ANALYSES OF VARIANT ACID β-GLUCOSIDASES: EFFECTS OF GAUCHER DISEASE MUTATIONS†
Benjamin Liou,1, Andrzej Kazimierczuk,1,† Min Zhang3, C. Ronald Scott3, Rashmi S. Hegde2 and Gregory Grabowski1
From The Children’s Hospital Research Foundation, 1Division and Program in Human Genetics, and the 2Division of Developmental Biology, Cincinnati, Ohio, Department of Pediatrics, University of Cincinnati College of Medicine, 3University of Washington, School of Medicine, Department of Pediatrics
Address correspondence to: Gregory A. Grabowski, M.D., Division and Program in Human Genetics, Children’s Hospital Research Foundation, 3333 Burnet Avenue, MLC 4006, Cincinnati, Ohio 45229-3039, Tel: 513-636-7290; Fax: 513-636-2261; E-Mail: greg.grabowski@cchmc.org

Acid β-glucosidase (GCase) is a 497 amino acid, membrane associated lysosomal exo-β-glucosidase whose defective activity leads to the Gaucher disease phenotypes. To move toward a structure/function map for disease mutations, 42 selected single amino acid substitutions were introduced into GCase, expressed in an insect cell system, purified and characterized for basic kinetic, stability and activator response properties. The variant GCases from Gaucher disease patients and selected variant GCases from the mouse had decreased relative kcat, and differential effects on active site binding and/or attachment of mechanism-based covalent (conduritol B epoxide) or reversible (deoxynojirimycin derivatives) inhibitors. A defect in negatively-charged phospholipid activation was present in the majority of variant GCases, but was increased in two, N370S and V394L. Deficits in saposin C, the natural GCase protein activator, enhancement of kcat were present in variant GCases involving residues 48-122, and ~2-fold increases were obtained with an L264I GCase. About 50% of variant GCases each had wild-type or decreased sensitivity to in vitro cathepsin D digestion. Mapping of these properties onto the crystal structures of GCase indicated wide dispersion of functional properties that can affect catalytic function and stability. Site-directed mutagenesis of cysteine residues showed that the disulfide bonds, C4-C16 and C18-C23, and a free C342 were essential for activity; the free C126 and C248 were not. Relative kcat was highly sensitive to a His substitution at R496, but not at R495. These studies and high phylogenetic conservation indicate localized and general structural effects of Gaucher disease mutations that were not obvious from the nature of the amino acid substitution, including those predicted to be non-disruptive (e.g., V→L). These results provide initial studies for the engineering of variant GCases and, potentially, molecular chaperones for therapeutic use.

Acid β-glucosidase (GCase, glucocerebrosidase, EC 3.2.1.45) is a membrane associated lysosomal hydrolase whose defective function leads to Gaucher disease (1,2). The mature glycoprotein has 497 amino acids that are derived from 517 or 536 amino acid precursors containing leader sequences that are removed during transit through the endoplasmic reticulum membrane (3,4). Cotranslational glycosylation occurs at 4 of 5 N-glycosylation sites (5). This glycosylation is essential for the development of a catalytically active conformer, specifically with N-glycosylation of the first sequon (5,6). The primary sequence does not provide clues to GCase’s tight membrane association as typical hydrophobic transmembrane domains are not present in the mature sequence (7). The newly synthesized enzyme is trafficked to the lysosome via mannose-6-phosphate and oligosaccharide independent pathways (8-10); the peptide sequence responsible for this trafficking remains to be defined. Once resident in the lysosome, the enzyme has a half-life of ~60 hours in fibroblasts and glycosylation is important to maintaining this
survival (8-10). GCase cleaves a variety of glucosyl ceramides and synthetic \( \beta \)-glucosides using detergent or phospholipid-based systems for \textit{in vitro} assays [for review, (2)]. The major natural substrate is the glycosphingolipid, glucosyl ceramide, with the deacylated analogue, glucosylsphingosine, being a minor, but pathologically important, substrate. GCase defects lead to accumulation of these substrates in a tissue specific manner (2).

GCase is a typical retaining \( \beta \)-glucosidase whose catalytic cycle proceeds through a two-step reaction mechanism requiring glucosylation of the active site by substrate followed by deglucosylation with a release of \( \beta \)-glucose (11-13). The nucleophile in this active site function is E340 and the presumptive acid/base is E235 (14-17). The O-glucosidic bond of substrates is protonated by the acid/base (E325) and is then attacked by the nucleophile (E340). Glucose becomes covalently attached and the leaving group is removed. This is followed by deprotonation of water by the acid/base with attack of the enzyme-glucose complex by the liberated nucleophile, and release of \( \beta \)-glucose. The enzyme requires membrane interfaces, with preference for negatively charged phospholipids, to enhance the \textit{in vitro} enzyme activity from near zero in a completely delipidated form. The presence of a naturally occurring 80 amino acid activator protein, saposin C, has additional effects on the activity of the negatively-charged phospholipids-activated GCase (18-22). The exact activation mechanisms of the negatively charged phospholipids and/or saposin C remain to be determined, but the lipids appear to alter the enzyme into a catalytically active conformer that is receptive to the function of saposin C \textit{in vivo} (19,20,23). The genetic absence of saposin C leads to deficiency of glucosyl ceramide cleavage and a Gaucher-like disease (24,25).

About 200 different point mutations encoding amino acid substitutions have been identified in patients with Gaucher disease, but few have been subjected to functional assessment (26). Many of these mutations seem obviously detrimental because the amino acid substitutions are predicted to be highly disruptive (e.g., large charge and/or side chain changes), whereas others do not (e.g., substitutions of two different branched-chain amino acids). Furthermore, the relationships of genotype to phenotype in Gaucher disease should be based on known disruptive functional changes derived from mutations. For example, highly disruptive mutations could produce more significant effects on catalytic function and/or enzyme instability leading to more severe or progressive disease manifestations. Also, knowledge of the distribution of the mutations and their functional effects on catalytic or stability properties of GCase could have significant implications for the design and/or development of new therapeutic agents for enzyme, chaperone, or genetic therapeutic approaches. The vast majority of point mutations occur as compound heterozygotes with two different mutant alleles expressed in the same cells, obviating the direct characterization of the specific GCase properties. Separation of these alleles in recombinant systems allows for \textit{in vitro} characterization of these properties.

