Identification of Glu-519 as the catalytic nucleophile in β-mannosidase 2A from *Cellulomonas fimi*

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Incubation of the β-mannosidase Man2A from *Cellulomonas fimi* with 2-deoxy-2-fluoro-β-D-mannosyl fluoride (2FManβF) resulted in time-dependent inactivation of the enzyme (inactivation rate constant $k_i = 0.57\ min^{-1}$, dissociation constant for the inactivator $K_i = 0.41$ mM) through the accumulation of a covalent 2-deoxy-2-fluoro-α-D-mannosyl–β-mannosidase 2A (2FMan–Man2A) enzyme intermediate, as observed by electrospray ionization mass spectrometry. The stoichiometry of inactivation was 1:1. Removal of excess inactivator and regeneration of active enzyme by transglycosylation of the covalently attached inhibitor to gentiobiose [Glcβ(1–6)Glc] demonstrated that the covalent intermediate was catalytically competent. Comparison by MS of the peptic digests of 2FMan–Man2A with peptic digests of native Man2A revealed a peptide of m/z 1520 that was unique to 2FMan–Man2A, and one of m/z 1036.5 that was unique to a Man2A peptide. Their sequences, determined by collision-induced fragmentation, were CSEFGFQGPPTW and FGFQGPPTW, corresponding to residues 517–528 and 520–528 of Man2A respectively. The difference in mass of 483.5 between the two peptides equals the sum of the masses of the tripeptide CSE plus that of 2-fluoromannose. It was concluded that in 2FMan–Man2A, the 2-fluoromannose esterified to Glu-519 blocks hydrolysis of the Glu-519–Phe-520 peptide bond, and that Glu-519 is the catalytic nucleophile in this enzyme. This residue is conserved in all members of family 2 of the glycosyl hydrolases. This represents the first ever labelling and identification of an active-site nucleophile in a β-mannosidase.

Key words: catalytic carboxyl, mechanism-based inhibitor.

INTRODUCTION

Hydrolysis of glycosides by retaining glycosidases occurs via a two-step mechanism involving two carboxy groups, one functioning as a nucleophile, the other as an acid-base catalyst [1]. In the first step of the reaction, the nucleophile attacks the anomeric centre of the substrate while the acid-base catalyst protonates the departing aglycone, leading to the formation of a covalent glycosyl-enzyme intermediate of inverted stereochemistry at the anomeric centre relative to the substrate. In the second step of the reaction, the acid-base catalyst promotes the attack of a water molecule on the opposite face of the anomeric centre, displacing the nucleophile and releasing the glycone with the same anomeric configuration as the substrate (Scheme 1, upper panel). The enzymes can also form glycosides by transglycosylation of the glycone moiety of the covalent glycosyl-enzyme intermediate to an acceptor aglycone rather than to water (Scheme 1, lower panel).

Mechanism-based inactivators, such as 2-deoxy-2-fluoroglycosides, in which the aglycone is a good leaving group, typically 2,4-dinitrophenolate or fluoride [2,3], can be used to trap the covalent glycosyl-enzyme intermediate. These reagents function in this manner because the replacement of the 2-hydroxy group of the glycone with fluorine destabilizes the oxocarbenium ion-like transition states, thereby slowing both the glycosylation and the deglycosylation steps. However, the presence of a good leaving group ensures that the glycosylation step is accelerated relative to the deglycosylation step, thus the intermediate accumulates. The intermediate is sufficiently stable to allow the labelled nucleophile to be identified by proteolysis and MS [4]. The inactivation is reversible; in the absence of free inhibitor, activity is recovered slowly by hydrolysis of the intermediate, or more rapidly by transglycosylation in the presence of a suitable acceptor.

Glycoside hydrolases are classified into families of either retaining or inverting enzymes on the basis of amino acid sequence similarities. For each glycoside hydrolase family a specific consensus pattern has been defined [5]. Using the fluorosugar approach the catalytic nucleophiles have been identified for members of family 1 [6], two members of family 2 [7,8], two members of family 3 [9,10], two sub-families of family 5 [11,12], family 7 [13], family 10 [14], family 11 [15], family 12 [16], family 13 [17], family 35 [18] and family 39 [19].

