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Potential Modifications to Enzyme Replacement Therapy in Anderson-Fabry Disease

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POTENTIAL MODIFICATIONS TO ENZYME REPLACEMENT THERAPY IN ANDERSON-FABRY DISEASE

By
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Abstract

POTENTIAL MODIFICATIONS TO ENZYME REPLACEMENT THERAPY IN ANDERSON-FABRY DISEASE
By
Mariam F. Meghdari

Advisor: David H. Calhoun, Ph.D.

Mutations in the GLA gene that encodes the lysosomal enzyme α-galactosidase A (αGal) result in the sphingolipidoses named Fabry disease. This enzymatic defect is inherited as an X-linked recessive disorder and is associated with a progressive deposition of glycosphingolipids, including globotriaosylceramide (GB3), galabioasylceramide, and blood group B substance in the cell. In affected males, and in some females, this leads to early death due to occlusive disease of the heart, kidney, and brain. This disease is currently treated by infusions of αGal, prolonging patients’ lives but producing antibodies against the enzyme reducing the treatment efficacy. Treatment also causes numerous and sometimes life threatening infusion related adverse reactions, including anaphylactic shock, and even death in rare occasions. Here we propose two potential improvements to the current therapeutic practices which would allow for more effective enzyme therapies. The first is constructing and analyzing potentially more active carboxyl-terminal deletions of αGal and the second focuses on targeting of αGal to the very high uptake scavenger receptor (SR) for improved transport to the lysosome.
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# Table of Contents

Abstract ................................................................................................................................. iv
Acknowledgments .................................................................................................................. v
List of Tables .......................................................................................................................... viii
List of Figures ........................................................................................................................ ix

Chapter 1 : Introduction ....................................................................................................... 1
  Lysosomal storage diseases .................................................................................................. 2
  Fabry disease ...................................................................................................................... 2
  α-Galactosidase A (αGal) ................................................................................................... 4
  Available therapies ........................................................................................................... 5
  Scope of work ...................................................................................................................... 8

Chapter 2 : Carboxyl-Terminal Truncations Alter the Activity of the Human α-Galactosidase A ................................................................. 11
  Abstract ............................................................................................................................... 12
  Introduction .......................................................................................................................... 13
  Materials and Methods ...................................................................................................... 16
    Cell strains and plasmids .................................................................................................. 16
    Bioreactor expression of recombinant αGal in *P. pastoris* .............................................. 17
    Construction of strains ..................................................................................................... 18
    Electrophoresis analysis .................................................................................................... 23
    Western blot analysis ....................................................................................................... 23
    Enzyme and protein assays .............................................................................................. 24
    Mass spectrometry of a purified mutant enzyme ............................................................ 25
    Thermostability and pH optimum of WT and mutant αGal ............................................. 26
    Characterization of kinetic properties .......................................................................... 26
    Protein structure analysis ................................................................................................. 28
  Results ................................................................................................................................. 28
    Purification of WT and mutant αGal ................................................................................ 28
    Mass spectrometry of a purified mutant enzyme ............................................................ 31
    Thermostability and pH optima of WT and deletion mutants of αGal ............................. 34
    Kinetic analysis of WT and C-terminal deletion mutants .............................................. 36
  Discussion .......................................................................................................................... 39
  Conclusions ........................................................................................................................ 45

Chapter 3 : Uptake of α-Galactosidase A by the Scavenger and Mannose Pathways ............................................................... 47
  Abstract ............................................................................................................................... 48
  Introduction .......................................................................................................................... 49
  Methods and Research Design .......................................................................................... 56
Purification of recombinant αGal ................................................................. 56
Enzyme assay .......................................................................................... 57
Protein assay ............................................................................................ 58
Electrophoresis analysis ........................................................................... 58
Cell culture ............................................................................................... 58
Evaluation of uptake using enzyme assay .................................................. 59
Evaluation of uptake using confocal microscopy ....................................... 60
Live cell imaging ......................................................................................... 60
Quantification of uptake in confocal images .............................................. 61
Quantification of modification by TNBS and fluorescamine assay .......... 62
2D electrophoresis ....................................................................................... 62
Results: .................................................................................................... 63
Purification of recombinant human αGal in P. pastoris ............................... 63
Uptake of αGal using confocal microscopy ............................................... 66
Live cell imaging ........................................................................................ 68
Time course confocal microscopy in fixed cells using IMFE1 endothelial cells 72
Uptake of αGal is dose dependent .............................................................. 75
Recombinant αGal produced in Pichia is taken up in higher levels in Fabry endothelial cells (IMFE1) compared to Fabry fibroblasts (FFB) .................................................. 77
Uptake of αGal in IMFE1 cells reaches a plateau after 3 hours .............. 78
Preliminary control experiments to demonstrate aconitylation of HSA .... 79
Aconitylation promotes uptake of HSA .................................................... 81
Aconitylation of HSA targets the enzyme to the scavenger receptor uptake pathway............ 84
Aconitylation of αGal ............................................................................... 86
Aconitylation of αGal targets the enzyme to the scavenger receptor uptake pathway in U2OS-SRA cells............................................................... 87
αGal is targeted to the scavenger receptor uptake pathway in U2OS-SRA cells ... 92
Aconitylation improves targeting of αGal to lysosomes in IMFE1 endothelial cells .. 93
Measurement of the degree of aconitylation of αGal .............................. 99
Discussion: .............................................................................................102
Conclusions: ...........................................................................................109
Literature Cited: ......................................................................................110
List of Tables

Table 2-1: Starins and Plasmids..............................................................................................................................21
Table 2-2: Primers Used for DNA Sequence Analysis..............................................................................................22
Table 2-3: Values of Km, Vmax, kcat and and the specificity constant (kcat/Km) for WT and C-Terminal Deletion Mutants of aGal. .................................................................................................................................27
Table 2-4: Purification Table for WT aGal Expressed in P. pastoris........................................................................29
Table 2-5: Literature Values for Kd and Vmax for the WT Human aGal.................................................................37
Table 3-1: Purification Table for aGal Expressed in P. pastoris..............................................................................65
Table 3-2: Overview of HSA/BSA acetylation samples.............................................................................................80
Table 3-3: ImageJ particle analysis of ACO (+, -) aGal in U2OS and U2OS-SRA cells from Figure 3-24............90
Table 3-4: ImageJ particle analysis of ACO (+, -) aGal time course uptake in IMFEI cells.................................94
Table 3-5: Overview of aGal acetylation samples.................................................................................................99
List of Figures

Figure 1-1: Pathways for the breakdown of GM1(monomiosialotetrahexosylganglioside) the "prototype" ganglioside, globo side and sphingomyeline to ceramide [40]......................................................................................3
Figure 1-2: Crystal structure of human αGal..............................................................................................................4
Figure 1-3: Relative enzyme activity of C-terminal deletions of human αGal [2].................................................................9
Figure 1-4: Introduction of a C-terminal deletion of 2 amino acids into αGal.........................................................19
Figure 1-5: Primers and αGal cDNA used to generate Δ 2, Δ 4, Δ 6, Δ 8 and Δ 10 mutant cDNAs .........................20
Figure 1-6: SDS-PAGE for purification of αGal.........................................................................................................30
Figure 1-7: Western Blot of purified WT and mutant αGal. .....................................................................................31
Figure 1-8: Mass spectrometry of purified Δ6 αGal....................................................................................................33
Figure 1-9: Thermostability profiles of WT and mutant αGal....................................................................................34
Figure 1-10: pH activity curves of WT and mutant αGal..........................................................................................35
Figure 1-11: Substrate saturation curves of WT and mutant αGal...............................................................................38
Figure 1-12: The C-termini of human and coffee αgalactosidase. .............................................................................42
Figure 1-13: C-terminal Distance from Secondary Binding Site and Opposite Active Site. ......................................44
Figure 3-1: Domain architecture of the different classes of scavenger receptors [22]. Their ligands, and expression profiles [23, 24].........................................................................................................................53
Figure 3-2: Reaction of surface Lys with cis-aconitic anhydride. .................................................................................54
Figure 3-3: Aconitylation is reversible at the acidic pH of the lysosome [4].................................................................54
Figure 3-4: Saturation of scavenger receptors in liver, spleen, kidney, and blood endothelium [6]..........................55
Figure 3-5: Fermentation profile of the bioreactor expression of recombinant human αGal in pichia following a methanol-limited strategy.................................................................64
Figure 3-6: αGal was purified as a single band on an SDS-PAGE gel..........................................................65
Figure 3-7: Uptake of αGal-Alexa using 3D confocal microscopy. ........................................................................67
Figure 3-8: Uptake of αGal-Alexa in fixed cells using normal fibroblast (NFB) cells (2hr incubation) ..............67
Figure 3-9: Uptake of αGal-Alexa using live cells. .................................................................................................69
Figure 3-10: Quantification procedure of confocal images with ImageJ.................................................................70
Figure 3-11: Particle analysis of live cell uptake of αGal-Alexa in Fabry fibroblast (FFB) cells..............................71
Figure 3-12: Time course uptake of αGal-Alexa in IMFE1 cells. ............................................................................73
Figure 3-13: Particle analysis of uptake of αGal-Alexa in IMFE1 cells from (Figure 3-12) .........................................74
Figure 3-14: Purified recombinant αGal produced in insect cells and P. pastoris is taken up by FFB (GM2775) in cell culture [7-9]........................................................................................................................................75
Figure 3-15: Dose dependent uptake of αGal in Fabry fibroblast (FFB) and Fabry endothelial (IMFE1) cells ....76
Figure 3-16: Comparison of uptake trends of αGal produced in pichia and mammalian cells in fibroblast and endothelial cells.................................................77
Figure 3-17: Comparison of time course uptake of αGal in FFB and IMFE1 cells. ...................................................78
Figure 3-18: Analysis of stability of αGal over high pH range ..............................................................................79
Figure 3-19: Analysis of charge of HSA pre-and post aconitylation ......................................................................80
Figure 3-20: 2D electrophoresis of HSA and Aco-HSA samples. .................................................................................81
Figure 3-21: Uptake of HSA-Alexa compared to Aco-HSA-Alexa in NFB. .................................................................83
Figure 3-22: Uptake of Aco-HSA-Alexa in U2OS and U2OS-SRA cells ................................................................85
Figure 3-23: Surface Lys residues of αGal................................................................................................................86
Figure 3-24: Uptake of αGal-Alexa and Aco-αGal-Alexa in U2OS and U2OS-SRA cells .........................................88
Figure 3-25: Quantification of uptake of αGal-Alexa and Aco-αGal-Alexa in U2OS and U2OS-SRA cells from Figure 3-24. ........................................................................................................................................89
Figure 3-26: Uptake of Aco-αGal-Alexa in U2OS-SRA cells (+, -) PolyI inhibition ...............................................91
Figure 3-27: Uptake of αGal-Alexa in U2OS-SRA cells: Representative images ..................................................92
Figure 3-28: Time course uptake of Aco-αGal-Alexa in IMFE1 cells.................................................................93
Figure 3-29: Particle analysis of time course uptake of Aco-αGal-Alexa in IMFE1 cells from Figure 3-13, 3-28) .95
Figure 3-30: Effect of inhibitors on uptake of non-Aco-αGal in IMFE1 cells .......................................................97
Figure 3-31: Effect of inhibitors on uptake of αGal in IMFE1 cells. ........................................................................98
Figure 3-32: Gel electrophoresis of representative αGal and Aco- αGal samples. ..................................................101
Figure 3-33: Uptake of moss αGal [1].......................................................................................................................103
Figure 3-34: Types of N-glycans. .........................................................................................................................104
Figure 3-35: Surface potential of αGal. .......................................................... 105
Figure 3-36: Isoelectric focusing gel of Fabrazyme and Replagal. [3]. ........................................ 106
Figure 3-37: Comparison of the sizes and charges of agalsidase beta [19]. ................................... 107
Figure 3-38: Comparison of the association of M6P-HSA liposomes and AcoHSA liposomes [5]. .......... 108
Figure 3-39: Proposed α-Gal A Conjugates ............................................................................. 109
List of Abbreviations

αGal: α-galactosidase A
Aco-αGal: Aconitylated α-galactosidase A
Aco-HSA: Aconitylated Human Serum Albumin
AOX: alcohol oxidase
ASA: Accessible surface area
BBB: blood brain barrier
BSA: bovine serum albumin
Con A: Concanavalin A Sepharose 4B
DAPI: 4′,6-diamidino-2-phenylindole
DEAE: Diethylaminoethyl
ERT: enzyme replacement therapy
FFB: Fabry Fibroblast
GB3: globotriaosylceramide
IEF: Isoelectric Focusing
IMFE1: immortalized Fabry endothelial cells
LDL: Low Density Lipoprotein
LSD: lysosomal storage disease
M6P: mannose-6-phosphate
MR: mannose receptor
MU: 4-methylumbelliferone
MUG: 4-methylumbelliferyl-α-D-galactoside
NFB: Normal Fibroblast
PAOX1: promoter for AOX1
PC: phosphatidylcholine
PEG: Polyethylene glycol
PolyI: polyinosinic acid
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SR: scavenger receptor
Thiogal: Immobilized-D-Galactose Gel
TNBS: trinitrobenzenesulphonic acid
U2OS: Human osteosarcoma cells
U2OS-SRA: human U2OS osteosarcoma cells over-expressing the murine scavenger receptor A
Chapter 1: Introduction
Lysosomal storage diseases

Lysosomal storage diseases (LSDs) are a group of approximately 60 inherited metabolic disorders caused by the absence or dysfunction of lysosomal enzymes (Figure 1-1). LSD’s lead to a number of clinical manifestations due to the accumulation of intermediate substrates of these enzymes in the lysosome. LSDs are individually classified as rare/orphan diseases, combined they make up anywhere between 1 in 4000 to 1 in 13,000 live births according to various published studies [31] with these numbers potentially being higher according to recent with recent newborn screening studies [32, 33].

Fabry disease

Fabry disease is a disorder of glycosphingolipid metabolism due to deficient or absent lysosomal of α-galactosidase A (αGal) activity (Figure 1-1) [34]. Numerous gene mutations of the protein have been described. Patients with mutations leading to the total absence of αGal suffer the classic form of Fabry disease, while those having missense mutations causing residual activity (2% to 25%) suffer from the milder forms of the metabolic disorder [35]. In contrast to many other LSDs, most patients remain clinically asymptomatic during the very first years of life. The first symptoms include chronic pain, skin lesions, myocardial infarction, gastrointestinal disorder, and vascular deterioration. Later in life, the complications associated with this disease are often life threatening [36, 37].

Fabry disease is the second most common LSD after Gaucher disease, initially thought to have a worldwide prevalence of approximately 1 in 40,000 to 1 in 117,000 live births for the classic form of the disease [38]. However, current newborn screening initiatives have found an
unexpectedly high prevalence of the disease, as high as 1 in approximately 3,100 newborns in Italy [39] and have identified a surprisingly high frequency of newborn males with Fabry disease, approximately 1 in 1,500 in Taiwan [38]. These findings suggest that Fabry disease may currently be under recognized, making the need for advancement in treatment more urgent.

Figure 1-1: Pathways for the breakdown of GM1(monoisialotetrahexosylganglioside) the "prototype" ganglioside, globoside and sphingomyelmine to ceramide [40]. Pathway leading to Fabry disease highlighted in red.
**α-Galactosidase A (αGal)**

The enzyme has a large amino-terminal catalytic domain, a small carboxyl-terminal domain, and is present as a head-to-tail homodimer that hydrolyses the terminal alpha-galactosyl moieties from glycolipids and glycoproteins [14]. The N-terminal domain is a classic (β/α)$_8$ barrel, and the C-terminal domain contains eight antiparallel β strands packed into a β sandwich (Figure 1-2) [14]. The authors of this structure conclude that the carboxyl-terminal amino acids are most likely not in contact with the catalytic site being “at least 45 Å from each active site and on the

![Figure 1-2: Crystal structure of human αGal.](image)

The α-galactosidase A polypeptide trace is shown with a rainbow from blue at the N-terminus to red at the C-terminus. N-linked carbohydrates are shown as bonds, and the galactose ligand is shown as spheres, marking the active site in the first domain [14].
opposite face of the molecule” and therefore most likely do not play a role in enzyme activity [10].

Each monomer contains three N-linked carbohydrate sites (Asn-139, Asn-192 and Asn-215) and five disulfide bonds. Elimination of any two glycosylation sites lead to an unstable enzyme and premature degradation, while Asn-215 was shown to be essential for protein solubility [41]. There are a large number of known mutations (over half of the residues) in the GLA gene which encode αGal causing Fabry disease. Most of these mutations lead to disruption of the hydrophobic core of the protein making Fabry disease primarily a disease of protein-folding [42].

**Available therapies**

The FDA has approved treatment of Fabry disease through intravenous administration of recombinant αGal known as enzyme replacement therapy (ERT) [3]. Two different forms of ERT are available for the treatment of Fabry disease; both are administered by intravenous infusion every other week and have been compared [3]. The first is genetically engineered in a human cell line by gene activation (agalsidase alfa, Replagal®, Shire Human Genetic Therapies, Inc., Cambridge, MA), the second produced in a Chinese hamster ovary cell line by recombinant techniques (agalsidase beta, Fabrazyme®, Genzyme Corporation, Cambridge, MA). The recombinant enzymes contain mannose-6-phosphate (M6P) residues that are recognized by the M6P receptor on the cell surface and incorporated into the cell [43]. However, the importance of the M6P uptake system as the sole mechanism of uptake for αGal has recently been questioned [15]. Uptake of αGal is mainly mediated by the high molecular weight endocytic receptor megalin in proximal tubule cells [44]. The sortilin receptor has also been identified as an αGal binding protein important in human glomerular endothelial cells [15]. The endocytic receptors
M6P, sortilin, and megalin have been identified to play a role in αGal cellular internalization in podocytes [45].

The clinical efficacy of ERT for Fabry disease has been shown through several clinical trials [3, 46-49]. Therapy was initially undertaken for males with the classic form of the disease (no detectable αGal activity) [36, 50, 51], but is now also underway for symptomatic heterozygous females [43, 52]. Treatment is also under consideration for adults and children with atypical (low levels of enzyme) Fabry disease. More than 80% of Fabry patients treated with agalsidase-beta and 56% treated with agalsidase-alfa developed an immune response [50, 51]. The antibodies are primarily of the IgG class, in addition, a fraction of the antibodies appear to exhibit neutralizing properties, which has been associated with an increase in urinary globotriaosylceramide (GB3) levels due to the uptake of immune-enzyme complexes by granulocytes in the bloodstream and macrophages in the tissues [53-55].