Here, analyses of 42 point mutations identified in Gaucher disease patients, 7 mutations of cysteine residues that are either involved in disulfide formation or are free, and 3 mutations of C-terminal histidines were conducted to gain insight into their effects on structure and function of GCase. These properties are correlated with the crystal structure of human GCase, along with comparisons of the properties of selected corresponding mutant mouse GCases to develop contextual insights into mutational effects.

**EXPERIMENTAL PROCEDURES**

**MATERIALS:** The following were from commercial sources: Quick-Change site-directed mutagenesis kit (Stratagene, CA) and Bac-N-Blue Transfection Kit (Invitrogen, CA); Sf9/Sf21 cells (BD Bios., Pharmingen, CA), SF900II serum-free medium (GIBCO/BRL), spinner tanks (Fisher/Kontes, PA). DTT, 2-mercaptoethanol, deoxynojirimycin, castanospermine, Triton X-100, sodium azide, ammonium sulphate, sodium acetate, and enzyme grade sodium citrate (Sigma, MO); Protease Inhibition Cocktail (cocktail III, CalBiochem, CA), Octyl-Sepharose (Amersham Bios., Sweden), butanol, glycerol and ethylene glycol (Fisher, PA). FPLC System (Phamacia, MI); 4-methylumbelliferyl-\( \beta \)-glucoside (4MUG\(^1\))
(Biosynth AG, Switzerland); cathepsin D, sodium taurocholate and conduritol B epoxide (CBE, Calbiochem, CA); brain phosphatidylserine (Avanti, AL). AP color developing kit, goat-anti-rabbit antibody (Bio-rad, CA). N-glycosidase F (New England Biolabs, MA), BCA protein concentration assay (Pierce, IL), YM10 Microcons (Amicon/Millipore, MA). Magnesium chloride heptahydrate (Acros Organics, NJ). Cerezyme was a gift from Genzyme Corp. (Cambridge, MA). Rabbit anti-human GCase (EC 3.2.1.45) was as described (27).

METHODS

Mutation identification: The mutations were identified in genomic DNA from enzymatically confirmed Gaucher disease patients, i.e., those with defective acid β-glucosidase (GCase), from a variety of ethnic and demographic backgrounds. Two approaches were used with amplified genomic DNA: 1) Exons alone were sequenced or 2) the GCase genes were amplified by long-range PCR and sequenced in their entireties. Sequences were analyzed by fluorescent labeling using the ABI capillary electrophoresis system. All sequence reads were verified by direct visualization following automatic base calls with Sequencher (GeneCodes, Ann Arbor, MA) or Mutation Surveyor (SoftGenetics, Inc., State College, PA). Many of the patients were submitted through the ICGG (International Collaborative Gaucher Group) program for GCase DNA sequence analysis. The majority of samples did not have readily identifiable clinical information and/or were heteroallelic for GCase mutations. Available phenotypic information is provided in Table 1. The objective of this work was to verify the pathogenic nature of the mutation, i.e., the predicted amino acid substitution, and its effects on in vitro enzymatic function. Additional mutagenesis was conducted to evaluate the effects of specific amino acids, e.g., the seven cysteines. Serine was chosen as an isosteric substitution for cysteines 4, 16, 18, 23, 126, and 248. The first four cysteines participate in disulfide bonds as follows: C4-C16 and C18-C23. C126, C248 and C342 are free (see Results). C342G and C16S are naturally occurring mutations from Gaucher disease patients (28,29). The mutant enzymes, R495H, R496H and R495H;R496H (a double mutant), were produced to evaluate the potential effects of the R495H substitution in the commercially available enzyme replacement product, imiglucerase™, and the mutation found in Gaucher disease patients, R496H, compared to the wild-type R495;R496 enzyme as determined by amino acid sequence analysis of the wild-type enzyme from human placenta (7).

Expression and purification of GCases: Codons for amino acid substitution were produced individually in the cDNA of wild-type GCase and cloned into the plasmid pBluescript 4.5 (Invitrogen, CA) following site directed mutagenesis (Quick-Change, Stratagene, TX). The GCase mRNAs from wild-type, N370S, V394L, D409H and D409V homozygous mice (30) were isolated, reverse transcribed, sequenced and cloned into the Bac-N-Blue vectors for recombination into baculovirus (see below). All cDNAs were completely resequenced to ensure that no spurious mutations were introduced. The wild-type and mutant cDNAs were inserted into the baculovirus by homologous recombination using the Bac-N-Blue system. The individual viruses were amplified in and purified from Sf9 cells as described (31,32). GCases expressed from these cDNAs were obtained from media of Sf21 cells that had been adapted to serum/protein-free medium. Each GCase was partially purified by batch hydrophobic chromatography following n-butanol extraction as follows: 1) delipidation (n-butanol, 1:4, v/v), 2) batch binding to octyl-sepharose, (1:10, v/v), 3) step batch washing (2 bed volumes) with 50 mM phosphate pH 5.8 containing 20% or 50% ethylene glycol; 4) GCase elution (100% ethylene glycol). All GCases had very similar purification properties requiring only minor modifications of the procedure. The only exception was the incorporation of protease inhibitor cocktail III into the medium and butanol extraction steps for the ∆36, F397S, D409H and D409V mutants, as they were rapidly digested without added cocktail III by proteases released from Sf21 cells into the SF900II medium. Following hydrophobic chromatography, all GCases could be stored for up to 3 mos without evidence of proteolytic digestion. The wild-type, N370S, V394L, D409H and D409V mouse GCases were expressed and purified as above to evaluate the potential effects of multiple amino
acid substitutions on GCase properties from different species. The comparative properties for these specific GCase variants were from Grace et al (31,32), although the properties of N370S were reverified here.

