Identification of Asp197 as the catalytic nucleophile in the family 38 α-mannosidase from bovine kidney lysosomes

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Abstract Bovine kidney lysosomal α-mannosidase is a family 38 α-mannosidase involved in the degradation of glycoproteins. The mechanism-based reagent, 5-fluoro-β-L-gulosyl fluoride, was used to trap a glycosyl–enzyme intermediate, thereby labelling the catalytic nucleophile of this enzyme. After proteolytic digestion and high performance liquid chromatography/tandem mass spectrometry (MS) analysis, a labelled peptide was localised, and the sequence: HIDPFGHSRE determined by fragmentation tandem MS analysis. Taking into consideration sequence alignments of this region with those of other α-mannosidases of the same family, this result strongly suggests that the catalytic nucleophile in this enzyme is Asp197.

Key words: Lysosomal α-mannosidase; Nucleophile; Mass spectrometry; Enzyme mechanism; Glycosidase

1. Introduction

Lysosomal α-mannosidase (EC 3.2.1.24) is a broad specificity exoglycosidase that catalyses the hydrolysis of α-mannoside linkages from the non-reducing end of N-linked glycans [1]. Deficiency of this enzyme causes the lysosomal storage disorder, α-mannosidosis, in which there is an accumulation of partially degraded oligosaccharides. This accumulation, seen in humans [2], cattle [3] and cats [4], results in varying degrees of neural, skeletal and immune defects. Genetic analysis of subjects with this disease shows that there are a number of possible point or deletion mutations that lead to the loss of enzymatic activity [5–7].

On the basis of amino acid sequence, this enzyme has been assigned to family 38 in Henrissat's glycosidase classification [8–10]. This family of α-mannosidases is characterised by its broad natural substrate specificity, its ability to cleave synthetic substrates such as *p*-nitrophenyl α-mannoside and its susceptibility to the inhibitor swainonine [11,12]. By following the reaction by ^{1}H-nuclear magnetic resonance, a representative enzyme from this family was shown to be a retaining glycosidase; that is, the α-stereochemistry at the mannose anomeric centre is retained in the hydrolysis product [13]. From this, it was deduced that these α-mannosidases catalyse hydrolysis by a double displacement mechanism (Fig. 1) in which a glycosyl–enzyme intermediate is formed and hydrolysed via oxacarbenium ion-like transition states. A carboxylic acid in the active site acts as the catalytic nucleophile in the formation of the covalent intermediate while a second carboxylic acid acts as a general acid catalyst. The same residue is thought to assist in the hydrolysis (deglycosylation) step, this time acting as a general base [14,15].

Fluorosugars have been useful mechanism-based inactivators in the study of retaining glycosidases [16–18]. Fluorine substitution at the 2- or 5-position slows down formation and hydrolysis of the covalent intermediate by inductively destabilising the cationic transition states. However, by having a good leaving group such as a dinitrophenyl group or a fluoride at the anomeric centre, the first step can be accelerated. This results in the accumulation of the covalent intermediate. This accumulated intermediate can be isolated, proteolytically digested and then analysed by liquid chromatography-mass spectrometry (LC-MS) to identify the labelled residue [19]. This approach has been used to label and identify the catalytic nucleophiles of a number of retaining glycosidases including an α-mannosidase from Jack Bean [20]. Although the Jack Bean α-mannosidase has many of the characteristics of a family 38 α-mannosidase, its primary sequence has not been determined and thus, it cannot be assigned to a particular family with full confidence. Because of the similarity in properties though, it was expected that the approach used to label the Jack Bean enzyme could also be used to identify the nucleophile of an α-mannosidase known to be in family 38. This paper describes the labelling and identification of the catalytic nucleophile of a known family 38 glycosidase, bovine kidney lysosomal α-mannosidase, using the inactivator 5-fluoro-β-L-gulosyl fluoride (5FgulF).

