SITE SPECIFIC LABELING OF CYTOPLASMIC PEPTIDE:N-GLYCANASE BY N,N’-DIACETYLCHITOBIOSE-RELATED COMPOUNDS*

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Running Title: Chitobiose binding to yeast PNGase

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Peptide:N-glycanase (PNGase)1 is the deglycosylating enzyme which releases N-linked glycan chains from N-linked glycopeptides and glycoproteins. Recent studies have revealed that the cytoplasmic PNGase is involved in the degradation of misfolded/unassembled glycoproteins. This enzyme has a Cys, His, and Asp catalytic triad which is required for its enzymatic activity and can be inhibited by “free” N-linked glycans. These observations prompted us to investigate the possible use of haloacetamidyl derivatives of N-glycans as potent inhibitors and labeling reagents of this enzyme. Using a cytoplasmic PNGase from budding yeast (Png1), Man6GlcNAc2-iodoacetoamide was shown to be a strong inhibitor of this enzyme. The inhibition was found to be through covalent binding of the carbohydrate to a single Cys residue on Png1, and the binding was highly selective. The mutant enzyme in which Cys191 of the catalytic triad was changed to Ala did not bind to the carbohydrate probe, suggesting that the catalytic Cys is the binding site for this compound. Precise determination of the carbohydrate attachment site by mass spectrometry clearly identified Cys191 as the site of covalent attachment. Molecular modeling of N,N’-diacetylchitobiose (chitobiose) binding to the protein suggests that the carbohydrate binding site is distinct from but adjacent to that of Z-VAD-fmk, a
peptide-based inhibitor of this enzyme. These results suggest that cytoplasmic PNGase has a separate binding site for chitobiose and other carbohydrates, and haloacetamide derivatives can irreversibly inhibit that catalytic Cys in a highly specific manner.

INTRODUCTION

Peptide:N-glycanase (PNGase: also referred to as N-glycanase or glycoamidase; peptide-N\textsuperscript{4}-(N-acetyl-β-D-glucosaminyl) asparagine amidase; EC 3.5.1.52) cleaves the amide bond between an Asn residue and the N-linked glycopeptide or glycoprotein (1). This enzyme activity was first found in plants (2) and subsequently in bacteria (3). Since those discoveries, this enzyme has been widely used as a powerful tool reagent for analyzing the structure and functions of N-linked glycan chains on glycoproteins. The occurrence of a cytoplasmic PNGase activity has been reported in a wide variety of animal origins as well as in yeast (4-8). The cytoplasmic PNGase was found to be quite distinct in terms of enzymatic properties from the PNGases of plant and bacterial origin (9). A gene encoding the cytoplasmic enzyme, PNG1, was first identified in S. cerevisiae (10). Structural elements of the gene are highly conserved throughout eukaryotes (10,11). The protein is categorized as a member of the transglutaminase superfamily (12) and contains a catalytic Cys, His, and Asp triad which is required for enzyme activity (1,13). The cytoplasmic PNGase has been shown to be involved in the degradation of misfolded/unassembled glycoproteins by a process called ER-associated degradation (ERAD) (14-19).

Previously the chemical synthesis of high-mannose glycans and its derivatives have been reported (20-23). Among these, the haloacetamidyl-sugar (CHO-XAc) is an attractive compound because it can generate de novo glycoproteins through Cys residues in the target proteins (22,24). It can also serve as a specific inhibitor for carbohydrate-processing enzymes, which harbor a catalytic Cys residue, as is the case with the cytoplasmic PNGase. In this study, we explored this possibility using purified yeast PNGase (Png1) and found that these compounds are very strong inhibitors of this enzyme. CHO-XAc was found to be covalently linked to this enzyme, and to our surprise the labeling was specific and under experimental conditions only a monovalent binding to the enzyme was observed, strongly suggesting that the labeling is against a specific Cys residue. This carbohydrate binding occurred with carbohydrates as short as N,N\textprime -diacetylchitobiose (GlcNAc\textsubscript{2} or chitobiose), indicating the existence of a binding pocket for chitobiose on Png1. The mutant enzyme in which the catalytic Cys191 was changed to Ala did not bind to CHO-IAc (idoacetamidyl sugar), suggesting that Cys191 is the binding site for this compound. This hypothesis was further
validated by precise analysis of the carbohydrate-binding site by mass spectrometry, which unequivocally demonstrated that the catalytic Cys is indeed the binding site. Recently the crystal structures of yeast and mouse PNGases were determined (25,26). Molecular modeling of chitobiose binding to yeast PNGase suggested the binding site for this carbohydrate, which was distinct from that of Z-VAD-fmk (25), a peptide-based inhibitor of cytoplasmic PNGases (18,27). Our studies suggest that these haloacetamidyl sugars could serve as novel, carbohydrate-based potent inhibitors of these enzymes, and also provide insight into the reaction mechanism of the cytoplasmic PNGase.

**EXPERIMENTAL PROCEDURES**

*Materials* - All chemicals used in this study were obtained either from Wako Pure Chemical Industries Ltd. (Osaka, Japan), Nacalai Tesque, Inc (Kyoto, Japan) or Sigma-Aldrich Japan (Tokyo, Japan) unless specified otherwise. pET28b-PNG1(C191A)-(his)₆ was prepared from pET28b-PNG1-(his)₆ (10) by site-directed mutagenesis as described previously (13).

*NMR* - ¹H NMR spectra were measured on JEOL EX-400 spectrometer.

*MALDI-TOF MS* – The MALDI-TOF MS spectra were recorded in the positive ion mode either on an AXIMA CFR (Shimadzu-KRATOS, Shimadzu Corp., Kyoto, Japan; for oligosaccharide-related compounds) or on an Ultraflex TOF/TOF (Bruker Daltonics Gmbh, Bremen, Germany; for CHO-labeled Png1) equipped with a nitrogen laser and an emission wavelength of 337 nm.

**Synthesis of Man₉GlcNAc₂-IAc (3 in Figure 1)** - Undecasaccharide 1 (Figure 1: 4.9 mg, 2.6 μmol), synthesized as described previously (20), was dissolved in saturated aqueous NH₄HCO₃ (1.5 ml) and stirred at 40°C for 72 h, then the mixture was concentrated and lyophilized with H₂O (3 times). The residue was dissolved in N,N-dimethylformamide (0.3 ml) and NaHCO₃ (8.3 mg, 0.098 mmol) and iodoacetic anhydride (7.9 mg, 0.024 mmol) was added. After stirring at 0°C for 20 min, H₂O (0.3 ml) was added and the mixture was purified by gel filtration [Sephadex LH20 (Amersham Biosciences, Uppsala, Sweden; 20 mmφ x 40 cm), eluted with H₂O] to give 4.1 mg (77%, 2 steps) of Man₉GlcNAc₂-IAc (3): ¹H NMR (400 MHz, D₂O) δ 1.88 (s, 3 H), 1.91 (s, 3 H), 3.42-4.00 (m, 68 H), 4.44 (d, J = 7.3 Hz, 1 H), 4.88 (s, 3 H), 4.89 (s, 1 H), 4.99 (s, 1 H), 5.15 (s, 1 H), 5.17 (s, 1 H), 5.25 (s, 1 H); MALDI-TOF mass m/z calculated for C₇₂H₁₂₀N₁₅O₆₅Na 2072.6 (M+Na)⁺, found 2073.4.

**Synthesis of Man₈GlcNAc₂-IAc (4 in Figure 1)** - Decasaccharide 2 (Figure 1: 4.6 mg, 2.7 μmol) was subjected to a series of reactions in a manner described for 3, to give 5.1 mg (quant., 2 steps) of Man₈GlcNAc₂-IAc (4): ¹H NMR (400 MHz, D₂O) δ 1.88 (s, 3 H), 1.90 (s, 3 H), 3.42-4.00 (m, 62 H), 4.08 (s, 1 H), 4.45 (m, 1 H), 4.73 (s, 1 H), 4.88 (s, 3 H), 4.94 (s, 1 H), 5.00 (s, 1 H), 5.16 (s, 1 H), 5.20
(s, 1 H); MALDI-TOF mass m/z calculated for C_{66}H_{110}N_{3}O_{51}Na 1910.5 (M+Na)^+, found. 1910.5.

**Synthesis of GlcNAc2-IAc (6 in Figure 1)** -
Chitobiose (5) (44.0 mg, 0.104 mmol) was subjected to a series of reactions in a manner as described for (3), to give 51.2 mg (83%, 2 steps) of GlcNAc2-IAc (6): \(^1\)H NMR (400 MHz, D_2O) δ 1.89 (s, 3 H), 1.93 (s, 3 H), 3.30-3.79 (m, 14 H), 4.43 (d, J = 7.3 Hz, 1H), 4.85 (d, J = 7.3 Hz, 1H); MALDI-TOF mass m/z calculated for C_{18}H_{30}N_{3}O_{11}Na 614.1 (M+Na)^+, found. 613.8.

**Synthesis of GlcNAc2-BrAc (7 in Figure 1)** -
Chitobiose (50.0 mg, 0.118 mmol) was dissolved in saturated aqueous NH_4HCO_3 (2.5 ml) and stirred at 40°C for 72 h then the mixture was concentrated and lyophilized with H_2O (3 times). The residue was dissolved in dioxane/H_2O (1/1, 2 ml) and NaHCO_3 (65.1 mg, 0.775 mmol) and bromoacetyl anhydride (211 mg, 0.811 mmol) was added to the solution. After stirring at 0°C for 15 min, solvents were removed by evaporation in vacuo. The residue was purified by Sep-Pak C18 cartridge (5 g, Waters adsorbed and washed with H_2O and eluted with H_2O/methanol = 20/1), followed by gel filtration [Amersham: Superdex 30 (20 mmφ x 60 cm), H_2O] to give 33.9 mg (58%, 2 steps) of GlcNAc2-ClAc: \(^1\)H NMR (400 MHz, D_2O) δ 1.87 (s, 3 H), 1.94 (s, 3 H), 3.30-3.79 (m, 12 H), 3.98 (m, 2H), 4.47 (d, J = 8.5 Hz, 1H), 4.95 (d, J = 9.8 Hz, 1H); MALDI-TOF mass m/z calculated for C_{18}H_{30}N_{3}O_{11}BrNa 522.2 (M+Na)^+, found. 522.2.

**Purification of yeast Png1 –**

His-tagged yeast Png1 was purified using a HiTrap Chelating column (Amersham). Briefly, a 50 ml overnight culture of E. coli bearing pET28b-PNG1-(His)_6 (10) was cultured with LB media containing kanamycin (50 µg/ml) and chloramphenicol (30 µg/ml), transferred to fresh 450 ml LB containing the same antibiotics, and further cultured at 37°C.
for 30 min. Then 5 ml of 0.1 M isopropylthiogalactoside was added to induce the expression of proteins and further cultured for 3 h. After collecting cells by centrifugation, the pellet was resuspended with 25 ml of Tris-HCl buffer (pH 8.0) containing 1 mM Pefabloc (Roche Diagnostics Corp., Indianapolis, IN) and 0.15 M NaCl, followed by addition of 500 µl of lysozyme (10 mg/ml in 20 mM Tris-HCl, pH 8.0). After incubation on ice for 30 min, 2.5 ml of 10% Triton X-100 (final 1%), and β-mercaptoethanol (final conc. 10 mM) were added, and further incubated for 20 min on ice. Sonication was performed using VC-750 (Sonics and Materials, Inc., Newtown, CT), 30% maximum power 2 times for 10 sec with at least a 1 min interval between sonication treatments. After clearing the solution by centrifugation at 12,000 x g (10 min at 4°C), the resulting supernatant was filtrated with a syringe driven 0.45 µm filter unit (Millex-HA, Millipore, Carrigtwohill, Co. Cork, Ireland).

Ten ml of extract were applied onto a 1 ml HiTrap chelating column, which was precharged with 0.5 ml of the 0.1 M NiCl2. After sample application, the column was washed with 10 ml of buffer A (20 mM sodium-phosphate buffer (pH 7.2), 150 mM NaCl). Elution was performed at a flow rate of 1 ml/min using an AktaPrime (Amersham) with a stepwise elution of increasing amounts of buffer B (20 mM sodium phosphate buffer (pH 7.2) 150 mM NaCl with 500 mM imidazole) as follows: 2% buffer B for 5 min, 20% buffer B for 10 min and 100% buffer B for 10 min. Fractions were collected every min, and dithiothreitol and EDTA were added to a final concentration of 5 mM and 1 mM, respectively. Fractions were monitored by SDS-PAGE and fractions containing purified PNGase (eluted at 100% buffer B solution) were collected, ultrafiltrated with Nanosep (Mol. Cutoff, 10K; Pall Gelman Corp., Ann Arbor, MI) in 20 mM sodium-phosphate buffer (pH 7.2), 150 mM NaCl and 5 mM dithiothreitol and used for enzyme assay. The protein content of the final enzyme preparation was determined by CBB staining using bovine serum albumin as a standard. A 0.2 mg/ml solution was used in the enzyme assay.

Assay for PNGase - PNGase activity was measured using RNase B (Sigma-Aldrich Corp., St. Louis, MO) as a substrate as described previously (28). Typically, the reaction mixture of 30 µl includes 9 µl of purified Png1 (final 60 μg/ml), 3 µl of RNase B (final concentration 0.2 mg/ml), and 18 µl of 200 mM Mes-NaOH buffer, pH 6.7/10 mM dithiothreitol. The reaction was performed for 3 min at 37°C and was stopped by adding 2 X sample buffer, followed by SDS-PAGE (28), and quantitated using Multi Gauge ver. 2.2 (Fujifilm Co., Tokyo, Japan).

Carbohydrate-Probe Binding Assay - For assessing the binding of various carbohydrate-related probes to purified Png1, 5 µl of the enzyme fraction (0.2 mg/ml) or E. coli extract expressing Png1 (2.0 mg/ml protein
content quantitated by Coomassie Protein Assay Reagent (Pierce Biotechnol. Inc., Rockford, IL)) were added to 4 µl of PBS buffer containing 10 mM dithiothreitol and 1 µl of the respective carbohydrate probes (final concentration of 50 µM unless indicated otherwise). The reaction was performed at 37°C and was stopped by adding 2X SDS-PAGE sample buffer. The sample was applied to SDS-PAGE or lectin blotting.

Effect of Man9GlcNAc2-IAc on caspase activity –

Caspase Combo II (caspase 2, 3 and 7) was purchased from Sigma-Aldrich Corp. and was suspended with 25 µl of PBS (1 U/µl). Caspase activity was assayed using N-acetyl-Val-Asp-Val-Ala-Asp-p-nitroanilide (Sigma-Aldrich Corp.) or N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Sigma-Aldrich Corp.) for caspases 2 and 3 and 7 in the presence and absence of 50 µM of Man9GlcNAc2-IAc, according to the manufacturer’s protocol. Also the labeling of Man9GlcNAc2-IAc with these caspases were examined after the incubation of 5 U of enzyme with or without 50 mM of Man9GlcNAc2-IAc at 37°C for 30 min, and the possible mobility shift of the enzymes on SDS-PAGE due to the labeling was assayed by silver staining (2D-silver stain II “Daiichi”, Daiichi Pure Chemicals Co., Tokyo, Japan).

Lectin Blotting -

Lectin blotting was carried out essentially as described previously for Western Blotting (Suzuki, et al. 2001). For lectin staining, horseradish peroxidase (HRP)-labeled Con A (Honen Corp., Tokyo, Japan) or HRP-labeled WGA (EY Laboratories Inc., San Mateo, CA) was used with a dilution of 1/5000. Sample detection was carried out using ECL-plus (Amersham). Gels were visualized using LAS-3000 mini (Fujifilm) and the bands detected were quantitated using Multi Gauge ver 2.2.

Effects of various GlcNAc2-related compounds on yeast PNGase activity -

Concentration required for 50% inhibition (IC50) of Png1 activity for various GlcNAc2-related compounds (GlcNAc2, GlcNAc2-IAc, GlcNAc2-BrAc, GlcNAc2-ClAc, Man9GlcNAc2-IAc, Man9GlcNAc2-IAc, and Man9GlcNAc2) was determined using varying concentrations of the inhibitors. No preincubation was performed for these compounds and reaction was initiated by adding Png1 in the reaction mixture. PNGase assay was carried out as described above.

Identification of the carbohydrate-binding site by LC-ESI MS -

For identification of the carbohydrate-binding cysteine residue, GlcNAc2-IAc (final conc. 50 µM) was reacted with yeast PNGase (70 µg in 700 µl) for 30 min and the mixture was ultrafiltrated with Nanosep (10 kDa MW cut off) to remove unreacted GlcNAc2-IAc, and the final retentate (400 µl in 20 mM Tris-HCl (pH 8.0)) was evaporated to dryness. The dried sample was dissolved in 20 µl of 0.5 M Tris-HCl/50 mM dithiothreitol/6 M guanidine HCl,
and incubated at room temperature for 1 h. 2 mg of chloropropionamide was added to the solution, and further incubated overnight at room temperature in the dark. The alkylated samples were ultrafiltrated with Nanosep and the buffer was exchanged to 50 mM NH₄HCO₃. Ten µl of modified trypsin (sequence grade; Promega Corp., Madison, WI; dissolved at 0.4 mg/ml with 50 mM acetic acid) were added to the final retentate (200 µl), and the digestion was carried out at 37°C for 8 h. The reaction was stopped by boiling the solution for 10 min, the reaction mixture was concentrated to 20 µl and samples were applied to LC-ESI MS.

A 2-µl aliquot of the tryptic peptides mixture was separated by reverse phase LC on a Cadenza CD-C18 column (2.0 mm x 150 mm, Imtakt, Kyoto, Japan) under the following gradient conditions: Mobile phases were (A) 0.1% formic acid and (B) 0.1% formic acid/ 80% acetonitrile. Gradient elution was performed from 5 to 50% B over 30 min, and then from 50 to 100% B over 10 min with a flow rate of 0.2 ml/min at 30°C. The eluates were continuously introduced into an electrospray ionization source (Esquire HCT, Bruker Daltonics) to obtain MS and MS/MS spectra.

Computer modeling of chitobiose binding to Png1 - AutoDock 3.05 (29) was used for the docking study using the Lamarckian genetic algorithm (LGA or GA-LS) to search for the global fit of chitobiose into the yeast PNGase structure (PDB ID:1X3Z) (25). The grid map consisted of a 60 by 60 by 60 grid points and the grid spacing was 0.375 Å in each dimension. The grid was centered at the coordinates of the Sγ atom of Cys191. The rest of the parameters were set to default values. After multiple docking iterations, models with the chitobiose locating in the proposed binding site of PNGase (25) were pooled. The model with the lowest binding energy was selected and analyzed in PyMol.

RESULTS

Man₉GlcNAc₂-IAc acts as a potent inhibitor of Png1

Recently convergent synthesis of high mannose-type oligosaccharides and their derivatives were reported (20-23). These homogeneous, structurally defined oligosaccharides can be of great use for determining the importance of carbohydrate structure, since subtle differences in glycan structures seem to be critical for the folding and/or degradation of glycoproteins (30-32). Among the oligosaccharides-related compounds synthesized, iodoacetamide-derivatives of oligosaccharides may be of particular interest since they can generate a neoglycoprotein through a Cys residue bearing a homogeneous oligosaccharide structure. Furthermore, we noticed that these compounds can also be very specific inhibitors for cytoplasmic PNGase, especially because (a) the enzyme can be
efficiently inhibited by free oligosaccharides due to a high affinity toward PNGase (9), and (b) PNGase has a catalytic Cys residue (1,13). To test this hypothesis, we first examined if these compounds can efficiently inhibit the enzyme. As shown in Figure 2, yeast PNGase (Png1) was strongly inhibited by the addition of Man₉GlcNAc₂-IAc at a concentration of 50 µM. The cytoplasmic PNGases was shown to be inhibited by alkylation reagents such as iodoacetamide (6,7,33), but under the experimental conditions (in the presence of 7.5 mM dithiothreitol), the inhibition effect is not significant (Figure 2). These results indicated that haloacetamidyl sugars such as Man₉GlcNAc₂-IAc could be very specific inhibitors of the cytoplasmic PNGase.

Characterization of the binding of haloacetamidyl sugars to Png1

Next we asked whether these compounds are actually covalently bound to the protein. To evaluate this, SDS-PAGE analysis was performed, as the attachment of Man₉GlcNAc₂ to the protein was expected to give rise to a distinct migration position. As shown in Figure 3A, a shift in the elution position of Png1 was observed, which is an indication of carbohydrate attachment to this protein. The reaction is very rapid since at the start of the experiment (i.e. immediately after the addition of Man₉GlcNAc₂-IAc, sample buffer was added to stop the reaction) attachment of carbohydrate was already observed. Glycosylated Png1 could be detected with Con A-staining (Figure 3B). It is interesting to note that although Png1 contains 14 cysteines, there was only a single reaction product which is ~2 kDa higher than the non-glycosylated form on SDS-PAGE (Figures 3A, B). These results suggest that only one Cys residue was reactive towards Man₉GlcNAc₂-IAc. Consistent with this observation, bovine serum albumin was not modified with the carbohydrate under the experimental conditions even after prolonged incubation (Figure 3A, B), supporting the idea that the modification with Man₉GlcNAc₂-IAc is very specific to a single Cys residue in Png1. To further confirm the specificity of Man₉GlcNAc₂-IAc binding, its incubation with an E. coli extract expressing Png1 was carried out. As shown by CBB staining, an apparent shift in the migration position of Png1 was observed, while most other visible proteins remained unchanged (Figure 3C). The shifted band could be detected specifically by Con A-staining (Figure 3D), confirming that Png1 was glycosylated in a highly selective manner. Moreover, 50 µM of Man₉GlcNAc₂-IAc was incubated with various caspases (2, 3 and 7), that are known to be inhibited by Z-VAD-fmk, a peptide-based inhibitor of cytoplasmic PNGases. In sharp contrast to the case with Png1, no binding was observed after 30 min incubation at 37°C (data not shown). Consistent with this result, 50 µM of
Man$_9$GlcNAc$_2$-IAc did not inhibit proteolytic activity of caspases 2, 3 and 7 (data not shown). Taken all results together, labeling of proteins with Man$_9$GlcNAc$_2$-IAc under the experimental conditions was very specific for a single Cys residue on Png1.

To ask if the binding was dependent on the folded conformation of Png1, the carbohydrate binding assay was also carried out in the presence of the chaotropic reagent, urea, at a concentration of 3.2 M. As shown in Figure 3E, although the reaction was slightly impaired (compare lane 3 (Man$_9$GlcNAc$_2$-IAc conc.: 5 µM)), the reaction still occurred at a higher substrate concentration (>100 µM of Man$_9$GlcNAc$_2$-IAc), indicating that folding around the carbohydrate binding site was, at least to some extent, resistant to urea up to 3.2 M. However, Png1 denatured in SDS-PAGE sample buffer did not bind to Man$_9$GlcNAc$_2$-IAc at a concentration of 1 mM for 10 min, indicating that proper folding is critical for its specific binding to Man$_9$GlcNAc$_2$-IAc (data not shown).

To examine the specificity of the carbohydrate structure in this reaction, other CHO-IAc with different carbohydrate structures (Man$_8$GlcNAc$_2$-IAc, GlcNAc$_2$-IAc) were reacted with Png1. Figure 4A shows that both compounds were able to bind to Png1, as judged by a shift in the migration pattern (compare lanes 1 and 2, 3 in Figure 4A). Attachment of GlcNAc$_2$ to Png1 was also confirmed by WGA-staining (Figure 4B). The reactivity of GlcNAc$_2$-IAc was similar to the corresponding BrAc (Figure 4C) or ClAc (Figure 4D) derivatives, with slightly faster kinetics for IAc (Figure 4E). However, the reactivity was apparently slower than either Man$_8$GlcNAc$_2$-IAc or Man$_9$GlcNAc$_2$-IAc, indicating that the Man residues result in a higher affinity to Png1 (Figure 4E).

To examine the inhibitory effects of haloacetamidyl sugars to Png1, the concentration required for 50% inhibition (IC$_{50}$) was determined for these compounds using various concentration of the inhibitors. As shown in Table 1, the inhibition of haloacetamidyl sugar was found to be very strong as compared to the original sugars, and even compounds of highest IC$_{50}$ values (GlcNAc$_2$-ClAc; 19 µM) was found to be more than 50-fold effective to GlcNAc$_2$. These results clearly show that the haloacetamidyl group of these compounds is important for their strong inhibitory property against Png1.

The Png1catalytic C191A mutant did not bind to Man$_9$GlcNAc$_2$-IAc

Since Png1 contains a catalytic Cys residue at position 191, this Cys is the obvious candidate for a specific binding site for Man$_9$GlcNAc$_2$-IAc. To examine this possibility, a mutant in which Cys191 was changed to Ala (C191A) was prepared. Png1(C191A) was previously shown to be an inactive enzyme (13). As expected, in sharp contrast to the case with
wild type Png1, no reaction for the mutant protein was observed even after overnight incubation (Figures 5A, B). Consistent with this finding, there was no Con A staining of the Png1 (C191A) sample, while a single band was detected for wild type Png1 (data not shown). These data further support the idea that the catalytic Cys residue is indeed the binding site for Man₉GlcNAc₂-IAc.

**LC-ESI MS analysis confirmed that Cys191 is the binding site for GlcNAc₂-IAc**

To unequivocally determine which Cys residue is involved in chitobiose binding, mass spectrometry (MS) analysis after derivatization with GlcNAc₂-IAc was carried out. First we examined the average masses of Png1 before and after the carbohydrate-binding reaction with MALDI-TOF MS. It was found that before the reaction the average mass was 44,260 Da which increased to 44,741 after the reaction (data not shown). This result indicated that roughly one GlcNAc₂-amidomethyl (AMGN2) group with a MW of 463 was bound per molecule, further supporting our assumption that there is only one binding site for GlcNAc₂-IAc. Next we performed digestion of CHO-Png1 with trypsin to search for the peptide containing AMGN2 group attached to the Cys. For this purpose, alkylation of other Cys residue was carried out to enhance the efficiency of the following tryptic digestion as well as the detection by MS. However, when iodoacetamide was used as alkylation reagent, it was not possible to distinguish the CHO-binding Cys from the other alkylated Cys residues (carbamidomethyl-Cys, C-CAM) since the GlcNAc residues came off easily during the tandem MS analysis (MS/MS). Consequently C-CAM could also be derived from C-AMGN2 (see below), making the distinction whether C-CAM resulted from C-AMGN2 very difficult. For this reason, we used chloropropionamide, which forms carbamidoethyl-Cys (C-CAE) instead of C-CAM as a modified free Cys, as an alternative alkylation reagent. It was found that, probably because of the additional methylene group, the peptides containing C-CAE could be detected by MS analysis in a highly sensitive manner (data not shown). After MS analysis, we attempted to identify precursor ions that matched the m/z value of the tryptic peptides. For Cys modification, either CAE or AMGN2 modification was assumed for this calculation. With the aid of the Mascot Search analysis (http://www.matrixscience.com/search_form_select.html) 34 tryptic peptides were identified with an overall sequence coverage of 43% (data not shown). Among those was the tryptic peptide 191-203 which contains two Cys residues, one at Cys191 (catalytic Cys) and the other at Cys195. Therefore for this peptide one Cys was predicted to be present as C-CAE and the other to be a C-AMGN2 derivative. As expected, we detected a corresponding precursor ion that matches the molecular mass of peptide 191-203 bearing
AMGN2 at a single Cys (calculated molecular mass: 2072.97). From the total ion chromatogram, we detected the peak of interest by two criteria: (1) The extracted ion chromatogram (EIC) revealed a 1036-1039 m/z for detection of the doubly charged precursor ion. (2) We detected a constant neutral loss of 202-204 m/z by auto MS/MS analysis, corresponding to the release of GlcNAc (m/z 203) from the target peptide. As shown in Figures 6A-C, there is only a single peak at 31.3 min, which meets both criteria, thus strongly suggesting that detected peptide is comprised of residues 191-203 in which the glycan is attached to one of the two Cys residues (i.e. Cys191 or Cys195). No other precursor ions were detected, which potentially contains C-AMGN2 (data not shown).

Next the MS/MS analysis of the precursor ion was extensively analyzed to determine the amino acid sequence of the peptide to identify the site of CHO-attachment. Figure 7A shows the scheme of fragment ions with y series (fragments from C-terminus) of the peptide 191-203, and Figures 7B-D shows the three Cys modifications observed in this study. As observed in Figure 7E, when Cys191 was assumed to be C-AMGN2 and Cys195 to be C-CAE, the fragment ions could be detected through peaks y3 to y12 (peptide 192-200), indicating that the assumption was correct. In addition loss of GlcNAc residues from precursor ions was observed, confirming the occurrence of chitobiose on this peptide. On the other hand, when Cys191 was predicted to be C-CAE and Cys195 to be C-AMGN2, only peptides from y3-y8 were observed (i.e. only peptide from 196-200 could be sequenced), strongly indicating that the underlying assumption was incorrect (Figure 7F). If Cys195 was assumed to be C-CAM following the release of GlcNAc2, the sequence was still not readable through Cys195 (Figure 7G). All of these data clearly identified Cys191 as the sole binding site for GlcNAc2-IAc.

Molecular Modeling of the GlcNAc2 binding site on Png1

To get additional insights into the specific binding of GlcNAc2 to Png1, molecular modeling was carried out using the AutoDock program (29). Previously the potential Man3GlcNAc2-binding site in yeast Png1 was proposed (25), but the atomic detail of the binding was not determined. The structure of yeast and mouse Png1-Rad23 complexes were solved previously (25,26) and the binding site for the peptide part of the substrate was identified using Z-VAD-fmk, a potent irreversible inhibitor of the Png1 enzyme (18,27). Based on the complex with the peptide bound, chitobiose was docked into the proposed oligosaccharide binding site of Png1, which is distant from the peptide binding site resulting in an estimated free energy of binding of -4.70 kcal/mol (Figure 8). The chitobiose molecule hydrogen bonds with Trp123, Asp217, His218, Cys237, Glu238 and Trp251 of
Png1, most of which form a large cleft in the molecule (25). Although no covalent attachment of the chitobiose to the peptide was assumed during docking, interestingly, the C1 position of the first GlcNAc is within hydrogen bonding distance of the Sγ atom of Cys191. In addition there is a stacking interaction between the sugar ring of the second GlcNAc of chitobiose and Trp251. It should be noted that based on this modeling, the binding site of the second GlcNAc largely overlaps with the glucose moiety of one of the sucrose-binding sites observed in the X-ray crystals (25). However, the GlcNAc adopts a somewhat flatter conformation and is closer to residues Trp251 and Lys253. Based on these data, it was concluded that there is a defined binding pocket for chitobiose on Png1 directly adjacent to the active site cysteine.

DISCUSSION

In this study, we have established that haloacetamidyl-derivatives of oligosaccharides are very specific labeling reagents and inhibitors of yeast Png1. Using GlcNAc2-IAc as a probe, only Cys191 was found to be modified among a total of 14 Cys residues in this protein, suggesting that the high affinity of Png1 towards chitobiose allowed for its specific binding. Molecular modeling confirmed that the reducing end of GlcNAc is in close proximity of Cys191, providing insights into the molecular mechanism of specific inhibition by this compound. The fact that no other Cys residues in Png1 or other proteins (BSA or proteins from an E. coli extract) were efficiently modified by this compound indicated that the non-specific reaction of these compounds with Cys residues is very slow, unless a high concentration of the carbohydrate probe is added.

It should be noted here that not only high mannose-type oligosaccharides derivatives such as Man₆,GlcNAc₂-IAc, but also GlcNAc₂-IAc was found to be a potent inhibitor of Png1. Although the affinity was not as strong as observed for Man₆,GlcNAc₂-IAc, the binding is still quite efficient. A GlcNAc₂-modified glycopeptide is generally not regarded as a good substrate for PNGase (9,34). However, GlcNAc₂ was previously found to be a competitive inhibitor of cytoplasmic PNGase (9), consistent with the current observation of its high affinity binding to Png1.

Thus far there is only one in vivo inhibitor, the tripeptide Z-VAD-fmk, available for the cytoplasmic PNGase. Z-VAD-fmk is also a widely used, broad spectrum caspase inhibitor (35), raising potential problems with respect to its specificity (27). Since the Man₆,GlcNAc₂-IAc did not have any effects on all caspases examined (caspase 2, 3 and 7), one would expect that the haloacetamidyl sugars, in sharp contrast to Z-VAD-fmk, could serve as highly selective inhibitors for cytoplasmic PNGases. Given that the CIAc-form of GlcNAc₂ is also a specific
inhibitor of Png1, this compound can be a good starting point for designing a specific \textit{in vivo} inhibitor for Png1, since the ClAc-form is generally much more stable than the IAc-form. It has been known that peracetylation of carbohydrate-related compounds renders them more permeable to cell membranes (36,37). Whether a peracetylated GlcNAc$_2$-ClAc compound can serve as a potent, \textit{in vivo} inhibitor of cytoplasmic PNGase is currently under investigation.

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FOOTNOTES

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The abbreviations used are: AMGN2, GlcNAc2-amidomethyl-; BrAc, bromoacetamido-; CAE, carbamidoethyl; CAM, carbamidomethyl, CBB, Coomassie Brilliant Blue R-250; CHO, carbohydrate; ClAc, Chloroacetamido-; Con A, Concanavalin A; ER, endoplasmic reticulum; ERAD, ER-associated degradation; HRP, horseradish peroxidase; IAc, iodoacetamido-; LC-ESI, liquid chromatography-electrospray ionization; MALDI-TOF, matrix assisted laser desorption/ionization-time of flight; Mes, 2-(N-morpholino)ethanesulfonic acid; MS, mass spectrometry, MS/MS. Tandem MS, PNGase, peptide:N-glycanase; WGA, wheat germ agglutinin; XAc, haloacetamidyl-.

LEGENDS FOR FIGURES

Figure 1: Structures of carbohydrate-related compounds used in this study.

Figure 2: Time dependency of inhibition of Png1 by iodoacetamide and Man9GlcNAc2-IAc. Png1 was incubated for the indicated times with these chemicals (50 μM) at 37°C before assaying the PNGase activity. Enzyme activities for incubation without Man9GlcNAc2-IAc at indicated time were set to 1.

Figure 3: Specific labeling of Png1 by Man9GlcNAc2-IAc. (A) 50 μM of Man9GlcNAc2-IAc was
incubated with Png1 and BSA (0.1 mg/ml each), and at the indicated times an aliquot was removed and analyzed by SDS-PAGE. (A) Con A blot analysis of the same samples as in (A). (C) Man$_9$GlcNAc$_2$-IAc (100 µM) was incubated with an E. coli extract expressing Png1. o/n; overnight incubation. (D) Con A blot analysis of the sample of 0 min and 30 min as in (C). (E) Effect of the chaotropic reagent, urea, on the labeling of Png1. Left: in the presence of 3.2 M urea; right: in the absence of urea. Different concentrations of Man$_9$GlcNAc$_2$-IAc were incubated with Png1 (0.1 mg/ml) and after a 10 min incubation the samples were analyzed by SDS-PAGE. Final concentration of Man$_n$GlcNAc$_2$-IAc; Lane 1: 1 mM; 2: 100 µM, 3: 5 µM; 4: 1 µM, 5: 200 nM; 6: 40 nM.

**Figure 4: Labeling of Png1 with various GlcNAc$_2$-derivatives.** (A) Png1 was incubated with 50 µM of GlcNAc$_2$-IAc (Lane 2) or Man$_n$GlcNAc$_2$-IAc (Lane 3) for 30 min and analyzed by SDS-PAGE. (B-D) 50 µM of GlcNAc$_2$-IAc (B), GlcNAc$_2$-BrAc (C), and GlcNAc$_2$-ClAc (D) were incubated with Png1 and analyzed by WGA-blotting. (E) The fraction of glycosylated Png1 obtained as in (A-D) was quantitated by CBB staining (Man$_n$GlcNAc$_2$-IAc and Man$_n$GlcNAc$_2$-IAc) or WGA staining (GlcNAc$_2$-IAc, GlcNAc$_2$-BrAc, and GlcNAc$_2$-ClAc). For the GlcNAc$_2$-series, the staining obtained after 30 min incubation, at which labeling was saturated, was set to 100.

**Figure 5: Reactivity of Man$_n$GlcNAc$_2$-IAc towards Png1 and mutant Png1(C191A).** (A) Png1 wild-type at a concentration of 0.1 mg/ml and Man$_n$GlcNAc$_2$-IAc (50 µM) was incubated at 37°C and at the indicated times aliquots were removed and analyzed by SDS-PAGE. Lane C: control (no incubation with Man$_n$GlcNAc$_2$-IAc). (B) Png1(C191A) at a concentration of 0.1 mg/ml was incubated with Man$_n$GlcNAc$_2$-IAc at 37°C and at the indicated times aliquots were analyzed by SDS-PAGE. o/n; overnight incubation.

**Figure 6: LC-ESI MS analysis of tryptic peptides obtained from GlcNAc$_2$-labeled Png1.** (A) Total ion chromatogram. (B) Extracted ion chromatogram around 1037.5, which is a calculated m/z value of peptide 191-203 where one of Cys is GlcNAc$_2$-amidomethyl Cys (C-AMGN2) and the other is Carbamidomethyl Cys (C-CAM). (C) Constant neutral loss of 202-204 m/z by auto MS/MS analysis suggesting occurrence of GlcNAc by MS/MS analysis.

**Figure 7 Sequence analysis of glycosylated peptide 191-203 by MS/MS analysis.** (A) Schematic representation of peptides obtained from fragmentations by MS/MS analysis. (B-D) Cys modification
observed in this experiment.  (B) GlcNAc₂-amidomethyl Cys (C-AMGN₂).  (C) Carbamidomethyl Cys (C-CAM), after loss of GlcNAc₂ from C-AMGN₂.  (D) Carbamidoethyl Cys (C-CAE) formed by alkylation with chloropropionamide.  (E-G) MS/MS sequence analysis of the peptide 191-203.  The sequence was determined based on the assumption that (1) Cys191 was the site for AMGN2 attachment (E), (2) Cys195 was the site for AMGN2 attachment (F), and (3) Cys195 was the site for attachment of AMGN2, but C-CAM was formed after the release of GlcNAc (G).  The major peaks labeled with asterisks in (E) are the doubly-charged ions of AMGN2-modified, intact tryptic peptide 191-203 from which a single GlcNAc (*) ; m/z 936.0) or GlcNAc₂ (**) ; m/z 834.5) are lost.

**Figure 8: Close up view of the molecular modeling of GlcNAc₂ (green) binding to Png1 in complex with Z-Val-AD-fmk (pink).**  Png1 coordinates with bound Z-Val-AD-fmk were taken from PDB entry 1X3Z (25).  Png1 is shown in a partially transparent surface representation with an underlying ribbon diagram.  Hydrogen bonds are shown as dashed lines in red and the active site cysteine is labeled in yellow.
Table 1. Effect of various GlcNAc₂-related compounds on Png1 activity.

| Compounds               | IC₅₀ (µM) |
|-------------------------|-----------|
| GlcNAc₂                 | > 1000    |
| GlcNAc₂-CIAc            | 19        |
| GlcNAc₂-BrAc            | 2.8       |
| GlcNAc₂-IAc             | 0.8       |
| Man₉GlcNAc₂             | 170       |
| Man₉GlcNAc₂-IAc         | 1.6       |
| Man₉GlcNAc₂-IAc         | 1.7       |

*IC₅₀, Concentration required for 50% inhibition.
Fig. 2 Suzuki et al.
Fig. 4 Suzuki et al.