Fabry disease patients with adverse reactions to the infusions are currently treated with antihistamines and antipyretics and the initial immune response is typically manageable [56]. It can be anticipated however, that life-long treatment required for these patients will lead to unacceptable levels of neutralizing antibodies. The drug cost of ERT is very high, running between $75,000 and $350,000 per patient-year, depending on the body weight of the patient as opposed to a generally accepted threshold for a new drug of $50,000–$150,000 [57]. The limitations of current approaches for ERT for Fabry disease and the need for improved techniques have been discussed [58, 59]. In addition, the effects of gene therapy [60-67] and pharmacological chaperones [68] are under investigation for the treatment of Fabry disease.
The recombinant enzymes prescribed for ERT are produced in mammalian cells (human or CHO cells). Producing adequate amounts of protein for ERT from mammalian cells is expensive. Mammalian cell culture is highly susceptible to viral infections, compromising the constant supply needed by patients. In 2009, a contamination in one of Genzyme’s plants forced a halt to the manufacturing of Fabrazyme, used to treat for Fabry patients, and Imiglucerase (Cerezyme®), for Gaucher patients. This caused a shortage in the United States and lasted until 2012, when the production was moved to a new plant [69]. These cells are used in part because the glycan structure is similar to that of native enzyme, allowing for the necessary M6P exposure and decreasing potential antigen production [43].

Alternative expression systems have been used for the production of αGal. Tobacco cell cultures [70] and the moss (Physcomitrella patens) [1] have both been successfully used to produce active forms of human αGal. Both of these systems have been engineered to perform human-like glycosylation. Recombinant αGal has also been produced in a modified yeast strains where Saccharomyces cerevisiae was manipulated to synthesize glycoprotein that lacks the outer chain of N-glycan, a structure that is specific to yeast but not humans [71]. αGal purified by that group was introduced into Fabry patient fibroblasts and a Fabry mouse model and successfully hydrolyzed accumulating substrates [71]. This study supports the use of yeast as a host for recombinant αGal production for ERT. These alternative systems could help lower the cost of treatments, increase manufacturing safety, and improve availability of ERT therapeutic options for patients decreasing the probability of shortages in the future.

In this work, we use the methylotrophic yeast Pichia pastoris. P. pastoris is the most highly developed of a small group of alternative yeast species chosen for their advantages over S. cerevisiae as expression hosts. This is due in part to the existence of well-established
fermentation methods and the presence of a tightly regulated methanol-inducible promoter $P_{\text{AOX1}}$. Alcohol oxidase (AOX) expression is undetectable in cells cultured on carbon sources such as glycerol, but constitutes up to 30% of total soluble protein in methanol-grown cells. Therefore, genes under the control of the $P_{\text{AOX1}}$ promoter can be maintained in an expression-off mode on a non-methanolic carbon source to minimize selection for non-expressing mutant strains during cell growth, and then efficiently switched on by shifting to methanol. We propose two new and innovative approaches allowing for more effective enzyme therapies and potentially circumventing adverse reactions.

**Scope of work**

**Aim 1: Carboxyl-Terminal Deletions of αGal.** Based on a previous report [2] indicating a possible correlation between C-terminal deletions of human αGal and enzyme activity; the first major goal of this research was to explore the effects of C-terminal deletions of the human αGal on enzyme activity. According to work done by Miyamura’s group, carboxyl-terminal deletions of the human αGal protein led to an increase of activity using a transient expression system [2]. This data was semi-quantitative and relied on comparison of the amounts of RNA present in northern blots of mRNA for αGal compared to β-actin (Figure 1-3). We constructed a set of even (2, 4, 6, 8, 10 amino acids) carboxyl-terminal deletion mutants, and characterized the $K_m$ and $V_{\text{max}}$ of these mutants.
Aim 2: Targeting of αGal to mannose and scavenger receptors of endothelial cells. The scavenger receptors (SRs) bind acetylated or oxidized low-density lipoprotein and macromolecules with negative charge, leading to subsequent degradation in the lysosome [72]. We hypothesized that αGal transported by way of functionalized liposomes or coupled to scavenger ligands would be delivered to lysosomes to degrade accumulated substrates in Fabry disease patients. We also hypothesized that mannose terminated αGal expressed in yeast would target the mannose receptors (MR) present on endothelial cells improving uptake in these cells.
These strategies have the potential to significantly reduce the number of infusions needed for the desired therapeutic effect. Currently there are more than 50 LSDs [50, 73] including Fabry disease that could potentially be treated through ERTs, making the possible contribution of this work to the field vast.
Chapter 2 : Carboxyl-Terminal Truncations Alter the Activity of the Human $\alpha$-Galactosidase A
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Abstract

Fabry disease is an X-linked inborn error of glycolipid metabolism caused by deficiency of the human lysosomal enzyme, αGal, leading to strokes, myocardial infarctions, and terminal renal failure, often leading to death in the fourth or fifth decade of life. The enzyme is responsible for the hydrolysis of terminal α-galactoside linkages in various glycolipids. ERT has been approved for the treatment of Fabry disease, but adverse reactions, including immune reactions, make it desirable to generate improved methods for ERT. One approach to circumvent these adverse reactions is the development of derivatives of the enzyme with more activity per mg. It was previously reported that carboxyl-terminal deletions of 2 to 10 amino acids led to increased activity of about 2 to 6-fold. However, this data was qualitative or semi-quantitative and relied on comparison of the amounts of mRNA present in Northern blots with αGal enzyme activity using a transient expression system in COS-1 cells. Here we follow up on this report by constructing and purifying mutant enzymes with deletions of 2, 4, 6, 8, and 10 C-terminal amino acids (Δ2, Δ4, Δ6, Δ8, Δ10) for unambiguous quantitative enzyme assays. The results reported here show that the $k_{\text{cat}}/K_m$ approximately doubles with deletions of 2, 4, 6 and 10 amino acids (0.8 to 1.7-fold effect) while a deletion of 8 amino acids decreases the $k_{\text{cat}}/K_m$ (7.2-fold effect). These results indicate that the mutated enzymes with increased activity constructed here would be expected to have a greater therapeutic effect on a per mg basis, and could therefore reduce the likelihood of adverse infusion related reactions in Fabry patients receiving ERT treatment. These results also illustrate the principle that in vitro mutagenesis can be used to generate αGal derivatives with improved enzyme activity.
Introduction

Mutations in the αGal gene result in the sphingolipidosis named Fabry disease [74]. The enzymatic defect is inherited as an X-linked disorder and is associated with a progressive deposition of the glycosphingolipids, including globotriaosylceramide, galabioasylceramide, and blood group B substance. In affected males, this leads to early death due to occlusive disease of the heart, kidney, and brain.

De Duve [75] first suggested that ERT might be a successful approach to the treatment of lysosomal storage defects such as Gaucher’s and Fabry disease. For Gaucher’s disease, ERT produced unequivocal clinical responses [76, 77] that were subsequently confirmed by others [78-80]. Classical Fabry disease patients lack detectable levels of αGal [74] so it should not be surprising that more than 80% of Fabry patients treated with agalsidase-beta [50] and more than 50% treated with agalsidase-alfa [51] developed an immune response. The antibodies produced are primarily of the IgG class and a fraction of the antibodies appear to exhibit neutralizing properties. These antibodies have been associated with an increase in urinary globotriaosylceramide levels due to the uptake of immune-enzyme complexes by granulocytes in the bloodstream and macrophages in the tissues [53-55].

ERT for Fabry disease patients was initially undertaken for males with the classic form of the disease (no detectable αGal activity) in a variety of clinical trials [34, 36, 37, 50, 51, 81], but therapy is now also underway for heterozygous females with Fabry disease [43, 46, 82] and is under consideration for children [48, 83, 84] and adults with atypical (low levels of enzyme) Fabry disease [85]. The two products used for ERT in Fabry disease patients have been compared [3]. The pattern of glycosylation on αGal has been analyzed [86] and its importance
for activity [41] and uptake by cells has been established [71, 87]. The limitations of current approaches for ERT for Fabry disease and the need for improved techniques have been discussed [55, 88, 89]. Efforts for gene therapy for Fabry disease are underway [60-65, 67, 90] and molecular chaperones are under investigation for specific alleles[91-93]. Substrate reduction therapy as an augmentation to ERT has been evaluated [94]. There are several reviews on the general topic of ERT for lysosomal storage diseases [95-99].

Expression of the human αGal has been reported in Escherichia coli [12], baculovirus [8, 9] Chinese hamster ovary cells [100] and human foreskin fibroblasts [101]. The highest levels of heterologous αGal expression was observed in P. pastoris [7]. Recombinant αGal has also been produced in a modified strain of S. cerevisiae that synthesized glycoprotein lacking the outer chain of N-glycan, a structure that is specific to yeast but not humans[71, 102]. When this αGal was introduced into Fabry patient fibroblasts or a Fabry mouse model, there was hydrolysis of accumulated substrates [71, 102].

The methylotrophic yeast P. pastoris is the most highly developed of a small group of alternative yeast species chosen for their advantages over S. cerevisiae as expression hosts [103, 104]. Two attributes critical in its selection are the existence of well-established fermentation methods and the presence of the tightly regulated methanol-inducible promoter. AOX expression is undetectable by enzyme assay or mRNA production in cells cultured on carbon sources such as glycerol, but constitutes up to 30% of total soluble protein in methanol-grown cells. Heterologous genes under the control of the P_{AOX1} promoter can be maintained in an expression-off mode on a non-methanolic carbon source in order to minimize expression of potentially toxic heterologous proteins during cell growth. The P. pastoris expression system has now been successfully used to produce a number of heterologous proteins at commercially useful
concentrations [105].

Lysosomal enzymes such as αGal are glycoproteins that are modified in the Golgi to contain N or O-linked carbohydrate structures [106]. The human αGal is glycosylated at Asp residues 139, 193, and 215 [41] with branched carbohydrate structures that vary in composition and sequence depending upon the host species and tissue type [86]. For example, the enzyme purified from humans contains variable amounts (5–15%) of asparagine linked complex and high mannose oligosaccharide chains [74]. Consequently, multiple forms are present in SDS gels and in isoelectric focusing experiments that correspond to the plasma and various tissue forms. The recombinant human αGal preparations used therapeutically are produced in human and CHO cells and these have distinct glycosylation patterns and differ in levels of sialic acid and M6P [3]. The recombinant αGal produced in insect cells [8, 9] and in P. pastoris [7] contain variable levels of mostly complex and high mannose side chains, respectively. Glycoproteins produced in P. pastoris typically contain from 6 to 14 mannose units (Man₆GlcNac₂ to Man₁₄GlcNac₂) that sometimes produces a Gaussian-like distribution of oligomannosides that may center near Man₁₂GlcNac₂ to Man₁₃GlcNac₂ [107].

These carbohydrate moieties serve a structural and functional role. For example, it has been demonstrated that glycosylation, particularly at Asn-215, is required for enzyme solubility [41]. Also, uptake of the enzyme by cells in vivo is affected by terminal M6P residues on the enzyme [87], and the 10–12 sialic acid residues on the plasma form of the enzyme accounts for the prolonged circulatory half-life of the enzyme compared to the tissue form with only one or two sialic acid residues [25]. The identification of these multiple forms as derivatives of the same protein in purified enzyme preparations can conveniently be monitored by treatment with
specific N-glycosidases or by Western blots.

Fabry disease patients with adverse reactions to the infusions are currently treated with antihistamines and antipyretics and the initial immune response has been manageable to date [56, 108], but it can be anticipated that life-long treatment required for these patients will lead to unacceptable levels of neutralizing antibodies. In this context, it is reasonable to devise approaches to circumvent these adverse reactions and the development of derivatives of the enzyme with more activity per mg is a logical approach. Miyamura and coworkers [2] reported that carboxyl-terminal deletions of 2 to 10 amino acids of αGal led to an increase in activity of about 4 to 6-fold as compared to wild type (WT). However, this data was qualitative or semi-quantitative. αGal activity was measured in cell lysates during transient infection of COS-1 cells using the artificial substrate MUG with a mock-transfection being used as a blank background subtraction. These results were then normalized by using the ratio of αGal to actin mRNA present in Northern blots. Here we use a P. pastoris expression system for the construction and purification of mutant enzymes with C-terminal deletions. The quantitative results reported here with purified enzymes reveal that C-terminal deletions results in an increase (Δ2, Δ4, Δ6, and Δ10) or decrease (Δ8) in enzyme activity.

**Materials and Methods**

**Cell strains and plasmids**

The *P. pastoris* host strain X-33 (No. K1740-01), E. coli strains TOP10 (No. C4040-50) and TOP10F’ (No. C665-11), plasmid pPICZαA (No. K1740-01), and TOPO XL PCR cloning kit (No. K4700-10) were purchased from Invitrogen.
Bioreactor expression of recombinant αGal in *P. pastoris*

High-cell-density fermentation was carried out as previously described [7] with a modified growth medium utilizing non-precipitating sodium hexametaphosphate as a phosphate source [109] and modified for a 7 L Applikon bioreactor. Fermentation medium of 3.5 L (0.93 g/l CaSO$_4$, 18.2 g/l K$_2$SO$_4$, 14.9 g/l MgSO$_4$.7 H$_2$O, 9 g/l (NH$_4$)$_2$SO$_4$, 40.0 g/l glycerol) was autoclaved at 121°C for 20 min in the vessel. After cooling to room temperature, filter sterilized sodium hexametaphosphate (25 g/l of fermentation basal salt medium dissolved in 500 ml of deionized water) and 0.435% PTM1 trace elements (CuSO$_4$.5 H$_2$O 6.0 g, NaI 0.08 g, MgSO$_4$. H$_2$O 3.0 g, Na$_2$MoO$_4$.2 H$_2$O 0.2 g, H$_3$BO$_3$ 0.02 g, CoCl$_2$ 0.5 g, ZnCl$_2$ 20.0 g, FeSO$_4$.7 H$_2$O 65.0 g, biotin 0.2 g, 5.0 ml H$_2$SO$_4$ per liter) were added to complete the fermentation medium. The pH was adjusted to 6.0 using ammonium hydroxide (28%).

Four frozen MGY cultures of 4 ml each were used to inoculate four 100 ml MGY cultures in 1-liter baffled flasks and grown at 250 rpm and 30°C until the OD$_{600}$ reached 2 to 6. The cultivation was divided into three phases, the glycerol batch, glycerol-fed batch, and methanol fed batch. The glycerol batch phase was initiated with 400 ml of inoculum shake-flask culture added to 4 L of the fermentation medium containing 4% glycerol and an initial value of 100% dissolved oxygen until a spike was observed indicating complete consumption of glycerol. Next, the glycerol-fed batch phase was initiated and a 50% w/v glycerol feed rate of 18.15 ml/h/liter initial fermentation volume and maintained until a cell yield of 180 to 220 g/liter wet cells was achieved. At this point the glycerol feed was terminated manually and a methanol-fed batch phase was initiated by starting a 100% methanol feed containing 12 ml PTM1 trace salts per liter. Methanol was initially fed at 3.6 ml/h/liter of initial fermentation volume, then increased to 7.3 ml/h/liter and finally increased to 10.9 ml/h/liter of initial fermentation volume for the
remainder of the fermentation. Dissolved oxygen spikes were used during the glycerol fed batch phase and methanol-fed batch phase and to monitor substrate levels. A dissolved oxygen level of 40%, pH of 6, and temperature of 25 °C were maintained by an ADI 1030 regulator. Sampling was performed at the end of each phase and at least twice daily and analyzed for cell wet weight and increased αGal activity over time. Cultivation was terminated once a plateau in αGal activity was observed.

Construction of strains

Plasmid pMS118 [12] contains the αGal cDNA cloned as an EcoRI fragment to the EcoRI site of plasmid pUC9. PCR primers (Figure. 2-1, 2-2) were used with plasmid pMS118 DNA and the PCR system (Roche, No. 11732641001) according to the vendor’s instructions. This generated cDNAs with a 5′ extension containing an XhoI site, Kex2 and Ste13 yeast signal cleavage sites, a 3′ end with an XbaI site, and a deletion of C-terminal amino acids to generate Δ2 to Δ10 mutants (Figure 2-1, 2-2). The PCR products were ligated to pCR-XL-TOPO to generate Δ2 to Δ10 plasmids (Table 2-1). These plasmids were used for electroporation [7] into E. coli strain TOP10 or TOP10F’ (Table 2-1).
Figure 2-1: Introduction of a C-terminal deletion of 2 amino acids into αGal.

The strategy shown here for the Δ2 mutant was used to generate all five deletion mutations (Figure 2-2). Plasmid pMS118 [12] contains the αGal cDNA cloned as an EcoRI fragment to the EcoRI site of plasmid pUC9. Primers AAF and AARD2 (Figure 2-2) were used as PCR primers for plasmid pMS118 DNA to generate cDNAs with a 5′ extension containing an XhoI site, Kex2 and Ste13 yeast signal cleavage sites, a 3′ end with an introduced XbaI site, and a deletion of C-terminal amino acids to generate the Δ2 mutant. Primer AAF anneals to the cDNA at the sequences encoding the N-terminal sequences of αGal and primer AARD2 anneals to the C-terminal sequences of αGal. Primer AARD2 anneals 12 nucleotides from the 3′ end of the cDNA and introduces a stop codon (UAA) after the aspartate codon three amino acids from the C-terminal end of the coding sequences of αGal resulting in a deletion of the two C-terminal amino acids (Leu-Leu) of the human enzyme (right panel). Cloning to the XhoI and XbaI sites of plasmid pPICZαA generates a protein fusion with the yeast signal peptide coding sequences in the vector. This signal peptide is removed by the Kex2 and Ste13 yeast signal peptidases through cleavage immediately upstream of the leucine corresponding the first amino acid of the mature form of αGal (left panel). This strategy was generalized to create the other deletion mutants using the primers in (Figure 2-2). In the left panel, the N-terminal peptide LDNGLR was identified in mass spectrometric analysis while EALDNGLR was not (Figure 2-5).
Figure 2-2: Primers and αGal cDNA used to generate Δ2, Δ4, Δ6, Δ8 and Δ10 mutant cDNAs.

