A kinetic study of the effects of galactocerebroside 3-sulphate on human spleen glucocerebrosidase

Evidence for two activator-binding sites

Elizabeth M. PRENCE, Kevin O. GARRETT and Robert H. GLEW*

Department of Microbiology, Biochemistry and Molecular Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, U.S.A.

Extraction of control human spleen glucocerebrosidase with sodium cholate and butan-1-ol reversibly inactivates the enzyme in terms of its ability to hydrolyse the water-soluble substrate 4-methylumbelliferyl β -D-glucopyranoside (MUGlc). The acidic brain lipid galactocerebroside 3-sulphate (sulphatide) reconstitutes β -glucosidase activity in a strongly concentration-dependent manner. In this study we show that sulphatide exhibits three critical micellar concentrations (CMCs): CMC₁, 3.72 μ M; CMC₂, 22.6 μ M; CMC₃, 60.7 μ M. We designate the aggregates formed at these CMCs as primary, secondary and tertiary micelles respectively. From the results of kinetic studies performed at various sulphatide concentrations (0.012–248 μ M), we found that sulphatide monomers (< 3 μ M) decreased the K_m (for MUGlc) of control glucocerebrosidase from 11 to 4.6 mM, and lowered the V_{max} , 2-fold. However, secondary and tertiary micelles were required for expression of high control glucocerebrosidase activities. Glucocerebrosidase prepared from the spleen of a patient with non-neuronopathic type 1 Gaucher's disease exhibited a very low K_m (2.8 mM) even in the absence of exogenous lipid, and sulphatide monomers had no effect on the mutant enzyme's K_m or V_{max} . However, secondary or tertiary micelles markedly increased the V_{max} of the type 1 glucocerebrosidase to 60% of the corresponding control enzyme value. In contrast, for the glucocerebrosidase of the neuronopathic type 2 case, although sulphatide decreased the K_m from 9.2 to 1.7 mM, the V_{max} never reached more than 5% that of the control enzyme, even at high concentrations of sulphatide. In addition, we found that secondary and tertiary sulphatide micelles enhanced the rate of inactivation of all three glucocerebrosidase preparations by chymotrypsin. Collectively, these results indicate the presence of two sulphatide-binding sites on glucocerebrosidase: one that enhances substrate binding, and another that enhances catalysis.

INTRODUCTION

Glucocerebrosidase (EC 3.2.1.45) is the lysosomal enzyme that catalyses the hydrolysis of glucocerebroside to glucose and ceramide. A deficiency of this enzymic activity is responsible for Gaucher's disease (Brady *et al.*, 1965; Patrick, 1965). The disease presents in non-neuronopathic (type 1) and neuronopathic (types 2 and 3) forms (Fredrickson & Sloan, 1972).

Various acidic lipids have been identified that markedly stimulate glucocerebrosidase activity (Ho & Light, 1973; Basu *et al.* 1984, 1985; Basu & Glew, 1984, 1985); these include phosphatidylserine, gangliosides and galactocerebroside 3-sulphate. In addition, an 11000-dalton protein, designated heat-stable factor (HSF) or co-glucosidase (Berent & Radin, 1981), has been identified (Ho & O'Brien, 1971) that enhances the activation of glucocerebrosidase by acidic lipids (Prence *et al.*, 1985). HSF increases the activity of control glucocerebrosidase, but only when acidic lipids are also present in the assay medium (Garrett *et al.*, 1985).

Although much has been done to identify the properties and structural features of lipids that activate glucocerebrosidase, little is known about the mechanism of activation. We have previously shown that activation

of rat liver glucocerebrosidase by phosphatidylserine or gangliosides and HSF was accompanied by an increase in the size of the enzyme (Prence *et al.*, 1985; Basu & Glew, 1985).

Further, we have demonstrated that galactocerebroside 3-sulphate (sulphatide) was very effective in reconstituting the β -glucosidase activity of a preparation of control human spleen glucocerebrosidase that had been depleted of endogenous lipids (Glew *et al.*, 1986). Also, when used as the lipid activator of glucocerebrosidase *in vitro*, sulphatide permitted us to distinguish cases of type 1 and type 2 Gaucher's disease; in the presence of sulpholipid the mutant glucocerebrosidase from the type 1 case was 60% as active as the control, whereas the corresponding enzyme from the type 2 case had a specific activity that was less than 5% of the control (Glew *et al.*, 1986).

In the present investigation we have extended our studies of the sulphatide–glucocerebrosidase interaction. Specifically, we have correlated the concentration-dependent aggregation of the lipid with its ability to reconstitute glucocerebrosidase from normal human spleen. In addition, we have determined the kinetic constants, K_m and V_{max} , of glucocerebrosidase preparations from controls and patients with type 1 or type 2 Gaucher's disease, in the presence of various sulphatide

Abbreviations used: MUGlc, 4-methylumbelliferyl β -D-glucopyranoside; CMC, critical micellar concentration; HSF, heat-stable factor.

