Human Acid β-Glucosidase

USE OF CONDURITOL B EPOXIDE DERIVATIVES TO INVESTIGATE THE CATALYTICALLY ACTIVE NORMAL AND GAUCHER DISEASE ENZYMES*

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Human acid β-glucosidase (glucosylceramidase; EC 3.2.1.45) cleaves the glycosidic bonds of glucosyl ceramide and synthetic β-glucosides, Conduritol B epoxide (CBE) and its brominated derivative are mechanism-based inhibitors which bind covalently to the catalytic site of acid β-glucosidase. Procedures using brominated CBE and monospecific anti-human placental acid β-glucosidase in a crude normal splenic preparation were developed to determine the molar concentrations of functional acid β-glucosidase catalytic sites in pure plasmid enzyme preparations from normal sources; \( k_{cat} \) values then were calculated from \( V_{max} = [E]k_{cat} \) using glucosylceramide substrates. Values for 6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-hexanoyl (3200 ± 410 min\(^{-1}\)) and undecanoyl (773 ± 44 min\(^{-1}\)) fatty acid acyl chains and the respective \( k_{cat} \) values for acid β-glucosidase in a crude normal splenic preparation were about 60% of these values. In comparison, the \( k_{cat} \) values of the mutant splenic acid β-glucosidase from two Type 1 Ashkenazi Jewish Gaucher disease (AJGD) patients were about 1.5-3-fold decreased and had \( k_{cat} \) values for each substrate which were similar to those for the normal acid β-glucosidase. The interaction of the normal and Type 1 AJGD enzymes with CBE in a 1:1 stoichiometry conformed to a model with reversible \( EI \) complexes formed prior to covalent inactivation. With CBE, the equal \( K_{m} \) values (maximal rate of inactivation) for the normal (0.051 ± 0.009 min\(^{-1}\)) and Type 1 AJGD (0.058 ± 0.016 min\(^{-1}\)) enzymes were consistent with the minor differences in \( k_{cat} \). In contrast, the \( K_i \) value (dissociation constant) (859 ± 64 \( \mu M \)) for the Type 1 AJGD enzymes was about 5 times the normal \( K_i \) value (166 ± 57 \( \mu M \)). These results indicated that the catalytically active Type 1 AJGD acid β-glucosidase had nearly normal hydrolytic capacity and suggested an amino acid substitution in or near the acid β-glucosidase active site leading to an in vitro instability of the mutant enzymatic activity.

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1 The abbreviations used are: GC, glucosylceramide; CBE, conduritol B epoxide; [\( \text{H} \)]B-CBE, bromo-trinitrated Conduritol B epoxide; AJGD, Ashkenazi Jewish Gaucher disease; SDS, sodium dodecyl sulfate; C\(_4\)-U-Glc, 4-alkyl-umbelliferyl-1-O-β-D-glucoside which the alkyl chain length (C\(_n\)) varied from \( n = 1 \) to \( n = 11 \); NBD-C\(_2\) or C\(_1\)-GC, 12-(N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl))-dodecanoyl or 6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-hexanoyl-GC.

