A thermodynamic assay to test pharmacological chaperones for Fabry disease

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Background: The majority of the disease-causing mutations affect protein stability, but not functional sites and are amenable, in principle, to be treated with pharmacological chaperones. These drugs enhance the thermodynamic stability of their targets. Fabry disease, a disorder caused by mutations in the gene encoding lysosomal alpha-galactosidase, represents an excellent model system to develop experimental protocols to test the efficiency of such drugs.

Methods: The stability of lysosomal alpha-galactosidase under different conditions was studied by urea-induced unfolding followed by limited proteolysis and Western blotting.

Results: We measured the concentration of urea needed to obtain half-maximal unfolding because this parameter represents an objective indicator of protein stability.

Conclusions: Urea-induced unfolding is a versatile technique that can be adapted to cell extracts containing tiny amounts of wild-type or mutant proteins. It allows testing of protein stability as a function of pH, in the presence or in the absence of drugs. Results are not influenced by the method used to express the protein in transfected cells.

General significance: Scarcity and dispersed populations pose a problem for the clinical trial of drugs for rare diseases. This is particularly true for pharmacological chaperones that must be tested on each mutation associated with a given disease. Diverse in vitro tests are needed. We used a method based on chemically induced unfolding as a tool to assess whether a particular Fabry mutation is responsive to pharmacological chaperones, but, by no means is our protocol limited to this disease.

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1. Introduction

The reduction in protein stability is the most common cause of monogenic diseases [1]. This knowledge paves the way to a new therapeutic approach. Pharmacological chaperones (PC) are small molecules that stabilize the mutant proteins, increase their intracellular concentration and consequently their intracellular activity. Because of their effect on the apparent stability of proteins they can also be described as “thermodynamic drugs”. The therapeutic approach with PC is currently being tested not only for lysosomal storage diseases [2] like Fabry [3,4], Gaucher [5,6] or Pompe [7,8], but also for other diseases like Phenylketonuria [9].

In order to develop new methodologies to test PC, we used Fabry disease (FD) as a model system. Fabry is an X-linked disease which affects not only male individuals, but also heterozygote female carriers, although in a milder form. It is due to mutations in the gene encoding lysosomal alpha-galactosidase (AGAL) [HGNC: GLA; UNIPROT: AGAL_HUMAN], a dimeric protein, synthesized and glycosylated in the endoplasmic reticulum and transported into lysosomes. The clinical picture of FD is rather complex because more than 400 missense mutations have been described and a good share of them is private. There is already an approved therapy for FD which consists in the infusion of the recombinant human protein (for a review on FD [10]). Unfortunately this therapy is very expensive and in many cases it causes the formation of antibodies against the exogenous protein [11]. For some mutations PC offer an alternative therapeutic approach. Chemicals to be used as PC can be found exploiting their ability to stabilize the wild type enzyme. Once they are found, they must be tested on each mutation because only a percentage of the cases will be responsive. This holds for FD and is in
cause they require milligrams of purified wild type enzymes, are not feasible for the screening of mutants, but tests in vitro are preferable. Classical methods to evaluate thermodynamic stability, which are very useful to assess protein stability for another purpose, that is testing drug responsiveness, and we suggest to use unfolding induced by urea [25]. Wild type AGAL was challenged with urea and results obtained monitoring unfolding with circular dichroism, pulse proteolysis or pulse proteolysis followed by western blot were compared. The last method which is designed for experiments on cell extracts containing low amounts of the protein of interest, was tested on four AGAL mutants. One of these, L300F-AGAL (c.898C→T, p.L300F) was produced using different transfection protocols in order to assess the reproducibility of results. Different pH buffers were used to mimic the conditions encountered by the protein in different cellular compartments.

2. Results

2.1. Chemically induced alpha-galactosidase unfolding

AGAL (Fabrazyme®) (6 μM per monomer concentration) was incubated at 20 °C in McIlvaine buffer pH 7.4 in the presence of various concentrations of urea until unfolding equilibrium was obtained and a 16–18 h incubation was considered adequate to reach equilibrium in the subsequent experiments. We find a strong stabilizing effect of DGJ against urea denaturation, a sharp transition in the case of apo-enzyme and a broad transition in the case of the complexed enzyme (Fig. 1) Although thermally or chemically induced unfolding describes different processes, the reduced steepness of the denaturation curve in the presence of the drug confirms the results obtained by Petsko and coworkers [28] who found a coincidence of calorimetric and van’t Hoff enthalpies for the free enzyme, but not for the complexed enzyme.

