X-ray Structure of Human Acid-β-Glucosidase Covalently Bound to Conduritol-B-Epoxide

IMPLICATIONS FOR GAUCHER DISEASE*

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Gaucher disease is an inherited metabolic disorder caused by mutations in the lysosomal enzyme acid-β-glucosidase (GlcCerase). We recently determined the x-ray structure of GlcCerase to 2.0 Å resolution (Dvir, H., Harel, M., McCarthy, A. A., Toker, L., Silman, I., Futerman, A. H., and Sussman, J. L. (2003) EMBO Rep. 4, 704–709) and have now solved the structure of GlcCerase conjugated with an irreversible inhibitor, conduritol-B-epoxide (CBE). The crystal structure reveals that binding of CBE to the active site does not induce a global conformational change in GlcCerase and confirms that Glu440 is the catalytic nucleophile. However, only one of two alternative conformations of a pair of flexible loops (residues 345–349 and 394–399) located at the entrance to the active site in native GlcCerase is observed in the GlcCerase-CBE structure, a conformation in which the active site is accessible to CBE. Analysis of the dynamics of these two alternative conformations suggests that the two loops act as a lid at the entrance to the active site. This possibility is supported by a cluster of mutations in loop 394–399 that cause Gaucher disease by reducing catalytic activity. Moreover, in silico mutational analysis demonstrates that all these mutations stabilize the conformation that limits access to the active site, thus providing a mechanistic explanation of how mutations in this loop result in Gaucher disease.

Gaucher disease, the most common lysosomal storage disorder (1), is caused by mutations in the gene encoding acid-β-glucosidase (GlcCerase), result which in intracellular accumulation of the lipid substrate, glucosylceramide (2, 3). These mutations diminish GlcCerase activity either by reducing enzyme activity or by reducing the lysosomal enzyme concentration. Enzyme activity is reduced in mutations that affect the turnover number, substrate affinity, or activator binding (4). Lysosomal enzyme concentration is reduced by mutations that compromise folding in the endoplasmic reticulum, resulting in proteasomal degradation of the protein (5).

We recently solved the three-dimensional structure of recombinant GlcCerase (Cerezyme®), the enzyme used in enzyme replacement therapy in Gaucher disease (6). The structure comprises three non-contiguous domains, with the catalytic site located in domain III (residues 76–381 and 416–430), a (β/α)8 (TIM) barrel. The function of the two non-catalytic domains is not known, but mutations that cause Gaucher disease are found in all three domains.

To determine whether substrate or inhibitor binding can induce conformational change(s) in GlcCerase, and to try to gain insight into possible roles of the non-catalytic domains, we have now determined the crystal structure of a conjugate of GlcCerase with an irreversible inhibitor, conduritol-B-epoxide (1,2-anhydro-myo-inositol; CBE) (7). Binding of CBE did not induce a global conformational change in the structure of GlcCerase but permitted us to assign a role to two surface loops found at the entrance to the active site and to suggest how mutations in one of these loops might reduce catalytic activity, thus leading to Gaucher disease.

EXPERIMENTAL PROCEDURES

Materials—Cerezyme® was obtained from patient leftovers. CBE was from Bioanalytical Systems (West Lafayette, IN), and N-glycosidase F was from Roche Applied Science (Mannheim, Germany). N-(6-(7-nitrobenzo-2-oxa-1,3-diazol-4-ylamino)hexanoyl)-β-erythro-α-(NBG-Glc) was prepared as described (8).

Crystallization and Data Collection—Cerezyme® was crystallized after partial deglycosylation using N-glycosidase F (6), which is capable of cleaving all types of asparagine bound N-glycans (9, 10), but did not produce complete deglycosylation of native Cerezyme® under the experimental conditions employed (6). GlcCerase-CBE crystals were obtained by soaking native GlcCerase crystals overnight at 19 °C in mother liquor (1 mM (NH₄)₂SO₄, 170 mM guanidinium HCl, 20 mM KCl, 100 mM acetate, pH 4.6) containing 1 mM CBE. The crystals were cryoprotected with a gradient of 5–25% glycerol. Data were collected...
"in-house" at 100 K on an R-AXIS IV++ imaging plate system mounted on a RU300 x-ray generator operating at 50 kV and 90 mA. Data were processed using XDS and XSCALE (11). Reflections were converted using XDSCONV to a format suitable for CNS, and a list of randomly generated test reflections was inherited from a master list for the native orthorhombic crystal form of GlcCerase (6). Table I summarizes data collection and processing.