DNA primers AAF, AARD2, AARD4, AARD6, AARD8, and AARD10 corresponding to the Δ2, Δ4, Δ6, Δ8 and Δ10 mutants were annealed to the cDNA of mature αGal contained within pMS118 to generate 3' end truncated PCR products for carboxy-terminal deleted enzymes. Primers AAF and primers AARD2 to AARD10 were used to introduce an XbaI site (indicated above) and a premature UAA stop codon via an antisense ATT triplet immediately downstream of nucleotides complementary to αGal (bold font) to produce cDNAs encoding for Δ2, Δ4, Δ6, Δ8, Δ10 mutants. The boxed LDNGLAR and SHNPTGTYLLQENPMOM protein sequences (indicated above) are peptide fragments that were identified through mass spectrometry of the Δ6 mutant (Figure 2-5).
DNA sequence analysis using the universal M13 primers (Table 2-2) confirmed the expected insert for these pCR-XL-TOPO derivatives. The modified cDNAs were excised from pCR-XLTOPO plasmids using XhoI and XbaI endonucleases and ligated into expression plasmid pPICZαA treated with the same two restriction enzymes to generate expression plasmids (Table 2-1) that were subsequently used for electroporation [7] into strain TOP10 or TOP10F′ (Table 2-1). The nucleotide sequence of mutant cDNAs in pPICZαA derivatives was analyzed (Genewiz) using 5′ AOX, 3′ AOX and α-factor sequencing primers (Table 2-2). These pPICZαA derivatives were used for electroporation of P. pastoris strain X-33 to generate yeast expression strains (Table 2-1).
Table 2-2: Primers Used for DNA Sequence Analysis.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' AOX</td>
<td>5' GACTGGTTCCAATTGACAAGC 3'</td>
<td>DNA sequencing primer for pPICZαA</td>
</tr>
<tr>
<td>3' AOX</td>
<td>5' GCAATGGCATTCTGACATCC 3'</td>
<td>DNA sequencing primer for pPICZαA</td>
</tr>
<tr>
<td>α-factor</td>
<td>5' TACTATTGCCAGCATGCTGC 3'</td>
<td>DNA sequencing primer for pPICZαA</td>
</tr>
<tr>
<td>M13: forward</td>
<td>5' GTAAAACGACGGCCAG 3'</td>
<td>DNA sequencing primer for pCR-XL-TOPO</td>
</tr>
<tr>
<td>M13: reverse</td>
<td>5' CAGGAAACAGCTATGAC 3'</td>
<td>DNA sequencing primer for pCR-XL-TOPO</td>
</tr>
</tbody>
</table>

Note. Primers were HPLC purified, 50 nmoles from Invitrogen

**Purification of αGal using double affinity chromatography**

Purification was as described [7, 110] with minor modifications (below). Bioreactor supernatant was passed through a 0.2 µm hollow fiber filter (Spectrum Labs, No. M22M-300-01N) and subjected to diafiltration using a 50 kDa pore size hollow fiber filter (Spectrum Labs, No. M25S-300-01N) against wash buffer (0.1 M sodium acetate buffer, pH 6.0, 0.1 M NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 1 mM MnCl₂). The resulting supernatant was applied to a Con A Sepharose 4B (GE Healthcare No. 17-0440-01) column, pre-equilibrated with wash buffer, and washed with 5 column volumes of wash buffer. It was observed that near-saturating sugar eluent concentrations do not improve glycoprotein recovery as compared to lower concentrations and that elution
phase pauses improve recovery [111]. In accordance with these findings, elution of αGal was carried out using modified elution buffer I (0.5 M methyl-α-D-mannopyranoside, 0.25 M methyl-α-D-glucopyranoside in wash buffer) over 1.5 column volume blocks separated by 12-hour interval soaks. Elution was discontinued when the absorbance at 280 nm and enzyme assays showed negligible presence of protein and αGal activity. No substantial difference in recovered enzyme was observed between purifications carried out with modified elution buffer I versus sugar saturated elution buffer I (data not shown). The Con A pool was subjected to diafiltration using a 50 kDa pore size hollow fiber filter (Spectrum Labs, No. M25S-30001N) against binding buffer (25 mM citrate-phosphate buffer, pH 4.8 containing 0.1 M NaCl).

The Con A pool was applied to an immobilized-D-galactose gel column (Thio-Gal, Pierce No. 20372) pre-equilibrated with binding buffer. The column was washed with 5 column volumes of binding buffer and αGal was eluted with elution buffer II (25 mM citrate-phosphate buffer, pH 5.5, 0.1 M NaCl, 0.1 M D-galactose) over 1.5 column volume blocks separated by 12 hour soaks. Fractions were assayed for enzyme activity and protein concentration and a peak tube with high specific activity was chosen as the sample to be used in a substrate saturation curve.

**Electrophoresis analysis**

Samples (8 µg) were mixed with an equal volume of reducing sample buffer (Bio-Rad Laemmli sample buffer with 5% β-mercaptoethanol) and heated for 5 minutes at 95 °C before loading on a Mini-Protean TGX Precast Gel 4–20% (w/v) (Bio-Rad No. 456-1094). Bands were visualized by Coomassie blue staining via the modified Fairbanks protocol [112].

**Western blot analysis**

Western blot analysis was performed using an anti-αGal polyclonal antibody produced in
chicken (Pierce/ThermoSci #PA1-9528) and horseradish peroxidase-conjugated anti-Chicken IgY antibody (Sigma #A9046). After SDS-PAGE (2 µg of samples loaded), the gel was incubated with a nitrocellulose membrane (Whatman, No. 10402594) for 15 minutes at room temperature in Transfer Buffer (48 mM Tris, 39 mM glycine, 20% MeOH, pH 9.2) and the proteins were then transferred to the nitrocellulose membrane using a Bio-Rad Trans Blot SD Semi-Dry Transfer Cell. The membrane was blocked with 8% (w/v) non-fat dried milk in PBST [10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl and 0.2% Tween 20 (pH 7.4)] at room temperature for 20 minutes. The membrane was then treated with primary antibody diluted in a milk/blot solution [1% (w/v) non-fat dried PBST] for 2 h at room temperature with mild shaking. After rinsing with PBST solution, the membrane was treated for 1 h at room temperature with secondary antibody diluted in the milk/blot solution. Protein bands were visualized on Kodak BioMax XAR film (VWR #IB1651454) with a Konica SRX-101A processor.

**Enzyme and protein assays**

Activity of αGal was assayed using the synthetic substrate, 4-methylumbelliferyl-α-D-galactopyranoside (MUG) as described [7] with modifications to a microtiter plate format (below). Enzyme activity is measured in units/ml where one unit is defined as the amount of enzyme required to convert 1 nmole of MUG to 4-methylumbelliferone in one hour at 37 °C. An aliquot of 3 µl was added to 27 µl of enzyme assay buffer (5 mM MUG in 40 mM sodium acetate buffer, pH 4.5). This mixture was incubated at 37 °C and 10 µl aliquots were taken at two time points and added to 290 µl of 0.1 M diethylamine in a microtiter plate to stop the reaction. Typically, time points were chosen as 1–4 minutes and values that were proportional to time were considered valid. The fluorescence of each sample was measured at an excitation
wavelength of 365 nm and an emission wavelength of 450 nm using a Tecan Infinite F200 microtiter plate reader. A standard curve of 10 µl of 0 – 0.5 nmol 4-methylumbelliferone dissolved in MeOH in 290 µl of 0.1 M diethylamine was used to quantitate MUG cleavage at specific time intervals. Analysis of the effects of MeOH indicated no effect on the 4-methylumbelliferone standard curve.

For samples containing higher protein concentrations, the BioRad DC Protein Assay (No. 500-0116) with a standard curve of (0.2 – 1.5) mg/ml was used according to the manufacturer’s specifications. For dilute samples of purified αGal, a more sensitive fluorescence-based fluorescamine assay [113] with a standard curve containing lower protein concentrations of (4.0–160) µg/ml was used. Briefly, 150 µl of 0.05 M sodium phosphate buffer and 50 µl of 1.08 mM fluorescamine dissolved in acetone were added to an aliquot of 50 µl of the sample and standards, mixed and incubated for 12 minutes. The fluorescence of each sample was measured at an excitation wavelength of 400 nm and an emission wavelength of 460 nm. Bovine serum albumin (Bio-Rad No. 500-0112) was used as the standard in both assays. Absorbance and fluorescence measurements were conducted on a Tecan Infinite F200 microplate reader using 96-well plates.

**Mass spectrometry of a purified mutant enzyme**

The Δ6 mutant was selected for mass spectrometry analysis conducted at the Rockefeller University Proteomics Resource Center in collaboration with M.T. Mark. SDS-PAGE gel slices were washed, de-stained, reduced using 10 mM dithiothreitol, alkylated using 100 mM iodoacetamide, and digested using trypsin. Peptides were then extracted from the gel two times, dried, and re-suspended in a 5% acetonitrile and 2% formic acid mixture. One third of each sample was loaded onto a C18 PepMap1000 micro-precolumn (300 µm I.D., 5 mm length, 5 µm
beads, Thermo Scientific) at a flow-rate of 5 µl/min, and subsequently onto an analytical C18 column (75 µm I.D., 3 µm beads, Nikkyo Technos Co.) at a flow rate of 300 nl/min. The gradient was 40 min long in the range 5 to 45% B (buffer A was 0.1% formic acid in water, and buffer B was 0.1% formic acid in acetonitrile). Eluted peptides were applied by electrospray directly into the LTQ-Orbitrap XL mass spectrometer from Thermo Scientific, operating in a 300 to 1800 m/z mass range. Tandem mass spectrometry was performed by collision induced dissociation using nitrogen as a collision gas. The resulting spectra were analyzed using Mascot and Proteome Discoverer 1.3 (Thermo Scientific) to identify the peptides in the sample.

Thermostability and pH optimum of WT and mutant αGal
Purified enzyme samples were diluted in 25 mM citrate-phosphate buffer, pH 5.5, 0.1 M NaCl, 0.01 M D-galactose. Samples of 50 µl were incubated in triplicate at 50 °C, 30 °C and 40 °C. Aliquots of 3 µl were removed for enzyme assays every 15 minutes for two hours. Samples were assayed in 0.02 M citrate buffer, pH 3.0-pH 6.5, containing 2 mM MUG.

Characterization of kinetic properties
Substrate saturation curves for αGal have been reported using MUG at concentrations up to 2 mM, 5 mM, and 10 mM (in the presence of 0.1% BSA and 0.67% EtOH [3]). We noted that under our experimental conditions MUG is fully soluble at 2 mM, partially soluble at 5 mM, and chemically oversaturated at higher concentrations. Other investigators reported the use of sonication or detergent treatment to increase the solubility of MUG (e.g., [26]) but we avoided this approach in order to avoid potential artifacts due to the use of these techniques. Substrate saturation curves using 2 mM and 5 mM MUG as the highest concentrations were carried out and the kinetic parameters for αGal were calculated separately obtaining similar values. The values reported here (Table 2-3A) were obtained using a substrate saturation curve of 0.3 to 2
mM MUG since this is the highest concentration that is fully soluble under our experimental conditions. The $K_m$ and $V_{\text{max}}$ values were calculated using Lineweaver-Burk and non-linear regression through the program Sigma-Plot (Systat Software, San Jose, CA).

Kinetic parameters were also determined using the colorimetric substrate, para-nitrophenyl-α-D-galactopyranoside (PNPaGal) (Table 2-3B). [114] Purified enzymes were diluted to approximately 20,000 units/mL as determined by fluorescent MUG assay. These diluted samples were then added at a proportion of 1:9 citrate-phosphate buffer (0.1 M) containing 7 - 50 mM PNPαGal. Aliquots of 20 µl of the enzymatic reaction were removed at 15 minute intervals to terminate the reaction over the course of an hour and added to 320 µl of borate buffer (pH 9.8) in

---

**Table 2-3: Values of $K_m$, $V_{\text{max}}$, $k_{\text{cat}}$ and and the specificity constant ($k_{\text{cat}}/K_m$) for WT and C-Terminal Deletion Mutants of $\alpha$Gal.**

<table>
<thead>
<tr>
<th>A) MUG</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Comments</td>
<td>$K_m$ (mM)</td>
<td>$V_{\text{max}}$ (mmole/hr/mg)</td>
<td>$k_{\text{cat}}$ (s$^{-1}$)</td>
<td>$k_{\text{cat}}/K_m$ (mM$^{-1}$ s$^{-1}$)</td>
</tr>
<tr>
<td>WT</td>
<td>2.44 ± 0.44</td>
<td>3.36 ± 0.29</td>
<td>84.0</td>
<td>34.4</td>
</tr>
<tr>
<td>Δ2</td>
<td>4.52 ± 0.62</td>
<td>5.56 ± 0.73</td>
<td>139</td>
<td>30.8</td>
</tr>
<tr>
<td>Δ4</td>
<td>3.51 ± 0.29</td>
<td>7.29 ± 0.74</td>
<td>182</td>
<td>51.9</td>
</tr>
<tr>
<td>Δ6</td>
<td>4.21 ± 0.52</td>
<td>4.89 ± 0.32</td>
<td>122</td>
<td>29.0</td>
</tr>
<tr>
<td>Δ8</td>
<td>3.89 ± 0.27</td>
<td>0.742 ± 0.21</td>
<td>16.6</td>
<td>4.78</td>
</tr>
<tr>
<td>Δ10</td>
<td>2.96 ± 0.29</td>
<td>6.90 ± 0.71</td>
<td>173</td>
<td>56.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B) PNPαGal</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Comments</td>
<td>$K_m$ (mM)</td>
<td>$V_{\text{max}}$ (mmole/hr/mg)</td>
<td>$k_{\text{cat}}$ (s$^{-1}$)</td>
<td>$k_{\text{cat}}/K_m$ (mM$^{-1}$ s$^{-1}$)</td>
</tr>
<tr>
<td>WT</td>
<td>15.0 ± 2.0</td>
<td>2.51 ± 0.17</td>
<td>62.8</td>
<td>4.18</td>
</tr>
<tr>
<td>Δ2</td>
<td>13.3 ± 1.2</td>
<td>5.14 ± 0.82</td>
<td>128</td>
<td>9.65</td>
</tr>
<tr>
<td>Δ4</td>
<td>15.7 ± 1.0</td>
<td>5.74 ± 0.39</td>
<td>143</td>
<td>9.13</td>
</tr>
<tr>
<td>Δ6</td>
<td>13.4 ± 1.1</td>
<td>1.89 ± 0.23</td>
<td>47.3</td>
<td>3.53</td>
</tr>
<tr>
<td>Δ8</td>
<td>13.0 ± 1.5</td>
<td>0.68 ± 0.08</td>
<td>17.0</td>
<td>1.31</td>
</tr>
<tr>
<td>Δ10</td>
<td>17.0 ± 3.0</td>
<td>6.24 ± 0.12</td>
<td>156</td>
<td>9.18</td>
</tr>
</tbody>
</table>

Note. The values given are for the human enzyme purified from *P. pastoris* and assayed in triplicate followed by Lineweaver-Burk and non-linear regression analysis. Comparison of both Lineweaver-Burk and non-linear regression kinetic parameters show good general agreement (data not shown). Non-linear regression results are displayed above. The $k_{\text{cat}}$ was calculated using 90 kDa as the MW of $\alpha$Gal. A) MUG was used as the substrate for enzyme assay. Mean and standard deviation measurements are from multiple assays of three independent enzyme preparations for the Δ8 enzyme, two independent enzyme preparations for the WT enzyme, and single enzyme preparations for the other mutant enzymes. B) PNPαGal was used as the substrate for enzyme assay.

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a microplate. Product formation was monitored by absorbance at 400 nm. Linear reaction velocities were observed for all measurements. A standard curve of 0–150 µM p-nitrophenylate in borate buffer (pH 9.8) [115] was used to quantitate product formation. \( K_m \) and \( V_{\text{max}} \) parameters were determined through non-linear regression using Sigma-Plot (Systat Software, San Jose, CA).

**Protein structure analysis**

The crystal structure of \( \alpha \text{Gal} \) (PDB 1R47) was viewed and analyzed in PyMOL (Delano Scientific). The MSLDKLL and QMSLKDLL peptides corresponding to the last 7 or 8 C-terminal amino acids of \( \alpha \text{Gal} \) were built in PyRosetta [116] and visualized in PyMOL [117]. Interatomic distances were measured using the PyMOL wizard distance command.

A homology model of the coffee bean \( \alpha \)-galactosidase was generated on the Phyre2 server [118]. The primary sequence of coffee bean \( \alpha \)-galactosidase (GenBank No. AAA33022.1) was set as the query. The crystal structure of rice \( \alpha \)-galactosidase (73% sequence identity to coffee \( \alpha \)-galactosidase, PDB# 1UAS) was set as the template. Superposition of the coffee homolog and human crystal structure of \( \alpha \text{Gal} \) (PDB# 1R47) was conducted in PyMOL [117]. Primary sequence alignments were carried out in ClustalOmega (EMBL-EBI).

**Results**

**Purification of WT and mutant \( \alpha \text{Gal} \)**

The WT and mutant enzymes were obtained from a 7 L bioreactor and purified (Table 2-4, Figure 2-3) using Con A and Thio-Gal tandem affinity chromatography. This two-column purification is simpler and faster than our previous purification methods that used three [9] or
four [7, 8] columns and the yield, degree of purity, and final specific activities were similar for all three methods.

Table 2-4: Purification Table for WT αGal Expressed in *P. pastoris*.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total Protein (mg)</th>
<th>Total Activity Units × 10³</th>
<th>Specific Activity Units/mg × 10³</th>
<th>Purification (Fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>10.928</td>
<td>134</td>
<td>610</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Con A Pool</td>
<td>138</td>
<td>30.4</td>
<td>221</td>
<td>18.1</td>
<td>22.8</td>
</tr>
<tr>
<td>Thio-Gal Pool</td>
<td>4.18</td>
<td>15.7</td>
<td>3,771</td>
<td>309</td>
<td>11.8</td>
</tr>
</tbody>
</table>

Note. 5 mM MUG was used as the substrate for enzyme assay.

