

FOCUS: MASS SPECTROMETRY AS A PROBE OF HIGHER ORDER PROTEIN STRUCTURE: RESEARCH ARTICLE

Substrate and Substrate-Mimetic Chaperone Binding Sites in Human α -Galactosidase A Revealed by Affinity-Mass Spectrometry

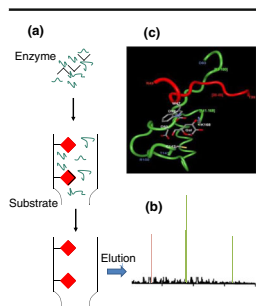
Adrian Moise,^{1,2} Stefan Maeser,¹ Stephan Rawer,³ Frederike Eggers,² Mary Murphy,⁴ Jeff Bornheim,⁴ Michael Przybylski^{1,2}

¹Steinbeis Center for Biopolymer Analysis and Biomedical Mass Spectrometry, 65428, Rüsselsheim am Main, Germany

²Laboratory of Analytical Chemistry, University of Konstanz, 78457, Konstanz, Germany

³ThermoFisher Scientific, Franfurter Strasse 129, 64269, Darmstadt, Germany

⁴Ametek-Reichert Technologies, 3362 Walden Avenue, 14043, Buffalo, NY, USA



Abstract. Fabry disease (FD) is a rare metabolic disorder of a group of lysosomal storage diseases, caused by deficiency or reduced activity of the enzyme α -galactosidase. Human α -galactosidase A (h α GAL) hydrolyses the terminal α -galactosyl moiety from glycosphingolipids, predominantly globotriaosylceramide (Gb3). Enzyme deficiency leads to incomplete or blocked breakdown and progressive accumulation of Gb3, with detrimental effects on normal organ functions. FD is successfully treated by enzyme replacement therapy (ERT) with purified recombinant h α GAL. An emerging treatment strategy, pharmacologic chaperone therapy (PCT), employs small molecules that can increase and/or reconstitute the activity of lysosomal enzyme trafficking by stabilizing misfolded isoforms. One such chaperone, 1-deoxygalactonojirimycin (DGJ), is a structural galactose analogue currently validated in clinical trials. DGJ is an active-site-chaperone that binds at the same or similar location as galactose; however, the molecular determination of chaperone binding sites in lysosomal enzymes represents a considerable challenge. Here we report the identification of the galactose and DGJ binding sites in recombinant α -galactosidase through a new affinity-mass spectrometry-based approach that employs selective proteolytic digestion of the enzyme–galactose or –inhibitor complex. Binding site peptides identified by mass spectrometry, [39–49], [83–100], and [141–168], contain the essential ligand-contacting amino acids, in agreement with the known X-ray crystal structures. The inhibitory effect of DGJ on galactose recognition was directly characterized through competitive binding experiments and mass spectrometry. The methods successfully employed in this study should have high potential for the characterization of (mutated) enzyme–substrate and –chaperone interactions, and for identifying chaperones without inhibitory effects.

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Keywords: Fabry disease, Human α -galactosidase, Chaperones, Binding sites, Proteolytic extraction, Affinity-mass spectrometry

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Introduction

Lysosomal enzymes are responsible for the degradation of a wide variety of substrates including glycoproteins and glycolipids. Mutations in the encoding genes may lead to inactive or misfolded and unstable enzyme configurations, which are targeted for endoplasmic reticulum-associated

protein degradation (ERAD) [1, 2]. The enzyme deficiency results in substrate accumulation and subsequent organ dysfunction. Fabry disease (FD) is characterized by accumulation of glycosphingolipids, particularly Gb3, in the vascular endothelium and visceral tissues throughout the body. The accumulation starts early in life and after 30–40 y, it leads to severe renal, cardiac, and neurologic complications [3, 4].

The deficient enzyme in FD is α -galactosidase A (GLA), which hydrolyses the terminal α -D-galactosyl moieties from glycolipids. The deficiency of GLA results in an inability to

catabolize glycolipids with terminal α -galactose. GLA is a homodimer of two approximately 50 kDa subunits, with each subunit containing 398 amino acid residues. The enzyme itself is glycosylated, the monomer having three N-linked glycosylation sites [3, 4]. The glycosylation is essential for enzyme trafficking to the lysosome [5]. Treatment of FD is currently carried out through enzyme replacement therapy (ERT) [6–8]. ERT employs injections of purified recombinant enzyme (r-h α GAL) in order to increase enzyme levels and reduce the amounts of stored substrate. Disadvantages of ERT are the immune response, bioavailability of recombinant enzymes, and high costs [9]. Two drugs have been approved in the EU for the treatment of FD: Agalsidase alfa (Replagal, Shire Human Genetic Therapies) and Agalsidase beta (Fabrazyme, Genzyme). The difference between the two products is the glycan structure as a result of the expression systems [10]. Agalsidase alfa, the enzyme employed in this work, is synthesized in a line of human fibroblasts and carries the normal human glycosylation profile.