* To whom all correspondence should be addressed.

concentrations. We have also determined these kinetic parameters in the presence of HSF and mixtures of HSF and sulpholipid. The results of this study provide insight concerning the differences between the two mutant glucocerebrosidases, and also suggest that glucocerebrosidase possesses two distinct activator-binding sites, one that enhances the enzyme's affinity for substrate, and another that enhances catalysis.

MATERIALS AND METHODS

Materials

Bovine sulphatide (monogalactosyl stearate) was obtained from Supelco. Sodium taurodeoxycholate and 4-methylumbelliferyl β -D-glucopyranoside (MUGlc) were purchased from Sigma Chemical Co. Chymotrypsin A was obtained from Boehringer Mannheim. *N*-Phenyl-1-naphthylamine was purchased from Aldrich Chemical Co. All other reagents used were of the highest purity available.

Tissue samples

Spleen obtained at autopsy (4–6 h after death) from individuals free of central-nervous-system disease served as the source of control human glucocerebrosidase. Spleen obtained at time of splenectomy from patients with type 1 Gaucher's disease served as the source of mutant glucocerebrosidase and HSF. Spleen from a person with neuronopathic Gaucher's disease was the source of type 2 glucocerebrosidase.

Isolation of HSF

HSF was purified from the spleen of a person with type 1 Gaucher's disease, as described by Peters *et al.* (1977).

Preparation of glucocerebrosidase

Glucocerebrosidase was isolated and partially purified from frozen (-70°C) tissue as previously described (Prence *et al.*, 1985), resulting in a 10–15-fold purification for all preparations used in this study. Spleen membranes were extracted with sodium cholate, and delipidated with butan-1-ol. The partially purified enzyme preparations were used throughout this study. Addition of conduritol B epoxide to all three glucocerebrosidase preparations used in the present study resulted in greater than 95% inhibition of β -glucosidase activity. Since conduritol B epoxide (an active-site irreversible inhibitor of glucocerebrosidase) has been used to generate an animal model of Gaucher's disease (Kanfer *et al.*, 1975), and does not inhibit the non-specific mammalian β -glucosidase that is also present in spleen (Daniels *et al.*, 1980), we conclude that the β -glucosidase activity that we measured, with MUGlc as the substrate, is attributable to glucocerebrosidase.

β -Glucosidase assay

The standard β -glucosidase assay was performed as previously described (Prence *et al.*, 1985), with 5 mM-MUGlc in 0.2 M-sodium acetate buffer, pH 5.5, with 7–10 μg of enzyme protein, in a final volume of 100 μl . As indicated in the Figure legends, lipid activators were added in various concentrations either in the absence or in the presence of HSF. One unit of enzyme activity is defined as the amount of enzyme that cleaves 1 nmol of glucose from the substrate/h at 37°C .

Determination of CMCs

The CMCs of sulphogalactocerebroside (sulphatide) were determined by using the fluorescent dye *N*-phenyl-1-naphthylamine as described by Kovatchev *et al.* (1981). CMC determinations were carried out at 37°C in 0.2 M-sodium acetate buffer, pH 5.5.

Protein determination

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

Preparation of aqueous solutions of sulphatide

Powdered sulphatide was dissolved in distilled deionized water, and the resulting suspension was sonicated for 1 min at 24°C with use of the standard microtip on a Heat-Systems Ultrasonics cell disruptor. Sulphatide concentration was determined by the phenol/ H_2SO_4 method of McKelvey & Lee (1969).

RESULTS AND DISCUSSION

Activation of spleen glucocerebrosidase by sulphatide

Table 1 shows new results that are similar to those of our initial study (Glew *et al.*, 1986), in which we investigated the ability of sulphogalactocerebroside to activate glucocerebrosidase from control spleen and patients with type 1 or type 2 Gaucher's disease. Although sodium taurodeoxycholate, a bile salt that is widely used to activate glucocerebrosidase, was effective in reconstituting the control enzyme, the β -glucosidase specific activities seen for the types 1 and 2 enzyme preparations were very low (2–5% of control). However, when sulphatide was used as the activator lipid, relatively high specific activities were seen with the type 1 glucocerebrosidase. In the presence of sulphatide and HSF, the type 1 enzyme was 60% as active as the control glucocerebrosidase, whereas the type 2 enzyme was only 2–3% as active as the control. These results are similar to those obtained previously with phosphatidylserine as the activator lipid (Glew *et al.*, 1982; Garrett *et al.*, 1985). In those studies, the types 1 and 2 glucocerebrosidase preparations could be differentiated when phosphatidylserine was used as the lipid activator, but not when sodium taurocholate was employed as the activator.

