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Participation of Asparagine 370 and Glutamine 235 in the Catalysis by Acid β -Glucosidase: The Enzyme deficient in Gaucher disease

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Abstract

The hydrolysis of glucosylceramide by acid β -glucosidase proceeds via a two-step, double displacement mechanism that includes cleavage of the O- β -glucosidic bond, enzyme-glucosylation and, then, enzyme-deglucosylation. Two residues that may impact this cycle are N370 and E235. The N370S mutant enzyme is very common in Gaucher disease type 1 patients. Homology and crystal data predictions suggested that E235 is the acid/base catalyst in the hydrolytic reaction. Here, the roles of N370 and E235 in hydrolysis were explored using mutant proteins with selected amino acid substitutions. Heterologously expressed enzymes were characterized using inhibitors, activators, and alternative substrates to gain insight into the effects on the glucosylation (single turnover) and deglucosylation (transglucosylation) steps in catalysis. Specific substitutions at N370 selectively altered only the glucosylation step whereas N370S altered this and the deglucosylation steps. To provide functional data to support E235 as the acid/base catalyst, progress curves with poor substrates with more acidic leaving groups were used in the presence and absence of azide as an exogenous nucleophile. The restoration of E235G activity to nearly wild-type levels was achieved using azide with 2,4-dinitrophenyl- β -glucoside as substrate. The loss of the acidic arm of the pH optimum activity curve of E235G provided additional functional support for E235 as the acid/base in catalysis. This study provides insight into the function of these residues in acid β -glucosidase active site function.

INTRODUCTION

Human acid β -glucosidase (GCase, glucosylceramidase, EC 3.2.1.45) is a membrane-associated lysosomal enzyme that cleaves the β -glucosidic linkage of its natural substrate, glucosylceramide, and synthetic β -glucosides [1,2]. Defective enzyme activity leads to the variants of Gaucher disease (types 1, 2 and 3), a prevalent lysosomal storage disease [1]. The mature GCase contains 497 amino acids with occupancy of 4 of 5 N-glycosylation sites [3]. Glycosylation is required to form a catalytically active conformer with occupancy of the first N-glycosylation site being essential to activity [3,4]. Molecular weight values of GCase vary from 59,000 – 67,000 in tissues due to differential glycosidic remodeling [5,6]. GCase requires hydrophobic agents, e.g., detergents (Triton X-100) or negatively charged lipids (e.g.,

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phosphatidylserine), acidic pH, and a protein cofactor, saposin C, for optimal hydrolytic activity [7–9]. Previous studies provided a kinetic model with subsites within the active site for the interaction of glycon head group, sphingosyl moiety, and fatty acid acyl chain of glucosylceramide [10,11]. Crystal structures support such a model [12]. The location of E340, the catalytic nucleophile [13], and E235 [14,15], a putative acid/base, are within the active site [12,16].

GCCase is a member of the retaining glycosidases whose catalytic cycle has been thought to proceed by a two-step, double displacement mechanism [17,18]. During the first step (glucosylation), a nucleophilic residue attacks the O-glycosidic anomeric linkage at C1 (Fig. 1). This is followed by protonic donation from an acid/base catalyst to the ceramide-glucosidic bond. Ceramide is released and the enzyme-glucosyl covalent complex is formed. This part of the mechanism has been challenged recently from data based on the crystal structure with bound N-alkyl deoxynojirimycins [19]. Such studies suggest a steric hindrance of E235 on the E340 nucleophilic attack and that a proton donation from E235 to the anomeric carbon of glucose produces a carbenion that is attacked by the nucleophile E340 for glucosylation of the enzyme [19]. The deglucosylation step, includes the addition of water to the enzyme-glucosyl complex in a base-catalyzed process assisted by an acid/base catalyst to release β -glucose while regenerating the acid of the acid/base and the nucleophile. The nucleophile in catalysis has been identified as E340 by tandem mass spectrometry following mechanism-based active site labeling [13]. The acid/base in catalysis has been suggested by structural studies, but functional studies are lacking [15,20,21].

Kinetic and *ex vivo* cell studies have provided characterization of some major effects of disease mutations on the enzyme. These are summarized as follows: 1) altered in the enzyme proteolytic stability, 2) decreased in catalytic rate constants [22] or 3) combined effects on catalytic function and enzyme stability [10,22,23]. For the wild-type enzyme, there is a high degree of specificity for the configuration of the glycon head group with glucose derivatives, but not its epimers, having significant binding [23,24]. Importantly, N-alkyl-amino- or -imino sugars [β -glucosylamines, 5-deoxy-5-imino-glucose (nojirimycins)] have some properties of transition state analogues [23]. Such analyses indicated that glycons with a basic group in the C-1 region likely mimic the transition state in which a carbenion develops at C-1 that is stabilized by interactions with E340 and the acid/base in catalysis (Fig. 1).

Alleles encoding the N370S mutant enzyme are quite common in non-neuronopathic Gaucher disease (type 1) patients from western countries. The presence of such alleles in affected patients appears to be protective against the development of early onset progressive nervous system disease [1]. The N370S has characteristic kinetic abnormalities that include a diminished binding of potent active site-directed inhibitors with ~4-fold increase in K_{app} [10,11,23]. Curiously, the K_{mapp} for various substrates are unchanged for N370S and many other mutant GCases. However, the K_{app} abnormalities for imino-glycosides seems unique to N370S and suggests a role for N370 in active site function. The initial crystal structures of acid β -glucosidase suggest little involvement of N370 in active site function because of its distance [12,25].

Computer-assisted hydrophobic cluster analysis has provided putative identification of the nucleophiles and acid/base catalysts for a group of glycosidic hydrolases [15]. Such comparisons identified a “(α/β)₈ barrel structure” motif in 2/8 families that predicts a structural relationship between the nucleophile and acid/base catalyst. Analyses of the 150 glycosyl hydrolase sequences from at least 8 protein families that are active against a great variety of substrates, suggest E235 of human GCCase as the acid/base catalyst [20,21]. The crystal structures of GCCase support this contention [12,25]. However, no functional data are available for verification. Substitution of a glycine (G) for glutamate (E) at position 235 leads to a loss

of enzyme activity [20,26], but several unrelated substitutions scattered throughout GCase have similar effects [22,26]. Thus, mechanistic conclusions are tentative when based on homology, and functional analyses would provide insight into the role of E235 into the catalytic cycle of GCase.

Here, systematic functional analyses of the selected mutant GCases were used to evaluate the effects of N370 and E235 on the catalytic cycle. Data are provided that indicate a role for N370 in catalysis and that E235 is the acid/base in this process.

MATERIALS AND METHODS

The following were from commercial sources: Quick-Change site-directed mutagenesis kit (Stratagene, CA) and BaculoGold and Sf9/Sf21 cells (BD Bios., Pharmingen, CA), SF900II serum-free medium (GIBCO/BRL), spinner tanks (Fisher, Kontes, PA; Octyl-sepharose (Amersham Bios., Sweden), n-butanol and ethylene glycol (Fisher, PA) and FPLC System (Pharmacia, MI); 4-methylumbelliferyl- β -D-glucose (4-MU-Glc) (Biosynth AG, Switzerland), taurocholate (TC) and conduritol B epoxide (CBE)(Calbiochem, CA), brain phosphatidylserine (BPS, Avanti, AL), deoxynojirimycin, castanospermine, Triton X-100 (TX), and sodium azide (Sigma, MO), 2,4-dinitrophenyl-2-fluoro- β -D-glucose (DNPG), o-nitrophenyl- β -glucose (o-NPG) and p-nitrophenyl- β -glucose (p-NPG), 2-mercaptoethanol, and dithiothreitol (DTT) (Sigma, MO). AP color developing kit, goat anti-rabbit antibody (BioRad, CA) and rabbit anti-human glucosidase polyclonal antibody [27], 2,4-dinitrophenyl- β -D-glucose (DNPG) was from Dr. Stephen Withers, CerezymeTM (imiglucerase) was from the Genzyme Corp. (Cambridge, MA).

Site-directed mutagenesis and GCase expression

Overlapping PCR was used to produce six NcoI-BamHI mutant fragments encoding the amino acids D (Asp), E (Glu), Q (Gln), S (Ser), A (Ala) or R (Arg) at amino acid 370 of the mature GCase sequence. Western blots of purified N370A (alanine) and R (arginine) mutants production of large amounts of proteolytically stable enzymes that were devoid of activity with any substrate; These were not studied further. Each NcoI-BamHI cassette was purified and cloned into the wild-type cDNA within the expression vector pAc610 (pAc610-GCase) [28, 29]. Following ligation the entire coding region of each resultant cDNA clone was sequenced. No spurious substitutions were found. Defective virus containing the mutant GCase cDNAs were obtained as described and co-transfected into the *Sf9* host cells. Single plaques from the infected cells were isolated and ~5 to 10 colonies for each construct were purified and amplified to titers of 10^{8-9} pfu/ml. The E235G containing virus was from Marie E. Grace, Ph.D. [20].

Sf9/Sf21 cells ($2.5-5 \times 10^6$ cells/ml) were adapted to serum-free medium (SF900II, GIBCO/BRL) in suspension spinner flasks, and were infected individually by each recombinant virus with a multiplicity of infection (MOI) =5. Western analyses with anti-human GCase polyclonal antibody and silver stain of SDS PAGE (12.5%) gels were performed with the cell lysates and media to monitor expression. The expression level of each mutant protein in media from spinner flasks was about 1 to 1.5 mg/L. Protein products in the serum-free medium were collected 72 hr post-infection.

GCase purification

GCase purification was at 4°C. GCase activity assays and immunoblots were conducted to monitor the level of GCase at each step. The media from cells infected with wild type baculovirus were used as control. Harvested media was mixed with n-butanol (20% v/v) and then centrifuged ($1000 \times g$, 30 min) to partition the aqueous and organic phases. The aqueous phase was harvested by aspiration. Octyl-sepharose beads were added into the isolated aqueous

phase and the mixtures were stirred gently for about 2 hr. Columns were packed and, washed with at least 10 column volumes of 20 mM sodium phosphate, pH 5.8. A step gradient of 25%, 50%, 75% and 90% ethylene-glycol in 20 mM sodium phosphate (EG/P, pH 5.8) was applied. GCCase was eluted in 90% EG/P pH 5.8 [30]. 2-Mercaptoethanol was added to all purification solutions to a final concentration of 4 mM to stabilize the enzyme activities. The 90% EG/P solution was dialyzed and concentrated (Amicon 150, YM10). GCCase activities recovered from the purified wild-type and mutants were assessed at about 65–79% of initial activity. Purity of expressed proteins was calculated by comparing total protein concentration to the specific GCCase on immunoblots using pure GCCase (99+%) as a standard. Each GCCase was estimated by silver staining to be >65–85±5% pure. The major contaminating protein was the insect cell GP60. In separate experiments, GP60 from media of WT virus infected cells was used to spike the pure WT GCCase at proportions from 0–60% (mg/mg protein). GP60 had no effect on activity or kinetic parameters as assessed with substrates or selected inhibitors in the TC/TX or BPS systems.

GCCase Assays and Kinetic Studies

GCCase assays were in triplicate or quadruplicate, and were repeated at least twice using 4MU-Glc (4 mM) as substrate. The reaction conditions were: 0.04M citrate/phosphate (pH 5.8), 0.25% (4.25 mM) TC/0.25% (4 mM) TX (TC/TX). BPS (0 to 32 µM) was substituted for TC/TX in selected assays [26]. In saposin C activation assay, various saposin C (0–200 nM) were tested at 0.5 µM BPS. Cross-reacting immunological material specific activities (CRIM SA) of purified proteins were calculated by comparing the density (Image Quant 5.2) of the intact enzyme protein on Western blots to standards of WT GCCase on the same gels. These values were used to estimate k_{cat} values from $V_{max} = k_{cat} [E]$, relative to that from the wild-type enzyme since the mutant enzymes were not fully purified. Recombinant mannose-terminated glucocerebrosidase (imiglucerase) were used to create linear standard curves on each gel for each mutant GCases.

dNM and CS are rapidly reversible competitive inhibitors of GCCase [10,24]. CBE is an irreversible active site-directed inhibitor [31]. Pilot studies of varying substrate (0.2–5 K_m) and inhibitor concentrations (0.3–10 K_i) showed identical inhibition patterns (competitive) for reversible inhibitors with the wild-type and all mutant enzymes. The K_m values for 4MU-Glc were 2.0 ± 0.5 mM for all active enzymes as determined from Lineweaver-Burk plots. pH dependent activity profiles were determined in 0.04M citrate/phosphate buffer pH 4.4 to 7.2 in the TC/TX or BPS systems. pK_1 and pK_2 values for each mutant were calculated based on pH profile data using PRISM and a diprotic model.

Assays with alternative substrates

Progress curves for substrate hydrolysis were generated using a temperature (15, 25, or 37 °C) controlled block with absorbance monitored every 1–20 sec. GCases were equalized by active-site concentration using DNPG as a single turnover substrate [17]. The release of DNP was monitored at $\lambda = 400$ nm for 1 to 4 hr at varying temperatures. Assays were repeated at least 3 times and analyzed using the PRISM software. Deglucosylation assays were conducted with 4MU-Glc or pNP-Glc as substrates with 0 to 200 mM pentanol in the reaction buffer [37 °C for 30 min]. Similar studies were conducted with the single turnover substrate DNPG [17].

Assays using E235G and wild-type GCases were in triplicates or quadruplicates, and were repeated at least three times using 4MU-Glc, DNPG, pNPG or o-NPG as substrates. The reaction conditions were the same as above for progress curves. The release of DNP, p-NP, or o-NP was monitored at $\lambda = 400$ nm for 1–4 hours at varying temperatures [18,32]. The pH dependence of E235G and wild-type enzymes was conducted using DNPG as substrate. Azide

concentrations were varied from 0 to 1000 μM . A ratio of 1:4 (M/M) GCase to azide was found to be optimal. IC_{50} values for E235G with dNM were determined in the presence of azide using DNPG as substrate.

RESULTS

N370 substituted GCases

For these studies, the only β -glucosidase present in the final enzyme preparations was completely inhabitable by CBE and was completely immunoprecipitated by rabbit anti-human GCase antibodies. GP60, an insect cell protein, was the only other significant protein present in the preparations (see Methods). Cross-reacting immunological material specific activities (CRIM-SA) were used to provide relative estimates of the k_{cat} for each GCase. CRIM-SAs were determined with TC/TX and BPS assays at two different pH values (pH 4.8 and 5.8). In the TC/TX system at pH 5.8, the CRIM-SA order was: N370D(139%)>N370(100%)>N370E (21%) $\geq$ N370S(15%)>N370Q(5%) (Table 1). N370E had slightly higher CRIM-SA at pH 4.8 than at 5.8. In BPS at pH 5.8, the CRIM-SA order was: N370(100%)>N370D(82%)>N370S (31%) $\geq$ N370E(32%)>N370Q(0.5%) (Table 1). Under either assay condition, the variation in CRIM-SA was $\leq 10\%$. Under more acidic conditions in BPS (pH 4.8), the CRIM-SA of N370D increased somewhat and became identical to that for the wild-type enzyme. The N370E had increased CRIM-SA at lower pH under either assay condition.

The enzymes were pre-incubated in either TC/TX or BPS containing citrate/phosphate buffers at varying pH values. The maximal enzyme activity was set to 100% for each mutant protein, thereby providing relative activity curves versus pH (Fig. 2A and B). In the TC/TX assay, the activities were maximal for N370, N370S and N370Q between pH 5.6 and 6.0 (Fig. 2A). The activity profile for N370S was broader with substantial activity at pH 6.5 – 7.0. The mutants with negative side chains (D and E) had an acidic shift in pH optimum from pH ~ 5.8 to ~ 4.8 and ~ 5.2 , respectively. In the BPS system, the optimal pH was slightly more basic (~ 5.7 to 6.0) for wild type and N370S (Fig. 2B). N370D was similar, but with a broader optimal level and maintenance of activity at slightly more acidic pHs. N370E and Q have an extra methylene group compared to N or D and had a significantly more acidic pH optima in the BPS system. The pH optimum curve for N370E was essentially the same in the TC/TX or BPS assay systems. The calculated pK_{a} s for these mutant diprotic enzymes are in Table 2.

Enzyme Activation by TC and BPS

To explore the relationship between activator and active site function, the GCase activation profiles of TC (0 to 8 mM) and BPS (0 to 8 μM) were evaluated with each mutant enzyme (Fig. 3). With TC, the fold activation of N370D (~ 22 fold) was similar to that for the wild-type enzyme (~ 25 fold). N370E (~ 8 fold) and N370Q (~ 1.5 fold) were more poorly activated by TC/TX compared to wild type enzyme (Fig. 3A). At low TC concentration, N370S responded poorly. After TC increased to ≥ 4 mM, activation was enhanced (~ 35 fold). The activation profiles for N370 (WT), N370S, N370D and N370E in the BPS system were similar to the respective patterns in the TC system. Using BPS as the activator, similar profiles were obtained except for a more prompt enhancement of N370S GCase activity compared to that obtained in TC (Fig. 3B). The properties of the amino acid side chains at position 370 significantly influence active site function. In combination with 0.5 μM BPS, saposin C enhanced N370 (WT) GCase ~ 3.5 -fold compared to ~ 2.4 -fold for N370S, and 1.8-fold for N370D or E. N370Q showed no effect (Fig. 3C). These data indicate that a negatively-charged side chain, i.e., D, at position 370 enhances active site function under certain conformational conditions (TC/TX). The very low CRIM-SA of N370Q shows the importance of proper spatial interaction at this residue, i.e., one methylene group extension in the side chain of an isofunctional amino acid (Q vs N) decreases the activity by $>95\%$. The addition of a charged group and a methylene

group, i.e., N370E, produced a GCase with 20–25% of the CRIM specific activity of the wild-type enzyme, but with a shifted in pH optimum. Thus, the negative charge can partially compensate for the adverse steric effects of the methylene group. In the BPS assay system at pH 5.5, the CRIM-SA order was N370>N370D suggesting differential conformational effects.

BPS had a differential activation effects on the CRIM-SA of N370E and N370Q indicating an important alignment of the side chains residues in this enzyme's active site. The greater effect of BPS on N370S than any other GCase tested here shows a unique effect of N370S on active site function. It is possible that the BPS partially reverts an N370S induced distortion within active site for substrate hydrolysis. These results suggest a conformational effect with direct influence on active site function by the 370 residue.

Active Site-directed inhibition studies

dNM and CS are rapidly reversible competitive inhibitors of wild-type GCase [10,26] and pilot studies showed this pattern of inhibition with each active mutant GCase (data not shown). CBE is an irreversible inhibitor [24,33]. Initial enzyme activities were equalized and set to 100% for each wild-type or mutant enzyme. The K_{iapp} (dNM and CS) or IC_{50} (CBE) values for each inhibitor with N370, N370D, N370E or N370Q enzymes were similar (Table 1). N370S was the exception with 2 to 4-fold increase in these respective values for dNM and CBE. These data also suggest that the basis for the decreased activities among mutant enzymes may involve steps in substrate hydrolysis, other than binding, i.e., glucosylation and/or deglucosylation.

Studies with Alternative Substrates

The goal of this study was to evaluate the separate steps of the hydrolytic process for these mutant enzymes. DNPG is a mechanism based active site-directed inhibitor, a single turnover substrate, that forms a covalent enzyme-glucosyl intermediate [17,18]. Preliminary studies showed the release of DNP is in 1:1 stoichiometry with the formation of a covalent GCase-2-deoxy-2-fluoro-glucose complex as evidenced by retention of inactivation in dilution experiments. The purified GCases were evaluated in TC and BPS assay systems. The GCase active sites were equalized by titration in separate experiments with DNPG. There is one "active" active site per mature polypeptide as estimated from CRIM SA values (data not shown). Experiments were at 25°C. The hydrolytic rates at 37°C were too fast to distinguish differences, and at 15°C hydrolytic rates were too slow to ensure enzyme stability (> 4 hour) (data not shown). The K_{mapp} values for DNPG for the N370, N370D, N370E and N370S enzymes were 90–120 μ M in either the TC/TX or BPS systems. The order of initial rates in the TC activation system was as follows: N370~N370D>>N370S~N370E>>N370Q (Fig. 4A). The order in BPS was: N370>N370D>>N370S>N370E>>N370Q (Fig. 4B). N370Q is not shown since the rates of DNP production were very low. The times for complete inhibition for N370S or N370E in TC/TX were 28 to 30-fold (3600–4500 sec) that for the wild-type enzyme (150–200 sec). This is somewhat greater than the estimates of CRIM-SA (k_{cat}) where N370S and N370E had 15–20% (a 5 to 7-fold decrease) of the activity of wild-type enzyme in TC/TX. Because N370S is highly activated by BPS, it is not surprising that the initial rate of N370S in BPS was increased, but relative to N370, the time to complete inhibition remained at 5 to 20-fold increased relative to wild-type. These data indicated that the N370S and E mutant enzymes have consistently abnormal glucosylation comparing to the wild-type enzyme. The N370D enzyme had nearly wild-type initial rates in the TC system and a ~5-fold decreased rate in the BPS system. These results show a significant sensitivity for the N370D enzyme that relates to the negative charge on the aspartate. However, all these mutant enzymes showed significant effects on active site function.

To determine the relative deglucosylation rates for each mutant enzyme, pentanol was chosen as a transglucosylation acceptor in the reaction [34]. 4-MU-Glc was the substrate. Greater fold-

activations of GCases would be predicted in those mutants with defective deglucosylation steps since a good acceptor would enhance deglucosylation to a greater extent (Fig. 5). N370D and E had similar fold enhancements relative to N370, although N370E required more pentanol to achieve wild-type enhancement levels. N370Q has very low activity, but had nearly wild-type response to pentanol in either TC/TX (Fig. 5A) or BPS (Fig. 5B) assay systems. N370S differed greatly from N370 or the other N370 mutant GCases in having an enhanced response to increase pentanol, indicating a significantly altered deglucosylation step in the N370S enzyme. This result and the abnormal glucosylation kinetics (Fig. 4) show that the glucosylation and deglucosylation steps both were altered for the N370S enzyme whereas only the glucosylation step was defective in the other mutants.

Functional Identification of E235G as the Acid/Base in Catalysis

Purified E235G and wild-type GCases were tested with different substrates (4MU-Glc, DNPG, p-NPG, and o-NPG). The E235G activities toward 4MU-Glc, p-NPG, o-NPG were less than 0.05% compared to the respective activities with the wild-type enzyme using TC (4.25 mM) as the activator. Under the same reaction conditions, DNPG, a substrate with an acidic leaving group, was slowly (~2% of wild-type levels) cleaved by E235G. This indicates that E235G has activity toward this substrate with a more acidic leaving group without the necessity of protonic assistance in the glucosylation step.

Different concentrations of azide, an exogenous nucleophile, were added into the reaction mixtures. A 1:4 molar ratio of enzyme to azide produced hydrolytic rates of DNPG by E235G that were nearly the same (~90%) as that of the wild-type enzyme (Fig. 6A). Azide at this concentration had no effect on the wild-type enzyme activity. Active site titrations were conducted using the E235G enzyme and DNPG in the TX/TC system, pH5.8. For equal amounts of purified E235G protein, the stoichiometry and numbers of “active” active sites were unchanged with azide from 10–100 μ M (Fig. 6B). Inhibition of the E235G enzyme with deoxynojirimycin was accomplished in the presence of azide using the DNPG substrate. The IC_{50} values for the E235G and the wild-type enzyme were the same (54 ± 11 μ M, E235G vs 48 ± 11 μ M, WT).

Using 1:4 (mole/mole) E235G to azide, the pH profile of E235G activity showed loss of pH dependency between pH 4.4 and pH 5.2. (Fig. 6C). Above pH 5.6, the activity pattern of pH dependency was nearly the same as that for the wild-type enzyme.

DISCUSSION

These studies show a significant role for the N370 residue in the catalytic cycle of GCase. Also, functional evidence is provided for the direct participation of E235 as the acid/base in the catalytic cycle of GCase. By placing multiple substitutions at the N370 residue, particular, well-documented [35] physiokinetic abnormalities were shown to be associated uniquely with the N370S substitution. These abnormalities include increased activation by phosphatidylserine, a normal K_{mapp} , abnormal inhibition constants for specific active site directed inhibitors, and normal stability as assessed by selected proteolytic sensitivity [26, 36]. Importantly, the glucosylation and deglucosylation steps of the catalytic cycle were both altered only with the N370S enzyme. Thus, this is a residue specific, not positional, effect. Importantly, these kinetic abnormalities indicate a mechanistic effect on catalysis.

The role of N370 in the catalytic cycle is significant, and is associated with local or isolated, not global, probably local conformational effects, at or near the active site. Examination of the pH activity profiles for the serine, glutamate, glutamine and aspartate substituted enzymes provide some insight into the potential effect of N370 at the active site. Specifically, the introduction of the acidic residues, glutamate and aspartate, showed an acidic shift in the pH

optimum for activity using the TC/TX assay system (Fig. 2). This is likely a conformational effect since in the brain phosphatidylserine assay system, the aspartate substituted enzyme did not show this acidic shift. In comparison, the isosteric substitutions, glutamate and glutamine, have an extra methylene group compared with either aspartate or asparagine, and do show an acidic shift in pH optimum. This indicates a rather exquisite sensitivity of the active site for steric or charge effects, in which residue 370 participates. Thus, the local conformational effects prescribe specific alignments in the active site that are modified by the assay environment, and indicate a substantial effect on the catalytic cycle. These functional effects might not be expected from crystal structures that position N370 outside of the active site pocket [12]. However, the crystal structure containing the potent inhibitor isofagomine indicates a significant loop conformational shift that alters this proximity.

Similar conclusions can be reached from the activation effects of taurocholate/Triton X-100 or phosphatidylserine with or without saposin C (Fig. 5C). With all of the mutant enzymes, some degree of activation was achieved although the N370Q enzyme had very poor activation. This is an isofunctional substitution for the asparagine at residue 370 and implies significant steric effects mediated by the environment surrounding this residue. In comparison, the substitution of an isosteric aspartate for asparagine at 370 leads to small effects as evidenced by essentially normal activation and the catalytic properties. When comparing the isofunctional groups, aspartate vs glutamate or asparagine vs glutamine, the significant changes in the degrees of achieved activation show that the residue at 370 has selective effects on active site function.

The glucosylation and deglucosylation steps in catalytic activity (Fig. 1) were evaluated to determine the effects of substitutions at N370 on the sequential steps in the catalytic cycle. Using the DNPPG, a single turnover substrate, very slow glucosylation rates were obtained with the N370S and N370E substituted enzymes in either assay system. The decrease in glucosylation rate was at least 10–12 fold. With N370D, the environment altered the rate significantly. Normal rates were obtained in the detergent system, whereas ~5-fold decreased rates were obtained in the BPS-based assay. In comparison, no effect was observed on the K_{app} for competitive inhibitors or the active site-directed inactivator, CBE. These data show direct effects on the glucosylation mechanism by the residue substitution at 370 that can be significantly altered by their environment. However, there was little overall effect on the kinetic parameters for active site inhibitor interaction. Again, these indicate a substantial local effect with direct influence on catalytic site function, but without severe disruption of the residues involved with substrate/competitive inhibitor binding. In the crystal structure of the isofagomine-GCase complex, the hydroxyl groups of isofagomine interact with Asp127, Trp179, Trp381 and Asn396. In comparison, the imino group that corresponds to the anomeric carbon of β -glucose is stabilized by E235 and E340. The residue Tyr313 is in loop 1 (residues 311–319) and plays a key role in moving this loop to open the active site for binding. N370 is located on the helical turn near loop1 and contributes to the hydrogen bonding network to stabilize the orientation of this loop [25] and, therefore, may control substrate access to the active site. Molecular modeling of this movement and stabilization of loop1 upon binding of isofagomine [25] reveals two troughs for binding of the hydrophobic sphingosyl and N-acyl chain of the glucosylceramide substrate. This is consistent with the proposed kinetic model for substrate binding and catalysis [26,37]. The presence of the C9-alkyl chain on N-nonyl-deoxynojirimycin and potential distortion of the active site to accommodate the imino group, present in oxygen position of the β -glucose ring, may contribute to the potential steric hindrance of E340 [19], since crystal structures with either covalently bound CBE [33] or non-covalently attached isofagomine [25] did not reveal such hindrance. Thus, the details of the exact mechanism of covalent attachment of glucose to the active site of GCase remain to be elucidated.

The second step in the regeneration of the enzyme, deglucosylation, was evaluated by transglucosylation using pentanol as an acceptor. The effects of substitutions at N370 were highly specific with the N370S substituted enzyme exhibiting large activation by increasing pentanol concentrations (Fig. 5). Such an effect would be expected if the deglucosylation step required significant assistance to facilitate completion of the catalytic cycle and regeneration of the active site residues. In comparison, the other substituted enzymes showed activation affects that were the same as that for the wild-type enzyme in either assay system. These results indicate an abnormality in the deglucosylation step of the N370S enzyme in either assay system that was unique and substitution specific. These results and those of the glucosylation studies show that the N370S enzyme has significant alterations in the mechanism of reaction involving both the glucosylation and deglucosylation steps, and that the proposal increase in loop 1 N370-containing helix distance caused by the S substitution has effects on the overall catalytic cycle. Other N370 substitutions have greater effects on the glucosylation step.

For mutant enzymes found in Gaucher disease patients, the K_{mapp} values have been normal or mildly changed [10,26,37–39]. For Michealis-Menten kinetics, the K_{mapp} approximates K_s , the thermodynamic binding constant. However, the discrepancy between selected K_{iapp} (2–5 fold increase) for potent competitive inhibitors, and the K_{mapp} for substrates indicates that for the N370S mutant GCCase, K_{mapp} is a kinetic constant, rather than a thermodynamic constant. This implies that the K_{iapp} values obtained with enzymes containing specific substituted residues at 370, more likely reflects active site interaction than the do the K_{mapp} values. These above studies provide guidance for interpretation of crystallization to evaluate the N370S residue and suggest that, although high resolution, the available crystal structures need correlation with functional analysis of GCCase. Additional structural functional studies of mutant GCases are needed to refine the alignment and anticipation of residues at the active site and their contributions to the catalytic cycle.

Previous studies have indicated that E235 is a putative acid/base in the catalytic cycle of GCCase. These conclusions were based on either the loss of enzyme activity or computational modeling [14,15,20,21]. The loss of enzyme activity is not a test of participation in active site function since all of the mutations that have been found in Gaucher disease patients lead to significant losses and, in some cases, absolute loss of enzyme activity. For all the mutant enzymes studied to date, the catalytic rate constants have been 7 to > 100-fold decreased [22,26,37]. Because of their chemical natures, e.g., V, L, I or G, many of these mutations cannot participate in the catalytic cycle/mechanism. Thus, functional studies were needed to support E235 as the acid/base in catalysis. Here, losses of activity to < 1% of wild-type were obtained with the E235G enzyme; glycine cannot have acid/base functions. Importantly, the restoration of some activity was obtained using a substrate with an acidic leaving group, i.e. DNPG. Indeed, enzyme activity could be restored to nearly wild-type levels by addition of exogenous nucleophiles, azide, to provide assistance in the cleavage of the glucosidic-enzyme bond [18]. Further support for E235 as the acid/base was the loss of the acidic arm of the pH optimum curve with DNPG substrate in the presence of azide (Fig. 6A and B). The near identity of the IC_{50} values for the wild-type and E235G enzymes with deoxynojirimycin indicates the presence of an intact active site with little distortion. This finding also implies that the E235 residue is not involved in initial collision complex for binding of this competitive inhibitor and, therefore, the deoxynojirimycins are unlikely to mimic transition state analogues. Similarly, the GCCase from *Paenibacillus* sp. TS12 was evaluated and found have similar properties using this approach [40]. Although this enzyme has no sequence similarity to the human enzyme, the complimentary data indicate that E235 is indeed the acid/base in the catalytic cycle of GCCase. These studies do not directly address, but do suggest, the development of a carbenion at the anomeric carbon of β -glucoside GCCase substrates, preceding covalent attachment of glucose to E340, as suggested from N-alkyl-dNM-bound crystal structures [19].

Studies presented here and additional studies with mutant enzymes in combination with refined crystal structures of active GCases should provide greater insight into potential for modeling GCases and their evolution to more active functional enzymes for therapy. Of importance is the lack of significant polymorphic variation of human GCases. The rare allele encoding E326K is the only variant described to date that is associated with a normal phenotype when in *trans* to another disease allele [41]. All other mutations, albeit found in affected individuals, lead to highly defective enzymes [26,39,42–44]. Studies shown here with variously substituted residues at 370 also indicate that it will be difficult to obtain GCase variants that retain or have enhanced function, and that only selected mutants with globally abnormal folding and/or normal catalytic mechanisms would be amenable to large scale effects with chaperone approaches. The mutations in Gaucher disease are spread throughout molecule and indeed involved all the domains predicted from crystal structures [12,19,22,25,33,45] and suggest rather global changes in the enzyme function or proteolytic stability will be obtained by various mutations [22].

Abbreviations

TC	Sodium taurocholate
TX	Triton X-100
o-NPG	p-NPG and DNPG, ortho-, para-, and 2,4-dinitrophenyl- β -D-glucoside, respectively
DNPG	2,4-dinitrophenyl-2-deoxy-2-fluoro- β -D-glucoside
4MU-Glc	4-methylumbelliferyl- β -D-glucoside
dNM	deoxynojirimycin
CS	castanospermine
DNP	2,4-dinitrophenol

References

1. Beutler, E.; Grabowski, GA. Gaucher disease. In: Scriver, CR.; Beaudet, AL.; Sly, WS.; Valle, D., editors. *The Metabolic and Molecular Bases of Inherited Diseases*. McGraw-Hill; New York: 2001. p. 3635-3668.
2. Brady RO, Kanfer JN, Shapiro D. Metabolism of glucocerebrosides. II. Evidence of an enzymatic deficiency in Gaucher's disease. *Biochem Biophys Res Commun* 1965;18:221–225. [PubMed: 14282020]
3. Berg-Fussman A, Grace ME, Ioannou Y, Grabowski GA. Human β -glucosidase: N-glycosylation site occupancy and the effect of glycosylation on enzymatic activity. *J Biol Chem* 1993;268:14861–14866. [PubMed: 8325864]
4. Grace ME, Grabowski GA. Human acid β -glucosidase: Glycosylation is required for catalytic activity. *Biochem Biophys Res Commun* 1990;168:771–777. [PubMed: 2110456]

5. Erickson AH, Ginns EI, Barranger JA. Biosynthesis of the lysosomal enzyme glucocerebrosidase. *J Biol Chem* 1985;260:14319–14324. [PubMed: 3932353]
6. Bergmann JE, Grabowski GA. Posttranslational processing of human lysosomal acid β -glucosidase: a continuum of defects in Gaucher disease type 1 and type 2 fibroblasts. *Am J Hum Genet* 1989;44:741–750. [PubMed: 2495719]
7. Basu A, Prence E, Garrett K, Glew RH, Ellingson JS. Comparison of N-acyl phosphatidylethanolamines with different N- acyl groups as activators of glucocerebrosidase in various forms of Gaucher's disease. *Arch Biochem Biophys* 1985;243:28–34. [PubMed: 3933429]
8. Basu A, Glew RH, Wherrett JR, Huterer S. Comparison of the ability of phospholipids from rat liver lysosomes to reconstitute glucocerebrosidase. *Arch Biochem Biophys* 1986;245:464–469. [PubMed: 3954363]
9. Qi X, Grabowski GA. Acid β -glucosidase: intrinsic fluorescence and conformational changes induced by phospholipids and saposin C. *Biochemistry* 1998;37:11544–11554. [PubMed: 9708990]
10. Osiecki-Newman K, Legler G, Grace M, Dinur T, Gatt S, Desnick RJ, Grabowski GA. Human acid β -glucosidase: inhibition studies using glucose analogues and pH variation to characterize the normal and Gaucher disease glycon binding sites. *Enzyme* 1988;40:173–188. [PubMed: 3234317]
11. Osiecki-Newman K, Fabbro D, Legler G, Desnick RJ, Grabowski GA. Human acid β -glucosidase: use of inhibitors, alternative substrates and amphiphiles to investigate the properties of the normal and Gaucher disease active sites. *Biochim Biophys Acta* 1987;915:87–100. [PubMed: 2956992]
12. Dvir H, Harel M, McCarthy AA, Toker L, Silman I, Futerman AH, Sussman JL. X-ray structure of human acid- β -glucosidase, the defective enzyme in Gaucher disease. *EMBO Rep* 2003;4:704–709. [PubMed: 12792654]
13. Miao S, McCarter J, Tull D, Grace ME, Grabowski GA, Withers SG, Aebersold R. Identification of Glu340 as the active site nucleophile in human glucocerebrosidase by use of electrospray tandem mass spectrometry. *J Biol Chem* 1994;269:10975–10978. [PubMed: 7908905]
14. Henrissat B. Sequence homology between a β -galactosidase and some β -glucosidases. *Protein Sequences & Data Analysis* 1991;4:61–62. [PubMed: 1924272]
15. Henrissat B, Callebaut S, Fabrega P, Lehn P, Rnon JP, Avies G. Conserved catalytic machinery and the prediction of a common fold for several families of glycosyl hydrolases. *Proc Natl Acad Sci USA* 1995;92:7090–7094. [PubMed: 7624375]
16. Premkumar L, Sawkar AR, Boldin-Adamsky S, Toker L, Silman I, Kelly JW, Futerman AH, Sussman JL. X-ray structure of human acid- β -glucosidase covalently bound to conduritol-B-epoxide. Implications for Gaucher disease. *J Biol Chem* 2005;280:23815–23819. [PubMed: 15817452]
17. Street IP, Kempton JB, Withers SG. Inactivation of a β -glucosidase through the accumulation of a stable 2-deoxy-2-fluoro- α -glucopyranosyl-enzyme intermediate: A detailed investigation. *Biochemistry* 1992;31:9970–9978. [PubMed: 1390781]
18. Kempton JB, Withers SG. Mechanism of *Argobacterium* β -glucosidase: Kinetic studies. *Biochemistry* 1992;31:9961–9969. [PubMed: 1390780]
19. Brumshtein B, Greenblatt HM, Butters TD, Shaaltiel Y, Aviezer D, Silman I, Futerman AH, Sussman JL. Crystal structures of complexes of N-butyl- and N-nonyl-deoxynojirimycin bound to acid β -glucosidase: insights into the mechanism of chemical chaperone action in Gaucher disease. *J Biol Chem* 2007;282:29052–29058. [PubMed: 17666401]
20. Grace ME, Desnick RJ, Pastores GM. Identification and expression of acid β -glucosidase mutations causing severe type 1 and neurologic type 2 Gaucher disease in non-Jewish patients. *J Clin Invest* 1997;99:2530–2537. [PubMed: 9153297]
21. Fabrega S, Durand P, Codogno P, Bauvy C, Delomenie C, Henrissat B, Martin BM, McKinney C, Ginns EI, Mornon JP, Lehn P. Human glucocerebrosidase: heterologous expression of active site mutants in murine null cells. *Glycobiology* 2000;10:1217–1224. [PubMed: 11087714]
22. Liou B, Kazimierczuk A, Zhang M, Scott CR, Hegde RS, Grabowski GA. Analyses of variant acid β -glucosidases: effects of Gaucher disease mutations. *J Biol Chem* 2006;281:4242–4253. [PubMed: 16293621]
23. Greenberg P, Merrill AH, Liotta DC, Grabowski GA. Human acid β -glucosidase: use of sphingosyl and N-alkyl- glucosylamine inhibitors to investigate the properties of the active site. *Biochim Biophys Acta* 1990;1039:12–20. [PubMed: 2141287]

24. Legler G. Glycoside hydrolases: Mechanistic information from studies with reversible and irreversible inhibitors. *Adv Carbo Chem Bioch* 1990;48:319–384.
25. Lieberman RL, Wustman BA, Huertas P, Powe AC Jr, Pine CW, Khanna R, Schlossmacher MG, Ringe D, Petsko GA. Structure of acid β -glucosidase with pharmacological chaperone provides insight into Gaucher disease. *Nat Chem Biol* 2007;3:101–107. [PubMed: 17187079]
26. Grace ME, Newman KM, Scheinker V, He GS, Berg-Fussman A, Grabowski GA. Analysis of human acid β -glucosidase by site-directed mutagenesis and heterologous expression. *J Biol Chem* 1994;269:2283–2291. [PubMed: 8294487]
27. Fabbro D, Desnick RJ, Grabowski GA. Gaucher disease: genetic heterogeneity within and among the subtypes detected by immunoblotting. *Am J Hum Genet* 1987;40:15–31. [PubMed: 3812484]
28. Summers, MD.; Smith, GE. A manual of methods for Baculovirus vectors and insect cell culture procedures, Texas Agricultural Experimental Station Bull. College Station; Tx: 1987.
29. Jarvis DL, Summers MD. Glycosylation and secretion of human tissue plasminogen activator in recombinant baculovirus-infected insect cells. *Mol Cell Biol* 1989;9:214–223. [PubMed: 2494430]
30. Furbish FS, Blair HE, Shiloach J, Pentchev PG, Brady RO. Enzyme replacement therapy in Gaucher's disease: Large-scale purification of glucocerebrosidase suitable for human administration. *Proc Natl Acad Sci USA* 1977;74:3560–3563. [PubMed: 269414]
31. Legler G. Glucosidases Methods. *Enzymol* 1977;46:368–381.
32. Wang Q, Trimbur D, Graham R, Warren RAJ, Withers SG. Identification of acid/base catalyst in *Agrobacterium faecalis* β -glucosidase by kinetic analysis of mutants. *Biochemistry* 1995;34:554–562.
33. Premkumar L, Sawkar AR, Boldin-Adamsky S, Toker L, Silman I, Kelly JW, Futerman T, Sussman JL. X-ray structure of human acid β -glucosidase covalently-bound to conduritol B epoxide: Implications for Gaucher disease. *J Biol Chem* 2005;280:23815–23819. [PubMed: 15817452]
34. Gopalan V, Vander Jagt DJ, Libell DP, Glew RH. Transglucosylation as a probe of the mechanism of action of mammalian cytosolic β -glucosidase. *J Biol Chem* 1992;267:9629–9638. [PubMed: 1577800]
35. Grabowski GA, Osiecki-Newman K, Dinur T, Fabbro D, Legler G, Gatt S, Desnick RJ. Human acid β -glucosidase. Use of conduritol B epoxide derivatives to investigate the catalytically active normal and Gaucher disease enzymes. *J Biol Chem* 1986;261:8263–8269. [PubMed: 3087971]
36. Prenc EM, Garrett KO, Panitch H, Basu A, Glew RH, Wherrett JR, Huterer S. Sulfogalactocerebroside and bis-(monoacylglycerol)-phosphate as activators of spleen glucocerebrosidase. *Clin Chim Acta* 1986;156:179–189. [PubMed: 3085988]
37. Grace ME, Berg A, He GS, Goldberg L, Horowitz M, Grabowski GA. Gaucher disease: heterologous expression of two alleles associated with neuronopathic phenotypes. *Am J Hum Genet* 1991;49:646–655. [PubMed: 1909090]
38. Grace ME, Graves PN, Smith FI, Grabowski GA. Analyses of catalytic activity and inhibitor binding of human acid β -glucosidase by site-directed mutagenesis. Identification of residues critical to catalysis and evidence for causality of two Ashkenazi Jewish Gaucher disease type 1 mutations. *J Biol Chem* 1990;265:6827–6835. [PubMed: 2324100]
39. Sa Miranda MC, Aerts JM, Pinto R, Fontes A, de Lacerda LW, Van Weely S, Barranger J, Tager JM. Activity of glucocerebrosidase in extracts of different cell types from type 1 Gaucher disease patients. *Clin Genet* 1990;38:218–227. [PubMed: 2225530]
40. Paal K, Ito M, Withers SG. *Paenibacillus* sp. TS12 glucosylceramidase: kinetic studies of a novel sub-family of family 3 glycosidases and identification of the catalytic residues. *Biochem J* 2004;378:141–149. [PubMed: 14561218]
41. Zhao H, Bailey L, Arnold GL, Grabowski GA. Gaucher's disease: identification of novel mutant alleles and genotype-phenotype relationships. *Clin Genet* 2003;64:57–64. [PubMed: 12791040]
42. Tager JM.; Aerts JM.; Jonsson MV.; Murray, GJ.; Van Weely, S.; Strijland, A.; Ginns, EI.; Reuser, JJ.; Schram, AW.; Barranger, JA. Molecular forms, biosynthesis and maturation of glucocerebrosidase, a membrane associated lysosomal enzyme deficient in Gaucher disease. In: Freysz, L.; Dreyfus, H.; Massarelli, R.; Gatt, S., editors. *Enzymes of Lipid Metabolism II*. Plenum Press; New York: 1986. p. 735-745.

43. Jonsson LM, Murray GJ, Sorrell SH, Strijland A, Aerts JF, Ginns EI, Barranger JA, Tager JM, Schram AW. Biosynthesis and maturation of glucocerebrosidase in Gaucher fibroblasts. *Eur J Biochem* 1987;164:171–179. [PubMed: 3549301]
44. Tsuji S, Choudary PV, Martin BM, Stubblefield BK, Mayor JA, Barranger JA, Ginns EI. A mutation in the human glucocerebrosidase gene in neuronopathic Gaucher's disease. *N Engl J Med* 1987;316:570–575. [PubMed: 2880291]
45. Brumshtein B, Wormald MR, Silman I, Futerman AH, Sussman JL. Structural comparison of differently glycosylated forms of acid- β -glucosidase, the defective enzyme in Gaucher disease. *Acta Crystallogr D Biol Crystallogr* 2006;62:1458–1465. [PubMed: 17139081]

Figure 1. Schematic of the double displacement mechanism of acid β -glucosidase hydrolysis of substrates

E340 and E235 are the nucleophile and putative acid/base within the active site. This retaining β -glucosidase forms a glucosyl-enzyme complex following attack of the acid of the acid/base residue (E235) at the anomeric carbon with the formation of a partially stabilized oxycarbonium structure. Ceramide (ROH) is released and a covalent glucosyl-enzyme is formed with the E340 nucleophile. The mechanism might include a carbenion rather than the partially stabilized intermediates as depicted [19]. This complex is then attacked by water that is base-assisted from the acid/base. β -glucose, the E235 and E340 are regenerated. δ designates a partial charge ($-$ or $+$).

Figure 2. pH activity profiles for N370 substituted acid β -glucosidases

In (A) the TC/TX or (B) brain phosphatidylserine (32 μ M)-based assays were used with the 4MU-Glc substrate. All activities were normalized to their individual maximal activities (100%) for profile comparative purposes. N370 is the wild-type enzyme. Citrate/phosphate (0.04M) buffers were used throughout. N370 (■); N370S (●); N370D (◆); N370E (▼); N370Q (▲), D=aspartate, E=glutamate, Q=glutamine, N=asparagine, S=serine.

Figure 3. Activation of N370 substituted acid β -glucosidases by negatively-charged lipids and/or saposin C

In (A) Triton X-100 (4 mM) was included in each assay with taurocholate varying from 0–8 mM at pH 5.6. In (B) each assay contained brain phosphatidylserine (BPS, 0–8 μ M) and the reactions were at pH 5.5. In (C) BPS and Saposin C (125 nM) were included in the assays (pH 5.5). For fold change the data were individually normalized to the respective activities without the effector(s). N370 (■); N370S (●); N370D (◆); N370E (▼); N370Q (▲).

Figure 4. Progress curves for the release of 2,4-dinitrophenol from the single turnover substrate, 2,4-dinitrophenyl-2-fluoro-2-deoxy- β -glucose

In (A) or (B) Triton X-100/taurocholate or BPS assay systems were used, respectively. “Active” active sites were equalized by prior titration with the DNPPG single turnover substrate/inhibitor. After about 3000–3600 sec., the absorbance from DNP plateaued at essentially equal levels for all GCases indicating an equal number of “active” active sites. Comparisons of the times to complete (2-fluoro)-glucosylation of the active sites provide relative rates of glucosylation since the K_m for all the mutants were the same ($108 \pm 17 \mu\text{M}$) with the DNPG substrate. The results are composites of triplicate assays (solid lines) and their respective 95% confidence intervals (dashed lines) smoothed by non-linear one phase association using least squares from >500 data points for each curve (<3000 sec). N370S and N370E showed rates of glucosylation 10–12 fold less than those for N370 in either system. Only the N370D enzyme showed a difference in glucosylation rates with the different assay systems, indicating steric/charge changes induced by the different negatively charged lipids that specifically altered this residue’s interaction in the active site.

Figure 5. Transglucosylation by N370 mutant enzymes using pentanol as the acceptor
 In either the TC/TX (A) or BPS-based (B) systems, N370S showed a greater fold-increase in transglucosylation with increasing pentanol than the N370 enzyme. In comparison, the other

N370substituted enzymes had very similar transglucosylation enhancements by pentanol. These results are consistent with defective deglucosylation of the glucosylated N370S enzyme compared to wild-type or the other N370-substituted mutant enzymes.

Figure 6. Activity of E325G in the presence of azide

In (A) E325G had little activity (<0.5% WT) toward the 2,4-dinitrophenyl- β -D-glucoside substrate in the absence of azide. At 10 μ M azide, the hydrolytic rate of 2,4-DNP- β -glucose by E235 ($\blacktriangle$ $\blacktriangle$) was that (95%) of the wild-type ($\blacksquare$ - $\blacksquare$). Except for an initial lag phase, the progress curve for generation of 2,4-dinitrophenol was parallel to that of wild-type, i.e., the same DNP generation rates. In (B) azide [50 (lower) and 100 (upper) μ M] were used and DNPPG was the substrate. The quantification of active sites was independent of [azide] >100 sec. The solid lines are means of two triplicate sets and the dashed lines are 95% confidence limits of the smoothed progress curves. In (C) the pH optimum curves for E325G show essentially no change in activity (<0.01 WT) in the absence of azide with DNPG as substrate ($\blacktriangle$ $\blacktriangle$). In the presence of azide the RA increased to 80–90% of wild-type with concomitant loss of the acidic arm ($\blacksquare$ --- $\blacksquare$). Azide had no effect on the wild-type enzyme ($\bullet$ - $\bullet$). RA=relative activity to wild-type.

Table 1
Activity and inhibition parameters for wild-type and mutant GCases

Source	CRIM-SA (% wild-type)			K _i app (μM)	IC ₅₀ (μM)
	TC/TX	BPS	Deoxynojirimycin	Castanospermine	Condurotol B Epoxide
N370	100	100	65±6.7	3.3±0.5	73±3
N370D	139±13	82±10	70.7±4.3	3.2±0.2	72±2
N370S	15±5	31±8	190±15	5.7±1.2	251±29
N370E	21±7	32±10	75±1.5	4±1	78±4
N370Q	5±2	0.5±3	62.3±3	3.5±2.2	75±2.5
N370A	0	0	-----	-----	-----
N370R	0	0	-----	-----	-----

Table 2Calculated pK_a values for mutated GCases

Source	Taurocholate/Triton X-100		Brain Phosphatidylserine	
	pK_1^*	pK_2	pK_1	pK_2
N370	4.9	6.7	5.4	7.2
N370D	<4.5	6.2	5.1	6.8
N370S	4.9	7.2	5.3	7.0
N370E	4.7	6.2	<4.5	6.38
N370Q	5.03	6.34	4.6	6.2
E235G	-----	6.8		7.1

* All values $\pm$ SE $\pm$ 0.25 pH units. Data fit to a diprotic model in Prism by least squares methods.