An alternative, novel approach for treatment of LSDs is pharmacologic chaperone therapy (PCT) [11–14]. Pharmacologic chaperones (PCs) are small molecules capable of binding to and stabilizing mutant enzymes. The stabilization function decreases ERAD and may result in increased catalytic function. PCT is a promising approach that could be used alone or in combination with ERT to correct for enzyme deficiency. A suitable candidate for PCT of FD, currently undergoing clinical trials, is 1-deoxygalactonojirimycin (DGJ) 2, [15–17]. DGJ is a specific active-site chaperone (ASSC), binding in the same location as galactose. The structures of GAL complexes with galactose and DGJ were determined by X-ray crystallography, and thus may be employed as model systems for the development of alternative and complementary analytical methods. In addition to X-ray crystallography, there is currently a considerable need for methods enabling the rapid molecular determination of chaperone binding sites in lysosomal enzymes, with high sensitivity and low requirements in sample purity.

Here we report a combination of proteolytic affinity extraction and mass spectrometry for the identification of the binding sites of galactose and DGJ in human α -galactosidase, and evaluation of the inhibitory effect of DGJ on the galactose binding. The effect on galactose recognition was also evaluated for Ambroxol (ABX), a compound well-known medically and used as an expectorant [12]. Recent work demonstrated therapeutic effects of ABX on β -glucocerebrosidase (GCCase), the deficient enzyme in Gaucher's disease; moreover, the application of ABX *in vivo* in patient-derived fibroblasts was shown to increase mutant GCCase activity [12, 18]. Proteolytic-affinity mass spectrometry approaches have been previously developed and successfully applied to the molecular identification of ligand binding sites in several biopolymers such as protein-peptide and antibody-antigen interactions, and to specific carbohydrate-binding peptides from galectins [19–24]. In this study, corresponding methods were successfully developed and demonstrated to provide structural details on binding sites with high specificity, sensitivity, and rapid analysis time.

Experimental

Immobilization of Galactose

Sepharose volumes described refer to volumes of drained medium. Covalent immobilization of galactose (Sigma, Taufkirchen, Germany) on Sepharose was carried out using divinyl sulfone (Sigma) activation as previously described [24]. Sepharose 4B (Sigma; 1 mL) was washed with 5 mL 0.5 M Na₂CO₃ pH 11, drained, and then resuspended in 1 mL 0.5 M Na₂CO₃ with 0.1 mL divinyl sulfone (DVS), and the suspension mixed end-over-end in a 90° rotary sample mixer for 70 min at 25 °C. The matrix was drained, washed with 5 mL 0.5 M Na₂CO₃ pH 11, and DVS-activated Sepharose resuspended in 1 mL 10% galactose solution in 0.5 M Na₂CO₃, and mixed for 18 h at 25 °C. The affinity matrix was then washed with 5 mL 0.5 M Na₂CO₃. Blocking of unreacted vinyl groups was carried out with 1 M ethanolamine for 1 h at 25 °C. The matrix was then washed sequentially with 0.1 M ammonium acetate containing 0.5 M NaCl, pH 4, and 0.1 M Tris-HCl/0.5 M NaCl, pH 8, and finally equilibrated in 25 mM Na₂HPO₄/20 mM NaCl, pH 7.5, and stored at 4 °C. The Sepharose blank used for control experiments was prepared by activating the matrix with DVS as described above, treatment with 1 M ethanolamine for 18 h at 25 °C, and washing with buffers as described above.

Immobilization of DGJ

DGJ (*N*-5-carboxypentyl-1-deoxygalactonojirimycin) was obtained from Toronto Research Chemicals (Toronto, Canada) and covalently coupled to Toyopearl HW-65-NH₂ (Tosoh Bioscience, Stuttgart, Germany). A 3 mL aliquot of matrix was washed sequentially with 30 mL water (MilliQ, Millipore, Eschborn, Germany) and 0.5 M NaCl pH 4.5–6.0 on a sintered glass filter. DGJ (1 mg) and 100 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were dissolved in 3 mL water, pH 5.5, mixed for 24 h at 25 °C, and the gel washed with 1 M NaCl. Unreacted amino groups were blocked with 0.2 M sodium acetate (0.8 mL/mL-gel) and acetic anhydride (0.4 mL/mL-gel) by shaking for 30 min at 0 °C. Additional acetic anhydride (0.4 mL/mL-gel) was added and the mixture shaken for 30 min at 25 °C. After blocking, the gel was washed sequentially with water and 0.1 M NaOH and stored at 4 °C in water containing 0.02% sodium azide.

Proteolytic Extraction-Mass Spectrometry

Recombinant human α -galactosidase A (GLA) was obtained from Sino Biological Inc. (Beijing, China). The enzyme was produced in human cells and, hence, has the identical amino acid sequence and glycosylation profile of the human natural enzyme. A 100 μ L GLA solution containing 100 μ g GLA was mixed with 100 μ L of 20 mM NH₄HCO₃ pH 8 containing 1 μ g/ μ L DTT. Trypsin was added to give an enzyme:substrate ratio of 1:100 and incubated for 12 h at 37 °C. Additional trypsin was then added to an enzyme:substrate ratio of 1:50, and the enzyme-

substrate mixture incubated overnight. Aliquots of 40 μ L peptide mixture containing the equivalent of 20 μ g enzyme were frozen and stored at -20 $^{\circ}$ C until use. An aliquot of digest was mixed with 160 μ L of 20 mM NH_4HCO_3 , pH 8, added over 200 μ L galactosyl-DVS-Sepharose and incubated for 12 h at 37 $^{\circ}$ C. Unbound peptides were removed by four washing steps with 400 μ L binding buffer, and the absence of peptide in the last fraction confirmed by MALDI-MS. The remaining affinity-bound peptides were eluted with 400 μ L of 60% ACN / 0.1% TFA (Figure 1a). Alternatively, the elution was performed with the same solvent with 1 μ g/ μ L DTT added. The specificity of binding peptides for galactose was ascertained by performing the extraction experiment on unmodified Sepharose, in which case no peptide was recovered.