Fig. 1. Urea-induced melting profile of wild-type lysosomal alpha-galactosidase (Fabrazyme®) recorded by circular dichroism. The protein (0.3 mg/ml in McIlvaine buffer at pH 7.4) was equilibrated with urea (from 0 to 6 M) in the presence of 1-deoxy-galactonojirimycin (DGJ) 40 μM or not, then ellipticity at 223 nm was recorded. Data were expressed as mean residue ellipticity.

Fig. 2. Urea-induced melting profile of wild-type lysosomal alpha-galactosidase (Fabrazyme®) recorded by pulse-proteolysis and SDS-PAGE analysis. The protein (0.3 mg/ml in McIlvaine buffer at pH 7.4) was equilibrated with urea (from 0 to 6 M) in the presence of 1-deoxy-galactonojirimycin (DGJ) 40 μM or not, then an aliquot of each sample was subjected to pulse proteolysis with thermolysin (1 min at 37 °C, 1:5 protease to substrate ratio) and then analyzed by SDS-PAGE. The protein was visualized by Coomassie Blue Staining (−DGJ, panel A; +DGJ, panel B). The intensity of the bands was quantified and expressed as relative intensity (panel C).
Fig. 3. Urea-induced unfolding profiles of wild-type lysosomal alpha-galactosidase present in raw cell extracts recorded by pulse-proteolysis and western-blot. Lysates of COS7 cells expressing wild-type lysosomal alpha-galactosidase were mixed with the denaturant to obtain final urea concentrations ranging from 0 to 6 M. The experiment was conducted in McIlvaine buffer at pH 4.5 (panel A), 5.2 (panel B) or 7.4 (panel C) either in the absence or in the presence of 1-deoxygalactonojirimycin (DGJ) 40 μM. Pulse proteolysis (1 min at 37 °C, 1:5 protease to substrate ratio) was performed after the equilibrium was reached to digest unfolded protein and then analyzed by western-blot. Thermolysin was used when operating at pH 5.2 or 7.4, pepsin when operating at pH 4.5. The intensity of the bands was quantified and data were expressed as fraction of the zero urea sample.
A two state model can account for the unfolding of the apo-enzyme, but not for that of the complex. For this reason our analysis is limited to the midpoint urea concentration $C_{0.5}$ of the normalized data as an operative measure of protein stability. This value can be determined quite accurately and is little affected by the mechanism of the unfolding. We are aware of the fact that a precise measure of the free energy of unfolding in water, which is outside the scope of this paper, would require the evaluation of the dependence of the free energy on denaturant concentration, i.e. the assessment of m-values [29]. However this value cannot be determined very accurately and its correlation with stability has been well documented mostly for monomeric proteins that unfold with a two-state mechanism and without ligands.

In the absence of DGJ $C_{0.5}$ is at $2.0 \pm 0.2$ M urea (Fig. 1) whereas, due to the strong stabilizing effect of the drug and consequently to the difficulty of determining a true endpoint of the melting curve, in the presence of DGJ only an approximate $C_{0.5}$ can be estimated above 4 M urea.

The same samples, which had been analyzed by circular dichroism (CD), were incubated with thermolysin (1:5 protease to Fabrazyme® ratio by weight). After having inactivated the protease by addition of EDTA, the samples were analyzed by SDS-PAGE (Fig. 2 panels A and B). The intensity of the bands was quantified and the profiles obtained (Fig. 2 panel C) confirm the stabilizing effect of DGJ. In particular we observe that the transition in the absence of DGJ is sharp whereas a more complex unfolding process occurs in the presence of DGJ. Hence the effect observed with a “classic” method, i.e. urea-induced denaturation monitored by circular dichroism (Fig. 1), is reproduced with pulse-proteolysis [30].

