Human Acid β-Glucosidase

N-Glycosylation Site Occupancy and the Effect of Glycosylation on Enzymatic Activity*

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Anat Berg-Fussman†, Marie E. Grace‡, Yiannis Ioannou‡, and Gregory A. Grabowski$§

From the ‡Division of Medical and Molecular Genetics, Department of Pediatrics, Mount Sinai School of Medicine, New York, New York 10029 and the §Division of Human Genetics, Children's Hospital Medical Center, Cincinnati, Ohio 45229

The five potential N-glycosylation sites (sequons) of human acid β-glucosidase were individually mutated to determine site occupancy and the effect of site occupancy on selected catalytic and stability properties of this enzyme. Each N-glycosylation consensus sequence [Asn-Xaa-(Ser/Thr)] was obliterated by individually substituting glutamine (Q) for asparagine (N). By expression of the normal and mutated cDNAs in insect (Sf9) and COS-1 cells and subsequent immunoblotting with anti-human acid β-glucosidase antibodies, the four sequons at Asn-19, Asn-59, Asn-146, and Asn-270 were shown to be glycosylated in either source. The sequon at Asn-462 was never occupied. The mutant enzymes N59Q, N146Q, and N270Q were catalytically active and had normal interactions with active site-directed inhibitors as well as with the activators, phosphatidylserine and saposin C. Of the occupied sequons, N-glycosylation of the first was critical to the synthesis of a catalytically active enzyme. Alteration of this sequon, Asn-19-Ala-20-Thr-21, by the substitutions N19Q, N19D, N19E, or T21G led to a lack of glycosylation at this site. Enzymes containing N19Q, N19E, or T21G had significant decreases (3- to 60-fold) in intrinsic enzyme activity. The N19D enzyme had nearly normal catalytic activity and had enhanced activation by phosphatidylserine. These results show that sequon occupancy as well as steric effects at residue 19 are important for the development of an active conformer of this enzyme. This is the first example of a lysosomal hydrolase that requires sequon occupancy for the synthesis of a catalytically active enzyme.

Human acid β-glucosidase (β-glucosyl-N-acylsphingosine glucohydrolase, EC 3.2.1.45) is the lysosomal hydrolase (1) that cleaves the β-glucosyl linkages of glucosylceramide as well as synthetic β-glucosides (2). This peripheral membrane glycoprotein is active as a monomer of Mr ~ 63,000-67,000 in mammalian sources (3). The hydrophobic nature of the enzyme requires the use of detergents, negatively charged lipids, and an activator protein, saposin C, for optimal hydrolysis of the natural or synthetic substrates (for review, see Ref. 3). Detailed kinetic studies of this enzyme indicate a complex enzyme with multiple subsites for interaction with phospholipids and saposin C. The active site also has subsites that accommodate the glycon, sphingosyl, and fatty acid acyl moieties of substrates and inhibitors (for review, see Ref. 3). Defects in the catalytic function, stability, and/or post-translational processing of acid β-glucosidase lead to the variants of Gaucher disease, the most prevalent lysosomal storage disease (4-9).

As is typical of lysosomal hydrolases (10), the normal enzyme is synthesized as a precursor containing a 19-amino acid secretory signal sequence that is proteolytically clipped during transit through the membrane of the endoplasmic reticulum (11). No additional proteolytic processing occurs during transport through the Golgi apparatus to the lysosome (11, 12). The mature, unglycosylated polypeptide has a calculated molecular weight of ~56,000. Co-translational glycosylation occurs at four of the five (11) predicted N-glycosylation sites, Asn-Xaa-(Ser/Thr) (13), but site occupancy has not been determined. Complete deglycosylation with N-Glycanase™ (14) or endoglycosidase F (11) indicated the presence of N-glycosylation. Complete characterization of the oligosaccharide moieties from the human placental enzyme demonstrated N-linked high mannosyl type and typical bi- and triantennary complex structures (15). Modification of the oligosaccharide composition has no effect on the stability or catalytic properties of the enzyme (16). However, prevention of glycosylation by expression of the human cDNA in bacterial systems or in tunicamycin-treated insect cells led to the synthesis of catalytically inactive enzyme forms (14). These results suggested that glycosylation at one or more sites may play a role in the formation of an active enzyme species (14).

