Identification of the Active Site Nucleophile in Jack Bean α-Mannosidase Using 5-Fluoro-β-L-Gulosyl Fluoride*

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Mannosidases play a key role in the processing of glycoproteins and thus are of considerable pharmaceutical interest and indeed have emerged as targets for the development of anti-cancer therapies. Access to useful quantities of the mammalian enzymes has not yet been achieved; therefore, jack bean mannosidase, a readily available enzyme, has become the model system. However, the relevance of this enzyme has not been demonstrated, nor is anything known about the active site structure of this, or any other, mannosidase. Hydrolysis by this enzyme occurs with net retention of sugar anomeric configuration; thus, a double displacement mechanism involving a mannosyl-enzyme intermediate is presumably involved. Two new mechanism-based inhibitors, 5-fluoro-α-D-mannosyl fluoride and 5-fluoro-β-L-gulosyl fluoride, which function by the steady state trapping of such an intermediate, have been synthesized and tested. Both show high affinity for jack bean α-mannosidase (Kᵢ⁺ = 71 and 86 μM, respectively), and the latter has been used to label the active site nucleophile. The labeled peptide present in a peptic digest of this trapped glycosyl-enzyme intermediate was identified by neutral loss scans on an electrospray ionization triple quadrupole mass spectrometer. Comparative liquid chromatographic/mass spectrometric analysis of peptic digests of labeled and unlabeled enzyme samples confirmed the unique presence of this peptide of m/z 1180.5 in the labeled sample. The label was cleaved from the peptide by treatment with ammonia, and the resultant unlabeled peptide was purified and sequenced by Edman degradation. The peptide identified contained only one candidate for the catalytic nucleophile, an aspartic acid. This residue was contained within the sequence Gly-Trp-Gln-Ile-Asp-Pro-Phe-Gly-His-Ser, which showed excellent sequence similarity with regions in mammalian lysosomal and Golgi α-mannosidase sequences. These mammalian α-mannosidases belong to family 38 (or class II α-mannosidases) in which the Asp in the above sequence is totally conserved. This finding therefore assigns jack bean α-mannosidase to family 38, validating it as a model for other pharmacologically interesting enzymes and thereby identifying the catalytic nucleophile within this family.

There has been widespread interest in mannosidases in recent years, largely due to their role in a multitude of biological systems and, as a result, their potential as therapeutic targets. In particular, mammalian Golgi α-mannosidase II is involved in glycoprotein biosynthesis and is currently an important therapeutic target for the development of anti-cancer agents (1). Mammalian lysosomal α-mannosidase has significant sequence similarity to the Golgi class II enzyme and is responsible for glycoprotein degradation (2, 3). The absence of this enzyme causes the genetic lysosomal storage disease α-mannosidosis in humans and cattle (4–6). These mannosidases have been categorized as class II mannosidases, based on sequence alignment, and clearly belong to family 38 in Henrisat’s glycosidase classification (2, 7–9). Class I α(1,2)-mannosidases have little similarity in sequence or substrate/inhibitor specificity to the class II enzymes and are classified as family 47 (2, 7, 10). While a number of these enzymes have been cloned and sequenced, relatively little success has been attained in their high level expression. This has severely limited structural and mechanistic studies on this important enzyme class, significantly slowing the search for potential therapeutic agents based upon their inhibition. In their absence, a suitable model enzyme is necessary, and the jack bean enzyme has been assumed to suit that role, although this choice has not been validated.

Jack bean α-mannosidase, although a commercially available enzyme, has not been characterized structurally, nor has its primary sequence been determined. Like the lysosomal enzyme, it hydrolyzes α(1,2), α(1,3), and α(1,6) linkages between mannose residues but also has a broad aglycone specificity (19). In common with class II α-mannosidases, the jack bean enzyme is a retaining enzyme (14), releasing α-D-mannose as first formed product. As such, it is presumed to follow a double-displacement mechanism in which a glycosyl-enzyme intermediate is formed and hydrolyzed via oxocarbenium ion-like transition states (11, 12, 15, 16). Formation of this intermediate is assisted by general acid catalysis from a carboxylic acid located in the active site. This same residue serves as the general base catalyst for the second, deglycosylation, step. A second active site carboxylic acid serves as the nucleophile that forms the covalent intermediate. The jack bean enzyme, like other class II mannosidases, is inhibited by swainsonine and mannostatin (17, 18) and accepts aryl-mannoside substrates. In contrast, the class I α(1,2)-mannosidases are inverting enzymes and possess none of these other characteristics (10, 13). Since only two families of α-mannosidases have been established to date, families 38 and 47, of which the former is retaining, it is quite possible that the jack bean enzyme, being a retaining enzyme, may well also be a member of family 38. However, sequence information will be necessary to confirm this prediction.

The nucleophilic and acid/base active site residues have not been labeled or identified in any α-mannosidase, nor has any crystal structure been obtained. Jack bean α-mannosidase was therefore chosen for use in the development of new methodol-
ogy aimed at labeling and identifying catalytically important residues in this enzyme class. 2-Deoxy-2-fluoro-β-D-glycosyl fluorides have proved to be valuable reagents for identifying the active site nucleophiles in a range of retaining β-glycosidases (20–22). However, the labeling of retaining α-glycosides with these compounds has been unsuccessful, leading to the development of 5-fluoroglycosyl fluorides to solve this problem (23, 24). These fluorosugars behave as mechanism-based inactivators. The good fluoride leaving group at C-1 serves to accelerate the first step of the double displacement reaction, whereas the C-5 or C-2 fluoride slows both steps via inductive acceleration of the first step of the double displacement reaction, thus trapping a 5-fluoro- or 2-deoxy-2-fluoroglycosyl-enzyme intermediate. 5-Fluoro-α-D-glycosyl fluoride and 5-fluoro-β-D-idosyl fluoride have been used to label and identify Asp-214 as the active site nucleophile in jack bean α-mannosidase. The identification of the catalytic nucleophile is then made possible by proteolytic digestion of the labeled enzyme followed by HPLC separation of the resultant peptides and localization of the labeled peptide using electrospray ionization tandem mass spectrometry to detect specific fragmentations associated with the glycosylated active site peptide (22, 25).

EXPERIMENTAL PROCEDURES

General Procedures and Synthesis—Jack bean α-mannosidase, all buffer chemicals, and other reagents were obtained from Sigma unless otherwise noted. Pepsin (from porcine mucosa) was obtained from Boehringer Mannheim. 2,4-Dinitrophenyl-α-D-mannopyranoside (DNPM) was synthesized in one step by treatment of α-mannose with 1-fluoro-2,4-dinitrobenzene (26). Synthesis of the inhibitors was performed according to Scheme 1, as follows.

2,3,4,6-Tetra-O-acetyl-5-fluoro-β-D-mannopyranosyl fluoride (4)—This was prepared from 2,3,4,6-tetra-O-acetyl-5-fluoro-β-D-mannopyranosyl fluoride (117 mg, 0.109 mmol) in an analogous fashion to 5FguloF to give 5FguloF (152 mg, 0.76 mmol) as a colorless syrup. Analysis for C_{12}H_{18}O_{7}F_{4}N: m/z 386 (M + NH}_{4}^+ (100%), 349 (M – F)^+ (68%).

2,3,4,6-Tetra-O-acetyl-5-fluoro-β-D-mannopyranosyl fluoride (5)—This was prepared from 2,3,4,6-tetra-O-acetyl-5-fluoro-β-D-mannopyranosyl fluoride (117 mg, 0.109 mmol) in a similar fashion to 5FguloF to give 5FguloF (152 mg, 0.76 mmol) as a colorless syrup. Analysis for C_{12}H_{18}O_{7}F_{4}N: m/z 386 (M + NH}_{4}^+ (100%), 349 (M – F)^+ (68%).

Active Site of Jack Bean α-Mannosidase

Here we describe the application of a new label, 5-fluoro-β-D-gulopyranosyl fluoride (5FguloF) to label the active site nucleophile in jack bean α-mannosidase. The identification of the catalytic nucleophile is then made possible by proteolytic digestion of the labeled enzyme followed by HPLC separation of the resultant peptides and localization of the labeled peptide using electrospray ionization tandem mass spectrometry to detect specific fragmentations associated with the glycosylated active site peptide (22, 25).
addition of a 10-μl aliquot of the inactivation mixture to a solution of DNPM (0.5 mM, 750 μl) in the above buffer and measurement of dinitropheolate release over a period of 30 s. Pseudo-first-order inactivation rate constants at each 5FguloF concentration (kobs) were determined by fitting the initial exponential phase of each curve to a first-order equation using the program GraFit (Leatherbarrow, R. J. GraFit version 3.0; Erithacus Software Ltd.: Staines, United Kingdom, 1990). The value of kobs, assuming inactivation according to the kinetic model shown in Scheme 2, was determined from the slope of a plot of kobs against time.

The kobs value for 5FguloF was determined by inactivation of the enzyme (0.01 mg/ml) in the presence of 0.8 mM 5FguloF followed by the addition of a 10-μl aliquot of the enzyme to a solution of DNPM (800 μl). The release of dinitropheolate was measured continually over consecutive 15-s periods up to ~7 min. The average rate of dinitropheolate release was calculated for the 15 s periods and plotted as a function of time, thereby providing a measure of the rate of return of activity due to turnover. A value for the first-order rate constant for reactivation (kreact) was determined by fitting the curve to Equation 1. This was repeated at various concentrations of DNPM. In addition, the kobs values for 5FguloF and 5FmanF were determined directly by monitoring the release of fluoride using an Orion 96-09 combination fluoride electrode.

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\frac{d[\text{ADP}]}{dt} = \frac{d[A]}{dt} \cdot (1 - e^{-kt}) \quad \text{(Eq. 1)}
\]

The apparent dissociation constants (K') for the interaction of 5FguloF and 5FmanF with the enzyme under steady-state reaction conditions were determined by continuous measurement of dinitropheolate release in the presence of DNPM and inhibitor. This was repeated at several different concentrations of inhibitor, the enzyme first being allowed to react for 5 min before the assay was run to ensure that a steady state was achieved. The observed rates were plotted in the form of a Dixon plot (1/ν versus [I]), and the K' values were determined from the intercept of this line with the horizontal line drawn through 1/νmax. Labeling and Proteolysis—Labeling of jack bean α-mannosidase was achieved by incubating the enzyme (1 mg/ml × 20 μl) with 5FguloF (1 μl × 40 mM) for 10 min at 50 min citrate buffer (pH 4.5). This sample was then used directly for mass spectrometric analysis. When prepared for proteolytic digestion purposes, the inactivation was repeated using a more concentrated enzyme sample (5 mM × 20 μl). After incubation for 10 min, the sample was diluted with pepsin solution (60 μl × 0.1 mg/ml pepsin; 50 mM sodium phosphate/His, pH 1.8), and the mixture was incubated at room temperature for 30 min. ESMS analysis and SDS-polyacrylamide gel electrophoresis of the proteolytic digest confirmed that the enzyme was completely digested.

Electrospray Mass Spectrometry—The analyses of the protein and peptide samples were carried out using a Sciex API-300 mass spectrometer interfaced with a Microm UMA HPLC system (Microm Bioreources, Inc., Auburn, CA). Intact jack bean α-mannosidase (10–20 μg, labeled or unlabeled) was introduced into the mass spectrometer through a microprobe PRLP column (1 × 50 mm) and eluted with a gradient of 20–100% solvent B at a flow rate of 50 μl/min over 5 min (solvent A, 0.06% trifluoroacetic acid, 2% acetonitrile in water; solvent B, 0.05% trifluoroacetic acid, 90% acetonitrile in water). The MS was scanned over a range of 400–2300 Da with a step size of 0.5 Da and a dwell time of 1 ms.

The peptides were analyzed by loading a 10-μl sample of the pepsin digest (1.25 mg/ml) onto a C18 column (Reliasil, 1 × 150 mm), which was eluted at a flow rate of 50 μl/min with a gradient of 0–60% B over 45 min. The proteolytic mixture was first examined in LC/MS mode and then in the neutral loss mode. In the single quadrupole (normal LC/MS) mode, MS conditions were as follows. The mass analyzer was scanned over the range of 300–2400 Da with a step size of 0.5 Da, a dwell time of 1.5 ms, an ion source voltage of 4.8 kV, and an orifice energy of 50 V. The neutral loss spectra were obtained in the triple quadrupole mode searching for the loss of m/z 60-3, which corresponds to the loss of the inhibitor label from a peptide that is triply charged. A scan range of 400–1800 Da was used with a step size of 0.5 Da and a dwell time of 1.5 ms. Other parameters were as follows: ion source voltage = 5.5 kV, orifice energy = 45 V, IQ2 = -50, Q0 = -10, CAD = 4.

Chemical Sequeuing—Partial purification of the labeled peptide was achieved by HPLC separation of the pepsin digest as described above and by collecting the appropriate fractions containing the partially purified labeled peptide via a postcolumn splitter. The label was cleaved by treatment with aqueous ammonia (100 μl of sample/10 μl of concentrated aqueous ammonia/10 min), and the resulting unlabeled peptide was purified by HPLC using the same conditions as above. Loss of the label increases the retention time by ~40 s, which allows isolation of pure material. The amino acid sequence of the labeled peptide was determined by S. Perry of the Nucleic Acid and Peptide Service at the University of British Columbia using standard pulsed liquid phase protocols and instrumentation on a Perkin-Elmer model 476A sequencer and model 120A phenylthiohydantoin analyzer (Applied Biosystems, Foster City, CA).

Methyl Esterification of Partially Purified Unlabeled Peptide—Partially purified unlabeled peptide (control sample) was mixed with a freshly prepared solution of 2 M methanolic HCl, and the mixture was incubated at room temperature for 30 min. The excess reagent was removed by concentration under vacuum (SVC 100 Speed Vac), and the product was dissolved in 50% acetonitrile/water.

RESULTS AND DISCUSSION

Synthesis—5FmanF (1) and 5FguloF (2) (Scheme 1 and Fig. 1) were synthesized from 2,3,4,6-tetra-O-acetyl-α-D-mannosyl fluoride (3) by four-step and three-step procedures as follows (Scheme 1). Radical bromination of 2,3,4,6-tetra-O-acetyl-α-D-mannosyl fluoride (3) in carbon tetrachloride generated the 5-bromo derivative (4). Displacement of the bromine at C-5 using silver fluoride in acetonitrile yielded the product of inverted configuration at C-5, namely 2,3,4,6-tetra-O-acetyl-5-fluoro-β-D-gulopyranosyl fluoride (5) in good yield. Subsequent treatment of this compound with boron trifluoride diethyl etherate in dichloromethane resulted in equilibration of the C-5 stereochemistry, with production of the more stable 2,3,4,6-tetra-O-acetyl-5-fluoro-α-D-mannopyranosyl fluoride (6). Deprotection of both 5 and 6 was achieved by treatment with ammonia in methanol to yield, after purification, analytically pure materials.

1H NMR analysis reveals that 5FguloF (2), the C-5 epimer of the mannosyl compound (1), adopts a “boat-like” conformation, whereas 5FmanF adopts a normal 1C1 chair conformation. This is supported by an x-ray crystal structure of the tetracetate derivative of 1 (not shown), which, again, clearly shows a normal chair conformation.

Kinetic Studies—5FguloF (2) inactivates jack bean α-mannosidase in a time-dependent fashion as shown in Fig. 2a, with inactivation at higher concentrations occurring too fast to measure, although the experiment was conducted at 4 °C (Fig. 2a). Inactivation is assumed to follow the model shown in Scheme 2. Pseudo-first-order rate constants were determined for inactivation at each concentration of inhibitor from the slopes of these plots, and a replot of these rate constants versus inhibitor concentration was found to be linear (Fig. 2b), allowing a second order rate constant, k/K', of 0.017 s⁻¹ mM⁻¹ to be obtained from the slope of this plot. The absence of any saturation behavior in this plot indicates a K' value in excess of 0.3 mM. Unfortunately, the rapid rate of inactivation precluded determination of either this parameter or the maximal inacti-
Fig. 2. Inactivation of jack bean α-mannosidase by 5FguloF (21). a, semilogarithmic plot of residual activity versus time at the following inactivator concentrations: 0.05 (●), 0.1 (■), 0.15 (□), 0.2 (○), and 0.3 mM (▲). Y, rate of DNPM hydrolysis. b, plot of pseudo-first-order rate constants from a versus [5FguloF].

Inactivation rate constant, $k_i$. However, evidence for active site binding was obtained from the protection against inactivation afforded by 0.18 mM mannojirimetazol, a known competitive inhibitor of α-mannosidases ($K_i = 0.18$ mM) (27). In its presence, the pseudo-first-order rate constant for inactivation at 0.19 mM 5FguloF was reduced from $1.9 \times 10^{-3}$ s$^{-1}$ to $0.75 \times 10^{-3}$ s$^{-1}$.

These data therefore indicate that, as seen in previous studies with 2-fluoroglycosyl fluorides on β-glycosidases (20–22), the 5-FguloF is inactivating the enzyme by trapping of a fluoroglycosyl-enzyme intermediate. Further insight was obtained by demonstrating the catalytic competence of the intermediate so trapped through the time-dependent reactivation observed when the labeled enzyme was freed from excess inhibitor (Fig. 3). Enzyme in the presence of 0.8 mM 5FguloF was diluted 80-fold into substrate (DNPM), and the reactivation was monitored continually over time. Such reactivation could be occurring via hydrolysis of the 5-fluorogulosyl-enzyme intermediate or via transglycosylation to DNPM. However, as can be seen in Fig. 3, the rate of reactivation was independent of substrate (DNPM) concentration. This suggests that DNPM does not bind significantly to the glycosyl-enzyme, thus that reactivation proceeds via a hydrolytic process. A first order rate constant for reactivation at 25 °C, $k_{cat}$, of 9.6 x 10$^{-3}$ s$^{-1}$ was calculated.

Confirmation of the value of this rate constant for the hydrolytic process was obtained by another method. Since turnover is observed, 5FguloF must be acting as a substrate, albeit a slow one. It was therefore possible to monitor substrate activity directly by monitoring fluoride release using a fluoride-selective electrode. The 5-fluorogluorase released rapidly decomposes with release of a second equivalent of fluoride; thus, all steady state kinetic data have been calculated on the basis of 2 eq of fluoride being released per substrate molecule hydrolyzed. Using this approach, the steady-state rate of release of fluoride was determined, and a value of $7.9 \times 10^{-3}$ s$^{-1}$ was calculated for $k_{cat}$. This is very close to the value determined from reactivation kinetics, showing that both methods are monitoring the same hydrolytic process. These data are therefore consistent with a model in which 5FguloF forms a short lived ($t_{1/2} = 72$ s) 5-fluorogulosyl-enzyme intermediate that accumulates in a time-dependent fashion, since the rate of formation (governed by $k_i$) is much greater than the rate of hydrolysis ($k_{cat}$).

Since 5FguloF forms a significant steady state concentration of glycosyl-enzyme intermediate, thereby blocking the active site, it can also be studied as a competitive inhibitor. The apparent dissociation constant, $K_i$ (Equation 2) can therefore be determined by measuring the rate of hydrolysis of DNPM in the presence of various concentrations of 5FguloF. The value of $K_i = 86 \pm 14$ μM determined for 5FguloF represents a minimum value for the true dissociation constant $K_i$, as defined below (28).

Somewhat different kinetic behavior was seen with 5FmanF (1), which did not show time-dependent inactivation behavior but rather was found to reversibly inhibit jack bean α-mannosidase with an apparent $K_i$ of 71 ± 13 μM. This behavior suggests that 5FmanF (1) is also acting as a slow substrate but that this time the turnover is too fast to allow trapping of the intermediate (therefore inactivation) on the time scale of the assay employed. Indeed, the enzyme was shown to slowly hydrolyze (1), the slow release of fluoride being measured with a fluoride-selective electrode. The rate of fluoride release was found to be independent of the concentration of 5FmanF at concentrations down to 0.7 mM, with a value of 0.025 ± 0.002 s$^{-1}$ being measured for $k_{cat}$. The apparent tight binding of 1 is thus once again a consequence of the accumulation of an intermediate.

Identification of the Site of Attachment of the Label.—Labeling studies with jack bean α-mannosidase to identify the catalytic nucleophile were carried out with 5FguloF, since it forms a longer lived glycosyl-enzyme intermediate than does 5FmanF. The stoichiometry of inactivation by 5FguloF was determined by application of electrospray mass spectrometry. Although native jack bean α-mannosidase has a molecular weight of 230,000 (29), SDS-polyacrylamide gel electrophoresis indicated two major subunits, one of molecular weight 66,000 (comparison with bovine serum albumin) and one of lower molecular weight around 40,000–50,000. Mass spectrometric analysis of native enzyme showed peaks in the 44,000 region and the 66,000 region (Fig. 4a). MS analysis of the inactive enzyme in the presence of 1 mM 5FguloF showed peaks at 43,846, 43,959, 66,533, and 66,708 (Fig. 4b). The increase in molecular weight of the larger subunit (average mass increase = 171) corresponds well, within experimental error, to the mass increase of 181 expected upon derivatization with a single molecule of 2 per active site. This suggests that catalytically competent active sites occur only in the larger subunit of this enzyme. The error in mass determination using this method is at least ±10 Da. This is primarily due to the broad, and somewhat irregular, profile of the peaks as determined by computer reconstruction of the initial mass spectrum. Reasons for the “doublet” nature of the peaks could include variation in glycosylation or other post-translational modification of the protein.

Peptic digestion of the labeled enzyme gave a complex mixture of peptides that was separated by HPLC using the ESMS as a detector in the LC/MS mode (Fig. 5a). The labeled peptide was identified by repeating this process using tandem mass spectrometry in neutral loss mode. In this mode, ions were subjected to limited fragmentation by collision with an inert gas, which caused selective homolytic fission of the labile glyco-
cosidic linkage between the label and the peptide. As a result, the labeled peptide loses a neutral sugar. The two quadrupoles were scanned in a linked mode so that only those ions differing by the mass of the label could be detected. For a singly charged peptide, this $m/z$ difference is the mass of the label ($m/z 181$); for the triply charged peptide, the $m/z$ difference is one-third of the mass of the label ($m/z 60.3$). Two charged fragments were seen to undergo a neutral loss of 60.3 (Fig. 5b) when a sample of the peptic digest of the labeled enzyme was subjected to this analysis. However, one of these fragmentations was observed in a control digest of unlabeled enzyme (Fig. 5c). This left only one charged fragment ($m/z 787.5$), which was not present in a control experiment (Fig. 5d). This corresponded to a labeled peptide fragment of mass 2359, which elutes with a retention time of 25.2 min.

The relevance of this neutral loss peak was checked by inspection of the peptide compositions of the digests of labeled and unlabeled enzyme in the standard LC/MS mode. Indeed, a peak corresponding to the labeled peptide ($m/z 1180.5$, doubly charged) was seen in the labeled digest that was not present in that from an unlabeled sample. Similarly, a strong peak of $m/z 1090$, doubly charged, corresponding to the nonlabeled peptide, was observed in the unlabeled digest and also in the labeled digest, although at much lower intensity.

Unfortunately, it did not prove possible to determine the sequence of this peptide by further tandem mass spectrometric analysis. It was therefore necessary to purify the peptide and subject it to Edman degradation. A sample of the labeled peptide was therefore partially purified using the conditions used for the profile in Fig. 4a. Using these conditions, it proved impossible to fully purify the peptide. The partially purified sample was therefore treated with aqueous ammonia to cleave the ester linkage between the sugar and the peptide, thereby shifting its retention time away from those of the contaminating peptides. Further HPLC of the mixture gave a pure sample of the unlabeled peptide, mass $52178$, which was observed as the doubly and triply charged peptides, $m/z 1090$ and 727, respectively. The pure, cleaved peptide was subjected to Edman degradation, yielding the sequence information shown in Table I. The sequence was determined to be NKIPRAGWQID-PFGHSAVQG (calculated mass $52178$), which contains 1 aspartic acid, 1 asparagine, and 2 glutamine residues.

It seemed probable that the aspartic acid, being the only carboxylic acid residue, was a good candidate for the catalytic nucleophile by analogy with all retaining glycosidases to date (11, 22). However, it is possible that, upon cleavage of the label with ammonia, the catalytic nucleophile (either Glu or Asp) in its ester form could be chemically modified to an amide functionality (Gln or Asn, respectively), thereby leaving some ambiguity, since the protein sequence was not known. To test this, the 2178-Da peptide fragment from the control, unlabeled, enzyme digest was treated with acidic methanol to esterify the carboxylic acid groups. The $m/z$ of this doubly charged peptide increased from 1090 to 1104, which corresponds to a peptide mass increase of 28. This was conclusive evidence that no chemical modification occurred due to cleavage of the labeled peptide with ammonia.
TABLE II
Active Site of Jack Bean α-Mannosidase

| Enzyme                        | Position            | Sequence             |
|-------------------------------|---------------------|----------------------|
| Jack bean α-mannosidase       | PRAGWIDPFGHSKA      |                      |
| Human Golgi α-mannosidase II  | 281 PRSGWIDPFHSP    |                      |
| Rat Golgi α-mannosidase II    | 248 PRSGWAIHDFHSP   |                      |
| Dictyostelium lysosomal α-mannosidase | 165 FKICWIDPFHSA |                      |
| Human lysosomal α-mannosidase  | 166 FVRAWIDPFHSP    |                      |

and that the catalytic nucleophile is indeed the aspartic acid.

This sequence showed excellent similarity with sequences from class II α-mannosidases, all of which belong to family 38 (Table II). In addition, this particular sequence contains one of the five aspartate residues that are conserved throughout the class II Golgi and lysosomal α-mannosidases. In particular, the GWQIDPFHGS sequence shows very high similarity throughout these Golgi and lysosomal α-mannosidases, and the DPFG sequence itself is completely conserved. It is reasonable to conclude that jack bean α-mannosidase is a class II enzyme and therefore also a member of family 38. It is also of interest that the active site nucleophile of yeast α-glucosidase is located within the sequence IDTAG, similar to that of DPFG seen here. Possibly this indicates some similarity in structure between family 38 α-mannosidases and family 13 α-glucosidases. The latter are known to have an (α/β)8 barrel structure.

Interesting comparisons with the molecular weights of other class II enzymes can also be drawn. Human lysosomal α-mannosidase is initially synthesized as a polypeptide of ~110 kDa that is subsequently processed into two subunits of 40–46 kDa and 63–67 kDa, which then constitute the native protein (molecular mass, 210 kDa) (3). The rat Golgi α-mannosidase II is a dimer composed of 124-kDa subunits (2). Treatment with chymotrypsin causes limited proteolysis to give a dimer of 110-kDa subunits that retains full activity. It seems likely that jack bean α-mannosidase is also synthesized as a polypeptide chain of ~110 kDa, which forms a dimer. The fragments of mass ~44 and ~66 kDa, observed by ESMS and SDS-polyacrylamide gel electrophoresis, could be due to limited proteolysis of the protein, which is still able to maintain its integrity unless exposed to denaturing conditions.

CONCLUSION

5FGuloF is an effective time-dependent inactivator of jack bean α-mannosidase, forming a relatively short lived (t1/2 = 72 s) 5-fluorogulosyl-enzyme intermediate. It therefore ultimately functions as a very slow substrate. 5FMAnF also acts as a slow substrate with a submillimolar K_m value but with a much faster turnover rate. The 5-fluorogulosyl-enzyme intermediate formed using 5FGulF was sufficiently stable to allow direct observation of the labeled enzyme by electrospray mass spectrometry and subsequent identification of the active site nucleophile by tandem mass spectrometric analysis of proteolytic digests coupled with Edman degradation. Inspection of the amino acid sequence surrounding the catalytic nucleophile showed excellent similarity with a series of class II Golgi and lysosomal α-mannosidases and suggests that the jack bean enzyme is closely related to this class of α-mannosidases. Further, the identification of the catalytic nucleophile as the aspartic acid within the sequence IDPFHG thereby identifies this as a key residue within family 38, a group of enzymes of considerable pharmaceutical interest. This result therefore opens the possibility of facile generation of inactive mutants of these medically important enzymes for use in “knockout” studies as well as providing information on the active site that is of value in understanding the mutations resulting in mannosidosis.

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