Table 1. Activation of human spleen glucocerebrosidase by various lipids

Glucocerebrosidase isolated from controls or patients with type 1 or 2 Gaucher's disease was assayed as described in the Materials and methods section, with the addition of the indicated amounts of activators per 100 μl of assay mixture

Effector	β -Glucosidase specific activity (units/mg of protein)		
	Control	Type 1	Type 2
None	35.1	1.41	0.96
Sodium taurodeoxycholate (0.2 mg)	510	27.4	9.39
Sulphatide (20 μg)	210	180	5.77
Sulphatide (20 μg) + HSF (0.2 μg)	317	192	6.30

However, considering the results of all three studies, we conclude that, though phosphatidylserine and sulphatide are both effective in reconstituting delipidated glucocerebrosidase preparations from controls and cases of type 1 Gaucher's disease, the sulphated glycolipid is much more effective at differentiating the glucocerebrosidase of type 1 and type 2 cases. However, phosphatidylserine activates control glucocerebrosidase to the same extent as sodium taurocholate (results not shown), whereas sulphatide is less effective than the bile salt in reconstituting control activity (Table 1).

Our goal in the present study was to correlate the aggregation state of the lipid with the concentration of sulphatide required to activate glucocerebrosidase. Furthermore, since we had found in our earlier study that inclusion of sulphatide in the β -glucosidase assay permitted one to distinguish glucocerebrosidase preparations from patients with non-neuronopathic and neuronopathic Gaucher's disease (Glew *et al.*, 1986), we also wanted to compare the effects of sulphatide on the mutant enzymes from the two types of patients.

Determination of the CMCs of sulphatide

As a first step in investigating the mechanism of glucocerebrosidase reconstitution by sulphatide, we wanted to define the CMCs of the lipid under the same conditions used to assay β -glucosidase activity, namely 0.2 M-sodium acetate buffer, pH 5.5, at 37 °C. To accomplish this, we employed the fluorescent indicator dye *N*-phenyl-1-naphthylamine and the technique described by Kovatchev *et al.* (1981). When lipid micelles form, the dye intercalates into the micelles, increasing the dye's fluorescence; when one plots relative fluorescence versus log(total lipid concentration), a sharp increase in fluorescence indicates a change in the state or degree of aggregation of the lipid, thereby revealing the CMC value. The results of such an analysis are shown in Fig. 1. Over a 20000-fold concentration range (0.001–20 $\mu\text{g}/100\ \mu\text{l}$), three distinct breakpoints were observed in the plot of fluorescence versus log(sulphatide concentration), indicating that sulphatide has three CMCs. From the results of three separate determinations, the mean CMC values for galactocerebroside 3-sulphate were found to be 0.30 $\mu\text{g}/100\ \mu\text{l}$ (3.72 μM), 1.82 $\mu\text{g}/100\ \mu\text{l}$ (22.6 μM) and 4.9 $\mu\text{g}/100\ \mu\text{l}$ (60.7 μM). In the present paper we refer to four forms of sulphatide: monomers (at sulphatide concentrations below 3.72 μM), primary micelles, secondary micelles and tertiary micelles. Since at low concentrations of sulpholipid the CMC curve is essentially flat, we assume that no lipid aggregation occurs in that concentration range, and therefore that the sulphatide exists as 'monomers'. We use the terms 'primary', 'secondary' and 'tertiary' to differentiate the various micellar forms detected by the fluorescent indicator. The number of sulphatide molecules in the various micellar forms, and their structures, are unknown.

Two previous studies performed with sulphatide dispersed in aqueous media by ultrasonic irradiation are in agreement with our CMC data and provide some information about the size of sulphatide micelles. In their study performed with 0.1 M-sodium acetate buffer, pH 5.6, Jeffrey & Roy (1977) found the CMC of sulphatide to be 70 μM . This value is in good agreement with the value obtained in the present study for CMC₃, namely, 60.7 μM . The fact that Jeffrey & Roy (1977) did

Fig. 1. CMC determination for sulphatide in aqueous solution

CMCs were determined by measuring the fluorescence of the indicator dye *N*-phenyl-1-naphthylamine in the presence of various concentrations of sulphatide. Shown is a representative result of three such experiments. Arrows indicate the mean values, and the hatched bars indicate the range of values obtained for each CMC. When the data shown were subjected to least-squares analysis, correlation coefficients of greater than 0.99 were obtained.

not observe additional CMCs at lower sulphatide concentrations may be due to differences in the sensitivity of the methods used by the two groups to determine CMC values; we used *N*-phenyl-1-naphthylamine, whereas Jeffrey & Roy (1977) used Rhodamine G to detect the presence of micelles.

