Biochem. J. (1989) 262, 541–548 (Printed in Great Britain)

Kinetic analysis of the interaction of alkyl glycosides with two human β-glucosidases

Venkatakrishnan GOPALAN,* Lydia B. DANIELS,* Robert H. GLEW*‡ and Marc CLAEYSSENS†
*Department of Microbiology, Biochemistry and Molecular Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, U.S.A., and †Laboratorium voor Biochemie, Rijksuniversiteit-Gent, B-9000 Gent, Belgium

This paper addresses the similarities and differences in the topology of the catalytic centres of human liver cytosolic β-glucosidase and placental lysosomal glucocerebrosidase, and utilizes well-documented reversible active-site-directed inhibitors. This comparative kinetic study was performed mainly to decipher the chemical and structural nature of the active site of the cytosolic β-glucosidase, whose physiological function is unknown. Specifically, analysis of the effects of a family of alkyl β-D-glucosides on the two glucosidases has led us to conclude that, relative to lysosomal glucocerebrosidase, the soluble β-glucosidase has a much more hydrophobic subsite in its catalytic centre. The alkyl β-D-glucosides consistently displayed 100–250-fold lower inhibition constants with the cytosolic broad-specificity β-glucosidase compared with the placental glucocerebrosidase; for example, with octyl β-D-glucoside the K_i values were 10 μM and 1490 μM for the cytosolic and lysosomal β-glucosidases respectively. Furthermore the higher affinity of the cytosolic β-glucosidase than glucocerebrosidase for the amphipathic alkyl β-D-glucosides was validated by the greater increase in the free energy of binding with increasing alkyl chain length [$\Delta\Delta G^0(K_i)/CH_2$: lysosomal enzyme, 2.01 kJ/mol (480 cal/mol); cytosolic enzyme, 3.05 kJ/mol (730 cal/mol)]. The implications of the presence of highly non-polar domains in the active site of the cytosolic β-glucosidase are discussed with regard to its potential physiological substrates.

INTRODUCTION

Human visceral organs such as liver, spleen and kidney contain two prominent β-glucosidases (Daniels *et al.*, 1981). The lysosomal β-glucosidase (glucocerebrosidase, glucosylceramidase, EC 3.2.1.45) catalyses the hydrolysis of glucocerebroside to glucose and ceramide. The cytosol contains a β-glucosidase that catalyses the hydrolysis of a variety of glycosides of *p*-nitrophenol and 4-methylumbelliferone, but not glucocerebroside; highest activity is expressed with aryl β-D-glucosides, followed by β-D-galactosides, α-L-arabinosides, β-D-fucosides and β-D-xylosides. The physiological function of this enzyme remains an enigma.

The lysosomal β-glucosidase has been extensively characterized; its deficiency causes the lipid-storage disorder known as Gaucher's disease (Glew *et al.*, 1988). In the absence of a clearly defined physiological substrate for the cytosolic β-glucosidase, speculations have been made with regard to its function. Previous investigations in our laboratory have implicated two possible roles. First, on the basis of the ability of glycosphingolipids to inhibit the broad-specificity β-glucosidase, LaMarco & Glew (1985) suggested that the enzyme is structurally related to the lysosomal β-glucosidase and that it plays a role in sphingolipid metabolism *in vivo*. Alternatively, as LaMarco & Glew (1986) demonstrated in their study of the guinea-pig liver cytosolic β-glucosidase, this enzyme may be involved in the hepatic detoxification of xenobiotic glycosides (e.g. L-picein, dhurrin and glucocheirolin). In addition, others (Williamson *et al.*, 1972*a*,*b*) have implicated a hepatic β-glucosidase in the metabolism and excretion of steroid glycosides.

In an effort to further our understanding of the structure and function of the human liver cytosolic β-glucosidase and also to investigate its similarity to the lysosomal β-glucosidase, a comparative kinetic study of the two enzymes was performed. This twofold objective was achieved with the use of amphiphilic alkyl β-D-glycosides as active-site-directed inhibitors of the two enzymes. De Bruyne & Yde (1977) were one of the first groups to use alkyl β-D-thiogalactopyranosides to obtain evidence of a hydrophobic pocket in the active site of *Escherichia coli* β-galactosidase. Osiecki-Newman *et al.* (1987) used an analogous series of lipoidal alkyl glucosides and alkyldeoxynojirimycins as inhibitors, and sphingosine derivatives as alternative substrates, to arrive at a three-domain model for the active site of lysosomal glucocerebrosidase. Use of some of these same inhibitors in a later study led Legler & Bieberich (1988) to speculate that the calf liver cytosolic β-glucosidase contains a hydrophobic cleft in its active site and that it could accommodate an n-alkyl chain composed of up to ten methylene groups.

These reports from different laboratories stimulated us to conduct a comparative study of the active sites of lysosomal and cytosolic β-glucosidases isolated from the same mammalian species, namely man. By so doing, we were able to minimize differences in assay conditions and experimental parameters for the two β-glucosidases. We undertook, therefore, a study of the interaction of a series of common active-site-directed reversible inhibitors with human liver cytosolic β-glucosidase and placental lysosomal glucocerebrosidases to explore the similarities and differences in the topologies of the two enzymes' active sites. Use of the alkyl β-D-glucoside series in this

‡ To whom correspondence should be addressed.

way enabled us to distinguish the two enzymes clearly as possessing distinct aglycone-binding subsites in their catalytic centres. In this paper we discuss the implications of our finding that the soluble cytosolic β-glucosidase has a more hydrophobic domain in its active site relative to the lysosomal glucocerebrosidase, an integral membrane protein. This comparative study has allowed us to draft a model of the active site of the cytosolic β-glucosidase. This model is based ideologically largely on one proposed by Osiecki-Newman *et al.* (1987) for lysosomal glucocerebrosidase.

