seyler's Z. Physiol. Chem. 321, 223 (1960).
23. Distler, J.J. and Jourdian, G.W., J. Biol. Chem. 248, 6772 (1973).