Molecular Genetics and Metabolism 96 (2009) 233–238

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

Molecular interaction of imino sugars with human α-galactosidase: Insight into the mechanism of complex formation and pharmacological chaperone action in Fabry disease

Kanako Sugawara [a], Youichi Tajima [b], Ikuo Kawashima [b], Takahiro Tsukimura [b], Seiji Saito [c], Kazuki Ohno [d], Kunihiko Iwamoto [e], Toshihide Kobayashi [e], Kohji Itoh [f], Hitoshi Sakuraba [a,*]

[a] Department of Analytical Biochemistry, Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose, Tokyo 204-8588, Japan
[b] Translational Research Project, The Tokyo Metropolitan Institute of Medical Science, Tokyo Metropolitan Organization for Medical Research, Tokyo, Japan
[c] Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan
[d] NPO for The Promotion of Research on Intellectual Property Tokyo, Tokyo, Japan
[e] Lipid Biology Laboratory, RIKEN (Institute of Physical and Chemical Research), Wako, Japan
[f] Department of Medicinal Biotechnology, Institute for Medicinal Science, Graduate School of Pharmaceutical Sciences, The University of Tokushima, Tokushima, Japan

ARTICLE INFO

Article history:
Received 17 November 2008
Received in revised form 24 December 2008
Accepted 24 December 2008
Available online 31 January 2009

Keywords:
Molecular interaction
Imino sugars
α-Galactosidase
Fabry disease
Isothermal titration calorimetry
Surface plasmon resonance biosensor assay

ABSTRACT

Enzyme enhancement therapy (EET) for Fabry disease involving imino sugars has been developed and attracted interest. It is thought that imino sugars act as pharmacological chaperones for wild-type and mutant α-galactosidases (GLAs) in cells, but the mechanisms underlying the molecular interactions between the imino sugars and the enzyme have not been clarified yet. We examined various kinds of imino sugars and found that galactostatin bisulfite (GBS) inhibited GLA *in vitro* and increased the enzyme activity in cultured Fabry fibroblasts as in the case of 1-deoxygalactonojirimycin (DGJ). Then, we analyzed the molecular interactions between the imino sugars and recombinant human GLA by means of isothermal titration calorimetry and surface plasmon resonance biosensor assays, and first determined the thermodynamic and binding-kinetics parameters of imino sugar and GLA complex formation. The results revealed that DGJ bound to the enzyme more strongly than GBS, the binding of DGJ to the enzyme protein being enthalpy-driven. In the case of GBS, the reaction was mainly enthalpy-driven, but there was a possibility that entropy-driven factors were involved in the binding. Structural analysis *in silico* revealed that both the chemicals fit into the active-site pocket and undergo hydrogen bonding with residues comprising the active-site pocket including the catalytic ones. The side chain of GBS was oriented towards the entrance of the active-site pocket, and thus it could be in contact with residues comprising the wall of the active-site pocket. Thermodynamic, kinetic and structural studies should provide us with a lot of information for improving EET for Fabry disease.

Lysosomal α-galactosidase (GLA, EC3.2.1.22) is an exoglycosidase that catalyzes the hydrolysis of the terminal α-D-galactosyl residues of glycoconjugates, predominantly globotriaosylceramide (GL-3). This enzyme, encoded by the *GLA* gene on the long arm of the X-chromosome, is synthesized on endoplasmic reticulum (ER)-bound ribosomes as a 429-amino acid precursor polypeptide with an N-terminal signal sequence of 31 amino acids that is cotranslationally removed. Upon entry into the ER, the enzyme is modified through the addition of *N*-linked oligosaccharides. Subsequently, the enzyme is transferred to the Golgi apparatus, where further modification of sugar chains and the addition of mannose 6-phosphate (M6P) residues at the non-reducing ends of the sugar chains occur. The enzyme is then transported to endosomes via M6P receptors, and moves to lysosomes, where it exerts its function as a mature form consisting of 398 residues, and the native enzyme in humans is thought to have a homodimeric structure [1]. According to the crystallographic structure of human GLA [2], the enzyme unit comprises two domains: an N-terminal $(\beta/\alpha)_8$-barrel domain and a C-terminal antiparallel β-sheet domain, and the active site is localized in the C-terminal of the β-sheet of the $(\beta/\alpha)_8$-barrel domain.

A genetic deficiency of GLA causes Fabry disease (MIM 301500). This disease exhibits a wide clinical spectrum from the classic form to the variant one. Patients with classic Fabry disease develop neuropathic pain in the extremities, angiokeratoma, hypohidrosis, corneal clouding, renal impairment, heart failure and strokes. On the other hand, there are also patients with variant Fabry disease with late-onset and milder clinical manifestations [1]. Recently, the results of newborn screening revealed that the incidence of Fabry

* Corresponding author. Fax: +81 42 495 8923.
E-mail address: sakuraba@my-pharm.ac.jp (H. Sakuraba).

1096-7192/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymgme.2008.12.017

disease, especially the variant form, is unexpectedly high, 1 in 3000–4000 male newborns [3]. So the clinical importance of this disease is increasing more and more.

As to therapy for Fabry disease, recombinant human GLAs produced in human fibroblasts and Chinese hamster ovary cells have been developed and are clinically available for enzyme replacement therapy (ERT) [4–6]. Despite its efficacy, ERT has some problems, i.e., the uptake of the enzyme by the brain, kidney and heart is low, and ERT with recombinant enzymes is expensive. Recently, enzyme enhancement therapy (EET) was developed as another potential approach for treating Fabry disease, and a clinical trial has been performed. This therapy is based on substrate analogues including 1-deoxygalactonojimycin (DGJ) that act as pharmacological chaperones and improve the stability or transportation of mutant GLAs in cells [7–10].

