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Chapter 1

Crystal structure of the enzyme acid β-glucosidase

Marc N Offman, Israel Silman, Joel L Sussman & Anthony H Futerman

Acid β-glucosidase (GCase) **(Figure 1)** is the defective protein in Gaucher disease. In 2003, the first crystal structure of GCase was solved; since then, additional crystal structures have been obtained for both the apoenzyme [1–7] and for complexes of the enzyme with a number of ligands [3,5,8–12] under various experimental conditions **(Table 1)**. Furthermore, the first crystal structure of the most common Gaucher disease mutation, N370S, was recently obtained, which, together with molecular dynamics studies, enhanced our understanding of the underlying molecular mechanisms that result in decreased enzymatic activity [7,13,14]. In this chapter, we give a general overview of the GCase crystal structures and summarize recent findings.

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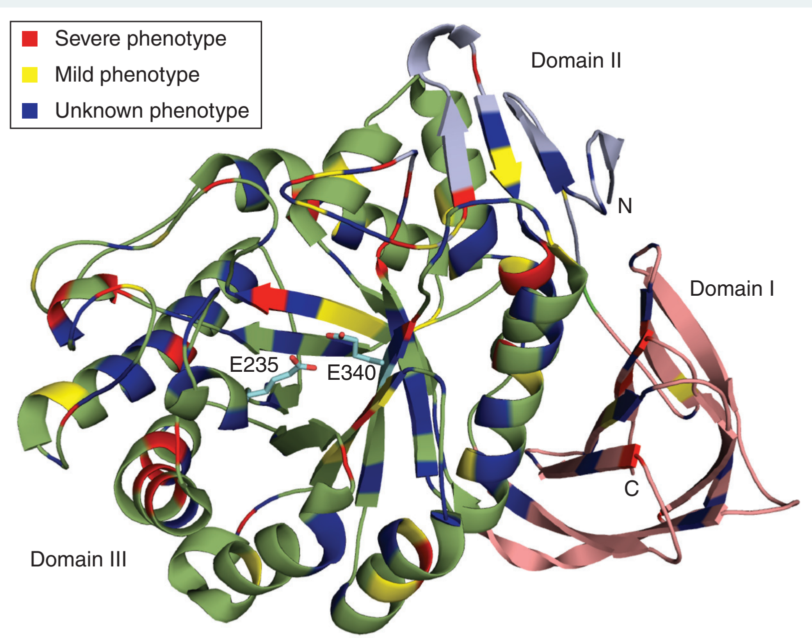
Aa **Saposin C:** saposin is an acronym for sphingolipid activator proteins. Sphingolipid activator proteins are localized primarily in the lysosome where they facilitate degradation of glycosphingolipids by activating the degrading enzymes.

Features of acid β-glucosidase

General

Acid β-glucosidase (GCase; EC 3.2.1.45) is a member of the glycoside hydrolase family 30. It consists of 497 amino acids and has a molecular weight of approximately 60 kDa. GCase is derived from a 516- or 536-residue precursor with the 5´ untranslated region sequence removed during transition through the endoplasmic reticulum (ER) membrane [1]. GCase is a membrane-associated enzyme located in lysosomes, where it hydrolyzes the β-glucosyl linkage of glucosylceramide (GlcCer). Its activity is enhanced by association with the activator protein saposin C, and upon interaction with negatively charged lipids at the lysosomal pH. Two clusters of sulfates have been identified, each containing two to three ions, of which the one close to residues 12, 44, 45 and 353–358 may be involved in membrane association due to its negative charge [15].

Figure 1. Overview of acid β-glucosidase.



All three domains are shown (domain I: light pink; domain II: light blue; domain III: green). Catalytic residues are colored cyan. Mutants that cause severe phenotype are colored red, mild phenotype-causing mutants are colored yellow and mutants causing unknown phenotypes are colored blue.