Gammack *et al.* (1964) and Jeffrey & Roy (1977) found that the sedimentation coefficient of sulphatide micelles was strongly dependent on buffer concentration. In water, Gammack *et al.* (1964) found the $s_{20,w}$ to be approx. 2 S; in dilute buffer solutions (e.g. 0.01 M-sodium formate), Jeffrey & Roy (1977) reported that the predominant micellar form of sulphatide has an $s_{20,w}$ value of 6 S. However, in 0.1 M-sodium formate, the sulphatide micelles sedimented as a heterogeneous population with an average $s_{20,w}$ of 26 S (Jeffrey & Roy, 1977). In the present study, the highest sulphatide concentration used was 25-fold below that used by Jeffrey & Roy (1977). Therefore a correlation between the sedimentation coefficients that they reported and the size of the micelles used in the assays in the present study may not be accurate.

Dependency of control spleen glucocerebrosidase reconstitution on the micellar form of sulphatide

Having shown (Fig. 1) that sulphatide occurs in one of four forms (monomer–tertiary micelles), depending on the total concentration of the lipid in the β -glucosidase assay medium, we wanted to know in which aggregation state the lipid activator was most effective in reconstituting glucocerebrosidase activity. For the glucocerebrosidase preparation from control spleen, as indicated by the $\circ$ symbols in Fig. 2, when the sulphatide concentration in

Fig. 2. Correlation between the activation of control glucocerebrosidase and sulphatide concentration

β -Glucosidase activity was determined as described in the Materials and methods section, with the control glucocerebrosidase preparation with the addition of various amounts of sulphatide in the absence (○) and in the presence (●) of 0.2 μ g of HSF/100 μ l. Hatched bars indicate sulphatide's three CMCs as determined in Fig. 1.

the assay medium reached CMC₁ there was an increase in β -glucosidase activity, and another abrupt increase when CMC₂ was reached. These results indicate that micelles, but not monomers, of sulphatide reconstitute control glucocerebrosidase. Furthermore, the fact that the slope of the plot of activity versus log(sulphatide concentration) is greater above CMC₂ than above CMC₁ suggests that secondary sulphatide micelles are more effective than primary sulphatide micelles in activating glucocerebrosidase.

The addition of HSF (Fig. 2, ● symbols) shifted the sulphatide activation curve upward, indicating that control glucocerebrosidase is responsive to this activator protein at sulphatide concentrations where primary, secondary or tertiary micelles exist. Maximal activity in the presence of HSF occurred at sulphatide concentrations in the range 30–40 μ g/100 μ l (results not shown). The specific activity of control glucocerebrosidase was not increased by HSF when the concentration of sulphatide was so low that only lipid monomers were present (0.001–0.30 μ g/100 μ l).

Reconstitution of mutant glucocerebrosidase preparations from spleens of type 1 and type 2 Gaucher's-disease cases

The same experiment was performed with similar cholate/butanol-extracted glucocerebrosidase preparations derived from cases of type 1 and type 2 Gaucher's disease. When the type 1 glucocerebrosidase preparation served as the source of enzyme (Fig. 3), β -glucosidase activity could be reconstituted extensively, so long as the sulphatide concentration in the assay medium exceeded CMC₁ (3.72 μ M); secondary micelles appeared to be more

Fig. 3. Effect of sulphatide concentration on the activity of glucocerebrosidase isolated from a patient with type 1 Gaucher's disease

β -Glucosidase was assayed as described for Fig. 2, with various sulphatide concentrations in the absence (○) and in the presence (●) of HSF. Hatched bars indicate the three CMCs of sulphatide.

Fig. 4. Effect of sulphatide concentration on the activity of glucocerebrosidase isolated from a patient with type 2 Gaucher's disease

Glucocerebrosidase was assayed as described for Fig. 2 in the absence (○) and in the presence (●) of HSF. Hatched bars indicate the three CMCs of sulphatide.

effective than primary micelles, as evidenced by the greater slope to the curve in the region where secondary micelles predominate. However, we have no explanation for the second set of breakpoints in the two curves in Fig. 3, which occur midway between CMC₁ and CMC₂, rather than at CMC₂.

An outstanding difference between the results presented in Figs. 2 and 3 is the lack of any marked effect of HSF on the activity of glucocerebrosidase derived from the

type 1 Gaucher's-disease patient. Thus, although the mutant type 1 enzyme was activated extensively by sulphatide (Table 1 and Fig. 3), this particular mutant enzyme was unresponsive to HSF. In contrast, reconstitution of the control glucocerebrosidase preparation was strongly influenced by both HSF and sulphatide (Table 1 and Fig. 2). This observation suggests that the mutation in this particular case of type 1 Gaucher's disease has impaired the enzyme's ability to interact with HSF or the putative HSF-sulphatide complex.

Fig. 4 shows the effect of sulphatide and HSF on the reconstitution of the mutant glucocerebrosidase prepared from the spleen of a child with the most severe form (neuronopathic, type 2) of Gaucher's disease. Although, as acknowledged above, the glucocerebrosidase of this type 2 case could only be reconstituted to less than 5% of the specific activity of control glucocerebrosidase (Table 1), the results presented in Fig. 4 demonstrate that this mutant enzyme is responsive to HSF at all concentrations of sulphatide. The results presented in Table 1 and Fig. 4 indicate that, although the mutation in this most severely affected case of Gaucher's disease has profoundly impaired the active site of glucocerebrosidase, it has not destroyed the site on the enzyme that interacts with HSF or HSF-acidic lipid complexes.