The non-glycosylated form of αGal (41.8 kDa) is isolated from cells as multiple glycosylated species with a predominant band of about 50 kDa and multiple higher molecular weight forms that differ in extent of glycosylation (Figure 2-3; See Introduction). We have previously demonstrated that high molecular weight glycoforms produced in insect cells and *P. pastoris* can be identified as derivatives of αGal rather than contaminants and these glycoforms are converted to a single band on SDS gels of about 41.8 kDa with endoglycosidase treatment [7-9]. In this report we also use a Western blot (Figure 2-4) to confirm that the high molecular weight forms seen on SDS gels for the WT and deletion mutants are all glycoforms of αGal. In some cases, lower molecular weight species present in purified enzyme preparations can be identified as αGal fragments in Western blots (e.g., Figure 2-4, lane 2). We quantitated the distribution of glycoforms in (Figure 2-3, S1 Fig, online supplement) and there is no obvious correlation between the glycosylation pattern and catalytic activity. It is well established that glycosylation affects enzyme stability and enzyme uptake (above) but to our knowledge there is no evidence that the glycosylation pattern affects the catalytic properties of this enzyme.
Figure 2-3: SDS-PAGE for purification of αGal.
Purified samples were run on a 4–20% polyacrylamide gel, under reducing conditions, and stained with Coomassie Brilliant Blue. The contents of the lanes are as follows: molecular weight marker (lane 1 and 8), WT(PC626) (lane 2), Δ2 (PC995) (lane 3), Δ4 (PC897) (lane 4), Δ6 (PC958) (lane 5), Δ8 (PC973) (lane 6), Δ10 (PC960) (lane 7). The minor bands present in the purified fraction are consistent with high molecular weight glycoforms seen previously when WT enzyme was purified from the same *P. pastoris* expression system [7].
Figure 2-4: Western Blot of purified WT and mutant αGal.
Purified WT and mutant enzymes were subjected to Western blotting using a polyclonal antibody raised against residues 55–64 and 396–407 of αGal. (a) Blot at shorter and (b) longer exposure.
Mass spectrometry of a purified mutant enzyme

Two possible amino terminal amino acids, glutamate or leucine, could be produced in *P. pastoris* depending upon the selection of the signal peptidase, Kex2 or Ste13 (Figure 2-1). Due to the fact that potential improper amino terminal processing may have an effect on kinetics, we selected one of the purified mutant enzymes (Δ6) for mass spectrometry analysis in order to identify the amino terminal sequence of this enzyme. This analysis also made it possible to provide independent verification of the expected changes in the C-terminal amino acid sequence predicted by in vitro mutagenesis (Figure 2-1, 2-2).

The mature form of the enzyme (signal peptide removed; [119]) produced in humans begins with a leucine codon (Figure 2-1, 2-2). Therefore, tandem mass spectrometry following tryptic digestion of the Δ6 αGal purified from *P. pastoris* could produce tryptic peptides EALDNGLAR or LDINGLAR, depending upon the use of the Kex2 or Ste13 protease sites (Figure 2-1, 2-2). A putative LDINGLAR peak was identified in the MS spectra with an m/z of 379.71, consistent with the (M+2H)\(^2+\) state of this peptide, while no peaks consistent with an EALDNGLAR peptide were found. We cannot eliminate what we consider to be the less likely possibility that the failure to detect the EALDNGLAR peptide may be due to the failure of the peptide to ionize in this MS experiment. Further fragmentation of the m/z = 379.71 associated peptide peak produced an MS/MS spectrum containing 4 of 7 possible y-ions and 4 of 7 possible b-ions from the expected fragmentation pattern of a hypothetical LDINGLAR peptide (Figure 1-5a). This result indicates that the Ste13 signal peptidase of *P. pastoris* generates an enzyme with an amino terminus identical to the enzyme produced in humans.
A search for a Δ6 C-terminal tryptic peptide of SHINPTGTVLLQLENTMQM (Figure 1-2) yielded a matching MS m/z = 1064.03 peak consistent with its (M+2H)$^{2+}$ state. Further fragmentation also produced an MS/MS spectrum containing 4 of 19 possible y-ions and 9 of 19 possible b-ions consistent with the anticipated sequence (Figure 1-5b). This result confirms the predicted C-terminal deletion of 6 amino acids and confirms the efficacy of the mutagenesis protocol used to produce this mutant enzyme.

Thus, the purified Δ6 αGal mutant possesses an N-terminal sequence corresponding to the mature form of αGal and a C-terminal sequence truncated by six amino acids.
Thermostability and pH optima of WT and deletion mutants of αGal

Figure 2-6: Thermostability profiles of WT and mutant αGal. Stability of recombinant WT and Δ2 to Δ10 mutant αGal at 30°C (a), 40°C (b), and 50°C (c) at pH 5.5 as monitored by fluorescent enzyme assay. Initial activities ranged from approximately 300 to 1,900 units/mL for all enzymes assayed. % Activity is normalized against activity at t = 0 mins. Data points for (a) and (c) are the mean of a triplicate measurement with error bars equivalent to ±1 standard deviation. Data points for (b) are the results of a single measurement. MUG was used as the substrate for enzyme assay.
Preparations of purified WT and mutant αGal show similar thermostability profiles at 30°C, 40°C, and 50°C, with activity half-lives of 30, 25 and 17 minutes, respectively (Figure 2-6). The general trend of these profiles agrees with previous results [120]. All enzymes show optimal activity near pH 4.5 (Figure 2-7) in accord with previous reports for WT αGal [16, 114, 121, 122], and there is no significant difference in the activity optima of purified WT and mutant αGal.

**Figure 2-7:** pH activity curves of WT and mutant αGal.

pH activity curves for WT and Δ2 to Δ10 mutant αGal. % Activity is normalized against each enzyme’s peak activity. Data points are the mean of a triplicate measurement and error bars are ± 1 standard deviation. MUG was used as the substrate for enzyme assay.
Kinetic analysis of WT and C-terminal deletion mutants

The values for $K_m$ and $V_{max}$ for WT enzyme (Table 2-3a) are in accord with published values (Table 2-5). The range of $K_m$ and $V_{max}$ values for the enzymes purified from several sources in various laboratories over a period of more than 30 years (Table 2-5) are in good agreement and the observed subtle variations are in the range expected. However, more precision is expected for measurements recorded for enzymes purified from the same source in a single laboratory at one given time (Table 2-3a). Substrate saturation curves (Figure 2-8a) and the calculated values for $K_m$, $V_{max}$, $k_{cat}$, and $k_{cat}/K_m$ using the MUG substrate (Table 2-3a) reveal differences in the enzyme activity of the mutants compared to WT. Deletions of 2, 4, 6 and 10 amino acids approximately double the $k_{cat}/K_m$ (0.8 to 1.7-fold effect; 29/34.4 = 0.8 and 58.3/34.4 = 1.7) while a deletion of 8 amino acids decreases the $k_{cat}/K_m$ (7.2-fold effect; 34.4/4.78 = 7.2). There are corresponding changes in the $V_{max}$ values and deletions of 2, 4, 6 and 10 amino acids approximately double the $V_{max}$ (1.5 to 2.2-fold effect; 4.89/3.36 = 1.5 and 7.29/3.36 = 2.2) while a deletion of 8 amino acids decreases the $V_{max}$ (4.5-fold effect; 3.36/0.742 = 4.5). There are also smaller differences in the $K_m$ values of the C-terminal deletion mutants compared to the WT (Table 2-3a). The $V_{max}$ data presented for the Δ8 (0.742 ± 0.21) and WT (3.36 ± 0.29) are derived from multiple assays from three and two independent enzyme samples, respectively, and this indicates the reliability of this data and adds strength to the interpretations of the data from the single enzyme preparations used for the other deletion mutants.
Table 2-5: Literature Values for $K_m$ and $V_{max}$ for the WT Human αGal.

<table>
<thead>
<tr>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (mmole/hr/mg)</th>
<th>Source</th>
<th>Reference</th>
<th>year</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.9</td>
<td>1.7</td>
<td>Liver</td>
<td>[16, 17]</td>
<td>1979</td>
</tr>
<tr>
<td>1.9</td>
<td>NA</td>
<td>Plasma</td>
<td>[25]</td>
<td>1979</td>
</tr>
<tr>
<td>2.5</td>
<td>NA</td>
<td>Spleen</td>
<td>[25]</td>
<td>1979</td>
</tr>
<tr>
<td>2.0</td>
<td>2.8</td>
<td>Spleen</td>
<td>[26]</td>
<td>1981</td>
</tr>
<tr>
<td>2.3</td>
<td>2.3</td>
<td>Sf9 insect cells</td>
<td>[27]</td>
<td>2000</td>
</tr>
<tr>
<td>2.0</td>
<td>4.8</td>
<td>Replagal</td>
<td>[3]</td>
<td>2003</td>
</tr>
<tr>
<td>4.0</td>
<td>3.3</td>
<td>Fabrazyme</td>
<td>[28]</td>
<td>2009</td>
</tr>
<tr>
<td>2.8</td>
<td>2.6</td>
<td>COS-7 cells</td>
<td>[29]</td>
<td>2007</td>
</tr>
<tr>
<td>4.5</td>
<td>3.3</td>
<td>COS-7 cells</td>
<td>[30]</td>
<td>2011</td>
</tr>
</tbody>
</table>

Note. The values given are for the human enzyme purified directly from human tissues or from the indicated recombinant sources. Replagal is produced in human foreskin fibroblasts and Fabrazyme is produced in CHO cells. The average from these literature values are $2.8 \pm 0.9$ mM ($K_m$) and $3.2 \pm 1.1$ mmole/h/mg ($V_{max}$). NA: not available. MUG was used as the substrate to determine the $K_m$ and $V_{max}$ values.

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The effects of the C-terminal deletions on the kinetic properties of the enzyme using the artificial substrate MUG (Table 2-3a) could be due to alterations in the inherent catalytic mechanism of the enzyme [21]. Alternatively, the altered kinetic properties could be due to changes in the
affinity of the enzyme for specific structural components of the artificial substrate, MUG. In this context, it is of interest to measure these kinetic parameters with an alternative substrate such as PNPαGal. The results (Table 2-3b, Figure 2-8b) indicate that there are similar changes in kinetic parameters using PNPαGal as the substrate, including increases (2.2-fold effect; 9.18/4.18 = 2.2) and decreases (3.2-fold effect; 4.18/1.31 = 3.2) in the $k_{cat}/K_m$ for the specific C-terminal deletion mutants ($\Delta 10$ and $\Delta 8$, respectively). Taken together, these results suggest that the C-terminal deletions likely affect some aspect of the inherent catalytic mechanism of the enzyme.

**Discussion**

This is the first report to establish in a quantitative manner that the C-terminal residues of αGal act as a modulator of catalytic activity. Our results confirm the general results of Miyamura et al. [2] that C-terminal deletions of 2, 4, 6 and 10 amino acids increase the $k_{cat}/K_m$ compared to WT. However, our results differ in that we find that a deletion of 8 amino acids results in a decrease of $k_{cat}/K_m$. It should be noted that there are numerous experimental differences between these two reports. For example, our analysis used purified enzymes expressed in *P. pastoris* and their experiments measured αGal enzyme activity during transient infection of COS-1 cells. There could be differences in αGal mRNA or protein stability between *P. pastoris* and COS-1 cells, and other proteins in the cytoplasm of *P. pastoris* or COS-1 cells could interact directly or indirectly with the αGal protein to affect its catalytic activity.

It is of interest that the two recombinant protein therapeutics, Fabrazyme (agalsidase-beta) and Replagal (agalsidase-alfa) contain C-terminal heterogeneity with truncated species lacking either one or two C-terminal residues [3]. Fabrazyme contains 69.7% full length protein with 7.6% $\Delta 1$ and 22.8% $\Delta 2$, while Replagal contains only 5.7% full length, with 73.2% $\Delta 1$ and 21.1% $\Delta 2$. 
These authors attribute the C-terminal heterogeneity to in vivo proteolytic processing of an undefined nature. These commercial enzymes do not differ significantly from the WT in $K_m$ and $V_{\text{max}}$ (Table 2-5) in spite of this degree of protein heterogeneity [3]. The significantly increased $V_{\text{max}}$ of some of the mutants with C-terminal deletions suggests the basis for an improved treatment for Fabry disease.

These results also illustrate the principle that in vitro mutagenesis can be used to generate αGal derivatives with improved enzyme activity. The potential for improved catalytic activity for this enzyme is illustrated by the existence of closely related enzymes with 3-fold to 250-fold higher activity (Table 2-6). A direct comparison of relevant amino acid residues between the human and related enzymes suggests the basis for rational in vitro mutagenesis to improve catalytic activity of the WT human enzyme. In this context, it is likely that mutants which show altered catalysis against the MUG substrate used here also have a correspondingly higher rate of catalysis against the natural substrate, but this possibility needs to be tested directly.

Clinical trials for ERT show seroconversion frequencies of more than 50% for males treated with 0.2 mg/kg agalsidase-alfa [51] and 88% for 1.0 mg/kg agalsidase-beta [50, 123]. Increasing the dose of administered enzyme in seroconverted patients raised antibody titers in some patients [123, 124]. IgG antibody status shows a strong association with serious infusion associated adverse reactions [124]. IgG positive serum from Fabry patients exhibits in vitro neutralization of enzyme activity [125] and lessens targeting to key disease organs in a Fabry mouse model [126]. The disease marker of urinary globotriaosylceramide levels is increased in seropositive patients as compared to seronegative patients [123, 125]. It has been proposed that higher doses of administered enzyme could overcome the inhibitory effect of antibodies on treatment
effectiveness [123]. By using an enzyme that is more active on a per mg basis, a therapeutic effect equivalent to WT is achievable through administration of a lower dose. The ability to administer a lower dose of the enzyme will intern decrease the amount of harmful antibodies produced in patients making this a better alternative to the current therapy. Future studies should include examination of these mutant enzymes relative to WT in cultured cells [15] and in mouse models [127, 128].

The specificity constant ($k_{\text{cat}}/K_m$) has a maximum possible value determined by the frequency at which enzyme and substrate molecules collide in solution [129] If every collision results in formation of an enzyme-substrate complex, diffusion theory predicts that $k_{\text{cat}}/K_m$ will attain a value of $10^8 - 10^9 \text{M}^{-1}\text{s}^{-1}$ [129]. The $k_{\text{cat}}/K_m$ of WT human αGal is approximately $5.49 \times 10^3 \text{M}^{-1}\text{s}^{-1}$ (Table 2-6) suggesting the possibility that altered forms of the human enzyme may exist that have higher catalytic activity. A BLAST analysis [10] identified the 33 sequences most closely related to αGal and kinetic parameters have been reported for 6 of these enzymes (Table 2-6). These enzymes share a high degree of sequence and structural similarities and are all in the same family 27 of glycosyl hydrolases [10]. There is a broad range in the values reported (Table 1.6) for $K_m$, $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$. Thus, a detailed structural comparison of these enzymes may permit the identification of key amino acid residues that influence these kinetic parameters.

Truncation of the C-terminus of the coffee bean α-galactosidase (Figure 2-9) showed that deletion of one or two amino acids decreases activity and deleting 3 or more residues abolished activity completely [130]. The results with the coffee bean enzyme contrast those presented here for the human enzyme. Both results however demonstrate that the C-terminus of αGal is critical for enzyme function.
We examined a superposition between the crystal structure of the human enzyme and a homology model of the coffee bean enzyme (see Methods) to explore a structural basis for this biochemical dissimilarity. The N-terminal catalytic (α/β)₈ domain superimposes very well (RMSD of 0.729 Å over 1126 total atoms) while the C-terminal antiparallel β-domain superimposes poorly (RMSD of 2.494 Å over 342 total atoms). A primary sequence alignment shows a comparable trend; the catalytic domain shows high sequence conservation while the C-terminal domain does not. An alignment of the C-terminal ends of the human and coffee bean enzymes is presented (Figure 2-9) indicating secondary structure alignment (left) and primary structure alignment (right).
sequence alignment (right). If the C-terminal domain governs a conserved mechanism of enzymatic regulation across the human and coffee homologs, then the deleterious effect of removing C-terminal amino acids from the coffee enzyme is consistent with observations made by Miyamura et al. [2] on the human enzyme.

Because of the low sequence homology observed in the C-terminal residues it can be hypothesized that the effect on the catalytic activity due to C-terminus deletions in both the human and coffee bean α-galactosidase is due to disruption of the enzyme’s three-dimensional structure. This disruption could have an effect on enzyme dimerization, the ability to bind substrate, or potential interactions with other molecules in the cell.

The most straightforward expectation of a series of C-terminal deletions is a direct correlation between the extent of the deletion and the effect on enzyme activity. In this sense, the reduced activity of the Δ8 mutant relative to the other C-terminal deletion mutants (Table 2-3) is unanticipated. However, we note that similar effects have been reported by others who carried out C-terminal deletion studies, including the IN269 mutation for the integrase of HIV [131], the Δ8 and Δ9 mutants of the thymidine kinase of Epstein-Barr virus [132], and the D5 and D10 mutants of the plant vacuolar H (+)-pyrophosphatase [133] Differential proteolysis may also explain why the Δ8 construct does not follow the same trend as the other mutants.
The crystal structure of αGal [10] revealed that the last visible residue of the C-terminus is separated by approximately 45 Å from the active site on the opposite monomer and is too far to have a direct effect on catalysis. However, within the same crystal structure (PDB 1R47) we measured C-terminal end to be only 20–25 Å away from a second ligand-binding site for β-D-galactopyranose on the same monomer [21] which is marked by Tyr 329 rendered as spheres. The C-terminus on monomer A is also separated by 45 Å to 50 Å from the active site of monomer B which is marked by the α-D-galactopyranoside ligand also rendered as spheres.

Figure 2-10: C-terminal Distance from Secondary Binding Site and Opposite Active Site.
The homodimeric crystal structure of αGal (PDB ID 1R47) solved by [10] is displayed in two different perspectives. Distance relationships relative to one out of the two possible C-termini are discussed. The carbon backbone is rendered in a ribbon format. The C-terminus on monomer A is separated by 20 Å to 25 Å from a secondary binding site for β-D-galactopyranose on the same monomer [21] which is marked by Tyr 329 rendered as spheres. The C-terminus on monomer A is also separated by 45 Å to 50 Å from the active site of monomer B which is marked by the α-D-galactopyranoside ligand also rendered as spheres.
distance of 22–26 Å (see Methods), bringing the C-terminus within potential contact distance of the secondary binding site. A crucial missing detail is the functional significance of the secondary site. It may serve as a site for small molecule chaperoning [21]. It also might participate allosterically in a manner similar to phosphofructokinase-1, that is allosterically activated by ADP [134, 135], a product of the enzyme’s ATPase function. The ligand that binds the secondary site on αGal is β-D-galactopyranose [21], which is the mutarotated product of the enzyme’s glycoside hydrolase function. A dynamic interplay may exist between the C-terminus, the secondary site and its ligand to affect the catalytic activity of αGal through allostery or structural stability of the protein. Further mechanistic studies will be needed to work out the exact relationships between these putative components and their relevance to in vitro catalysis.

