Detailed Studies on Substrate Structure Requirements of Glycoamidases A and F

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Glycoamidases (peptide-\(N^\alpha\)-(N-acetyl-\(\beta\)-glucosaminyl)-asparagine amidase, EC 3.5.1.52; also known as peptide: N-glycanases (PNGases) release \(N\)-linked oligosaccharides from glycopeptides and/or glycoproteins by hydrolyzing the glycosylated \(\beta\)-amide bond of the asparagine side chain. The most widely used glycoamidases are those from Flavobacterium meningosepticum (glycoamidase F or PNGase F) and almond emulsin (glycoamidase A or PNGase A). To study the substrate structure requirement of these enzymes systematically, we synthesized \(>30\) glycopeptides containing cellobiose, lactose, GlcNAc, and di-\(N,N^\prime\)-acetylchitobiose (CTB). The length of the peptide was varied from one to five amino acids, and glycosylamines were linked to either Asn or Gln located at different positions in the peptide, including \(N\)-linked oligosaccharides of the peptide was varied from one to five amino acids, \(N\)-glycosylation of glycoproteins in mammalian cells (13, 14).

Although the biological function of the enzyme is increasingly attracting attention, the most popular use of the enzyme currently remains to be \(N\)-glycosylation of glycoproteins/glycopeptides, most often for structural elucidation of glycoconjugates (15, 16). The commercially available glycoamidases are from sweet almond (glycoamidase A) (2) and Flavobacterium meningosepticum (glycoamidase F) (4). Both enzymes act on all three types of \(N\)-linked oligosaccharide chain, i.e. high mannose, hybrid, and complex types (17, 18). However, the \(N\)-glycoside chain with a Fuc\((\alpha1,3)\)GlcNAc-Asn segment, found in glycoproteins from plants and insects (19–22), could be liberated only by glycoamidase A, but not by glycoamidase F (23). It is also acknowledged that a certain length of peptide is needed for the enzyme activities of both enzymes. Recently, Altmann et al. (24) showed, by stepwise degradation of a biantennary glycopeptide with exoglycosidases, that the size of the carbohydrate moiety in the substrate has little influence on the enzyme activities of both glycoamidases and that the hydrolysis rates of both enzymes may be primarily determined by the length of the peptide. However, the glycopeptides used in this study contained at least three amino acids, and the glycopeptides with one or two amino acids were not studied.

To study the influence of the length and sequence of the peptide part on the glycoamidase activity, we synthesized four series of glycopeptides, with each series containing cellobiose, lactose, GlcNAc, or di-\(N,N^\prime\)-acetylchitobiose and containing one or two aromatic amino acids with the sugar attached to either Asn or Gln. The results obtained with these substrates are presented below.

**EXPERIMENTAL PROCEDURES**

**Materials**—Glycoamidase F (PNGase F) either was purchased from Glyko, Inc. (Novato, CA) or was a gift from Takara Shuzo Co. (Ohtu, Japan). Glycoamidase A was from Seikagaku Kogyo Co. (Tokyo, Japan). GlcNAc, lactose, and cellobiose were from Pfanstiehl Laboratories, Inc. (Waukegan, IL). Di-\(N,N^\prime\)-acetylchitobiose was prepared by a published method (25). All amino acid derivatives were from Advanced ChemTech (Louisville, KY). Man\(_9\)-GlcNAc\(_2\)-Asn-Phe and Man\(_9\)-GlcNAc\(_2\)-Asn were prepared from soybean agglutinin by Pronase digestion, followed by gel filtration on Sephadex G-50 and purification by HPLC\(^t\) on a graphitized carbon column (26). Tyr-Ile-(Man\(_9\)-GlcNAc\(_2\))-Asn-Ala-Ser-N\(_2\)-acetylglucosaminidase A using Man\(_9\)-GlcNAc\(_2\)-Asn as a donor and GlcNAc, lactose, and cellobiose were from Pfanstiehl Laboratories, Inc. (Waukegan, IL). Di-\(N,N^\prime\)-acetylchitobiose was prepared by a published method (25). All amino acid derivatives were from Advanced ChemTech (Louisville, KY). Man\(_9\)-GlcNAc\(_2\)-Asn-Phe and Man\(_9\)-GlcNAc\(_2\)-Asn were prepared from soybean agglutinin by Pronase digestion, followed by gel filtration on Sephadex G-50 and purification by HPLC\(^t\) on a graphitized carbon column (26). Tyr-Ile-(Man\(_9\)-GlcNAc\(_2\))-Asn-Ala-Ser-N\(_2\)-acetylglucosaminidase A using Man\(_9\)-GlcNAc\(_2\)-Asn as a donor and GlcNAc as an acceptor (27). All other reagents and solvents were of ACS grade.

\(^t\) All sugars used were of the \(\alpha\)-configuration.

\(^t\) The abbreviations used are: HPLC, high performance liquid chromatography; HPAEC-PAD, high performance anion-exchange chromatography with a pulsed amperometric detector; Boc, \(t\)-butoxycarbonyl; CTB, di-\(N,N^\prime\)-acetylchitobiose; Bzl, benzyloxycarbonyl; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; DMF, \(N,N\)-dimethylformamide.

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Substrate Requirements of Glycoamidases

General Methods—Unless otherwise specified, reactions were carried out at 22–24 °C. Organic layers from various extractions were dried over anhydrous sodium sulfate. Evaporation was performed with a rotary evaporator at 20–45 °C under diminished pressure. For thin-layer chromatography, Silica Gel 60 F254 (coated on an aluminum sheet, layer thickness of 0.25 mm; E. Merck AG, Darmstadt, Germany) was used. Amino Acid Analysis—The synthetic glycopeptides purified by HPLC were recorded with a Brucker WH 360 spectrometer using solutions in CDCl3 (for acetylated sugars) or Me2SO-d6 (for protected glycopeptides).

Enzymatic Reaction with Glycoamidases A and F—A mixture of 10 nmol of substrate and 0.2 millimolar of glycoamidase A or 0.5 millimolar of glycoamidase F in 15 μl of 0.1 mM ammonium acetate buffer (pH 5.0 for glycoamidase A) and pH 8.0 for glycoamidase F) was incubated at 37 °C for 18 h. The mixture was evaporated using a SpeedVac and redissolved in 200 μl of H2O. One-fourth of the sample was analyzed using the HPAEC-PAD system (see below), and the enzymatic reaction product was quantified by comparison of peak areas with standard compounds analyzed in a separate run. One enzyme unit is defined as the quantity of enzyme that releases 1 nmol of substrate and 0.2 milliunit of glycoamidase A or 0.5 milliunit of glycoamidase F in 1 hour.

Direct-Connect guard cartridge column (Alltech Associates Inc., Deerfield, IL) were used for separation depending on the hydrophobicity of the compounds. The eluent was 25 mM ammonium acetate buffer (pH 6.0) with a linear gradient of CH3CN (10–40% for ODS columns and 0–20% for the graphitized carbon column) developed in 40 min at 40 °C.

RESULTS

Synthesis of Cellulose- and Lactose-containing Glycopeptides

The synthetic strategy for cellulose glycopeptides is summarized in Scheme 1. The synthesized glycopeptides are listed in Table I. For celllobiose (32) by a one-pot reaction (29) and then converted to per-celllobiose azide (34) by reacting with NaN3. The azide was reduced to 35 by hydrogenation and subsequently condensed with Boc-O-Ala-Bzl to provide 36. The expected β-anomeric configuration of celllobiose linkage was confirmed by 1H NMR (J β-α = 7.8 Hz) in CDCl3. Compound 36 was de-O-benzylated and coupled with H-Ala-O-Bzl to give a dipptide derivative (37), which was de-O-benzylated and condensed with H-Ser-OMe to yield a celllobiose tridepide (38). De-O-acetylation of 36, 37, and 38 by the Zemplén procedure afforded 1, 2, and 3 in quantitative yields. Celllobiose (4) and lactose (8)