2. Materials and methods

2.1. General

2,4-Dinitrophenyl α-D-mannoside (DNP-Man) [21] and 5FgulF [20] were synthesised as previously published. Pepsin (from porcine mucosa) was obtained from Boehringer Mannheim. All other buffer chemicals and reagents were obtained from Sigma Chemical Co. unless otherwise noted.

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Abbreviations: 5FgulF, 5-fluoro-β-L-gulopyranosyl fluoride; DNP-Man, 2,4-dinitrophenyl α-D-mannopyranoside; ESI-MS, electrospray ionisation mass spectrometry; MS-MS, tandem mass spectrometry; CID, collision-induced dissociation

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2.2. Protein purification

The purification of bovine kidney lysosomal α-mannosidase was published elsewhere [5] with the addition of the following step. Following the Q-Sepharose column, the enzyme was loaded on a CM-Sepharose (Pharmacia) column (1.5×8 cm) equilibrated with 0.02 M Tris–HCl, pH 7.6 buffer. Using a stepwise gradient of NaCl, the α-mannosidase activity eluted off between 0.8 and 1.0 M NaCl. The eluate was concentrated using an Amicon ultrafiltration unit fitted with a YM 30 membrane and applied to a Superdex 200 column as was previously reported.

2.3. Steady state kinetic analysis

All studies were carried out at 30°C in 100 mM sodium acetate buffer, pH 4.5 containing 0.1% bovine serum albumin. Determination of kinetic parameters for DNP-Man was performed by following the initial increase in absorbance at 400 nm upon the addition of enzyme (typically 3 nM final concentration) to a range of concentrations (0.25–5 mM) of DNP-Man. Measurements were made in 1 cm path length cuvettes with a UNICAM UV/Vis spectrophotometer attached to a circulating water bath. Rates were calculated using an extinction coefficient of $\Delta\varepsilon = 8439\ M^{-1}\ cm^{-1}$ and kinetic parameters were estimated by direct fit of the data to the Michaelis–Menten equation using the programme GraFit 3.09b (Leatherbarrow, R.J., Erithacus Software Ltd.). A K_i value for 5FgulF was determined by measuring rates of hydrolysis of a range of DNP-Man concentrations (0.25–1.0 mM) at each of a series of 5FgulF concentrations (10–200 μM). The data were plotted in the form of a Dixon plot (1/rate vs. [inhibitor]). Linear regression of each line was done using the programme GraFit. The K_i was determined from the intersection of these lines ($K_i = -$intersection). Analysis of 5FgulF as a substrate was carried out by monitoring fluoride ion release using an ORION 96-04 combination fluoride electrode interfaced to a Pentium 133 MHz personal computer.

2.4. Labelling and proteolysis

Enzyme stock (25 μl of 8 mg/ml in 100 mM sodium acetate, pH 4.5) was mixed with 1 μl of 5FgulF (40 mM in water) and incubated for 3 min at room temperature. The sample was diluted with 60 μl of pepsin solution (0.3 mg/ml in 200 mM sodium phosphate buffer, pH 2.0) and incubated for 60 min at room temperature. The sample was then frozen until ready for analysis and was used immediately upon thawing.

2.5. Electrospray ionisation mass spectrometry (ESI-MS)

All mass spectra (LC-MS, tandem mass spectrometry (MS-MS) neutral loss, and collision-induced dissociation (CID) spectra) were