To gain further insight into glycosylation site occupancy and the role of glycosylation in the development of active enzyme forms, site-directed mutagenesis was used to individually destroy each potential glycosylation consensus sequon or sequon. Expression of these cDNAs in insect and/or mammalian cells showed the oligosaccharide site occupancy and that occupancy of the first site is important for development of a catalytically active enzyme.

EXPERIMENTAL PROCEDURES

Materials—The following were from commercial sources: restriction endonucleases, Taq polymerase, T4-DNA ligase, and Prime-A-Gene labeling system (Promega); Sequenase version 2.0 kit (U.S. Biochemical Corp.); oligonucleotide directed in vitro mutagenesis system, 32P- and 35S-nucleotides, and Rainbow™ protein molecular weight markers (Amersham); agarose and low melting point agarose (Life Technologies Inc.); Grace’s medium, Dulbecco’s modified Ea-
### Designation | Amino acid substitution | Oligonucleotide
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N19Q | Asn-19 → Gin | Normal: 5'GGTGTTGTCTGCTGGCAGGCACATATCTGAC3'  Mutant: 5'GGTGTTGTCTGCTGGCAGGCACATATCTGAC3'  
N19D | Asn-19 → Asp | Normal: 5'GGTGTTGTCTGCTGGCAGGCACATATCTGAC3'  Mutant: 5'GGTGTTGTCTGCTGGCAGGCACATATCTGAC3'
N14E | Asn-14 → Gin | Normal: 5'GATTCAGTGTCAGTACCCCA3'  Mutant: 5'GATTCAGTGTCAGTACCCCA3'
N19Q | Asn-19 → Gin | Normal: 5'GGGCCCATCCAGGCTCAGCACACGGGCACA3'  Mutant: 5'GGGCCCATCCAGGCTCAGCACACGGGCACA3'

**Table 1**

Oligonucleotides for site-directed mutagenesis of acid-β-glucosidase.

1 The abbreviations used are: 4MU-Glc: 4-methylumbelliferyl-β-D-glucopyranoside; DNase, deoxyribonuclease; N-Me: N-methyl; N-Glc: N-glycosidase cDNAs were purified by plaque hybridization (5, 25). The levels of acid β-glucosidase activity and protein expressed from independent recombinant viral isolates for each cDNA were monitored by enzyme assay and immunoblots, 3 days after infection. For these studies, titers were adjusted to achieve maximal specific activities (5).

**Construction of Recombinant Baculovirus—** Recombinant baculovirus containing the normal or mutant cDNAs were produced by homologous recombination (5). Viral DNA (BaculoGold™) was used for preparation of recombinant viruses which contained acid β-glucosidase cDNAs encoding the N19E, T21G, S207Q, and N19D enzymes. Infection of Spodoptera frugiperda (Sf9) cells with wild type or recombinant *A. californica* nuclear polyhedrosis virus, determination of viral titers, and calcium phosphate-mediated transfections were done as described (5).

Recombinant viruses containing the acid β-glucosidase cDNAs were purified by plaque hybridization (5, 25). The resultant polymerase chain reaction product contained the coding sequence from the most 3' in-frame initiation ATG to the stop codon. Ligation of the 1.6-kilobase EcoRI-Sac1 fragment into the p91023(B) EcoRI site, transformation, and tetranuclease activity and protein expressed from independent recombinant viral isolates for each cDNA were monitored by enzyme assay and immunoblots, 3 days after infection. For these studies, titers were adjusted to achieve maximal specific activities (5).

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purified by cesium chloride gradients prior to use (24).