Glycosylation

Cotranslational glycosylation of GCase occurs at four out of five putative N-glycosylation sites (N19, N59, N146 and N270) [15], and is essential for the development of a catalytically active conformer. Newly synthesized GCase is targeted to the lysosome via mannose 6-phosphate and oligosaccharide-independent pathways, and has a half-life of approximately 60 h [2].

GCase activity is enhanced by the activator protein saposin C, and by negatively charged lipids.

Since Gaucher disease is a monogenetic disease, the current preferred therapy is enzyme replacement therapy.

Catalytic mechanism

Glu235 serves as the acid/base and Glu340 as the nucleophile in the catalytic cycle [1]. Two different catalytic mechanisms have been proposed. It was originally suggested that GCase acts as a typical retaining β-glucosidase for which the catalytic cycle proceeds via a two-step reaction mechanism: glucosylation of the active site by substrate, and deglucosylation with release of β-glucose [2]. New findings, however, suggest a different catalytic mechanism due to steric hindrance within the active site. In this latter mechanism, since the anomeric carbon of the glucose is not susceptible to nucleophilic attack by Glu340, the glycosidic bond must be hydrolyzed via a carbenium ion intermediate [9].

Mutations

Currently, more than 300 distinct mutations of GCase are known to cause Gaucher disease **(Figure 1)** [16,17]. Little is known, however, about the structural consequences of these mutations, their effects on GCase activity and their influence on the targeting of the mutant protein to the lysosome. In general, the diminished GCase activity observed can be ascribed either to reduced catalytic activity of the mutant enzyme or to a reduced lysosomal concentration of the enzyme [15]. In the first case, the mutation affects the turnover number, the substrate affinity and/or binding of the activator. In the second case, mutations compromise the correct folding of GCase in the ER, resulting in proteasomal degradation of the defective protein.

GCase in enzyme replacement therapy

Since Gaucher disease is a monogenetic disease, an effective therapy for treating non-neuropathic types is enzyme replacement therapy (ERT). This involves intravenous administration of GCase, which needs to be targeted to the macrophages,

Cerezyme®: also called imiglucerase. Approved for the treatment of Gaucher disease. An analog of human acid β-glucosidase (GCase) produced with recombinant DNA. Other approved GCase products for enzyme replacement therapy are velaglucerase and taliglucerase.

Table 1. Summary of crystal structures of acid β-glucosidase under different conditions.

Study (year)	PDB code	pH	Chains	Resolution (Å)	R-factor†	Ligand AS	Ref.
Dvir *et al.*(2003)	1ogs	4.5	A,B	2.00	0.195		[1]
Premkumar *et al.* (2005)	1y7v	4.6	A,B	2.40	0.242	CBE	[8]
Liou *et al.*(2006)	2f61	6.0	A,B	2.50	0.196		[2]
Brumshtein *et al.* (2006)	2j25	5.5	A,B	2.90	0.218		[15]
Lieberman *et al.*(2007)	2nsx 2nt0 2nt1	4.5 4.5 7.5	ABCD ABCD ABCD	2.11 1.79 2.30	0,193 0.181 0.178	IFG	[3]
Brumshtein *et al.* (2007)	2v3d 2v3e	6.5 6.5	AB AB	1.96 2.00	0.157 0.163	NB-DNJ NN-DNJ	[9]
Shaaltiel *et al.*(2007)	2v3f	6.5	AB	1.95	0.154		[4]
Kacher *et al.*(2008)	2vt0	6.5	AB	2.15	0.154	CBE	[10]
Brumshtein *et al.* (2009)	2wcg	6.5	AB	2.30	0.138	6N-NOI-NJ	[11]
Brumshtein *et al.* (2010)	2wkl	7.0	A,B	2.70	0.176		[6]
Brumshtein *et al.* (2011)	2xwd 2xwe	6.5 6.5	AB AB	2.66 2.31	0.153 0.153	NOI-NJ 6S-NOI-NJ	[12]
Lieberman *et al.*(2009)	3gxd 3gxf 3gxi 3gxm	4.5 7.5 5.5 4.5	ABCD ABCD ABCD ABCD	2.50 2.40 1.84 2.20	0.208 0.182 0.193 0.223	 IFG	[5]
Wei *et al.*(2011)	3ke0 3keh	5.4 7.1	A,B A,B	2.70 2.80	0.175 0.215		[7]
	3rik		ABCD	2.48	0.165	3RI	[21]
	3ril		ABCD	2.40	0.174	3RK	[21]

