Human glucocerebrosidase catalyses transglucosylation between glucocerebroside and retinol

Dorothy J. VANDERJAGT,* Donald E. FRY† and Robert H. GLEW*‡

*Department of Biochemistry and †Department of Surgery, University of New Mexico School of Medicine, Albuquerque, New Mexico 87131, U.S.A.

The basal activity of human placental glucocerebrosidase is elevated 16-fold by n-pentanol when assayed using *p*-nitrophenyl β -D-glucopyranoside (*p*NPGlc) as the β -glucosidase substrate. This enhancement of activity is the result of the formation of a transglucosylation product, n-pentyl β -D-glucoside, in rate-determining competition with the hydrolytic reaction. The transglucosylation product accounts for ~80% of the reaction product generated in the presence of n-pentanol (0.18 M) when either glucocerebroside or *p*NPGlc was used as the substrate. This stimulatory effect can be increased an additional 3-fold by the inclusion of phosphatidylserine (20 μ g/ml) or sodium taurodeoxycholate (0.3%, w/v) in the incubation medium. In the presence of retinol, glucocerebrosidase also catalyses the synthesis of a novel lipid glucoside, retinyl glucoside, when either glucocerebroside or *p*NPGlc serves as the substrate. The reaction product was identified as retinyl β -D-glucoside, based on its

susceptibility to hydrolysis by almond β -D-glucosidase and the subsequent release of equimolar amounts of retinol and glucose. The rate of retinyl- β -glucoside formation is dependent on the concentration of retinol in the incubation medium, reaching saturation at ~0.3 mM retinol. Retinyl β -D-glucoside is a substrate for two broad-specificity mammalian β -glucosidases, namely the cytosolic and membrane-associated β -glucosidases of guinea pig liver. However, retinyl β -D-glucoside is not hydrolysed by placental glucocerebrosidase. These data indicate that the glucocerebrosidase-catalysed transfer of glucose from glucocerebroside to natural endogenous lipid alcohols, followed by the action of a broad-specificity β -glucosidase on the transglucosylation product, could provide mammals with an alternative pathway for the breakdown of glucocerebroside to glucose and ceramide.

INTRODUCTION

Glucocerebrosidase (EC 3.2.1.45) catalyses the hydrolysis of glucocerebroside to ceramide and glucose [1]. The enzyme exhibits optimal activity at pH 4.5–5.5 and is specific for the β -glucosidic linkage in glucocerebroside. In addition, glucocerebrosidase will catalyse the hydrolysis of aryl β -D-glucosides such as 4-methylumbelliferyl β -glucoside [2] and *p*-nitrophenyl β -D-glucoside [3]. In leucocytes, spleen and liver, the enzyme occurs *in situ* bound to the lysosomal membrane and its solubilization requires extraction with detergents (e.g. sodium cholate) or solvents (e.g. n-butanol) which results in the extensive but reversible inactivation of the enzyme [4,5]. However, the *in vitro* activity of solubilized lysosomal-derived glucocerebrosidase can be restored by the inclusion of certain bile salts [e.g. sodium taurodeoxycholate (TDC)] or acidic phospholipids [e.g. phosphatidylserine (PtdSer)] in the reaction mixture [4,5]. A marked deficiency of glucocerebrosidase activity in humans is the cause of Gaucher disease, an autosomal recessive disorder in which glucocerebroside accumulates in phagocytic cells, primarily in the liver, spleen, and bone marrow of affected individuals [1,3]. A second, non-lysosomal glucocerebrosidase was recently reported in various human cell types; however, although it too hydrolyses glucocerebroside, it is not deficient in patients with Gaucher disease [6].

Most mammalian tissues contain another β -glucosidase which is located in the cytosol and has a neutral pH optimum. In contrast to glucocerebrosidase, the cytosolic β -glucosidase does not cleave glucocerebroside. However, it catalyses the hydrolysis of certain plant glycosides (e.g. amygdalin and prunasin) [7,8]

and various non-physiological aryl glycosides, including the *p*-nitrophenyl and the 4-methylumbelliferyl derivatives of β -D-glucose, β -D-galactose, α -L-arabinose, β -D-xylose, and β -D-fucose [9,10]. Although the physiological function of the cytosolic broad-specificity β -glucosidase has not been elucidated, the enzyme has been isolated from various sources [7,11–13] and its kinetic properties have been described extensively [14].