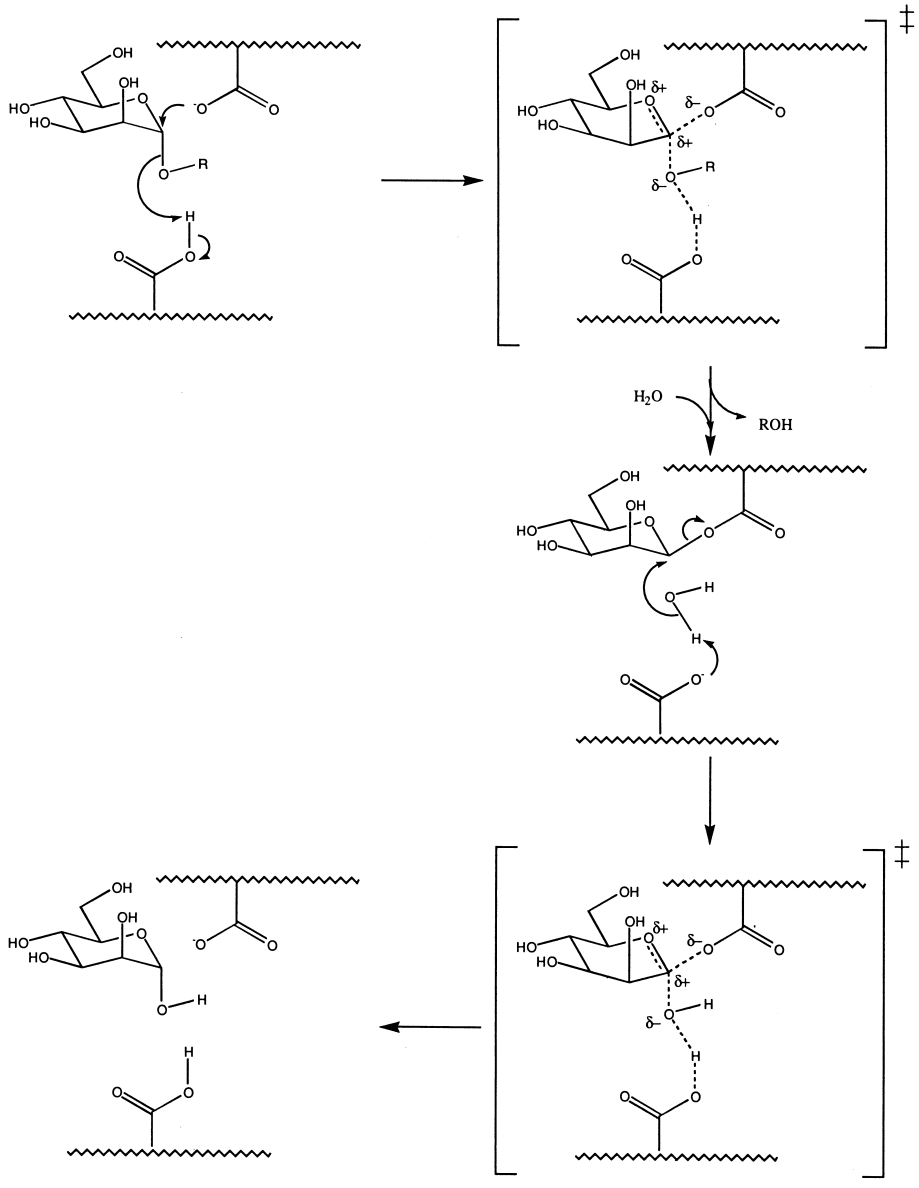

Fig. 1. Proposed mechanism for family 38 α-mannosidases.

recorded on a PE-Sciex API 300 triple quadrupole mass spectrometer (Sciex, Thornhill, Ont., Canada) equipped with an ESI source. Peptides were separated by reverse-phase high performance liquid chromatography (HPLC) onto an Ultra Microprotein Analyzer (Michrom BioResources Inc., Pleasanton, CA, USA) directly interfaced with the mass spectrometer. In each MS experiment, the proteolytic digest was loaded on to a C_{18} column (Reliasil; 1 mm × 150 mm) and then eluted with a gradient of 0–60% solvent B over 60 min at a flow rate of 50 μl/min (solvent A was 0.05% (v/v) trifluoroacetic acid/2% (v/v) acetonitrile in water; solvent B was 0.045% (v/v) trifluoroacetic acid/90% (v/v) acetonitrile in water). In the single quadrupole mode, the MS conditions were as follows. The mass analyser was scanned over the range of 300–2200 amu with a step size of 0.5 amu, a dwell time of 5.0 ms, an ion source voltage of 5 kV, and an orifice voltage of 50 V. The neutral loss spectra were obtained in the triple quadrupole mode searching for the loss of *m/z* 90.5, which corresponds to the loss of the inhibitor label from a peptide that is doubly charged. A scan range of 400–1800 amu was used with a step size of 0.5 amu and a dwell time of 1.5 ms. Other parameters were as follows: ion source voltage = 4.8 kV; orifice energy = 45 V; IQ2 = −42; Q0 = −10; the collision gas was argon/N_2 (9:1).