The lysosomal enzyme, acid β-glucosidase (N-acylsphingosyl-1-O-β-D-glucoside glucohydrolase, EC 3.2.1.45) cleaves the β-glycosidic linkage of glucosylceramide (GC) (1, 2) as well as synthetic β-glucosides which are water soluble (3–5). For optimal hydrolysis of GC or synthetic substrates, this membrane-associated enzyme requires detergents, negatively charged lipids, and/or a "co-glucosidase" (3–9). Kinetic studies of the purified normal human acid β-glucosidase have indicated the presence of at least three domains within its active site which participate in substrate binding and hydrolysis: 1) the catalytic site which recognizes β-glucoside moieties of substrates and may participate in a proton transfer for activation of substrate or for cleavage of the oxirane ring of conduritol B epoxide (CBE) for covalent binding (9); 2) an aglycon-binding site which has specificity for acyl moieties of substrates and inhibitors (9); and 3) a third domain which interacts with cationic sphingosyl derivatives as well as negatively charged lipids (9) and shares some of the properties of the "allosteric" site described by Erickson and Radin (10). An additional domain on the enzyme may be required for binding of a "co-glucosidase" (6, 7), a naturally occurring glycoprotein effector. In contrast, interest in the kinetic properties of acid β-glucosidase derives from the fact that different mutations in the structural gene encoding for human acid β-glucosidase result in the subtypes and variants of Gaucher disease, the most prevalent lysosomal storage disease (11). Recent kinetic (in vitro and ex vivo) and immunologic studies of the residual enzyme in tissue extracts from Gaucher disease patients indicate that two major classes of defective enzymes resulting from allelic mutations in the acid β-glucosidase structural gene: 1) mutations resulting in altered proteins with abnormal active site function (12, 13); and 2) mutations causing decreased enzyme stability (13), biosynthesis, or processing (14) but intact active site function (12, 13). The mutations which give rise to these two major classes of defective enzymes are heterogeneous (13). Of particular interest is the residual acid β-glucosidase in Type 1 Ashkenazi Jewish Gaucher disease (Type 1 AJGD), the most prevalent genetic disease among this Jewish population (11), which had abnormal function of the active site (13). Studies with the residual enzyme in crude fibroblast extracts and delipidated splenic preparations from Type 1...
AJGD patients (8, 13) revealed abnormal interactions with third domain probes (e.g. glucosylphosphingosine, sphingosine, and phosphatidylserine). Based on these results, it was postulated that a mutation (e.g. single base substitution) altered this domain accounting for the proposed 10-20-fold decreased $V_{\text{max}}$ of acid $\beta$-glucosidase in this form of Gaucher disease (15).

Since the decreased $V_{\text{max}}$ was estimated from specific activities based on the amount of cross-reacting immunologic material in Type 1 AJGD sources (13, 15, 16), the large differences in $V_{\text{max}}$ between the normal and Gaucher disease enzymes may not reflect a decreased $k_{\text{cat}}$ of the active enzyme species, but rather the presence of catalytically inactive enzyme or acid $\beta$-glucosidase with altered avidity for the antibodies (13). In an effort to overcome these difficulties and to determine $k_{\text{cat}}$ directly, [H]Br-CBE, a catalytic site-directed covalent inhibitor of acid $\beta$-glucosidase (17), was used to quantitate the catalytically active enzyme in preparations of homogeneous normal placental acid $\beta$-glucosidase, partially purified normal spleenic enzyme, and mutant enzymes from the spleens of two Type 1 AJGD patients. These patient enzymes were representative of Type 1 AJGD, since previous studies of the residual acid $\beta$-glucosidase activity in fibroblast extracts from these two and 10 other Type 1 AJGD patients demonstrated essentially identical physical, kinetic, and immunologic properties (13). These studies provide support for a mutation in the structural gene for acid $\beta$-glucosidase which results in a specific active site defect which also leads to a decreased intracellular stability of acid $\beta$-glucosidase activity in Type 1 AJGD.

**EXPERIMENTAL PROCEDURES**

**Materials**

The following were from commercial sources: Triton X-100, $\beta$-n-glucose (Sigma), sodium taurocholate (Calbiochem), sodium dodecyl sulfate (SBS, British Drug House, Poole, UK), NBD-dodecanoyl and NBD-hexanoic acids (Molecular Probes, Junction City, OR), and 4-methylumbelliferyl-1-O-$\beta$-glucoside (4-C$_9$-UGlc) (RPI, Mount Prospect, IL). 4-C$_9$-UGlc was recrystallized from ethanol prior to use. All other reagents were used directly.