**Immunoelectroblotting—**Immunoelectroblotting was conducted (17) using SDS or Tricine (26) PAGE. Briefly, Sf9 cells, infected with wild type (A. californica nuclear polyhedrosis virus) or pure recombinant virus as well as COS-1 cells mock-transfected or transfected with p91023(B) containing acid β-glucosidase inserts, were harvested by ultrasonic irradiation of the washed pellets in 0.1% Triton X-100 (525 g, 10 min) and resuspension in 0.9% NaCl. Pellets were stored at −20 °C until use. Acid β-glucosidase was solubilized at 4 °C by ultrasonic irradiation of the washed pellets in 0.1% Triton X-100 and 0.1% sodium taurocholate using a cup sonicator (Breson Cell Disruptor 200, 80 watts, pulse times of 30 s, 20 s, and 26 s) (8). The sonicates were clarified by centrifugation (875 × g, 20 min), and the resulting supernatants were used in the immunoelectroblotting studies (17). Immunoblots were conducted using the anti-human acid β-glucosidase IgG monoclonal antibodies, MC-1 and MC-38; which recognize different epitopes on the enzyme or polyclonal rabbit anti-human acid β-glucosidase antibodies (17). Extracts of Sf9 cells infected with wild-type virus or mock-transfected COS-1 cells gave no signals on immunoblots using the above antibodies.

N-Linked deglycosylation of normal (NL) and mutated human acid β-glucosidase expressed in Sf9 cells was achieved by treatment with N-Glycosidase F or N-Glycanase™ under denaturing conditions, according to the manufacturer’s instructions. With N-Glycosidase F, octyl β-glucoside was used as the nonionic detergent to optimize electrophoretic separations on Tricine PAGE.

**Enzyme Assays—**Acid β-glucosidase hydrolytic rates were determined fluorometrically using NBD-GC or 4MU-Glc substrates (27). Unless specified, the final reaction mixtures (0.2 ml) contained 0.04 M phosphate/citrate, pH 5.5, 4 mM β-mercaptoethanol, and 1.25 mM EDTA as well as 0.25% Triton X-100 and 0.25% mM sodium taurocholate (Buffer A). Assays were done using crude sonicates prepared as described above for the immunoblotting studies. For the inhibition studies using DNM or Br-CB, the inhibitor was added as a concentrated solution in water to the assay solution. The enzyme source was added last. IC₅₀ values, the concentration of inhibitor which results in a 50% decrease of initial enzyme activity, were determined as described (5, 29). The amount of normal or mutant enzyme source, COS-1 or Sf9 cell sonicates, was adjusted to ensure that <5% of the substrate was hydrolyzed during the reaction. Reactions were terminated after 0.5–2 h at 37 °C (29).

Activation by phosphatidylserine was determined as described (8, 27). Briefly, aliquots of crude sonicates in 0.06% Triton X-100 were incubated at room temperature for 15 min in 0.04 M citrate/phosphate buffer, pH 5.5, containing 0.06% Triton X-100 and varying concentrations of phosphatidylserine. Activity to 4 mM 4MU-Glc was assayed as described above with final phosphatidylserine concentrations ranging from 0 to 3 mM. The final concentration of Triton X-100 in the assay mixtures was 0.06%. Solutions containing phosphatidylserine (Buffer B) and Triton X-100 were prepared from concentrated stock solutions in chloroform/methanol (2:1). The appropriate aliquots of each stock solution (5 mg/ml phosphatidylserine; Triton X-100) were transferred to a glass tube, dried under N₂, and then under vacuum for 2 h. The dried compounds were then dispersed into citrate/phosphate buffer using a cup sonicator. Response to the protein activator, saposin C, was determined by assaying activity to 4MU-Glc in 0.2 M sodium acetate, pH 4.7, containing 20 μg/ml (0.025 mM) phosphatidylserine. Saposin C was prepared from the spleen of a Gaucher disease patient (18, 28).

For heat inactivation studies, the above clarified supernatants from Sf9 cells were diluted 10-fold in Buffer A and incubated for 0 to 30 min at 50 °C. At each time point, the tubes were removed and placed immediately on ice. Activity toward the 4MU-Glc substrate was determined as above. The times of incubation at 50 °C that resulted in 50% loss of the initial activity (t₅₀) were determined (5).