2.6. Sequencing of labelled peptide by ESI-MS-MS

The CID spectrum of the isolated, labelled peptide was obtained by selectively introducing the *m/z* 688 peptide from the first quadrupole (Q1) into the collision cell (Q2) and then observing the daughter ions in the third quadrupole (Q3). The following settings were used: Q1 was locked on *m/z* 688; Q3 scanned over the range *m/z* 50–1390; the step size was 0.5 and the dwell time 2.0 ms; the ion source potential was 4.8 kV; the orifice potential was 45 V; the focusing ring voltage was 200 V; the Q0 potential was −10 V; the Q2 potential was −42 V; the collision gas was N_2.

3. Results and discussion

In contrast to what was seen with the Jack Bean α-mannosidase, incubation of bovine kidney lysosomal α-mannosidase with 5FgulF did not result in time dependent inactivation of the enzyme. Instead, this compound was found to be a tight binding competitive inhibitor of the lysosomal α-mannosidase with an apparent K_i value of 67 μM (Fig. 2). Considering that the substrate, DNP-Man, has a K_m value of 1.2 mM (results not shown), 5FgulF binds considerably tighter than might be expected for a very simple substrate analogue; especially considering its minimal aglycone (fluoride) and the inverted configuration at C-5. This result is suggestive, however, of 5FgulF acting as a slow substrate with a rate-limiting deglycosylation step, as is seen with some other 5-fluoro sugars [20,22]. Under such conditions, a covalent intermediate accumulates, but is still sufficiently short-lived to reactivate

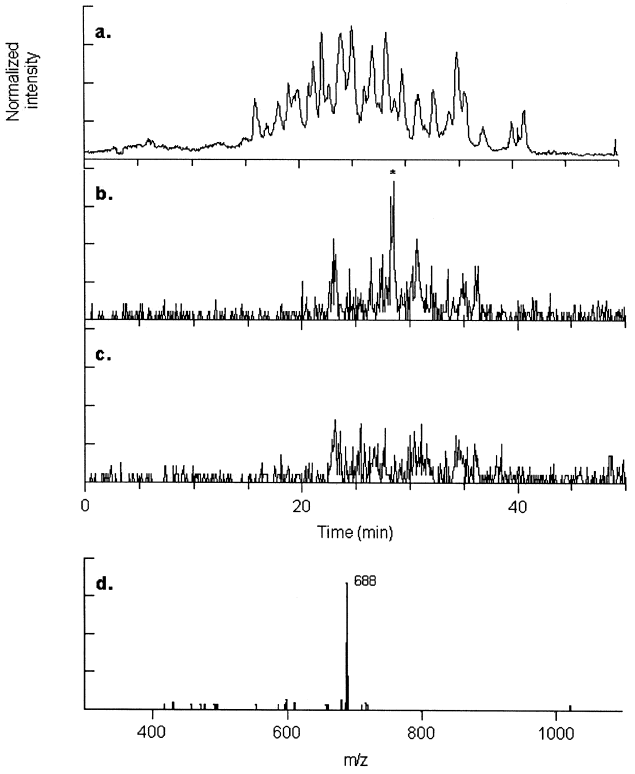

Fig. 3. ESI-MS experiments on peptic digests of bovine lysosomal α-mannosidase. (a) Enzyme incubated with 5FgulF, total ion current in normal MS mode. (b) Enzyme incubated with 5FgulF, total ion current in the neutral loss mode. The labelled peptide of interest is indicated with an asterisk. (c) Unlabelled enzyme, total ion current in neutral loss mode. (d) Mass spectrum of the peptide eluting at 28.37 min.