†A measure of the agreement between the crystallographic model and the experimental x-ray diffraction data.
6N-NOI-NJ: 6-amino-6-deoxy-5-*N*,6-*N*-[*N*′-(*n*-octyl)iminomethylidene]nojirimycin; 6S-NOI-NJ: 5-*N*,6-*S*-[*N*′-(*n*-octyl)iminomethylidene]-6-thionojirimycin; AS: Active site; CBE: Conduritol B-epoxide; IFG: Isofagomine; NB-DNJ: *N*-butyl-deoxynojirimycin; NN-DNJ: *N*-nonyl-deoxynojirimycin;
NOI-NJ: 5-*N*,6-*O*-[*N*′-(*n*-octyl)iminomethylidene]nojirimycin; PDB: Protein Data Bank; R-factor: Reliability factor.

the cells mainly affected in Gaucher disease [18]. Accordingly, production of Cerezyme® (Genzyme Corporation [a Sanofi company], MA, USA; one of the

Velaglucerase alfa, a Gene-Activated™ human GCase, has a twofold increased internalization rate compared with imiglucerase.

approved pharmaceutical formulations used for ERT; generic name: imiglucerase) involves sequential deglycosylation so as to expose terminal mannose residues. The remaining core glycan, an oligosaccharide, which consists of five sugars, causes enhanced internalization by mannose receptors on the surface of the macrophage.

Other pharmaceutical formulations used in ERT include velaglucerase alfa (Gene-Activated™ human GCase; Shire Human Genetic Therapies, Inc., MA, USA), which differs in the glycosylation pattern due to the inhibition of α-mannosidase in the ER, and taliglucerase alfa, a GCase with natural α-mannoses, which is produced in carrot cells.

Structural aspects of GCase

General observations: the first crystal structure (1OGS [2003])