Additional GCase sequences from other mammalian species (Chimpanzee, Orangutan, Porcine, Fugu, partial Rat, Mouse, Honey Bee, and C. elegans) were obtained from public databases and aligned using DNASIS 3.7 (Hitachi Software Engineering Co., San Francisco, CA). The canine GCase cDNA sequence was determined from a clone isolated from a constructed cDNA library using canine brain total RNA (Lambda ZAP kit).

**Enzyme Purification and Crystallization:**
Wild-type GCase for crystallization was purified from Cerezyme™ to remove non-protein components. During the conduct of these experiments, Sussman and coworkers (17,33) solved the GCase crystal structure at ~2.0 Å level. Our crystal structure was highly similar, except that the active site region had greater resolution than that of Dvir et al (33). The deglycosylation procedure developed here provides fully active GCase prior to crystallization. To achieve this, deglycosylated GCase was purified on Octyl sepharose and active fractions (70-90% ethylene glycol) were pooled and exchanged into 10 mM sodium acetate, pH 5.2 immediately before the crystallization. This allowed for recoveries of 85-93% of initial enzyme activity. In comparison, the exchange into MES, pH 6.6 (33) was shown in preliminary experiments to irreversibly inactivate GCase. Final protein concentration was estimated by BCA protein assays to be 10 mg/ml. Activities for GCase to be crystallized were determined with 4MUG (4 mM) as substrate in the presence of brain phosphatidylserine and purified recombinant human saposin C in 25 mM citrate/50 mM phosphate, pH 5.5. Recombinant human saposin C was expressed and purified as described (34). Crystals of N-glycanase treated GCase were obtained by vapor diffusion at room temperature.

**Relative Catalytic Activity:**
Comparisons of the catalytic activity, relative kcat, were determined by referencing to cross-reacting immunological material (CRIM) and expressed as CRIM specific activity (CRIM SA). This approach was needed since the degree of purity of each GCases was not identical, but all activity toward the 4MU-Glc substrate within each preparation was completely inhabitable by CBE, consistent with being a GCase. CRIM SA for each GCase variant was determined using immunoblots developed with anti-human GCase polyclonal antisera (27), and assuming equal reactivity of the GCase variants and wild-type GCase. Predetermined amounts of GCase activity were applied to SDS gels in amounts equivalent to standards of known masses of homogeneous human GCase (2.5, 5 and 10 ng). These amounts of GCase were known to provide readings in the linear range of the densitometer. The CRIM SA was determined from duplicates for each sample based on the standard curve densities.

**Wild-type and Mutant GCase Characterization:**
Immunoblot analyses were as described using rabbit anti-human GCase polyclonal antiserum produced against purified human placental GCase (27). The pH optimum profiles were developed using citrate/phosphate buffers over the range of pH values from 4.4 to 7.2. pKa values were estimated from a diprotic model and varied by ±0.3-0.6 pH units (n=3). Preliminary studies showed deoxynojirimycin and castanospermine to be rapidly reversible competitive inhibitors of all the active enzymes used here, and Kᵢ values were determined from the equation, IC₅₀=Kᵢ(1+Kₘ/[S]). Conduritol B epoxide (CBE) was shown by dilution experiments to be an irreversible inhibitor of all the active enzymes used here. IC₅₀ values were determined under standard assay conditions. The standard GCase activity assay contained citrate/phosphate, pH 5.5, 0.25% sodium taurocholate/0.25% Triton X-100, and 4 mM 4MUG. Assays in the presence of phosphatidylserine and/or saposin C did not contain either taurocholate or Triton X-100. For these assays, the GCases were preincubated at pH 5.5 with phosphatidylserine for 30 min at room temperature prior to the addition of saposin C.

Cathepsin D sensitivity for selected GCases was determined as follows: 2.9 pmol of each GCase variant were digested in the presence of Cathepsin D (2 µM, Calbiochem, CA) in 20 µl of 25 mM Na Acetate pH 4.8, 50 mM NaCl, 1.25 mM EDTA, and 1.25 mM DTT 37°C. After 48 hrs, the reaction mixtures were subjected to
Western analyses using rabbit anti-human GCase polyclonal antiserum (27). The band densities of intact GCase (Mr~60,000) were quantified in digested and undigested aliquots using ImageQuant 5.2 (Molecular Dynamics, CA). The results are expressed as the percentage of remaining intact GCase. The final enzyme preparations for the majority of GCases contained small Mr contaminant proteins that varying between 10 to 30% of the total protein as estimated from stained SDS-PAGE. To ensure that the sensitivity toward Cathepsin D was not altered by such contaminants, the pattern of fragments obtained on immunoelectroblotting was shown to be unaltered during the purification procedure, and by doping, of selected GCases from crude medium through final purification steps, with pure wild-type GCase in different concentrations. The pattern/sensitivity of wild-type GCase was unaltered under these conditions, indicating that sufficient Cathepsin D was used to avoid interference by non-specific proteins. The amounts of variant GCases were estimated from densitometric quantification of immunoblots using pure wild-type GCase from imiglucerase as standard.

RESULTS

Mutations, point substitutions and characteristics of mutant proteins: The variant GCases properties are in Tables 1 and 2, and Figures 1 to 6. Of the listed GCases, in Tables 1 and 2, the following have not been discovered in samples from Gaucher disease patients: the serine for cysteines at 4, 18, 23, 126 and 248, and R495H or R496H. The majority of the natural variant substitutions occur in conjunction with another variant GCase allele, i.e., they are heteroallelic. All variants were expressed independently and no interaction between GCase variants has been observed in double infections in the baculovirus systems (35).