Effects of the different micellar forms of sulphatide on the kinetic constants, K_m and V_{max} , of various glucocerebrosidase preparations

Having determined the effect of sulphatide concentration on the specific activity of the three different glucocerebrosidase preparations, we next compared the K_m values of all three enzymes, with MUGlc as the substrate, in the presence of concentrations of sulphatide where monomers or primary, secondary or tertiary micelles predominate. The results of this study are summarized in Table 2. Our primary purpose in this set

of kinetic studies was to determine which forms of the acidic lipid bind to control and mutant glucocerebrosidases: we assumed that, if the K_m was changed by the inclusion of sulphatide in the β -glucosidase assay medium, the lipid was interacting with the enzyme. The sulpholipid is probably not interacting with the substrate, since the kinetic parameters of the broad-specificity cytosolic β -glucosidase are not affected when sulphatide is present in the MUGlc assay mixture (K. LaMarco & R. H. Glew, unpublished work).

For the control glucocerebrosidase preparation, two pieces of information indicated that the enzyme was capable of binding monomers of sulphatide: the K_m decreased from 11 to 4.6 mM and the V_{max} decreased by half ($P < 0.001$) when sulphatide monomers were present in the assay medium. In addition, Table 2 shows that primary micelles, while supporting a relatively low K_m (2.5 mM), were incapable of raising the V_{max} above the basal value (84.4 versus 67.3 units/mg). In contrast, as we saw in Fig. 2, secondary and tertiary sulphatide micelles are the most effective in reconstituting glucocerebrosidase, raising the V_{max} significantly.

The V_{max} of the glucocerebrosidase of the type 1 case was also markedly increased by secondary and tertiary micelles. A surprising result was that the K_m of the lipid-depleted mutant glucocerebrosidase from the case of type 1 Gaucher's disease was characterized by a relatively low K_m value (2.8 mM), even when sulphatide was not included in the β -glucosidase assay medium. Inclusion of monomers or micelles of sulphatide in the assay medium did not significantly alter the K_m of the type 1 glucocerebrosidase preparation (K_m 1.1–1.9 mM). It appears that the mutation in this particular case of type 1 Gaucher's disease has affected the site on glucocerebrosidase where sulphatide binding would normally result in a lowered K_m .

Because of the very low specific activity of the

Table 2. Effect of sulphatide on kinetic parameters of human spleen glucocerebrosidase

β -Glucosidase activity of control and types 1 and 2 Gaucher's-disease spleen glucocerebrosidase preparations was determined with the addition of the indicated amounts of sulphatide. For each condition, β -glucosidase determinations were performed in triplicate at ten different MUGlc concentrations covering a 20-fold range, as described by Segel (1975). K_m (for MUGlc) and V_{max} were determined by fitting data to a rectangular hyperbola by using a computer analysis method described by Cleland (1979). K_m values are expressed as mM, and V_{max} values as units/mg of protein.

Source of glucocerebrosidase	Concn. of sulphatide ($\mu\text{g}/100 \mu\text{l}$)...	0	0.08 (monomers)	0.8 (primary micelles)	3.5 (secondary micelles)	8.0 (tertiary micelles)
Control						
V_{max}		84.4*	46.5*	67.3	126	239
K_m		11	4.6	2.5	2.8	1.6
V_{max}/K_m		7.6	10	26	45	150
Type 1						
V_{max}		2.47	2.12	9.84	80.1	148
K_m		2.8	1.9	1.1	1.4	1.2
V_{max}/K_m		0.88	1.1	8.9	57	123
Type 2						
V_{max}		2.03	N.D.†	2.60	5.14	6.48
K_m		9.2	N.D.†	1.6	1.9	1.5
V_{max}/K_m		0.22	N.D.†	1.6	2.7	4.3

* Significantly different from each other at $P \leq 0.001$ by Student's t test.

† Owing to the very low activity of the type 2 glucocerebrosidase when assayed under these conditions, the V_{max} and K_m values could not be determined (N.D.).

glucocerebrosidase preparation from the patient with type 2 Gaucher's disease, we could not determine the K_m and V_{max} values in the presence of sulphatide monomers (Table 2). Nevertheless, we were able to show that the various micellar forms of sulphatide all caused the enzyme to exhibit a lower K_m (1.5–1.9 mM) than that obtained in the absence of exogenous activator lipid (9.2 mM). This observation indicates that, although the V_{max} of the type 2 glucocerebrosidase was not increased greatly by sulphatide, the micellar forms of the acidic lipid do interact with this mutant enzyme.