**RESULTS**

To determine the N-glycosylation sequon occupancy for acid β-glucosidase, the normal and mutant cDNAs were individually expressed in insect (Sf9) and COS-1 cells. The expressed proteins were analyzed by immunoblotting. To distinguish the mobilities of the different mutant enzymes from the normal, long electrophoretic run times (up to 20 h) were used with resultant fuzziness of some of the detected bands. Fig. 1A shows typical immunoblots obtained using Sf9 cells. The N19Q, N59Q, N146Q, N270Q, and N19D enzymes migrated faster than the enzyme expressed from the normal cDNA (NL). Similarly, the N19E and T21G enzymes migrated faster than the normal enzyme (Fig. 1B). These results indicated that the glycosylation consensus sequences at the first four positions are normally occupied in Sf9 cells. Essentially identical results were obtained in COS-1 cells (Fig. 1C). In either cell type, N462Q (Fig. 1C) or N462D (Fig. 1B) migrated normally which indicated that this glycosylation site is not normally occupied. N146Q consistently had slower migration than the mutants N19Q, N59Q, N270Q, and N19D.
but this altered migration was only slightly different from normal. To conclusively demonstrate occupancy at Asn-146, the cDNAs encoding N146Q or with only the third glycosylation site (G-3) intact were expressed in Sf9 cells and deglycosylated with N-Glycosidase F. Faster migration of N146Q before deglycosylation again was obtained on Tricine PAGE. Treatment of the G-3 enzyme with N-Glycosidase F produced a shift in migration of the acid β-glucosidase so that the migration was identical with that for the mutant enzyme with all glycosylation sites obliterated (Fig. 2). The deglycosylated G-3 also migrated with the fastest band obtained by N-Glycosidase F treatment of the normal enzyme expressed in Sf9 cells (Fig. 2). This result shows that glycosylation occurs at this site. The cDNA containing mutations at all glycosylation consensus sequences (multiple mutant) migrated identically with the normal enzyme which had been completely deglycosylated with N-Glycanase™ (data not shown) or N-Glycosidase F (Fig. 2) or the unglycosylated human protein expressed in E. coli (data not shown).

The specific activities of the expressed normal and mutant enzymes were determined in Sf9 cells and COS-1 cells. Of the glutamine-substituted enzymes, detectable activities were obtained in Sf9 cells with N59Q, N146Q, and N270Q (Table II). In contrast, the activities with the N19Q, N462Q, and N462D mutant enzymes were very low even when large amounts of immunologically reactive protein were expressed. These results demonstrate a very decreased catalytic rate constant for these mutant enzymes. The effects of different amino acid substitutions within the first glycosylation site were evaluated by creating and expressing enzymes containing N19D, N19E, or T21G. In comparison to N19Q, these enzymes were catalytically active but had variably decreased specific activities based on the amount of cross-reacting immunologic material (CRIM) to anti-human acid β-glucosidase antibodies (5). These estimates indicated that the CRIM specific activities for the N19Q, N19E, and T21G enzymes were >60, ~30, and ~3-fold decreased, respectively, compared to the normal human enzyme expressed in Sf9. The corresponding values for the N19D enzyme were nearly normal but depended on the assay conditions (see below). Essentially identical results were obtained in COS-1 cells (data not shown).

To determine if any of the enzymes were secreted, media from either Sf9 or COS-1 cell cultures expressing the normal or active mutant enzymes were assayed for enzyme activity and the ratios were determined for the enzyme activity in the media compared with the total enzyme activity within cells and in the media. For cells infected or transfected with the normal cDNA and for Sf9 cells, under low lysis conditions, little enzyme was secreted into the media (<25% of total enzyme). This ratio was the same for all mutant enzymes tested. Similarly, little enzyme could be detected by immunoblotting of concentrated media from the respective COS-1 cells.

To evaluate additional effects of glycosylation site occupancy on active site function or stability, inhibition (DNM and Br-CBE) and activation (phosphatidylserine and saposin C) studies were conducted (Table III). The potent active site-directed inhibitors (29), DNM and Br-CBE, had normal or nearly normal IC₅₀ values with all tested mutant enzymes. The exceeding low activity of the N462Q or N462D enzymes is due to its location near catalytically important regions of the enzyme. Indeed, a mutation, R463C, has been identified as causing Gaucher disease (31, 32). The interactions of acid β-glucosidase with its activators, phosphatidylserine and saposin C, were tested with the normal and selected active mutant enzymes. The hydrolysis of 4MU-Glc by mutant enzymes, except N19D, was enhanced normally, or nearly so, by these compounds. The N19D enzyme was hyper-responsive to phosphatidylserine. About 2.6-fold greater degrees of activation were achieved with this enzyme than with the normal