The CRIM specific activity (CRIM SA) assessments using polyclonal antibodies provide a comparison of kcat relative to that from wild-type. For the GCase variants found in Gaucher disease patients, three groups of CRIM specific activities were evident (Fig. 1): 1) Those with 35-50% wild-type CRIM SA: Δ36 and E326K. E326K was a stable enzyme with about 40-50% of wild-type CRIM SA, even though the mutation resulted in addition of a bulky group with a complete charge change (i.e., - to +). This variant has been found in phenotypically normal individuals as the heteroallele to L444P or G202R encoding alleles (36). The Δ36 GCase, an in-frame codon deletion of T36, could be partially purified in the presence of cocktail III, and that enzyme had significant CRIM specific activity (~35% of wild-type). 2) Fifteen variants with <10% CRIM SA, and 3) 25 variants had ≤5% CRIM SA. The M416V and C342G enzymes were stable in medium and could be produced in significant amounts, but were essentially devoid of activity. The variants K198E, F397S, and R463P were rapidly degraded in medium and the CRIM SAs were estimated directly from medium in the presence of protease inhibitors.

Several GCase variants were produced by “knock-in” approaches in mice (30). The corresponding mutant mRNAs were isolated from cultured skin fibroblasts of the respective homozygous mice, expressed in the baculovirus system, purified from media, and characterized (Table 1). These variants corresponded to the human Gaucher disease mutations encoding N370S, V394L, D409H and D409V. The murine and human D409H and D409V GCases were unstable in medium surrounding Sf21 cells. For these murine GCases, cocktail III was added to preserve significant GCase signals. The CRIM SA of the mutant murine GCases was ~11-18%. Those in human were comparable only for N370S and V394L (~12-15%). The values for human D409H and D409V were very low (<0.2%) (32,37). The large standard deviations for estimates of mouse D409H or V derive from variations in the culture conditions, inhibition of proteases, and the preservation of these unstable mutant GCases. These results show that the specific mutations in mice and humans have highly similar enzymatic effects even with the 15% difference in amino acid context of the amino acid backbone (Fig. 8).

The results of active site interaction studies of the above variant GCases with CBE, deoxyojirimycin and castanospermine are summarized in Table 1, and Figures 2 and 3. CBE is a covalent inactivator of GCases with a two-step
mechanism involving a reversible complex of CBE-GCase, and a subsequent modification to the covalent complex. In comparison, deoxynojirimycin is a competitive inhibitor that forms a rapidly reversible inhibitor-GCase complex.

By comparing the effects of these inhibitors, one can dissect the binding and/or covalent complex formation components that could be significantly altered by the amino acid substitutions. The IC50 or Ki values segregate into 5 groups: 1) variant GCases with large increases of IC50 or Ki values for both classes of inhibitors including: F259L, N370S and V394L. This result and the decreases in CRIM SA implicate alterations in formation of the binary collision complex and the covalent attachment of glucose to the active site. 2) Variants with moderate increases of IC50 values for CBE and deoxynojirimycin including: R48Q, K79N, R120Q, I161N, L174F, and Y363C, and similar implications as for class 1. 3) The variant GCases L185F, G193E, L264I, Y305C, E326K, E349K, R353W, L371V and G390R had increases in Ki(deoxynojirimycin) by 2-3 fold (p<0.03) with no effect on IC50(CBE). This result implies a moderate change in binding at the active site for deoxynojirimycin, but not CBE. In general, the Ki(castanospermine) paralleled Ki(deoxynojirimycin) for about half of these variants with about 1.5 to 2.2 fold increases for L185F, G193E, E349K and R353W (p<0.05). Castanospermine is a derivative of deoxynojirimycin that contains a bridge between C6 and the N of deoxynojirimycin, thereby making a 5 member ring that is out of the plane of the glycan ring. The inhibition values for this reversible competitive inhibitor had a mean Ki value of 8.26±2.45[SD] for all mutant GCases compared to the wild-type value of 5.34±1.65[SD] (Table 1). These results imply a significant specificity to the various structures of inhibitors for formation of the binary complex. These data indicate a further, but minor, change in active site recognition of these nojirimycin derivations. 4) The GCases Δ36 and D399N have CBE IC50 values ~50% of wild-type values, whereas the corresponding values for deoxynojirimycin are not altered. For these enzymes, the covalent attachment of CBE could be altered, leading to greater inactivation rates at this fixed time point. This was not directly assessed due to limited enzyme stability and availability. 5) The other variant GCases were not different than wild-type. These GCases in Group 5, probably, have their major pathologic effects by altering the stability and/or trafficking properties of the enzyme in cells (see below).

Substrate (4MUG) K_{m}app for all GCase variants had a mean value of 2.2±0.33[SD] mM. This compares with a mean value for the wild-type GCase of 1.98±0.25[SD] mM, and with those from cell extracts of Gaucher disease patients [(e.g., (32,37)]. The individual values did not correlate with either the CBE or deoxynojirimycin inhibition values. Indeed, the K_{m} value is likely a kinetic parameter, rather than a binding constant.

**Activation of Variant GCases by Phosphatidylserine or Saposin C:** There was a generalized defect in the phosphatidylserine (4 µM) activation of variant GCases from Gaucher patients (Fig. 4). This deficit was about 5-fold, i.e., enhancements of 15-fold in wild-type vs. 2 to 5 fold in the mutants. The exceptions included E326K, N370S and V394L. The latter two GCases, either human or mouse (Table 1), had excess activation compared to wild-type (~2-3 fold) as previously described (32,37), whereas E326K exhibited ~50% of WT activation. These results indicate that most of the Gaucher disease mutations lead to variant GCases that cannot develop an optimal phosphatidylserine induced conformational change for catalysis.