Due to the lack of direct contact between the carboxyl-terminal amino acids and the catalytic site of αGal, the explanation for the effect of the deletions of the carboxyl-terminal amino acids is not obvious. Another hypothesis to be tested is that αGal is in a class of enzymes like the E. coli dihydrofolate reductase [136-140], in which tunneling and coupled motion accounts for the effects of mutations distal from the catalytic site on enzyme function.

Conclusions

C-terminal truncation mutants of αGal were constructed, expressed and purified from P. pastoris using Con A and Thio-Gal affinity column chromatography. Michaelis-Menten parameters were measured on the purified mutants. Deletion of 2, 4, 6 and 10 amino acids approximately doubles $k_{cat}/K_m$ relative to WT (0.8-1.7-fold effect) while deleting 8 amino acids decreases $k_{cat}/K_m$ (7.2-fold effect). Mutants with increased activity are proposed as an improved alternative therapy over WT enzyme for Fabry disease patients. These results also illustrate the principle that in vitro
mutagenesis can be used to generate αGal derivatives with improved enzyme activit
Chapter 3 : Uptake of α-Galactosidase A by the Scavenger and Mannose Pathways
Abstract

ERT for Fabry disease has so far proved only partially successful in preventing adverse outcomes, presumably due to the lack of efficient delivery of therapeutic αGal to many affected tissues. ERT improves patient symptoms but fatal outcomes persist and storage of the toxic enzyme substrate, GB3, remains in many of the affected organs. Our approach is the development of derivatives of the enzyme with improved uptake properties targeting disease affected tissues such as the brain and in particularly endothelial cells, a major site of accumulation of GB3. A relatively unexplored possibility is to target therapeutic enzyme to the ubiquitous SR uptake system. SRs are abundant on endothelial cells that are not effectively targeted by current ERT mediated by the M6P uptake system used for treatment of LSDs such as Fabry disease. In addition, these treatments do not pass the blood-brain barrier (BBB). The scavenger receptors SR-A3, SR-A5, and SR-BI are expressed in brain capillary endothelial cells and promote receptor-mediated endocytosis and transcytosis for delivery of drugs across the BBB. Production of αGal in Pichia results in mannose terminated recombinant enzyme. This enzyme is hypothesized to target the MR that is known to be expressed in many cell types including endothelial, smooth muscle, and kidney mesangial cells.

We introduced negative charges on HSA and the recombinant human αGal by treatment with cis-aconitic anhydride to generate terminal carboxyl groups (Aco-αGal). We tested the uptake of αGal using Fabry fibroblasts and endothelial cells. Targeting to the SR was tested by monitoring uptake in cells overexpressing the murine scavenger receptor A (U2OS-SRA). Enzyme uptake in cells in culture was monitored by direct enzyme assay and by confocal microscopy using Alexa-tagged-αGal (Alexa-αGal) using fixed cells and a live-cell assay for continuous uptake analysis. Our preliminary results indicate targeting of αGal produced in Pichia to both the SR
and MR uptake pathways with enhanced uptake of aconitylated αGal after short incubation times (30 min) in Fabry endothelial cells. The ability to target αGal to the MR and SR pathways would permit enhanced effectiveness of the currently available M6P-dependent ERT. These results may lead to improved approaches for ERT for Fabry disease patients and for other LSD patients including targeting accumulated substrate in endothelial and neurological tissues dependent upon transit of the BBB.

**Introduction**

The relative tissue and cellular distribution of agalsidase alfa (Replagal™, therapeutic produced in a cultured human cells), was shown to be heterogeneous following a single intravenous injection in a mouse knockout model of Fabry disease [141]. Therefore, in addition to antibody formation, enzyme access to certain sites of tissue substrate storage may be another limiting factor of ERT. Currently approved ERT for Fabry disease [97, 142] was assumed to depend upon the M6P receptor for uptake of αGal. However, the M6P receptor is present on some but not all cell types that are affected in Fabry disease. Endothelial cells, a major site of GB3 accumulation (Figure 3-1), lack M6P receptors [15, 143]. Despite recent studies suggesting uptake of phosphorylated enzyme by the M6P receptor at the BBB in newborn mice [144], or in adult mice using epinephrine as an inducer [145] or in the presence of much higher doses that used in ERT [146] in the LSD Mucopolysaccharidosis type IIIA (MPS IIIA). This uptake however was shown to be very low[147], in addition maintaining high plasma levels of the lysosomal enzyme used in ERT to achieve this uptake would most likely result in a severe immune response. As is the M6P pathway is known to not operate substantially at the BBB [58, 148, 149]. It was suggested that M6P mediated uptake of αGal is not representative of the mechanism of uptake of the enzyme in
cells that are a focus of storage and injury in Fabry disease [15]. Recombinant human αGal produced in plants removes accumulated GB3 even though these enzymes lack terminal M6P residues [1, 70], indicating that M6P receptors are not essential for enzyme uptake. Patients with I-cell disease lack M6P groups on lysosomal enzymes but still transport some enzymes to the lysosome, including αGal, using other uptake pathway(s) [150, 151].

SRs were first defined in 1979 by Brown and Goldstein as macrophage receptors that mediate endocytosis of modified low density lipoprotein (LDL) such as acetylated LDL (Ac-LDL) and oxidized LDL leading to foam cell formation and were shown to play a role in the pathogenesis of atherosclerosis. Currently this definition has been broadened such that SRs are now categorized as a family of molecules sharing the ability to bind polyanionic ligands. Structurally diverse, including both membrane bound and soluble proteins, they are involved in the recognition and/or endocytosis of negatively charged molecules [152]. SRs are grouped into eight different classes (Class A-H) based on shared structural domains (Figure 3-1)[153]. Despite these structural differences, mutagenesis studies have shown that the ligand-binding domain of many of these receptors contains arginine or lysine clusters mediating electrostatic interactions with the negatively charged ligands [154]. Ligand-binding can activate signaling cascades leading to diverse cellular functions or the SR/ligand complexes can undergo endocytosis leading to degradation or accumulation of the ligand in the endosome-lysosome system[154].

The ligands for the SRs involve negatively charged proteins, such as aconitylated, maleylated and succinylated albumins, modified low-density lipoproteins, and polynucleotides [155]. Negatively charged albumins can participate as drug carriers for delivery to SRs [156]. Successful receptor-mediated delivery to macrophages in vitro used low-molecular-weight antitumor agents conjugated with maleylated albumin [157-159]. Maleylated albumin was used
as a targeting carrier for a photosensitizer [160] and a macrophage activating peptide [161, 162] using SRs. In addition, direct succinylation targeted delivery of catalase to liver nonparenchymal cells [156].

The delivery of an enzyme by way of the SR for the therapy of LSDs builds on previous techniques for specific delivery of molecules to cells. For example, sterically stabilized liposomes derivatized with polyethylene glycol were used to deliver doxorubicin to squamous cell lung carcinoma cells by means of specific antibodies attached to the liposome surface [163]. In addition, others have demonstrated massive targeting of liposomes that are surface modified with anionized albumins, one of the techniques we propose here as a future study, to hepatic endothelial cells [164]. This group also demonstrated the successful targeting to the SR of formaldehyde treated HSA [165] and oxidized human LDL [166]. In addition, efficient hepatic uptake of negatively charged proteins and uptake of soluble antigens by dendritic cells through targeting to the SR has been shown [4, 167].

The SR family of uptake systems are highly active on several tissue types including macrophages, monocytes, platelets, endothelial, smooth muscle, and epithelial cells (Figure 3-1)[154]. An important site of accumulation of αGal substrates, including GB3 and other related compounds, is in endothelial cells. In a study of the effect of these SRs, the delivery of acetylated HSA to endothelial cells was described as “massive”, with complete clearance from the blood in 30 minutes, while in the control 80% was still in the blood [164].

SR-AI and SR-BI are expressed at brain capillary endothelial cells and they have been shown to have a very important role at the BBB [17, 24]. The current ERT is prevented from accessing the central nervous system because of the BBB. The SR-BI receptor has been shown to play a role
in the transport of cholesteryl esters at the BBB. This makes these receptors suitable for targeting ERT in Fabry disease to the brain [168].
Figure 3-1: Domain architecture of the different classes of scavenger receptors [22]. Their ligands, and expression profiles [23, 24].
Aconitylation was hypothesized to be a good means of adding negative charge and targeting to the SR for several reasons. Firstly, with aconitylation a net negative charge of three is added for each surface lysine, which is modified; maximizing the potential of adding negative charge to our enzyme (Figure 3-2). Secondly, aconitylation is shown to be reversible at acidic pH (Figure 3-3) and this will generate a normal and fully active αGal at the low pH of the lysosome after ERT in Fabry disease patients. Chloroquine, an inhibitor of lysosomal acidification, blocked deacylation of aconitylated ovalbumin after lysosomal uptake in vivo [4]. Lastly, saturating SRs with aconitylated human serum albumin (Aco-HSA), in vivo, produces no immune response (Figure 3-4)[6].

Figure 3-2: Reaction of surface Lys with cis-aconitic anhydride. Cis-aconitic anhydride coupling to Lys residues introduces a net three negative charges for each Lys residue.

Figure 3-3: Aconitylation is reversible at the acidic pH of the lysosome [4]. Treatment of succinylated (Suc-Ova), maleylated (Mal-Ova), and aconitylated (Aco-Ova) ovalbumin (OVA) at 37° in phosphate buffered saline at pH 5.0 (a) or 7.4 (b)[4]. Reversal of aconitylation will generate a normal and fully active αGal at the low pH of the lysosome after ERT.
A SR system present in the mononuclear phagocytic system on endothelial and Kupffer cells (Figure 3-4) is responsible for the uptake and clearance of Aco-HSA [6]. High plasma concentrations (up to 1 mg/ml) of ¹²⁵I-Aco-HSA did not affect blood coagulation or lymphocyte proliferation, and showed no acute or sub-acute toxicity [6]. The lack of an immune response to Aco-HSA (Figure 3-4) was indicated by sensitive lymphocyte proliferation assays showing that lymphocyte functions were not significantly altered following immunization of rats with complete Freund's adjuvant (an immunopotentiator) for a four-week period [6]. Liver and spleen uptake of ¹²⁵I-Aco-HSA was selectively blocked by the specific SR inhibitors polyinosinic acid (PolyI) or formaldehyde-treated human serum albumin [6]. These results demonstrate the presence of high levels of Aco-HSA distributed throughout the body.

![Figure 3-4: Saturation of scavenger receptors in liver, spleen, kidney, and blood endothelium [6].](image)

Tissue distribution of ¹²⁵I-Aco-human serum albumin 10 min after intravenous injection to male rats in the absence (control) and presence of the scavenger receptor inhibitors Poly-I or formaldehyde-treated human serum albumin (Form-HSA) [6]. There was no detectable immune response to Aco-HSA following immunization of rats for four weeks using complete Freund's adjuvant.
Aco-αGal can potentially target diverse sites of accumulation of GB3 substrate in Fabry disease patients (Figure 3-1), including kidneys, lungs, cardiomyocytes [169, 170], virtually all microvascular endothelial cells, and potentially the brain. Development of an efficient and widespread delivery system for αGal can minimize ERT dose and frequency and this could reduce the immune response to αGal [34, 37, 50, 51, 53-55, 81, 99, 171-173].

Because of the amount of genetic, clinical, and structural information available for Fabry disease, it can serve as model for the entire family of LSDs [14]. Therefore, if successful, these approaches could be applied to enzyme therapy for other LSDs. While ERT can be expected to extend life spans, most patients suffer from ill-defined infusion-associated reactions. Therefore, any improvement in treatment that leads to more effective therapeutic effects or less frequent infusions would markedly improve the lives of Fabry disease patients and other patients receiving ERT for LSDs.

**Methods and Research Design**

**Purification of recombinant αGal**

High-cell-density fermentation was carried out as previously described using a 7L bioreactor [20]. Bioreactor supernatant was passed through a 0.2 µm hollow fiber filter (Spectrum Labs, No. M22M-300-01N) and subjected to diafiltration using a 50 kDa pore size hollow fiber filter (Spectrum Labs, No. M25S-300-01N) against Buffer A (10 mM NaHPO₄, pH 6.5, 0.02% NaN₃, and 0.05 mM PMSF). The resulting supernatant was applied to a DEAE Sepharose Fast Flow (GE Healthcare, No.17-0709-01) column, pre-equilibrated with 5 column volumes Buffer A. Elution of αGal was carried out using a 0 to 1M NaCl gradient in Buffer A. Elution was
discontinued when the absorbance at 280 nm and enzyme assays showed negligible presence of protein and αGal activity. The DEAE elution tubes with sufficient αGal activity were pooled and subjected to diafiltration using the previously described 50 kDa pore size hollow fiber filter (Spectrum Labs, No. M25S-30001N) against binding buffer (25 mM citrate-phosphate buffer, pH 4.8 containing 0.1 M NaCl). The diafiltrated product was applied to an immobilized-D-galactose gel column (Thio-Gal, Pierce No. 20372) pre-equilibrated with binding buffer. The column was washed with 5 column volumes of binding buffer and αGal was eluted with elution buffer II (25 mM citrate-phosphate buffer, pH 5.5, 0.1 M NaCl, 0.1 M D-galactose) over 1.5 column volume blocks separated by 12-hour soaks. Fractions were assayed for enzyme activity and protein concentration and pooled, this pool was chosen as the sample to be used in uptake studies. This two-column purification is simpler and faster than our previous purification methods that used two [20] three [9] or four [7, 8] columns and the yield, degree of purity, and final specific activities were similar for all three methods.

**Enzyme assay**

Activity of αGal was assayed using the synthetic substrate MUG as described [7] with modifications to a microtiter plate format (below). Enzyme activity is measured in units/ml where one unit is defined as the amount of enzyme required to convert 1 n mole of MUG to 4-methylumbelliferone in one hour at 37°C. An aliquot of 3 µl was added to 27 µl of enzyme assay buffer (5 mM MUG in 40 mM sodium acetate buffer, pH 4.5). This mixture was incubated at 37°C and 10 µl aliquots were taken at two time points and added to 290 µl of 0.1 M diethylamine in a microtiter plate to stop the reaction. The fluorescence of each sample was measured at an excitation wavelength of 365 nm and an emission wavelength of 450 nm using a Tecan Infinite F200 microtiter plate reader. A standard curve of 10 µl of 0 – 50 nM 4-methylumbelliferone
dissolved in MeOH in 290 µl of 0.1 M diethylamine was used to quantitate MUG cleavage at specific time intervals. Analysis of the effects of MeOH indicated no effect on the 4-methylumbelliferone standard curve.

**Protein assay**

Absorbance at 280nm on A NanoDrop™ 2000/2000c was used to quantify purified recombinant protein. An Extinction coefficient of 117,475 M⁻¹ cm⁻¹ was calculated for αGal [174] and used for A280 measurements with a molecular weight of the monomer, 45 kDa. A standard Bradford protein assay was also used to confirm results. In short, 10 µl of the sample/standard was combined with filtered 4X diluted Coomassie dye and incubated for 5 minutes. The absorbance was measured at 595 nm using a Tecan Infinite F200 microtiter plate reader. A standard curve of 0 - 400 µg/ml BSA was used.

**Electrophoresis analysis**

Samples were mixed with an equal volume of reducing sample buffer (Bio-Rad Laemmli sample buffer with 5% β-mercaptoethanol) and heated for 5 minutes at 95°C before loading on a Mini-Protean TGX Precast Gel 4–20% (w/v) (Bio-Rad No. 456-1094). Bands were visualized by Coomassie blue staining via the modified Fairbanks protocol [112].

**Cell culture**

FFB (GM2775, Coriell Institute for Medical Research, Camden, NJ) and healthy non-fetal tissue fibroblasts (NFB) (GM04390, Coriell Institute for Medical Research, Camden, NJ) used as a control were grown to confluency in MEM, GlutaMAX™ (Gibco) supplemented with 10% FBS,
Antibiotic-Antimycotic, and MEM nonessential amino acid solution (ThermoFisher) in T-75 flasks at 37 °C and 5% CO₂.

Human Dermal Microvascular Endothelial Cells (HMVEC, Lonza, Walkersville, MD) and the immortalized Fabry endothelial cell line-1 (IMFE-1) cells [175] were generously donated by Prof. R. Schiffmann (Baylor Research Institute), and were plated into T-75 flasks and grown to confluency in the EGM-2MV kit (Lonza, Walkersville, MD) at 37 °C and 5% CO₂ as per that labs recommendation before the experiment.

Human osteosarcoma U2OS (HTB-96TM) cells from ATCC were grown in McCoy’s 5A medium with 1% Pen-Strep and 10% FBS in tissue culture treated T-75 flasks. U2OS-SRA cells (human U2OS osteosarcoma cells over-expressing the murine scavenger receptor A) were generously donated by Professor F. Maxfield (Weill Cornell Medical College) and grown in McCoy’s 5A medium with 1% Pen-Strep, 1 mg/ml G418 and 10% FBS on non-tissue culture treated petri plates at 37 °C and 5% CO₂.

**Evaluation of uptake using enzyme assay**

Fibroblast and endothelial cells were grown till 80-90% confluence on 6-well plates. The medium was removed and 2ml of culture medium containing BSA (5 mg/ml) and appropriate concentration of the purified recombinant αGal was added. plates were then incubated at 37°C and 5% CO₂ for indicated times. Following this incubation, the medium was removed, followed by three washes with PBS. Cells were then harvested using trypsin/EDTA and precipitated by centrifugation. Each cell pellet was re-suspended in 50 µl of 1X Buffer A. Cells were then ruptured by freeze-thawing three times and protein and enzyme assays done on the cell extracts.
Uptake of human αGal was measured by enzyme assay using the synthetic substrate MUG.

