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Molecular mechanism for stabilization of a mutant α-galactosidase A involving M51I amino acid substitution by imino sugars

Takahiro Tsukimura [a], Yasunori Chiba [b,c], Kazuki Ohno [d], Seiji Saito [e], Youichi Tajima [c,f], Hitoshi Sakuraba [a,c,*]

[a] Department of Analytical Biochemistry, Meiji Pharmaceutical University, Kiyose, Tokyo, Japan
[b] Research Center for Medical Glycoscience, National Institute of Advanced Industrial Science and Technology, Tsukuba, Ibaraki, Japan
[c] Department of Clinical Genetics, Meiji Pharmaceutical University, Kiyose, Tokyo, Japan
[d] NPO for the Promotion of Research on Intellectual Property Tokyo, Chiyoda-ku, Tokyo, Japan
[e] Graduate School of Agricultural and Life Science, The University of Tokyo, Bunkyo-ku, Tokyo, Japan
[f] Department of Molecular Medical Research, The Tokyo Metropolitan Institute of Medical Science, Tokyo Metropolitan Organization for Medical Research, Setagaya-ku, Tokyo, Japan

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ABSTRACT

Small molecules including imino sugars are expected to act as chaperones for a mutant α-galactosidase A (GLA), which will be useful for pharmacological chaperone therapy for Fabry disease. However, there is little detailed information about the molecular mechanism. We paid attention to an M51I mutant GLA which had been reported to strongly react to an imino sugar. The predicted structural change caused by this amino acid substitution is very small and located on the surface of the molecule. We produced the mutant enzyme in yeast, and determined its enzymological characteristics. The enzymological parameter values are almost the same as those of the wild-type GLA, although the mutant enzyme is unstable not only under neutral pH conditions but also under acidic ones. Then, we directly examined the effect of imino sugars including 1-deoxygalactonojirimycin and galactostatin bisulfite on the purified mutant enzyme. The imino sugars apparently improved the stability of the mutant enzyme under both neutral and acidic pH conditions. The results of surface plasmon resonance biosensor assaying suggested that the imino sugars retained their binding activity as to the mutant enzyme under both neutral and acidic pH conditions. This information will facilitate improvement of pharmacological chaperone therapy for Fabry disease.

1. Introduction

Fabry disease (MIM 301500) is an X-linked genetic disease caused by deficient activity of α-galactosidase A (GLA; EC 3.2.1.22), one of the lysosomal hydrolases. So far, more than 400 *GLA* mutations have been reported (Human Gene Mutation Database; http://www.hgmd.org) and more than 50% of them are missense. The enzyme deficiency leads to the accumulation of glycolipids, predominantly globotriaosylceramide (Gb3), and elevated plasma globotriaosylsphingosine (Lyso-Gb3), resulting in systemic manifestations including cardiac, renal and cerebrovascular disorders [1,2]. The results of newborn screening revealed that the incidence of Fabry disease is unexpectedly high, and the clinical importance of this disease is increasing more and more [3].

As to therapy for Fabry disease, two different recombinant human GLAs produced by cultured human fibroblasts or Chinese hamster ovary cells have been developed and are available for enzyme replacement therapy [4,5]. But there are some problems, i.e., low uptake of them by the brain, kidneys, and heart, and the very high cost. Recently, pharmacological chaperone therapy with small molecules is being investigated as a potential therapy for various diseases including Fabry disease [6]. GLA is synthesized and folded in the endoplasmic reticulum (ER), exported to the Golgi apparatus and subsequently transported to lysosomes, and the enzyme has a homodimeric structure. However, many GLAs having an amino acid substitution are misfolded and unstable, although they exhibit residual enzyme activity. Such mutant enzyme proteins appear to be recognized by the ER quality control system and degraded before sorting into lysosomes. Pharmacological chaperones are expected to bind to a mutant GLA protein and thereby facilitate its folding process and stabilize it [7–10]. Successful application of such pharmacological chaperone therapy would help to resolve the problems of enzyme replacement therapy, i.e., inexpensive chemical synthesis of small molecules, and their effective incorporation into systemic organs. As such potential pharmacological chaperones for GLA, imino sugars including 1-deoxygalactonojirimycin (DGJ) [11] and galactostatin bisulfite (GBS) [12] are known to increase the enzyme activity in Fabry cells having specific missense mutations; about 50 missense mutations for which imino sugars are effective have been reported [7–18]. Animal experiments revealed that DGJ reduced the tissue Gb3 levels in Fabry mice [19,20], and a clinical trial involving DGJ for pharmacological chaperon therapy is now in progress. But these chemicals are essentially potent inhibitors of GLA. So, a washout period

* Corresponding author at: Department of Analytical Biochemistry, Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose, Tokyo 204-8588, Japan. Fax: +81 42 495 8923.
E-mail address: sakuraba@my-pharm.ac.jp (H. Sakuraba).