As these small molecules can diffuse broadly in tissues, improvement of the stability and correction of the trafficking defect of mutant GLAs with these chemicals would be beneficial for the treatment of Fabry disease, and EET with these chemicals would be economic. However, these chemicals are usually potent inhibitors of GLA. Some of them strongly inhibit GLA activity *in vitro* and *in vivo*. So, the development of improved chemicals suitable for EET is strongly required. For this purpose, information on the molecular interaction between the enzyme and the chemicals is very important. But, there is little such information. Thermodynamic approach should provide us with key information involving protein and sugar interaction as a powerful tool.

In this study, we examined substrate analogues including imino sugars as to binding to GLA, and investigated the binding parameters and thermodynamics of the molecular interactions of these chemicals with GLA. Furthermore, we constructed structural models of the active-site pocket of GLA with the chemicals bound to its active site to clarify the mechanisms of complex formation.

Materials and methods

Materials

Recombinant human GLA produced in Chinese hamster ovary cells was purchased from Genzyme Japan (Tokyo, Japan). DGJ, galactostatin bisulfite (GBS), 1-deoxy-L-altronojirimycin hydrochloride (Altro-DNJ), *N*-butyldeoxygalactonojirimycin (NB-DGJ), *N*-methyldeoxynojirimycin (NM-DNJ), 1-deoxy-L-idonojirimycin hydrochloride (Ido-DNJ), isofagomine hydrochloride (IFG), *N*-nonyldeoxynojirimycin (NN-DNJ), *N*-dodecyldeoxynojirimycin (ND-DNJ), deoxyfuconojirimycin hydrochloride (DFJ), and deoxymannojirimycin hydrochloride (DMJ) were obtained from Toronto Research Biochemicals (North York, Canada). 1-Deoxynojirimycin (DNJ) and 1,4-dideoxy-1,4-imino-D-arabinitol hydrochloride (D-AB1) were purchased from WAKO Chemicals (Osaka, Japan). *N*-Ethyldeoxynojirimycin hydrochloride (NE-DNJ) and *N*-butyldeoxynojirimycin hydrochloride (NB-DNJ) were purchased from BIOMOL (Plymouth Meeting, PA). Conduritol-B-epoxide (CBE) and castanospermine (CAST) were obtained from Sigma–Aldrich (St. Louis, MO). All of the compounds were of reagent grade.

α-Galactosidase assaying and determination of enzymatic properties of human GLA

α-Galactosidase activity was measured fluorometrically with 4-methylumbelliferyl-α-D-galactopyranoside (Calbiochem, LaJolla, CA) as a substrate and *N*-acetyl-D-galactosamine (Sigma–Aldrich) as an inhibitor for α-*N*-acetylgalactosaminidase according to the method previously reported [11]. Protein determination was performed with a Micro BCA Protein Assay Kit (PIERCE, Rockford, IL), using bovine serum albumin as a standard.

A kinetic experiment was performed with 0.06 μg of the recombinant human GLA, various concentrations (1–8 mmol l^{-1}) of the substrate and 117 mmol l^{-1} *N*-acetyl-D-galactosamine in 0.1 mol l^{-1} citrate-phosphate buffer, pH 4.6, to determine the maximum velocity (V_{max}) and Michaelis constant (K_m) values. The specific enzyme activity of GLA was determined with 4 mmol l^{-1} substrate. To determine the optical assay conditions, the reaction mixture (total volume: 50 μl) was incubated at 37 °C for 5–50 min, and thereafter the reaction was performed for 10 min. The reaction was stopped by adding 950 μl of 0.2 mol l^{-1} glycine buffer (pH 10.7). Then, the released 4-methylumbelliferone was measured with a Wallac 1420 ARVO MX Multilabel Counter (Perkin-Elmer, Waltham, MA) at excitation and emission wavelengths of 355 and 460 nm, respectively.

Examination of inhibitory activity of imino sugars toward GLA

For the inhibition assay, imino sugars, i.e., DGJ, GBS, Altro-DNJ, NB-DGJ, DNJ, NM-DNJ, NE-DNJ, NB-DNJ, Ido-DNJ, D-AB1, IFG, CAST, NN-DNJ, ND-DNJ, CBE, DFJ and DMJ, were added at various concentrations to the assay mixture, according to the method described previously [12]. The inhibitory constant (K_i) values were calculated from plots of slope versus imino sugar concentration.

Examination of the effects of DGJ and GBS on cultured Fabry fibroblasts

Cultured fibroblasts from a patient with Fabry disease having the Q279E mutation [13–15] were established and maintained in our laboratory. The cells were cultured in Ham's F-10 medium containing 10% fetal calf serum and antibiotics at 37 °C in a humidified incubator flushed continuously with a 5% CO_2–95% air mixture. The study involving the cultured human fibroblasts was approved by the Ethical Committee of our institute (No. 1908). To examine the effects of the representative imino sugars including DGJ and GBS on the Fabry fibroblasts, the chemicals were added to the culture medium of the cells to give concentrations of 0, 20, 100, 400, and 1000 μmol l^{-1}. After 4 days culture, the cells were harvested mechanically, washed three times with phosphate-buffered saline (PBS), pH 7.4, and then collected as a pellet by centrifugation. An appropriate amount of water was then added to the pellet and the cells were sonicated, the resulting homogenate being used for α-galactosidase assay and protein determination.