In the presence of negatively charged phospholipids, the natural protein activator, saposin C, enhances wild-type GCase activity by an additional ~2-fold (Fig. 5). The variant GCases R120Q, P122L, and L444P showed little effect of saposin C (150 nM), and variably deficient activations were noted over a narrow range of mutations that included R48Q, K79N, R120Q, P122L, and D127V. L264I showed about twice wild-type activation levels. These results imply additive activation effects of phosphatidylserine and saposin C that can function independently.

**pH Optima for Variant GCase Activities:** The optimum pH for activity of the mutant GCases and the estimated pK_{a} values for this diprotic
enzyme are listed in Table 2 and 3. The majority of the active enzymes have wild-type values for these parameters, irrespective of the specific substitution. The D127V, E349K, G390R and D399N have distinctly acidic shifts in pH optima with considerable acidic shifting of the entire curves, a mean 0.4-0.6 pH unit leftward shift for both pK_a's. These results suggest changes in the solvent environment near the active site residues and/or their interactions. Importantly, the variant GCases remain diprotic without loss of either arm of the relatively symmetric bell-shaped curves.

**Sensitivity of Variant GCases to Cathepsin D**: Cathepsin D sensitivity was used to evaluate the conformational effects of the specific Gaucher disease mutations (Fig. 6). Thirteen variants displayed extreme sensitivity to this treatment with little or no intact enzyme and/or enzyme activity remaining following the 48 hr incubation period. Nine GCases had nearly wild-type or slightly improved resistance including, E326K and E349K. R48Q, L174F, L264I and N382K had ~25-50% of the wild-type resistance.

**Additional Mutations in GCase and their effect on GCase Properties**: The disulfide bonds in wild-type GCase are between residues C4 and C16, and C18 and C23. These are located in an isolated loop structure (Domain 1) in the crystal (33) (Fig. 7). To evaluate the importance of these and the free cysteines at positions 126, 248 and 342, GCases containing singly mutated cysteines at each position were produced and characterized. Substitutions of serine for cysteine, an isosteric replacement, at 4, 16, 18 or 23 produced enzymes with severe decreases in catalytic activity and were easily detected on immunoblots, i.e., they were stable proteins. Similarly, C342G GCase had <0.2% CRIM SA. In comparison, the GCases C126S and C248S had ~80% and ~30% of wild-type GCase CRIM SA. These results indicated that any one of the cysteines involved in disulfide formation is essential to formation and/or preservation of an active enzyme. C342 is near the active site nucleophile, E340 (14), with potentially significant conformational and local effects. The C126S and C248S GCases had wild-type values for K_i and K_m (Table 2). C126S had wild-type levels of activation by PS. The comparable value for C248S was ~35%. Both these GCases had pH optimum curves that were indistinguishable from wild-type (Table 2).

The GCase variants with substitutions of histidine at positions 495 and/or 496 indicated that the carboxy tail of the enzyme has important functions in catalytic activity. The histidine substitutions led to stable enzymes in the Sf21 medium. The R495H and R496H GCase variants had wild-type and severely diminished levels of CRIM SA, respectively. The R495H is present in the porcine GCase, but the R496H was discovered as a heteroallele in a patient with Gaucher disease (38). Curiously, the double mutant enzyme R495H;R496H showed recovery of enzyme activity to nearly normal levels with wild-type K_i and K_m values (Table 2). R495H and the double mutant had 100% and ~60% of wild-type activation by PS, respectively, and comparable pH optima curves (Table 2).

**Crystal structure of partially deglycosylated GCase**: The active site residues E340 and E235 are in the center of the domain 3 β-barrel. The region around these residues is the least ordered in the crystal structure. In particular, very weak electron density is observed for residues 313-319 and 342-346, both of which are in loops that form part of the opening to the active site. The closest approach between the side-chains of active site residues Glu340 and Glu235 is 4.4Å in molecule A and 3.9Å in molecule B, the two asymmetric monomers in the GCase crystal. This is closer than the reported 5.2Å (33). Also, Y313 that was reported to form a hydrogen bond with the side-chain carboxyl oxygen of E235 is poorly ordered in molecule A of the present structure, and is too far to form a strong hydrogen-bond with E235 in molecule B.

**DISCUSSION**

The overall objective of this research was to confirm the pathogenicity of the various mutations detected in patients with Gaucher disease, to determine in vitro properties that were altered by the specific point mutations, and to gain insight into the structure/function correlations for GCase. The rationale for the in vitro studies was that most of the mutations occurring in Gaucher disease patients are heteroallelic and, thus, precluding
determination of their unique properties. This is true for the kinetic properties and sensitivity of the mutant GCase to proteases, and also is relevant to the misfolding properties induced by GCase mutations as assessed in cultured cells (39,40). Thus, the overall concept was to determine the degree of abnormality induced by the specific point mutation and to develop a correlation map with our crystal structure of GCase.

Essentially all of the mutant enzymes listed in Table 1 have significantly attenuated catalytic rate constants, $k_{\text{cat}}$, as estimated by the CRIM specific activity. This approach was used since equal degrees of purity could not be obtained for all mutant GCases. For these analyses, polyclonal anti-human GCase antibody was used to quantify the amount of enzymatic protein present in each normal or mutant sample under the assumption that for the majority, if not all, of the mutant GCases, the polyclonal antibody would have equal avidity and interaction with the proteins. Thus, within these limitations, the catalytic rate constants of the majority of the variant GCases were decreased to below 10% of wild-type; several had no activity. This shows that these mutations could be pathogenic to the patients. This diminished catalytic capacity was coupled mostly to abnormalities in kinetics, inhibition, activation by phosphadylserine or saposin C, or in vitro proteolytic sensitivity to Cathepsin D. Using the Cathepsin D assay as an assessment tool of the overall conformational changes of the mutant GCases, about half of the mutants lead to an increased sensitivity of the protein to in vitro Cathepsin D digestion. Some of the proteins, e.g., the $\Delta$36, were highly sensitive to endogenous insect cell proteases, but in the presence of protease inhibitors, could be purified and had substantial residual activity and high sensitivity to Cathepsin D. Thus, the majority of the mutant GCases with point mutations from Gaucher patients led to major changes in proteolytic sensitivity in vitro and catalytic deficits.