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would be required in pharmacological chaperone therapy [16,20], and a chemical is expected to strongly bind to a mutant GLA in the ER and quickly dissociate from that in lysosomes. For further improvement of pharmacological chaperone therapy for Fabry disease, detailed information on the molecular interaction between a small chemical and a mutant GLA is strongly required. However, there have been few reports for this purpose [12,21].

We paid attention to a mutant GLA having an M51I amino acid substitution, which had been reported to strongly react to an imino sugar [3]. In this study, we produced the mutant GLA and examined its enzymological characteristics, the effect of imino sugars on the mutant enzyme, and the molecular interaction between them.

2. Materials and methods

2.1. Structural analysis of the M51I mutant GLA

Structural modeling of the M51I mutant GLA was performed using molecular modeling software TINKER [22–26]. The crystal structure of human GLA (PDB:1R46) [27] was used as a template, and energy minimization was performed. The root mean square gradient value was set at 0.05 kcal/mol. The M51I mutant GLA model was then superimposed on the wild-type GLA structure based on Cα atoms by the least square mean fitting method [28–31]. In this study, we defined that the structure was affected by an amino acid substitution when the position of an atom in a mutant differed from that in the wild-type by more than 0.015 Å, not the 0.15 Å usually used as the cutoff distance in the previous studies [32,33], to detect even a small structural change. To determine the influence of the amino acid substitution geographically and semiquantitatively, coloring of affected atoms in the three-dimensional structure of GLA was performed based on the distance between a wild-type atom and the corresponding M51I mutant one.

The root mean square distance (RMSD) values of all atoms in the M51I mutant GLA were determined according to Weiner's method [34] to predict the degree of the structural change.

The solvent-accessible surface area (ASA) value of the M51 in the wild-type GLA was calculated using ACCESS [35] to predict the position of the substituted amino acid residue in the GLA molecule.

2.2. Construction of an expression plasmid

pCXN$_2$Gal [36] including the human GLA cDNA sequence was used as a template. The coding region for the GLA was amplified by PCR and introduced into the pOMEA1 vector [37]. The resultant vector was named pOMEA1-GLA. To generate an M51I mutant GLA, site-directed mutagenesis was performed with a Gene Tailor site-directed mutagenesis kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. pOMEA1-GLA was used as a template with the following primers, M51I sense, 5′-GGCTGCACTGGGAGCGCTTC*ATA*TG-CAACCTTGA-3′; and M51I antisense, 5′-GAAGCGCTCCCAGTGCAGC-CAGCCCATGGT-3′, the italicized nucleotides being the mutated site. The mutation was confirmed by nucleotide sequencing analysis.

Then, the coding region was amplified by PCR and introduced into the pEE14.4 vector (Lonza Biologics, Allendale, NJ).

2.3. Effects of imino sugars on the M51I mutant GLA expressed in COS-7 cells

COS-7 cells were cultured in Dulbeco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY) containing 10% fetal calf serum and antibiotics at 37 °C in an incubator containing 5% CO_2. Transient expression of the M51I mutant GLA was carried out using Lipofectamine LTX (Invitrogen) according to the manufacturer's instructions. After transfection, the cells expressing the M51I mutant GLA were cultured for 5 h in the same medium, which was then replaced by the medium containing either 20 or 100 μM DGJ or GBS (Toronto Research Biochemicals, North York, Canada). After 72 h culture, the cells were harvested, washed three times with phosphate-buffered saline (PBS), and then the cell homogenates were used for the GLA assay, protein determination and Western blotting.

2.4. GLA assaying and protein determination

GLA activity was fluorometrically measured with 4-methylumbelliferyl (MU)-α-D-galactopyranoside (Calbiochem, LaJolla, CA) as a substrate in the presence of *N*-acetyl-D-galactosamine (Sigma-Aldrich, St. Louis, MO), a specific inhibitor of *N*-acetylgalactosaminidase in 0.1 M citrate-phosphate buffer [pH 4.6], as described previously [38]. The reaction mixture (total volume: 50 μl) was incubated at 37 °C for 10 min. Then, the reaction was stopped by adding 0.2 M glycine buffer [pH 10.7], and the released MU was measured with a Wallac 1420 ARVO MX Multilabel Counter (Perkin-Elmer, Waltham, MA) at excitation and emission wavelengths of 355 nm and 460 nm, respectively. Protein determination was performed with a Micro BCA Protein Assay Kit (Thermo Scientific, Rockford, IL), using bovine serum albumin as a standard.