2.2. Scaling down chemically induced alpha-galactosidase unfolding for the analysis of mutants

AGAL was produced in COS7 cells transiently transfected with an expression vector. Whole cell extracts were aliquoted and incubated with variable concentration of urea in the presence or in the absence of DGJ.
Fig. 6. Urea-induced unfolding profiles of D244H, Q280K and R301P lysosomal alpha-galactosidase mutants present in raw cell extracts recorded by pulse-proteolysis and western-blot. Lysates of COS7 cells expressing D244H (panel A), Q280K (panel B) or R301P (panel C) lysosomal alpha-galactosidase were mixed with the denaturant to obtain final urea concentrations ranging from 0 to 5 M. The experiment was conducted in McIlvaine buffer at pH 7.4 either in the absence or in the presence of DGJ 40 μM. Pulse proteolysis (1 min at 37 °C, 1:5 thermolysin to substrate ratio) was performed after the equilibrium was reached to digest unfolded protein and then analyzed by western-blot. The intensity of the bands was quantified and data were expressed as fraction of the zero urea sample.
of 40 μM DGJ. After 16–18 h of equilibration, the unfolded proteins were digested proteolytically and the residual AGAL was quantified by western blot. Three sets of experiments were conducted at different pHs and are summarized in Fig. 3.

Thermolysin was added (1:5 ratio with total protein in the extracts by weight) when the denaturation had been carried out at pH 7.4 or 5.2. Pepsin was added (1:5 ratio with total protein in the extracts by weight) when the denaturation had been carried out at pH 4.5.

Fig. 3 panel C shows that at neutral pH DGJ stabilizes the protein shifting the midpoint concentration from 1.8 ± 0.1 M to 3.5 ± 0.2 M urea. C_{0.5} is slightly lower for the human enzyme expressed in COS7 cells than for Fabrazyme®. This can be explained by a different glycosylation of the enzyme. In Fig. 4 we compare AGAL produced in COS7 cells to Fabrazyme®. Their electrophoretic mobilities are different, but become the same upon treatment with N-Glycosidase F.

The enzyme is more stable at pH 5.2 (Fig. 3 panel B) compared to pH 7.4 (Fig. 3 panel C) with a C_{0.5} approximately at 4 M urea in the absence of the DGJ and it is resistant to denaturation even at 6 M urea in the presence of the drug. On the other hand, we observe a reduction in the stability at pH 4.5 and C_{0.5} values drop to 0.1 M in the absence of DGJ or to 1.2 M urea in the presence of DGJ.

L300F-AGAL [31] is a mutant with less than 10% of the wild type activity. COS7 cells expressing this mutant recover activity when they are exposed to DGJ [27]. We tested thermodynamic stability of L300F-AGAL by challenging it with urea. A single transfection in COS7 cells was required to produce the extract. 50 μg proteins in total, that was aliquoted and treated with different concentrations of urea in the presence or in the absence of DGJ. Fig. 5 shows the results obtained at pH 5.2 (panel A) or 7.4 (panel B).

This experiment demonstrates that the mutant is less stable than wild type both at neutral and at acidic pH, yet it becomes as stable as the wild type when treated with DGJ. Moreover, we observe that L300F-AGAL is more stable at pH 5.2 than at pH 7.4.

Besides L300F-AGAL, other mutations associated with classic clinical manifestations, Q280K-AGAL (c.838C>A, p.Q280K) [32], D244H-AGAL (c.730G>C, p.D244H) [33], R301P-AGAL (c.902G>C, p.R301P) [34] were tested at pH 7.4 with increasing amounts of urea, limited proteolysis and western blot. Results are shown in Fig. 6. We chose neutral conditions because they reflect the environment of the endoplasmic reticulum and the same expression protocol based on Lipofectamine®

3. Discussion

Pharmacological chaperones work because they stabilize mutant proteins and the method based on thermodynamic analysis is designed to assess this property straightforwardly.