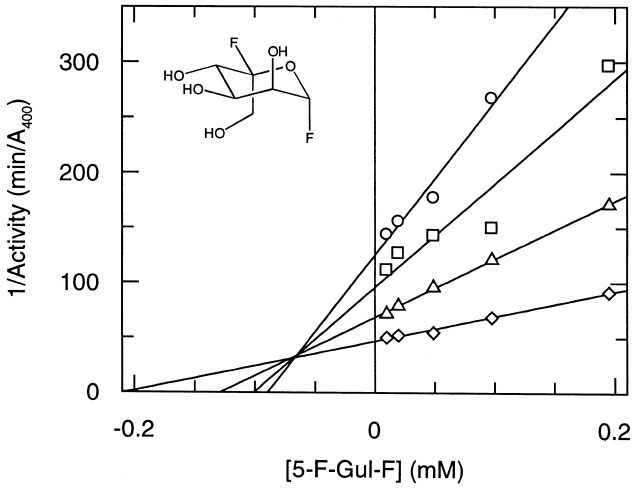

Fig. 2. Dixon plot of the inhibition of bovine lysosomal α-mannosidase by 5FgulF (inset). Enzyme was incubated with the following concentrations of substrate (DNP-Man): 0.25 mM (○), 0.365 mM (□), 0.5 mM (△), 1.0 mM (◇).

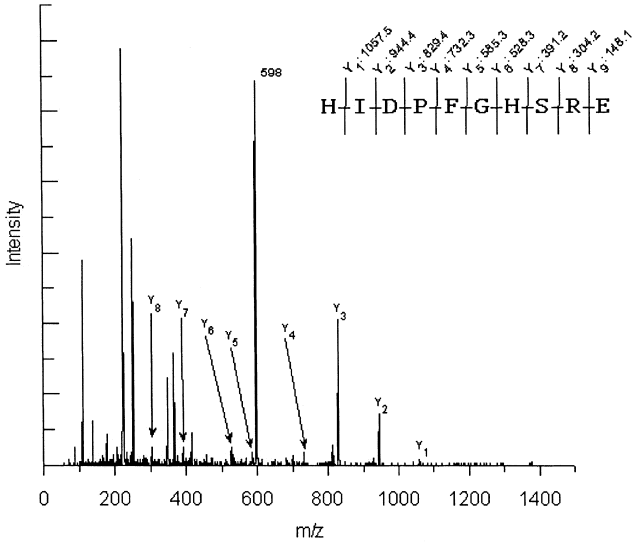

Fig. 4. ESI-MS-CID spectrum of the 5Fgul peptide (*m/z* 688, in doubly charged state). Observed Y series fragments are shown above the peptide sequence.

MA2B_BOVIN	VAWHI**D**PFGHSR**E**QASLF
MA2B_HUMAN	VAWHI**D**PFGHSR**E**QASLF
MAN2_HUMAN	SGWAI**D**PFGHSP**T**MAYLL
MANA_DICDI	IGWHI**D**PFGHSA**T**QARIF
MAN2_DROME	ASWAI**D**PFGHSP**T**MPYIL
MANE_PIG	FSWQV**D**PFGASA**T**TPTLL
MAN1_RAT	EFWLP**D**TFGYSA**Q**LPQIM
MAN1_YEAST	IFWLP**D**TFGYSS**Q**MPQLC
Jack Bean	RAGQI**D**PFGHSA**V**QG