MATERIALS AND METHODS

Materials

4-Methylumbelliferyl β-D-glucoside, glucosylsphingosine and a series of n-alkyl β-D-glucosides (C_6–C_{12}) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Phosphatidylserine was obtained from Supelco Co., Bellefonte, PA, U.S.A. All other reagents were obtained from Fisher Scientific Co., Pittsburgh, PA, U.S.A., and were of analytical-purity grade. Alkyl β-D-thioxylosides, alkyl β-D-glucosides (C_4 and C_5) and octyl β-D-galactoside were synthesized as described elsewhere (De Bruyne & van der Groen, 1972). The placental lysosomal glucocerebrosidase was generously given by Dr. J. A. Barranger, Children's Hospital, Los Angeles, CA, U.S.A. This enzyme was purified to homogeneity. Details of the purification scheme have been reported in Aerts *et al.* (1986).

Preparation of cytosolic β-glucosidase

Human liver was obtained at the time of autopsy from individuals free of liver disease. The broad-specificity β-glucosidase was purified to homogeneity after chromatography on an anion-exchange column and an octyl-Sepharose affinity column as described elsewhere (DePetro, 1987). The cytosolic β-glucosidase was demonstrated to be free of glucocerebrosidase activity by its resistance to inhibition by conduritol B epoxide, a specific irreversible inhibitor of the lysosomal β-glucosidase (Daniels & Glew, 1982).

Enzyme assays

All enzyme assays were performed at 37 °C for 15–30 min.

The standard incubation medium for the cytosolic β-glucosidase contained 0.2 M-sodium acetate buffer, pH 5.5, 5.0 mM-4-methylumbelliferyl β-D-glucoside and various amounts of protein in a final volume of 0.1 ml. 2-Mercaptoethanol (2.0 mM) and bovine serum albumin (2.0 mg/ml) were also included in the assay medium to stabilize the enzyme.

The standard assay medium for glucocerebrosidase contained 0.2 mM-sodium acetate buffer, pH 5.5, 5.0 mM-4-methylumbelliferyl β-D-glucoside and 25 μg of phosphatidylserine in a final volume of 0.1 ml.

Both enzyme assays were terminated by the addition of 2.9 ml of 0.2 M-glycine/NH_3 buffer, pH 10.5, and 4-methylumbelliferone release was determined fluorimetrically (Peters *et al.*, 1975). One unit of enzyme activity represents 1 nmol of glucose released/h at 37 °C.

Kinetic analyses

The kinetic parameters K_m and $V_{max.}$ were determined from Lineweaver–Burk plots (Segel, 1975). The initial reaction rates and their corresponding substrate concentrations were subjected to a computer analysis that fits the data to a rectangular hyperbola by using the mathematical procedure described by Cleland (1979) and one that places greater weight on values obtained at the higher substrate concentrations. The computer program calculates K_m, $V_{max.}$ and the standard errors for these values. The means of the standard errors for K_m and $V_{max.}$ values in all our kinetic analyses averaged 10% and 6% respectively. Determination of K_i values was made by using standard methods by replotting of the slope of each line from the Lineweaver–Burk analysis versus inhibitor concentration. The replots were accepted only when they yielded correlation coefficients greater than or equal to 0.99. The standard free energy of inhibitor binding $[\Delta G^0(K_i)]$ was calculated by using the Gibbs equation:

$$\Delta G^0 = -RT \cdot \ln K_{eq.}$$

where $K_{eq.} = 1/K_i$ (M^{-1}), $T = 310$ K and $R = 8.3144$ $J \cdot mol^{-1} \cdot K^{-1}$ (1.9872 $cal \cdot mol^{-1} \cdot K^{-1}$). Correlation of the free energy of binding $[\Delta G^0(K_i)]$ of the alkyl β-D-thioxylosides and alkyl β-D-glucosides with the free energy of octanol/water partitioning $[\Delta G^0(K_p)]$ and with carbon number followed the analysis procedure of De Bruyne & Yde (1977) and Deleyn *et al.* (1980).

Protein determination

Protein was determined by the method of Bradford (1976), with bovine serum albumin as standard.

RESULTS

Inhibition of the cytosolic β-glucosidase by alkyl β-D-thioxylosides

LaMarco & Glew (1985) reported that the broad-specificity β-glucosidase of human liver is inhibited by amphipathic β-D-glycosides, glucosylsphingosine and galactosylsphingosine and argued for the presence of glycone and hydrophobic aglycone domains at the enzyme's active site. In addition, Legler & Bieberich (1988), in their extensive kinetic analysis of the active site of calf liver cytosolic β-glucosidase with the use of aliphatic and cycloaliphatic alkyl glucosides and n-alkyl derivatives of β-glucosylamine and l-deoxynojirimycin, obtained convincing evidence of a hydrophobic cleft in its catalytic centre. In order to learn more about the hydrophobic domain in the active site of the human liver cytosolic β-glucosidase, we first investigated the inhibition of the enzyme by a homologous series of n-alkyl β-D-thioxylosides in which the length of the alkyl chain was varied from C_4 to C_7.

Lineweaver–Burk analyses of the inhibition data obtained with these thioxylosides revealed purely competitive inhibition kinetics (results not shown). The K_i values were obtained by replotting the slope of each Lineweaver–Burk plot versus inhibitor concentration. The finding of a linear slope replot in each case confirmed that a single-site interaction was occurring between inhibitor and the enzyme.