**Evaluation of uptake using confocal microscopy**

Recombinant αGal was labeled with Alexa Fluor 488 according to the instructions of the manufacturer (Molecular Probes). Alexa tagging was done in Modified Buffer A at a pH of 7.5. Degree of Labeling (DOL = moles dye / moles protein) of the sample was determined based on obtaining the protein concentration by absorbance at 280 nm and at 494 nm in a NanoDrop™ 2000/2000c.

FFB, IMFE1 and U2OS-SRA cells were grown to near confluence on an 8-well Nunc™ Lab-Tek™ II Chambered Coverglass (Thermo Fisher Scientific, Catalog# 155409) incubated at 37°C and 5% CO₂ with Alexa Fluor 488-labeled αGal (5 µg/ml) for variable time lengths with or without inhibitors in the presence of 5 mg/ml BSA to increase enzyme stability. Cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min at room temperature. Cells were then washed with PBS 3 times and covered with the ProLong® Diamond Antifade Mountant with the nuclear stain DAPI (4',6-diamidino-2-phenylindole) before imaging in a Zeiss Confocal LSM 710. Cells were counterstained with LysoTracker Red DND-99 (L-7528; Molecular Probes) for 2 hours before fixation for co-localization analysis. Uptake was quantified using ImageJ.

**Live cell imaging**

Live cell imaging was performed using a ZEISS LSM 880 Super-Resolution confocal laser-scanning microscope with Airyscan and CO₂ incubator was used. Cells were incubated with Alexa488 tagged enzyme in FluoroBrite™ DMEM and Prolong Live Antifade Reagent
(Invitrogen) in the presence of 5 mg/ml BSA to increase enzyme stability. Images were acquired every 10 minutes. ImageJ was also used to quantify live cell images. Cells were incubated with LysoTracker Deep Red to visualize lysosomes and evaluate uptake of enzyme to lysosomes (co-localization).

**Quantification of uptake in confocal images**

ImageJ was used to quantify the fluorescence intensity of enzyme and lysosomes and to determine colocalization. Images were loaded on the ImageJ software and the channels separated. Particle analysis was performed on both the green and red channel images (Figure 3.6). Co-localization of the LysoTracker Red and αGal particle images was visualized using the co-localization highlighter plugin. (Figure 3-9) Particle analysis was performed on the co-localized image generated as well as the Alexa and LysoTracker channels (Figure 3-10).

**Aconitylation**

Aconitylation was carried out as per published methods with minor changes to accommodate αGal instability at high pH (Figure 3-19) [165, 167, 176]. The surface lysine residues of samples in buffer at a pH of 7.5 were reacted with excess cis-aconitic anhydride and stirred at 4°C for 1hr. The degree of modification of lysine residues was assessed by estimating the loss of free amino groups as measured by trinitrobenzenesulphonic acid assay (TNBS) [18] or fluorescamine [177] assay post removal of excess anhydride and using a glycine standard curve. In addition, the pre-and post aconitylated samples were analyzed using native PAGE and 2D protein electrophoresis, where the first dimension is an isoelectric focusing gel (IEF) and the second dimension is an SDS-PAGE. IEF gels can be used to detect minor changes in the protein charge.
Quantification of modification by TNBS and fluorescamine assay

The TNBS component reacts readily with primary amino groups of amino acids in aqueous format at basic pH to form yellow adducts which can be monitored at 345 nm. Assay samples were dialyzed in 0.1 M sodium bicarbonate buffer (pH 8.5). The supplied 5% TNBS solution (Thermo Fisher Scientific Inc.) was diluted 500-fold in 0.1 M sodium bicarbonate buffer (pH 8.5). 0.5 mL of the diluted TNBS solution was added to 1mL of protein solution (approximately 1 mg), mixed and incubate at 37 °C for 2 hours. 0.5 mL of 10 % SDS and 0.25 mL of 1 N HCl was added to each sample/standard to stop and stabilize the reaction. Absorbance of the solution was measured using a Tecan infinite F200 at 335nm.

Fluorescamine also reacts with primary amino groups of amino acids [177]. 150 µl of 0.05 M sodium phosphate buffer pH 8.5 and 50 µl of 1.08 mM fluorescamine dissolved in acetone were added to an aliquot of 50 µl of the sample and standards, mixed and incubated at room temperature for 12 minutes. The fluorescence of each sample was measured at an excitation wavelength of 400 nm and an emission wavelength of 460 nm.

2D electrophoresis

Samples were loaded on isoelectric focusing gels (pH 3.0 to 10.0) (Novex, San Diego, CA). The gels were run at 100 V for 1 hour and then at 200 V for 1 hour and 500 V for 30 minutes in an XCM II Mini-Cell per company instructions (Novex, San Diego, CA). The gel was washed with distilled water for 30 seconds and fixed and stained with Crocein Scarlet 7B (Sigma) and Coomassie brilliant blue R-250 (Sigma) to determine the isoelectric point (pI) via the modified Fairbanks protocol [112] with minor modification to incorporate fixation by Crocein Scarlet 7B. Briefly, the proteins separated on IEF Gels were subjected to 2D electrophoresis using
NuPAGE® 4-12% Bis-Tris Protein Gels with a 2D-well to separate focused proteins by mass according to manufacturer specifications. The IEF gel was incubated in 100 mL 20% ethanol for 10 minutes, the desired lane cut out and incubated for 3-5 minutes in 1) 2 mL 2X SDS sample buffer and 0.5 mL ethanol, 2) 0.25% 2-Mercaptoethanol βME in 1X sample buffer, and 3) 125 mM Alkylation Solution consisting of iodoacetamide in 1X sample buffer. Each incubation step was followed by rinsing with 1X NuPAGE® MES SDS Running Buffer. The IEF gel strip was transferred into the 2D well of the NuPAGE gel and run at 200V. A pre-stained low MW marker (Bio-Rad 161-0305) was used.

**Results:**

**Purification of recombinant human αGal in *P. pastoris***

αGal was obtained from a 7 L bioreactor (Figure 3-5) and purified (Table 3-1, Figure 3-6) using the DEAE and Thio-Gal columns. Due to the high concentration and volume of enzyme needed for the uptake studies planned, the more efficient DEAE column was substituted for the Con A Sepharose 4B (GE Healthcare No. 17-0440-01) column used in earlier experiments. The ConA column required lengthy soaks which caused a loss of enzyme. The DEAE column however, has much greater efficiency, being much quicker, and results in over 80% recovery of sample. Side-by-side comparison of the two procedures showed they both result in pure enzyme being produced, observed in specific activity and on SDS-PAGE.
The non-glycosylated monomeric form of αGal is 41.8 kDa. However, the form isolated from cells contains multiple glycosylated species with a predominant band of about 50 kDa and multiple higher molecular weight forms that differ in extent of glycosylation (Figure 3-6). We have previously demonstrated that high molecular weight glycoforms produced in insect cells and P. pastoris can be identified as derivatives of αGal rather than contaminants and these glycoforms are converted to a single band on SDS gels of about 41.8 kDa with endoglycosidase treatment [7-9, 20] and all appear in a western blot [20].
Figure 3-6: αGal was purified as a single band on an SDS-PAGE gel. Purified samples were run on a 4–20% polyacrylamide gel, under reducing conditions, and stained with Coomassie Brilliant Blue. The contents of the lanes are as follows: Bio-Rad unstained low molecular weight ladder (lane 1), Supernatant (lane 2), DEAE pool (lane 3), and Thiogal pool (lane 4). The minor bands present in the purified fraction are consistent with high molecular weight glycoforms seen previously when WT enzyme was purified from the same P. pastoris expression system. [7] [20]

Table 3-1: Purification Table for αGal Expressed in P. pastoris

<table>
<thead>
<tr>
<th>Step</th>
<th>Total Protein (mg)</th>
<th>Total Activity (Units × 10^6)</th>
<th>Specific Activity (Units/mg × 10^3)</th>
<th>Purification (Fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>55,550</td>
<td>208</td>
<td>3.7</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>DEAE Pool</td>
<td>653</td>
<td>137</td>
<td>211</td>
<td>56.2</td>
<td>66.0</td>
</tr>
<tr>
<td>Thio-Gal Pool</td>
<td>9.92</td>
<td>25.0</td>
<td>2,504</td>
<td>668</td>
<td>11.9</td>
</tr>
</tbody>
</table>


**Uptake of αGal using confocal microscopy**

Confocal microscopy was used as a reliable and sensitive method of monitoring uptake of αGal (Figure 3-7, 3-8, 3-12). The recombinant αGal was tagged with Alexa 488 according to manufacturer specifications. Initial experiments showed that uptake could be accurately monitored in both IMFE1 and FFB cells after fixation for short time periods as well as overnight (Figure 3-7, 3-8, 3-13). Untagged recombinant αGal was used as a negative control to account for auto-fluorescence. In these experiments, LysoTracker Red DND-99 was used to identify lysosomes allowing for monitoring uptake as well as co-localization/ targeting to the lysosomes of αGal (Figure 3-7, 3-8, 3-12). The nuclear stain DAPI was also used to visualize the nucleus.

In addition to 2D confocal images (Figure 3-8), 3D images using z-stacks were generated in a Zeiss LSM 710 Confocal microscope to better visualize co-localization of αGal with the lysosomes (Figure 3-7).
Figure 3-7: Uptake of αGal-Alexa using 3D confocal microscopy. Laser-scanning microscope 3D images of uptake of αGal-Alexa in normal fibroblast cells. Cells are labeled with LysoTracker Red (red) to identify lysosomes, DAPI nuclear stain (blue) and αGal with AlexaFluor488 fluorescent tag (green; yellow represents co-localization) from a LSM 710 Super-Resolution confocal laser-scanning microscope.

<table>
<thead>
<tr>
<th></th>
<th>ALEXA</th>
<th>LysoTracker Red</th>
<th>DAPI</th>
<th>Co-localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>αGal (no Alexa) (- Control)</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>αGal-Alexa</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Figure 3-8: Uptake of αGal-Alexa in fixed cells using normal fibroblast (NFB) cells (2hr incubation). αGal-Alexa (5 ug/ml) was added to wells containing NFB cells and incubated for 2-hour at 37°C and 5% CO₂. Cells were fixed post incubation with Alexa tagged αGal and counterstained with LysoTracker Red DND-99 for co-localization analysis. Post fixation the cells were covered with the ProLong® Diamond Antifade Mountant with DAPI before imaging on a LSM 710 confocal laser-scanning microscope.
Live cell imaging

Live cell imaging provides for continuous monitoring of uptake compared to images acquired in fixed cell confocal experiments. We carried out live cell imaging over an 80-minute time period (Figure 3-9). For this live cell assay a ZEISS LSM 880 Super-Resolution confocal laser-scanning microscope with Airyscan and live cell temperature and CO₂ incubator attachment was used. Cells were incubated with αGal-Alexa in FluoroBrite™ DMEM and Prolong Live Antifade Reagent in the presence of 5 mg/ml BSA to increase enzyme stability. By monitoring uptake in one cell over a period of time we hoped to be able to draw conclusions as to the dynamics of uptake in that cell and could potentially use this as a means to compare uptake of our sample pre- and post aconitylation.

We initially observed excessive bleed through of LysoTracker Red into the green Alexa (490/525) channel during preliminary live cell experiments, which made accurate quantification of the green channel impossible. To resolve this issue LysoTracker Deep Red (647/668) was substituted for the more commonly used LysoTracker Red (577/590) marker to identify lysosomes. We also observed bleed through of the live cell nuclear stain, Hoechst 33342 (360/460) into the green Alexa (490/525) channel. We were unable to resolve this issue and therefore omitted the nuclear dye from our live cell experiments to maintain the integrity of the quantification of the green channel (Figure 3-9).
**Figure 3-9: Uptake of αGal-Alexa using live cells.**

αGal-Alexa (5ug/ml) was incubated in Fabry fibroblast in the live cell attachment of a ZEISS LSM 880 confocal microscope at 37°C and 5% CO₂. Images were acquired every 10 minutes.
ImageJ was used to quantify the uptake of the Alexa tagged enzyme in confocal studies as described in the methods (Figures 3-10). N=5 cells were initially chosen at random (more cells would need to be imaged to draw substantial conclusions in future studies) and the images from this live experiment were analyzed and uptake quantified with ImageJ (Figure 3-11). ImageJ allows quantification of an otherwise subjective data set. It also identifies the existence of the cell-to-cell variability consistent with variations seen when measuring uptake by fixed cell confocal microscopy.

**Figure 3-10: Quantification procedure of confocal images with ImageJ.**

a) Particle analysis of the LysoTracker (red) channel in ImageJ identifies lysosome particles, b) Particle analysis of the Alexa-488 (green) channel in ImageJ identifies enzyme particles c) Co-localized image generated by ImageJ. d) zoomed in view showing co-localized particles e) zoomed in view showing co-localization in original confocal image. f) Particle analysis in ImageJ on co-localized image identified co-localized particles. g) Zoomed in view showing numbered co-localized particles.
Quantification of uptake in n = 5 cells (Figure 3-11), highlights the dramatic cell to cell variability initially observed in fixed cell experiments but allows us to still observe the general trend in the increase of uptake of Alexa tagged αGal over the 80-minute incubation period. In future studies flow cytometry can be used to address this cell to cell variability. By using flow cytometry to monitor the presence of the Alexa tagged enzyme we would be able to analyze millions of cells and therefore draw more concrete conclusions as to the trends observed. The dip in uptake observed at 80 minutes is due to a shift in focus of the LSM 880 lens from the optimal position do to repeated plate movements necessary to visualize all selected cells.

**Figure 3-11: Particle analysis of live cell uptake of αGal-Alexa in Fabry fibroblast (FFB) cells.**

αGal-Alexa (5μg/ml) was incubated in FFB cells in the live cell attachment of a LSM880 confocal microscope. Distinct fields/cells (n=5) were chosen for continued analysis and confocal images of these fields were acquired every 10 minutes. Uptake was quantified using ImageJ.
Time course confocal microscopy in fixed cells using IMFE1 endothelial cells

Uptake of αGal was monitored in IMFE1 immortalized endothelial cells over different time periods; 0, 0.5, 2 and 19 hours (Figure 3-12). Cells were incubated with enzyme for the indicated time periods and then fixed before imaging in a confocal microscope. An average of 15 cells was imaged in each instance and the images analyzed using ImageJ (Figure 3-13). These results indicated an increase in both uptake of αGal-Alexa as well as co-localization of the enzyme with the lysosomes over time. Untagged recombinant αGal was used as a negative control to account for auto-fluorescence.

This time course analysis (Figure 3-13) indicates that αGal is initially taken up at a higher rate (t <3hrs) and that uptake plateaus with longer incubation times (19 hours). These trends were confirmed in uptake studies done where enzyme assay was used to quantify uptake in both FFB and IMFE1 cells (Figure 3-17).
<table>
<thead>
<tr>
<th>Incubation</th>
<th>αGal-Alexa</th>
<th>LysoTracker Red</th>
<th>DAPI</th>
<th>Co-localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 hr</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>2 hr</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
<tr>
<td>19 hr</td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**Figure 3-12: Time course uptake of αGal-Alexa in IMFE1 cells.**

αGal-Alexa (5 ug/ml) in IMFE cells post 0.5, 2, and 19-hour incubation at 37°C and 5% CO₂. Cells were incubated with Alexa tagged αGal and counterstained with LysoTracker Red DND-99 for 2 hours before fixation for co-localization analysis. Post fixation the cells were covered with the ProLong® Diamond Antifade Mountant with DAPI before imaging on a LSM 710 confocal laser-scanning microscope.
Figure 3-13: Particle analysis of uptake of αGal-Alexa in IMFE1 cells from (Figure 3-12). Quantification with ImageJ of uptake and co-localization with lysosomes of αGal-Alexa (5 ug/ml) in IMFE1 cells post 0.5, 2, and 19-hour incubation at 37°C and 5% CO₂. (mean and standard deviation from n=15 cells)
Uptake of $\alpha$Gal is dose dependent

It has been previously reported that FFB in cell culture take up the recombinant human $\alpha$Gal produced in insect cells and *P. pastoris* in a dose dependent manner as measured using direct enzyme assays (Figure 3-14) [7-9].

Uptake of the recombinant human $\alpha$Gal produced in the 7L bioreactor and purified using the DEAE and Thio-Gal columns was measured by direct enzyme assay in FFB and IMFE1 cells in replicates and was confirmed to be dose dependent in an overnight incubation with enzyme (Figure 3-15).
Figure 3-15: Dose dependent uptake of αGal in Fabry fibroblast (FFB) and Fabry endothelial (IMFE1) cells.
Cells were incubated with indicated doses of αGal overnight (19 hours) in replicates, then lysed and uptake was quantified using enzyme assay with the artificial substrate MUG as described in the methods.
Recombinant αGal produced in *Pichia* is taken up in higher levels in Fabry endothelial cells (IMFE1) compared to Fabry fibroblasts (FFB)

In a recent study analyzing the lysosomal delivery of the available therapeutic αGal in cell models of Fabry disease, it was shown that total endothelial uptake was less than fibroblast uptake at concentrations relevant to the therapeutic situation (≤ 3 µg/ml) (Figure 3-16B) [15]. This is in accord with the currently approved ERT being mediated by the M6P receptors which are generally absent from endothelial cells, a major site of GB3 accumulation in Fabry disease.

![Graph A: Recombinant Pichia αGal (Meghdari, unpublished)](image1)

![Graph B: Therapeutic (Replagal or Fabrazyme) [15]](image2)

**Figure 3-16: Comparison of uptake trends of αGal produced in pichia and mammalian cells in fibroblast and endothelial cells.**

A) Recombinant αGal produced in *Pichia* was incubated with FFB and IMFE1 overnight at varying concentrations. Uptake was quantified using direct enzyme assay. B) Uptake of αGal in normal fibroblasts and blood outgrowth endothelial cells. Uptake after 3-hour incubation at varying enzyme concentration of Replagal (Shire Human Genetic Therapies) or agalsidase beta - Fabrazyme (Genzyme Corporation – a Sanofi subsidiary) [15].
In order to investigate these results, the recombinant αGal was incubated overnight at varying concentrations in Fabry fibroblast and endothelial cells (Figure 3-16A). Published results (Figure 3-16B) [15] indicate the current M6P terminated therapeutic is taken up in greater abundance in FFB as opposed to endothelial cells. Our mannose terminated enzyme, rectifies this issue with observed increased uptake in disease-relevant IMFE1 as opposed to FFB (Figure 3-16A). Our Mannose terminated recombinant αGal was taken up at much higher levels in endothelial cells, a major disease-relevant cell type in Fabry disease.