β-Mannosidase 2A (Man2A) from the bacterium *Cellulomonas fimi* is a member of glycosidase family 2 [20]. This family contains β-glucuronidases, β-galactosidases and β-mannosidases. β-Mannosidases do not completely correspond with the family 2 consensus pattern. Therefore they are proposed to form a distinct family 2 sub-family. All family 2 enzymes are nevertheless assumed to adopt similar three-dimensional structures, as has certainly been shown already in the X-ray structures of the *Escherichia coli* (lac Z) β-galactosidase [21] and the human lysosomal glucuronidase [22]. The catalytic nucleophile has been identified by use of the fluorosugar approach for a family 2 β-galactosidase (*E. coli* β-galactosidase) and a family 2 β-glucuronidase (human β-glucuronidase) [7,8]. However, there has been no such identification with a member from the β-mannosidase sub-family. Indeed no β-mannosidase in any family has been subjected to such an analysis. This paper describes the experimental identification of Glu-519 as the nucleophile in

Abbreviations used: Man2A, β-mannosidase 2A; 2FManβF, 2-deoxy-2-fluoro-β-D-mannosyl fluoride; 2FMan–Man2A, 2-deoxy-2-fluoro-α-D-mannosyl–Man2A enzyme intermediate; ESI, electrospray ionization.

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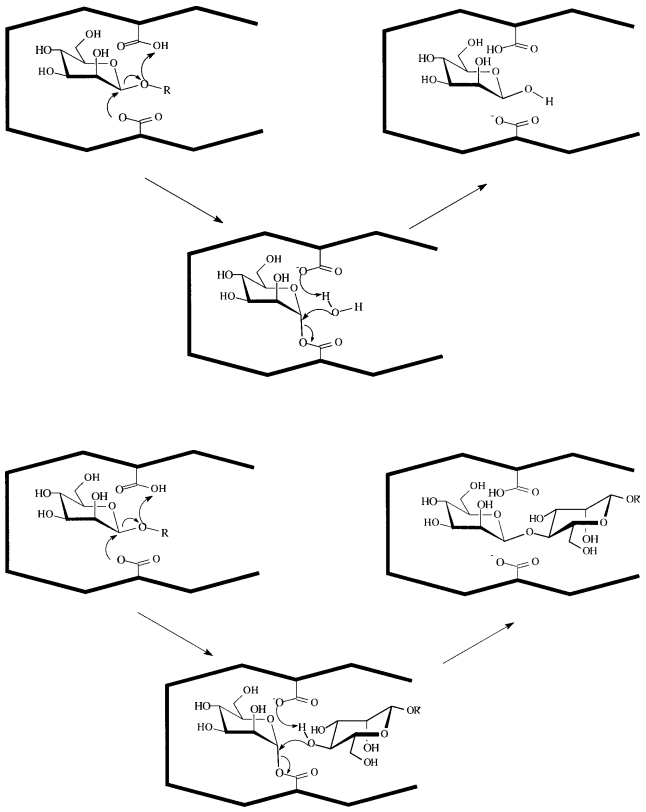


Scheme 1 Hydrolysis mechanism (upper panel) and transglycosylation mechanism (lower panel) of a retaining β-mannosidase

Man2A using the fluorosugar approach, thereby confirming that the mannosidase sub-family is correctly aligned within family 2.

MATERIALS AND METHODS

Materials

Man2A was purifed as described previously [20]. *p*-Nitrophenyl β-D-mannoside and BSA were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 2-Deoxy-2-fluoro-β-D-mannosyl fluoride (2FManβF) [4] was synthesized as described previously [23].

Reaction of Man2A with 2FManβF

Reaction mixtures contained 50 mM phosphate, pH 7.0, 0.1 % BSA, 8.3 μM Man2A and 19.5–520 μM 2FManβF in a final volume of 0.3 ml. The mixtures were incubated at 37 °C and the release of fluoride was measured with a 9606BN Ionplus Orion fluoride ion electrode (Fisher Scientific, Nepean, Ontario, Canada). Pseudo-first-order rate constants (k_{obs}) at each concentration of inhibitor were determined by fitting each inactivation curve to a first-order rate equation with GraFit 3.0 [24]. Values for the inactivation rate constant (k_i) and for the dissociation constant (K_i) of the inhibitor were determined by non-linear regression analysis of the data according to the equation [12]:

$$k_{obs} = k_i[I]/(K_i + [I])$$

Re-activation of inactivated Man2A

Man2A inactivated completely by reaction with 2FManβF was freed of excess inhibitor by buffer exchange (50 mM phosphate, pH 7.0) in a Microsep microconcentrator, using a 10 kDa cut-off membrane (Filtron; Pall Gelman Sciences, Mississauga, Ontario, Canada). Re-activation mixtures contained 8.3 μM inactivated Man2A in 50 mM phosphate, pH 7.0, containing 0.1 % BSA, plus 0–80 mM gentiobiose [Glcβ(1–6)Glc] in a final volume of 0.5 ml. Man2A activity was measured at intervals by transferring samples (7 μl) to 2 mM *p*-nitrophenyl β-D-mannoside in the same buffer in a final volume of 0.6 ml. The release of *p*-nitrophenol was followed by $A_{400\,nm}$. The re-activation rate constants (k_{obs}) were determined from plots of ln[(rate before inactivation)/(observed rate)] versus time. The rate constant (k_{trans}) and dissociation constant (K_{trans}) for re-activation were determined from a reciprocal plot of k_{obs} versus gentiobiose concentration as described previously [12].

Proteolysis of Man2A

Man2A (1.2 mg) was inactivated with 2FManβF as described above, then, along with a control sample of untreated Man2A, was digested with 120 μg of pepsin in 1 ml of 50 mM phosphate buffer, pH 2.0, at room temperature. Digestion was stopped by freezing the samples at −70 °C. The products were analysed by HPLC–MS immediately after thawing.

Electrospray ionization (ESI)–MS

Mass spectra were obtained with a PE-Sciex API 300 triple quadrupole mass spectrometer (Sciex, Thornhill, Ontario, Canada) equipped with an Ionspray ion source. Errors in the reported masses are 0.1 %. Peptides were separated by reverse-phase HPLC on an Ultrafast Microprotein Analyzer (Michrom BioResources Inc., Pleasanton, CA, U.S.A.) interfaced directly with the mass spectrometer. Each proteolytic digest was loaded on to a C18 column (Reliasil, 1 mm × 150 mm) equilibrated with 0.05 % trifluoroacetic acid and 2.0 % (v/v) acetonitrile in water (solvent A). The peptides were eluted with a gradient of 0 to 60 % solvent B (0.045 % trifluoroacetic acid and 80 % acetonitrile in water) over 60 min, followed by 100 % solvent B for 2 min. Solvents were pumped at a constant flow rate of 50 μl/min. Spectra were obtained in the single-quadrupole scan mode (HPLC–MS) or in the tandem MS-product scan mode (MS–MS). In the HPLC–MS mode, the quadrupole mass analyser was scanned over a mass to charge ratio (m/z) range of 300 to 2200 amu, with a step size of 0.5 amu and a dwell-time of 1.5 ms per step. The ion source voltage was set at 5.5 kV and the orifice energy was 45 V. In the tandem MS daughter-ion scan mode, the spectrum was obtained by selectively introducing the parent ion (m/z = 1036.5 or 1520) from the first quadrupole (Q1) into the collision cell (Q2) and observing the product ions in the third quadrupole (Q3). Thus, Q1 was either locked on m/z 1036.5, the Q3 scan range was 50–1050, or Q1 was locked on 1520, the Q3 scan range was 50–1550; the step size was 0.5; dwell time was 1 ms; ion source voltage was 5 kV; orifice energy was 45 V; Q0 = −10; Q2 = −48.

RESULTS

Inactivation of Man2A

The reaction of Man2A with 2FManβF was monitored directly by measuring the release of fluoride using a fluoride-specific electrode. Figure 1 shows that inactivation followed pseudo-first-order kinetics, allowing a pseudo-first-order rate constant for