For identification of DJG binding peptides, a solution of 20 μ g of α -galactosidase A was digested with trypsin (enzyme:substrate ratio 1:40) in 100 μ L 25 mM NH_4HCO_3 pH 8, 1 μ g/ μ L DTT, for 24 h at 37 $^{\circ}$ C, followed by another addition of trypsin (to an enzyme:substrate ratio of 1:20) and digested for another 24 h at 37 $^{\circ}$ C. The peptide mixture was lyophilized, dissolved in 200 μ L of 10 mM ammonium acetate, pH 4.5, and DTT (1 μ g/ μ L), and incubated overnight at 25 $^{\circ}$ C. Unbound peptides were removed by washing with 4 mL binding buffer in 400 μ L fractions. Affinity-bound peptides were then eluted under strong shaking with 400 μ L ACN:0.1%TFA 2:1, DTT 1 μ g/ μ L for 15 min at 37 $^{\circ}$ C, and this procedure was repeated twice. The supernatant, washing, and elution fractions were collected and analyzed by MALDI-MS. To evaluate unspecific binding of peptides to Toyopearl HW-65, a control experiment was performed using unmodified matrix; no peptide was recovered.

Inhibition of Galactose Recognition Using DGJ and ABX

The inhibitory effect of DGJ and ABX was evaluated by proteolytic extraction-mass spectrometry in six parallel experiments, including several controls. Trypsinized enzyme (120 μ g) was split between six affinity columns (Table 1), with columns 1–5 containing 200 μ L galactosyl-DVS-Sepharose and the column 6 a 200 μ L Sepharose blank. DGJ, ABX, galactose, and fucose were added over columns 2–5 to final concentrations of 2 mM, respectively; the final buffer volume was 200 μ L. The columns were incubated for 12 h at 37 $^{\circ}$ C, and unbound peptides removed by four steps of washing with 400 μ L binding buffer. The remaining affinity-bound peptides were eluted with 400 μ L 60% ACN/0.1% TFA, containing 1 μ g/ μ L DTT.

Mass Spectrometry

MALDI-TOF mass spectrometry was carried out with a Bruker Biflex linear TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen UV laser (337 nm), a dual channel plate detector, and a 26-sample SCOUT source. A saturated solution of α -cyano-4-hydroxy-cinnamic acid in ACN/0.1 TFA in water (2:1 v/v) was used as matrix. Aliquots of 1 μ L sample solution and matrix solution were mixed on the stainless steel MALDI target and allowed to dry. Spectra were acquired at an acceleration voltage of 20 kV and a detector voltage of 1.5 kV.

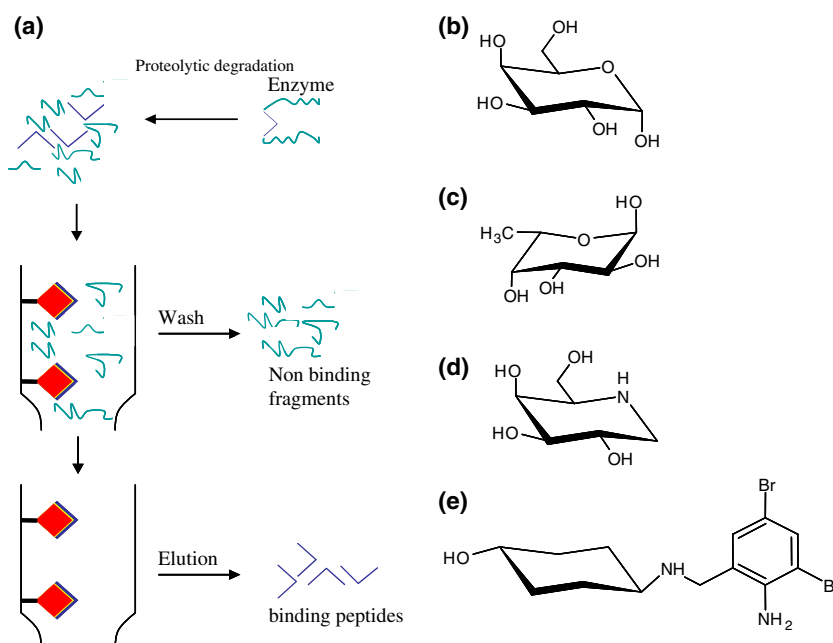


Figure 1. (a) Scheme of proteolytic-extraction mass spectrometry for the identification of carbohydrate binding structures in proteins. The protein is first digested in solution and the resulting fragments bound on a column with immobilized carbohydrate. Non-binding peptides are removed by washing and the binding fragments are then recovered. (b) α -D-galactose; (c) α -L-fucose; (d) DGJ, 1-deoxygalactonojirimycin; (e) ABX, trans-4-(2-amino-3,5-dibromobenzylamino)-cyclohexanol