Uptake of αGal in IMFE1 cells reaches a plateau after 3 hours

To compare uptake rates in a dynamic phase, IMFE1 and FFB cells were incubated with recombinant αGal overnight (19 hours) and for a shorter time period of 3 hours and uptake quantified by enzyme assay (Figure 3-17). Uptake in IMFE1 cells was substantially higher than FFB at both time points with maximum uptake being reached after 3-hours of incubation with αGal. We hypothesize that this plateau is most likely due to saturation of uptake by the MR, in

![Figure 3-17: Comparison of time course uptake of αGal in FFB and IMFE1 cells. αGal was incubated with FFB and IMFE1 cells overnight and at 3hours at a concentration of 6 μg/ml. Uptake was quantified using enzyme assay.](image-url)
which all accessible MR have bound enzyme. These results agree with trends observed in Confocal studies (Figure 3-13) and indicate the need to monitor uptake at lower time points in IMFE1 cells in order to study kinetics of uptake.

**Preliminary control experiments to demonstrate aconitylation of HSA**

HSA was aconitylated as per published methods with minor changes to accommodate αGal instability at high pH (Figure 3-18) [165, 167, 176]. We synthesized Aco-HSA by reacting the surface lysine residues of HSA in buffer at a pH of 7.5 with excess cis-aconitic anhydride and stirring at 4°C for 1hr. The degree of modification of lysines was assessed by estimating the loss of free amino groups as measured by TNBS [18] or fluorescamine [177] assay post removal of excess anhydride and using a glycine standard curve (Table 3-2). In addition, the pre-and post aconitylated samples were analyzed using native PAGE, isoelectric focusing (IEF), and 2D isoelectric focusing to measure aconitylation. (Figure 3-19, 3-20).

![Figure 3-18: Analysis of stability of αGal over high pH range.](image)

Conjugation with cis-aconitic anhydride is recommended under basic conditions (pH 8.5) [18]. In preliminary experiments, we measured the stability of αGal at pH 6.5 to 8.5 at incubations times of 15, 30, and 60 min. The results indicated that conjugation at a pH of 7.5 for 30 or 60 min was optimal to prepare Aco-αGal.
Table 3-2: Overview of HSA/BSA aconitylation samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>conc. Anhydride added</th>
<th>% Aconitylation Fluorescamine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1X</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>1X</td>
<td>43</td>
</tr>
<tr>
<td>Aco-HSA</td>
<td>0.5X</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>2X</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>2X</td>
<td>52</td>
</tr>
<tr>
<td>Aco-BSA</td>
<td>1X</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>2X</td>
<td>42</td>
</tr>
</tbody>
</table>

A) native PAGE

B) Isoelectric focusing gel

Figure 3-19: Analysis of charge of HSA pre-and post aconitylation.
A) Native PAGE separates HSA/Aco-HSA samples based on size to charge ratio and B) IEF gel separates HSA/Aco-HSA samples solely based on their charge. The more negatively charged Aco-HSA samples migrates farther on both gels confirming aconitylation of the sample.
Aconitylation promotes uptake of HSA

It was reported in the literature that the introduction of negative charges on the surface of proteins by chemical modifications such as acetylation or aconitylation targets proteins for uptake by the scavenger receptor family of integral membrane proteins [4, 167]. Specifically, aconitylation of HSA was reported to result in massive uptake in vivo and in vitro to the lysosomes of endothelial cells [164, 178]. An essential step in enzyme therapy for Fabry disease and other lysosomal storage diseases depends upon enzyme uptake and lysosomal transport by multiple cell types and using several uptake pathways. HSA has a circulating half-life of 20 days due to its size and protection from intracellular degradation in the lysosome, making it a good
control for the aconitylation experiments with αGal. In preliminary experiments, we modified HSA by aconitylation and tagged it with the fluorescent dye Alexa488 and monitored uptake into normal human fibroblast cells using confocal microscopy (Figure 3-21).

No detectable fluorescence of Alexa488 (green fluorescence) was detected in a control experiment for lysosomes (labeled with LysoTracker Red) with unmodified and unlabeled HSA (Figure 3-21A, Frames 1, 2) and very low levels of uptake were detected with unmodified HSA-Alexa488 (Figure 3-21B, Frames 1,2). In contrast, significant accumulation of Aco-HSA-Alexa488 was detected in lysosomes as indicated by co-localization of LysoTracker Red (red fluorescence) with Alexa488 (green fluorescence) in the formation of yellow lysosomal compartments (Figure 3-21C, Frames 1,2,3,4). These results confirm literature reports that Aco-HSA is successfully targeted to the lysosome and demonstrate the efficacy of our aconitylation protocol.
Figure 3-21: Uptake of HSA-Alexa compared to Aco-HSA-Alexa in NFB.
Cells were labeled with LysoTracker (red) to identify lysosomes, DAPI nuclear stain (blue) Cells were incubated for 3 hours with A) HSA (Control) B) HSA and C) Aco-HSA with AlexaFluor488 fluorescent tag (Green); yellow represents co-localization) from a LSM 710 Super-Resolution confocal laser-scanning microscope. Multiple frames shown for clarification. D) Individual channels of Frame 1, cells incubated with HSA-Alexa E) Individual channels of Frame 1, cells incubated with Aco-HSA-Alexa.
**Aconitylation of HSA targets the enzyme to the scavenger receptor uptake pathway**

U2OS-SRA cells are human U2OS osteosarcoma cells over-expressing the murine scavenger receptor A. Because of the dramatic increase of scavenger receptors present in these cells in contrast to their control (U2OS cells) they are used as a tool for identifying uptake to the scavenger receptor uptake pathway [179].

Confocal uptake studies were done with Aco-HSA in U2OS cells as well as U2OS-SRA cells overexpressing scavenger receptor type A. Aco-HSA was taken up more readily in U2OS-SRA cells than in U2OS cells after a short incubation time of 2 hours. This difference practically disappears after overnight incubation with the fluorescently labeled Aco-HSA when uptake seems to plateau in both cells types (Figure 3-22).
**A) 2hr incubation**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Aco-HSA-Alexa</th>
<th>LysoTracker Red</th>
<th>DAPI</th>
<th>Co-localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>U2OS</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>U2OS-SRA</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

**B) Overnight incubation**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Aco-HSA-Alexa</th>
<th>LysoTracker Red</th>
<th>DAPI</th>
<th>Co-localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>U2OS</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>U2OS-SRA</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

**Figure 3-22: Uptake of Aco-HSA-Alexa in U2OS and U2OS-SRA cells.**
Aco-HSA-Alexa (5 µg/ml) in U2OS and U2OS-SRA cells A) post 2-hour incubation and B) post overnight incubation at 37°C and 5% CO₂. Cells were incubated with Alexa tagged αGal and counterstained with LysoTracker Red DND-99 for 2 hours before fixation for co-localization analysis. Post fixation the cells were covered with the ProLong® Diamond Antifade Mountant with DAPI before imaging on a LSM 710 confocal laser-scanning microscope.
Aconitylation of $\alpha$Gal

The first step in forming SR-targeted $\alpha$Gal is to construct aconitylated and fluorescently-labeled $\alpha$Gal. $\alpha$Gal has 33 lysines per subunit; the programs ASA-View [180] and GetArea[181] were used to visualize and analyze the relative surface accessibility of residues of the Protein data bank (PDB) structure of human $\alpha$Gal (1R46)[10]. Relative solvent accessibility was determined by dividing the accessible surface area of a residue for the maximum exposed surface of the same residue type in a Gly–X–Gly oligopeptide, residues with relative solvent accessibility of more than 50% are considered to be solvent exposed. Using this calculation $\alpha$Gal contains approximately 20 surface lysines in the monomer, which will be used for aconitylation and fluorescent labeling (Figure 3-23). Cis-aconitic anhydride coupling to lysine residues introduces a net three negative charges for each lysine residue (Figure 3-2).

Figure 3-23: Surface Lys residues of $\alpha$Gal.
The surface structure of the $\alpha$Gal dimer contains about 20 well-exposed Lys residues (red). Aconitylation of $\varepsilon$-NH$_2$ groups (Figure 3-2) introduces a net three negative charges for each Lys residue.
Aconitylation of αGal targets the enzyme to the scavenger receptor uptake pathway in U2OS-SRA cells

Uptake of αGal and Aco-αGal was analyzed in U2OS cells (human bone osteosarcoma) as well as U2OS-SRA cells (overexpressing murine scavenger receptor type A). Aco-αGal was more readily taken up in U2OS-SRA cells with an average of 100 ± 74 particles of Aco-αGal-Alexa per field than in U2OS cells with an average of 12 ± 9 particles of Aco-αGal-Alexa per field after an incubation time of 2 hours with enzyme (Figure 3-24, 3-25, Table 3-3). This is similar to the trend seen in the control with Aco-HSA (Figure 3-22). This is in contrast to the trend in uptake of non-Aco-αGal in which uptake is similar in both cell types with slightly more uptake being observed in U2OS-SRA cells indicating that non-Aco-αGal may also be slightly targeted to the SR uptake pathway (159 ± 104 in U2OS vs 192 ± 20 in U2OS-SRA).

It is important to note that the overall uptake of non-Aco-αGal is higher than Aco-αGal in both of these cells types. This is most likely due to the loss of approximately 44% in specific activity of the enzyme during the aconitylation procedure. We also hypothesize that aconitylation may be blocking another uptake pathway of non-Aco-αGal in U2OS cells but this hypothesis needs to be tested further.
<table>
<thead>
<tr>
<th>Cells</th>
<th>αGal-Alexa</th>
<th>LysoTracker Red</th>
<th>DAPI</th>
<th>Co-localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>U2OS</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td>U2OS-SRA</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cells</th>
<th>Aco- αGal -Alexa</th>
<th>LysoTracker Red</th>
<th>DAPI</th>
<th>Co-localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>U2OS</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td>U2OS-SRA</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
</tbody>
</table>

**Figure 3-24:** Uptake of αGal-Alexa and Aco-αGal-Alexa in U2OS and U2OS-SRA cells.

U2OS and U2OS-SRA cells were incubated with Alexa tagged αGal-Alexa and Aco-αGal-Alexa at 37°C and 5% CO₂ for 2 hours. Cells were counterstained with LysoTracker Red DND-99 for 2 hours before fixation for co-localization analysis. Post fixation the cells were covered with the ProLong® Diamond Antifade Mountant with DAPI before imaging on a LSM 710 confocal laser-scanning microscope.
A) Average particles per field for cells incubated with αGal-Alexa

B) Average particles per field for cells incubated with Aco-αGal-Alexa

Figure 3-25: Quantification of uptake of αGal-Alexa and Aco-αGal-Alexa in U2OS and U2OS-SRA cells from Figure 3-24. Confocal images were analyzed using ImageJ. A) cells incubated with αGal-Alexa, B) cells incubated with Aco-αGal-Alexa. Values included in table are average particles per Field for n = 20 cells imaged.
Table 3-3: ImageJ particle analysis of Aco (+, -) αGal in U2OS and U2OS-SRA cells from Figure 3-24.

<table>
<thead>
<tr>
<th></th>
<th>Aco</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Cells</td>
<td>U2OS</td>
<td>U2OS-SRA</td>
<td>U2OS</td>
<td>U2OS-SRA</td>
</tr>
<tr>
<td>Alexa</td>
<td>12 ± 9</td>
<td>100 ± 74</td>
<td>159 ± 159</td>
<td>192 ± 20</td>
</tr>
<tr>
<td>LysoTracker</td>
<td>159 ± 104</td>
<td>192 ± 20</td>
<td>217 ± 96</td>
<td>185 ± 31</td>
</tr>
<tr>
<td>Co-localized</td>
<td>8 ± 7</td>
<td>45 ± 14</td>
<td>66 ± 52</td>
<td>109 ± 18</td>
</tr>
</tbody>
</table>

Note. Values included in table are average particles per Field (n = 20 cells imaged).

In order to confirm targeting of Aco-αGal to the SR in U2OS-SRA cells, uptake was analyzed in the presence and absence of the SR inhibitor PolyI (Figure 3-26). This experiment showed that addition of PolyI completely inhibited uptake of Aco-αGal-Alexa in U2OS-SRA cells with no green fluorescence being detectable in the presence of the inhibitor after a 2-hour incubation (Figure 3-26B). This inhibition indicates that the primary pathway of uptake of Aco-αGal-Alexa in these cells is through the scavenger receptor uptake pathway.
A) Confocal Image of representative field

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Aco-αGal-Alexa</th>
<th>LysoTracker Red</th>
<th>DAPI</th>
<th>Co-localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>- PolyI</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>+PolyI</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
</tbody>
</table>

B) ImageJ particle analysis of all fields

<table>
<thead>
<tr>
<th>PolyI</th>
<th>Alexa</th>
<th>LysoTracker</th>
<th>Co-localized</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>172 ± 72</td>
<td>22 ± 23</td>
</tr>
</tbody>
</table>

Average Per Field: 30 ± 26, 0, 172 ± 72, 89 ± 26, 22 ± 23

Figure 3-26: Uptake of Aco-αGal-Alexa in U2OS-SRA cells (+, -) PolyI inhibition.
Aco-αGal-Alexa was incubated in U2OS-SRA cells for 2 hour with and without 100 µg/ml PolyI at 37°C and 5% CO2. Cells were counterstained with LysoTracker Red DND-99 for 2 hours before fixation for co-localization analysis. A) Representative image taken with a ZEISS LSM 880 confocal laser-scanning microscope B) ImageJ particle analysis of all fields, mean and Standard deviation for n = 30-35 cells.
αGal is targeted to the scavenger receptor uptake pathway in U2OS-SRA cells

Uptake of Alexa488 tagged recombinant αGal was monitored in U2OS-SRA cells as a control. U2OS-SRA cells are human U2OS osteosarcoma cells over-expressing the murine scavenger receptor, making them an ideal candidate for analyzing targeting to the scavenger receptors. αGal-Alexa was incubated for two hours with cells with and without the scavenger receptor inhibitor PolyI. Images were acquired of approximately 20 cells per well and analyzed. (Figure 3-27) Interestingly the addition of PolyI to the wells seems to have completely inhibited uptake of the recombinant αGal in this cell type. This suggests that the enzyme produced in P. pastoris may already be targeting to the scavenger receptor to some extent.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>αGal-Alexa</th>
<th>LysoTracker Red</th>
<th>DAPI</th>
<th>Co-localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>-PolyI</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>+PolyI</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**Figure 3-27:** Uptake of αGal-Alexa in U2OS-SRA cells: Representative images. Uptake of αGal-Alexa (5 ug/ml) in U2OS-SRA cells post 2 hour incubation at 37°C and 5% CO2. Cells were incubated with Alexa tagged αGal and counterstained with LysoTracker Red DND-99 for 2 hours before fixation for co-localization analysis. Post fixation the cells were covered with the ProLong® Diamond Antifade Mountant with DAPI before imaging on a LSM 710 confocal laser-scanning microscope.
Aconitylation improves targeting of αGal to lysosomes in IMFE1 endothelial cells

In order to compare the uptake and targeting to the lysosomes of aconitylated and nonaconitylated αGal in IMFE1 endothelial cells, a time course uptake study was done using Aco-αGal-Alexa (Figure 3-28) to mimic what was done with non-Aco-αGal (Figure 3-12).

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Aco-αGal-Alexa</th>
<th>LysoTracker Red</th>
<th>DAPI</th>
<th>Co-localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 hr</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>2 hr</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
<tr>
<td>19 hr</td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**Figure 3-28: Time course uptake of Aco-αGal-Alexa in IMFE1 cells.**
Aco-αGal-Alexa (5 ug/ml) in IMFE1 cells post 0.5, 2, and 19-hour incubation at 37°C and 5% CO₂. Cells were counterstained with LysoTracker Red DND-99 for 2 hours before fixation for co-localization analysis and images taken on a LSM 710 confocal laser-scanning microscope.
Table 3-4: ImageJ particle analysis of Aco (+, -) αGal time course uptake in IMFE1 cells.

<table>
<thead>
<tr>
<th>Average # of particles per field</th>
<th>Aco</th>
<th></th>
<th></th>
<th>Incubation (hr)</th>
<th>0.5</th>
<th>2</th>
<th>19</th>
<th>0.5</th>
<th>2</th>
<th>19</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa</td>
<td></td>
<td></td>
<td></td>
<td>Incubation (hr)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>18 ± 3</td>
<td>24 ± 29</td>
<td>96 ± 45</td>
<td></td>
<td>10 ± 10</td>
<td>73 ± 27</td>
<td>110 ± 35</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Co-localized</td>
<td>4 ± 5</td>
<td>10 ± 20</td>
<td>50 ± 30</td>
<td></td>
<td>6 ± 8</td>
<td>17 ± 19</td>
<td>42 ± 13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Co-localized*</td>
<td>22 ± 27</td>
<td>36 ± 32</td>
<td>50 ± 18</td>
<td></td>
<td>58 ± 43</td>
<td>24 ± 23</td>
<td>41 ± 13</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note. Values included in table are average particles per field (n = 15-20 cells imaged).

*% Co-localized = \( \frac{\text{Co-localized}}{\text{Alexa}} \) × 100

Initial time course analysis with αGal (Figure 3-13, 3-29A) indicates that αGal is initially taken up at a higher rate (t <3hrs) and that uptake plateaus with longer incubation times (19-hours) in IMFE1 cells. In the case of Aco-αGal however this relationship seems to be more linear with uptake increasing steadily over time (Figure 3-29B). Compression of the uptake of aconitylated and non-aconitylated αGal shows that at t = 0.5 hours, Aco-αGal is taken up better (18 ± 3 Alexa particles) than non-Aco-αGal (10 ± 10 Alexa particles) in IMFE1 cells with the difference being close to 4-fold once the ~50% loss in specific activity of the aconitylated sample is taken into account.

Comparing the amount of Aco (+, -) αGal co-localized with the lysosomes, we see that a higher percentage of internalized enzyme are targeted to the lysosomes (% co-localized) in the case of Aco-αGal with this percentage steadily increasing with the increase of overall uptake over the three time points measured (22% → 36% → 50%). In contrast, the growth of % co-localization seems to be much slower in non-Aco-αGal (58% → 24% → 41%) (Table 3-4).
A) Uptake of αGal-Alexa in IMFE1 cells

![Graph](image1)

B) Uptake of Aco-αGal-Alexa in IMFE1 cells

![Graph](image2)

Figure 3-29: Particle analysis of time course uptake of Aco-αGal-Alexa in IMFE1 cells from Figure (3-13, 3-28).