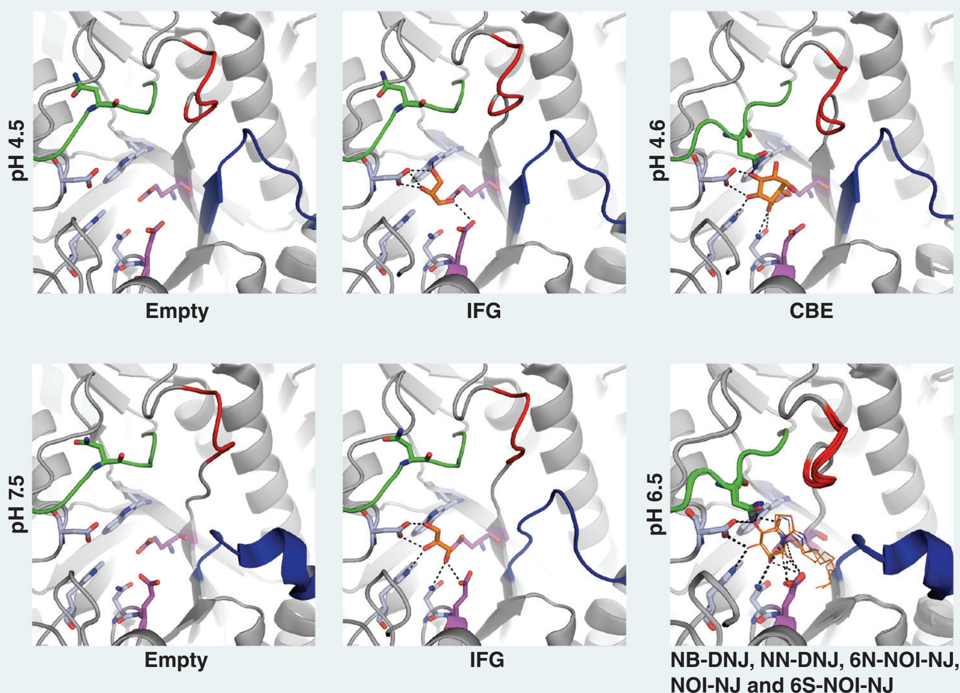
The first crystal structure of GCase was resolved at 2.0 Å **(Table 1)**, and contained two GCase molecules per asymmetric unit [1]. The enzyme was seen to contain three distinct noncontinuous domains [1]. Domain I is the smallest, and includes residues 1–27 and 383–414; it consists of one three-stranded antiparallel β-sheet, which is flanked orthogonally by a loop and by the C-terminal strand **(Figure 1)**. Two disulphide bridges (residues 4–16 and 18–23) within domain I may be important for correct folding to occur. Residue N19 is glycosylated, and is essential for catalytic activity *in vivo*. Domain II includes residues 30–75 and 431–497, and consists of two interacting β-sheets, a structure that resembles an immunoglobulin (Ig) fold. Domain III (residues 76–381 and 416–430), the largest domain, contains the catalytic site, three free cysteines (126, 248 and 342), and has the architecture of a $(\beta/\alpha)_8$ triosephosphate isomerase barrel. A tight interaction between domains I and III exists; by contrast, domains II and III are only connected by a flexible hinge.

Active site & ligands

As already mentioned, E235 is the acid/base, and E340 serves as the nucleophile in the catalytic site, assignments which have been confirmed by site-directed mutagenesis and by homology modeling [19]. The average distance between the two was shown to be approximately 5.2 Å, consistent with retention of the anomeric carbon upon cleavage. Docking of the substrate showed that only the glucose moiety and glycoside bond fit within the active-site pocket **(Figure 1)**. Therefore, the two GlcCer hydrocarbon chains either interact with saposin C or remain in the lipid bilayer during catalysis. This notion is also supported by the presence of a cluster of hydrophobic residues that surround the entrance to the

active site and may facilitate interaction of GCase with the lysosomal membrane or with saposin C. Inside the active-site pocket, seven aromatic side chains (F128, W179, Y244, F246, Y313, W381 and F397) are aligned, and may be involved in substrate recognition, as has been observed for other β-glycosidases. An overview of the GCase active site under various conditions and in GCase–ligand complexes is presented in **Figure 2**. A summary of the principal structural properties can be found in **Box 1**.

Figure 2. Close-up of the active site.



Active-site residues are colored in magenta. Loop 1 is colored in red, loop 2 in green and loop 3 in blue. Various complexes are shown at various pH values. Interacting residues are colored light blue and H-bonds grey. Compounds are colored orange. Several similar compound/acid β-glucosidase complexes in which no large structural changes are seen are presented as a single entity in the lower right panel.
6N-NOI-NJ: 6-amino-6-deoxy-5-*N*,6-*N*-[*N*′-(*n*-octyl)iminomethylidene]nojirimycin; 6S-NOI-NJ: 5-*N*,6-*S*-[*N*′-(*n*-octyl)iminomethylidene]-6-thionojirimycin; CBE: Conduritol B-epoxide; IFG: Isofagomine; NB-DNJ: *N*-butyl-deoxynojirimycin; NN-DNJ: *N*-nonyl-deoxynojirimycin; NOI-NJ: 5-*N*,6-*O*-[*N*′-(n-octyl)iminomethylidene]nojirimycin.

What have we learned from the various crystal structures?

Apoenzyme: free enzyme that is not in complex with the ligand.