Thermodynamic analysis allows the association of a quantifiable parameter to the stability of proteins in a given state. Melting temperatures, free energies or enthalpies of unfolding, midpoint denaturant concentration, and m-values help us evaluate the stability of wild type and mutant forms of a protein under different environmental conditions, pH or ligands. In particular the thermal shift assay proved to be a useful method to test pharmacological chaperones on purified proteins [35]. This method, however, cannot be exploited to analyze mutants, unless they are expressed in large amounts and purified. Such a pre-requisite is unfeasible in the majority of cases. On the other hand, urea induced denaturation can be monitored by limited proteolysis followed by electrophoresis separation exploiting the fact that unfolded proteins are more sensitive to proteases than their folded counterparts. The method can be adapted to raw extracts. We demonstrated that results obtained by limited proteolysis are consistent with those obtained by more conventional optical techniques in the case of Fabrazyme®.

Urea-induced unfolding allows the assignment of a useful measure of stability to a protein in a given state that is the half-denaturation urea concentration, C_{0.5}. We demonstrated that urea-induced unfolding can be exploited to test the stability of wild type or mutant AGAL in cell extracts and the capacity of chaperones to stabilize the enzyme.

![Fig. 7. Correlation between urea C_{0.5} and alpha galactosidase increase. Urea concentration was from Figs. 5 and 6 (this paper); alpha galactosidase activity is expressed as mut_activity + DGJ / WT_activity − DGJ × 100. The Pearson correlation coefficient is 0.94.](image-url)
Usually the efficacy of PC is evaluated on the patient’s cells or in cells transfected with an expression vector carrying the mutation and cultured in a medium supplemented or not with the drug. We compared the data obtained in different labs with this method. We found 15 responsive mutations which have been tested in different labs [19,20,22,24].

We obtained the responsiveness in percentage defined as the AGAL activity of a given mutant after administration of DGJ to the cells, divided by the activity of wild type AGAL expressed in the same cells non-exposed to the drug, multiplied by 100, i.e. \( \text{resp} = \frac{\text{mut\_activity + DGJ}}{\text{WT\_activity} - \text{DGJ}} \times 100 \). We calculated an average responsiveness in percentage (resp), a standard

![Fig. 8. Alpha-galactosidase activity resulting from 1-deoxy-galactonojirimycin administration in transfected cells. Comparison between different cell hosts and transfection methods. COS7 or HEK293 cells were co-transfected with pCMV6-AC harboring L300F-AGAL gene and with pMIR vector harboring the luciferase reporter gene. Transfections were performed with Lipofectamine® ltx, Fugene® HD or CalPhos™ Mammalian Transfection Kit, either using cell in adhesion (A) or in suspension (S). Cells were than cultivated with (+) or without (−) 0.02 mM DGJ. After cell lysis AGAL activity and luciferase activity were measured. Four independent experiments were performed in the presence of DGJ, two without DGJ. Alpha galactosidase activity (expressed as \( \frac{\text{mut\_activity + DGJ}}{\text{WT\_activity} - \text{DGJ}} \times 100 \)) was normalized by protein concentration in panel A and by luciferase activity in panel B.]

![Fig. 9. Stability enhancement of lysosomal alpha galactosidase by 1-deoxy-galactonojirimycin. Comparison between different cell hosts and transfection methods. Lysates of COS7 or HEK293 cells transfected with different methods and expressing L300F (panels A, B, and C) or wild type (panels D and E) lysosomal alpha-galactosidase were mixed with the denaturant to obtain final urea concentrations ranging from 0 to 6 M. The experiment was conducted in McIlvaine buffer at pH 7.4 either in the absence or in the presence of DGJ 40 \( \mu \text{M} \). Pulse proteolysis (1 min at 37 °C, 1:5 thermolysin to substrate ratio) was performed after the equilibrium was reached to digest unfolded protein and then analyzed by western-blot. The intensity of the bands was quantified and data were expressed as fraction of the zero urea sample.]
A

L300F - Hek293
adherent cells
lipofectamine

B

L300F - Hek293
adherent cells
calcium phosphate

C

L300F – Cos7
adherent cells
calcium phosphate

D

WT - Hek293
adherent cells
lipofectamine

E

WT - Hek293
adherent cells
calcium phosphate
deviation (SDresp) and an average standard deviation. The results are in qualitative accordance, but quantitatively the extent of responsiveness varies, with an average standard deviation, $\sum_{i=1}^{n} (SDresp_i)$, as large as $15 \pm 10$.