**Methods**

**Enzyme Sources**—Normal acid $\beta$-glucosidase was purified from human placenta by affinity chromatography on N-alkyl-deoxynojirimycin-Sepharose (21). Homogeneity of the preparation was documented by the presence of a single protein species on SDS-polyacrylamide electrophoresis or reverse-phase high pressure liquid chromatography (17) and by a single N-terminal amino acid sequence (21). Normal spleen was obtained at surgery from a patient with idiopathic thrombocytopenic purpura and stored at $-20^\circ$ C for 6 months. The acid $\beta$-glucosidase from this spleen was purified through the butanol delipidation step (5) and was about 60-fold enriched. The Type 1 AJGD spleens were obtained at splenectomy from 25- and 54-year-old patients and stored at $-20^\circ$ C until used (6-12 months). The mutant spleenic $\beta$-glucosidase was purified either by hydrophobic (3) or $N$-alkyl-deoxynojirimycin-Sepharose chromatography (21). These final preparations were about 500- and 7500-fold enriched and several protein bands were observed on SDS-polyacrylamide gel electrophoresis. The 500-fold enriched Type 1 AJGD enzyme also was delipidated with 40% of a 1:1 (v/v) mixture of butanol-ether to ensure that intrinsic lipids were removed (6). All enzyme preparations were stable for several months when stored at $4^\circ$ C in 0.04 M citrate, 0.05 M phosphate, pH 5.5, 4 mM $\beta$-mercaptoethanol, and 1 mM EDTA (buffer A) containing 60-80% ethylene glycol. These four different enzyme preparations of varying purity were used in comparative studies to control for the effect of contaminants on the kinetic measurements since homogeneous Type 1 AJGD acid $\beta$-glucosidase could not be obtained in an active form.

Using the pure normal placental acid $\beta$-glucosidase and the partially purified normal or Type 1 AJGD splenic enzymes (which had been deglycosylated with N-glycanase (Genzyme, Boston, MA)), identical immunoblot molecular weights (56,000) were obtained with monoclonal antibodies specific for normal placental acid $\beta$-glucosidase. In addition, immunoblot fainter profiles of cross-reacting immunologic material from the glycosylated enzymes in splenic fibroblast extracts from several normal individuals or other Type 1 AJGD patients, although the profiles in each tissue source were different. These studies indicated that the partially purified mutant enzymes examined had relatively identical molecular weight to the normal placental or spleenic enzymes and they were similar to those from several other Type 1 AJGD patients.

**Kinetic Studies**—Hydrolysis of NBD-GC derivatives (9) or 4-C$_9$-U-Glc (9, 18) was determined fluorometrically. The typical reaction mixture (0.2 ml) contained 0.05 M phosphate, 0.04 M citrate, pH 5.5, Triton X-100 (4 mM), 4.65 mM taurocholate, 4 mM $\beta$-mercaptoethanol, 1 mM EDTA, substrate, $<$1% ethylene glycol, and enzyme. Assays with the highly purified Type 1 AJGD enzyme contained 0.6% human serum albumin in the incubation mixtures to maintain enzyme stability. Under these conditions, human serum albumin binding to the $k_{\text{cat}}$ or $V_{\text{max}}$ values of the more stable normal placental or delipidated splenic acid $\beta$-glucosidase, respectively. The lipoidal substrates in chloroform/methanol (2:1, v/v) were added to dry tubes, and then the solvents were evaporated under nitrogen and then with ethanol. The reaction mixture for 2-4 $\mu$mol of substrate was dried and redissolved containing Triton X-100, and the reactions were initiated by the addition of enzyme. The amount of enzyme was adjusted to ensure that less than 5% of the substrate was hydrolyzed. Reactions at 37 $^\circ$ C were terminated after 0.5-2 h. Protein concentrations were estimated by the method of Lowry et al. (22).