Pharmacological chaperone: small molecule that is easily taken up by cells, where it aids the correct folding and targeting of otherwise misfolded mutant proteins.

Since the first determination of the crystal structure of the GCase apoenzyme, more than 20 more GCase crystal structures have been solved, including complexes with various ligands as well as additional apoenzyme structures **(Table 1)**. Each of them sheds light on a different aspect of GCase specificity and activity, and may impact therapeutic approaches for Gaucher disease. Below is a brief summary of the new insights obtained from these crystal structures.

1Y7V (2005): the first GCase structure with an inhibitor & identification of lid loops

The first crystal structure of a ligand–GCase complex, at 2.4 Å resolution, was obtained at pH 4.6 **(Table 1 & Figure 2)** [8]. This crystal structure was of a complex of deglycosylated imiglucerase with conduritol B-epoxide (CBE). No global structural changes were seen relative to the apoenzyme. Furthermore, Glu340 was confirmed as the catalytic nucleophile. Binding of CBE helped to define the role of two surface loops found at the entrance of the active site (loop 1: 345–349; and loop2: 394–399 as defined in [8]). For these loops, only one of two possible conformations was found for the CBE–GCase complex compared with the apoenzyme, with the active site being accessible in the complex. Comparison of the various conformations suggested that the loops served as a lid over the active site. Interestingly, a cluster of Gaucher disease-causing mutations occur in loop 2. *In silico* investigation of these mutants predicted stabilization and limited access to the active site, and thus provide a mechanistic explanation of how these particular mutations can result in Gaucher disease.

In 2005, *N*-butyl-deoxynojirimycin (NB-DNJ), a low-molecular-weight compound used for substrate reduction therapy of Gaucher disease, was shown to act as a pharmacological chaperone that increases the GCase activity of several mutants, including the most common mutant, N370S, by raising the lysosomal concentration of the enzyme [20]. The crystal structure of the GCase–NB-DNJ complex was resolved subsequently (see below) [9].

2J25 (2006): structure of unmodified imiglucerase

The first crystal structures reported were obtained for deglycosylated imiglucerase. In

Pharmacological chaperones can be used to increase GCase activity of several mutants, including N370S.

order to obtain the structure of unmodified GCase, new crystallization conditions were developed. In the crystal structure of the intact imiglucerase, the glycans bound to three asparagines via *N*-glycosylation were resolved, and it was shown that there were no significant structural differences between the intact and the deglycosylated imiglucerase. All of the glycosylation sites are located adjacent to empty cavities within the crystal, so that the presence of the sugar residues in these spaces does not result in steric clashes that could hinder crystallization. Furthermore, additional variability was observed for the lid loops surrounding the active site, including the identification of a third loop spanning residues 312–319 (loop 3 as defined in [15]).

2NSX (2007): structure of the isofagomine/GCase complex

Another crystal structure obtained for a complex of GCase with a pharmacological chaperone able to bind at the active site was its complex with isofagomine (IFG; **Figure 2**). IFG, which is a competitive inhibitor of GCase, increased levels of the enzyme in cell lysates, and restored lysosomal trafficking of N370S. The crystal structure of the IFG–GCase complex at low pH was compared with that of glycerol-bound GCase at low pH, and with the first GCase apoenzyme structure obtained at pH 7.4. IFG binds in a well-ordered chair conformation, and is stabilized by several hydrogen (H)-bonds with the enzyme. The hydroxyl groups of IFG interact with residues D127, W179, W381 and N396, and the imino group is stabilized by E235 and E340. Binding of IFG, together with lowering of the pH, results in a number of structural changes; thus, some critical movements can be seen in two of the three lid loops – loop 1 (defined as loop 2 in [3]) and loop 3 (defined as loop 1 in [3]). For loop 3, a helical turn is observed at lysosomal pH upon binding of IFG, which is stabilized by a new H-bonding network involving N370. This crucial network further generates well-ordered side chains in loop 3, an effect observed upon binding of IFG but not of glycerol. For loop 1, a significant shift in conformation is observed when comparing the structures at neutral and acidic pH (root mean square deviation: ~1.5 Å^2).