The approach based on chemically induced unfolding gives results that are little influenced by the method chosen to express the protein. The results are consistent with those obtained measuring the enzymatic activity in cells exposed or not exposed to the drug [27] (Fig. 7). The method based on chemically induced unfolding offers some advantages. For example it consents to test proteins at different pHs. This is important considering that, in general, chaperones are inhibitors of the enzymatic activity. The ideal drug for lysosomal storage disorders such as Fabry disease, should bind the protein in the neutral compartment where it folds and dissociates in the acidic compartment where AGAL exerts its function. Moreover the method based on chemically induced unfolding can be used to test lead compounds on mutants expressed in tiny amounts and unpurified. In this case the molecules might be yet unable to cross the cell membrane and administration in the cell medium would be not effective.

Diverse methods to test the efficacy of pharmacological chaperones on individual mutations of a given disease are urgently needed. In particular this is true for Fabry disease, a pathology characterized by hundreds of different mutations each affecting a scarce and dispersed population. Clinical trials with control groups are difficult as exemplified by studies carried out to test the efficacy of DGJ on male [12,26] or female [36] populations. Twenty-one out of 450 sense-nonsense mutations have been tested and only in few cases was it possible to enroll more than one patient. Independent positive results obtained in vitro with the thermodynamic approach and other methods [37] might be the only support to choose the best therapy, PC or enzyme replacement therapy for individual Fabry patients.

4. Materials and methods

4.1. Cell cultures

The clone SC319065, which contains the full length cDNA for wild type human AGAL inserted into the expression vector pCMV6-AC, was purchased from Origene (Rockville, MD, USA). Q280K-AGAL, D244H-AGAL, L300F-AGAL and R301P-AGAL were obtained by site directed mutagenesis in the same vector [27]. Human embryo kidney 293 (HEK293) and African green monkey kidney (COS7) cells were maintained in Dulbecco’s Modified Eagle’s Medium (GIBCO BRL) with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO2.

COS7 cells were transfected with individual plasmids using the Lipofectamine®2000 (Invitrogen Molecular Probes, lifetecnologies.com) cationic lipid reagent as previously described [27]. Transfected cells were plated onto a 100 mm dish and cultured in DMEM containing 10% FBS in the presence of 0.02 mM DGJ at 37 °C and 5% CO2. After a 48 h incubation, the cells were washed in PBS (5 times), scraped and harvested by centrifugation. Dry pellets were resuspended in water and lysed by freeze-thawing. The efficiency of the transfection was calculated by cotransfecting a 1:4 ratio of pMiR vector (Applied Biosystems/Ambion, Italy) containing the luciferase gene and assaying the reporter gene activity under standard conditions using ONE-Glo™ Luciferase Assay System (Promega, Italy). AGAL activity was measured by adding 2 μl of the cell lysates to 38 μl of AGAL assay buffer (sodium citrate 27 mM-sodium phosphate dibasic 46 mM, 4-methylumbelliferyl-alpha-D-galactopyranoside 5 mM and N-acetyl-D-galactosamine 100 mM, pH 4.5) and incubated for 1 h at 37 °C. All chemicals were obtained from Sigma (SIGMA, Milan, Italy). The reaction was stopped by adding 360 μl of 1 M sodium carbonate buffer [20]. Fluorescence was detected using a fluorescence spectrophotometer (Cary Eclipse-Varian) at 355 nm excitation and 460 nm emission. A 4-methylumbelliferone standard curve ranging from 5 nM to 25 μM was run in parallel for conversion of fluorescence data to AGAL activity expressed as nmol/mg protein per hour.