Table 2 also shows that the catalytic efficiency (V_{max}/K_m) of the glucocerebrosidase from the patient with type 1 Gaucher's disease, when determined in the presence of secondary or tertiary sulphatide micelles, is almost as high as that of the corresponding control enzyme (45 versus 55, and 150 versus 123). In contrast, the catalytic efficiency of the type 2 glucocerebrosidase, determined under the same conditions, was less than 6% that of the control enzyme.

Effects of HSF on the kinetic parameters, K_m and V_{max} , of various glucocerebrosidase preparations

Since HSF had a profound effect on the activation of control and type 2 glucocerebrosidase by sulphatide (Figs. 2 and 4), we wanted to know what effect HSF might have on the kinetic parameters, K_m and V_{max} , of the various glucocerebrosidase preparations. Table 3 shows the results of such a kinetic study performed on glucocerebrosidase isolated from controls and patients with type 1 or type 2 Gaucher's disease. K_m and V_{max} values were determined in the presence of HSF alone, or with HSF plus either sulphatide monomers or tertiary micelles. As shown in Table 3, HSF alone had the same effect on K_m and V_{max} as did the sulphatide monomers (Table 2); HSF decreased the K_m of both control and type 2 glucocerebrosidase preparations, while also significantly decreasing the V_{max} value. When HSF and sulphatide monomers were present together in the assay medium, the resulting V_{max} and K_m values were similar to those obtained with either activator alone. These results suggest that HSF and the sulphatide monomers interact with the same site on glucocerebrosidase, resulting in lowered K_m and V_{max} values. In contrast, HSF had no significant

effect on the kinetic parameters of the glucocerebrosidase of the patient with type 1 Gaucher's disease. This result is consistent with our observation that HSF had very little effect on the activation of type 1 glucocerebrosidase over the entire range of sulphatide concentrations (Fig. 3).

The results presented in this study support the concept that there are two activator-binding sites on glucocerebrosidase: one site interacts with HSF or sulphatide monomers or primary micelles and modulates the enzyme's affinity for the MUGlc substrate (i.e. the K_m); the second site, which binds secondary and tertiary sulphatide micelles, affects the rate of the β -glucosidase reaction (i.e. the V_{max}). We propose that control glucocerebrosidase operates most efficiently (as evidenced by the high V_{max}/K_m ratio calculated from data in Table 3) when both activator sites are occupied: the first by sulphatide monomers or HSF, and the second by secondary or tertiary micelles.

Effect of sulphatide on the rate of chymotrypsin inactivation of glucocerebrosidase

In an effort to show, by means other than kinetic, that sulphatide binds to the various glucocerebrosidases, we investigated the effect of sulphatide on the inactivation of glucocerebrosidase by chymotrypsin (Fig. 5). A similar approach has been taken by investigators studying other enzymes that have a lipid requirement (Maurer *et al.*, 1985). All three glucocerebrosidase preparations, control (Fig. 5a), type 1 (Fig. 5b) and type 2 (Fig. 5c) were incubated with chymotrypsin in the absence (● symbols) or in the presence (○ symbols) of sulphatide at a lipid concentration that corresponded to tertiary micelles. As shown in Fig. 5, in the absence of sulphatide all three glucocerebrosidase preparations were relatively resistant to inactivation by the proteinase. However, when sulphatide was present (30 μ g/100 μ l), a rapid loss of β -glucosidase activity ensued. The chymotrypsin inactivation of all three glucocerebrosidases occurred in two phases: an initial rapid loss of activity was followed by a second, slower, loss. This observation suggests that there are two different chymotrypsin-sensitive sites on glucocerebrosidase.

By assaying chymotrypsin activity with the non-physiological substrate benzoyl-tyrosine ethyl ester (Rao

Table 3. Effect of HSF on kinetic parameters of human spleen glucocerebrosidase

Glucocerebrosidase isolated from spleens of human controls and patients with type 1 or type 2 Gaucher's disease was assayed with the indicated amounts of HSF and sulphatide. K_m (for MUGlc) and V_{max} values were determined as described for Table 2. For each condition, β -glucosidase determinations were performed in duplicate, except as indicated in the footnotes. K_m values are expressed as mM and V_{max} values as units/mg of protein.

Additions to the assay medium	Control		Type 1		Type 2	
	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
None	84.4*	11	2.47	2.8	2.03	9.2
HSF (0.2 μ g)	26.0*	3.0	2.2	1.0	0.7	1.6
HSF + sulphatide monomers	35.6	2.6	3.58	1.0	N.D.†	N.D.†
HSF + sulphatide tertiary micelles	357	1.9	135	1.3	5.69	1.0

* Significantly different from each other at $P \leq 0.001$ by Student's *t* test. These determinations were performed in triplicate.

† Not determined.