** Determination of $k_{\text{cat}}$ and $V_{\text{max}}$**—The $k_{\text{cat}}$ values for alternate substrates with the normal and Type 1 AJGD acid $\beta$-glucosidase were calculated from $V_{\text{max}} = [E]$, $k_{\text{cat}}$ assuming that only those active sites which retained their respective full catalytic activity were labeled by [H]Br-CBE (see below). $V_{\text{max}}$ values for the 4-C$_9$- and -C$_{11}$-U-Glc and NBD-C$_9$- or NBD-C$_{11}$-GC substrates were obtained from linear Lineeweaver-Burk plots which had been evaluated by the least squares method. The graphic method of Cleland (23) was used to determine $K_m$ and $V_{\text{max}}$ for the 4-C$_9$- and -C$_{11}$-U-Glc, since substrate inhibition was observed. The $k_{\text{cat}}$ values were based on six separate experiments performed in duplicate for each substrate and enzyme source. For studies which determined $k_{\text{cat}}$ from the relationship of the enzyme activity to the number of catalytic sites (i.e. Fig.1), enzyme activities were determined with subsaturating amounts of substrate (4 mM 4-C$_9$-U-Glc or 0.2 mM NBD-C$_9$-GC) to avoid the problem of substrate insolubility at high concentrations; the $V_{\text{max}}$ was calculated from the amount of concentration dependent $k_{\text{cat}}$ as $V_{\text{max}}$ = [E], was estimated by quantitating the number of catalytic sites in each enzyme preparation using [H]Br-CBE (8000 cpm/pmol) as follows. Various amounts of enzymatic activity or protein from the different enzyme preparations in buffer A were incubated with a large excess of [H]Br-CBE (2-8 $\mu$l; 10 $\mu$mol final concentration) in 0.6% human serum albumin. Complete inactivation of each enzyme was achieved with this concentration of [H]Br-CBE by 2 h at 22 $^\circ$ C. To ensure that all [H]Br-CBE-binding sites were saturated, the mixtures were incubated at 22 $^\circ$ C for 24 h. Human serum albumin was required to maintain enzyme stability under these conditions for up to 24 h. The following protocol (24, 25) for the reaction of [H]Br-CBE with the enzyme-Br-CBE complexes were immunoprecipitated quantitatively with monospecific rabbit anti-human acid $\beta$-glucosidase IgG and Staphylococcus aureus Protein A (13). The resultant supernatants were reprecipitated successively with additional IgG and Protein A until no additional increase in precipitated radiolactivity was observed; immunoprecipitation was usually quantitative after a single cycle. The resultant pellets were washed by resuspension and centrifugation (10,000 x g, 40 min) twice in phosphate-buffered saline, containing 1% human serum albumin, 0.5 M NaCl, and 0.05% Tween 20 and then twice in phosphate-buffered saline, containing 0.05% Tween 20. The washed pellets were dissolved (24 h, 22 $^\circ$ C) with 100 $\mu$l of Protosol™ (New England Nuclear) in 900 $\mu$l of water and the radiolactivity determined. With a fixed amount of the pure placental or crude spleenic enzymes (<2 nmol of 4-C$_9$-U-Glc hydrolyzed)
lyzed/min) from normal sources, the number of catalytic sites was constant when the concentration of $[^{3}H]$Br-CBE was varied between 2 and 20 $\mu$M. With similar amounts of enzymatic activity from the Type 1 AJGD enzyme preparations, the number of catalytic sites remained constant when the concentration of $[^{3}H]$Br-CBE was varied between 5 and 20 $\mu$M. Based on these results, a final concentration of 10 $\mu$M $[^{3}H]$Br-CBE was used in these experiments.

The concentration of catalytic sites/mg of protein of original enzyme solution was determined from the radioactivity in the precipitates. The total enzyme concentration, $[E]$, was based on a molecular weight of 56,000 for the pure unglycosylated normal enzyme which was calculated from amino acid composition (21). This protocol provided a 1:1 mol/mol stoichiometry of $[^{3}H]$Br-CBE in the precipitates and pure normal placental enzymatic protein (17).