In the following kinetic analyses performed with these alkyl glycosides we have proceeded under the basic assumption that these compounds are extremely poor substrates for the β-glucosidases and hence that K_m would be equal to K_s. This assumption is supported by

Table 1. Summary of the free energy of binding [$\Delta G^0(K_i)$] to human liver cytosolic β-glucosidase and the free energy of octanol/water partitioning [$\Delta G^0(K_p)$] of alkyl thioxylosides

C_n denotes the number of carbon atoms in the alkyl chain. The data in this Table are presented in graphical form in Fig. 1.

C_n alkyl thioxyloside	K_i (μM)	$-\Delta G^0(K_i)$ [kJ/mol (cal/mol)]	$-\Delta G^0(K_p)$ [kJ/mol (cal/mol)]
C_4	4720	13.81 (3300)	0.21 (50.0)
C_5	1810	16.28 (3890)	3.14 (750)
C_6	564	19.29 (4610)	5.86 (1400)
C_7	120	23.26 (5560)	8.79 (2100)

the report by Legler & Bieberich (1988), wherein using calf liver cytosolic β-glucosidase they demonstrated that alkyl β-D-glucosides exhibit $K_{cat.}$ values that were less than 5% of that of 4-methylumbelliferyl β-D-glucoside. There is no report of the hydrolysis of alkyl β-D-glucosides by the lysosomal β-glucosidase, glucocerebrosidase. However, we assume the binding of alkyl β-D-glucosides to this enzyme inhibits the rate-determining step in the hydrolysis of 4-methylumbelliferyl β-D-glucoside.

The competitive nature of the inhibition caused by each of the alkyl thioxylosides led us to assume the inhibition constant, K_i, to be the reciprocal of the equilibrium constant, $K_{eq.}$, for the formation of the enzyme–inhibitor complex E–I ($E+I \rightleftharpoons E\text{–}I$). Hence the standard free energy of binding [$\Delta G^0(K_i)$] of each thioxyloside to the β-glucosidase could be calculated by substituting $1/K_i$ for $K_{eq.}$ in the Gibbs equation:

$$\Delta G^0 = -RT \cdot \ln K_{eq.}$$

Furthermore, the importance of the hydrophobicity of the inhibitor's alkyl chain to inhibitor binding was assessed by comparing the standard free energy of binding [$\Delta G^0(K_i)$] of each inhibitor to the enzyme with the standard free energy of partitioning [$\Delta G^0(K_p)$] of the same thioxyloside in an octanol/water two-phase system.

The free energy of binding [$\Delta G^0(K_i)$] and the free energy of partitioning [$\Delta G^0(K_p)$] of the C_4–C_7 alkyl thioxylosides reported in Table 1 were used to derive a relationship between $\Delta G^0(K_i)$, $\Delta G^0(K_p)$ and the carbon number of the alkyl chain of the thioxylosides. As shown in Fig. 1, for the C_4–C_7 alkyl thioxylosides there was a strictly linear increase between the negative free energy of binding to the cytosolic β-glucosidase [$\Delta G^0(K_i)$] and their negative free energy of octanol/water partitioning [$\Delta G^0(K_p)$] versus the alkyl chain length of the thioxylosides. These results support the hypothesis that the strength of the thioxyloside–enzyme interaction is largely a function of the hydrophobicity of the inhibitor.

The changes in both the standard free energies of binding [$\Delta G^0(K_i)$] caused by the inclusion of an additional methylene group in the alkyl chain of the thioxyloside series [$\Delta\Delta G^0(K_i)/CH_2$ and $\Delta\Delta G^0(K_p)/CH_2$] are strikingly similar [−3.14 kJ/mol (−750 cal/mol) and −2.85 kJ/mol (−680 cal/mol) respectively]. This strong correlation (slope = 1.1, $r = 0.994$) is apparent in the replot of $\Delta G^0(K_i)$ versus $\Delta G^0(K_p)$ for the alkyl thioxyloside series (see inset to Fig. 1).

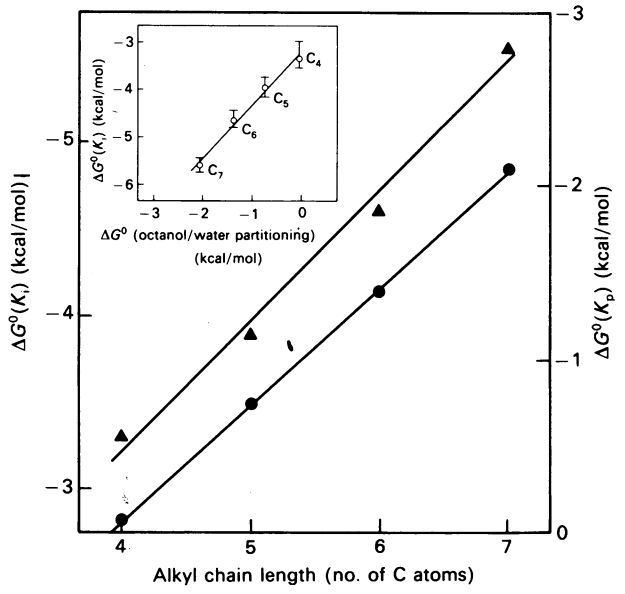

Fig. 1. Plots of the free energy of binding [$\Delta G^0(K_i)$] of alkyl β-D-thioxylosides to human liver cytosolic β-glucosidase (▲) and their free energy of partitioning [$\Delta G^0(K_p)$] in octanol/water (●) versus the number of carbon atoms in the alkyl chains

The inset shows the correlation by linear-regression analysis of the $\Delta G^0(K_i)$ of binding of alkyl β-D-thioxylosides to human liver cytosolic β-glucosidase, as calculated from the K_i values, to the $\Delta G^0(K_p)$ of octanol/water partitioning of the alkyl chains. Conversion factor: 1 cal = 4.184 J.