About 50% of the various point mutants had <5% of the wild-type GCase activity. These were scattered throughout our crystal structure, but more were interior in hydrophobic regions and around the active site. Since the mutations were selected from patients with Gaucher disease, it might be anticipated that low catalytic activity would be observed. It is interesting that the interior location produced significant conformational changes that led to severe catalytic deficits that were not detected with active site-directed inhibitors or substrates. Also, throughout phylogeny (Fig. 8), none of the identified Gaucher mutations occurs at a conserved residue region from man to worm.

The E326K GCase had about 50% of the wild-type CRIM specific activity and a significantly diminished activation by phosphadylserine. However, compound heterozygosity with G202R/E326K, and L444P/E326K genotypes, and half normal GCase activity levels indicated that normalcy for periods of one to four decades can exist in the presence of an E326K allele (36). Interestingly, the E349K GCase and E326K are approximately equally placed from the active site in the crystal structure, but E349K had much greater effects on the CRIM specific activity and other enzymic properties than did E326K. Both enzymes, however, had equal, and nearly wild-type, in vitro sensitivity to Cathepsin D digestion. Thus, although the $E \rightarrow K$ change would be predicted to have major disruptive affects, such alterations were position dependent.

Interestingly, the overall impact of most of the Gaucher disease mutations on phosphatidylserine activation was substantial. Only three of the point mutants had normal to increased responsiveness to phosphatidylserine, indicating that the vast majority of mutations affect the global conformation of the enzyme leading to decreases in catalytic power. Interestingly, N370S, V394L and I161N flank the active site and are >7 Å from the active center residues (Fig. 7). However, the binding of deoxynojirimycin and conduritol B epoxide was altered by 7- to 10-fold. Other N370 mutations, i.e., D, E or Q substitutions, for N, decrease catalytic capacity without concomitant changes in active site binding functions (15). This indicates that the disease mutation in humans, N370S, is residue specific with local affects on active site function. Interestingly, an apparently non-disruptive substitution, L371V, alters the CRIM specific activity, but has no effect on active site interaction with the inhibitors, whereas the neighboring N370S substitution does. N370 is not
in proximity to the deeper aspects of the active site and has no obvious direct interaction at the active site based on its placement in the crystal structure (Fig. 7).

Five valine→leucine and one leucine→valine substitutions have been discovered in patients with Gaucher disease (28,41-45). The V394L GCase has major kinetic changes similar to those in N370S. V394L homozygotes have not been reported in humans, but as a compound heterozygotes with L444P or 84GG (a null) have neuronopathic Gaucher disease (46-48) indicating a major disruption of the glucosylceramide pathway. V394 is located in domain 1 opposite the active site relative to N370 and forms part of a ring of amino acids surrounding the entrance to the active site (Fig. 7) adjacent to R395 that extends out from the surface. It is possible that the V394L substitution alters active site access. Indeed, during the final preparation of this manuscript, the crystal structure of GCase with bound CBE showed this to result from destabilization of an open loop conformation of residues V394-D399 (17). V15L also leads to very severe disease. V15 is in a hydrophobic pocket made up of F9, L354 and Y412, where replacement with the larger leucine side chain would be likely to result in steric clashes. V15 is also located in domain 1 near the first glycosylation site and could interfere with disulfide formation, similar to the lack of N-glycosylation at N19 (5). Thus, replacement of the β-branched hydrophobic residue, V, with the larger γ-branched leucine leads to substantive, and major, alterations in enzyme function suggesting a very limited target region for acceptable changes within the GCase molecule.

None of the mutations had major impact on saposin C activation of the mutant GCases, except for those residues from 48 through 122. With these residue changes, there was some diminished responsivenes to saposin C with preserved interactions at the active site. Thus, no obvious binding site for saposin C is evident from such analyses.

GCase also appears to have somewhat malleable properties within the context of the mutations. For example, human and mouse N370S, V394L and D409H/V were produced in mice to mimic human mutations. These mice developed glucosylceramide storage and/or have diminished enzyme activity in their specific cell types (30). Also as shown here, the properties of the isolated mutant mouse enzymes are very similar to those in humans. For example, the N370S and V394L GCases have essentially identical kinetic properties, and responses to phosphatidylinerine and saposin C, even though the background amino acid sequence differs by ~15% across the entire protein. Interestingly, the physiologic effects of N370S mutation in the mouse are quite different than those in human (30). N370S homozygosity in humans is associated with less severe Gaucher disease, whereas in the mouse it is lethal. Since the properties of N370S are essentially identical in mouse and human, the lethality must relate to the differences in substrates that are present in mouse and human (49,50). The lethality of the N370S mutation in mice results from a defect in the skin permeability barrier, similar to that observed in the GCase null mouse (30,51). It is interesting that the composition of glucosylceramide in the skin of mice differs from that in a human by having longer average chain fatty acid acyl chain lengths (52). This suggests that the longer acyl chain glucosylceramides may be poor substrates for N370S enzymes with resultant effects on the skin permeability barrier, the cause of lethality in N370S/N370S mice (30). Indeed, in vitro hydrolysis of glucosylceramides with varying fatty acid acyl chains by partially purified GCases from unaffected and Ashkenazi Jewish Gaucher disease type 1 patients showed a progressively more efficient cleavage (~2-fold) of C12-C18 glucosylceramides by wild-type GCase than the N370S GCase (32).