**Determination of $K_{d}$ and $k_{max}$ Values for CBE**—For the determination of $K_{d}$ (dissociation constant) and $k_{max}$ (maximal rate of inactivation) values with CBE, the normal or Type 1 AJGD enzymes in buffer A were incubated in the presence of the required amount of CBE and 0.5% each of Triton X-100 and human serum albumin (120 $\mu$L, final volume) at 22 $^\circ$C. This temperature was used since at 37 $^\circ$C, the acid $\beta$-glucosidase is the different preparations was not equally stable. At indicated times, 5-$\mu$L aliquots of these mixtures were immediately diluted with 0.34 M citrate, 0.05 M phosphate, pH 5.5, containing 4 mM $\beta$-mercaptoethanol and 1 mM EDTA to a final CBE concentration of less than 1 $\mu$M for the normal enzymes and 1-4 $\mu$M for the Type 1 AJGD enzymes. Control experiments demonstrated that the respective enzymes were stable under these conditions. An additional control included the respective enzyme incubated in the absence of CBE for the indicated time. Then the desired concentration of CBE (2.5 $\mu$L) was rapidly mixed with a 2.5-$\mu$L aliquot of the incubated enzyme and immediately diluted to the final concentrations of CBE indicated above. These controls were used as zero time points for the CBE inactivation curves of the respective enzymes; they differed from true zero time points (i.e. no CBE) by less than 10%. Enzymatic activities were determined after incubation of the diluted enzyme-CBE mixture with 4-C1-U-Glc (4 mM) for 2 h at 37 $^\circ$C (9, 18).

The $K_{d}$ and $k_{max}$ of the respective enzymes for CBE were determined from the model

$$E + \frac{K_{d}}{E} \rightarrow EI$$

where $EI$ is a reversible complex and $E*I$ is the inactivated enzyme with the inhibitor covalently bound. This model is described by

$$1/k_{cat} = K_{d}/k_{max} \cdot 1/[I] + 1/k_{max}$$

where $k_{max}$, the apparent inactivation rate constant, was determined at each [I], CBE concentration, by

$$\ln[E/E_{0}] = -k_{app} t$$

$E$ and $E_{0}$ were the remaining enzyme activity at time $t$ and the original enzyme activity with the 4-C1-U-Glc (4 mM) substrate, respectively (9, 18). The constants in Equations 1 and 2 were determined from weightless linear regression curves evaluated by the method of least squares. The results are reported as means and ranges from five separate experiments performed in triplicate for the normal placental enzyme and three separate experiments in triplicate with the Type 1 AJGD splenic enzyme preparations.

**RESULTS**

**Determination of $k_{cat}$ Values**—To determine the $k_{cat}$ values for the normal and Type 1 AJGD active enzymes, the concentration of catalytic sites in each preparation was determined using $[^{3}H]$Br-CBE and nonospecific rabbit anti-human acid $\beta$-glucosidase IgG. As shown in Fig. 1, the amount of enzymatic activity (4-C1-U-Glc) was directly related to the number of catalytic sites which were specifically labeled by $[^{3}H]$Br-CBE in the respective enzyme preparations; similar results were obtained using NBD-C12-GC (200 $\mu$M; $K_{m} = 30$ $\mu$M (Table I)) as substrate (data not shown). The $k_{cat}$ values for the normal and Type 1 AJGD enzymes with 4-C1-U-Glc were obtained directly from the slopes of the respective curves in Fig. 1 and the corresponding data with NBD-C12-GC as substrate. The $k_{cat}$ values for the normal homogeneous placental enzyme, with the 4-C1-U-Glc and NBD-C12-GC substrates, were 2360 min$^{-1}$ (range 2240-2480) and 2440 min$^{-1}$ (range 2320-2560), respectively. Using the delipidated normal splenic extract, the $k_{cat}$ values were 1380 min$^{-1}$ (range 1370-1390) for the 4-C1-U-Glc substrate (Fig. 1) and 1480 min$^{-1}$ (range 1410 to 1510) with NBD-C12-GC. The corresponding $k_{cat}$ values for the Type 1 AJGD splenic enzymes were 920 min$^{-1}$ (range 830-1000) with 4-C1-U-Glc and 980 min$^{-1}$ (range 880-1080) with NBD-C12-GC.