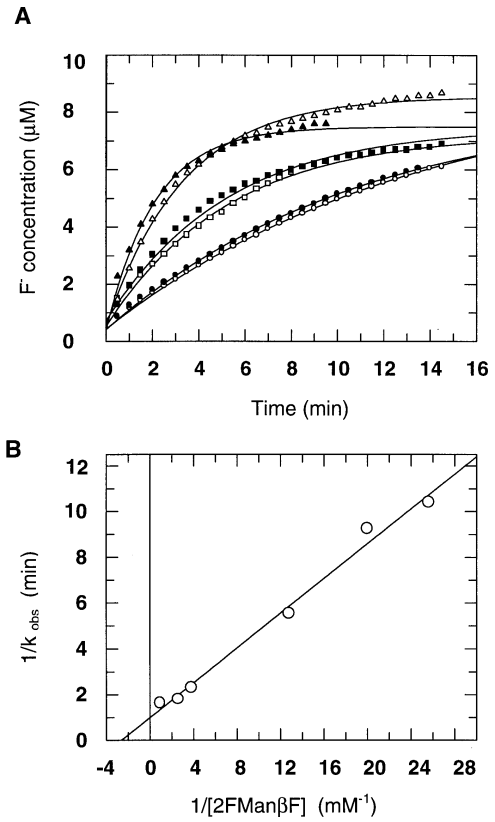


Figure 1 Inactivation of Man2A with 2FManβF

(**A**) Kinetics of the release of fluoride ion with 8.3 μM Man2A and 39 μM (○), 50 μM (●), 78 μM (□), 260 μM (■), 390 μM (△) and 520 μM (▲) 2FManβF. (**B**) Double-reciprocal plot of the first-order rate constants for inactivation (k_{obs}) versus concentration of 2FManβF.

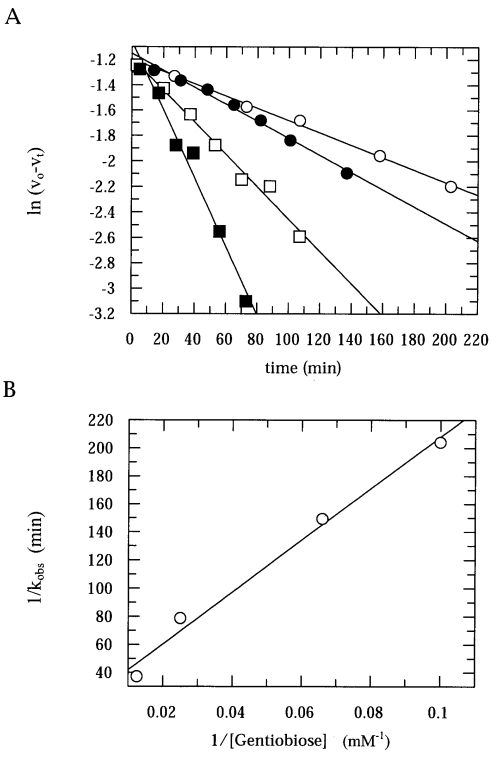


Figure 2 Re-activation of the Man2A–2FMan covalent complex by gentiobiose

(**A**) Semi-logarithmic plot of activity versus time for 10 mM (○), 15 mM (●), 40 mM (□) and 80 mM (■) gentiobiose. (**B**) Double-reciprocal plot of first-order rate constants from (**A**) versus gentiobiose concentration.

inactivation at each inactivator concentration to be determined. Analysis of the data was carried out according to the kinetic model shown in Scheme 2.

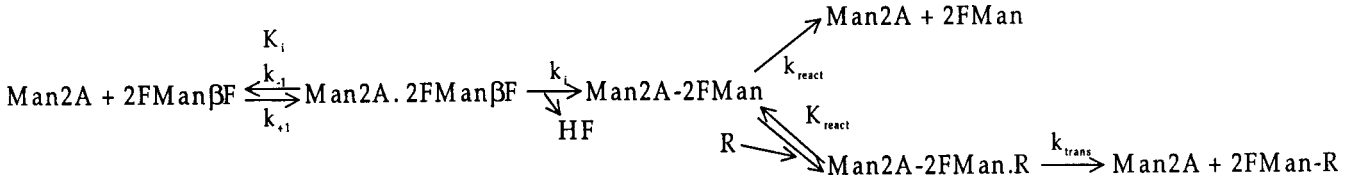
Reciprocal replots of these data (Figure 1B) yielded an inactivation rate constant, k_i, of 0.57 ± 0.02 min^{-1} and a dissociation constant, K_i, of 0.41 ± 0.03 mM. The data in Figure 1(A) clearly show the stoichiometry of the reaction since each plot levels off around 8 μM fluoride, which is the concentration of enzyme present in the reaction mixture.

Re-activation of inactivated Man2A

Upon incubation of inactivated Man2A, from which excess inhibitor had been removed by dialysis, activity was slowly recovered according to pseudo-first-order kinetics with an apparent rate constant, k_{react}, of 0.002 min^{-1}. This corresponds to a half-life for the 2FMan–Man2A covalent intermediate of 344 min. Re-activation was accelerated by gentiobiose in a concentration-dependent manner, according to pseudo-first-order kinetics (Figure 2A), presumably by transglycosylation of 2-fluoromannose from the inactivated enzyme to gentiobiose [25]. A re-activation rate constant, k_{trans}, of 0.043 ± 0.014 min^{-1} and a dissociation constant, K_{trans}, of 78 ± 25 mM were obtained from a plot of $1/k_{obs}$ versus 1/gentiobiose concentration (Figure 2B).