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**Table I**

| Specific activities of acid β-glucosidase N-glycosylation mutants | cDNA | 4MU-Glc | Glucosylceramide |
|-------------------|-----|---------|-----------------|
| Normal            | 4061 ± 2355 | 2855 ± 1138 |
| N19Q              | 25     | 75      |
| N19D              | 415 ± 80  | 2112 ± 103 |
| N19E              | 176    | 35      |
| T21G              | 554    | 162     |
| N59Q              | 1183 ± 83 | 3123 ± 127 |
| N146Q             | 1843 ± 126 | 2912 ± 388 |
| N270Q             | 1253 ± 180 | 2859 ± 97  |
| N462Q             | 30     | 12      |
| N462D             | 29     | 0.42    |
| G-3               | 24     | 0.14    |
| Multiple mutant   | 24     | 0.2     |
| AcNPVb            | 27     | 0.1     |

* Multiple mutant = acid β-glucosidase cDNA containing all five potential N-glycosylation sites altered with Asn (N)-substituted with Gln (Q).
* Sf9 cells infected with A. california nuclear polyhedrosis virus (AcNPV).

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2. M. E. Grace, K. M. Newman, V. Scheinker, G. S. He, A. Berg, and G. A. Grabowski, submitted for publication.
or other mutants. Heat inactivation studies demonstrate that only the destruction of the third glycosylation site (N146Q) had a significant effect (t95 = 9.5 min versus 15.5 for normal) on decreasing the thermostability of the enzyme.

**DISCUSSION**

The present studies show that the first four N-glycosylation consensus sequences, sequons, of human acid β-glucosidase are normally occupied and that the catalytic activity of this enzyme depends on the occupancy and/or the nature of amino acid 19. The expression of the normal cDNA encoding acid β-glucosidase in bacteria or in insect cells exposed to tunicamycin resulted in the synthesis of stable but catalytically inactive enzymes (14). In addition, deglycosylation of purified acid β-glucosidase in the native state suggested that a single oligosaccharide moiety was easily removed and that its presence was needed to maintain the activity of the enzyme (14). In comparison, the trimming of glycosidic residues to the mannosyl core had little, if any, influence on catalytic activity (33). In fibroblasts, the type or degree of glycosylation of acid β-glucosidase did not appear to influence either the stability or catalytic activity when inhibitors of glycosidic trimming enzymes were used to alter the type of glycosylation (16). However, site occupancy was not evaluated in these studies. The studies presented here show that the catalytic activity of a lysosomal hydrolase is dependent upon specific glycosylation site occupancy.

The activity of only a few other enzymes is modulated by glycosylation site occupancy, and this was due to either the presence or absence of oligosaccharide moieties at a particular consensus sequence (34–36). The influence of sequon occupancy of tissue plasminogen activator has been extensively and thoroughly analyzed in natural cells (34, 37) and following heterologous expression (35). Tissue plasminogen activator has three glycosylation sequons (37). Occupancy of all three, but especially that at Asn-184, results in diminution of catalytic activity (34). The nature of the glycosylation at Asn-117 and Asn-448 also influenced the kinetic properties of this enzyme (34, 35). Complete lack of glycosylation at all three sites leads to a higher activity enzyme which was more sensitive to enhancement of activity by a fibrinogen fragment (34).