The activity of the broad-specificity β -glucosidase from guinea pig liver is stimulated markedly by n-alkanols [15]. This apparent increase in β -glucosidase activity results from the rate of alcoholysis of the glucosyl-enzyme intermediate being greater than the rate of its hydrolysis [16]. Alcoholysis results in the formation of alkyl-glucosides. A similar transglucosylation reaction has been previously reported for glucocerebroside. Mumford et al. [17] synthesized glucocerebroside from 4-methylumbelliferyl β -D-glucopyranoside and *N*-[¹⁴C]oleoylsphingosine (ceramide) in the presence of glucocerebrosidase from calf brain. The synthesis of glucocerebroside was the result of a transglucosylation reaction and not a reversal of the hydrolytic reaction as it did not take place when the enzyme was incubated in the presence of free glucose and ceramide. However, this particular study was performed using only a partially purified glucocerebrosidase preparation and the relative rates of transglucosylation and hydrolysis were not reported.

In this paper, we examine the transglucosylating activity of a human lysosomal glucocerebrosidase using a highly purified enzyme preparation from human placenta. We show that glucocerebrosidase is capable of catalysing transglucosylation between glucocerebroside and n-alkanols. More importantly, we document the ability of human glucocerebrosidase to catalyse a

transglucosylation reaction between glucocerebroside and retinol, a naturally occurring amphipathic alcohol.

MATERIALS AND METHODS

Materials

p-Nitrophenyl β -D-glucopyranoside (*p*NPGlc), *p*-nitrophenyl β -D-galactopyranoside (*p*NPGal), amyl β -D-glucopyranoside (pentyl glucoside), hexyl β -D-glucopyranoside (hexyl glucoside), almond β -glucosidase, α - and β -D-glucose, TdC, hexokinase and glucose 6-phosphate dehydrogenase (mixed enzymes from Baker's Yeast) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. *n*-Pentanol was obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A. PtdSer was purchased from Supelco Inc., Bellefonte, PA, U.S.A. The trimethylsilyl reagent (TriSil) for derivatizing glucose and alkyl glucosides was obtained from Pierce, Rockford, IL, U.S.A.

Retinol (ICN Biochemicals, Cleveland, OH, U.S.A.) was purified by reversed-phase h.p.l.c. The concentration of retinol was determined spectrophotometrically at 325 nm using a molar absorption coefficient of $53008 \text{ M}^{-1} \cdot \text{cm}^{-1}$. All procedures involving retinol were carried out under reduced light.

Glucocerebroside was prepared from the spleen of a patient with type 1 Gaucher disease and purified to homogeneity as described elsewhere [18]. All other chemicals were of reagent grade and purchased from Fisher Scientific Co., Pittsburgh, PA, U.S.A.

Enzyme preparations

Ceredase, a preparation of human placental glucocerebrosidase, was the gift of Dr. Scott Furbish, Genzyme Corporation, Cambridge, MA, U.S.A. The glucocerebrosidase preparation was more than 98% pure and ran as a single 62 kDa band on SDS/PAGE. The concentrated enzyme preparation was stored in 1% (w/v) human serum albumin in order to stabilize the glucocerebrosidase activity and was diluted immediately before use in kinetic studies.

The cytosolic β -glucosidase was purified from guinea pig liver as described previously [7]. Another non-lysosomal broad-specificity β -glucosidase was obtained from guinea pig liver after extraction of the high-speed centrifugation pellet of a liver homogenate with Triton-X100 (0.5%, v/v). This enzyme, designated TX-100 β -glucosidase, was purified through the same DEAE-cellulose and octyl-Sepharose affinity steps used in purifying the cytosolic β -glucosidase (R. H. Glew and W. Hays, unpublished work). The TX-100 β -glucosidase does not catalyse glucocerebroside hydrolysis, regardless of whether or not TdC is present in the incubation medium, and it is not inhibited by conduritol β -epoxide. Micromolar concentrations of this compound do inhibit glucocerebrosidase.