Detection of a 2FMan–Man2A covalent complex

The molecular mass of native Man2A, determined by ESI–MS, was 94980 Da (predicted 94960 Da) while that of Man2A after inactivation with 2FManβF was 95139 Da (predicted 95124 Da). The mass difference of 159 Da between the inactivated and native enzyme corresponds, within error, to the difference of



Scheme 2 Kinetic model for the inactivation and re-activation of Man2A with 2FManβF

R, re-activating ligand.

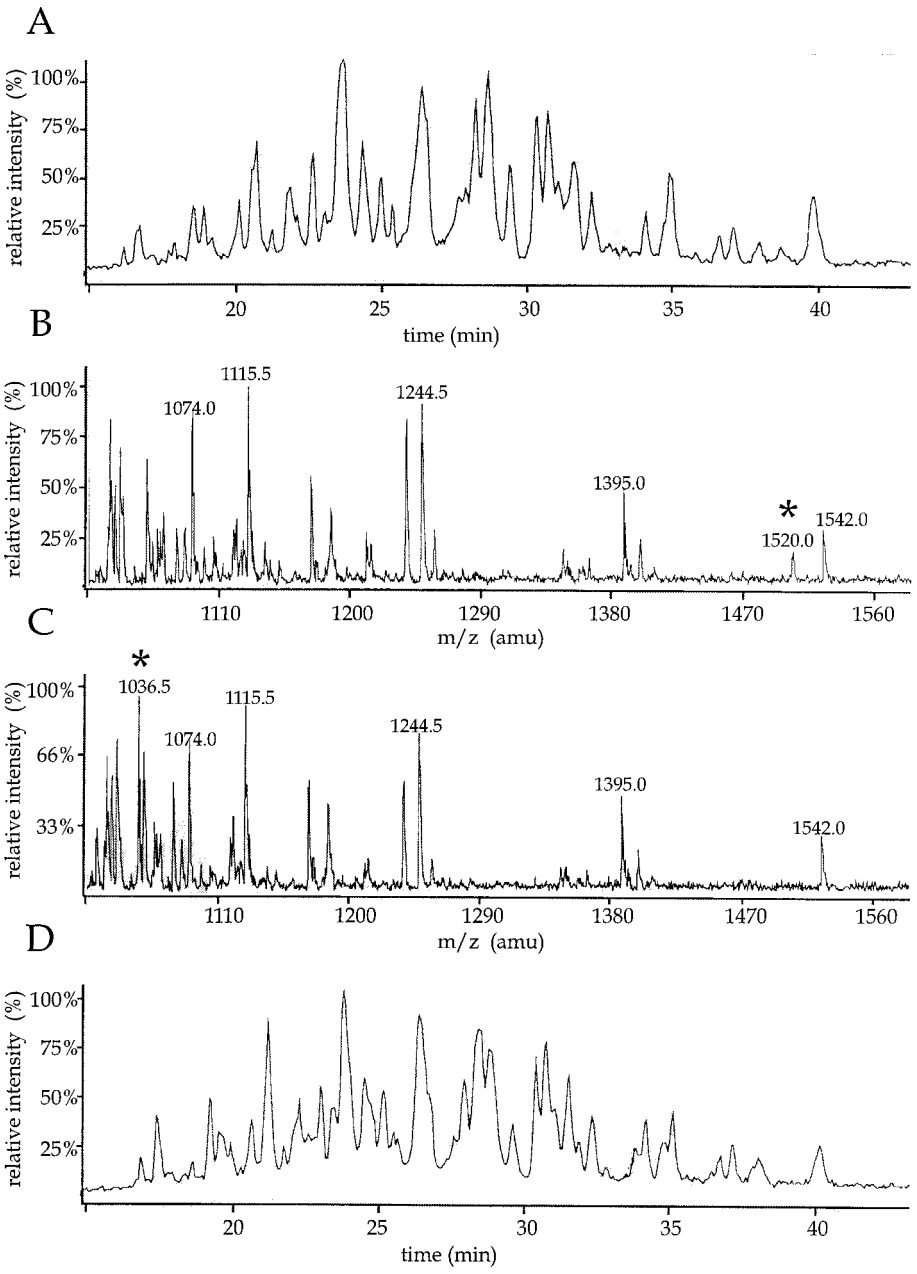


Figure 3 Detection of the 2-fluoromannose-labelled peptide by comparative MS mapping