Structure Determination and Refinement—The GlcCerase-CBE structure was solved using the difference Fourier technique, exploiting the native isomorphous crystal structure of GlcCerase (Protein Data Bank ID code 1OG8). The 1OG8 coordinates were used as a starting model for rigid body refinement (30–2.4 Å resolution) in CNS (12). After a round of simulated annealing, Fo – Fc and 2Fo – Fc maps were used to fit the CBE molecule, two carbohydrate moieties, and 10 sulfate molecules. XtalView (13) was used for all model building and repositioning of atoms in the model structure. The chemical model of cyclohexitol was built using CS Chem3D Pro (version 5.0), and energy minimization was performed on the inhibitor model using the molecular dynamics utilities of CS Chem3D Pro (www.camsoft.com). These models were used to generate suitable topology and parameter files for CNS using the PRODRG2 server (14) prior to map fitting. 2-Fold non-crystallographic symmetry restraints were applied in all the refinement cycles. As the map phases improved with subsequent simulated annealing and individual B-refinements, additional water molecules were added. The model was further refined by positional maximum likelihood minimization and by individual B-factor refinement. The results are summarized in Table II. Figs. 2, 5, and 6 were generated using PyMOL (www.pymol.org). Side chain modeling for the catalytic nucleophile was performed using CS Chem3D Pro (version 5.0), and energy minimization was performed on the inhibitor model using the molecular dynamics utilities of CS Chem3D Pro (www.camsoft.com). These models were used to generate suitable topology and parameter files for CNS using the PRODRG2 server (14) prior to map fitting.

RESULTS

Structure of the GlcCerase-CBE Complex—Initial experiments were performed to determine whether CBE was able to bind to GlcCerase crystals. Upon redissolving in the GlcCerase reaction buffer, GlcCerase crystals were fully active, but crystals into which CBE had been soaked were devoid of catalytic activity (Fig. 1).

X-ray data for the GlcCerase-CBE complex were collected (Table I) and refined to 2.4 Å resolution (Table I). From the initial Fo – Fc and the 2Fo – Fc maps, it is apparent that CBE binds to the active site of GlcCerase (Fig. 2). More specifically, the distance between the epoxide oxygen of CBE, oriented similarly to the cyclohexitol ring, and Glu340 (E235), the residues in proximity to CBE are displayed in CPK colors.
hydrogen-bonding distance, consistent with the role of Glu^235 as the acid/base catalyst (18) (Fig. 2b).

Additional residues in proximity to the cyclohexitol in GlcCerase (Fig. 4a) were compared with those found in a plant β-D-glucan glucohydrolase (19) (Fig. 4b), a member of the same glycohydrolase family. In contrast to the chair conformation adopted by the cyclohexitol upon binding to a plant β-D-glucan glucohydrolase (19), the cyclohexitol is found in a boat conformation in GlcCerase, with hydrogen bonds to Asn^234O/H9254, N/H9254, Glu^340O/H9280, Trp^179N/H9280, and Asp^127O/H9254 and O/H9254 (Fig. 4a). The boat conformation is stabilized by tight hydrogen-bonding interactions between C4-OH of the cyclohexitol and Asp^127 and between C3-OH and Asp^127 and Trp^179.

Characterization of a Lid at the Entrance to the Active Site—Superimposition of the native GlcCerase structure on that of GlcCerase-CBE revealed an root mean square deviation of only ~0.32 Å, demonstrating that GlcCerase does not undergo a global structural change upon binding CBE. However, examination of the root mean square deviations of individual residues revealed a significant difference between native GlcCerase and GlcCerase-CBE with respect to the conformation of two loops, Ser^345–Glu^349 (loop 1) and Val^394–Asp^399 (loop 2). In native GlcCerase, the two molecules in the asymmetric unit display two alternative conformations (6) for both of these loops (Fig. 5a). In loop 2, a major conformational difference is seen in the positions of Asn^396 and Phe^397 in the two asymmetric units (Fig. 5c), and in loop 1 a more modest difference is seen in the conformations of Lys^346 and Glu^349 (Fig. 5b). In contrast, the two molecules in the asymmetric unit of the GlcCerase-CBE crystals adopt only one of these two conformations.

Interestingly, the two loops are located on the surface of GlcCerase at the entrance to the active site. In the conformation adopted in the GlcCerase-CBE structure, Asn^396 and Phe^397 are positioned such that access to the active site is not restricted (Fig. 6a). However, in the other alternative conformation, which is displayed only in the native GlcCerase structure, the side chains of Asn^396 and Phe^397 swing over and block the entrance to the active site (Fig. 6b), suggesting that this
loop serves as a lid regulating access to the active site in GlcCerase. Thus, these two loops allow GlcCerase to exist in either an open (Fig. 6a) or closed (Fig. 6b) conformation, depending on the orientation of the loops. The movies in supplemental Figs. 1 and 2 illustrate the dynamics of the movement of these two loops (29).