Determination of thermodynamic parameters of imino sugar and GLA complex formation by means of isothermal titration calorimetry (ITC)

To examine the complex formation between imino sugars and GLA thermodynamically, ITC involving a Miocrocal VP-ITC high sensitivity titration calorimeter (MicroCal, Northampton, MA) was performed according to the method described previously [14–16]. DGJ and GBS were added to the GLA solution, independently. The heats of dilution were determined as the heats of injection after the titrations reached plateaus. The imino sugar/GLA ratio (N), binding constant (K_a) value, free energy change (ΔG), enthalpy change (ΔH), and entropy change (ΔS) were calculated by curve-fitting analysis as described previously [12].

Determination of thermodynamic and binding-kinetics parameters of imino sugar and GLA complex formation by means of surface plasmon resonance (SPR) biosensor assays

Assaying of biospecific interactions between imino sugars and GLA was performed using a BIAcore T100 (Biacore AB, Uppsala, Sweden) biosensor system [16,17]. GLA (62 μg ml^{-1} in 10 mmol l^{-1} phosphate buffer, pH 5.0) was coupled to a certified grade CM5 chip at a flow rate of 10 μl min^{-1} at 25 °C using the amine coupling

kit supplied by the manufacturer. The unreacted species on the surface of the chip was blocked with ethanolamine. The average level of the immortalized GLA was 3.4×10^3 RU (standard deviation, SD: 0.2×10^3 RU; $n = 8$). All measurements were performed using 50 μmol l^{-1} phosphate buffer, pH 7.0. For the analysis, the solution of the ligands was passed over the chip (DGJ, 62.5, 125 and 250 nmol l^{-1}, temperature, 24, 28, 30, 32, 36, 38 and 40 °C; GBS, 0.098, 0.20, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25 and 50 μmol l^{-1}, temperature, 24, 28, 30, 32, 34, 36, 38, and 40 °C) at a flow rate of 30 μl min^{-1}, and the parameters at 32 °C were obtained. Data analysis was performed according to the method previously described [16,17].

Structural modeling of the complexes of the catalytic domain of human GLA with imino sugars bound to its active site

AutoDock 4.0, a grid-based docking program, was used for docking of the ligands including DGJ and GBS to GLA. The crystal structure of human GLA (PDB: 1R47) [2] was used as a template, and galactose and water molecules were removed from the protein. The polar hydrogens and united atom Kollman charges were assigned for the protein. Then, these ligands were docked within the structure. The default parameter sets were used for docking calculations.

Results

Enzymological character of GLA

Enzymological analysis revealed that the specific activity, V_{max} and K_m values of the enzyme were 2.3 mmol h^{-1} mg $protein^{-1}$, 65 μmol l^{-1} min^{-1}, and 4.0 mmol l^{-1}, respectively, for 4-methylumbelliferyl-α-D-galactopyranoside as a substrate.

Inhibitory activity of imino sugars toward GLA

Various kinds of imino sugars were examined as to their inhibitory actions against GLA. A Lineweaver–Burk plot of the inhibition of GLA by the imino sugars was prepared, and their K_i values were calculated. Among these imino sugars, DGJ and GBS exhibited strong inhibition of GLA, followed by Altro-DNJ and NB-DGJ, which exhibited moderate inhibitory activity (Table 1). All of these imino sugars competitively inhibited the enzyme judging from the Lineweaver–Burk plot (date not shown). The structures of these chemicals are shown in Fig. 1. Other chemicals including NM-DNJ, Ido-DNJ, IFG, NN-DNJ, ND-DNJ, DFJ, DMJ, DNJ, D-AB1, NE-DNJ, NB-DNJ, CBE and CAST did not exhibit any inhibitory effect on GLA.

Effects of DGJ and GBS on GLA activity of cultured Fabry fibroblasts

The results of the inhibition assaying suggested that DGJ and GBS bound to GLA strongly, and thus thereafter these chemicals were used as samples for the experiments. They were separately added to the culture medium of fibroblasts derived from a patient with Fabry disease having Q279E mutation. After incubation in this medium for 4 days, the cells were harvested and the GLA activity was determined. The results are summarized in Fig. 2. The GLA activity in untreated Fabry fibroblasts was almost nil, and it increased to 20% of that in a healthy control when DGJ or GBS was added to the medium at the concentration of 20 μmol l^{-1}. The addition of DGJ increased the enzyme activity up to 50% of normal control when the DGJ concentration was 200 μmol l^{-1}, although it apparently decreased when the DGJ concentration was $\geqslant$400 μmol l^{-1}. Regarding to GBS, the GLA activity was decreased when the GBS concentration was $\geqslant$100 μmol l^{-1}.

Table 1
Inhibitory activities of the imino sugars toward GLA.

Imino sugar	K_i (μmol l^{-1})
DGJ	0.041 ± 0.002 (3)
GBS	0.10 ± 0.01 (3)
Altro-DNJ	31 ± 1 (3)
NB-DGJ	81 ± 1 (3)

The K_i values are expressed as means ± SD (n).