Total ion chromatograms of pepsin digests of labelled (**A**) and unlabelled (**D**) Man2A. Mass spectrum of the digest from the labelled enzyme taken at 28.6 min (**B**) and from the unlabelled enzyme taken at 28.8 min (**C**). *, indicates the peptides unique to each digest.

164 Da predicted for the attachment of 2-fluoromannose during inactivation of the enzyme, thereby confirming the 1:1 stoichiometry of inactivation and the covalent attachment of the inhibitor to the enzyme.

Identification of the amino acid labelled by 2FβManF

Peptic proteolysis of samples of labelled and unlabelled Man2A proceeded without difficulty, yielding digests within which the labelled peptide was located by comparative HPLC–MS mapping. The total ion chromatograms showed large numbers of ions, each of which correspond to one or more peptides. Comparative analysis of the two digests revealed that essentially all the peptides in the labelled digest had a corresponding partner in the unlabelled digest, with the exception of one pair. A peptide of molecular mass 1520 Da (peptide 1520) was present only in the labelled digest while a peptide of molecular mass 1036.5 Da (peptide 1036.5) was present only in the unlabelled digest (Figure 3). The mass difference of 483.5 Da between these peptides, however, does not correspond to the 164 Da expected for the attachment of the inhibitor, indicating that some additional amino acids must also be present. Samples of these two peptides were therefore partially purified and the sequences of both peptides were determined by collision-induced fragmentation,

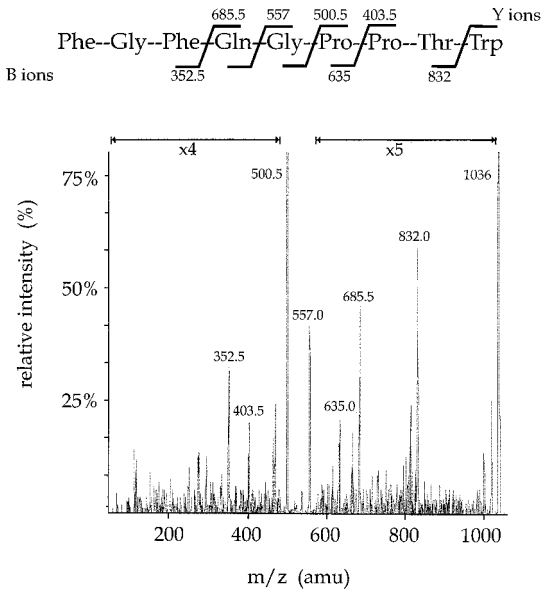


Figure 4 Sequencing the unique peptide (*m/z* 1036.5) from unlabelled Man2A by ESI–MS–MS

Daughter-ion mass spectrum for the peptide of *m/z* 1036.5. The 'B' and 'Y' ions corresponding to the sequence FGFQGPPTW are indicated.

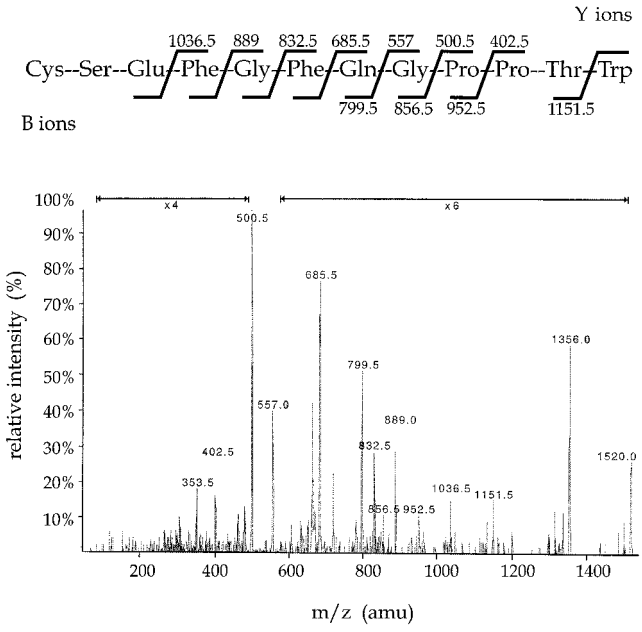


Figure 5 Sequencing the unique peptide (*m/z* 1520) from labelled Man2A by ESI–MS–MS

