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Brief Communication

The Molecular Basis of Pharmacological Chaperoning in Human α-Galactosidase

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SUMMARY

Fabry disease patients show a deficiency in the activity of the lysosomal enzyme α-galactosidase (α-GAL or α-Gal A). One proposed treatment for Fabry disease is pharmacological chaperone therapy, where a small molecule stabilizes the α-GAL protein, leading to increased enzymatic activity. Using enzyme kinetics, tryptophan fluorescence, circular dichroism, and proteolysis assays, we show that the pharmacological chaperones 1-deoxygalactonojirimycin (DGJ) and galactose stabilize the human α-GAL glycoprotein. Crystal structures of complexes of α-GAL and chaperones explain the molecular basis for the higher potency of DGJ over galactose. Using site-directed mutagenesis, we show the higher potency of DGJ results from an ionic interaction with D170. We propose that protonation of D170 in acidic conditions leads to weaker binding of DGJ. The results establish a biochemical basis for pharmacological chaperone therapy applicable to other protein misfolding diseases.

INTRODUCTION

α-Galactosidase (α-GAL, also known as α-galactosidase A or α-GAL A; Enzyme Commission number 3.2.1.22) is a lysosomal glycosidase that breaks down complex macromolecules for cellular reuse. α-GAL catalyzes the hydrolysis of terminal α-linked galactosides from macromolecules. In humans, deficiency of the α-GAL enzyme causes Fabry disease, a lysosomal storage disease characterized by the progressive accumulation of metabolites in the cells, leading to tissue damage and eventual organ failure (Brady et al., 1967; Desnick et al., 2001). Many Fabry disease-causing mutations have been identified in the *GLA* gene encoding the α-GAL protein (Human Gene Mutation Database, http://www.hgmd.org), most of which disrupt the hydrophobic core of the protein, presumably leading to protein misfolding and degradation in the endoplasmic reticulum (ER) (Eng and Desnick, 1994; Fan et al., 1999; Garman and Garboczi, 2002, 2004; Okumiya et al., 1995; Romeo et al., 1975). Thus, Fabry disease is primarily a protein misfolding disease.

The only currently approved treatment for Fabry disease is enzyme replacement therapy (ERT), where recombinant enzyme is intravenously administered into patients to restore the missing enzymatic function. ERT has demonstrated reduction of accumulated substrate in tissues, leading to clinical improvement of Fabry disease patients (Eng et al., 2001; Schiffmann et al., 2001), and has been proposed for many inherited metabolic diseases (Beutler, 2006).

An alternative treatment, pharmacological chaperone (PC) therapy, has been proposed for Fabry disease and other protein misfolding diseases (Fan and Ishii, 2007; Parenti, 2009; Sawkar et al., 2005; Suzuki et al., 2009; Tan et al., 1991). In contrast to using nonspecific small molecules for "chemical chaperone therapy," PC therapy for Fabry disease uses an active-site-specific chaperone, such as the catalytic product galactose (Frustaci et al., 2001), or a product analog, such as the imino sugar 1-deoxygalactonojirimycin (DGJ, currently in phase III clinical trials) (Asano et al., 2000). In PC therapy, the small molecule is hypothesized to stabilize the folded enzyme, shifting the folding equilibrium toward properly folded protein, and reducing removal of the polypeptide through ER-associated degradation (ERAD) (Cohen and Kelly, 2003; Fan et al., 1999; Yam et al., 2006; Yam et al., 2005). PCs such as DGJ and galactose are promising clinical candidates, but their biochemical mechanism is not well understood; they have been proposed to accelerate the folding of their target, to slow the unfolding of the target, to stabilize the target, to allow for proper folding, to promote posttranslational modification, and/or to allow binding of a partner to the target (Fan et al., 1998; Lieberman et al., 2009). Additionally, how competitive enzymatic inhibition leads to increased activity remains unresolved. Because of their potential for treating a wide range of protein misfolding diseases (Cohen and Kelly, 2003), PCs have attracted intense clinical attention.

In this study, we examined the biochemical and biophysical basis for PC binding to human α-GAL. We showed via biochemical assays that DGJ binds to and stabilizes α-GAL with higher potency than galactose. We investigated the effect of pH on the binding affinities of DGJ and galactose and showed that the chaperones stabilize α-GAL better at near-neutral pH than at acidic pH. Crystal structures of α-GAL in complex with the PCs DGJ and galactose revealed a key ionic interaction critical for the increased potency of DGJ. Finally, we performed biochemical studies on a D170A variant of α-GAL, unambiguously identifying the atomic interaction responsible for the increased potency of DGJ over galactose.

RESULTS AND DISCUSSION

Binding of Pharmacological Chaperones

To measure binding of the PCs DGJ and galactose, we examined the enzymatic activity of α-GAL in the presence of the chaperones. Both DGJ and galactose act as competitive inhibitors of α-GAL. We determined the K_i for DGJ to be 39 nM and for galactose to be 16 mM (Figure S1; Table S1 available online). The enzymatic assays showed that DGJ is 400,000-fold more potent than galactose at inhibiting α-GAL, corresponding to 7.6 kcal/mol of additional binding energy, a remarkable difference for molecules that differ in only two functional groups.

Resistance to Unfolding Monitored by Trp Fluorescence

To measure the unfolding rate of α-GAL in 7.5 M urea, we used intrinsic tryptophan fluorescence. The fluorescence signal of α-GAL shows a decrease in fluorescence intensity and a red shift in λ_{max} from 335 nm to 350 nm as the protein denatures. In the absence of chaperone, α-GAL denatures with a $t_{½}$ of 2.2 hr at pH 6.5 and 1.3 hr at pH 4.5 (Figures 1 and S2), indicating that α-GAL chemically denatures slightly faster at a lower pH.

Next, to test the effect of DGJ on the unfolding rate, we repeated the fluorescence assay after preincubation with DGJ. The rate of unfolding of α-GAL is slowed considerably by the addition of PCs, particularly at pH 6.5. At pH 6.5, the addition of 50 μM DGJ slows the unfolding of α-GAL to a $t_{½}$ greater than 24 hr, with little change in the fluorescence spectrum over 24 hr. At pH 4.5, the addition of 50 μM DGJ decreases the rate of unfolding of α-GAL from a $t_{½}$ of 1.3 hr to 6.5 hr.

Third, to test the effect of galactose, we repeated the fluorescence assay. The addition of 50 mM galactose also slows the urea unfolding of α-GAL, increasing the $t_{½}$ of unfolding from 2.2 hr to 8.0 hr at pH 6.5 and from 1.3 hr to 2.5 hr at pH 4.5.

We conclude from these experiments that (1) PCs are able to slow the rate of unfolding of α-GAL, (2) DGJ is more potent than galactose at preventing unfolding of α-GAL, and (3) both chaperones slow the unfolding more at pH 6.5 than pH 4.5.

Increase in Apparent Melting Temperature of α-GAL Measured by Circular Dichroism

To examine the thermal stability of α-GAL, we measured the apparent melting temperature T_m(app) of α-GAL using the circular dichroism (CD) signal at 222 nm in thermal denaturation experiments. To investigate the effects of the PCs DGJ and galactose and of pH on the stability of α-GAL, we repeated the thermal denaturations in the presence of the chaperones and at three pH values. Upon the addition of 50 μM DGJ, the T_m(app) of α-GAL increases by 13.2°C to 22.0°C, depending on the pH (Figure 2; Table S2). In contrast, 50 μM galactose has no effect on the T_m(app) of α-GAL. However, upon increasing the concentration of galactose 1,000-fold to 50 mM, the T_m(app) of α-GAL increases by 5.3°C to 8.5°C.

We also compared the T_m(app) as a function of pH. In the absence of pharmacological chaperone, the T_m(app) of α-GAL is unchanged between pH 4.5 and 6.5 (60.7°C and 60.8°C, respectively) but is lower at pH 7.2 (56.1°C), indicating that the protein is less stable at higher pH values.

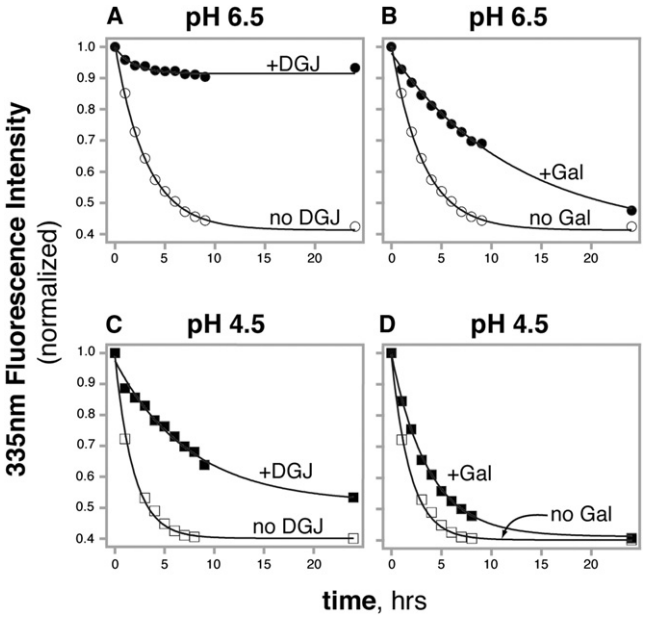

Figure 1. Pharmacological Chaperones Slow the Unfolding Kinetics of α-GAL (Measured by Trp Fluorescence)

(A–D) Unfolding of α-GAL at pH 6.5 (A and B) and pH 4.5 (C and D) in the absence (open symbols) and presence (filled symbols) of 50 μM DGJ (A and C) and 50 mM galactose (B and D). See also Figure S2.

Resistance to Protease Digestion Monitored by Proteolysis

To examine the effect of the PCs on the resistance of α-GAL to protease digestion, we performed proteolysis experiments. In the presence of a protease and urea, the amount of undigested α-GAL protein represents a measure of the protein's stability.

To measure the effect of chaperones at pH 6.5, we digested α-GAL with thermolysin at pH 6.5 and quantitated the amount of α-GAL resistant to the protease. In the presence of DGJ, α-GAL becomes increasingly resistant to thermolysin digestion starting at 500 nM DGJ (Figures 3 and S3). In the presence of galactose, α-GAL becomes resistant to protease digestion starting at 10,000-fold higher concentration, approximately 5 mM galactose.

To examine the effect at pH 4.5, we digested α-GAL with pepsin and quantitated the undigested α-GAL. In the presence of DGJ, α-GAL becomes increasingly resistant to protease starting at 500 nM DGJ (Figure 3). In the presence of galactose, α-GAL becomes resistant to protease starting at 10,000-fold higher concentration, approximately 5 mM galactose.

The proteolysis experiments mirror the results of the fluorescence and CD experiments, showing that both DGJ and galactose are able to stabilize the α-GAL protein. In all three assays, the potency of DGJ is much higher than galactose. In general, the stabilizing effects of DGJ are more pronounced at near-neutral pH.

Structural Basis for Improved Potency of DGJ

To examine the structural effects of PC binding to α-GAL, we determined high-resolution crystal structures of two complexes: the α-GAL:DGJ complex at 2.1 Å resolution and the

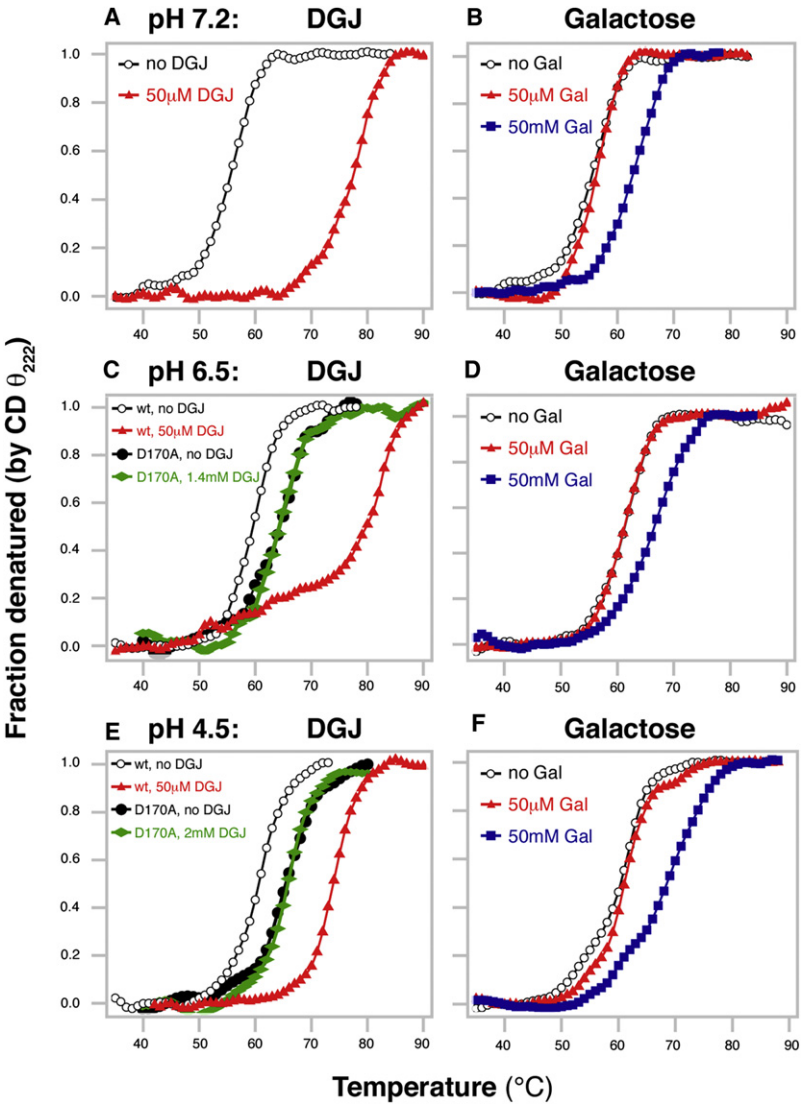

Figure 2. Increased Apparent Melting Temperature T_m(app) of α-GAL (Monitored by CD)

(A–F) DGJ (A, C, and E) and galactose (B, D, and F) were tested at pH 7.2 (A and B), pH 6.5 (C and D), and pH 4.5 (E and F) in the absence (white symbols) and presence of 50 μM (red symbols) or 50 mM (blue symbols) DGJ or galactose. In (C) and (E), the D170A mutant is also shown in the absence (black symbols) and presence (green symbols) of 1.4 or 2 mM DGJ. The D170A mutant does not respond to even 30- or 40-fold higher concentrations of DGJ. See also Table S2.

α-GAL:galactose complex at 2.0 Å resolution, allowing us to examine the atomic basis for the differences in potency between the chaperones.

The crystal structures show that both DGJ and galactose bind similarly in the active site of α-GAL, as expected for binding of a catalytic product and a product analog (Figure 4). There is a more favorable interaction between D170 and the DGJ ligand compared to the galactose ligand. In order to act as a nucleophile in the α-GAL reaction mechanism (Guce et al., 2010), the D170 side chain must be deprotonated and negatively charged. The DGJ ligand contains a protonatable heterocyclic nitrogen atom, allowing for an energetically favorable hydrogen bond. Because the pK_a of DGJ is 7.1 (Legler and Pohl, 1986), the nitrogen is likely protonated in the pH 5.1 crystals, leading to a highly favorable charged interaction between the DGJ and the D170 side chain. Galactose functions as a chaperone by mirroring the binding of the galactoside substrate, and the K_i for the PC is close to the K_M for substrate (5–20 mM). The galactose ligand contains an unprotonated heterocyclic oxygen, which makes either weak van der Waals interaction with the deprotonated D170 side chain, or a hydrogen bond if D170 is protonated.

Effect of D170 on the Interaction with Chaperones

The crystal structures of the complexes of α-GAL with DGJ and galactose led us to hypothesize that the higher potency of DGJ derives mainly from an interaction between the heterocyclic nitrogen of the ligand and the carboxylate of the catalytic nucleophile D170. To test this hypothesis, we made a D170A variant of human α-GAL (lacking the carboxylate) and examined the ability of DGJ to bind and stabilize this variant. Because the D170A variant lacks enzymatic activity, we used biochemical and crystallographic assays to test PC binding.

First, we repeated the CD thermal denaturation experiments at pH 6.5 and pH 4.5 with the D170A α-GAL variant. Consistent with our hypothesis, the D170A α-GAL showed no increase in T_m(app), even in the presence of 1.4 or 2 mM DGJ (Figure 2), whereas the wild-type (WT) α-GAL showed a 13°C to 21°C increase in T_m(app) with 30- or 40-fold less DGJ. Thus, the D170 carboxyl is critical to the stabilizing effect of DGJ.

Second, we repeated the proteolysis experiments on the D170A mutant with DGJ and galactose. In contrast to WT α-GAL, the D170A variant requires a much higher concentration of DGJ to protect from digestion. The removal of the D170 carboxylate group increases the DGJ concentration threshold for protection by over 1,000-fold (Figure 3, S3, and S4). Thus, the D170 carboxylate group is primarily responsible for the much higher potency of DGJ. Using galactose as a PC in the protease assay shows that in the D170A variant, DGJ is no better than galactose as a PC, with protection occurring at millimolar concentrations of galactose. These results indicate that the D170 carboxylate is more critical to the DGJ interaction than it is to the galactose interaction, and that the increased potency of DGJ is entirely due to interaction with the D170 side chain.

Third, we determined a crystal structure of DGJ bound to the D170A mutant α-GAL, which showed that DGJ binds to the D170A active site identically to WT α-GAL (Figure 4). Thus,

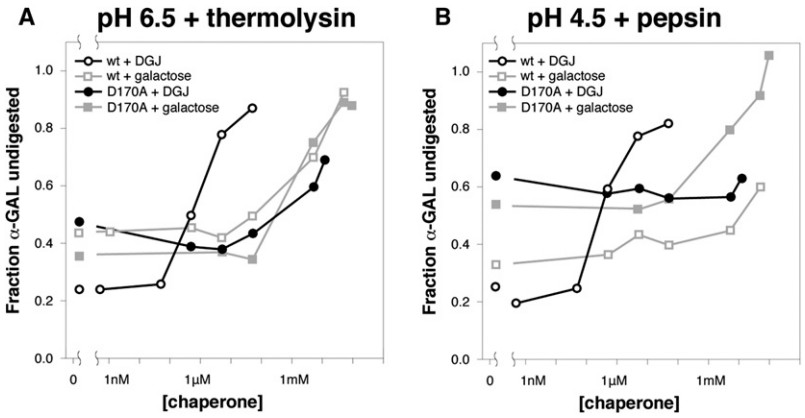

Figure 3. DGJ and Galactose Confer Protease Resistance upon α-GAL

(A and B) Thermolysin (A) and pepsin (B) digestion of WT α-GAL (open symbols) and D170A α-GAL (filled symbols) in urea after incubation with DGJ (black lines) and galactose (gray lines), respectively. WT and D170A α-GAL band intensities were quantitated at multiple chaperone concentrations. The D170A mutant responds only to high concentrations of chaperone. See also Figures S3 and S4.

the high potency of DGJ for WT α-GAL derives from its interaction with the D170 side chain.

Conclusions

Our study shows that PC binding confers thermodynamic stability to α-GAL and dramatically slows the unfolding of the protein. In the equilibrium between native and unfolded states of α-GAL, PC binding to the native state slows the rate of unfolding and shifts the equilibrium toward the native state. This property is particularly valuable in the ER, where the folding of the nascent polypeptide helps it to avoid the ERAD pathway. Stabilization of the native state of the protein increases the fraction of enzyme that traffics out of the ER and travels to the lysosome.

For a small molecule to be an effective PC, it must be able to selectively bind to the active site of an enzyme but then dissociate, allowing the enzyme to turnover substrate (Fan, 2003, 2008). Different models exist for the force driving low pH dissociation, including protonation of the PC, protonation of an active site residue, competition by excess substrate, etc. (Fan, 2003, 2008; Fantur et al., 2010; Jo et al., 2010; Suzuki et al., 2009). For lysosomal enzymes, the pH dependence of affinity of the PC is important, as the chaperone must dissociate from the active site at low pH for the enzyme to function. The heterocyclic nitrogen of DGJ has a pK_a of 7.1, and the protonation state of PCs has been hypothesized to cause the pH-dependent release (Fantur et al., 2010). We propose an alternative hypothesis for the pH dependence of DGJ binding to α-GAL: that the protonation state of the active site nucleophile D170 causes pH dependence. At pH 6.5 and pH 7.2, the D170 carboxylate is expected to deprotonate and the DGJ nitrogen to protonate, leading to a highly favorable ionic interaction between them. In contrast, at pH 4.5, the carboxylate of D170 is more likely to protonate, losing its ionic interaction with the nitrogen of DGJ, leading to weaker binding. In our model, the protonation state of the D170 carboxylate affects the affinity of α-GAL for DGJ, and removal of the carboxylate in the D170A α-GAL variant leads to much weaker binding to DGJ. Our experiments do not support an alternative model where protonation of the nitrogen in DGJ leads to weaker binding of the PC (Fantur et al., 2010).

In conclusion, we have made the following observations about the interaction of α-GAL and PCs. First, DGJ binds to the WT α-GAL and stabilizes the enzyme, as shown by the CD, fluorescence, and proteolysis experiments. Second, in all the biochemical experiments, the protective effect of DGJ is greater at neutral

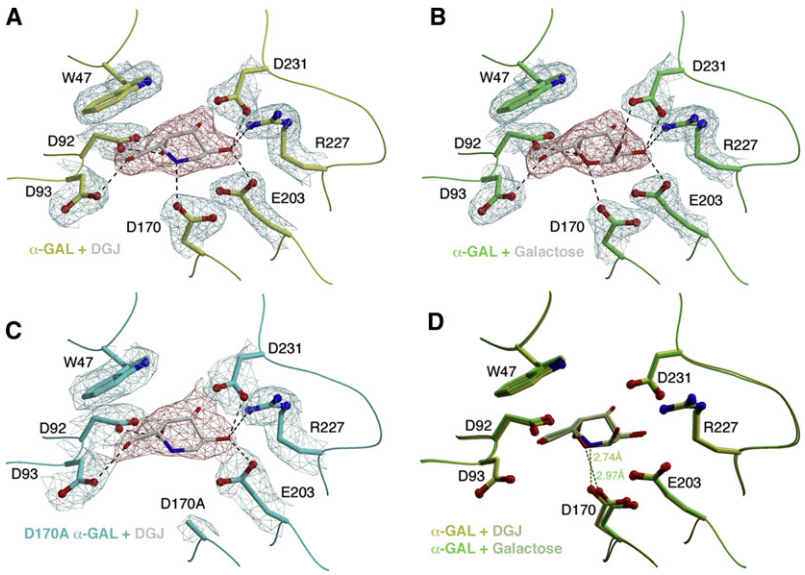

Figure 4. Crystal Structures of Human α-GAL Bound to Pharmacological Chaperones

(A–C) σ_A-Weighted 2Fo-Fc electron density maps of DGJ- and galactose-soaked crystals of WT human α-GAL (A and B) and the D170A mutant α-GAL with DGJ bound (C). All maps are contoured at 1.8 σ with the ligand density colored red for clarity.

(D) A superposition of (A) and (B) highlights the key interaction between the ligand and the D170 carboxylate. See also Table S3.

pH than at pH 4.5. Third, galactose is capable of PC activity but requires 10,000- to 100,000-fold higher concentrations than DGJ, consistent with the differences in K_i measured in enzymatic assays. Fourth, crystal structures show that the PCs bind exclusively to the active site, and the protective effect of the chaperones derives from specific interactions with active site residues. As a counter example, glucose binds weakly to α-GAL away from the active site (Guce et al., 2010) but does not show the same chaperoning effect as DGJ or galactose. Fifth, the enhanced potency of DGJ results from interactions with the D170 carboxylate. When the carboxylate is removed in the D170A variant, DGJ chaperones no better than galactose. We have identified the key atomic interaction responsible for the increased potency of DGJ. These results can be generalized to the entire family of active-site-specific chaperones, allowing for development of improved chaperones.

SIGNIFICANCE

Using a pharmacological chaperone to treat a protein folding disease presents a molecular paradox: to increase the activity of the enzyme, a competitive inhibitor of the enzyme is used. We probe the molecular mechanism of the paradox using biochemical and biophysical approaches on human α-GAL, including enzyme kinetics, chemical denaturation monitored by fluorescence, thermal denaturation monitored by circular dichroism, protease susceptibility, and X-ray crystallography. Our studies show that 1-deoxygalactonojirimycin (DGJ), which is only two functional groups different from galactose, is a 400,000-fold better binder. We hypothesize that a single ionic interaction is responsible for the higher potency of DGJ. We test the hypothesis using a D170A mutant α-GAL lacking the ionic interaction, which loses the high potency of DGJ. We explore the pH dependence of pharmacological chaperone binding, as the chaperones must dissociate from α-GAL in the low pH of the lysosome. In this article, we refute one proposed mechanism of action (that protonation of the small molecule leads to weaker binding in the lysosome) and propose that protonation of the catalytic nucleophile D170 causes weaker DGJ binding at low pH.

ACCESSION NUMBERS

Coordinates and structure factors have been deposited in the Protein Data Bank under codes 3S5Y, 3S5Z, and 3TV8.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2011.10.012.

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