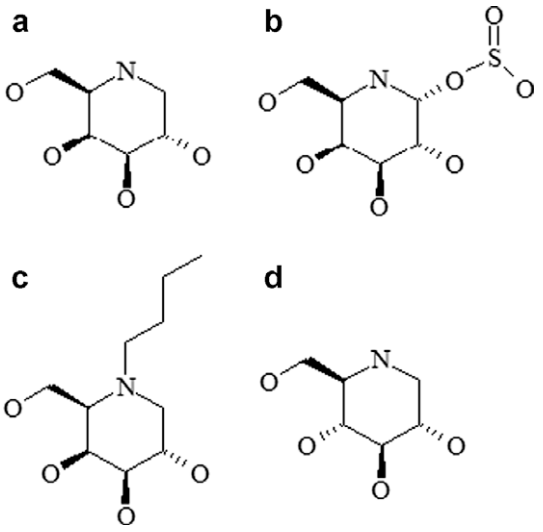

Fig. 1. Structures of the imino sugars. (a) DGJ, (b) GBS, (c) NB-DGJ, and (d) Altro-DNJ.

Thermodynamic and binding-kinetics parameters of imino sugar and GLA complex formation

The formation of complexes of GLA with imino sugars comprising DGJ and GBS bound to its active site was thermodynamically examined by means of ITC and SPR biosensor assays, the results being shown in Table 2. ITC revealed that the imino sugar/enzyme ratio (N) was approximately one in both cases, indicating that the

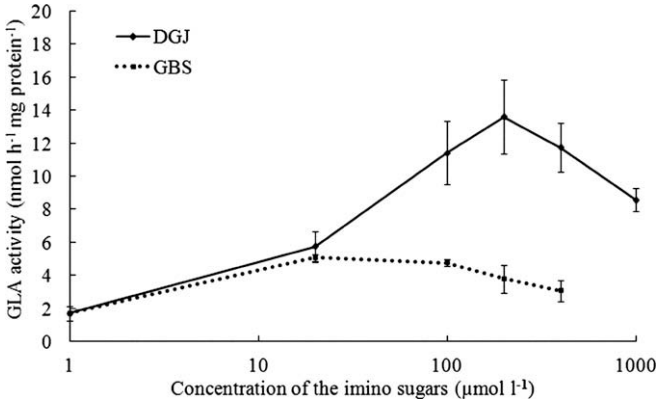

Fig. 2. Effects of DGJ and GBS on GLA activity in cultured Fabry fibroblasts. Fabry fibroblasts having the Q279E mutation were cultured in culture medium containing DGJ and GBS independently at concentrations of 0, 20, 100, 200, 400 and 1000 μmol l^{-1}. The cells were cultured for 4 days, and then GLA activity in the cells was measured. The experiment was performed three times, and the GLA activities in the Fabry cells were expressed as means ± SD. The GLA activity in a healthy control was 26 nmol h^{-1} mg $protein^{-1}$.

Table 2
Thermodynamic parameters of imino sugar/GLA complex formation determined by SPR (BIAcore) biosensor assaying and ITC.

Imino sugar	Method	N (IS GLA^{-1})	K_a (l mol^{-1})	ΔG (kcal mol^{-1})	ΔH (kcal mol^{-1})	$-T\Delta S$ (kcal mol^{-1})	ΔS (cal mol^{-1} deg^{-1})
DGJ	BIAcore	–	–	−11	−23	11.8	−40
	ITC	1.3	1.0×10^{8}	−11	−14	2.1	−6.8
GBS	BIAcore	–	–	−9.1	−12	2.4	−8.2
	ITC	1.0	3.9×10^{6}	−9.4	−6.8	−2.6	8.4

IS, imino sugar; GLA, α-galactosidase; N, IS/GLA ratio; K_a, binding constant; ΔG, free energy change; ΔH, enthalpy change; $T\Delta S$, entropy term change; ΔS, entropy change.

imino sugars bind to GLA one to one. The binding constants (K_a) obtained for DGJ and GBS as to GLA were 1.0×10^8 and 3.9×10^6 l mol^{-1}, respectively. These results show that DGJ binds to the enzyme more strongly than GBS. As to changes in free energy, enthalpy and entropy term, the results of the analyses are summarized in Fig. 3. The results revealed that the binding of DGJ to the enzyme molecule was apparently enthalpy-driven. The parameters obtained through SPR biosensor assays were basically in good agreement with those obtained through ITC measurements. The agreement between the SPR biosensor assay data and ITC ones substantiates the monophasic nature of the binding interaction. There was a difference between the entropy change value on the complex formation between GBS and the enzyme obtained on SPR biosensor assaying and that on ITC. Judging from the results, it was thought that the reaction was mainly enthalpy-driven, although there was a possibility that entropy-driven factors were involved in the interaction. SPR biosensor analysis revealed that the equilibrium constants (K_D) for DGJ and GBS were 1.1×10^{-8} and 3.2×10^{-7} mol l^{-1}, respectively. DGJ exhibited a relatively low K_D value and high k_a value, indicating high affinity due to fast complex formation, as shown in Table 3.

Table 3
Rate constants of complex formation and dissociation (k_a and k_d), and equilibrium constants (K_D) for binding between GLA and imino sugars.

	K_D (mol l^{-1})	k_a (l mol^{-1} s^{-1})	k_d (s^{-1})
DGJ	1.1×10^{-8}	1.4×10^{5}	1.5×10^{-3}
GBS	3.2×10^{-7}	5.7×10^{3}	1.8×10^{-3}

Structural models of the complexes of the catalytic domain of GLA with imino sugar bound to its active site

To elucidate the interaction between the imino sugars and the enzyme, we built structural models of representative imino sugars, DGJ and GBS, strongly bound to the active-site pocket of the enzyme protein, as shown in Figs. 4 and 5. Fig. 4 shows the docking structures of the DGJ/GLA and GBS/GLA complexes. DGJ is docked to the active-site pocket (Fig. 4a, left). All the hydroxyl groups and the nitrogen of DGJ take part in the hydrogen bonding with GLA (Figs. 4a, center and 5a). Residues K168, D92, D93 and D170 (nucleophile) are predicted to bind to the hydroxyl groups at O4, O5, O6 and N1 of DGJ through hydrogen bonding, and the hydroxyl group at O3 is predicted to bind to both R227 and D231 (acid/base). All together, there are six hydrogen bonds between GLA and DGJ. It is deduced that these residues contribute to the substrate binding specificity. Residues W47, Y134, and C142 are thought to be involved in the hydrophobic interaction with DGJ (Figs. 4a, right and 5a). The active-site pocket is deduced to be suitable for DGJ as to both space and binding. As in the case of DGJ, GBS is docked to the active-site pocket (Fig. 4b, left). All the hydroxyl groups and the nitrogen of GBS take part in the hydrogen bonding with GLA, like those of DGJ (Figs. 4b, center, and 5b). This binding mode is almost as the same as that in the case of DGJ, and there are also six hydrogen bonds between GLA and GBS. The SO_3 group of GBS lies along the inner wall of the active-site pocket (Fig. 4b, right), which suggests that the side chain of GBS influences the interaction with GLA. The estimated binding energy is −5.55 kcal mol^{-1}, and that of DGJ being −6.09 kcal mol^{-1}.

Discussion

In lysosomal diseases, some of the catalytically active mutant enzyme proteins appear to be misfolded, recognized by the ER's quality control system, and degraded before sorting into lysosomes. Hence, efforts have been made to correct the trafficking defect of catalytically active mutant enzymes using pharmacological chaperones, i.e., DGJ for mutant GLAs responsible for Fabry disease [7–10], NB-DNJ for mutant acid α-glucosidases responsible for Pompe disease [18,19], and NN-DNJ for mutant β-glucosidases responsible for Gaucher disease [20]. However, these chemicals basically inhibit the corresponding enzymes *in vitro* and *in vivo*, and some of these compounds affect the enzymes involving in the first step of glycosylation of glycosphingolipids [21]. So, it is strongly required to develop new derivatives that bind to mutant enzymes and transport them to lysosomes without any inhibitory

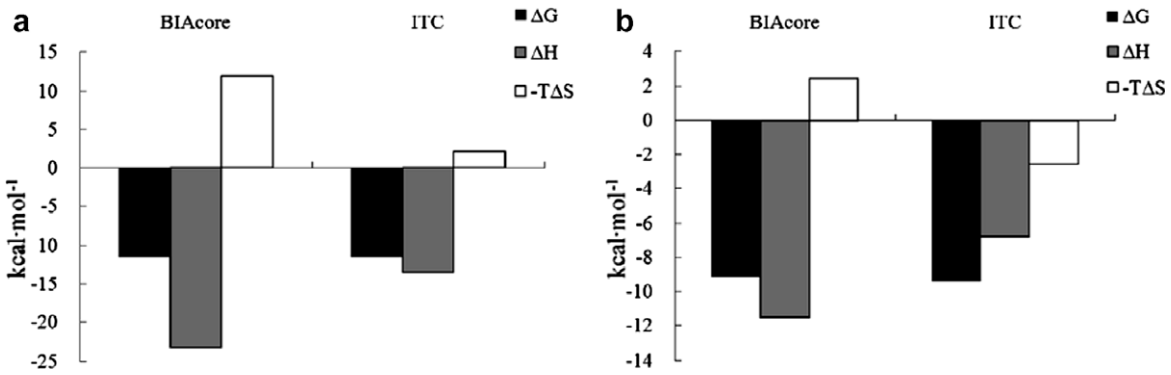

Fig. 3. The contribution of changes in enthalpy and entropy to the change in free energy determined on SPR biosensor assaying (BIAcore) and ITC. ΔG, free energy change; ΔH, enthalpy change; $T\Delta S$, entropy term change. (a) Complex formation of DGJ and GLA, (b) Complex formation of GBS and GLA.

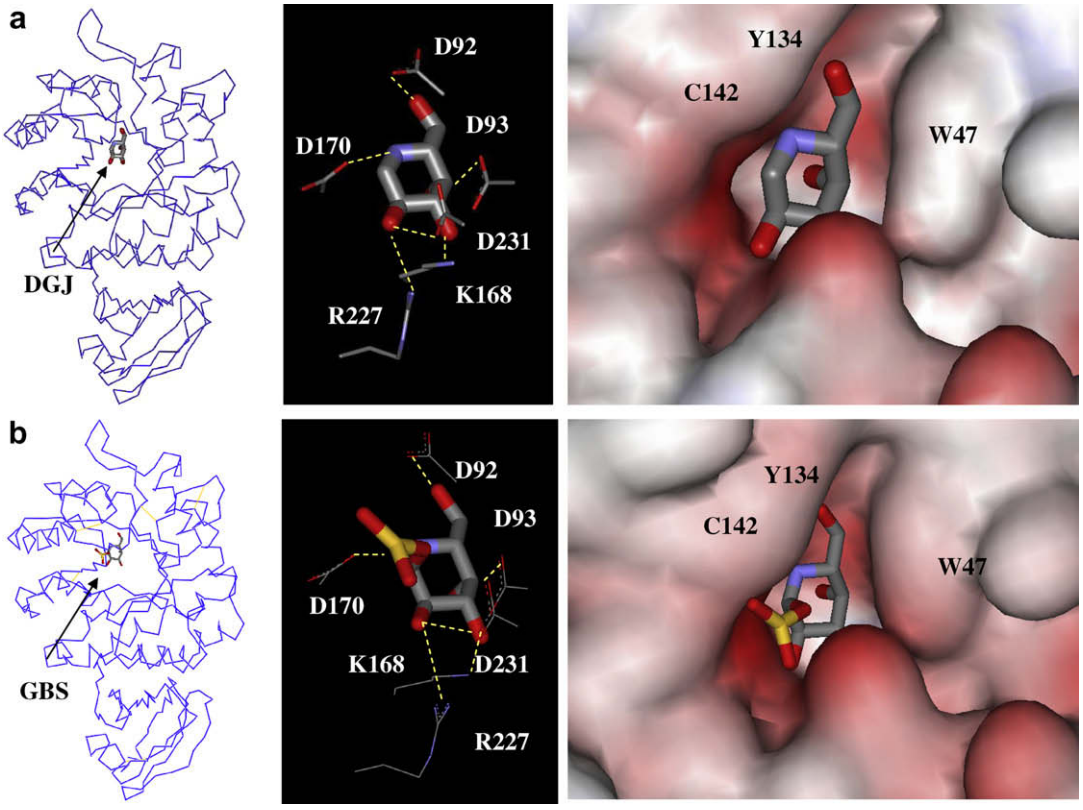

Fig. 4. Docking models of the DGJ/GLA and GBS/GLA complexes. (a) Docking model of the DGJ/GLA complex. Overview (left), residues contributing to the interaction through hydrogen bonding (center), and surface structure of the active-site pocket of GLA and DGJ (right). In (a) (center), yellow lines indicate the hydrogen bonding. (a) (Right) was drawn using WebLab Viewer Lite. (b) Docking model of the GBS/GLA complex. Overview (left), residues contributing to the interaction through hydrogen bonding (center), and surface structure of the active-site pocket of GLA and GBS (right). In (b) (center) yellow lines indicate the hydrogen bonding. (b) (Right) was drawn using WebLab Viewer Lite.

activity toward the enzymes, and for that purpose information on the molecular interactions between the enzymes and chemicals is very important.

In this study, we examined various kinds of imino sugars available and found that GBS exhibited a strong inhibitory effect on human recombinant GLA like DGJ, which had been commonly used as a pharmacological chaperone for mutant GLAs, followed by Altro-DNJ and NB-DGJ. These chemicals resemble each other structurally. However, it is deduced that the hydroxyl groups and the nitrogen of Altro-DNJ and NB-DGJ do not take any part in the hydrogen bonding with GLA, and their estimated binding energies are apparently lower than those of DGJ and GBS (data not shown), suggesting that the binding activities of Altro-DNJ and NB-DGJ are lower than those of DGJ and GBS.

Then, we examined the effects of DGJ and GBS on cultured Fabry fibroblasts having the Q279E mutation. It is known that Q279E is one of the mutations for which DGJ is capable of normalizing intracellular processing [10]. It is thought that Q279E causes a small structural change on the surface of the enzyme molecule [22], and the mutant enzyme protein with Q279E is unstable, especially at neutral pH conditions [15]. This result revealed that the addition of a small amount of GBS to the culture medium of Fabry fibroblasts having Q279E increased the GLA activity as in the case of DGJ, although the effective dose and the degree of the recovery

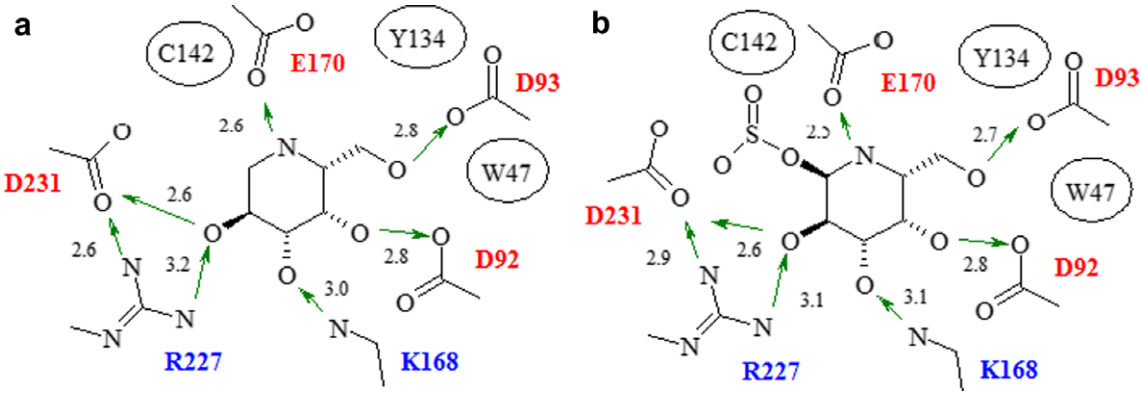

Fig. 5. Schematic representation of the DGJ/GLA (a) and GBS/GLA (b) interactions. Green arrows indicate hydrogen bonding. Red and blue characters indicate acid residues and basic ones, respectively. The illustrations were drawn based on the docking model.

in GLA activity are different from those in the case of DGJ. This suggests that there is a difference in their binding activities as to GLA.