Analysis of the distribution of mutant forms of GlcCerase that cause Gaucher disease reveals a cluster of mutations in loop 2, including V394L (2), R395P (20), N396T (21), V398L/F (22, 23), and D399N (24). In silico analysis of these mutants is consistent with either destabilization of the open conformation or stabilization of the closed conformation (Table III), thus limiting substrate access to the active site. For instance, V394L, one of the six most common mutations in Gaucher disease (2), results in enhanced hydrophobic interactions of Leu394 with Trp393 and Phe246 in the closed conformation; conversely, this same mutation destabilizes the open conformation, since the larger side chain cannot interact with the aromatic side chains of Trp393 and Phe246. Likewise, R395P destabilizes the open conformation due to the loss of a stabilizing salt bridge with Glu388. N396T results in additional hydrogen bonding between Thr396 and the carbonyl oxygens of residues Glu388 and Gly389 in the closed conformation but in reduced H-bonding with Asp127 in the open conformation. Thus, the structure of GlcCerase complexed to CBE gives insight into the mechanism by which catalytic activity is reduced by mutations in this newly identified lid that controls access to the active site.

DISCUSSION

In the current study we have solved the x-ray structure of GlcCerase covalently bound to CBE. Using this structure, we confirmed earlier assignments, based on electrospray tandem mass spectrometry, that Glu340 is the nucleophile and Glu235 is the acid/base catalyst (25). Since catalytic activity was completely inhibited by binding of the cyclohexitol to Glu340, and since we saw no binding to any other residues, we can also exclude the possibility that the inhibitory effect of CBE on GlcCerase activity is due to binding to Asp443 and Asp445, as proposed earlier (17).

There are no global structural changes between the GlcCerase-CBE structure and that of native GlcCerase, suggesting that binding of inhibitors to the active site, and presumably also binding of the lipid substrate, does not induce a major conformational change. This also serves as proof of concept that the GlcCerase structure can be used as a starting point for designing structure-based drugs aimed at restoring the activity of defective GlcCerase (3). Important among these are the small molecular weight chaperones that, upon binding to the active site, restore trafficking of improperly folded GlcCerase out of the endoplasmic reticulum and through the secretory pathway to lysosomes, thereby restoring normal, or near-normal, lysosomal GlcCerase levels (5, 26).

Although there are no global structural changes, we did detect a significant structural change in two surface loops at the entrance to the active site that appear to act as a lid controlling access to the active site. Color coding is the same as described for Fig. 5.

![Fig. 6. Surface of GlcCerase illustrating the open and closed conformations. a, the open conformation in which CBE is bound to the active site. b, the closed conformation in which the surface lid restricts access to the active site.](http://www.jbc.org/)

| Mutation | Closed conformation | Open conformation |
|----------|---------------------|-------------------|
| V394L    | Val394              | Contacts with Phe246 and Trp393 |
|          | Leu394              | Contacts with Phe246, Phe246, Arg396, and Trp393 |
| R395P    | Arg395              | No interaction |
|          | Pro396              | No interaction |
| N396T    | Asn396              | No H-bond |
|          | Thr396              | H-bonds to Glu388(O) and Gly389(O) |
| V398L    | Val398              | Contacts with Phe128 and Phe397 |
|          | Leu398              | Contact with Phe397 |
| V398F    | Val398              | Contacts with Phe128 and Phe397 |
|          | Phe398              | Contact with Phe397 |
| D399N    | Asp399              | Salt bridge to Arg359 and H-bonds to Gln414(N), Arg354(O), and Ser400(N) |
|          | Asn399              | H-bonds to Met118(Sδ), Val343(O), and Ser400(N) |
|          |                     | Contacts with Phe246 and Trp393 |
|          |                     | Contact with Pro395 |
|          |                     | Salt bridge to Glu388 |
|          |                     | No interaction |
|          |                     | H-bond to Asp127 |
|          |                     | No interaction |
|          |                     | H-bond to Asp127 |
|          |                     | Contact with Phe128 |
|          |                     | No interaction |
|          |                     | Contact with Phe128 |
|          |                     | No interaction |
|          |                     | Salt bridge to Arg359 |
|          |                     | H-bond to Gln414(N), Ser400(N), and Arg354(N) |
|          |                     | H-bonds to Ser400(N) and Gln414 |
strate binding site undergo a marked conformational change from an open to a closed conformation upon binding the donor substrate. The flexibility of these loops is crucial for the catalytic activity of the glycosyltransferases (27).

In GlcCerase, the role of the two surface loops in governing access to the active site is supported by the reduced affinity of GlcCerase for CBE in various GlcCerase mutations, such as V394L (4), R395P, and N396T (20), although V394L also displays reduced thermostability and reduced stability at low pH (4), implying a disruption of structural integrity that may result in rapid lysosomal degradation. Thus, loop mutations may both decrease the catalytic activity of GlcCerase and reduce its lysosomal enzyme concentration. Unfortunately, sufficient amounts of GlcCerase mutated in residues in these loops are not available for crystallization studies, but in silico mutational analysis is entirely consistent with the lid being in the closed conformation in the mutated proteins, which would prevent access of GlCer to the active site. The discovery of a lid regulating access to the GlcCerase active site provides the first mechanistic insight into how GlcCerase mutations result in reduced catalytic activity and as a consequence cause Gaucher disease.

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