Table 1. Results of Proteolytic Extraction-MS Experiments with Added Inhibitors and Affinity Determinations of Ligand Epitope Peptides

No.	Column	Inhibitor	Peptides identified	Inhibitory effect	K_D^a
1	Immobilized galactose	-	[83–100], [141–168]	N/A	5.4 9.2
2	Immobilized galactose	DGJ	-	Yes	
3	Immobilized galactose	ABX	[83–100], [141–168]	No	
4	Immobilized galactose	Gal	-	Yes	0.0031
5	Immobilized galactose	Fuc	[83–100], [141–168]	No	
6	Sepharose blank	-	-	N/A	

^a $\times 10^{-3}$ M

Synthesis and Purification of Epitope Peptides

The aGal ligand epitope peptides aGal [83–100] and [141–168], and analogous peptides were synthesized by solid phase peptide synthesis (SPPS) on a semiautomatic peptide synthesizer (ResPepSL; Intavis Bioanalytical Instruments, Köln, Germany) according to the Fmoc strategy. Syntheses were carried out on a Rink Amide resin (GL Biochem Ltd., Shanghai, China), with a loading capacity of approximately 0.5 mmol/g. The aGal peptides and the reaction mixture were purified by reversed phase high performance liquid chromatography (RP-HPLC) on a semipreparative Vydac C12 column, using the mobile phases, 80% acetonitrile, 0.1% TFA in milliQ water (eluent B), and 0.1% TFA in milliQ water (eluent A). A linear gradient of 0–60% eluent B was used in 60 min at a flow rate of 3 mL/min. Purified peptides were characterized by ESI-MS and MALDI-MS.

Affinity Determinations

Affinity K_D determinations were performed with a Dual Channel Ametek-2Ch-7500 SPR biosensor (Ametek-Reichert Technologies, Buffalo, NY, USA). Epitope peptides were immobilized on gold chips as previously described [23, 24], and different concentrations were injected. K_D determinations were performed as previously described [25], providing a pseudo first order kinetic constant. Plotting k_{obs} against the concentration provided association (k_{on}) and dissociation constants (k_{off}), from which $K_D = k_{off}/k_{on}$ was determined.

Results and Discussion

Identification of the Galactose Binding Site of α -Galactosidase

N-terminal sequence analysis of GAL showed the lack of a signal sequence, the first amino acid being Leu32. For better comparison with literature data, the sequence numbering of GAL was kept identical as in the intact protein. Identification of the galactose binding site was performed using the proteolytic extraction MS approach. The enzyme was digested with trypsin in solution, and the resulting peptide mixture was added over a column with galactose immobilized on Sepharose using divinyl sulfone activation (Figure 1). The supernatant was collected and analyzed by MALDI-MS. After washing away any unbound peptides, the remaining affinity-bound peptides were eluted with an ACN-based solvent [23]. The mass spectrum of the elution fraction revealed two specific peptides, GAL [83–100] and GAL [141–168] (Figure 2). Peptide [83–100] was also found as an oxidation product with an internal disulfide bond, (Cys90–Cys94). This

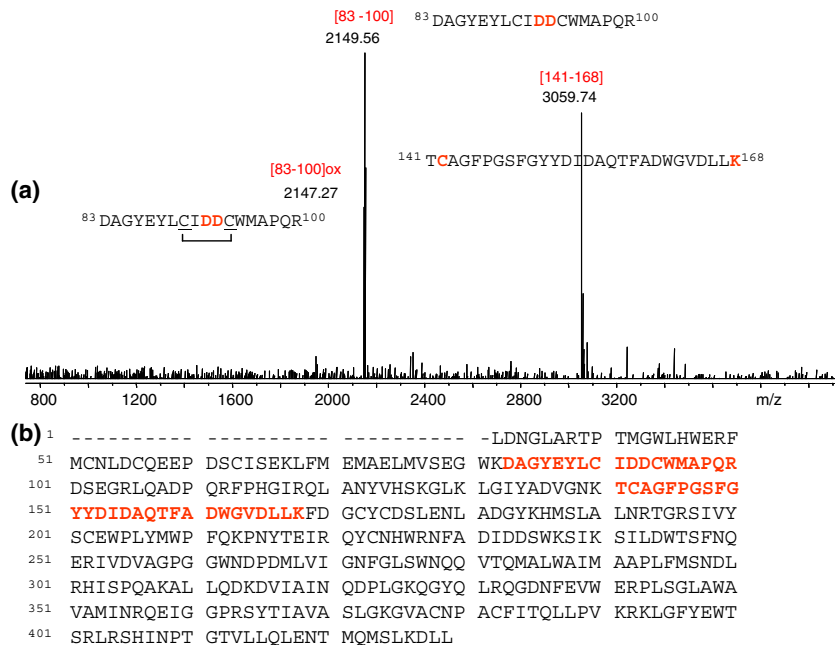


Figure 2. (a) MALDI-TOF mass spectrum of the elution fraction from tryptic extraction of haGAL on immobilized galactose. Monoprotonated ions of peptides [83–100], [83–100]ox, and [141–168] were identified. (b) Sequence of haGAL with identified peptides highlighted in red.