In separate sets of experiments similar to those in Fig. 1, the number of catalytic sites in the normal or Type 1 AJGD acid $\beta$-glucosidase sources was determined as a function of total protein concentration in the respective enzyme preparations. These data indicated that $[^{3}H]$Br-CBE bound to the homogeneous normal placental enzyme in a 1:1 molar ratio with enzymatic protein (17). The normal splenic acid $\beta$-glucosidase was estimated to represent about 0.4-0.6% of the total protein in the preparation. Similarly, the Type 1 AJGD active enzymatic protein was 0.7 and 10.5% of the total protein in the two different preparations. From these data, the total enzyme concentrations, $[E]$, were calculated to be between 0.5 and 15 $\mu$M, i.e. about 20,000-700-fold less than the concentration of $[^{3}H]$Br-CBE (10 $\mu$M) used in these experiments.

The $k_{cat}$ values (Table I) for the pure normal placental enzyme and the Type 1 AJGD splenic enzymes with each substrate were determined from $V_{max} = [E]k_{max}$ assuming that only enzyme molecules which had retained full catalytic activity had been quantitated by the $[^{3}H]$Br-CBE method. For each substrate, the $k_{cat}$ values were about 1.5-3-fold lower for the Type 1 AJGD enzymes than the respective values for pure
normal placental acid β-glucosidase. Essentially identical $k_{\text{cat}}$ values were obtained with either Type 1 AJGD splenic preparation. As indicated above, the $k_{\text{cat}}$ values for 4-C$_3$-U-Glc and NBD-C$_{12}$-GC as substrates with the crude normal splenic preparation were about 1.3–1.7-fold greater than those for the Type 1 AJGD splenic enzymes. The respective $K_m$ values for each substrate were similar in the Type 1 AJGD and normal enzyme preparations (Table I).

**Determination of $K_m$ and $k_{\text{cat}}$ Values for CBE**—In order to obtain independent confirmation that the $k_{\text{cat}}$ values for the Type 1 AJGD enzymes were about 1.5–3-fold lower than the normal values, the interaction of CBE, the parent compound of [$^3$H]Br-CBE, with these enzymes was evaluated. Our previous finding that increasing concentrations of δ-glucosalone (9), sphingosine (12), or substrates (4-C$_3$-U-Glc or NBD-C$_{12}$-GC (17)) directly increased the $k_{\text{cat}}$ values for CBE with the normal enzyme suggested that a noncovalent intermediate was formed prior to covalent attachment of CBE to the enzyme. To evaluate this model, a series of inactivation rate curves was developed using the pure normal placental and Type 1 AJGD acid β-glucosidases.

The data, plotted according to Equation 2, were linear, indicating a first-order process. To determine the $K_m$ (dissociation constant) and the $k_{\text{cat}}$ (maximal rate of inactivation) values, the data in Fig. 2 were re-plotted according to Equation 1 (Fig. 3). The $1/k_{\text{app}}$ intercept provides $1/K_m$ and the $1/[\text{CBE}]$ intercept is $-1/K_m$. The $k_{\text{cat}}$ values for the normal placental enzyme (0.051 min$^{-1}$, range = 0.042–0.060 min$^{-1}$) and the Type 1 AJGD enzymes (0.058 min$^{-1}$, range = 0.042–0.074 min$^{-1}$) were not different. In contrast, the $K_m$ value for the Type 1 AJGD enzymes (839 μM, range = 775–903) was about five times that for the normal enzyme ($K_m = 166$ μM, range = 109–233).

Using these values and Equation 1, the predicted inactivation rate curves showed a close correspondence to those obtained experimentally (Fig. 2). Furthermore, the predicted inactivation rate curves at infinite [CBE] were identical for the normal and Type 1 AJGD enzymes. In comparison, if the $k_{\text{cat}}$ values for the Type 1 AJGD enzymes were 10-fold less than those for the normal enzyme, completely different inactivation rate curves would be obtained (Fig. 4). In this case, the inactivation rate curve at infinite [CBE] would correspond closely to that observed with the Type 1 AJGD enzymes at 80 μM CBE (Fig. 4). Compared to normal acid β-glucosidase, a 1.5–3-fold decrease in the $k_{\text{cat}}$ value of the Type 1 AJGD enzymes would not be readily apparent. However, the close correlation of the predicted and experimental curves suggests that such a difference in $k_{\text{cat}}$ was unlikely.