The present structure, and that of Dvir, et. al (33), show disulfide bonds between cysteine 4 and 18 and 16 and 23. The remaining cysteines at residues 126, 248 and 342 are free. The remaining cysteines at residues 126, 248 and 342 are free. Interestingly, the first four cysteines are conserved in all species, as is cysteine 342 that is near the catalytic nucleophile, E340. Cysteine 126 is conserved in all species except the honeybee, and cysteine 248 is conserved only in mammals (Fig. 8). Since the disulfide structures occur within a separate domain, domain 1, on the periphery of the main GCase structure and glycosylation occurs at
residue N19, the folding of GCase has been proposed to be a vectoral process determined by proper formation of this first domain 1 (5). The lack of glycosylation at N19 during synthesis leads to an inactive enzyme as does substitution of serine at any one of the first four cysteines (5). However, as shown here, nearly complete deglycosylation under appropriate conditions can produce an enzyme with wild-type activity. This implies that co-translational glycosylation plays an important role in disulfide formation. These data suggest that the glycosylation and the formation of the disulfides in this part of domain 1 provide a nidus for the directed folding of normal GCase.

If mutated, the cysteines at residues 126, 248 and 342 might be expected to have differential effects on GCase based on different conservation in phylogeny. Indeed, substitution of a glycine at C342 leads to a catalytically inactive enzyme that has been found in Gaucher disease patients. The proximity of this residue to the catalytic nucleophile might have predicted significant effects on the active site function. C126S had nearly wild-type properties. C248S produced significant decreases in CRIM specific activity that might result from its proximity to the active site, but not from phylogenetic comparisons (Fig. 8). Recent studies have suggested that the reduction state of the three free cysteines is critical to the preservation of catalytic activity and prevention of the enzyme aggregation (T. Edmunds, unpublished observation/personal communication). Thus, these free cysteines have dual roles in the preservation of enzyme activity that might result from being nearly equidistant from and surrounding the active site.

Also of interest were the carboxy terminal histidines. Curiously, the R496H occurs as a pathogenic mutation in Gaucher disease and its neighbor, R495H is present in the enzyme replacement product, imiglucerase™. The R495H GCase has normal properties while the R496H GCase mutation has little, if any, in vitro catalytic activity. More surprising is that the double mutant R495H;R496H leads to ~90% reconstitution of enzymatic activity. R496 forms hydrogen bonds with D474 stabilizing the fold of domain 2, while the side chain at position 495 is on the surface and unlikely to participate in stabilizing interactions.

In mammals, substitutions can occur at R495 with C and/or H in the orangutan and pig, respectively. In comparison, R496 is conserved in all mammals. These two arginine residues at the carboxy terminal are not known to form salt bridges or participate in a lid of the active site as occurs in other lipases. Certainly, these are not evident from the crystal structure. Two loops involving S345-E349 and V394-D399 apparently control active site access (17), but their roles in enzyme stability or in catalytic activity remain to be elucidated.

While Gaucher-disease associated mutations are not restricted to a particular surface or the active site of the enzyme, some generalizations can be drawn in correlating the mutational analyses reported above and the three-dimensional structure of GCase. In particular, we discuss here three classes of residues: those on the enzyme surface, residues at domain interfaces and residues in the hydrophobic core of the individual domains.

Of the residues reported in Table 2, side-chains of K79, K198, Y304, E326, E349 and R463 are surface accessible. Of these Y304C and E326K retain >10% activity. K198E and R463P were inactive. Mutations at domain interfaces are: R48Q (domain 1:2 interface), N382K (domain 1:3 interface), D399N, D409H/V and M416V (domain 1:3 interface), and L371V, L461P and R463P (domain 2:3 interface). Only M416V and R463P completely inactivate the enzyme. Interestingly, mutation of residues between the alpha and beta portions of the domain 3 β-barrel reduce enzymatic activity to below 10% as seen for the mutations M123V, I161N, L185F, G193E, F259L, L264I, R353W, Y363C, D380H. Similarly, changes in the β-barrel core of domain 3 in the active site space, R120Q and C342G, and in the active site opening, D127V, V394L and F397S, also substantially reduce activity. Both R120 and C342 are in close proximity to the active site and might be expected to have direct catalytic roles. The D127V mutation leads to modest changes in Ki values for CBE, deoxynojirimycin, and castanospermine, consistent with D127 participating in tight hydrogen bonding to the cyclohexitol of CBE bound to E340 (17). The most modest reduction in enzymatic activity was associated with the surface mutation E326K (42% activity) in domain 3, and localized to a surface of
the enzyme that has relatively few identified mutations in this group.

Our crystal structure provides some different information compared to that of the original structure proposed by Dvir et al. (33). Premkumar et al. (17) have redissolved such crystals and shown them to retain activity. However, in our hands the published procedure leads to a completely inactivated GCase and, consequently, some direct effects on the structure in and around the active site. Indeed, the vast majority of differences in the crystal structure reported here and that previously reported by Dvir et al (33) are near the active site. Clearly, more work on the structure of GCase remains, including the full elucidation of the active site structure and the docking of substrates to the enzyme. The available crystal structures do not provide insight into potential subsites for substrate or alkyl glycon binding to the active site as have been proposed from kinetic studies (31,53).

From the distribution of the mutations in Gaucher disease and the heterogeneity of the physical and kinetic properties of GCase resulting from those mutations, it is not clear that predictions can be made from GCase structure to function. There appear to be major sensitive areas leading to significant alterations in catalytic activity and, in particular, the minor changes of branched chain amino acids and other such changes that are not compatible with active forms of the enzyme.