4.2. Urea-induced unfolding

Fabrazyme® (Genzyme, Cambridge, MA) (0.3 mg/ml) was induced to unfold by urea in McIlvaine buffer at pH 7.4, with or without 0.04 mM DGJ in a final volume of 0.150 ml. Dilutions of the enzyme reconstituted in water, buffer and 8 M urea were carried out in separate tubes in order to obtain the desired final conditions where urea concentration varied from 0 to 6 M. Molar ellipticity per mean residue [θ]290/deg cm2 mol−1 was calculated from the equation $[\theta]_{290/deg-cm^2-mol^{-1}} = 100 \times [\theta]_{290}/[C]$ where $[\theta]_{290}$ is the ellipticity measured in degrees on a JASCO J-815 spectropolarimeter. l is the pathlength of the cell in cm and C is the protein concentration referred to the mean residue molecular weight. Aliquots were withdrawn from the same samples prepared for CD measurements, supplemented with CaCl2 (10 mM final concentration) and with the appropriate amount of thermolysin necessary to realize a 1:5 protease to protein ratio. After 1 min incubation at 37 °C the reaction was stopped by addition of EDTA (40 mM final concentration). Folded undigested proteins were quantified with a ChemiDoc XRS (Bio-Rad laboratories, Hercules, CA-USA) system after having been separated by SDS-PAGE and colored by Coomassie Blue Staining.

Urea-induced unfolding of COS7 or HEK293 cell lysates containing wild-type AGAL or L300F-AGAL, or Q280K-AGAL, or D244H-AGAL, or R301P-AGAL was conducted in McIlvaine buffer at pH 4.5, 5.2 or 7.4, in a total volume of 17 μl containing 0.05 mg/ml protein, urea ranging from 0 to 6 M, in the presence or in the absence of 0.04 mM DGJ. The samples were incubated for 16–18 h at 20 °C.

Pulse proteolysis was performed after the equilibrium was reached to digest unfolded proteins. The samples at pH 5.2 or 7.4 were treated with appropriate amount of thermolysin in order to realize a 1:5 protease to substrate (total proteins) ratio. CaCl2 was also added to the reaction mixture up to 10 mM. After 1 min incubation at 37 °C the reaction was stopped by addition of EDTA (40 mM final concentration). The samples at pH 4.2 were treated with the appropriate amount of pepsin in order to realize a 1:5 protease to substrate (total proteins) ratio. After 1 min incubation at 37 °C the protease was inactivated by addition of Tris 1 M pH 9.0.

Aliquots (1 μg) were loaded onto a 12% SDS-PAGE gel and transferred to PVDF membrane (Bio-Rad Laboratories, Hercules, CA-USA) for 2 h at 100 V in Tris-Glycine buffer containing 20% methanol. The membrane was blocked with 5% (w/v) non-fat dried skimmed milk in TBS containing Tween20 0.05% (TTBS) at 4 °C overnight, and then treated with the primary antibody (polyclonal antibody produced in rabbit, Abcam 70520) diluted in TTBS 1:500 for 1 h at room temperature. After washing with an excess of TTBS, the
membrane was treated with the secondary antibody (HRP-conjugated anti-rabbit IgG antibody produced in goat, Bio-Rad 1706515) diluted in the TBBS solution 1:2000 for 1 h at room temperature. After washing, the detection was performed by using the Immun-Star WesternC chemiluminescence detection kit (Bio-Rad Laboratories, Hercules, CA, USA).

4.3 Miscellaneous

N-Glycosidase F was purchased by Roche (Roche Diagnostics GmbH, Mannheim, Germany). Deglycosylation of Fabrazyme® or AGAL expressed in COS7 cells (9 μg, 0.3 mg/ml) was performed according to the producer’s instructions. Briefly, EDTA and SDS were added to the proteins (final concentrations were 20 mM and 0.1% respectively). The samples were boiled for 5 min, immediately cooled before the addition of NP-40 to a final concentration of 0.7% and N-Glycosidase F (1 unit) and incubated overnight at 37 °C. Controls were run in parallel without the addition of N-Glycosidase F. Aliquots were analyzed by SDS-PAGE and western blotting.

SDS-PAGE was performed using standard procedures [38]. Protein concentrations were routinely estimated using the Bio-Rad Protein System, with bovine serum albumin as the standard. Protease concentrations were determined by using the appropriate extinction coefficient (ε1%280 = 17.6 μmolds⁻¹cm⁻¹). Graph plotting and curve fitting were carried out with KaleidaGraph (Synergy Software, PA).

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