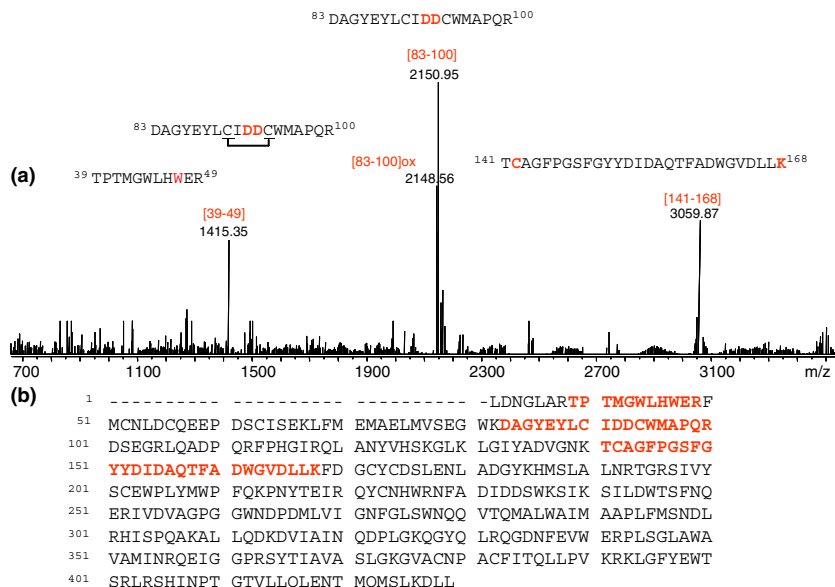


Figure 3. (a) MALDI-TOF mass spectrum of the elution fraction from the tryptic extraction of haGAL on immobilized DGJ showing the monoprotonated ions of binding peptides [39–49], [83–100], [83–100]ox, and [141–168]. (b) Sequence of haGAL with identified peptides highlighted in red

disulphide bond was not found in the native protein structure and, hence, was formed during or after the elution, but was eliminated by employing DTT in the elution.

The identified peptides [83–100] and [141–168] contain several specific amino acids that are in agreement with the X-ray crystallography determination to interact with galactose [3]. The sequence [83–100] contains Asp92 and Asp93, which form hydrogen bonds with the galactose 5-OH and 6-OH groups, respectively. The peptide [141–168] contains Cys142 which participates in van der Waals interactions with galactose, and Lys168, which forms hydrogen bonds with the 3-OH and 4-OH groups of galactose. The peptide [39–49] containing the amino acid Trp47 involved in substrate selection was not found but identified in the binding of DGJ as described below. The lack of this peptide in the binding with galactose is not fully

understood. It may be due to differences in binding affinity of the peptide, and/or due to the hydrophobic C5 linker in DGJ that influences this peptide's binding.

The lack of peptides spanning the amino acids D170, E203, R227, and D231 may be explained by the shielding of these residues due to the glycosylation site of aGal at N215. Corresponding shielding of proteolytic cleavage sites near glycosylated sequences have been observed in previous studies [26]. Missed tryptic cleavage sites have been in fact found between residues 192 and 240.

Identification of the DGJ Binding Site

The binding site of DGJ was identified following the same protocol as employed for galactose. The mass spectrum of the

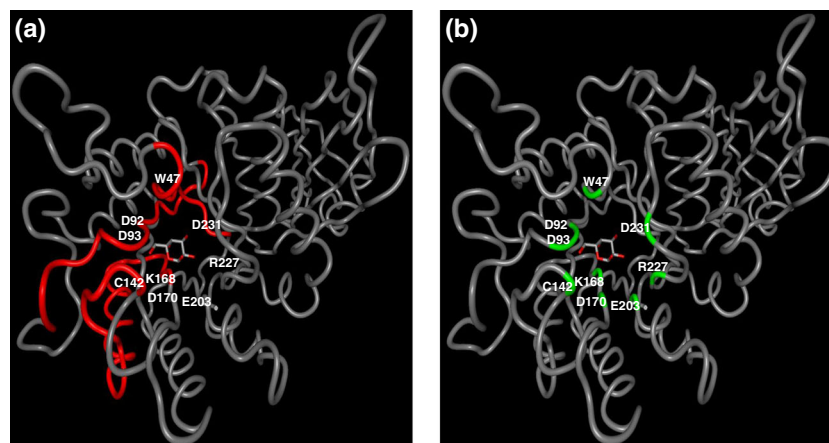


Figure 4. X-ray crystal structure of haGAL in complex with ga-lactose (PDB entry 1R47). The MS-identified galactose and DGJ binding peptides [39–49], [83–100], and [141–168], are shown in red (a), whereas the substrate binding site identified by X-ray crystallography is shown in green (b). Amino acids that are in direct contact with the carbohydrate are keyed in bold. Galactose is represented as stick model