Fig. 5. Effect of sulphatide on the rate of chymotrypsin inactivation of glucocerebrosidase

Glucocerebrosidase from control (a), type 1 Gaucher's disease (b) and type 2 Gaucher's disease (c) spleens was incubated with chymotrypsin (10%, w/w) in 0.1 M-Tris buffer, pH 8.3, at 24 °C in the absence (●) and in the presence (○) of 0.37 mM-sulphatide. Samples were taken at the indicated times, placed on ice and assayed immediately for β -glucosidase activity by the standard assay with the inclusion of 0.2% (w/v) sodium taurodeoxycholate. Activity is expressed as the percentage of initial activity (at time zero) remaining after incubation with chymotrypsin at 24 °C. Incubation of the glucocerebrosidase in the presence of sulphatide alone (minus chymotrypsin) at 24 °C resulted in no loss of β -glucosidase activity after 2 h (results not shown).

& Lombardi, 1975), we were able to rule out the possibility that the sulphatide-enhanced inactivation of glucocerebrosidase was the result of sulphatide directly activating chymotrypsin. No effect on chymotrypsin hydrolysis of the ester was observed when sulphatide was included in the assay medium (results not shown). Therefore we attribute differences in the rate of inactivation of glucocerebrosidase by chymotrypsin in the presence and in the absence of sulphatide (Fig. 5) to the binding of the lipid to glucocerebrosidase. HSF had no effect on the inactivation of glucocerebrosidase by chymotrypsin (results not shown).

Correlations between sulphatide concentration and chymotrypsin inactivation of glucocerebrosidase

Having shown in Fig. 5 that high concentrations of sulphatide (corresponding to tertiary micelles) had a

Fig. 6. Effect of sulphatide concentration on the inactivation of glucocerebrosidase by chymotrypsin

Glucocerebrosidase from control (a), type 1 Gaucher's-disease (b) and type 2 Gaucher's-disease (c) spleens was incubated in the absence and in the presence of 5% (w/w) chymotrypsin, with the addition of the indicated amounts of sulphatide, in 0.1 M-Tris buffer, pH 8.3. Incubations were done at 37 °C for 30 min, after which time all samples were assayed for β -glucosidase activity by the standard assay with the addition of 0.2% (w/v) sodium taurodeoxycholate. Results are expressed as percentages of control activity (no chymotrypsin added) present in the chymotrypsin-treated samples. Hatched bars indicate the range of sulphatide concentrations that correspond to the three CMCs.

profound effect on the rate of chymotrypsin inactivation of glucocerebrosidase, we were interested in determining what effect the aggregation state of sulphatide might have on this process. To this end, the three glucocerebrosidase preparations were incubated with chymotrypsin in the presence of various concentrations of sulphatide (Fig. 6). The control (Fig. 6a), type 1 (Fig. 6b) and type 2 (Fig. 6c) glucocerebrosidase preparations were resistant to chymotrypsin at sulphatide concentrations below 1.0 $\mu\text{g}/100 \mu\text{l}$. However, as the second CMC was reached, a marked decline in β -glucosidase activity was observed for all three enzymes. These results indicate that sulphatide monomers and primary micelles, although capable of decreasing the K_m (Table 2), do not affect the chymotrypsin sensitivity of control glucocerebrosidase. However, secondary and tertiary sulphatide micelles, which dramatically increase V_{max} (Table 2), appear to

expose a chymotrypsin-sensitive site upon binding to the enzyme. Inclusion of HSF in the preincubation medium had no effect on the slope or breakpoint of the curves in Fig. 6 (results not shown). It is noteworthy that each of the mutant enzymes behaved like control glucocerebrosidase in this experiment.

In addition to the above-mentioned evidence supporting the hypothesis that glucocerebrosidase possesses two activator-binding sites, the results of the present study allow us to draw conclusions about the nature of the mutations in the glucocerebrosidases isolated from the non-neuronopathic and neuronopathic cases of Gaucher's disease. Glucocerebrosidase from the patient with the non-neuronopathic (type 1) form of the disease exhibited a very low K_m (2.8 mM) in the absence of activators (Table 2). Unlike the control enzyme, the K_m and V_{max} values of the type 1 glucocerebrosidase were not affected by HSF or sulphatide monomers. However, a large increase in V_{max} was seen when secondary or tertiary micelles were present in the β -glucosidase assay medium (Tables 2 and 3). These results indicate that the first activator-binding site (which interacts with HSF or sulphatide monomers or primary micelles) is altered in this mutated form of the enzyme isolated from a type 1 Gaucher's-disease case. However, the second site, which regulates V_{max} through interactions with secondary and tertiary sulphatide micelles, appears to be intact in the type 1 glucocerebrosidase.