The inactivation rate curves at several CBE concentrations also were used to determine the stoichiometry of binding with the normal and Type 1 AJGD enzymes (27). Fig. 5 is a replot of log(1/$t_{1/2}$) versus log[CBE]. The slopes of these Hill plots were 0.94 and 1.08 for the normal and Type 1 AJGD enzymes, respectively, indicating a 1:1 stoichiometry between CBE and either enzyme. These results confirm our previous studies of the normal enzyme based on protein concentration (17) and establish the binding relationship for these Type 1 AJGD enzymes.

**DISCUSSION**

In this communication, we report studies which provide evidence for a defect of active site function of acid β-glucosidase from patients with Type 1 AJGD. Compared to the normal enzyme, the defect in the Type 1 AJGD enzyme resulted in a small decrease (1.5–3-fold) in the $k_{\text{cat}}$ values for several substrates and a 5-fold decreased affinity for CBE, a covalent catalytic site inhibitor. These results imply that the deficiency of enzymatic activity in Type 1 AJGD cannot be explained by a major alteration in the catalytic capacity of the active mutant enzyme. This explanation for the nature of the defect in Type 1 AJGD differs from that previously suggested by others (15) and by us (12) which proposed that the defect in the Type 1 AJGD enzyme resulted from a large decrease in $V_{\text{max}}$ or $k_{\text{cat}}$. Based on comparative immunologic studies of normal and Type 1 Gaucher disease enzymes in highly purified splenic preparations Pentchev et al. (15) suggested a mutation in the acid β-glucosidase structural gene which resulted in a 10–20-fold decrease in $V_{\text{max}}$ and, by

### Table 1

**Comparison of kinetic constants of the normal and type 1 AJGD acid β-glucosidase with alternate substrates**

<table>
<thead>
<tr>
<th>Substrate aglycon</th>
<th>$K_m$ (mM)</th>
<th>$k_{\text{cat}}$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>AJGD</td>
<td>Normal placental</td>
</tr>
<tr>
<td>2-N-(NBD-C$_{12}$)-sphingosyl</td>
<td>0.03 ± 0.01*</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td>2-N-(NBD-C$_{6}$)-sphingosyl</td>
<td>0.05 ± 0.02</td>
<td>0.05 ± 0.03</td>
</tr>
<tr>
<td>4-C$_3$-U</td>
<td>0.05 ± 0.02</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td>4-Glu</td>
<td>0.17 ± 0.03</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td>4-Glu</td>
<td>0.12 ± 0.02</td>
<td>0.28 ± 0.05</td>
</tr>
<tr>
<td>4-Glu</td>
<td>1.9 ± 0.3</td>
<td>2.9 ± 0.5</td>
</tr>
</tbody>
</table>

*Method 1: $k_{\text{cat}}$ was derived from the slopes of enzymatic activity as a function of [3H]Br-CBE in the immunoprecipitates (see Fig 1 and text).

*Method 2: $k_{\text{cat}}$ was derived from $V_{\text{max}} = [E_i]k_{\text{cat}}$ where $[E_i]$ was determined from the radioactivity of [3H]Br-CBE in immunoprecipitates after complete inactivation of a fixed amount of enzyme activity by 10 μM [3H]Br-CBE (see text).

*These results supersede previous reports in which a 10-fold arithmetic error was made in the $K_m$ values (5, 8, 9, 12, 13).
Interaction of Conduritol B Epoxide and Acid β-Glucosidase

Fig. 2. Rates of inactivation of pure normal placental acid β-glucosidase (A) and partially purified Type 1 AJGD splenic enzyme (B) by varying concentrations of CBE. The data were plotted according to Equation 2 (see text) where $E$ and $E_0$ represent the remaining enzymatic activity at time $t$ and the original enzymatic activity, respectively. The theoretical curves (---) for the respective enzymes were calculated from Equation 2 based on the $K_i$ and $k_{max}$ values obtained from Fig. 3 and Equation 1 (see text). The $I = \infty$ curves were theoretical curves where $k_{app} = k_{max}$, $I^*$ refers to the theoretical curve at $I = \infty$ when $k_{max}$ for the AJGD Type 1 enzyme was one-tenth of the normal value.

Inference, $k_{app}$ of the Type 1 Gaucher disease enzyme. Using immunologic, inhibitor, and inactivation (CBE) studies of the residual enzyme in fibroblast extracts, we proposed a specific active site defect which could account for this $V_{max}$ abnormality in Type 1 AJGD (8). More recently, we suggested that the abnormal in vitro and ex vivo inactivation rates of the Type 1 AJGD enzyme by CBE or Br-CBE may be due to a defective proton transfer process required for activation of the CBE oxirane ring (12). However, this latter suggestion was based on the assumption that, similar to fungal β-glucosidases, the affinity of CBE for the human enzyme was weak (i.e., $I \gg K_i$). The observed rate constant under these conditions would be $k_{app}/K_i$. Because of the nearly identical $K_i$ or $K_i$ values for 4-C6-U-Glc or β-glucose, respectively, with the normal and Type 1 AJGD enzymes as well as the structural similarity of CBE and β-glucose, we assumed that the affinity (i.e., $K_i$ value) of CBE for either enzyme would be the same. The observed difference in the inactivation rates, therefore, was ascribed to a lower $k_{max}$ and, by inference, a decreased $k_{app}$ for the Type 1 AJGD enzyme (12). In contrast, the present data conform well to a model that predicts that the normal or Type 1 AJGD acid β-glucosidase and CBE form reversible EI complexes prior to inactivation and that the dissociation constant ($K_i$) of CBE was about 1500-fold lower than that for β-glucose ($K_i = 220$ mM). These results indicate that the $K_i$ value for CBE was abnormal in Type 1 AJGD while the $k_{max}$ was normal.

Previous studies have been limited by the lack of homogeneous Type 1 AJGD enzyme which retained full catalytic activity to measure $k_{app}$ directly and by assumptions of antigenic identity of the normal and Type 1 AJGD enzymes (13, 15, 16). To overcome these limitations, we used the covalent inhibitor, [3H]Br-CBE, to determine the $k_{app}$ values from $V_{max}$ values and the concentration of acid β-glucosidase catalytic
interaction of Coniduritol B Epoxide and Acid β-Glucosidase

sites, \([E_i]\), in pure normal placental acid β-glucosidase, delipidated crude normal splenic extracts, and the Type 1 AJGD enzyme preparations. This approach obviated the need for homogeneous mutant enzyme which retained full catalytic activity and only required that all enzyme which bound \([^3H]Br-CBE\) could be completely immunoprecipitated. Thus, the results of these studies were dependent upon the degree of labeling obtained with the Type 1 AJGD enzyme. Complete labeling of the active Type 1 AJGD enzyme was supported by the following findings. 1) \([^3H]Br-CBE\) was recovered from SDS-polyacrylamide electrophoretic gels of the pure normal enzyme and the corresponding protein band in the Type 1 AJGD enzyme. The calculated \(k_{on}\) from these curves for the pure normal placental enzyme agreed well with the observed value based on protein determinations (Fig. 1 and Table I). 5) The maximal rate of inactivation (\(k_{max}\)) of the Type 1 AJGD enzyme by CBE was similar to that for the normal placental acid β-glucosidase. This result is inconsistent with a major alteration in \(k_{max}\), if the steps required for covalent binding of \([^3H]Br-CBE\) and substrate hydrolysis are the same (26). In addition, we assumed that only active enzyme could bind \([^3H]Br-CBE\) covalently and that these molecules had retained their respective full catalytic activities. Thus, the finding of a 1:1 stoichiometry of inhibitor binding to the normal enzyme based on protein (17) or activity determinations supports this assumption. The normal \(k_{max}\) values and minor decrease in \(k_{on}\)

\(4\) G. A. Grabowski, unpublished observation.

**REFERENCES**


Interaction of Conduritol B Epoxide and Acid β-Glucosidase