2.5. Western blotting

The homogenate (0.5 μg protein) of the COS-7 cells expressing the M51I mutant GLA was separated on a Tris-glycine polyacrylamide gel (Multigel II Mini 8/16; Cosumo-bio, Tokyo, Japan), and then electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Co., Bedford, MA). Immunoblotting analysis was performed using rabbit polyclonal antibodies to human GLA [39] and peroxidase-conjugated donkey anti-rabbit secondary antibodies (GE Healthcare, Piscataway, NJ). Finally, the blot was visualized with an ECL plus Western Blotting Detection System (GE Healthcare) as a chemiluminescent substrate and measured using ImageQuant (GE Healthcare). Actin was analyzed as a control using mouse monoclonal antibody to actin (Millipore Co.) and peroxidase-conjugated donkey anti-mouse secondary antibodies (GE Healthcare).

2.6. Production and purification of GLAs

Methylotrophic yeast *Ogataea minuta* TK5-3/Ura cells (provided by Kyowa Hakko Kirin Co., Tokyo, Japan) were transfected with the pOMEA1-GLA vector as described previously [40]. The transfected *O. minuta* cells were precultured in 5 ml of YPD medium (1% yeast extract, 2% peptone, and 2% glucose) and then transferred to 250 ml of BMGY medium (2% peptone, 1% yeast extract, 1.34% yeast nitrogen base without amino acids, 0.5% glycerol and 0.1 M potassium phosphate [pH 6.0]). When glycerol had been completely consumed, 1% methanol was added every 6 h as a carbon source and inducer. Methanol induction was performed at 28 °C and continued until the GLA activity in the culture broth reached saturation. After the induction, the supernatant of the cultured medium was concentrated ~30-fold with an ultrafiltration system, Amicon 8400, equipped with a 30-kDa cutoff YM30 ultrafiltration membrane (Millipore Co.) at 4 °C. Then, ammonium sulfate was added slowly to the supernatant to a final concentration of 55%. The precipitate was re-dissolved in 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer [pH 6.0], and then dialyzed against the same buffer. All column materials used in this experiment were purchased from GE Healthcare. A sample was applied to a HiLoad Q 26/10 Sepharose HP column equilibrated with the same buffer. After washing the column, GLA was eluted with a 0–0.5 M NaCl gradient in the same buffer. Fractions containing GLA activity were dialyzed against 20 mM MES buffer [pH 6.0] containing 300 mM ammonium sulfate. A sample was applied to a HiLoad Phenyl 26/10 Sepharose HP column equilibrated with the same buffer. After washing the column, GLA was eluted with 20 mM MES buffer [pH 6.0]. Fractions containing GLA activity were dialyzed against 20 mM MES buffer [pH 6.0] containing 150 mM NaCl,

and then concentrated with a Centriprep YM30 (Millipore Co.). A sample was then applied to a HiLoad 16/60 Superdex 200 pg column, and the fractions containing GLA activity were pooled.

2.7. Enzymatic deglycosylation of the GLAs

An enzymatic deglycosylation kit was purchased from Prozyme (San Lendodro, CA). Four micrograms of aliquots of the purified GLAs were incubated with 1 mU of *N*-glycanase F for 14 h at 37 °C in 10 μl of incubation buffer (supplied with the kit). The enzymatic digestion was terminated by the addition of 10 μl of 2× sodium dodecylsulfate (SDS) sample buffer (125 mM Tris–HCl [pH 6.8], 4% SDS, 20% glycerol, and 5% β-mercaptoethanol) at 100 °C for 5 min, and then 20 μl aliquots containing 4 μg of the purified GLAs were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). They were separated on a Tris–glycine polyacrylamide gel, and then stained with Coomassie brilliant blue R.

2.8. Kinetic and inhibition examination of the purified GLAs

A kinetic experiment on the purified GLA samples was performed using various concentrations (1.0–8.0 mM) of MU-α-D-galactopyranoside as a substrate to determine the maximum velocity (*Vmax*) and Michaelis constant (*Km*) values.

For inhibition assays, DGJ and GBS were added at various concentrations (DGJ, 0, 50, 100, and 200 nM; and GBS, 0, 100, 200, and 400 nM) to the assay mixture, and then the GLA assays were performed. The inhibitory constant (*Ki*) values were calculated from plots of slope versus imino sugar concentration.

2.9. Effects of imino sugars on the stability of the GLAs

To compare the stability of the wild-type GLA and that of the M51I mutant under various pH conditions in a buffer, 1 μg/ml GLA was incubated in either 10 mM citrate-phosphate buffer [pH 4.0–6.5] or 10 mM sodium phosphate buffer [pH 6.75–8.0] at 37 °C for 1 h. After incubation, GLA activity was assayed immediately under pH 4.6 condition.

To assess the effects of imino sugars on the stability of the wild-type and M51I mutant GLAs, various concentrations (DGJ, 10, 100 nM and 1.0 μM; and GBS, 0.10, 1.0, and 10 μM) of the imino sugars were added to 1.0 μg/ml enzyme in 10 mM sodium phosphate buffer [pH 7.5], followed by incubation at 37 °C for various times (0, 10, 30, and 60 min).