Fig. 5. Partial multiple sequence alignments of eight representative family 38 members and Jack Bean α-mannosidase. The two potential nucleophiles have been shown in bold. Swiss Prot identifiers are indicated in parentheses. The sequences shown are: bovine lysosomal α-mannosidase (Q29451), human lysosomal α-mannosidase (O00754), *Dictyostelium* lysosomal α-mannosidase (P34098), human Golgi α-mannosidase II (Q16706), *Drosophila* Golgi α-mannosidase II (Q24451), pig epididymis-specific α-mannosidase (Q28949), rat endoplasmic reticulum α-mannosidase (P21139), yeast vacuolar α-mannosidase (P22855), Jack Bean α-mannosidase [20]. The sequences were aligned by hydrophobic cluster analysis (courtesy of B. Henrissat CNRS-CERMAV, Grenoble, France).

during the assay time; thus it does not function as an inactivator. The accumulation of the intermediate is, however, seen as a lowering of the apparent K_i relative to the true binding constant. Indeed, when the compound was tested as a substrate by measuring rates of fluoride ion release, 5FgulF was found to be a substrate with a $k_{cat} = 0.022\ s^{-1}$ (by way of comparison, the k_{cat} value of DNP-Man is 9.9 s^{-1}). Consistent with the K_i value of 67 μM determined, the rate was independent of 5FgulF concentration down to at least 0.7 mM.

These results suggested that it should be possible to generate a labelled peptide containing the active site nucleophile by rapid proteolytic digestion of the mannosidase preincubated with 5FgulF such that a glycosyl–enzyme accumulated. The mannosidase was therefore incubated with 1.5 mM 5FgulF followed by pepsin digestion at pH 2 generated a mixture of peptides which was separated by HPLC attached to an ESI-MS detector (Fig. 3a). The labelled peptide was identified by repeating this process but running the MS-MS in neutral loss mode. In this mode, peptides are subjected to mild fragmentation by collision with an inert gas in the second quadrupole. This collision causes a homolytic cleavage of the labile ester bond between the sugar and the peptide, resulting in loss of a neutral sugar. The first and third quadrupoles are scanned in a linked fashion so that only ions whose mass changes by exactly the mass of the label lost in the second quadrupole can pass through the third quadrupole to the detector. In this way, it is possible to identify the ion that contained the ester-linked sugar. In the present case in which 5Fgul is lost, this difference is *m/z* 181 for a singly charged ion and *m/z* 90.5 for a double charged ion. No peptides of note were observed when scanned for a loss of 181. However, when scanned for a loss of 90.5, several peptides were identified (Fig. 3b). Fortunately, only one of these fragments (*m/z* 688) was not seen in the control digest of the unlabelled enzyme (Fig. 3c,d). This doubly charged peptide fragment corresponds to a peptide of mass 1376, which elutes with a retention time of 28.37 min. To confirm the neutral loss analysis results, comparative mapping was done on the peptide digests of the unlabelled and labelled enzyme. As was expected, the identified peptide was not present in the unlabelled digest. Instead, the corresponding unlabelled peptide (*m/z* = 598) was found at around the same retention time. This unlabelled peptide was also found at much lower intensity in the labelled digest due to incomplete labelling.

Both the labelled and corresponding unlabelled peptides were isolated from the digest mixture by HPLC and subsequently sequenced using ESI-MS-MS. The two samples gave very similar mass spectra. Analysis of this fragmentation pattern as shown in Fig. 4 yields the sequence HIDPFGHSRE. Unfortunately, we were unable to identify the specific amino acid residue labelled since no fragments still bearing the label are seen in the CID spectrum. However, this sequence contains only two carboxylic acids, an aspartate and a glutamate. Such residues have been shown to act as the nucleophile in all retaining glycosidases functioning via this mechanism seen to date. Sequence alignment of this fragment with other members of the family 38 α-mannosidase grouping (Fig. 5) shows that only the aspartate, and not the glutamate, is conserved throughout this family. These two results combined strongly suggest that the catalytic nucleophile in bovine kidney lysosomal α-mannosidase is this aspartate 197. The results of this paper confirm the previous tentative assignment of this residue in family 38 α-mannosidases.

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