Daughter-ion mass spectrum for the peptide of *m/z* 1520. The 'B' and 'Y' ions corresponding to the sequence CSEFGFQGPPTW are indicated.

with mass data collected in the daughter-ion scan mode. Peptide 1036.5, derived from the unlabelled digest, had the sequence FGFQGPPTW, corresponding to amino acids 520–528 of Man2A (Figure 4). Although this sequence does not contain a glutamic acid or aspartic acid that might act as the catalytic nucleophile, the amino acid (519) immediately preceding in Man2A is a glutamic acid residue. Sequencing of peptide 1520 derived from the labelled digest revealed a sequence of CSEFGFQGPPTW, corresponding to amino acids 517–528 of Man2A (Figure 5). The difference in mass of 483.5 Da between the two peptides equals the sum of the masses of the three amino acids at the N-terminus of peptide 1520 plus the 2-fluoromannosyl moiety.

DISCUSSION

Stoichiometric labelling of Man2A by 2FβManF is clearly revealed both by the release of one equivalent of fluoride per equivalent of enzyme inactivated and by the protein mass increase corresponding to one 2-fluoromannosyl residue. This is entirely consistent with the trapping of a 2-fluoromannosyl–enzyme intermediate. The catalytic competence of this intermediate is shown by the fact that the inactivated enzyme recovers activity upon incubation via spontaneous hydrolysis of the glycosyl-enzyme intermediate and more importantly by the fact that the addition of a suitable acceptor sugar, in this case gentiobiose increases the re-activation rate considerably. Indeed, the rate of re-activation of Man2A by transglycosylation to gentiobiose (k_{trans}) was 21-fold higher than the rate of spontaneous re-activation (k_{react}). This increase compares favourably with the enhanced rates of re-activation seen for other enzymes: 13-fold for the exo-β-1,3-glucanase from *Candida albicans* with benzyl-thio-β-D-glucopyranoside as acceptor [12] and 530-fold for a β-glucosidase from an *Agrobacterium* sp. with benzyl-β-glucoside as acceptor [26]. The dissociation constants (K_{trans}) of the acceptors, which reflect binding in the +1 sites, were similar for the three enzymes: 78 ± 25 mM for Man2A, 59 ± 3 mM for the exo-β-1,3-glucanase and 56 ± 10 mM for the β-glucosidase.

The enhanced re-activation by transglycosylation arises because acceptor–enzyme interactions in the +1 site promote reaction by transglycosylation, presumably by increasing the effective concentration of the acceptor and stabilizing the transition state leading to glycosyl transfer. This phenomenon was analysed in detail for the *Agrobacterium* sp. β-glucosidase where it was shown that a 2-deoxy-2-fluoroglycosyl β-glucoside product was formed and released upon re-activation [26]. Transglycosylation processes such as this are entirely expected and consistent with a catalytically competent intermediate.

The observation of a pair of peptides in peptic digests of labelled and unlabelled Man2A differing not only in the presence of the 2-fluoromannosyl residue but also in the presence of three additional amino acid residues is unusual. In no other case to date has such a difference been observed in comparative mapping studies of peptides derived from trapped intermediates on glycosidases. In all the other cases the only difference observed was that of the label, indicating that the proteolytic cleavage was not affected by the presence of the label in those cases. However, in the case of Man2A it is apparent that the presence of the ester-linked sugar blocked the action of pepsin at this site. This is probably a simple consequence of the fact that pepsin cleaves, *inter alia*, the Glu-519–Phe-520 bond in the free enzyme, while in none of the other cases studied was there a preferred cleavage site immediately following the nucleophile itself. It is quite probable that this particular amide bond would be the most occluded by the presence of the sugar.

Glu-519 is entirely conserved, not only within the mannosidase sub-family, but also within the whole of family 2. This is the residue that has been identified as the nucleophile within a β-galactosidase and a β-glucuronidase in the family. This result

therefore confirms that, despite the mannosidases forming a subfamily, the alignment proposed at this site is reasonable and correctly identifies this important residue. This work represents the first ever labelling and identification of an active-site nucleophile in a β-mannosidase.

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