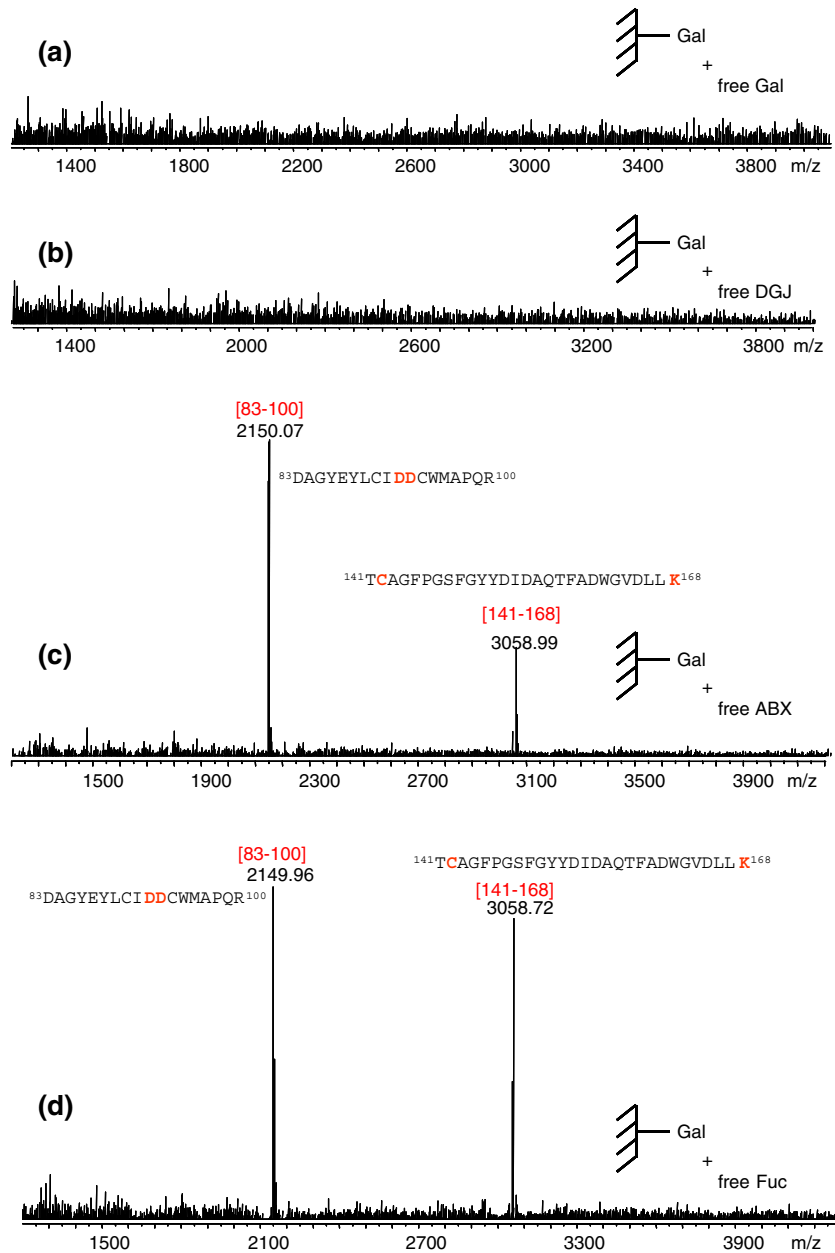


Figure 5. MALDI mass spectra of the elution fractions with immobilized galactose and added inhibitors. The free galactose **(a)** and free DGJ **(b)** inhibited binding of peptides [83–100] and [141–168] to immobilized galactose. Free ABX **(c)** and free fucose **(d)** did not interfere with the binding of peptides [83–100] and [141–168] to galactose

elution fraction (Figure 3) showed the presence of three peptides, GAL [39–49], GAL [83–100], and GAL [141–168], in complete agreement with the results obtained for the binding of galactose. DGJ is a structural analogue of galactose and was found to bind to the original substrate binding site [12]. Several hydrogen bonds established with the galactose binding were also formed when DGJ was bound. The Asp92 and Asp93 residues were shown to interact with O5 and O6 of the DGJ molecule, whereas Lys168 interacts with the O4 group. The additional peptide found for DGJ, GAL [39–49] contains the Trp47 residue that stacks against C4–C5–C6 of galactose [3] and is important for substrate selection. This tryptophane residue was not expected to directly interact with DGJ, but should

have a stabilizing function on the surface of the binding pocket near the Asp92 and Asp93 residues (Figure 4).

Inhibitory Effects of DGJ and ABX on Galactose Binding

To assess the influence of chaperone binding on the activity of GAL, the proteolytic extraction-MS experiments were performed in the presence of chaperones (DGJ, ABX) and control monosaccharides (galactose, fucose) (Figure 1). Since GAL is specific for the immobilized galactose and, as shown above, produces galactose-binding peptides, mixing various small molecules with the tryptic digest may lead to competition for

the tryptic peptides between the immobilized galactose and the free ligands. However, competition will only occur if the peptides have affinity for the ligands and will result in specific peptides missing in the elution fraction. Comparing the results of the proteolytic extraction experiments in the absence and in the presence of other ligands can give information on the specificity of ligands that cannot be easily immobilized. For this study, five affinity microcolumns with immobilized galactose and a sixth column with unmodified Sepharose were prepared and tested. On four of the columns with immobilized galactose, a different ligand (Figure 1) was added together with the GAL tryptic digest. On the other two columns that served as controls (one with immobilized galactose, the other with unmodified Sepharose), only the tryptic digest was added.