Inhibition of cytosolic β-glucosidase and lysosomal glucocerebrosidase by n-alkyl β-D-glucosides

On the basis of the result obtained with the alkyl β-D-thioxylosides, we were interested in determining the relationship between the free energy of binding [$\Delta G^0(K_i)$] and the size of the alkyl glycoside inhibitors whose alkyl chains extended beyond C_7. For this study we used n-alkyl β-D-glucosides ranging in chain length from C_6 to C_{12}, mainly because the higher-chain-length n-alkyl β-D-thioxylosides were not available to us. In addition, this C_6–C_{12} alkyl β-D-glucoside series enabled us to carry out a comprative kinetic analysis of the cytosolic and lysosomal β-glucosidases. Despite their ability to inhibit the broad-specificity cytosolic β-glucosidase, the thioxylosides do not inhibit the lysosomal glucocerebrosidase, since the active site of the latter has a highly specific glycone domain that recognizes only β-D-glucosides.

The same kinetic approach that was used with the thioxylosides series of inhibitors was used to determine K_i values for the alkyl β-D-glucosides. Lineweaver–Burk analyses and the corresponding replots of slope versus inhibitor concentration for the two β-glucosidases and a representative alkyl β-D-glucoside are shown in Figs 2 and 3. Fig. 2 illustrates the competitive nature of the inhibition obtained with pentyl β-D-glucoside and the cytosolic β-glucosidase; the K_i value calculated from the slope replot (see inset to Fig. 2) was 243 μM. An identical pattern was observed when nonyl β-D-glucoside was

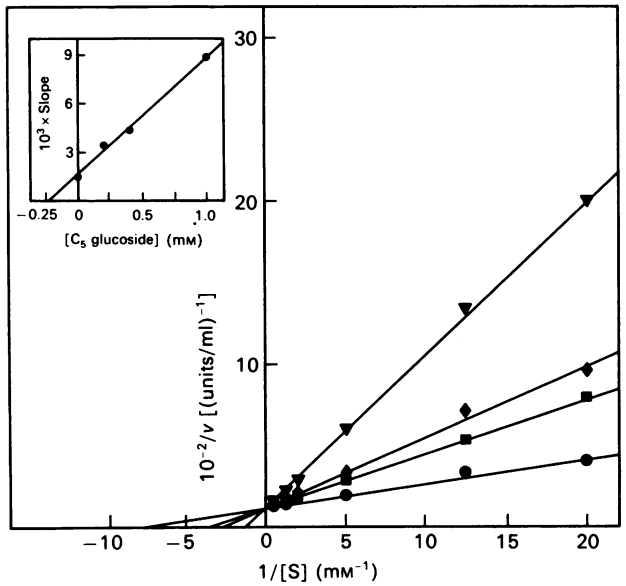

Fig. 2. Lineweaver–Burk analysis of cytosolic β-glucosidase activity measured in the presence of various concentrations of pentyl β-D-glucoside

The activity was measured by using the standard β-glucosidase assay over a 40-fold range of 4-methylumbelliferyl β-D-glucoside concentrations in the absence (●) or in the presence of 2 mM- (■), 4 mM- (◆) and 10 mM-pentyl β-D-glucoside (▼). The inset is a replot of the slope of each line from the Lineweaver–Burk plot as a function of inhibitor concentration and was used to calculate the K_i of the inhibitor.

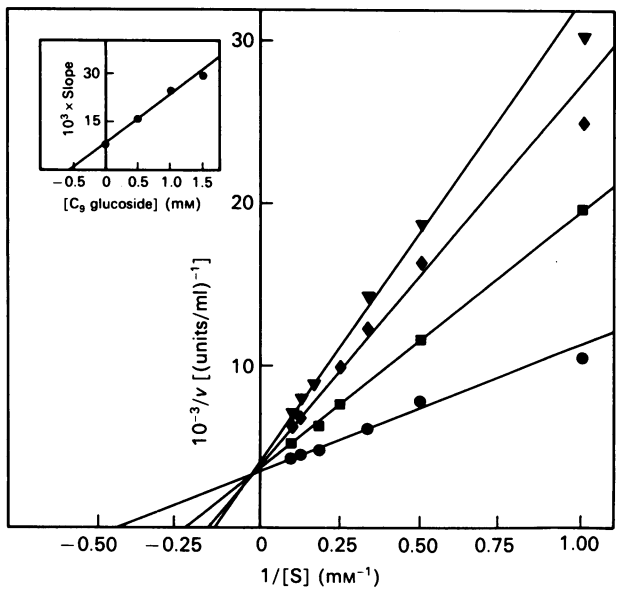

Fig. 3. Lineweaver–Burk analysis of glucocerebrosidase activity measured in the presence of various concentrations of nonyl β-D-glucoside

The activity was measured by using the standard β-glucosidase assay in the presence of 25 μg of phosphatidylserine/0.1 ml assay mixture over a 20-fold range of 4-methylumbelliferyl β-D-glucoside concentrations in the absence (●) or in the presence of 5 mM- (■), 10 mM- (◆) and 15 mM-nonyl β-D-glucoside (▼). The inset is a replot of the slope of each line from the Lineweaver–Burk plot as a function of inhibitor concentration and was used to calculate the K_i of the inhibitor.

used to inhibit the lysosomal glucocerebrosidase (Fig. 3); the linear replot of slope versus inhibitor concentration (see inset to Fig. 3) yielded a K_i value of 60 μM. These K_i values, as well as those reported below, are at least 10–20-fold below the critical micellar concentrations of these alkyl β-D-glucosides (Batz, 1985). Thus the inhibition that we have observed is the result of the binding of monomers and not micelles or aggregates to the β-glucosidases.

The inhibitions observed for the alkyl β-D-glucoside series (from hexyl to dodecyl) with the lysosomal and cytosolic β-glucosidases were markedly different in several respects (Fig. 4). First, the K_i values for the two β-glucosidases for any particular alkyl glucoside were markedly different. Specifically, the K_i values obtained with all of the alkyl β-D-glucosides and the lysosomal glucocerebrosidase were 100–250-fold greater than those of the cytosolic β-glucosidase (Table 2). For example, the K_i values for the octyl β-D-glucoside with the lysosomal and cytosolic β-glucosidases were 1490 μM and 10 μM respectively. To obtain a better understanding of the biophysical process involved in the enzyme–inhibitor interaction, these values were expressed as standard free energies of binding [$\Delta G^0(K_i)$] for each alkyl glucoside (from hexyl to dodecyl), and these free energies in turn were plotted versus the number of carbon atoms in the alkyl chain of the inhibitor. As shown in Fig. 4, the cytosolic β-glucosidase had a more negative standard free energy of binding for every alkyl glucoside studied, as is to be expected from its lower K_i values. This indicates that the alkyl glucosides bind more tightly to the cytosolic β-glucosidase relative to glucocerebrosidase. The different slopes observed with the two β-glucosidases are reflective of the distinct changes in the standard free energy of binding on increasing the alkyl chain length of the alkyl glucoside [$\Delta\Delta G^0(K_i)/CH_2$]. Finally, with the cytosolic β-glucosidase, the linear increase in the standard free energy of binding observed as the chain length of the alkyl glucoside is increased exhibits an abrupt break at C_9. The C_{10} and C_{12} alkyl glucosides displayed higher K_i values (5 μM and 24 μM respectively) and consequently lower free energies of binding than the values one would predict by extrapolating the linear plot from butyl β-D-glucoside to nonyl β-D-glucoside. This pattern was not observed with the lysosomal glucocerebrosidase (Fig. 4).

Inhibition of cytosolic and lysosomal β-glucosidases by octyl β-D-galactoside and glucosylsphingosine

To determine the effect of altering the glycone moiety without changing the hydrophobic alkyl chain of a particular alkyl glycoside, we determined the K_i values for octyl β-D-galactoside and the cytosolic and lysosomal β-glucosidases (Table 3). In each case competitive inhibition kinetics were observed (results not shown). For the cytosolic β-glucosidases the K_i for octyl β-D-galactoside was nearly the same as that of its glucosyl equivalent (for octyl β-D-galactoside $K_i = 9.0$ μM; for octyl β-D-glucoside $K_i = 10.0$ μM). In contrast, the K_i value for this particular alkyl galactoside with the lysosomal β-glucosidase was 4.4-fold higher than the K_i value for the corresponding alkyl glucoside (for octyl β-D-galactoside $K_i = 6.6$ mM; for octyl β-D-glucoside K_i

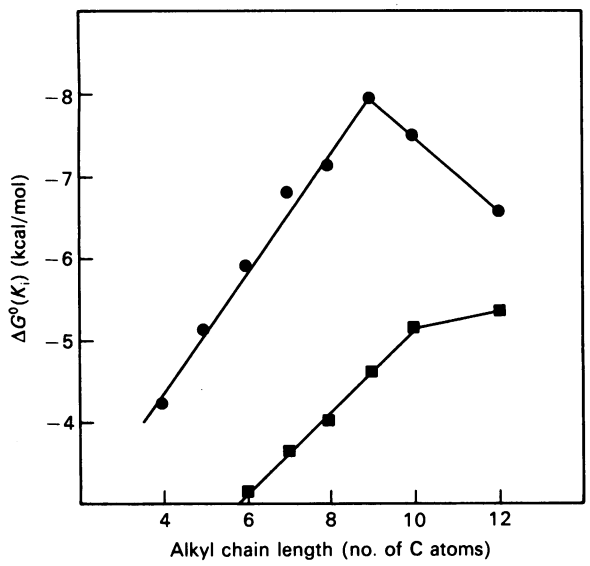

Fig. 4. Plots of free energy of binding [$\Delta G^0(K_i)$] of various alkyl β-D-glucosides to human liver cytosolic β-glucosidase (●) and placental glucocerebrosidase (■) as a function of the number of carbon atoms in their alkyl chains

Conversion factor: 1 cal = 4.184 J.

= 1.5 mM). These observations are in agreement with those made by Daniels *et al.* (1982), who showed that the cytosolic β-glucosidase exhibits broad specificity with regard to the glycone moiety, as oppposed to the lysosomal enzyme, which has strict specificity for glucosyl derivatives.

Glucosylsphingosine is also a potent inhibitor of the two β-glucosidases used in this study. Though Osiecki-Newman *et al.* (1987) and Legler & Bieberich (1988) have reported the inhibition constants, we have determined these K_i values since the assay conditions in those studies and the present one were different. Competitive and mixed-type inhibition kinetics were observed with the lysosomal and cytosolic β-glucosidases respectively. The mixed-type inhibition observed for the soluble β-glucosidase has previously been interpreted as indicating binding of the inhibitor with two sites on the enzyme. The nature of the inhibition caused by glucosylsphingosine is consistent with that described by others cited above. The K_i values for glucosylsphingosine with the cytosolic and lysosomal β-glucosidases were 26.0 μM and 13.1 μM respectively (Table 3).

Effect of phosphatidylserine on the K_i of heptyl β-D-glucoside for the cytosolic β-glucosidase

Since the activity of glucocerebrosidase is nearly absolutely dependent on acidic lipids, assays of the purified

Table 2. Summary of alkyl glucoside(s) inhibition study with human liver cytosolic β-glucosidase and placental lysosomal glucocerebrosidase

C_n indicates the number of carbon atoms in the alkyl chain. The data in this Table are presented in graphical form in Fig. 4. Abbreviation: N.D., not determined.

	Cytosolic enzyme		Lysosomal enzyme	
C_n alkyl glucoside	K_i (μM)	$-\Delta G^0(K_i)$ [kJ/mol (kcal/mol)]	K_i (mM)	$-\Delta G^0(K_i)$ [kJ/mol (kcal/mol)]
C_4	1050	17.70 (4.23)	N.D.	N.D.
C_5	243	21.46 (5.13)	N.D.	N.D.
C_6	67.4	24.77 (5.92)	6.20	13.10 (3.13)
C_7	15.6	28.53 (6.82)	2.70	15.23 (3.64)
C_8	9.98	29.71 (7.10)	1.49	16.78 (4.01)
C_9	2.47	33.30 (7.96)	0.60	19.12 (4.57)
C_{10}	5.26	31.38 (7.50)	0.24	21.51 (5.14)
C_{12}	23.7	27.45 (6.56)	0.17	22.38 (5.35)

Table 3. Comparison of K_i values for octyl β-D-glucoside, octyl β-D-galactoside, D-glucose and β-D-glucosylsphingosine with cytosolic and lysosomal β-glucosidases

	K_i (mM)		
Inhibitor	Cytosolic β-glucosidase (*A*)	Glucocerebrosidase (*B*)	K_i ratio (*B*/*A*)
Octyl β-D-glucoside	0.010	1.49	149
Octyl β-D-galactoside	0.009	6.60	733
D-Glucose	223	115	0.52
β-D-Glucosylsphingosine	0.026	0.013	0.50

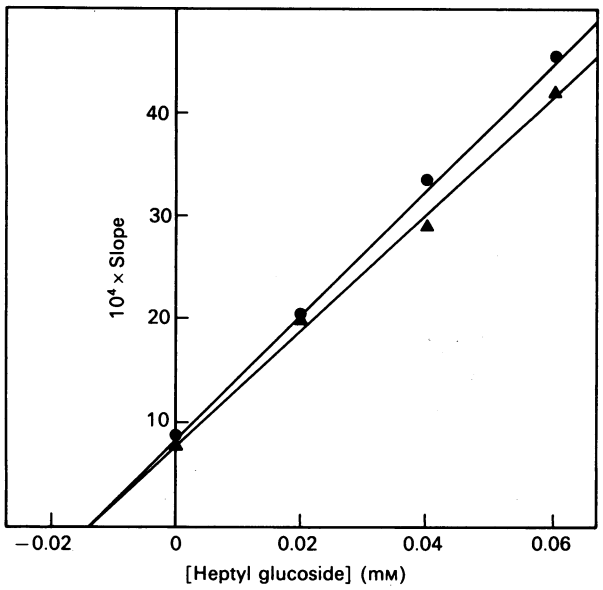

Fig. 5. Effect of phosphatidylserine (25 μg/0.1 ml assay mixture) on the inhibition constant (K_i) of heptyl β-D-glucoside for human liver cytosolic β-glucosidase

Slope replots of the inhibition kinetic data in the absence (▲) and in the presence (●) of phosphatidylserine are shown.

enzyme *in vitro* require the presence of a suitable lipid activator in the assay medium (Glew *et al.*, 1988). In the present study phosphatidylserine was routinely used in the lysosomal β-glucosidase assay (25 μg/0.1 ml). However, the activity of the soluble cytosolic enzyme does not depend upon any lipid activator. Hence all of the K_i determinations described above for the cytosolic enzyme were performed in the absence of phosphatidylserine. Since phosphatidylserine forms lipid bilayers in aqueous media, it was important to rule out the possibility that phosphatidylserine might have affected the K_i values obtained for glucocerebrosidase, presumably by altering the effective concentration of the alkyl β-D-glucosides. As a representative control experiment, we determined the K_i values for heptyl β-D-glucoside with the cytosolic β-glucosidase in the presence and in the absence of phosphatidylserine. As shown in Fig. 5, the inclusion of phosphatidylserine (25 μg/0.1 ml) in the cytosolic β-glucosidase assay medium had no effect on the K_i values obtained with heptyl β-D-glucoside and the cytosolic β-glucosidase (K_i = 13.8 μM in the absence of phosphatidylserine; K_i = 13.9 μM in the presence of phosphatidylserine). We conclude that the large differences in the K_i values between the cytosolic and lysosomal β-glucosidases are attributable to inherent differences in the two enzymes and not to the presence or the absence of phosphatidylserine in the assay medium.

DISCUSSION

This study exploits the ability of a series of alkyl β-D-glycosides to serve as active-site-directed inhibitors of both lysosomal and cytosolic β-glucosidases and documents differences between hydrophobic subsites in the catalytic centres of these two prominent human β-glucosidases.

The affinity of the alkyl glycosides for both the β-glucosidases increases on increasing the alkyl chain length, as is evident from the increasingly negative standard free energies of binding (Fig. 4). A comprehensive study by De Bruyne & Yde (1977) on the binding of alkyl β-D-thiogalactosides to *Escherichia coli* β-galactosidase revealed no variation in the enthalpy of binding (ΔH^0) on increasing the length of the alkyl chain of the inhibitor. Assuming that this applies to the two alkyl glycoside series used in the present study, namely the alkyl β-D-thioxylosides and alkyl β-D-glucosides, the increasingly negative free energies of binding probably arise from an increasingly positive entropy change. Since there is no variation in the hydrophilic glycone moiety of the alkyl glycoside series used here, the entropic contribution must be the result of the increase in hydrophobicity of the inhibitor as the alkyl chain is lengthened. The change in free energy introduced by a non-polar solute molecule in water should be proportional to the average number of water molecules that are forced to lie in contact with the solute's surface (Tanford, 1980). The accessible surface area of the alkyl glucosides exposed to water increases as their alkyl chain is lengthened, leading to contact with a greater number of reoriented water molecules. Hence the redistribution of a C_8 alkyl glycoside relative to a C_5 alkyl glycoside from water to the apolar domain in the active site of a particular enzyme should result in release of a greater number of reoriented water molecules to the less organized bulk water phase. Consequently the entropic contribution to the free energy of binding of C_8 alkyl glucoside is higher than that of C_5 alkyl glucoside.

The strong positive correlation that we observed between the standard free energies of binding of the C_4–C_7 alkyl thioxylosides to the cytosolic β-glucosidase and their standard free energies of partitioning between octanol and water (see inset to Fig. 1) points to an active site whose interior is apolar and probably similar to that of octanol in terms of hydrophobicity. This correlation establishes that the driving force for these two processes is the same, namely the transfer of the aglycone moiety of these alkyl glycosides from an aqueous phase to an apolar environment.

To appreciate fully the results of the comparative kinetic study of the lysosomal and cytosolic β-

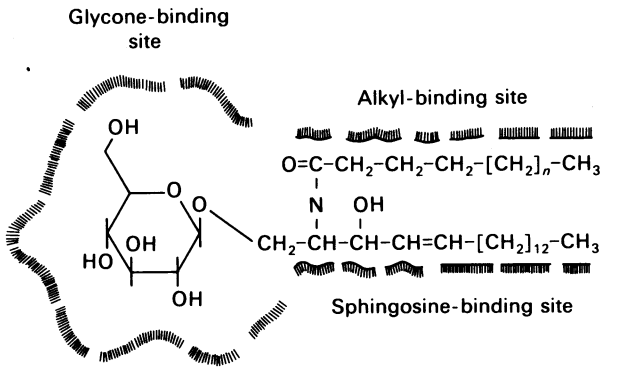

Fig. 6. Model for the active-site structure of mammalian β-glucosidase

It has been modified from Osiecki-Newman *et al.* (1987); the glycone-binding site, the hydrophobic alkyl-binding site and the sphingosine-binding site are indicated.

glucosidases, it is useful to refer to a three-domain model recently proposed for the active site of the lysosomal β-glucosidase. On the basis of the extensive kinetic studies performed with reversible competitive inhibitors, substrate analogues and glycosyl derivatives, Osiecki-Newman *et al.* (1987) constructed the model shown in Fig. 6. The binding sites were specified after taking into consideration the various moieties of the physiological substrate: the fatty acyl group of glucocerebroside (*N*-acylglucosylsphingosine) interacts with the proposed aglycone-binding site, glucose with the glycone-binding site, and sphingosine with the hydrophilic domain and the 'third site' (Fig. 6). We refer to this third site as the sphingosine-binding domain.

With reference to this model, we assume it is the aglycone-binding site of the lysosomal β-glucosidase that binds the hydrophobic moiety of the alkyl glucosides and that it is the glycone-binding subsite of the active site that binds the glucose moiety. On the basis of our comparative kinetic study with glucosylsphingosine and alkyl β-D-glucosides, we wish to adopt this same model and apply it to the active site of the cytosolic β-glucosidase.

The K_i values for glucosylsphingosine and the two β-glucosidases differed by only 2-fold (lysosomal enzyme, 13.1 μM; cytosolic enzyme, 26.0 μM). The higher K_i value for the cytosolic β-glucosidase relative to the lysosomal β-glucosidase is due to differences in the affinities of the two active sites for glucose, as is evident from the 2-fold difference in the K_i values for D-glucose (Table 3). Hence it seems likely that the sphingosine-binding domains of the two β-glucosidases are very similar. It is known that the sugar-binding sites of the two β-glucosidases are different, since the cytosolic enzyme acts on a variety of glycosides whereas glucocerebrosidase is highly specific for glucosides (Daniels *et al.*, 1982).

The alkyl β-D-glucosides (from butyl to dodecyl) acted as active-site-directed inhibitors of the lysosomal and cytosolic β-glucosidases. These competitive inhibitors were bound much more tightly by the cytosolic β-glucosidase than by the lysosomal glucocerebrosidase, as reflected by the 100–250-fold difference in their K_i values. We have shown in the present paper that differences in assay media cannot explain these large differences in K_i values (Fig. 5). Since it is the non-polar moiety of the alkyl glucosides that is presumed to interact with the aglycone-binding site in each of the β-glucosidases, we conclude that the aglycone-binding subsite in the catalytic domain of the soluble cytosolic β-glucosidase is considerably more hydrophobic than that of the lysosomal glucocerebrosidase.

The contribution of each additional methylene group to the free energy of binding of alkyl β-D-glucosides to the lysosomal β-glucosidase is less than that of the cytosolic β-glucosidase [$\Delta\Delta G^0(K_i)/CH_2$: lysosomal enzyme, 2.01 kJ/mol (480 cal/mol); cytosolic enzyme, 3.05 kJ/mol (730 cal/mol)]. This observation reinforces the contention that the broad-specificity β-glucosidase has a more apolar domain in its active site than its lysosomal counterpart.

In part because the broad-specificity β-glucosidase is located in the cytosol, previous research has been directed largely towards discovering some water-soluble β-glucoside as its physiological substrate. The fact that the active site of the soluble β-glucosidase is even more apolar than that of the lysosomal β-glucosidase that hydrolyses glucocerebroside prompts us to suggest that future studies of this type should consider amphipathic β-glucosides as potential substrates for the cytosolic β-glucosidase, especially those that have a very hydrophobic aglycone moiety. In this regard it would be worthwhile determining if β-D-glycosyl-1,2-di-*O*-acylglycerols are substrates for the broad-specificity β-glucosidase. These sugar-containing lipids are major structural components of the membranes of many plants (Quinn & Williams, 1983). The presence of two hydrophobic moieties and a varied polar glycosyl moiety would make them ideal substrates for the β-glucosidase.

This paper has shown that long-chain alkyl glucosides (e.g. octyl β-D-glucoside) inhibit the lysosomal β-glucosidase (glucocerebrosidase), but with K_i values that are 100–250-fold higher than those for its cytosolic counterpart, the broad-specificity β-glucosidase. Furthermore, we have taken advantage of the differing sugar-specificities of the two β-glucosidases and amplified this difference in K_i values by altering the glycone moiety of the alkyl glycoside inhibitor. Since the glycone domain in the active site of the lysosomal enzyme is highly specific for glucosyl derivatives, we expected to increase this difference in K_i values by using a long-chain alkyl galactoside. As shown in Table 3 and as expected, the lysosomal/cytosolic β-glucosidase K_i value ratio of 149:1 for octyl β-D-glucoside increased nearly 5-fold to 733:1 when octyl β-D-galactoside was used in place of octyl β-D-glucoside. This appears to be the first case of a β-glucosidase inhibitor that is orders of magnitude more effective against the cytosolic β-glucosidase than the lysosomal β-glucosidase.

Finally, a practical application of the results of the present study may be in the design of a new β-glucosidase assay with greater specificity for the detection of Gaucher heterozygotes. Diagnostic assays currently in use often employ the non-physiological fluorigenic 4-methylumbelliferyl β-D-glucoside as the substrate to detect the deficient lysosomal (acid) β-glucosidase. Interference by the cytosolic enzyme has been a contributing factor in the misclassification of controls with relatively low glucocerebrosidase activities and heterozygotes with high enzymic activities (Daniels & Glew, 1982). The inclusion of a specific inhibitor of the cytosolic β-glucosidase, such as octyl β-D-galactoside, in the diagnostic assay medium might lessen the likelihood of misidentification of heterozygotes. Such a specific inhibitor of the cytosolic enzyme could also serve as a probe to investigate its function *in vivo*. Comparative analyses of the metabolism and composition of mammalian cells cultured in the presence and in the absence of octyl β-D-galactoside could possibly provide clues to the physiological role of the cytosolic β-glucosidase.

This work was supported by the National Institutes of Health Grant no. AM31357.

REFERENCES

Aerts, J. M. F. G., Donker-Koopman, W. E., Murray, G. J., Barranger, J. A., Tager, J. M. & Schram, A. W. (1986) Anal. Biochem. **154**, 655–663

Batz, H.-G. (1985) Tensides as Aids in Biochemical Research (Technical Bulletin), Boehringer Mannheim, Tukzing

Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254

Cleland, W. W. (1979) Methods Enzymol. **63**, 103–138

Daniels, L. B. & Glew, R. H. (1982) Clin. Chem. **28**, 569–577
Daniels, L. B., Coyle, P. J., Chiao, Y.-B., Glew, R. H. & Labow, R. S. (1981) J. Biol. Chem. **256**, 13004–13012
Daniels, L. B., Gnarra, J. R. & Glew, R. H. (1982) in Gaucher Disease: A Century of Delineation and Research (Desnick, R. J., Gatt, S. & Grabowski, G. A., eds.), pp. 333–355, Alan R. Liss, New York
De Bruyne, C. K. & van der Groen, G. (1972) Carbohydr. Res. **25**, 59–65
DeBruyne, C. K. & Yde, M. (1977) Carbohydr. Res. **56**, 153–164
Deleyn, F., Claeyssens, M. & De Bruyne, C. K. (1980) Can. J. Biochem. **58**, 5–8
DePetro, J. J. (1987) M.S. Dissertation, University of Pittsburgh
Glew, R. H., Basu, A., La Marco, K. L. & Prence, E. M. (1988) Lab. Invest. **58**, 5–25
LaMarco, K. L. & Glew, R. H. (1985) Arch. Biochem. Biophys. **260**, 669–676
LaMarco, K. L. & Glew, R. H. (1986) Biochem. J. **237**, 469–476
Legler, G. & Bieberich, E. (1988) Arch. Biochem. Biophys. **260**, 427–436
Osiecki-Newman, K., Fabbro, D., Legler, G., Desnick, R. J. & Grabowski, G. A. (1987) Biochim. Biophys. Acta **915**, 87–100
Peters, S. P., Lee, R. E. & Glew, R. H. (1975) Clin. Chim. Acta **60**, 391–396
Quinn, P. J. & Williams, W. P. (1983) Biochim. Biophys. Acta **737**, 223–266
Segel, I. H. (1975) Enzyme Kinetics, pp. 100–160, Wiley–Interscience, New York
Tanford, C. (1980) The Hydrophobic Effect: Formation of Micelles and Biological Membranes, 2nd edn., Wiley–Interscience, New York
Williamson, D. G., Layne, D. S. & Collins, D. C. (1972*a*) J. Biol. Chem. **247**, 3286–3288
Williamson, D. G., Layne, D. S., Nilsen, M. & Hobkirk, R. (1972*b*) Can. J. Biochem. **50**, 958–962

Received 17 January 1989/31 March 1989; accepted 14 April 1989