β -Glucosidase assays

The standard glucocerebrosidase assay medium contained 5 mM substrate (*p*NPGlc or glucocerebroside), 0.1 M sodium acetate, pH 5.5, glucocerebrosidase, and the specified amount of activator (*n*-pentanol, PtdSer, TdC) in a final volume of 0.1 ml [19]. Incubations were initiated by the addition of enzyme and reactions were carried out for 20 or 30 min at 37 °C. For the studies in which *p*NPGlc served as substrate, the incubations were terminated by the addition of 0.9 ml of 0.02 M sodium borate (pH 9.2); the amount of *p*NP liberated was calculated

using a molar absorption coefficient of $18300 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 400 nm. One unit of β -glucosidase activity corresponds to the release of 1 nmol of *p*NP per h at 37 °C. All enzyme assays were performed in triplicate (coefficient of variation 8.1 %).

Determination of D-glucose and alkyl glucosides

D-Glucose liberated by the various β -glucosidases was determined using three coupling enzymes: hexokinase, glucose-6-phosphate dehydrogenase and diaphorase, as described elsewhere [16]. For samples in which the presence of detergent interfered with the enzymic assays, glucose was determined by g.l.c. as described below.

For the determination of glucose and alkyl glucosides by g.l.c., aliquots (0.1 ml) of the enzyme incubation mixtures were transferred to reactivials (Pierce) to which a known amount of hexyl β -D-glucoside (internal standard) was added. The samples were then dried *in vacuo*. The residue was treated with 0.1 ml of chloroform and dried under a stream of nitrogen to ensure the removal of the last traces of water. TriSil reagent (50 μ l) was added to each vial and the samples were vortexed vigorously. Following a 30 min incubation at 22 °C, samples were centrifuged and an aliquot (1 μ l) of the clear supernatant was injected into a Hewlett Packard 5690A g.l.c. system equipped with a DB-1 capillary column (0.055 cm $\times$ 1500 cm). Samples were eluted with the following gradient: 1 min at 150 °C, followed by a linear gradient from 150 to 250 °C over 20 min, and a final 3 min at 250 °C. The helium flow rate was 16.3 ml/min. The areas of the eluted peaks were determined using a Hewlett Packard 3396 integrator. The amounts of glucose and pentyl glucoside produced were calculated from standard curves generated using known amounts of glucose and pentyl β -D-glucoside in the presence of hexyl β -D-glucoside, the internal standard. The assays were performed in duplicate (coefficient of variation 5.3 %).

Hydrolysis of retinyl β -glucoside

Incubations of retinyl glucoside with the cytosolic and TX-100 β -glucosidases were carried out in 0.2 M sodium citrate, pH 6.0, for 60 min at 37 °C. Incubations with almond β -glucosidase (5 units) were carried out for 60 min at 37 °C in 0.1 M sodium acetate, pH 5.5. Incubations with glucocerebrosidase were carried out using the standard glucocerebrosidase assay conditions in the presence of 0.5% (w/v) TdC as described above.

H.p.l.c.

The separation of reactants and products contained in assay mixtures that included retinol was accomplished using reversed-phase h.p.l.c. Analyses were performed using a Waters 600E solvent delivery system equipped with a variable absorbance detector (Model 484) and an Econosphere C_{18} column (particle size 5 μ m, 0.46 cm $\times$ 25 cm; Alltech, Deerfield, IL, U.S.A.). Samples were eluted with a mobile phase consisting of methanol/ H_2O (85:15, v/v) at a flow rate of 0.8 ml/min. Retinol and retinyl derivatives were monitored at 325 nm using an integrating recorder (Spectra Physics SP4270) and peak areas were used for quantification.

RESULTS

Activation of glucocerebrosidase by *n*-pentanol

In preliminary experiments conducted using purified human spleen and placental glucocerebrosidase, we found that C_4 – C_6 *n*-

Figure 1 The effect of *n*-pentanol on the glucocerebrosidase-catalysed hydrolysis of *p*NPGlc

The rate of release of *p*NP from *p*NPGlc by placental glucocerebrosidase was determined in the presence of increasing concentrations of *n*-pentanol in the absence (○) and presence (●) of PtdSer (20 µg/ml). The incubation medium contained *p*NPGlc (5 mM), *n*-pentanol (0–0.18 M), and placental glucocerebrosidase (66 units) in 0.1 ml of sodium acetate buffer (0.1 M, pH 5.5). All reactions were carried out for 20 min at 37 °C. Note that the range of activities obtained for incubations containing *n*-pentanol and PtdSer (left-hand ordinate) are ~ 10-fold those obtained in the presence of *n*-pentanol alone (right-hand ordinate).

Table 1 The effect of various activators alone and in combination on human placental glucocerebrosidase activity

The glucocerebrosidase activity expressed refers to an incubation volume of 0.1 ml; *p*NPGlc was used as substrate. One unit of activity corresponds to the release of 1 nmol of *p*NP/h per incubation. Concentrations of activators used: *n*-pentanol, 0.18 M; PtdSer 20 µg/ml; TdC, 0.5%, w/v. +, activator present; –, activator absent from reaction mixture.

Activator			10 ⁻³ × Activity (units)
<i>n</i> -Pentanol	PtdSer	TdC	
–	–	–	3
+	–	–	48
–	+	–	95
+	+	–	440
–	–	+	167
+	–	+	477

alkanols markedly stimulated (4–6-fold) the hydrolytic activity of glucocerebrosidase in the absence of any exogenous lipid activators. The order of potency, both on a concentration basis and in terms of fold stimulation of glucocerebrosidase activity, was *n*-hexanol > *n*-pentanol > *n*-butanol (results not shown). Because of the relatively high solubility of *n*-pentanol in water, we elected to examine the stimulatory effect of *n*-pentanol on the purified placental enzyme using *p*NPGlc as the substrate in the absence and presence of two lipids which are commonly used to reconstitute glucocerebrosidase activity, namely TdC and PtdSer.

A progressive increase in the rate of *p*NP production was obtained for *n*-pentanol concentrations between 0.02 and 0.18 M, with a 16-fold activation over baseline being achieved in the presence of 0.18 M *n*-pentanol (Figure 1). The inclusion of a constant amount of PtdSer (20 µg/ml) in the incubation medium

over the same range of *n*-pentanol concentration resulted in a further increase in the reaction rate as well as a leftward shift in the dose–response of glucocerebrosidase activity to *n*-pentanol. Whereas apparent saturation of glucocerebrosidase was not observed even at the highest concentration of *n*-pentanol used (0.18 M), the inclusion of PtdSer in the incubation mixture not only increased the rate of *p*NPGlc hydrolysis for the same *n*-pentanol concentrations, but also resulted in a maximization of glucocerebrosidase activity at *n*-pentanol concentrations greater than 80 mM.

The rate of *p*NPGlc hydrolysis obtained when *n*-pentanol (0.18 M) and PtdSer (20 µg/ml) were present simultaneously in the incubation medium was ~ 3-fold higher than the sum of the activities obtained when each activator was added separately (Table 1). When TdC replaced PtdSer as the lipid activator, the rate of *p*NPGlc hydrolysis was increased 2.2-fold over the sum of the activities obtained when *n*-pentanol and TdC were used separately. In combination with *n*-pentanol, both lipid activators gave the same rate of hydrolysis when included at optimal concentrations (Table 1).

Glucocerebrosidase catalyses transglucosylation from *p*NPGlc and glucocerebroside to *n*-pentanol

G.l.c. analysis of the incubation medium that contained *p*NPGlc, *n*-pentanol (0.18 M), and glucocerebrosidase revealed a prominent peak eluting at 13.2 min, which corresponded exactly to the elution time of the *n*-pentyl β-D-glucoside standard (Figure 2b). This peak was absent from reaction mixtures that did not contain *n*-pentanol (Figure 2a) or when boiled glucocerebrosidase was used in place of the native enzyme (results not shown). This observation provides evidence of the ability of glucocerebrosidase to catalyse transglucosylation between a non-physiological β-glucosidase substrate (*p*NPGlc) and *n*-pentanol to produce pentyl β-D-glucoside. We have previously demonstrated that *n*-octyl β-glucoside can be separated from the corresponding α isomer by g.l.c. [16].

Pentyl β-glucoside accounted for a significant fraction of the glucose released from *p*NPGlc for all concentrations of *n*-pentanol used. The *n*-pentyl β-D-glucoside/glucose product ratio ranged from 2.6 to 6.9 in the presence of increasing concentrations of *n*-pentanol. Similar results were obtained when PtdSer was added to the assay medium, although the ratios of the transglucosylation product to the hydrolysis product were generally lower (0.5–3.2) for all concentrations of *n*-pentanol tested.

Because *p*NPGlc is not a physiological substrate, we wanted to determine whether the glucocerebrosidase-catalysed transglucosylation reaction would take place if glucocerebroside were used as the glucoside substrate in place of *p*NPGlc. Incubations of glucocerebrosidase with *n*-pentanol were therefore carried out using the natural substrate glucocerebroside, whose solubility and dispersion were ensured by including TdC (0.5%, w/v) in the incubation medium. G.l.c. analysis of the derivatized reaction products (Figure 3b) indicated the presence of a prominent peak at 13.2 min which corresponded to the pentyl β-D-glucoside standard. This experiment demonstrates that glucocerebroside is capable of supporting a transglucosylation reaction with *n*-alkanols in the presence of placental glucocerebrosidase and its natural substrate, glucocerebroside.

The *n*-pentyl glucoside/glucose ratio obtained using glucocerebroside as the glucose donor in the presence of TdC and *n*-pentanol was 2.6, i.e. approximately the same as that obtained when *p*NPGlc served as the glucose donor. This indicates that the ratio of the transglucosylation rate to the hydrolysis rate is

Figure 2 Demonstration of a transglucosylation reaction catalysed by human glucocerebrosidase

G.I.C. analysis of (a) a standard glucocerebrosidase reaction mixture and (b) the same reaction mixture supplemented with 0.18 M n-pentanol. The standard reaction mixture contained *p*NPGlc (5 mM), and glucocerebrosidase (66 units) in 0.1 M sodium acetate buffer, pH 5.5. After a 20 min incubation at 37 °C, samples were dried and derivatized for g.i.c. analysis as described in the Materials and methods section. The peaks at retention times of 2.5, 8.7, 10.4, 13.2, and 22.2 min correspond to *p*NP, α -D-glucose, β -D-glucose, n-pentyl β -D-glucoside and *p*NPGlc respectively. Note that the peak at 13.2 min (n-pentyl β -D-glucoside) is observed only in (b).

not very dependent on the nature of the β -glucoside substrate that serves as the glucose donor.

Glucocerebrosidase catalyses transglucosylation from *p*NPGlc and glucocerebroside to retinol

The ability of glucocerebroside to catalyse transglucosylation between glucocerebroside and a normal alcohol such as n-pentanol raises the interesting question of whether a physiologically relevant hydrophobic alcohol such as retinol could serve in place of n-pentanol as the glucose acceptor in such a reaction. Enzyme incubations were therefore performed in the presence of retinol (0.5 mM) and glucocerebrosidase with *p*NPGlc (5 mM) serving as the second substrate, i.e. the glucose donor. Analysis of the reaction products by h.p.l.c. revealed the presence of a unique peak at 12.3 min (Figure 4b) which was not seen when retinol was not included in the incubation mixture (Figure 4a). The rate of formation of this new product was dependent on the concentration of retinol in the incubation medium; maximum production of the retinyl derivative was obtained with ~0.3 mM retinol (results not shown).

Identical incubations using the natural substrate glucocerebroside in place of *p*NPGlc also resulted in the formation of a retinyl glucoside product in the presence of retinol (results not shown). However, no retinyl glucoside was obtained when *p*-nitrophenyl β -D-galactose was used in place of *p*NPGlc as the substrate, or when the enzyme preparation was inactivated by boiling for 5 min before being added to the incubation mixture.

Figure 3 Demonstration of the transglucosylation reaction between glucocerebroside and n-pentanol catalysed by glucocerebrosidase

G.I.C. analyses of reaction mixtures containing (a) 5 mM glucocerebroside, TdC (0.5%, v/v), and glucocerebrosidase (66 units) and (b) glucocerebroside, TdC, glucocerebrosidase, and n-pentanol (0.18 M). All reactions were carried out for 30 min at 37 °C. The peaks at retention times of 8.7, 10.4, 13.2, 14.6, and 18.1 min correspond to α -D-glucose, β -D-glucose, n-pentyl β -D-glucoside, n-hexyl β -D-glucoside, and glucocerebroside respectively. The arrow indicates the elution position of n-pentyl β -D-glucoside which was observed only in reaction mixtures which contained n-pentanol (b).

Glucocerebrosidase is highly specific for the β -glucoside linkage and will not catalyse the hydrolysis of *p*NPGal.

Hydrolysis of retinyl glucoside

Fractions corresponding to the h.p.l.c. peak at 12.3 min were pooled and used for structural determinations. The absorption spectrum of the reaction product was compared with that of a retinol standard and was found to have an absorption spectrum typical of retinoid compounds (results not shown). The product was then subjected to hydrolysis by almond β -glucosidase and the hydrolysis mixture was analysed by reversed-phase h.p.l.c. Following hydrolysis by almond β -glucosidase, the retention time of the starting material shifted from 12.3 min to 19.0 min, the latter peak corresponding precisely to the retention time of the retinol standard. In addition, the amount of glucose produced by the incubation of the retinol transglucosylation product with almond β -glucosidase was estimated using an enzymic assay, and the amount of retinol was estimated by calibration of the h.p.l.c. column with a retinol standard; the concentration ratio of glucose to retinol obtained was 0.99. From these data and given the known specificity of almond β -glucosidase, we conclude that the product formed in the transglucosylation reaction between retinol and *p*NPGlc or glucocerebroside is retinyl β -D-glucoside.

Having demonstrated that the broad-specificity β -glucosidase from almond hydrolyses retinyl β -glucoside, we next examined the ability of the two prominent broad-specificity mammalian β -

Figure 4 Demonstration of the glucocerebrosidase-catalysed transglucosylation reaction between pNPGlc and retinol

H.p.l.c. analysis of a reaction mixture containing pNPGlc (5 mM), TdC (0.5%, w/v), retinol (0.5 mM), and glucocerebrosidase (66 units). In (a), the glucocerebrosidase had first been inactivated by boiling; in (b) the glucocerebrosidase had not been heat-treated. The samples were chromatographed on a C_{18} column using a mobile phase consisting of methanol/water (85:15, v/v) at a flow rate of 0.8 ml/min. Under these conditions, pNP and pNPGlc coeluted at ~4 min and retinol eluted at 20.2 min. The peak at 12.3 min was obtained only for the incubation depicted in (b) and was determined to be retinyl-glucoside.

Figure 5 Comparison of the susceptibility of retinyl β -D-glucoside to hydrolysis by glucocerebrosidase and two guinea pig β -glucosidases

Retinyl β -glucoside formed in the transglucosylation reaction catalysed by glucocerebrosidase was incubated for 1 h with (b) guinea pig cytosolic β -glucosidase (60 units), (c) guinea pig TX-100 β -glucosidase (60 units), or (d) human placental glucocerebrosidase (2000 units). A control incubation containing retinyl β -glucoside in 0.2 M sodium citrate buffer without enzyme is shown in (a). The placental glucocerebrosidase incubation was carried out in 0.1 M sodium acetate, pH 5.5, containing 0.5% (w/v) TdC; the guinea pig β -glucosidase incubations were carried out in 0.2 M sodium citrate, pH 6.0. The appearance of a peak corresponding to the retention time of retinol was used to estimate the extent of retinyl β -glucoside hydrolysis.

glucosidases, namely the cytosolic and the TX-100 β -glucosidases, to hydrolyse this retinyl derivative which is synthesized by glucocerebrosidase. Equal amounts of retinyl β -glucoside

were subjected to hydrolysis by incubation with equivalent amounts (60 units) of cytosolic or TX-100 β -glucosidase from guinea pig liver. As shown in Figures 5(b) and 5(d), both the cytosolic and the TX-100 β -glucosidases hydrolyse retinyl β -glucoside. No hydrolysis was observed when retinyl glucoside was incubated for 1 h with 2000 units of glucocerebrosidase in the presence of detergent (TdC; 0.5%, w/v) (Figure 5d).

DISCUSSION

The main finding of this report is that human placental glucocerebrosidase catalyses a transglucosylation reaction *in vitro* between glucocerebroside and alcohols such as n-pentanol and retinol. The glucocerebrosidase-catalysed transglucosylation reaction with retinol results in the synthesis of a novel glucoside, retinyl β -D-glucoside.

Using highly purified human glucocerebrosidase, we have demonstrated stimulation of the hydrolytic activity of the enzyme by n-pentanol. Based on analogy to other glycosidases whose mechanisms have been more extensively studied [20–22], including the broad-specificity mammalian β -glucosidase [18], the probable reaction pathway for hydrolysis by the lysosomal β -glucosidase is that shown in Figure 6. Following the formation of the Michaelis complex, glucocerebroside–enzyme (CerGlc–E), and the expulsion of the aglycone, ceramide (Cer), the enzyme–glucosyl intermediate (Glc–E) is formed. Deglucosylation of the enzyme can then occur either by hydrolysis (k_3) to produce free glucose or by alcoholysis (k_4) to produce a transglucosylation product, in this example n-pentyl β -D-glucoside. The apparent stimulation of hydrolytic activity by n-pentanol is a consequence of k_3 being the rate-limiting step in the absence of n-pentanol. k_4 is greater than k_3 because, relative to water, n-pentanol is the preferred nucleophilic acceptor of glucose.

Although both the lysosomal β -glucosidase, glucocerebrosidase, and its cytosolic counterpart, the broad-specificity β -glucosidase, are both activated by n-alkanols [14], there are some marked differences in the kinetic effects of alcohols on the two enzymes. First, whereas activation of the cytosolic β -glucosidase is observed at relatively low n-pentanol concentrations (< 0.10 M), inhibition of a competitive nature is observed at alcohol concentrations greater than 0.10 M [15]. We hypothesized that this biphasic response reflects the presence of two distinct alcohol-binding sites on the cytosolic enzyme, a high-affinity site, occupation of which results in activation of the enzyme, and a low-affinity site [15]. This second, lower-affinity alkanol-binding site may be part of a hydrophobic domain at or close to the active

Figure 6 A proposed reaction pathway for the degradation of glucocerebroside in the absence and presence of n-pentanol

Cer, ceramide; CerGlc, glucocerebroside; E, glucocerebrosidase; Glc, glucose.

centre of the enzyme. In contrast, glucocerebrosidase is not inhibited by n-pentanol at concentrations up to 0.18 M, indicating that n-alkanols bind at only one site on the lysosomal β -glucosidase (Figure 1).

Morimoto et al. [23] have proposed a four binding site model for glucocerebrosidase which includes sites for the sugar and hydrophobic aglycone portions of the substrate, as well as separate sites for stimulatory acidic lipids, e.g. PtdSer, and the sphingolipid activator protein, SAP-2. It has previously been reported that PtdSer and inhibitory glucosylsphingosine compete for the same site on the enzyme [24]. However, Ohashi et al. [25] examined the kinetic properties and stability of six different mutant alleles produced by site-directed mutagenesis and found that glucocerebrosidase mutated at amino acid position 463 exhibited a normal response to glucosylsphingosine but an abnormal response to PtdSer. Based on these results, they concluded that additional lipid-binding sites exist on glucocerebrosidase that have yet to be identified. The n-pentanol (glucose acceptor)-binding site may be such a binding site.

Of particular interest to us is the observation that a higher rate of hydrolysis was obtained when n-pentanol and PtdSer were present simultaneously in the incubation medium than when the enzyme was incubated in the presence of either activator separately. The ability of n-pentanol to further increase the hydrolytic activity of glucocerebrosidase suggests that PtdSer and n-pentanol affect the enzyme's catalytic activity by different mechanisms. Using n-butanol-delipidated glucocerebrosidase from rat liver and human spleen, Basu and Glew [26] reported that PtdSer enhances catalytic activity by inducing a conformational change in the enzyme which results in a more favourable orientation of the catalytic site. The synergistic effects obtained when PtdSer and n-pentanol are used as co-activators (Figure 1) may result from the alcohol-binding site of glucocerebrosidase being brought into close proximity to the catalytic site by the PtdSer-induced conformational change. A similar conformational change was recently reported for another PtdSer-dependent enzyme, protein kinase C [27]. Activation of protein kinase C by PtdSer results from a reversible conformational change in the enzyme which leads to the exposure of the autoinhibitory pseudosubstrate domain, resulting in increased susceptibility to proteolysis by arginase C.

It is not known whether glucocerebrosidase catalyses transglucosylation reactions *in vivo*, and if so, whether such a transglucosylating activity is physiologically significant. However, we have demonstrated that the physiologically relevant amphipathic alcohol, retinol, can serve as a glucose acceptor for the glucocerebrosidase reaction *in vitro*. These results raise the possibility that such a reaction could occur *in vivo* in pathophysiological states in which the requisite concentrations of substrate (glucocerebroside) and acceptor (e.g. retinol) exist. A retinyl-glucoside product was obtained when either the non-physiological substrate *p*NPGlc or the naturally occurring glucocerebroside was used as the glucose donor. The structure of the retinyl sugar formed in both reactions was determined to be retinyl β -D-glucoside, based on its susceptibility to hydrolysis by three specific β -glucosidases: almond β -glucosidase and two broad-specificity β -glucosidases derived from guinea pig liver. In addition, equimolar amounts of retinol and glucose were obtained following exhaustive hydrolysis of the product by almond β -glucosidase.

In a recent study of 356 individuals, we reported that there is a wide range of cytosolic β -glucosidase activity in human leucocyte preparations [28]. In addition, no correlation was observed between the cytosolic β -glucosidase and glucocerebrosidase activities, ($r = 0.035$), indicating essentially total in-

dependence of expression of the two β -glucosidases in leucocytes. Knowing now that two mammalian broad-specificity β -glucosidases can catalyse the hydrolysis of retinyl β -glucoside, the transglucosylation product of the glucocerebrosidase reaction, we speculate that the natural variability in the level of cytosolic β -glucosidase activity in human tissues may be a factor in the phenotypic variation observed in Gaucher disease. Markedly reduced levels of cytosolic β -glucosidase activity have been reported in several patients with the more severe neurological form of Gaucher disease [29,30].

In summary, this report documents the occurrence of a glucocerebrosidase-catalysed transglucosylation reaction between glucocerebroside and two alcohols, n-pentanol and retinol. This reaction occurs both in the presence and absence of PtdSer and TdC, known glucocerebrosidase activators. In addition, the retinyl derivative synthesized in the presence of the lysosomal β -glucosidase is readily hydrolysed by two broad-specificity β -glucosidases widely distributed in mammalian tissues. These observations suggest a possible role of the transglucosylating activity of glucocerebrosidase in the pathology of Gaucher disease. The physiological implications of this observation in the normal metabolic state and in Gaucher disease and other lysosomal storage disorders warrant further investigation.

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