A summary of the results obtained with the combination of columns, samples, and inhibitors is shown in Table 1. Mass spectra of the elution fractions from each column are shown in Figure 5. From the control column with immobilized galactose, two peptides were recovered, [α GAL83-100] and [α GAL141-168]. From the blank column no peptide was recovered, indicating that none of the α GAL tryptic peptides exhibit unspecific binding to Sepharose. The elution fraction from the column with added galactose did not contain any peptides due to the galactose in solution interfering with the binding of peptides to the immobilized galactose. The mass spectrum of the elution fraction from the column with added fucose showed the same two peptides (α GAL [83-100] and α GAL [141-168]) as the control column, indicating that the binding of these peptides to galactose is not prevented by fucose. α GAL does not bind to fucose and it was reasonable to expect that α GAL-derived peptides also would not bind. No peptides were recovered from the column with added DGJ indicating that peptides α GAL [83-100] and α GAL [141-168] have affinity for DGJ. The two peptides were recovered from the column with added ABX, showing that ABX does not compete with galactose for binding of α GAL [83-100] and α GAL [141-168]. These results were in agreement with affinity data of the synthetic peptide ligands [83-100] and [141-168] determined by SPR (Table 1). Peptide affinities, albeit being only in the low mM range compared to the intact enzyme (ca. 3 μ M), were found to bind with high specificity as previously observed for lectin-carbohydrate interactions [23, 24].

Conclusions

Proteolytic extraction affinity-MS of human α -galactosidase A on affinity columns with immobilized galactose identified two galactose-binding peptides, α GAL [83-100] and α GAL [141-168], which contain the essential amino acids involved in galactose binding, in agreement with previously reported X-ray crystallography data on the enzyme in complex with galactose. The binding of peptides α GAL [83-100] and α GAL [141-168] to galactose could be inhibited in a proteolytic extraction experiment by addition of DGJ to the digest mixture, indicating affinity of the α GAL peptides for DGJ. These

results show affinity-mass spectrometry as a powerful tool for obtaining relevant information on carbohydrate-binding peptide sites, providing a molecular complement to the traditional X-ray and NMR approaches.

The present binding site study was performed with an active chaperone on a single enzyme as a model system, with a crystal structure available. However, using the same methodology identifications of ligand binding epitopes were successfully performed on a series of lectins without available crystal structure [23, 25]. We therefore expect that the proteolytic extraction method can be well extended to binding studies with low molecular chaperone derivatives of different structures, using the same general immobilization procedures.

Although the affinity of the ligand peptides was found to be substantially lower compared with the protein, their binding specificity suggests some structural elements maintained in agreement with NMR studies of galectin peptides that indicate high flexibility [23, 24]. Further work on synthetic mutant peptides [39-49] and [83-100] is being carried out at present by affinity-MS and bioaffinity determination, in comparison with the wild-type Gal sequences. In summary, affinity-mass spectrometry is shown here to be a promising approach for the identification of binding sites, validation, and screening of substrates and chaperones in the development of new LSD treatment strategies.

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References

1. Lemansky, P., Bishop, D.F., Desnick, R.J., Hasilik, A., von Figura, K.: Synthesis and processing of α -galactosidase A in human fibroblasts. Evidence for different mutations in Fabry disease. *J. Biol. Chem.* **262**, 2062-2265 (1987)
2. Yam, G.H.-F., Zuber, C., Roth, J.: A synthetic chaperone corrects the trafficking defect and disease phenotype in a protein misfolding disorder. *FASEB J.* **19**, 12-18 (2005)
3. Bekri, S.: In: Mehta, A., Beck, M., Sunder-Plassmann, G. (eds.) *Fabry Disease: Perspectives from 5 Years of FOS*. Oxford Pharma-Genesis, Oxford (2006)
4. Beck, M.: Agalsidase alfa—a preparation for enzyme replacement therapy in Anderson-Fabry disease. *Expert Opin. Investig. Drugs* **11**(6), 851-858 (2002)
5. Garman, S.C., Garboczi, D.N.: The molecular defect leading to Fabry disease: structure of human α -galactosidase. *J. Mol. Biol.* **337**, 319-335 (2002)
6. Ioannou, Y.A., Zeidner, K.M., Grace, M.E., Desnick, R.J.: Human α -galactosidase A: glycosylation site 3 is essential for enzyme solubility. *Biochem. J.* **332**, 789-797 (1998)
7. Desnick, R.: Enzyme replacement and enhancement therapies for lysosomal diseases. *J. Inherit. Metab. Dis.* **27**, 385-410 (2004)
8. Valayannopoulos, V., Brassier, A., Chabli, A., Caillaud, C., Lemoine, M., Odent, T., Arnoux, J.B., de Lonlay, P.: Enzyme replacement therapy for lysosomal storage disorders. *Arch. Pediatr.* **18**, 1119-1123 (2011)
9. Wilcox, W.R., Banikazemi, M., Guffon, N., Waldek, S., Lee, P., Linthorst, G.E., Desnick, R.J., Germain, D.P.: Long-term safety and efficacy of