The results of inhibition and thermodynamic analyses revealed that both DGJ and GBS bound to GLA one to one, and that they competitively inhibited the enzyme activity. The binding of DGJ to GLA is enthalpy-driven, suggesting that hydrogen bonding and van der Waals interactions contribute significantly to the complex formation. The results of the structural analysis *in silico* well correlate with those of the thermodynamic examination. In the DGJ/GLA complex model, the DGJ molecule seems to fit into the active-site pocket, and there is no steric hindrance between DGJ and the enzyme. Residues D92, D93, K168, D170, R227, and D231 directly bind to a DGJ molecule through hydrogen bonds. N1 and the hydroxyl group at O3 of DGJ undergo hydrogen bonding with the nucleophile, D170, and the acid/base, D231, respectively. Furthermore, there is a possibility that the protonated nitrogen of DGJ undergoes an electrostatic interaction with the nucleophile, D170. Such an interaction would inhibit the enzyme activity strongly.

As to the GBS/GLA complex formation, the entropy change (ΔS) and entropy term change ($T\Delta S$) values determined on SPR are different from those determined on ITC. This might be due to the fact that SPR reflects the surface-base and ITC the solution phase. However, the free energy change (ΔG) determined on SPR is almost the same as that determined on ITC. The binding mode of GBS with GLA is thought to be basically similar to that of DGJ. However, the results of the thermodynamic examination suggested that there was a possibility that entropy-driven factors were involved in the GBS/GLA complex formation. Judging from the results of the structural analysis, the side chain of GBS was oriented towards the entrance of the active-site pocket. The side chain was half buried in the protein, resulting in a decrease of the hydrophilic surface area and an increase of the entropy of water molecules. Furthermore, GBS is more hydrophilic than DGJ is, and GBS is predicted to exhibit higher solubility and specificity for GLA than DGJ does. Although the affinity constant for GBS is lower than that for DGJ, GBS might be advantageous as a drug in these points. Furthermore, the kinetic study suggested that the GBS/GLA complex formation is slower than the DGJ/GLA one. It is likely that there is room for improvement of GBS as to the rate of complex formation. In the cases of both DGJ and GBS, there is an enough space in the enzyme protein to bind the chemicals in contact with the bottom of the active-site pocket, which is composed of hydrophobic residues, and it would be possible to modify the chemicals to bind to the enzyme strongly but not to exhibit any inhibitory effect on the enzyme, based on the structural and thermodynamic information.

In conclusion, we examined the molecular interaction of GLA and imino sugars including DGJ and GBS. The thermodynamic characteristics are very important in the field of drug discovery [23]. In general, a molecule with high binding enthalpy is thought to be a good lead compound, and the measurement of the thermodynamic characteristics makes the drug discovery process efficient. The thermodynamic study provided us with a lot of information involving the complex formation of GLA with these imino sugars, i.e., the mode of the complex formation and the binding activity of the chemicals. The structural investigation gave us further information to elucidate the mechanisms of complex formation. They should facilitate the improvement of EET for Fabry disease.

Acknowledgments

This work was partly supported by grants from the Japan Society for the Promotion of Science, the High-Tech Research Center Project of the Ministry of Education, Science, Sports and Culture of Japan, the Ministry of Health, Labor and Welfare of Japan, the Japan Science and Technology Agency, and CREST.

References

[1] R.J. Desnick, Y.A. Ioannou, C.M. Eng, Alpha-galactosidase A deficiency: Fabry disease, in: C.R. Scriver, W.A. Sly, A.L. Beaudet, D. Valle (Eds.), The Metabolic and Molecular Bases of Inherited Disease, eighth ed., McGraw-Hill Inc., New York, 2001.
[2] S.C. Garman, D.N. Garboczi, The molecular defect leading to Fabry disease: structure of human alpha-galactosidase, J. Mol. Biol. 337 (2004) 319–335.
[3] M. Spada, S. Pagliardini, M. Yasuda, T. Tukel, G. Thiagarajan, H. Sakuraba, A. Ponzon, R.J. Desnick, High incidence of later-onset Fabry disease revealed by newborn screening, Am. J. Hum. Genet. 79 (2006) 31–40.
[4] R. Schiffmann, G.J. Murray, D. Treco, P. Daniel, M. Sellos-Moura, M. Myers, J.M. Quirk, G.C. Zirzow, M. Borowski, K. Loveday, T. Anderson, F. Gillespie, K.L. Oliver, N.O. Jeffries, E. Doo, T.J. Liang, C. Kreps, K. Gunter, K. Frei, K. Crutchfield, R.F. Selden, R.O. Brady, Infusion of alpha-galactosidase A reduces tissue globotriaosylceramide storage in patients with Fabry disease, Proc. Natl. Acad. Sci. USA 97 (2000) 365–370.
[5] C.M. Eng, M. Banikazemi, R.E. Gordon, M. Goldman, R. Phelps, L. Kim, A. Gass, J. Winston, S. Dikman, J.T. Fallon, S. Grodie, C.B. Stacy, D. Mehta, R. Parson, K. Norton, M. O'Callaghan, R.J. Desnick, A phase 1/2 clinical trial of enzyme replacement in Fabry disease: pharmacokinetic, substrate clearance, and safety studies, Am. J. Hum. Genet. 68 (2001) 711–722.
[6] C.M. Eng, N. Guffon, W.R. Wilcox, D.P. Germain, P. Lee, S. Waldek, L. Caplan, G.E. Linthorst, R.J. Desnick, Safety and efficacy of recombinant human alpha-galactosidase A-replacement therapy in Fabry's disease, N. Engl. J. Med. 345 (2001) 9–16.
[7] J.-Q. Fan, A contradictory treatment for lysosomal storage disorders: inhibitors enhance mutant enzyme activity, Trends Pharmacol. Sci. 24 (2003) 355–360.
[8] G.H.-F. Yam, C. Zuber, J. Roth, A synthetic chaperone corrects the trafficking defect and disease phenotype in a protein misfolding disorder, FASEB J. 19 (2005) 12–18.
[9] G.H.-F. Yam, N. Bosshard, C. Zuber, B. Steinmann, J. Roth, Pharmacological chaperone corrects lysosomal storage in Fabry disease caused by trafficking-incompetent variants, Am. J. Physiol. Cell Physiol. 290 (2006) C1076–C1082.
[10] S. Ishii, H.-H. Chang, K. Kawasaki, K. Yasuda, H.-L. Wu, S.C. Garman, J.-Q. Fan, Mutant alpha-galactosidase A enzymes identified in Fabry disease patients with residual enzyme activity: biochemical characterization and restoration of normal intracellular processing by 1-deoxygalactonojirimycin, Biochem. J. 406 (2007) 285–295.
[11] J.S. Mayes, J.B. Scheerer, R.N. Sifers, M.L. Donaldson, Differential assay for lysosomal alpha-galactosidases in human tissues and its application to Fabry's disease, Clin. Chim. Acta 112 (1981) 247–251.
[12] M. Yoshimizu, Y. Tajima, F. Matsuzawa, S. Aikawa, K. Iwamoto, T. Kobayashi, T. Edmunds, K. Fujishima, D. Tsuji, K. Itoh, M. Ikekita, I. Kawashima, K. Sugawara, N. Ohyanagi, T. Suzuki, T. Togawa, K. Ohno, H. Sakuraba, Binding parameters and thermodynamics of the interaction of imino sugars with a recombinant human acid alpha-glucosidase (alglucosidase alfa): insight into the complex formation mechanism, Clin. Chim. Acta 391 (2008) 68–73.
[13] S. Ishii, H. Sakuraba, Y. Suzuki, Point mutations in the upstream region of the alpha-galactosidase A gene exon 6 in an atypical variant of Fabry disease, Hum. Genet. 89 (1992) 29–32.
[14] T. Okumiya, S. Ishii, T. Takenaka, S. Kamei, H. Sakuraba, Y. Suzuki, Galactose stabilizes various missense mutants of alpha-galactosidase in Fabry disease, Biochem. Biophys. Res. Commun. 214 (1995) 1219–1224.
[15] R. Kase, U. Bierfreund, A. Klein, T. Kolter, K. Utsumi, K. Itoh, K. Sandhoff, H. Sakuraba, Characterization of two alpha-galactosidase mutants (Q279E and R301Q) found in an atypical variant of Fabry disease, Biochem. Biophys. Acta 1501 (2000) 227–235.
[16] K. Bachhawat, C.J. Thomas, M.V. Krishnasastry, M.I. Khan, A. Surolia, On the stringent requirement of mannosyl substitution in mannooligosaccharides for the recognition by garlic (*Allium sativum*) lectin. A surface plasmon resonance study, J. Biol. Chem. 276 (2001) 5541–5546.
[17] C.F. Shuman, M.D. Hämäläinen, U.H. Fanielson, Kinetic and thermodynamic characterization of HIV-1 protease inhibitors, J. Mol. Recognit. 17 (2004) 106–119.
[18] T. Okumiya, M.A. Kroos, L. Van Vliet, H. Takeuchi, A.T. Van der Ploeg, A.J.J. Reuser, Chemical chaperones improve transport and enhance stability of mutant alpha-glucosidases in glycogen storage disease type II, Mol. Genet. Metab. 90 (2007) 49–57.
[19] G. Parenti, A. Zuppaldi, M. Gabriela Pittis, M. Rosaria Tuzzi, I. Annunziata, G. Meroni, C. Porto, F. Donaudy, B. Rossi, M. Rossi, M. Filocamo, A. Donati, B. Bembi, A. Ballabio, G. Aridria, Pharmacological enhancement of mutated alpha-glucosidase activity in fibroblasts from patients with Pompe disease, Mol. Ther. 15 (2007) 508–514.
[20] A.R. Sawker, W.C. Cheng, E. Beutler, C.H. Wong, W.E. Balch, J.W. Kelly, Chemical chaperones increase the cellular activity of N370S beta-glucosidase: a therapeutic strategy for Gaucher disease, Proc. Natl. Acad. Sci. USA 99 (2002) 15428–15433.
[21] F.M. Platt, G.R. Neises, G.B. Karlsson, R.A. Dwek, T.D. Butters, *N*-Butyldeoxygalactonojirimycin inhibits glycolipid biosynthesis but does not affect *N*-linked oligosaccharide processing, J. Biol. Chem. 269 (1994) 27108–27114.
[22] K. Sugawara, K. Ohno, S. Saito, H. Sakuraba, Structural characterization of mutant alpha-galactosidases causing Fabry disease, J. Hum. Genet. 53 (2008) 812–824.
[23] E. Freire, Do enthalpy and entropy distinguish first in class from best in class? Drug Discov. Today 13 (2008) 869–874.