In comparison to the normal enzyme, the acid β-glucosidase mutant, N19D, which lacked glycosylation at the first sequon, had greater enhancement of activity by phosphatidylycerine. Since N19Q was nearly inactive and N19E and T21G had decreased CRIM specific activities, these results indicate that the nature of the amino acids within the first sequon as well as occupancy of this sequon influence the formation of a catalytically active species. Steric influences were more important than functional effects of substituted amino acids for the synthesis of a fully active enzyme. Isofunctional substitutions at amino acid 19 led to major decreases in catalytic activity; i.e., the ratio of CRIM specific activities for the enzymes N19N (normal) and N19Q was ~60, and the ratio for the enzymes N19D and N19E was ~30. In comparison, isostructural substitutions had smaller effects on enzyme activity; i.e., the ratio of CRIM specific activities for the enzymes N19N and N19D was ~1 and for the enzymes N19E and N19Q was ~2 to 3. However, the normal thermostability and the enhanced effects of phosphatidylycerine with N19D suggested that the conformation assumed by this mutant, while not grossly distorted, is not that of the normal enzyme. Computer calculations (38) showed that the presence of an aspartate or a glutamate at residue 19 significantly altered the predicted secondary structure in this region. In comparison, the presence of a glutamine, substituted for an asparagine, did not (data not shown). Thus, the nature of the amino acid and, potentially, the degree or type of glycosylation at amino acid 19 may influence the maximal catalytic activity of the enzyme. By comparison, the lesser effect of the T21G substitution on catalytic activity suggests additional positional effects. Although not extensively investigated, tissue-specific (spleen versus brain) differences in sensitivity of acid β-glucosidase to exogenous negatively charged lipids (39) might be based in alternative glycosylation site compositions. Present results suggest that differences in the degree of glycosylation at a single site were present only at the sequon Asn-146 which may have smaller oligosaccharide moieties (Figs. 1 and 2). Prevention of glycosylation at sequon Asn-146 produced an enzyme with increased thermostability. These findings suggest that selection of the specific cDNA and/or cell type for expression of recombinant acid β-glucosidase with altered glycosylation may have significant therapeutic implications (34, 40).

By substitution of Ala or Gln for Asn-43, human lipoprotein lipase was shown to require glycosylation of a single sequon for catalytic activity (36). The more conservative substitution of an Asp for Asn (41) was not investigated. In addition, glycosylation at residue 43 was needed for secretion of the lipoprotein lipase into the culture media (36). With acid β-glucosidase, very little enzyme was secreted normally or catalytically active species. Steric influences were more important than functional effects of substituted amino acids for the synthesis of a fully active enzyme. Isofunctional substitutions at amino acid 19 led to major decreases in catalytic activity; i.e., the ratio of CRIM specific activities for the enzymes N19N (normal) and N19Q was ~60, and the ratio for the enzymes N19D and N19E was ~30. In comparison, isostructural substitutions had smaller effects on enzyme activity; i.e., the ratio of CRIM specific activities for the enzymes N19N and N19D was ~1 and for the enzymes N19E and N19Q was ~2 to 3. However, the normal thermostability and the enhanced effects of phosphatidylycerine with N19D suggested that the conformation assumed by this mutant, while not grossly distorted, is not that of the normal enzyme. Computer calculations (38) showed that the presence of an aspartate or a glutamate at residue 19 significantly altered the predicted secondary structure in this region. In comparison, the presence of a glutamine, substituted for an asparagine, did not (data not shown). Thus, the nature of the amino acid and, potentially, the degree or type of glycosylation at amino acid 19 may influence the maximal catalytic activity of the enzyme. By comparison, the lesser effect of the T21G substitution on catalytic activity suggests additional positional effects. Although not extensively investigated, tissue-specific (spleen versus brain) differences in sensitivity of acid β-glucosidase to exogenous negatively charged lipids (39) might be based in alternative glycosylation site compositions. Present results suggest that differences in the degree of glycosylation at a single site were present only at the sequon Asn-146 which may have smaller oligosaccharide moieties (Figs. 1 and 2). Prevention of glycosylation at sequon Asn-146 produced an enzyme with increased thermostability. These findings suggest that selection of the specific cDNA and/or cell type for expression of recombinant acid β-glucosidase with altered glycosylation may have significant therapeutic implications (34, 40).

The mechanism by which the presence of an oligosaccharide moiety or the nature of the amino acid at the first sequon (residue 19) confers a catalytic conformer is unknown. However, it is interesting that all four occupied glycosylation sites are present in the amino-terminal 50% of the enzyme. In addition, the carboxy-terminal 50% of the acid β-glucosidase contains most of the hydrophobic amino acids and, probably, major components of the active site (30). Thus, sequon occupancy or the specific amino acid at residue 19 must affect catalytic activity by altering global conformation during the emergence of the nascent enzyme through the endoplasmic reticular membrane. It seems likely, that, under normal circumstances, glycosylation of Asn-19 provides a nidus for the vectorial folding of this enzyme during its synthesis.

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