Furthermore, the effects of the imino sugars on the M51I mutant GLA under various pH conditions were examined. The imino sugars (DGJ, 100 nM; and GBS 1.0 μM) were added to 1.0 μg/ml of the M51I mutant GLA in either 10 mM citrate-phosphate buffer [pH 4.0–6.5] or 10 mM sodium phosphate buffer [pH 7.0–8.0], followed by incubation at 37 °C for 1 h. After the incubation, GLA activity was assayed immediately under pH 4.6 condition. The stability of the GLA was expressed as the ratio (percent) of the enzyme activity at a particular incubation time point to the value at the time zero.

2.10. Surface plasmon resonance (SPR) biosensor assays

To examine the complex formation between the imino sugars and the GLAs, SPR biosensor assays were performed using a BIAcore X100 (GE Healthcare) biosensor system. GLA (60 μg/ml in 10 mM acetate buffer [pH 5.0]) was coupled to a research grade CM5 chip at a flow rate of 10 μl/min 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. Measurements were performed using 50 mM sodium acetate buffer [pH 5.0] containing 150 mM NaCl and 50 mM sodium phosphate buffer [pH 7.0] containing 150 mM NaCl. For the analysis, the solution of the ligands was passed over the chip (DGJ, 31, 63, 125, and 250 nM [pH 5.0 and 7.0]; and GBS, 0.13, 0.25, 0.50, and 1.0 μM [pH 5.0], and 0.31, 0.63, 1.3, and 2.5 μM [pH7.0]) at a flow rate of 30 μl/min at 28 °C, as described previously [12].

2.11. Statistical analyses

Data are expressed as means ± standard deviation (SD). We performed statistical analyses with a Student's *t*-test. Values were considered statistically significant at $P<0.05$.

3. Result

3.1. Predicted structural change in the M51I mutant GLA

We built a structural model of the M51I mutant GLA and calculated the number of atoms affected by the amino acid substitution. Here, we used 0.015 Å as the cutoff distance to detect small structural changes. The numbers of affected atoms in the main and side chains were calculated to be 23 and 45, respectively, under the conditions used. Coloring of the atoms affected by the amino acid substitution was shown in Fig. 1. The RMSD value was 0.007 Å and the ASA value is 115 $Å^2$. These results indicate that the structural change in GLA due to the M51I amino acid substitution is very small and is localized on the molecular surface far from the active site.

3.2. Effects of imino sugars on the M51I mutant GLA expressed in COS-7 cells

We constructed an expression vector for the M51I mutant GLA, introduced it into COS-7 cells and then examined the effects of the imino sugars on the transiently expressed M51I mutant GLA. The results are summarized in Fig. 2. The cells were cultured in medium containing DGJ and GBS. Then the cells were washed and the enzyme activity and the amount of the M51I mutant GLA protein in cells were measured. They apparently increased when the imino sugars were added to the culture medium at the concentration of 20 μM. On the other hand, the enzyme activity decreased when the concentration of the imino sugars was increased to 100 μM, although the amount of protein remained the

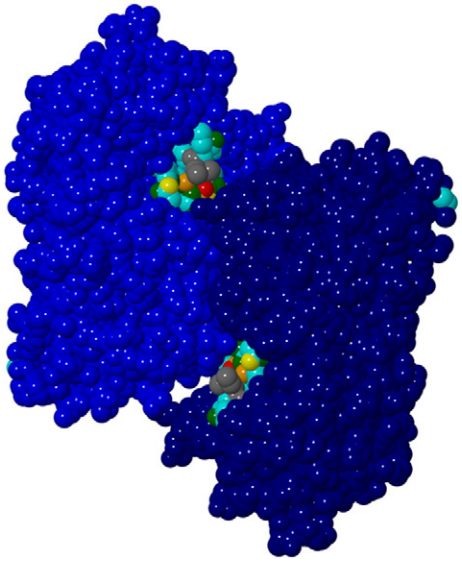

Fig. 1. Predicted structural change in the M51I mutant GLA. The GLA dimer is presented as a ball model. Each atom is colored according to the distance between the atom in the M51I GLA structure and the corresponding atom in the wild-type one. The colors of the atoms show the distances as follows: blue < 0.015 Å, 0.015 Å ≤ cyan < 0.030 Å, 0.030 Å ≤ green < 0.045 Å, 0.045 Å ≤ yellow < 0.060 Å, 0.060 Å ≤ orange < 0.075 Å, and red ≥ 0.075 Å.

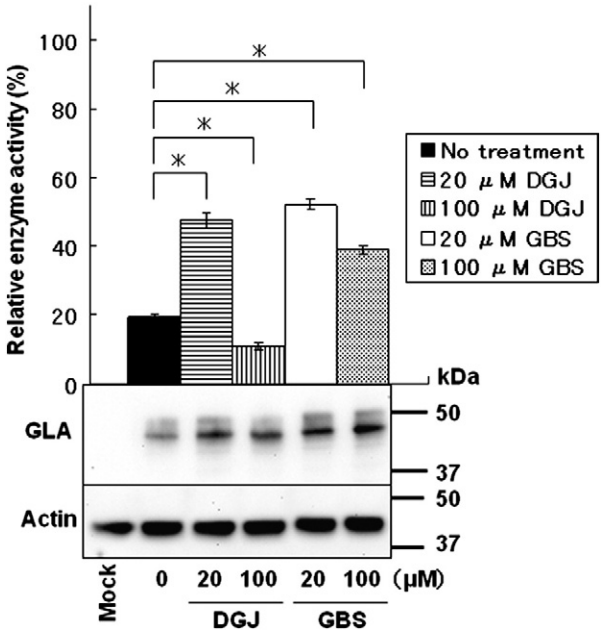

Fig. 2. Effects of imino sugars on the M51I mutant GLA expressed in COS-7 cells. COS-7 cells were transfected with the expression vector including the M51I mutant GLA using Lipofectamine LTX Plus. The cells expressing the M51I mutant GLA were cultured in medium containing either 20 or 100 μM imino sugar (DGJ or GBS). After 72 h culture, the cells were harvested, washed, and then GLA assaying and Western blot analysis were performed. The results of GLA assaying (top) and Western blotting (middle), and those of Western blotting for actin (bottom) were shown. The relative enzyme activity is exhibited as a percentage of the wild-type GLA. Values are expressed as means ± SD ($n = 3$). *$P < 0.05$.

same. This suggests that the imino sugars act as both pharmacological chaperones and inhibitors for the M51I mutant GLA.

3.3. Production and purification of GLAs

To produce a sufficient amount of GLAs, the genes encoding the wild-type and M51I mutant GLAs were individually introduced into the *O. minuta* strain and cells secreting the GLAs were obtained. The productivity was estimated to be approximately 1.5 and 0.6 mg/l for the wild-type GLA and M51I mutant, respectively.

Then, the GLAs were purified from the medium by means of column chromatography. The purification degree was approximately 200-fold, and the recovery of the GLAs was 40% (Table 1). The purified GLAs were detected as three or more broad bands on SDS-PAGE, but they gave one band after deglycosylation, suggesting that they have heterogeneous sugar chains (Fig. 3).

3.4. Enzymological properties of the GLAs

The purified GLAs were enzymologically characterized (Table 2). The *Vmax* values of the wild-type and M51I mutant GLAs produced in *O. minuta* were 3.3 and 2.6 mmol/h/mg, respectively. The *Km* values of the wild-type and M51I mutant GLAs were 4.5 and 4.0 mM, respectively. The results suggest that there is no large difference in the enzyme activity or the substrate affinity between them.

Imino sugars were examined as to their inhibitory action against the GLAs and their *Ki* values were determined (Table 2). DGJ exhibited stronger inhibition of the wild-type and M51I mutant GLAs than GBS. There was no great difference in inhibition by imino sugars between the wild-type and M51I mutant GLAs. They competitively inhibited the enzymes judging from a Lineweaver–Burk plot (data not shown).

The stability of the wild-type and M51I mutant GLAs under various pH conditions was examined (Fig. 4). The results revealed that the stability of the M51I mutant GLA was lower than that of the wild-type GLA under both neutral (pH 7.0–8.0) and acidic (pH 4.0–4.5) pH conditions.

Table 1
Purification of the wild-type GLA (A) and M51I mutant GLA (B) from the culture medium of *O. minuta* cells.

Sample	Total protein (mg)	Total activity (mmol/h)	Specific activity (mmol/h/mg)	Relative purity	Yield (%)
A.					
Concentrated supernatant	1.5×10^{3}	14	1.1×10^{-2}	1	100
Dialyzed 55% $(NH_4)_2SO_4$ fraction	3.6×10^{2}	14	3.9×10^{-2}	4	98
Q-Sepharose	82	13	1.5×10^{-1}	14	89
Phenyl-Sepharose	4.8	8.8	1.8	1.6×10^{2}	62
Superdex 200 pg	3.2	6.1	1.9	1.7×10^{2}	43
B.					
Concentrated supernatant	1.4×10^{3}	7.4	5.4×10^{-3}	1	100
Dialyzed 55% $(NH_4)_2SO_4$ fraction	3.0×10^{2}	5.9	2.0×10^{-2}	4	80
Q-Sepharose	83	5.3	6.4×10^{-2}	12	72
Phenyl-Sepharose	4.8	4.9	1.0	1.9×10^{2}	67
Superdex 200 pg	2.4	3.3	1.4	2.6×10^{2}	44

3.5. Effects of imino sugars on the stability of the GLAs

To assess the effects of the imino sugars on the stability of the GLAs at pH 7.5, various concentrations of DGJ and GBS were added to the enzymes in buffers, and then the changes in the enzyme activity were examined (Fig. 5A). The stability of the M51I mutant GLA was apparently improved dose dependently by the addition of DGJ (10 nM to 1.0 μM) and GBS (1.0–10 μM). As to the wild-type GLA, improvement in stability was found when DGJ was added at a high concentration, 1.0 μM.

Then the effects of imino sugars on the stability of the M51I mutant GLA under various pH conditions were examined, the concentrations of DGJ and GBS being 100 nM and 1.0 μM, respectively (Fig. 5B). Both DGJ and GBS improved the stability of the M51I mutant GLA under neutral (pH 7.0, 7.5, and 8.0) and acidic (pH 4.0 and 4.5) pH conditions, and the effect was stronger in the case of DGJ than that of GBS.

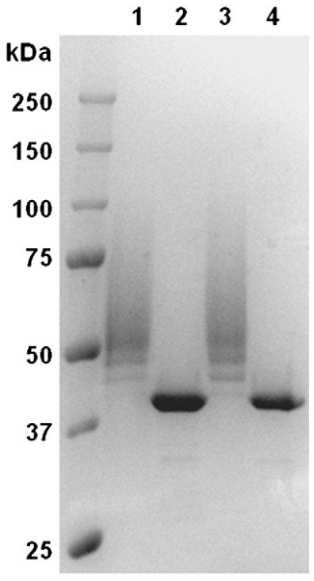

Fig. 3. Coomassie brilliant blue R staining of the GLAs on SDS-PAGE. The wild-type GLA (lanes 1 and 2), and M51I mutant GLA (lanes 3 and 4). Before the electrophoresis, the enzyme was treated (lanes 2 and 4) or not treated (lanes 1 and 3) with *N*-glycanase F. The left-side lane contains molecular standards.

Table 2
Enzymological parameters of the wild-type and M51I mutant GLAs, and inhibitory constant values of the imino sugars.

	Specific activity (mmol/h/mg)	*Vmax* (mmol/h/mg)	*Km* (mM)	*Ki* (nM)	
				DGJ	GBS
Wild-type	1.9	3.3	4.5	38 ± 4	95 ± 4
M51I	1.4	2.6	4.0	47 ± 2	122 ± 1

The enzyme activity of GLA was fluorometrically determined with MU-α-D-galactopyranoside as a substrate. The *Ki* values are expressed as means ± SD ($n = 3$).

3.6. Binding kinetics parameters of the imino sugar and GLA complex formation

The molecular interaction of the imino sugars with the GLAs was examined by means of SPR biosensor assays. The results are summarized in Table 3. DGJ exhibited lower equilibrium constant (K_D) and higher rate constant of complex formation (k_a) values as to the GLAs than GBS under both neutral and acidic pH conditions. These results show that DGJ binds to the GLAs more strongly than GBS, which results from fast complex formation.

There was no large difference in the complex formation with the imino sugars between the wild-type and M51I mutant GLAs. The rate constant of complex dissociation (k_d) value at pH 5.0 was almost the same as that at pH 7.0 for both DGJ and GBS, indicating that there was no difference in dissociation of the imino sugars between under neutral and acidic pH conditions.

4. Discussion

The majority of responsible mutations for Fabry disease are missense ones and they result in the biosynthesis of mutant GLAs with single amino acid substitutions. These mutant GLAs tend to be unstable and degraded in the neutral pH environment of the ER. Imino sugars are expected to act as chaperones to overcome the impaired trafficking of misfolded GLAs, and it has been predicted that they bind to mutant GLAs at neutral pH, such as in the ER, increase their stability, and then dissociate from the enzyme proteins at acidic pH, such as in lysosomes [9–11]. However, direct proof has not been obtained. Although there have been many reports that the addition of an imino sugar to the culture medium increases the GLA activity in cells expressing Fabry mutant GLA proteins with some amino acid substitutions [7–20], there have been few on the molecular interaction of a mutant GLA molecule and an imino sugar.

We paid attention to the M51I mutant GLA. Imino sugars including DGJ and GBS apparently increased the enzyme activity and the amount of the mutant GLA protein in COS-7 cells transfected with an expression vector. The results suggested that the imino sugars improved the stability of the catalytically active enzyme expressed in cells, although excessive administration of the imino sugars reversely inhibited the enzyme activity. Then, we produced a sufficient amount of the M51I mutant GLA protein in yeast cells. A purification study followed by biochemical characterization revealed that the M51I mutant GLA had heterogeneous *N*-type sugar chains and almost the same enzymological parameters as in the case of the wild-type GLA. The results of structural analysis revealed that the change in the M51I mutant GLA is very small and located in a restricted region far from the active site pocket, indicating that it does not affect the active site and that it is well correlated with the biochemical data. Even such a small structural change on the surface of the molecule probably causes misfolding of the protein and decreases its stability. In this study, the stability of the mutant enzyme was examined under various pH conditions, and the results revealed that the M51I mutant GLA was unstable not only under neutral pH conditions but also at acidic ones.

Recently, Lieberman et al. [21] investigated the molecular interaction between DGJ and the wild-type GLA by means of differential scanning calorimetry. They reported that GLA was more stable at lysosomal pH with and without DGJ and that the thermodynamic parameters indicate two-state, van't Hoff unfolding in the absence of DGJ, under neutral and lysosomal pH conditions, and non-two-state unfolding in the presence of DGJ. Furthermore, they compared the structures of the wild-type GLA and the wild-type GLA/DGJ complex by means of X-ray crystallography and reported that DGJ-dependent conformational changes in GLA were not seen. Sugawara et al. [12] reported that the binding of DGJ to the wild-type GLA was enthalpy-driven and that of GBS was mainly enthalpy-driven, but there was a possibility that entropy-driven factors were also involved in the binding. These reports involving the interaction between the wild-type GLA and imino sugars provide us with useful information for considering molecular mechanism for stabilization of a mutant GLA by imino sugars.

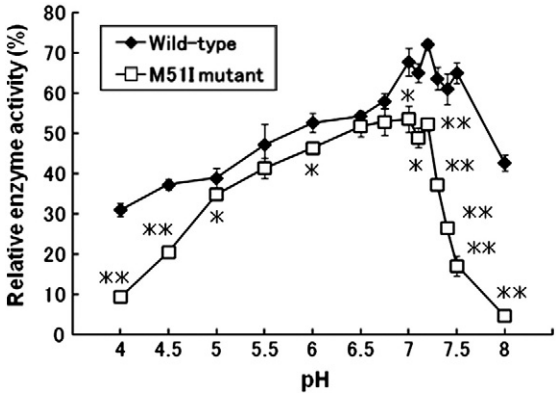

Fig. 4. Stability of the wild-type and M51I mutant GLAs under various pH conditions. The purified wild-type and M51I mutant GLAs were incubated under the indicated pH conditions in a buffer at 37 °C for 1 h. Then, enzyme assaying was performed under pH 4.6 condition. The relative enzyme activity is shown as a percentage of the value at the time zero. Values are expressed as means ± SD ($n = 3$). $^{*}P<0.05$, $^{**}P<0.001$.

On the basis of the information, we examined the effect of imino sugars on the stability of the M51I mutant GLA. The results revealed that they improved the stability of the mutant enzyme under both neutral and acidic pH conditions at relatively low concentrations. SPR biosensor assaying revealed that DGJ bound to the mutant enzyme more strongly than GBS. There were at least no overt differences in the binding-kinetics parameter values of DGJ and the mutant GLA complex formation between neutral and acidic pH conditions, suggesting that DGJ retains its binding ability as to the mutant enzyme also in lysosomes. Hence, accumulated substrates including Gb3 should compete with imino sugar as to the binding with the mutant GLA and probably play a role in dissociation of the imino sugar and the mutant enzyme in lysosomes. DGJ had been thought to bind to the mutant enzyme in the ER, where the pH is neutral, and be released from the enzyme in lysosomes, where the pH is acidic [9–11]. However, our study revealed that the k_d value of the mutant GLA/DGJ complex at pH 7.0 is almost the same as that at pH 5.0. This indicates that there is still room for improvement, i.e., modification of the chemical for its quick dissociation from a mutant GLA at acidic pH conditions.

In conclusion, we examined the molecular interaction of imino sugars and a mutant GLA. The results provided us with a lot of information on the mechanism of stabilization of the mutant GLA by imino sugars. They should facilitate the improvement of pharmacological chaperone therapy for Fabry disease.

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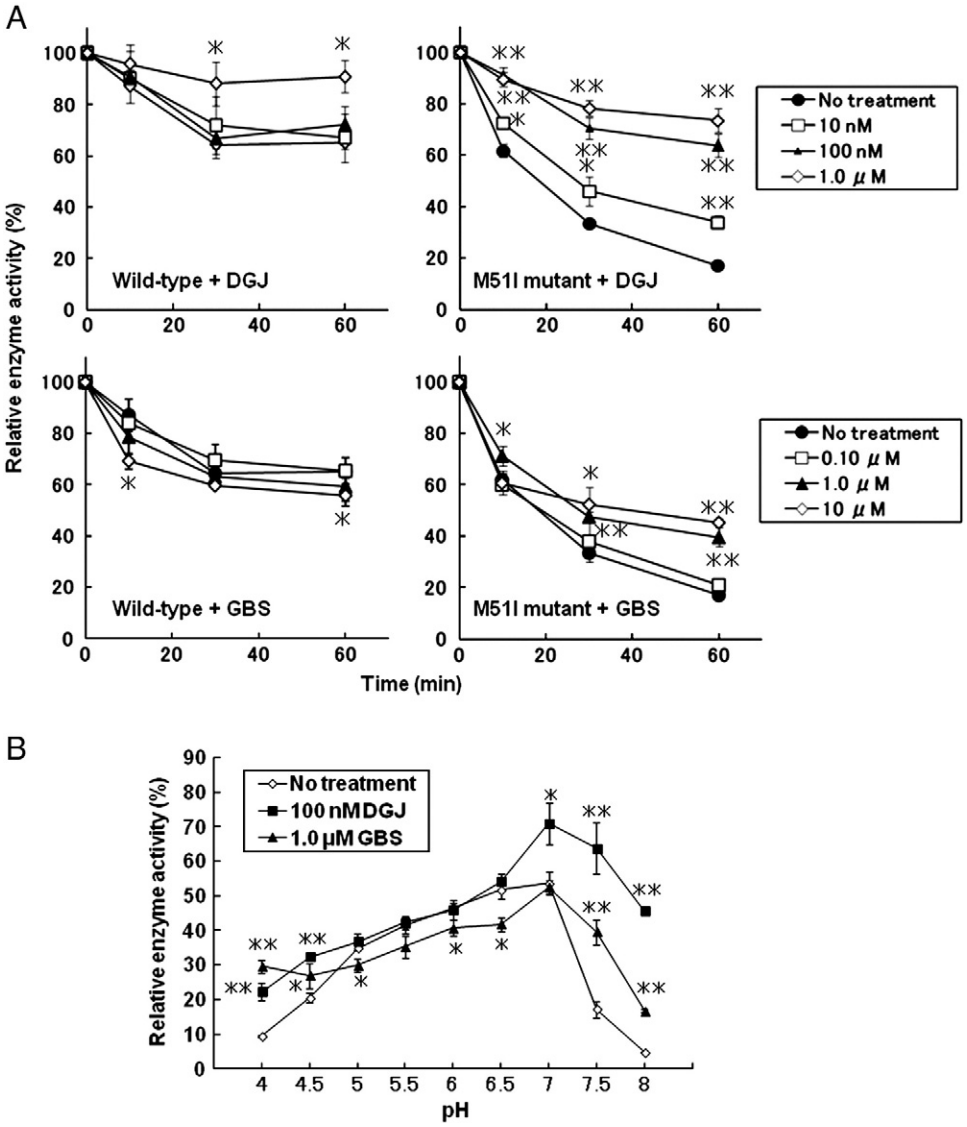

Fig. 5. Effects of imino sugars on the stability of the purified M51I mutant GLA in a buffer. (A) Effects of DGJ and GBS on the stability of the wild-type and M51I mutant GLAs under the pH 7.5 condition in a buffer. The purified wild-type and M51I mutant GLAs were incubated in 10 mM phosphate buffer [pH 7.5] with or without DGJ and GBS at 37 °C for the indicated times. (B) Effects of DGJ and GBS on the stability of the M51I mutant GLA under the indicated pH conditions in a buffer. The purified M51I mutant GLA was incubated under the indicated pH conditions in a buffer with 100 nM DGJ or 1.0 μM GBS at 37 °C for 1 h. Then, enzyme assaying was performed under pH 4.6 condition. The relative enzyme activity is shown as a percentage of the value at the time zero. Values are expressed as means ± SD ($n = 3$). $^{*}P<0.05$, $^{**}P<0.001$.

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Table 3
Rate constants of complex formation and dissociation (k_a and k_d) and equilibrium constants (K_D) for binding between the imino sugars and the GLAs under the pH 7.0 and 5.0 conditions.

		pH 7.0			pH 5.0		
		K_D (mol/l)	k_a (l/mol·s)	k_d (1/s)	K_D (mol/l)	k_a (l/mol·s)	k_d (1/s)
DGJ	Wild-type	3.7×10^{-9}	1.5×10^{5}	5.5×10^{-4}	6.0×10^{-9}	1.2×10^{5}	7.2×10^{-4}
	M51I	3.1×10^{-9}	7.3×10^{4}	2.7×10^{-4}	6.9×10^{-9}	7.4×10^{4}	5.1×10^{-4}
GBS	Wild-type	1.9×10^{-7}	7.0×10^{3}	1.3×10^{-3}	3.5×10^{-8}	3.6×10^{4}	1.3×10^{-3}
	M51I	1.2×10^{-7}	4.6×10^{3}	5.5×10^{-4}	3.8×10^{-8}	2.0×10^{4}	7.5×10^{-4}

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