Results obtained with glucocerebrosidase from the neuronopathic (type 2) Gaucher's-disease case indicate that both activator-binding sites are intact in this mutant form of the enzyme. HSF and primary sulphatide micelles were very effective in lowering the K_m of the type 2 glucocerebrosidase (Tables 2 and 3). In addition, secondary and tertiary sulphatide micelles were capable of enhancing the chymotrypsin inactivation of the mutant type 2 glucocerebrosidase (Figs. 5 and 6). Therefore the glucocerebrosidase isolated from the type 2 Gaucher's-disease case appears to possess relatively competent activator-binding sites. However, the binding of these activators to both sites on the type 2 glucocerebrosidase did not produce a large increase in V_{max} (Tables 2 and 3), indicating that the mutation in this more severely affected neurological case probably involves some modification of a key functional group at the enzyme's active site.

The presence of functionally distinct sites on the glucocerebrosidase molecule has been previously proposed by Grabowski *et al.* (1984), who suggested the existence of a catalytic site, an aglycone (substrate)-binding site and an allosteric (acidic-lipid-binding) site. The results of the present study indicate that the above model should be modified to include a total of two activator-binding sites: one that affects primarily the K_m and another that affects V_{max} .

This work was supported by Grant AM 31357 from the U.S. Public Health Service, National Institutes of Health, and the Summer Science Research Program for Medical Students, Grant no. 8-85-23 from the March of Dimes Birth Defects Foundation.

REFERENCES

- Basu, A. & Glew, R. H. (1984) *Biochem. J.* **224**, 515–524
- Basu, A. & Glew, R. H. (1985) *J. Biol. Chem.* **260**, 13067–13073
- Basu, A., Glew, R. H., Daniels, L. B. & Clark, L. S. (1984) *J. Biol. Chem.* **259**, 1714–1719
- Basu, A., Prence, E., Garrett, K., Glew, R. H. & Ellingson, J. S. (1985) *Arch. Biochem. Biophys.* **243**, 28–34
- Berent, S. L. & Radin, N. S. (1981) *Arch. Biochem. Biophys.* **208**, 248–260
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Brady, R. O., Kanfer, J. N. & Shapiro, D. (1965) *Biochem. Biophys. Res. Commun.* **18**, 221–225
- Cleland, W. W. (1979) *Methods Enzymol.* **63**, 103–138
- Daniels, L. B., Glew, R. H., Radin, N. S. & Vunnam, R. R. (1980) *Clin. Chim. Acta* **106**, 155–163
- Fredrickson, D. S. & Sloan, H. R. (1972) in *The Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. B. & Fredrickson, D. S., eds.), pp. 730–759, McGraw-Hill, New York
- Gammack, D. B., Perrin, J. H. & Saunders, L. (1964) *Biochim. Biophys. Acta* **84**, 576–586
- Garrett, K. O., Prence, E. M. & Glew, R. H. (1985) *Arch. Biochem. Biophys.* **238**, 344–352
- Glew, R. H., Daniels, L. B., Clark, L. S. & Hoyer, S. W. (1982) *J. Neuropathol. Exp. Neurol.* **41**, 630–641
- Glew, R. H., Basu, A., Prence, E., Garrett, K. & Cooper, R. (1986) *Proc. CNRS-INSERM International Symposium and NATO Workshop on Enzymes of Lipid Metabolism*, in the press
- Grabowski, G. A., Gatt, S., Kruse, J. & Desnick, R. J. (1984) *Arch. Biochem. Biophys.* **231**, 144–157
- Ho, M. W. & Light, N. D. (1973) *Biochem. J.* **136**, 821–823
- Ho, M. W. & O'Brien, J. S. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2810–2813
- Jeffrey, H. J. & Roy, A. B. (1977) *Aust. J. Exp. Biol. Med. Sci.* **55**, 339–346
- Kanfer, J. N., Legler, G., Sullivan, J., Raghavan, S. S. & Mumford, R. A. (1975) *Biochem. Biophys. Res. Commun.* **67**, 85–90
- Kovatchev, S., Vaz, W. L. C. & Eibl, H. (1981) *J. Biol. Chem.* **256**, 10369–10374
- Maurer, A., McIntyre, J. O., Churchill, S. & Fleischer, S. (1985) *J. Biol. Chem.* **260**, 1661–1669
- McKelvy, J. F. & Lee, Y. C. (1969) *Arch. Biochem. Biophys.* **132**, 99–110
- Patrick, A. D. (1965) *Biochem. J.* **97**, 17c–18c
- Peters, S. P., Coyle, P., Coffee, C. J., Glew, R. H., Kuhlen-schmidt, M. S., Rosenfeld, L. & Lee, Y. C. (1977) *J. Biol. Chem.* **252**, 563–573
- Prence, E., Chakravorti, S., Basu, A., Clark, L. S., Glew, R. H. & Chambers, J. A. (1985) *Arch. Biochem. Biophys.* **236**, 98–109
- Rao, K. N. & Lombardi, B. (1975) *Anal. Biochem.* **65**, 548–551
- Segel, I. H. (1975) *Enzyme Kinetics*, pp. 100–160, 161–226, Wiley-Interscience, New York