2V3F (2007): production of plant recombinant GCase (taliglucerase alfa) in a carrot cell suspension culture, followed by crystallization & x-ray structure determination

In 2007, a new production pipeline of recombinant human GCase in a carrot cell suspension culture was introduced, which produced enzyme with terminal mannose residues on its complex glycans, and showed biological activity similar to that of imiglucerase. Its crystallization yielded crystals that diffracted to 1.95 Å. Compared with the crystal structures of unbound and

CBE-bound imiglucerase, the root mean square deviation values are 0.64 and 0.60 Å, respectively. The active site residues E235 and E340 and the neighboring residues in the active site are structurally conserved [4].

2V3D & 2V3E (2007): structures of complexes of GCase with NB-DNJ & *N*-nonyl-deoxynojirimycin

The crystal structures of the complexes of GCase with the pharmacological chaperones NB-DNJ and *N*-nonyl-deoxynojirimycin (NN-DNJ) revealed that both bind to the active site of GCase. The imino sugar moiety H-bonds to side chains of active-site residues, and the alkyl chains are oriented toward the entrance of the active site where they are involved in hydrophobic interactions. A >300-fold difference was observed in the inhibition constant (K_i) values for the two inhibitors (116 µM for NB-DNJ and 0.3 µM for NN-DNJ). Since the entrance to the active site favors hydrophobic interactions, the lower K_i observed for NN-DNJ can be ascribed to its elongated hydrophobic alkyl chain. In all three crystal structures involving reversible interaction with a noncovalent inhibitor (NB-DNJ, NN-DNJ and IFG), a H-bond is formed between Y313 and E340; this requires loop 3 to be in a helical conformation. By contrast, in the covalent complex in which CBE is covalently bound to E340, Y313 can only form a H-bond with E235, and loop 3 is in a coiled conformation, suggesting that loop 3 is an integral component of the catalytic machinery. By contrast, loop 1 does not undergo major changes in its backbone angles or in its secondary structure. Most importantly, however, the study on the NB-DNJ and NN-DNJ complexes proposed an alternative three-step reaction mechanism, different from that involving direct attack of the carboxylate oxygen of E340 on the anomeric carbon of GlcCer. In this alternative mechanism, an intermediate involving a planar carbon atom attack by E340 was suggested. It was further suggested that E235 must be protonated, and that a water molecule is required for hydrolysis of the covalent intermediate [9].

2VT0 (2008): structure of an additional CBE–GCase complex

In 2008, another crystal structure of the CBE–GCase complex was resolved at pH 6.5, rather than at the value of pH 4.6 that had been employed earlier. It was shown that processing of GCase never results in deglycosylation of the essential N19 residue; thus, *N*-glycosidase F only partially deglycosylated imiglucerase by removing two and occasionally three sugar chains [10].

3GXD, 3GXF, 3GXI & 3GXM (2009): complexes of GCase with IFG at neutral & acid pH values

The structure and stability of GCase were studied at neutral and low pH, pH values intended to reflect that of the environment in the ER and the

lysosome, respectively. Furthermore, the structure of the IFG–GCase complex was also compared at both pH values, revealing information important for the continued design of pharmacological chaperones. Differential scanning calorimetry revealed pH dependence of the stability of the IFG–GCase complex, with increased stability being observed at the lysosomal pH. However, the active-site loop conformations are not pH sensitive and only change upon ligand binding [5].

2WKL (2010): structure of velaglucerase alfa

In this study, the crystal structure of velaglucerase alfa, a Gene-Activated human GCase, was determined. In contrast to imiglucerase and taliglucerase alfa, velaglucerase alfa contains the intact native human enzyme sequence. It was shown that the distances between the carboxylate oxygens of the catalytic residues E340 and E235 are consistent with distances proposed for acid–base hydrolysis. However, the glycosylation patterns found in velaglucerase alfa are distinctly different from those found in imiglucerase and taliglucerase alfa. For velaglucerase alfa, a high-mannose-type glycan with nine mannose units is detected, whereas imiglucerase contains complex-type glycans with core fucosylation, which terminate with the chitobiose trimannosyl core. This different glycosylation pattern results in at least a twofold increase in the rate of internalization into human macrophages relative to imiglucerase [6].

Further crystal structures deposited

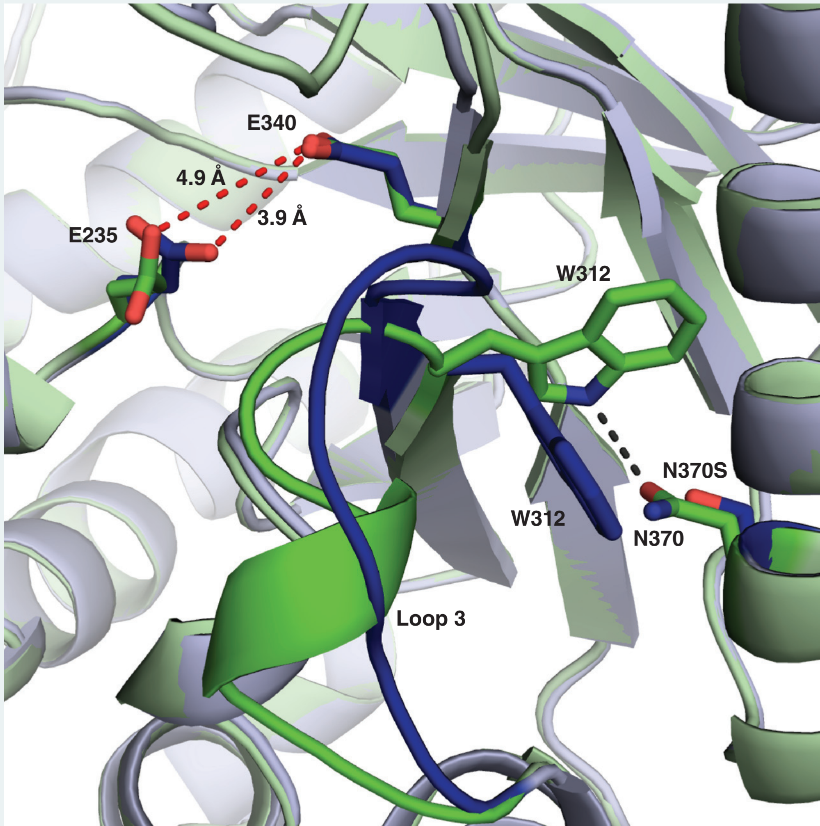
- 2WEG (2009) [11]: GCase structure with 6-amino-6-deoxy-5,6-di-*N*-(*N*′-octyliminomethylidene (6N-NOI-NJ) nojirimycin;
- 2XWD and 2XWE (2011) [12]: GCase with 5-*N*,6-*O*-[*N*′-(*n*-octyl)iminomethylidene]nojirimycin (NOI-NJ) and 5-*N*,6-*S*-[*N*′-(*n*-octyl)iminomethylidene]-6-thionojirimycin (6S-NOI-NJ), two potent inhibitors with pharmacological chaperone activity;
- 3RIK and 3RIL (2011): binding of 3,4,5,6-tetrahydroxyazepanes to the GCase active site: implications for pharmacological chaperone design for Gaucher disease.

Mutational analysis

3KE0 & 3KEH (2011): analysis of the crystal structure of the most common mutant GCase, N370S

The first crystal structure of a Gaucher disease mutant was that of N370S. The mutant and wild-type (WT) enzymes were investigated at physiological and lysosomal pH, and overall folding of the mutant was

Figure 3. Comparison between wild-type and N370S crystal structures.



Wild-type (WT) (green) and N370S (blue) structures are superimposed. The amino acid N370 and N370S, and W312, which interacts with N370 in WT acid β-glucosidase, are shown in stick representation. H-bonds are in dark grey. Loop 3 is shown in saturated colors; it is in the helical conformation for the WT, and in the extended conformation for N370S. The distance between the two active sites residues (E235 and E340) is depicted in red.

identical to that of the WT. However, small changes were observed for the lid loops, the catalytic residues (E235 and E340), and the H-bonding networks **(Figure 3)**. In general, this was reflected in a reduced maximum velocity (V_{max}) and in an increased Michaelis constant (K_m) for N370S relative to the WT enzyme, and it was also more stable and less flexible. Finally, it was found that the N370S mutant at pH 7.4 showed properties similar to those of the WT protein at pH 4.5. These findings confirmed predictions made earlier in a molecular dynamics study, which also

Box 1. Summary of structural properties.

- Contains three domains
- Domain I includes residues 1–27 and 383–414, two disulfide bridges (4–16 and 18–23) and one glycosylation site (N19)
- Domain II includes residues 30–75 and 431–497, and resembles an immunoglobulin fold
- Domain III includes residues 76–381 and 431–497 and has a (β/α) 8 triosephosphate isomerase barrel architecture; it includes the catalytic site and three free cysteines (126, 248 and 342)
- E235 is the acid/base
- E340 is the nucleophile
- A cluster of hydrophobic residues surrounding the entrance to the active site may stabilize the interaction of acid β-glucosidase with the lysosomal membrane or with saposin C
- Seven aromatic side chains (F128, W179, Y244, F246, Y313, W381 and F397) are linearly aligned in the active site and may be involved in substrate recognition
- Three lid loops surround the active site and control access to it (loop 1: 345–349; loop 2: 394–399; loop 3: 312–319)

revealed further details, such as a suboptimal distance between E235 and E340 in the N370S mutant enzyme. In the molecular dynamics study, it was also predicted that NB-DNJ is able to stabilize a GCase–saposin C complex, which is a requirement for formation of a substrate–enzyme complex. A more thorough analysis of the predicted and experimental results was presented [14], which showed the potential of molecular dynamics simulations of GCase mutants to provide valuable information in the context of mutation-specific drug design [7,13,14].

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Summary.

- Acid β-glucosidase (GCase) is the defective protein in Gaucher disease. It is a member of the glycoside hydrolase family 30 and consists of 497 amino acids with an Mr of approximately 60 kDa. GCase hydrolyzes the β-glucosyl linkage of glucosylceramide in lysosomes.
- It comprises three distinct, noncontinuous domains. Domain I, the smallest, includes residues 1–27 and 383–414; it consists of one three-stranded antiparallel β-sheet. Domain II includes residues 30–75 and 431–497 and consists of two interacting β-sheets, a structure that resembles an immunoglobulin fold. Domain III (residues 76–381 and 416–430), the biggest domain, contains the catalytic site, three free cysteines (126, 248 and 342) and has the architecture of a (α/β) 8 triosephosphate isomerase barrel.
- For activity, the following features are important: E235 acid/base; E340 nucleophile; three flexible lid loops flanking the entrance to the active site; various disulfide bridges and free cysteines; glycosylation of N19.
- More than 20 GCase crystal structures under different conditions and with various ligands bound are available.
- More than 300 mutants spread all over the protein can cause Gaucher disease, but structural insights have thus far helped little regarding genotype–phenotype correlations.
- Generally, two effects are thought to cause diminished activity: reduced catalytic activity and reduced lysosomal concentration. In the former case, mutations affect the turnover rate, substrate affinity and/or activator binding. For the latter, mutants compromise the correct folding of GCase in the endoplasmic reticulum, which results in proteasomal degradation of the defective protein.
- Several small chemical chaperones are able to aid folding, increase activity and assure correct targeting in some Gaucher disease mutants.

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