A) αGal-Alexa (5 ug/ml) B) Aco-αGal-Alexa (5 ug/ml) in IMFE1 cells post 0.5, 2, and 19-hour incubation at 37°C and 5% CO₂. Uptake was quantified using ImageJ as described in methods.
αGal produced in *Pichia* is targeted to the mannose receptor

To further investigate potential receptors involved in the uptake of the aconitylated and non-aconitylated αGal produced in yeast, uptake of these enzymes was monitored in the presence of M6P and Mannan and scavenger receptor inhibitors PolyI, Aco-HSA, and Ac-LDL in an overnight uptake experiment (Figure 3-30A, 3-31A) by enzyme assay and with a combination of Mannon and PolyI during a 2-hour incubation (Figure 3-30B, 3-31B) using confocal microscopy. Our results indicated a reduction of approximately 80% in uptake overnight and approximately 40% after 2 hours when the MR is inhibited indicating that in IMFE1 cells this is the major pathway for uptake of non-Aco-αGal (Figure 3-30).

SR inhibitors PolyI, Aco-HSA, and Ac-LDL had no effect on uptake of non-aconitylated αGal in IMFE1 cells (Figure 3-30). Therefore, despite our previous results indicating αGal ability to target to scavenger receptors (Figure 3-27), the scavenger receptor does not contribute very much to the overall uptake of αGal uptake in IMFE1 cells. Unexpectedly, these inhibitors increased uptake in the case of Aco-αGal both in overnight studies measured with enzyme assay (Figure 3-31A) as well as confocal studies where uptake was analyzed using imageJ (Figure 3-31B). The reason for this increase in uptake when the scavenger receptor is inhibited is unknown but one reason may be due to competition between the mannose and scavenger receptors for Aco-αGal. This is further demonstrated when both receptors are inhibited (Figure 3-31B). In this case, the uptake of Aco-αGal is decreased more than with mannan alone indicating both receptors are playing a role in the uptake of Aco-αGal.
A) Overnight (19 hours) incubation

B) 2-hour incubation

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>No Inhibitor</th>
<th>Mannan</th>
<th>PolyI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Aco-αGal</td>
<td>78 ± 27</td>
<td>46 ± 55</td>
<td>86 ± 41</td>
</tr>
</tbody>
</table>

Figure 3-30: Effect of inhibitors on uptake of non-Aco-αGal in IMFE1 cells.
A) αGal (6 µg/ml) were incubated overnight (19 hours) at 37°C and 5% CO₂ in IMFE1 cells post. The effect of 5mM M6P, 2 mg/ml Mannan, 10 µg/ml Aco-HSA, and 100 µg/ml Poly(I) were tested in duplicate. Uptake was quantified using enzyme assay, B) Uptake of αGal-Alexa (5 µg/ml) in IMFE1 cells post 2-hour incubation at 37°C and 5% CO₂. The effect of 2 mg/ml Mannan and 100 µg/ml Poly(I) were tested. Uptake analyzed by confocal microscopy. Results are displayed as % of control (No Inhibitor = 100%).
A) Overnight (19 hours) incubation

B) 2-hour incubation

<table>
<thead>
<tr>
<th>Inhibitor(s)</th>
<th>No Inhibitor</th>
<th>Mannan</th>
<th>PolyI</th>
<th>PolyI + Mannan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aco-αGal</td>
<td>56 ± 68</td>
<td>41 ± 69</td>
<td>69 ± 55</td>
<td>20 ± 22</td>
</tr>
</tbody>
</table>

**Figure 3-31: Effect of inhibitors on uptake of Aco-αGal in IMFE1 cells.**
A) Aco-αGal (6 µg/ml) were incubated overnight (19 hours) at 37°C and 5% CO2 in IMFE1 cells post. The effect of 5mM M6P, 2 mg/ml Mannan, 10 µg/ml Aco-HSA, and 100 µg/ml Poly(I) were tested in duplicate. Uptake was quantified using enzyme assay, B) Uptake of Aco-αGal-Alexa (5 µg/ml) in IMFE1 cells post 2-hour incubation at 37°C and 5% CO2. The effect of 2 mg/ml Mannan and 100 µg/ml Poly(I) were tested. Uptake analyzed by confocal microscopy. Results are displayed as % of control (No Inhibitor = 100%).
Measurement of the degree of aconitylation of αGal

We synthesized Aconitylated αGal by reacting the ε-NH₂ group of lysine residues of approximately 1 mg/ml of purified αGal in Buffer A at a pH of 7.5 with excess cis-aconitic anhydride (SIGMA) as preformed with the positive control HSA and described in literature [165, 167, 176]. The pH of the reaction mixture was adjusted to 7.5 post anhydride addition with 1M NaOH and was then stirred at 4°C for approximately 1.5 hours. Either dialysis or a Sephadex G-25 column were used to remove excess anhydride.

The degree of modification of lysines was assessed by estimating the loss of free amino groups and using a glycine standard curve as measured by TNBS assay [182] and the more sensitive fluorescamine assay [113] post removal of excess anhydride. In addition, 2D electrophoresis was done on the pre-and post aconitylated samples as described in the previous section for HSA (Figure 3-19, 3-20).

Table 3-5: Overview of αGal aconitylation samples

<table>
<thead>
<tr>
<th>Aco-αGal sample</th>
<th>Conc. Anhydride added</th>
<th>% Aconitylation Fluorescamine</th>
<th>% Aconitylation TNBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMII112</td>
<td>1X</td>
<td>NA</td>
<td>47</td>
</tr>
<tr>
<td>63.61</td>
<td>1X</td>
<td>-6</td>
<td>NA</td>
</tr>
<tr>
<td>63.66</td>
<td>1X</td>
<td>-5</td>
<td>NA</td>
</tr>
<tr>
<td>63.71</td>
<td>5X</td>
<td>-53</td>
<td>-183</td>
</tr>
<tr>
<td>63.73</td>
<td>2X</td>
<td>-15</td>
<td>NA</td>
</tr>
<tr>
<td>63.74</td>
<td>2X</td>
<td>-35</td>
<td>-378</td>
</tr>
<tr>
<td>63.83</td>
<td>2X</td>
<td>11</td>
<td>-32</td>
</tr>
<tr>
<td>60.28</td>
<td>2X</td>
<td>-20</td>
<td>NA</td>
</tr>
<tr>
<td>40.22 (Δ4)</td>
<td>2X</td>
<td>-16</td>
<td>NA</td>
</tr>
</tbody>
</table>
Aconitylation was performed on multiple preparations of recombinant αGal. Initially it was observed by TNBS assay that the αGal sample was successfully aconitylated (Table 3-5). These aconitylated samples were tagged with Alexa488 and confocal studies were subsequently performed. Upon further testing with the TNBS assay it was observed that there is large variability between replicates in this assay with the signal being very low above background. Therefore, the more sensitive fluorescamine assay was used to confirm aconitylation of samples. This assay indicated that no aconitylation had taken place in either of the preparations (Table 3-5). Because of the puzzling nature of this result IEF was done on all aconitylated and non-aconitylated samples to clarify the issue.

IEF gels were run as described earlier, in all gels except for one, non-aconitylated αGal presented as a broad band as opposed to a sharp band as we had anticipated indicating our recombinant sample is present as a mixture containing varying negative charge (Figure 3-32A). There was no difference observed in the native PAGE (3-32B) and IEF gels (Figure 3-32A) for aconitylated and non-aconitylated αGal. When these bands were run on the second dimension SDS-PAGE it further confirmed that this method could not be used to confirm aconitylation of our samples (Figure 3-32C, 3-32D). This inability to confirm aconitylation using TNBS assay, fluorescamine assay, and IEF gels conflicts with the results we see in confocal uptake studies with aconitylated and non-aconitylated αGal.
Figure 3-32: Gel electrophoresis of representative αGal and Aco-αGal samples. A) IEF and B) Native PAGE were performed under non-denaturing conditions on αGal and Aco-αGal samples. Lanes containing C) αGal and D) Aco-αGal were cut out of the IEF gel and run on a NuPAGE® 4-12% Bis-Tris Protein Gels with a 2D-well to separate focused proteins by mass according to manufacturer specifications.
Discussion:

Our findings suggest that the recombinant αGal produced in *P. pastoris* is taken up by the MR and by the SR uptake pathways and that aconitylation of αGal increases the uptake by the SR resulting in better delivery of enzyme to lysosomes in Fabry endothelial cells. Our results also confirm that aconitylation of HSA improves uptake and delivery to the lysosomes.

The two therapeutics currently available for ERT are expressed in Chinese Hamster Ovary (CHO) cells used by Genzyme and HT1080 (fibroblasts derived from fibrosarcoma) used by Shire. The use of these mammalian cell lines is due in part to their ability to perform human-like glycosylation containing M6P, promoting uptake of the therapeutic by the M6P pathway. It was initially assumed that targeting to the M6P pathway is necessary for ERT therapy in LSD such as Fabry disease. However, more recent results indicate that mannose terminated forms of human αGal produced in moss (moss-αGal) [1] as well as tobacco cells [70] (PRX-102, chemically modified, resulting in a cross-linked dimer of PEGylated subunits increasing enzyme stability) are taken up by different cells types and are successful in clearing substrate in both in-vitro and in-vivo studies. Although the mechanism of uptake for PRX-102 is unknown, the mannose terminated moss-αGal is shown to be taken up by the MR (Figure 3-33). Shen et. al. suggested that mannose-terminated enzymes can be more effective than M6P terminated enzymes in the treatment of Fabry disease, and that M6P residues may not always be a prerequisite for ERT as previously assumed. These newly developed recombinant enzymes are among 122 other Fabry disease therapies currently in clinical trials (ClinicalTrials.gov).
Unlike mammalian glycosylation patterns which are phosphorylated and composed of sugars terminated with sialic acid residues, enzymes produced in P. pastoris contain a simpler form of glycan known as high mannose (Figure 3-34). These glycans are composed of two core N-acetylglucosamine residues and ramified mannoses (Figure 3-34) The MR has been shown to be present on endothelial cells [1] a major site of accumulation of GB3 in Fabry disease. Our results show that our recombinant high mannose αGal produced in P. pastoris is mainly targeting the MR as indicated by a reduction of approximately 80% in uptake overnight and approximately 40% after 2 hours of incubation in the presence of mannan (Figure 3-29). We also show that αGal expressed in P. pastoris is taken up more readily in immortalized Fabry endothelial cells (IMFE1) than in Fabry fibroblast cells (Figure 3-16A) which contain low levels of MR[1]. Published results indict the therapeutic αGal with M6P regulated uptake is more efficient in
fibroblast than endothelial cells (Figure 3-16B). This better delivery to endothelial cells could prove to be tremendously advantageous in ERT in both Fabry disease and other LSDs.

**Figure 3-34: Types of N-glycans.**
The three different types (High Mannose, Complex and Hybrid) share a common core structure including the first two N-acetylglucosamine residues and the first three mannose residues.[11]

SRs are a diverse group comprised of membrane proteins and soluble secreted extracellular domain isoforms with the ability to recognize common ligands such as poly-ionic ligands including lipoproteins, apoptotic cells, cholesterol esters, phospholipids, proteoglycans, ferritin, and carbohydrates. SRs are grouped into subclasses with little to no primary sequence similarity between each class. HSA was aconitylated and the change in charge of the protein was confirmed by TNBS Assay as well as isoelectric focusing gels (Figure 3-19, 3-20). This negatively charged Aco-HSA was fluorescently tagged with Alexa 488 and was shown to target more readily than non-Aco-HSA to the lysosomes in NFB cells, using confocal microscopy (Figure 3-21). In addition, confocal studies in U2OS as well as U2OS-SRA cells showed that
aconitylated HSA is taken up more readily in U2OS-SRA cells than in U2OS cells after a short incubation time of 2 hours. This difference is narrowed after overnight incubation where uptake seems to plateau in both cells types (Figure 3-22). U2OS cells contain low levels of the type A scavenger receptor SRABA5 [183], this may be the reason we are able to see uptake of Aco-HSA in U2OS cells overnight.

Mimicking trends seen in the HSA control, aconitylation of αGal also resulted in targeting of the enzyme to the scavenger receptor as demonstrated by complete inhibition of uptake in U2OS-SRA cells post incubation with the scavenger receptor inhibitor PolyI (Figure 3-26). Interestingly, non-Aco-αGal was also shown to have the potential to target to the scavenger receptor uptake pathway (Figure 3-27). αGal is a negatively charged enzyme (Figure 3-35) which is most likely why it is targeted to the scavenger receptors in this cell type. This is however not the major uptake pathway in the more therapeutically relevant IMFE1 cells.

Inhibition studies done overnight and analyzed using enzyme assay as well as with 2-hour

![Image](image_url)
incubation (analyzed using confocal microscopy) in IMFE1 cells, revealed that Poly I had no major effect on uptake of non-Aco-αGal in these cells (Figure 3-30). In contrast, addition of SR inhibitors such as PolyI, Aco-HSA, and Ac-LDL cause an increase in uptake of Aco-αGal in IMFE1 cells (Figure 3-31). Our results indicate that in the case of Aco-αGal the mannose and scavenger receptor uptake pathways compete for internalization of Aco-αGal with the MR resulting in more efficient internalization into the cell while the SR results in faster targeting to the lysosomes.

Further analysis with our αGal sample using confocal uptake studies done on live cells (Figure 3-9, 3-11) and time course studies in fixed cells (Figure 3-12, 3-13, 3-17) indicate that uptake in IMFE1 cells is initially rapid at t < 3 hours and plateaus with a longer incubation time of 19 hours (Figure 3-13, 3-17). This is in contrast to general trends observed with Aco-αGal in which overall uptake as well as percentage of internalized enzyme targeted to the lysosomes (% co-localized) increases steadily over a period of 19 hours (Figure 3-28B) (Table 3-4). This steady increase indicates the potential of Aco-αGal as an improved form of therapy and should therefore be further analyzed in longer experiments in vitro as well as in vivo.

In contrast to HSA we were unable to confirm successful aconitylation of our recombinant αGal. TNBS and fluorescamine assays had varying results with variable preparations and were consistently inconclusive with the number of free surface lysines being identified post aconitylation being higher than

![Figure 3-36: Isoelectric focusing gel of Fabrazyme and Replagal. [3]
Lane 1, Pharmacia IEF standards, pH 2.5–6.5; lane 2, 10 mg Fabrazyme; lane 3, 10 mg Replagal.](image)
pre aconitylation. In addition, IEF gels showed no difference between the aconitylated and non-Aco-αGal samples (Figure 3-32A). Both of these samples appeared as a broad band or as a set of bands in the pH range of 4-5. This result poses questions of its own as one would expect the non-aconitylated αGal sample to appear as a single band on an IEF gel. The therapeutics currently being produced in mammalian cells appear to be similar on an IEF gel to the gels performed in this lab (Figure 3-32A) [3]. This is consistent with αGal being produced in mammalian cells containing negatively charged carbohydrates with M6P and sialic acid components accounting for the lower pH range and the variability in charge seen on published IEF gels (Figure 3-36, 3-37) [19]. Further studies with our recombinant αGal are needed to analyze the reason for the pattern we see on our IEF gels. It is unclear how high mannose glycans would have any effect on the recombinant protein charge. Another explanation for the multiple charged proteins observed could be protein deamidation which may occur during the purification process.

![Image](image_url)

**Figure 3-37: Comparison of the sizes and charges of agalsidase beta [19].**

(A) Sizes of agalsidase beta (Fbz; Fabrazyme) were compared in SDS-PAGE before and after deglycosylation using PNGase F digestion. Here, M represents the molecular weight marker. (B) Isoelectric patterns were analyzed in IEF gel with/without a sialidase treatment.
The inability to show aconitylation $\alpha$Gal could also be due to the fact that $\alpha$Gal contains only approximately 20 exposed surface lysines while HSA contains about 60 lysines with most of them having well-exposed lysine –NH groups making it possible to produce strongly negatively charged poly-anionic molecules by reaction with cis aconitic anhydride.

The argument can therefore be made that the current available therapeutic for ERT, which due to the presence of M6P and sialic acid residues is more negatively charged than the recombinant $\alpha$Gal produced here, may also be targeted to the scavenger receptor uptake pathway to some extent.

![Graph](image)

**Figure 3-38: Comparison of the association of M6P-HSA liposomes and AcoHSA liposomes [5].**

Liver endothelial cells were incubated with $[^{3}H]$cholesteryloleyl ether (3H-COE) labelled M6P-HSA liposomes (closed bars) and AcoHSA liposomes (open bars) in the absence or presence of 0.1 mg/ml M6P-HSA, AcoHSA or 10 mg/ml polyinosinic acid. The association of M6P-HSA liposomes and AcoHSA liposomes without treatment was taken as 100%. mean±SEM of 3–4 experiments. **P<0.001 versus M6P-HSA liposomes without treatment, #P<0.05, ##P<0.001 versus AcoHSA liposomes without treatment.
extent. These results corroborate published data suggesting that coupling M6P-HSA to liposomes targets them to both the M6P and scavenger receptor uptake pathways in hepatic stellate and endothelial cells (Figure 3-38) [5]. Therefore, enhancing the negative charge on αGal should increase this targeting and result in a more efficient enzyme for ERT.

**Conclusions:**

Our overall results indicate the potential for the development of a therapeutic produced in *Pichia* allowing for targeting to the MR with an increase in negative charge resulting in better internalization to the lysosomes by targeting to the SRs. The aconitylation procedure results in the loss of approximately 50% in specific activity of αGal and therefore is not ideal for the addition of negative charge. One possible solution to adding more negative charge to αGal is to construct multi-arm–PEG-αGal-Aco-HSA conjugates, and liposomes encapsulating αGal with surface Aco-HSA-targeting (Figure 3-39) Further studies involving construction of these conjugates and multi-molecular assemblies composed of recombinant human αGal and aconitylated HSA should be evaluated for targeting αGal to the SR uptake system without the loss in specific activity seen with aconitylation, offering the potential for a better enzyme for ERT.

**Figure 3-39: Proposed α-Gal A Conjugates.**
Literature Cited:


