Synthesis of a novel photoaffinity derivative of 1-deoxynojirimycin for active site-directed labeling of glucosidase I

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Glucosidase I releases the distal α1,2-glucosyl residue in the Glc3Man9GlcNAc2 precursor immediately after its transfer from the dolichol-P-P-linked intermediate in the endoplasmic reticulum and triggers the processes for the posttranslational remodeling, folding, and maturation of N-linked glycoproteins. The enzyme has been purified and characterized from several eukaryotic systems. Its cDNA and the gene have also been cloned. The enzyme is a target for the development of drugs for several pathological conditions. A structural analysis on the biochemically purified enzyme has been hampered because of its low abundance and unstable character. The recombinant enzyme has not been obtained in quantity and characterized. Glucosidase I is strongly inhibited by the glucose analog 1-deoxynojirimycin (DNM). To gain an insight into the architecture of the active site of the enzyme, we have report the synthesis of a photoactive derivative of DNM, viz. 4-(α-azidosalicylamido)butyl-5-amido-pentyl-1-DNM (ASBA-P-DNM). With an IC50 of 0.42 μM, it is nearly nine times stronger inhibitor than DNM (IC50 = 3.5 μM). On photolysis, the bound [132I]ASBA-P-DNM specifically labels the native enzyme, which yields a 24-kDa peptide after treatment with V8 protease, apparently representing the region around its active site. Thus ASBA-P-DNM should serve as a novel reagent to conduct structure-function analysis on glucosidase I.

Key words: glucosidase I/1-deoxynojirimycin/photoactive probe

Introduction

The biosynthesis of N-linked glycoproteins begins at the rough endoplasmic reticulum (ER), where a preassembled branched oligosaccharide, Glc(1)2Glc(1)3Glc(1)3Man(1)2Man(1)2Man(1)3[Man(1)2Man(1)2Man(1)6] Man(1)6Man(1)4GlcNAcβ1,4GlcNAc, attached to dolichol pyrophosphate, is transferred en bloc to the nascent polypeptide in the ER lumen (Kornfeld and Kornfeld, 1985). Remodeling of the oligosaccharide on the newly synthesized glycoprotein begins immediately after transfer.

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The enzyme glucosidase I triggers this process by cleaving the outermost α1,2-linked glucosyl residue even as the polypeptide is still undergoing synthesis on the polyosomes and is being translocated into the lumen of the ER (Moremen et al., 1994). In an alternate processing pathway, an endomannosidase removes Glc3Man fragment from the glycosylated polypeptide and provides a bypass to glucosidase I (Anumula and Spiro, 1983). The action of glucosidase I is followed by glucosidase II and additional members of the posttranslational processing, folding and maturation machinery in the secretory pathway (Helenius and Aebi, 2001). Subsequently, depending on the molecular features encoded in the structure, the mature glycoprotein is transported to its ultimate destination within the cell or exported as part of the cellular secretion.

Two recent findings have further brought the significance of glucosidase I into focus. A deficiency in the enzyme resulting from a compound heterozygosity resulted in a congenital disorder of glycosylation, designated type IIb (Volk et al., 2002). This had severe physiological and clinical consequences and resulted in the death of the host early in infancy. Allelic mutations in the GSC locus of Arabidopsis that encode for glucosidase I give rise to mutants that accumulate Glc3Man9GlcNAc2-containing glycans (Boisson et al., 2001; Gilimor et al., 2002). These mutants are severely impaired during embryo morphogenesis and exhibit dramatic alterations in their cellular differentiation during development.

Inhibition of glucosidase I has been shown to interfere with the normal folding, transport, and egress of glycoproteins from the ER and can lead to aggregation and degradation of the improperly folded glycoproteins (Sears and Wong, 1998). Furthermore, it has been shown to affect the infectivity of viruses, for example, HIV and hepatitis types B and C (Block et al., 1998; Fleet et al., 1998; Zitzmann et al., 1999). The use of glucosidase I inhibitor, 1-deoxynojirimycin (DNM) and its N-butyl and N-nonyl derivatives was shown to affect the assembly of viral glycoproteins in HIV-infected cells, impairing the infectivity and release of virus from the cells (Mehta et al., 1998). DNM binds to the substrate-binding region of the active site of glucosidases I and II and acts as a competitive inhibitor during catalysis by these enzymes (Hettkamp et al., 1984). Its N-alkyl derivatives are more selective for inhibiting glucosidase I than glucosidase II (Tan et al., 1991).

Photoaffinity labeling is a powerful and sensitive approach for gaining an insight into ligand-protein interactions and has been often employed in identifying the ligand and substrate binding domains of receptors and enzymes, respectively (Gartner, 2003; Zhang et al., 2003). A critical requirement for the interaction is that the probe must be highly specific for its target site in the macromolecule. In the...
context of glycobiology, such probes of sugar-nucleotides have served as important tools for identifying and characterizing a number of glycosyltransferases (for example, Shailubhai et al., 1990; Wang-Gillam et al., 2000).

Glucosidase I from several sources has been purified to homogeneity and characterized (Bause et al., 1989; Shailubhai et al., 1987); however, the 3D structure of the enzyme has not been elucidated, and the nature of its active site remains to be defined. An understanding of the structure–function relationship of the enzyme would provide a scaffold for designing therapeutics to treat diseases involving glycoprotein interactions. As a first step toward gaining an insight into the active site of glucosidase I, we report the synthesis of a novel photoactive probe, β-azidosalicylamido-buty1-5-amido-pentyl-1-DNM (ASBA-P-DNM) from DNM; it was rendered radioactive by iodination with $^{125}$I for use in labeling the active site of the enzyme. The compound showed high affinity for the native enzyme, and its photolysis provided an easy means to label the enzyme.

Results

Glucosidase I inhibition by derivatives of DNM

DNM and a series of its homologous N-alkyl derivatives are potent competitive inhibitors of glucosidase I (Mellor et al., 2002; Tan et al., 1991); however, the alkyl groups of these compounds do not have a functional group that would allow modification to render them photoactive for tagging the enzyme at the substrate binding site. This study was undertaken to synthesize a photoactive derivative of DNM, examine its ability to inhibit the enzyme, and then test its radiiodinated form to label the native enzyme. The overall plan for the synthesis of these compounds is outlined in Scheme I; the thin-layer chromatography (TLC) analysis of the purified products is shown in Figure 1. Inhibitory effects of DNM and the different derivatives were examined with the affinity-purified glucosidase I using the [Glc-$^3$H]Glc$_3$Man$_9$GlcNAc$_2$ substrate (Figure 2).

The data in Table I represent IC$_{50}$ values, that is, the concentrations of DNM and its derivatives required to inhibit 50% of the enzyme activity under the conditions of assay, calculated from the results in Figure 2. Substitution of alkyl groups on DNM that have a polar group in close proximity to the ring nitrogen dramatically lowered the inhibitory action of DNM on the catalytic activity of glucosidase I. The IC$_{50}$ values for N-(2-aminoethanoyl) (AE)-DNM, N-2-carboxymethyl (CM)-DNM, and N-(2-aminoopropanoyl) (AP)-DNM were 146, >500, and >500 μM, respectively, compared to 2.75 μM for DNM.

An alkylation of DNM, as in N-methyl-DNM, 4-azoanilino-N-pentyl (AA-P)-DNM and ASBA-P-DNM enhances the potency of DNM (IC$_{50}$ 0.33, 0.30, and 0.42 μM, respectively). The introduction of a charged group, even on a relatively small alkyl chain as in N-5-carboxypropyl (CP)-DNM lowers the inhibitory potential of the compound for the enzyme (compare IC$_{50}$ 7.16 μM for CP-DNM versus 2.75 μM for DNM). The compound AA-P-DNM contains a six-carbon hydrophobic arm separating the DNM moiety from the azidoanilino photoactive center and has a favorable IC$_{50}$ (0.30 μM) for tagging glucosidase I after photolysis. However, we were unsuccessful in obtaining the radiiodinated form of this compound in good yield under mild iodination conditions; apparently the azido group sufficiently deactivates the phenyl ring and its presence does not favor further substitution. Under strong oxidizing conditions, the DNM moiety became unstable and yielded several products with much reduced level of inhibitory potency toward glucosidase I. Therefore, AA-P-DNM was not found to be suitable for use in radiolabeling the enzyme at the substrate-binding site.

Synthesis and characterization of $[^{125}$I]ASBA-P-DNM

A radiolabeled photoactive derivative of DNM, $[^{125}$I]ASBA-P-DNM was prepared by coupling ASBA to CP-DNM through amidation (Materials and methods). In contrast to AA-P-DNM, the ASBA-P-DNM could be readily iodinated with $^{125}$I because of the activating influence of the -OH group on the phenyl ring. The UV absorption spectrum of a 25 mM aqueous solution of ASBA-P-DNM is shown in Figure 3. Without irradiation, ASBA-P-DNM had a maximum absorption at 270 nm with a shoulder at 315 nm, characteristic of an aryl azide. UV irradiation of this compound for different time intervals with 254 nm UV light gradually decreased its absorption at 270 nm (Figure 3, inset), where the peak disappeared after 20 s of exposure to UV, indicating the photolysis of the aryl azide.

The results of Table I show, the IC$_{50}$ value of ASBA-P-DNM (0.42 μM) for glucosidase I was nearly nine times lower than the IC$_{50}$ of DNM. When tested with glucosidase II, it gave an IC$_{50}$ value of 4.5 μM (not shown), similar to the IC$_{50}$ of DNM (3.5 μM). Thus, $[^{125}$I]ASBA-P-DNM appeared to be suitable to radiolabel the active site of glucosidase I.

$[^{125}$I]ASBA-P-DNM photolabeling of crude microsomal extract

Photolabeling of a crude detergent extract of bovine microsome sample with $[^{125}$I]ASBA-P-DNM showed a high affinity and specific labeling of glucosidase I (85 kDa, Figure 4A, lane 3) enzyme in the concentrated protein mixture. Notably, in these conditions of labeling with 0.5 mM $[^{125}$I]ASBA-P-DNM, only the purified glucosidase I was labeled; the hetrodimeric glucosidase II, expressed as recombinant enzyme Glc II-α-MycHis$_6$ and Glc II-β, was not labeled (Figure 4B). This is consistent with the observation that the IC$_{50}$ for the inhibition of glucosidase II with ASBA-P-DNM has a value nearly 10-fold higher than that for glucosidase I.

Photolabeling of glucosidase I with $[^{125}$I]ASBA-P-DNM

Glucosidase I was photolabeled with $[^{125}$I]ASBA-P-DNM as described under Materials and methods. Autoradiogram (Figure 5B, lane 1) of the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel of the labeled protein showed that the major radiolabeled polypeptide migrated with an $M_r$ of 85 kDa, identical to the migration of the enzyme polypeptide in the Coomassie blue–stained band (Figure 5A, lane 1). To determine the specificity of

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[125I]ASBA-P-DNM photolabeling at the active site of the enzyme, labeling experiments were performed in the presence of different concentrations of N-methyl-DNM and also by using thermally denatured enzyme as control. As shown (Figure 5B, lanes 3–5), the presence of increasing concentrations of N-methyl DNM caused a decline in the photoaffinity labeling of the 85 kDa band, indicating specific labeling of the enzyme at its active site by [125I]ASBA-P-DNM. The radiolabeling of thermally denatured enzyme caused significant reduction in the incorporation of
radioactivity in the enzyme (Figure 5B, lane 2). The dye staining in the different lanes on the gel (Figure 5A, lanes 1–5) shows that equal amount of the enzyme was loaded in all the lanes. The low-molecular-weight peptide bands visible in A and B are most likely the degradation products of the enzyme and possibly contain the active site of the enzyme because the radiolabeling of these bands could be competed out with the inclusion of N-methyl-DNM (not shown).

It was earlier shown that DNM protection of the active site of glucosidase I prevented its inactivation by phenylglyoxal, a reagent that chemically modifies the arginine residues in proteins. This and other observations led us to propose the ERHLDLRCW motif at the active site of the enzyme (Romaniouk and Vijay, 1997). An experiment was conducted to modify the arginine residues in the enzyme with phenylglyoxal (Materials and methods). This modification blocked labeling of the enzyme with

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<th>Inhibitor</th>
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<td>DNM</td>
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<td>N-methyl-DNM</td>
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<td>AA-P-DNM</td>
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<td>ASBA-P-DNM</td>
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The IC50 values were calculated from the data in Figure 2.
[125I]ASBA-P-DNM was used as a competitor and phenyl glyoxal as the enzyme modifier. Four milligrams glucosidase I was incubated with 0.5 mM [125I]ASBA-P-DNM and UV irradiated in the absence (lanes 1A, 1B) or presence of 3 mM (lanes 3A, 3B), 10 mM (lanes 4A, 4B), and 100 mM (lanes 5A, 5B) N-methyl-DNM. The photolabeled samples were subjected to 10% SDS–PAGE and stained with Coomassie blue (A) and subjected to autoradiography (B). Lanes 2A and 2B contain temperature-denatured enzyme. Arrow indicates the 85-kDa band of glucosidase I.

(C) Autoradiogram of glucosidase I (Glc I) photolabeled with the radioactive probe before and after modification with phenylglyoxal. Ten milligrams of Glc I was incubated without (lane 1) and with (lane 2) 20 mM phenylglyoxal, dialyzed, and photolabeled with 0.5 mM [125I]ASBA-P-DNM. Protein samples were precipitated with 5% TCA, run on 10% SDS–PAGE, and autoradiographed.
Discussion

Among several inhibitors of \( \alpha \)-glucosidases, DNM and its N-alkyl derivatives have found potential application as pharmacological agents in pathological conditions, such as diabetes (Bollen et al., 1988; Sels et al., 1999), malignancy (Ranes et al., 2001), Gaucher’s disease (Priestman et al., 2000), hepatitis B (Block et al., 1998) and C (Zitzmann et al., 1999), and AIDS (Fleet et al., 1988). The architecture of the active site of a number of \( \alpha \)-glucosidases, particularly the glycoprotein processing glucosidase I, has not been defined. Due to its critical juxtaposition in the pathway of protein N-glycosylation, it is important to have this information for a more rational design of specific therapeutics.

A large-scale purification of the enzyme from animal or plant tissues that would be amenable for crystallization to conduct structural analysis is difficult given its low abundance and unstable character. The cDNA and the gene of the enzyme have been cloned, but structural studies or analysis of the active site of the recombinant enzyme have not been reported. On transient overexpression in COS1 cells, the cDNA of the human hippocampus enzyme showed only a fourfold increase in the enzyme activity over the background, endogenous enzyme activity (Kalz-Fuller et al., 1995). In our studies with the expression of the mouse cDNA in SF9 cells, the recombinant enzyme requires the cotransfection of cDNAs of the chaperones calnexin, calreticulin, and ERp57 to obtain significant catalytic activity (unpublished data). Apparently, the recombinant enzyme does not fold well after expression. Until a structural model for the enzyme can be developed after crystallization or through modeling and computational analysis, an investigator must try strategies of solution biochemistry to minimally gain some insight into the active site of the enzyme. Photoaffinity labeling at or near the substrate-binding region of the purified enzyme offers a useful approach for such an effort. Importantly, the probe must be specific for the native enzyme and highly reactive to reach out within a reasonable distance from the binding region within the active site.

Several laboratories have shown that N-alkylation of DNM increases its inhibitory potential for glucosidase I (Mellor et al., 2002; Tan et al., 1991). Consistent with the previous studies, we observed that CP-DNM, which has a five-carbon spacer between its carboxyl group and the nitrogen in the ring component, was more effective than several other alkyl derivatives of DNM and could serve...
well for the attachment of a photoactive group. Its extension with 4-azidoaniline (AA) or ASBA enhanced the IC$_{50}$ of DNM for the enzyme. This indicates that the N-substitution of DNM that gives rise to AA-P-DNM and ASBA-P-DNM allows alignment and interaction of these derivatives with the hydrophobic region surrounding the catalytic pocket of the enzyme. However, only ASBA-P-DNM was found to be suitable for radiolabeling with $^{125}$I because of the mild conditions under which it could be rendered radioactive without destroying its integrity.

The photoaffinity labeling of glucosidase I with $^{[125]}$IASBA-P-DNM was directed toward the active site because labeling decreased significantly in the presence of N-methyl DNM (Figure 5B) and was not observed after modifying the enzyme with phenylglyoxal (Figure 5C). There was also a remarkable reduction in the photolabeling of the 24-kDa peptide fragment obtained after digestion of the enzyme with V8 protease that had been labeled with $^{[125]}$IASBA-P-DNM in the presence of DNM (Figure 6A, lane 3). When thermally denatured enzyme was labeled with the probe, again there was very little labeling of any specific region of the enzyme. These observations support the view that the active site of the enzyme resides within the 24-kDa peptide and the probe can gain entry into it under the conditions employed in this study. The positive western blot of the V8-digested 24-kDa peptide with under the conditions employed in this study. The positive monospecific antipeptide 582±598 antibody (Figure 6C) further supports this observation. Earlier, we had observed that glucosidase is a multidomain protein in which the active site resided within a 39-kDa segment (Shalubhai et al., 1991).

In conclusion, our data show that $^{125}$IASBA-P-DNM can serve as a useful tool to isolate and gain an insight into the active site of glucosidase I. The nitrene generated from the photolyzed probe gives a maximum distance of 25 Å between the ring N of DNM and the N atom at the end of the unstable radical for insertion into glucosidase I. This distance is well within the span for a probe to sweep the active site of an enzyme. Further studies on the analysis of peptides of the enzyme obtained by tagging with both radioactive and nonradioactive ASBA-P-DNM can then be combined with 3D modeling or X-ray diffraction analysis on the conserved sequence of the enzyme from different species. Mammalian as well as non-mammalian sources such as bovine, human, Caenorhabditis elegans, and Aribdopsis should reveal the architecture of the active site of the enzyme. Recently, it was shown that mutations in Arg$_{468}$ and Phe$_{652}$, both residing in exon 4 of the human enzyme, contribute to congenital disorder of glycosylation IIb (Volker et al., 2002) indicating that these amino acids are essential for glucosidase I activity. This observation is consistent with our earlier proposal that the motif E$_{590}$RHLDDLRCW$_{602}$ represented the substrate binding motif in the enzyme in the rat mammary gland. This proposal was developed by identifying cysteine, arginine, and tryptophan at the active site by modifying the amino acid residues of the enzyme after loading DNM into the active site of the enzyme (Pukhazhenthi et al., 1993; Romaniouk and Vijay, 1997). An analysis of the enzyme, as given, provides a direct means of verifying this proposal. Our current experiments are designed to test this hypothesis.

Materials and Methods

Materials

All reagents and chemicals used in this study were purchased from commercially available sources and were of the highest purity. DNM was a kind gift from Drs. D. Schmidt and Scangos (Bayer AG, Wuppertal, Germany). N-methyl-Dowex, Dowex 1 × 4 resin, Concanavalin A-Sepharose, Lubrol PX, Triton X-100, protease inhibitors, phenylglyoxal, and all other reagents used in the synthesis of DNM derivatives were purchased from Sigma/Aldrich (St. Louis, MO) unless otherwise noted. Ni-NTA agarose was purchased from Invitrogen (Carlsbad, CA), and alkaline phosphatase–conjugated secondary antibody was from Promega (Madison, WI). ASBA, IODO-GEN iodinating tubes, N-hydroxysuccinimide (NHS), and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) were purchased from Pierce (Rockford, IL). 4-Azidoaniline hydrochloride was obtained from Fluka (St. Louis, MO). V8 protease was from Promega. Na$_{25}$I (specific activity 15 Ci/mg) and $^{[4]}$C-methylated protein markers were from Amersham (Little Chalfont, U.K.). Affi-Gel 102 gel and gel electrophoresis reagents were purchased from BioRad (Hercules, CA). Silica gel TLC plates were from EM Science (Gibbstown, NJ); all other chromatographic reagents were purchased from Fisher (Silver Spring, MD). Bovine udders were obtained from a local slaughterhouse. Radioactive substrates, [Glc-$^{3}$H]Glc$_{3}$Man$_{3}$GlcNAc$_{2}$ and [Glc-$^{3}$H]Glc$_{2}$Man$_{2}$GlcNAc$_{2}$ for the assay of glucosidases I and II, respectively, were prepared in our laboratory according to the published procedures (Vijay and Perdew, 1982). The peptide 590TAERHLDLRCWVA$_{602}$ was synthesized and purified by Sigma-Genosys (Woodlands, TX).

Synthesis of DNM derivatives

CM-DNM was prepared by treating DNM (0.120 mmol) with 2-bromo-acetic acid (0.2 mmol) in 0.1 ml water and incubating the mixture at 50°C for 24 h. The mixture was dried and excess 2-bromo-acetic acid was removed by several extractions with diethyl ether. The contents were dissolved in water and applied to a Dowex 1 × 4 anion exchange column prepared in the OH$^{-}$ form. The column was washed with water and the bound CM-DNM was eluted with 2 M acetic acid. The eluate was dried, dissolved in water, and stored at −20°C. Purity of CM-DNM was determined by TLC analysis, and its structure was characterized by $^{1}$H-nuclear magnetic resonance (NMR) in D$_{2}$O (chemical shifts: 3.20–4.19 ppm [H1–8, DNM]; 3.67 ppm [H9,10].

CP-DNM was synthesized according to the published procedure (Hettkamp et al., 1984). Its structure was characterized by $^{1}$H-NMR in D$_{2}$O (chemical shifts: 2.98 ppm [H1]; 3.49 ppm [H2]; 3.43 ppm [H3]; 3.25 ppm [H4]; 3.56 ppm [H5]; 3.69 ppm [H6]; 3.87 ppm [H7]; 3.98 ppm [H8]; 3.11 ppm [H9]; 1.50 ppm [H10]; 1.26 ppm [H11,12]; 1.65 ppm [H13,14]; 2.21 ppm [H15,16].

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AP-DNM was synthesized by conjugating N-(tert-butoxycarbonyl)-L-alanine (t-Boc-alanine) to DNM through a three-step procedure. The carboxyl group of t-Boc-alanine (42 mmol, 8 g) was first activated by reacting with pentafluorophenol (54 mmol, 10 g) and dicyclohexylcarbodiimide (1.1 M) dissolved in 5 ml dimethyl sulfoxide (DMSO) and incubating the mixture at 0 °C for 24 h. Reaction mixture was filtered and washed successively with a dilute solution of H2SO4, water, 5% NaHCO3, and water. The filtrate was dried and crystallized in ethanol. Activated t-Boc-alanyl-o-pentafluorophenol (1.2 mmol, 0.43 g) was then reacted with DNM (0.8 mmol, 0.16 g) in 2 ml DMSO at room temperature for 24 h, and the reaction products were analyzed by TLC using ethyl acetate:acetic acid (15:1) as the solvent. The reaction mixture was loaded on a silica gel column; the column was washed with hexane, and t-Boc-alanyl-DNM was eluted with a hexane:ethyl acetate (1:4) solution. AP-DNM was obtained by removal of the t-Boc group from t-Boc-alanyl-DNM (0.3 mmol) by treatment with trifluoroacetic acid (2 ml) in 2 ml methanol at room temperature for 1 h. The reaction mixture was dried, and t-Boc was removed by extraction with diethyl ether. Purity of the AP-DNM product was determined through TLC analysis, using the solvent system 1-butanol:acetic acid:water (4:1:1), and the structure of the compound was verified by 1H-NMR in D2O (chemical shifts: 3.00±4.37 ppm 8H [H1±8, DNM]; 1.25 ppm 3H [-CH3]; 3.20 ppm 1H [H9]).

AE-DNM was prepared from t-Boc-glycine and DNM according to the protocol used to prepare AP-DNM. The purity of the AE-DNM compound was analyzed by TLC using solvent system 1-butanol:acetic acid:water (4:1:1). Its structure was verified by 1H-NMR in D2O (chemical shifts: 3.23 ppm 1H [H1]; 3.57 ppm 1H [H2]; 3.75 ppm 1H [H3]; 3.65 ppm 2H [H4,5]; 3.82 ppm 1H [H6]; 4.02±4.05 ppm 2H [H7,8]; 3.48 ppm 2H [H9,10]).

AA-P-DNM was synthesized by conjugating 4-azidoanilino to CP-DNM by the amidation procedure previously described (Sehgal and Vijay, 1994). All steps were performed under subdued, indirect light. A 0.035 mmol of 4-azidoaniline was incubated at room temperature for 24 h and stopped by treatment with trifluoroacetic acid (1.1 M) dissolved in 5 ml dimethyl sulfoxide (DMSO) and incubating the mixture at 0 °C for 24 h. Reaction mixture was filtered and washed successively with a dilute solution of H2SO4, water, 5% NaHCO3, and water. The filtrate was dried and crystallized in ethanol. Activated t-Boc-alanyl-o-pentafluorophenol (1.2 mmol, 0.43 g) was then reacted with DNM (0.8 mmol, 0.16 g) in 2 ml DMSO at room temperature for 24 h, and the reaction products were analyzed by TLC using ethyl acetate:acetic acid (15:1) as the solvent. The reaction mixture was loaded on a silica gel column; the column was washed with hexane, and t-Boc-alanyl-DNM was eluted with a hexane:ethyl acetate (1:4) solution. AP-DNM was obtained by removal of the t-Boc group from t-Boc-alanyl-DNM (0.3 mmol) by treatment with trifluoroacetic acid (2 ml) in 2 ml methanol at room temperature for 1 h. The reaction mixture was dried, and t-Boc was removed by extraction with diethyl ether. Purity of the AP-DNM product was determined through TLC analysis, using the solvent system 1-butanol:acetic acid:water (4:1:1), and the structure of the compound was verified by 1H-NMR in D2O (chemical shifts: 3.63±4.20 ppm 8H [H1±8, DNM]; 1.39–1.78 ppm 6H [H11–16]; 2.26 ppm 2H [H17, 18]; 3.51 ppm 2H [H20, 21]; 1.80–1.82 ppm 4H [H22±25]; 3.12 ppm 2H [H26, 27]; 6.63 ppm 2H [H1−2”, phenyl]; 7.84 ppm 1H [H3, phenyl]). UV-visible spectra of AP-DNM samples (25 mM) in water were analyzed before and after exposure to 254 nm UV light at 5, 10, and 20-s time intervals. ASBA-P-DNM samples were exposed to UV light at a 7 cm distance.

[125I]ASBA-P-DNM was prepared by radioiodinating ASBA-P-DNM using IODO-GEN tubes. [125I] was first activated by incubating 1 mCi Na2125I in 20 ml 25 mM phosphate buffer (pH 7.2) in an IODO-GEN tube for 6 min while shaking every 30 s. Activated iodine solution was transferred to 2 ml ASBA-P-DNM (25 mM) solution in water and was left for 10 min at room temperature. [125I]ASBA-P-DNM was purified by preparative silica gel TLC on aluminum-backed plates that were developed in chloroform:methanol:water (10:10:1). The position of the radioiodinated compound was located by autoradiography of the TLC plate, and the product was purified by elution from silica gel with methanol:water (1:1) solution. [125I]ASBA-P-DNM (specific activity 2.26 Ci/mmol) was stored at −20°C.

Purification of glucosidase I and assay of enzyme activity
Crude detergent extract of microsomal proteins of the lactating bovine mammary gland was obtained by extracting with 0.8% Lubrol in 200 mM phosphate buffer (pH 6.0). Glucosidase I was purified from this extract as previously described (Shailubhai et al., 1987). The enzyme activity was measured with the radioactive substrate, [Glc125H] GlcP,ManpGlcNAc2 (13,000 cpm per assay), dissolved in 10 mM phosphate buffer (pH 6.8) in a final volume of 100 ml. The reaction was incubated at 37°C for 1 h. The contents of incubation were applied to a column of Concanavalin A-Sepharose, washed with buffer, and the released [125H] glucose was measured by scintillation counting.

Expression of glucosidase II and measurement of its activity
Glucosidase II-α, as fusion protein Glc-α-MycHis6, and glucosidase II-β subunit were cloned and expressed in a...
baculovirus system (Feng et al., unpublished data). The coexpressed forms of the subunits of the enzyme were purified on Ni-NTA-agarose column. The fusion protein was catalytically active. Its enzyme activity was determined as given for glucosidase I except that 7000 cpm of the radioactive oligosaccharide [Glc-3H] Glc-Man3GlcNAc2 was used as the substrate. It showed a specific activity of 2170 units per mg (1 unit of enzyme activity is defined as the amount of enzyme protein required to hydrolyze 10% of the corresponding substrate in 30 min at 37°C).

Modification of glucosidase I with phenylglyoxal

A 10 mg aliquot of glucosidase I enzyme sample was incubated with 100 mM phosphate buffer (pH 7.5) and 20 mM phenylglyoxal for 1 h at room temperature (Romaniouk and Vijay, 1997). The modified enzyme was dialyzed and used for photolabeling with [125I]ASBA-P-DNM.

Photolabeling of glucosidase I

A 10 mg aliquot of purified glucosidase I in 2.3 ml of 25 mM phosphate buffer (pH 6.8) was incubated at room temperature for 10 min in the dark with 0.5 mM [125I]ASBA-P-DNM in the absence or presence of different concentrations of DNM, N-methyl-DNM, or 0.5 mg glucosidase I peptide 590TAERHLDLRCWVA602 as competitors. Subsequently, the mixture was irradiated with 254 nm UV light for 1 min from a distance of 7.5 cm. Phenylglyoxal modified glucosidase I sample was also incubated with 0.5 mM [125I]ASBA-P-DNM for 10 min in the dark and UV irradiated as described. The enzyme samples were precipitated with 10% trichloroacetic acid (TCA), kept on ice for 1 h, and centrifuged at 15,000 × g. The protein pellets were washed several times with ice-cold acetone to remove unbound [125I]ASBA-P-DNM and TCA and subjected to 10% SDS–PAGE. Alternatively, the washed protein pellets were dissolved in 5 M urea for digestion with V8 protease followed by 16.5% SDS–PAGE. The gels were autoradiographed to determine the labeling of the enzyme or its digested peptide fragments labeled with the photolyzed [125I]ASBA-P-DNM.

The crude microsomal extract was photolabeled with 0.5 mM [125I]ASBA-P-DNM using the same procedure as for glucosidase I but without the use of any competitors.

V8 digestion

A 20-mg portion of glucosidase I dissolved in 100 ml 4 M urea was diluted with 100 ml 100 mM ammonium bicarbonate buffer, pH 7.8. Fifty milliliters of immobilized Staphylococcus aureus V-8 protease was added to enzyme buffer mixture, and glucosidase I was digested at 37°C for 24 h.

Monospecific antibody to peptide fragment of glucosidase I

Through the genome database search, we identified a conservative peptide fragment of human glucosidase I, 582DDYPRASHPSVTERHLD598, that is immunogenic and specific for the glucosidase I enzyme. The immunogenic peptide 582DDYPRASHPSVTERHLD598 and the monospecific antibody to the peptide sequence were prepared by Research Genetics (Huntsville, AL). Anti-rabbit IgG was used as secondary antibody for western analysis.

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Abbreviations

AA-P-DNM, 4-azidoanilido-N-pentyl-1-deoxyojirimycin; AE-DNM, N-(2-aminoethanol)-1-deoxyojirimycin; AP-DNM, N-(2-aminopropanoyl)-1-deoxyojirimycin; ASBA, p-azido-salicyl-butyl-amine; ASBA-P-DNM, 4-(p-azido-salicylamido)butyl-5-ami-no-1-deoxyojirimycin; CM-DNM, N-2-carboxymethyl-1-deoxyojirimycin; CP-DNM, N-5-carboxypentyl-1-deoxyojirimycin; DMSO, dimethyl sulfoxide; DMF, N,N′-Dimethylformamide; DNM, 1-deoxynojirimycin; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; ER, endoplasmic reticulum; MES, 2-(4-morpholine)-Ethane Sulfate Acid; NHS, N-hydroxysuccinimide; NMR, nuclear magnetic resonance; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TLC, thin-layer chromatography.

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