- enzyme replacement therapy for Fabry disease. *Am. J. Hum. Genet.* **75**, 65–74 (2004)
10. Germain, D.P.: Fabry disease: recent advances in enzyme replacement therapy. *Expert Opin. Investig. Drugs* **11**, 1467–1476 (2002)
 11. Boyd, R.E., Lee, G., Rybczynski, P., Benjamin, E.R., Khanna, R., Wustman, B.A., Valenzano, K.J.: Pharmacological chaperones as therapeutics for lysosomal storage diseases. *J. Med. Chem.* **56**, 2705–2725 (2013)
 12. Ortolano, S., Vieitez, I., Navarro, C., Spuch, C.: Treatment of lysosomal storage diseases: recent patents and future strategies. *Recent Patents Endocr. Metabol. Immune Drug Discov.* **8**, 9–25 (2014)
 13. Parenti, G.: Treating lysosomal storage diseases with pharmacological chaperones: from concept to clinics. *EMBO Mol. Med.* **1**, 268–279 (2009)
 14. Valenzano, K.J., Khanna, R., Powe, A.C., Boyd, R., Lee, G., Flanagan, J.J., Benjamin, E.R.: Identification and characterization of pharmacological chaperones to correct enzyme deficiencies in lysosomal storage disorders. *Assay Drug Dev. Technol.* **9**, 213–235 (2011)
 15. Guce, A.I., Clark, N.E., Rogich, J.J., Garman, S.C.: The molecular basis of pharmacological chaperoning in human α -galactosidase. *Chem. Biol.* **18**, 1521–1526 (2011)
 16. Giugliani, R., Waldek, S., Germain, D.P., Nicholls, K., Bichet, D.G., Simosky, J.K., Bragat, A.C., Castelli, J.P., Benjamin, E.R., Boudes, P.F.: A Phase 2 study of migalastat hydrochloride in females with Fabry disease: selection of population, safety and pharmacodynamic effects. *Mol. Genet. Metab.* **109**, 86–92 (2012)
 17. Khanna, R., Soska, R., Lun, Y., Feng, J., Frascella, M., Young, B., Brignol, N., Pellegrino, L., Sitaraman, S.A., Desnick, R.J., Benjamin, E.R., Lockhart, D.J., Valenzano, K.J.: The pharmacological chaperone 1-deoxygalactonojirimycin reduces tissue globotriaosylceramide levels in a mouse model of Fabry disease. *Mol. Ther.* **18**, 23–33 (2010)
 18. Bendikov-Bar, I., Maor, G., Filocamo, M., Horowitz, M.: Ambroxol as a pharmacologic chaperone for mutant β -glucocerebrosidase. *Blood Cells Mol. Dis.* **50**, 141–145 (2013)
 19. McLaurin, J., Cecal, R., Kierstad, M.E., Tian, X., Phinney, A.L., Manea, M., French, J.E., Lambermon, M.H., Darabie, A.A., Brown, M.E., Janus, C., Chishti, M.A., Horne, P., Westaway, D., Fraser, P.E., Mount, M.T., Przybylski, M., St. George Hyslop, P.S.G.: Therapeutically effective antibodies against amyloid- β -peptide target amyloid- β -residues 4–10 and inhibit cytotoxicity and fibrillogenesis. *Nat. Med.* **8**, 1263–1269 (2002)
 20. Juszczak, P., Paraschiv, G., Szymanska, A., Kolodziejczyk, A.S., Rodziewicz-Mtowidlo, S., Grzonka, Z., Przybylski, M.: Binding epitopes and interaction structure of the neuroprotective protease inhibitor cystatin C with β -amyloid revealed by proteolytic excision mass spectrometry and molecular docking simulation. *J. Med. Chem.* **52**, 2420–2428 (2009)
 21. Stefanescu, R., Born, R., Moise, A., Ernst, B., Przybylski, M.: Epitope structure of the carbohydrate recognition domain of asialoglycoprotein receptor revealed by high resolution proteolytic excision mass spectrometry. *J. Am. Soc. Mass Spectrom.* **22**, 148–157 (2012)
 22. Stefanescu, R., Iacob, R.E., Damoc, E.N., Marquardt, A., Amstalden, E., Manea, M., Perdivara, I., Maftai, M., Paraschiv, G., Przybylski, M.: Mass spectrometric approaches for elucidation of antigen-antibody recognition structures in molecular immunology. *Eur. Mass Spectrom.* **13**, 69–75 (2007)
 23. Moise, A., Andre, S., Eggers, F., Krzeminski, M., Przybylski, M., Gabius, H.J.: Towards bioinspired galectin mimetics: identification of ligand contacting peptides by proteolytic excision mass spectrometry. *J. Am. Chem. Soc.* **133**, 14844–14847 (2011)
 24. Jimenez-Castells, C., Defaus, S., Moise, A., Przybylski, M., Andreu, D., Gutierrez-Gallego, R.: Surface-based and mass spectrometric approaches to deciphering sugar-protein interactions. *Anal. Chem.* **84**, 6515–6520 (2012)
 25. Moise, A.: Identification and quantification of carbohydrate interacting structures in proteins using affinity-mass spectrometry. Dissertation, University of Konstanz (2014)
 26. Perdivara, I., Deterding, L.E., Cozma, C., Tomer, K.B., Przybylski, M.: Glycosylation profiles of epitope-specific anti- β -amyloid antibodies revealed by liquid chromatography-mass spectrometry. *Glycobiology* **19**, 958–970 (2009)