REFERENCES


**FOOTNOTE**

*These authors contributed equally.
†These studies were supported by NIH grants to GAG (DK36729) and to RH (EY014648).
1Abbreviations: GCase, glucocerebrosidase, acid β-glucosidase (EC3.2.1.45); 4MUG, 4-methylumbelliferyl-β-D-glucopyranoside; CBE, conduritol B epoxide

**FIGURE LEGENDS**

**Figure 1:** CRIM Specific Activities (SA) of GCase Variants: The CRIM SAs were determined using anti-human GCase polyclonal antiserum detection of intact enzyme, M_r~60,000, on immunoblots. Densitometric scans were conducted on equal amounts GCase variant and wild-type activities applied to each well. The results are shown as the percentage of wild-type activity. The GCases were purified from media of Sf21 cells expressing the specific GCase. The shaded rectangle represents the variation in wild-type CRIM SA about the mean (100%). The *GCase variants required a protease cocktail to protect intact GCase from digestion. The CRIM SA for these enzymes was assessed directly from aliquots of media. ∆36 was highly purified in the presence of protease inhibitors.

**Figure 2:** IC50 values for Conduritol B Epoxide of GCase Variants: The shaded rectangle represents the range of values for the wild-type (±SD). The *GCase variants had little or no enzyme activity. CBE was shown in preliminary studies to be an irreversible inhibitor of all GCase variants.
Figure 3: IC50 values for Deoxynojirimycin of GCase Variants: The shaded rectangle represents the range of values for the wild-type (±SD). The *GCase variants had little or no enzyme activity. Deoxynojirimycin was shown in preliminary studies to be a rapidly reversible competitive inhibitor of all GCase variants.

Figure 4: Phosphatidylserine activation of GCase Variants: Delipidated GCase variants were assayed in the absence and presence of phosphatidylserine (4 µM) dispersions. The enzymes were preincubated for 30 minutes prior to addition of substrate.

Figure 5: Saposin C activation of GCase variants: Activities were assessed following the addition of recombinant human saposin C (150 nM) and substrate to GCase/phosphatidylserine (4 µM) preincubated (30 min) dispersions. The horizontal line represents the mean wild-type activation level.

Figure 6: Cathepsin D digestion of GCase variants: GCase variants, 2.9 pmoles as determined by immunoblots, were incubated in the presence of cathepsin D (2 µM) for 48 hrs at room temperature. The remaining intact GCase variants (M~60,000) were quantified on immunoblots and are represented as the percentage of original remaining. The horizontal line represents the mean for wild-type (~65% of initial).

Figure 7: Gaucher mutations of GCase mapped onto the 3-D structure. The three domains of the enzyme are shown in blue (domain 1), pink (domain 2) and gold (domain 3). The active site residues E340 and E235 are shown in red. The distribution and selected properties of variant GCases are as indicated. For clarity the specific substitutions are not indicated, but the wild-type residue is, e.g., N370* refers to the N370S protein as having CRIM SA ≥10% of wild type. The reader is referred to Table 1 for specific substitutions. The substitutions indicated with a * are those GCases with ≥10% of wild type. Those in boxes (N370S and V394L) have increased responsiveness to phosphatidylserine, and those in green font had wild type resistance to in vitro cathepsin D digestion.

Figure 8: Amino acid sequence comparison of several species. A = Human (GeneID: 2629), B = Chimpanzee (GeneID:449571), C = Orangutan (Protein ID =CAH90774.1), D = Porcine(GeneID: 449572), E = Canine (GeneID: 612206), F = Mouse(GeneID: 14466), G = Partial Rat, H = Fugu(SINFRUT 59858), I = Honeybee(GeneID: 409708), J = C. elegans(GeneID: 178535). Identity of amino acids is indicated in black. Alignments were with DNasis and, then, manually.
<table>
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<th>Source</th>
<th>Type</th>
<th>CRIM SA</th>
<th>IC50 (µM) ±SD</th>
<th>Km (mM)</th>
<th>pH optimum (pKₐ, pKₙ)</th>
<th>Activity Stability</th>
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*dNM = deoxynojirimycin, CS=castanospermine. NA or ND = not applicable or not done; *Data from (32)
Table 2: Properties of GCase Cysteine and COOH Terminal Histidine variants

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<td></td>
<td></td>
<td>DNM (Kᵢ)</td>
<td>CS (Kᵢ)</td>
<td>CBE (IC50)</td>
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<td>1.8±0.6</td>
<td>82±12</td>
<td>5±3</td>
<td>76</td>
</tr>
<tr>
<td>C248S</td>
<td>0.30±0.06</td>
<td>2.7±1.0</td>
<td>65±6</td>
<td>4±1</td>
<td>68</td>
</tr>
<tr>
<td>C342G</td>
<td>&lt;0.1</td>
<td>nd</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>R495; R496</td>
<td>1.0±0.10</td>
<td>2.0±0.5</td>
<td>68±4</td>
<td>4.1±0.2</td>
<td>75±4</td>
</tr>
<tr>
<td>R495H; R496</td>
<td>0.98±0.10</td>
<td>1.8±0.6</td>
<td>71±3</td>
<td>3.3±0.1</td>
<td>65±6</td>
</tr>
<tr>
<td>R495; R496H</td>
<td>0.04±0.03</td>
<td>2.6±1.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>R495H; R496H</td>
<td>0.8±0.05</td>
<td>1.7±0.8</td>
<td>65±5</td>
<td>7.3±0.6</td>
<td>71±3</td>
</tr>
</tbody>
</table>

¹R495H GCase was purified and characterized in the Baculovirus system and, also, imiglucerase was purified from Cerezyme™ and contains R495H. The properties of these two GCases with R495H were identical. All other GCases were purified from medium of Sf21 cells infected with the corresponding baculovirus construct. PS fold activation was for equal molar amounts of intact enzyme, (E),~2 nM, in 8 µM, or no PS at their optimal pH values.
Figure 3

IC 50 (µM)

GCase Variant
Figure 7
Figure 8
Analyses of variant acid β-glucosidases: effects of gaucher disease mutations
Benjamin Liou, Andrzej Kazmierczuk, Min Zhang, C. Ronald Scott, Rashmi S. Hegde and Gregory A. Grabowski

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