| Glycopeptide | Synthetic method | Carbohydrates | Structures of Peptide |
|--------------|-----------------|---------------|----------------------|
| 1            | A               | Cel′           | Boc-Asn-OMe          |
| 2            | A               | Cel′           | Boc-Asn-Ala-OMe      |
| 3            | A               | Cel′           | Boc-Asn-Ala-Ser-OMe  |
| 4            | B               | Cel′           | Boc-Tyr-Ile-Asn-Ala-Ser-OMe |
| 5            | A               | Lac            | Boc-Asn-OMe          |
| 6            | A               | Lac            | Boc-Asn-Ala-Ser-OMe  |
| 7            | A               | Lac            | Boc-Asn-Ala-Ser-OMe  |
| 8            | A               | Lac            | Boc-Tyr-Ile-Asn-Ala-Ser-OMe |
| 9            | A               | GlcNAc         |Cbz-Asn-OMe           |
| 10           | A               | GlcNAc         |Cbz-Asn-Ala-Ser-OMe   |
| 11           | A               | GlcNAc         |Cbz-Asn-Thr-OMe       |
| 12           | A               | GlcNAc         |Cbz-Asn-Ala-Gly-OMe   |
| 13           | B               | GlcNAc         |Boc-Tyr-Ile-Asn-Ala-Ser-OMe |
| 14           | A               | GlcNAc         |NH2-Asn-Ala-Ser-OMe   |
| 15           | A               | CTB            | Boc-Asn-OMe          |
| 16           | A               | CTB            | Boc-Asn-Ala-OMe      |
| 17           | A               | CTB            | Boc-Ile-Asn-OMe      |
| 18           | A               | CTB            | Boc-Phe-Asn-OMe      |
| 19           | A               | CTB            | Boc-Asn-Ala-Ser-OMe  |
| 20           | A               | CTB            | Boc-Asn-Thr-OMe      |
| 21           | A               | CTB            | Boc-Ala-Gly-OMe      |
| 22           | A               | CTB            | Boc-Tyr-Ile-Asn-Ala-Ser-OMe |
| 23           | A               | CTB            | Boc-Tyr-Ile-Asn-OMe  |
| 24           | A               | CTB            | Boc-Ile-Asn-Ala-OMe  |
| 25           | A               | CTB            | Boc-Tyr-Ile-Asn-Ala-Ser-OMe |
| 26           | A               | CTB            | Boc-Ile-Asn-Ala-Ser-OMe |
| 27           | A, B            | CTB            | Boc-Tyr-Ile-Asn-Ala-Ser-OMe |
| 28           | B               | CTB            | Boc-Tyr-Ile-Asn-Ala-Ser-OMe |
| 29           | A               | CTB            | Boc-Gln-OMe          |
| 30           | B               | CTB            | Boc-Gln-Ala-Ser-OMe  |
| 31           | B               | CTB            | Boc-Tyr-Ile-Gln-Ala-Ser-OMe |

a In Method A, a glycosylamine derivative was condensed with Boc-O-Ala-Bzl, followed by stepwise elongation of the peptide portion. In Method B, a glycosylamine was coupled to the preformed peptide.
b All carbohydrates are attached to the β-amide group of Asn or to the γ-amide group of Gln.
c Cel, celllobiose; Lac, lactose.
pentapeptides were prepared by Method B described below. Synthesis of lactose glycopeptides was based on the same strategy as shown in Scheme 1.

**Synthesis of GlcNAc- and CTB-containing Glycopeptides**

GlcNAc and CTB glycopeptides were synthesized by either of the following two procedures, as exemplified by the CTB glycopeptides.

Method A (Stepwise Elongation)—This synthetic strategy for CTB glycopeptides is summarized in Scheme 2. Di-N,N'-acetylchitobiose (39) was per-O-acetylated in pyridine, and then the isolated crystalline peracetate (40) was converted to glycosyl azide (42) via its oxazoline derivative (41) according to a published method (30). Compound 42 was reduced to chitobiosylamine (43) by hydrogenation and subsequently condensed with Boc-Asp-O-Bzl to provide 44. Compound 44 served as the building block for synthesis of various CTB glycopeptides. A stepwise elongation strategy by cycles of COOH- or NH2-terminal deprotection and condensation with the desired amino acids followed by de-O-acetylation with methanolic ammonia led to a series of CTB glycopeptides.

Method B (En Bloc Synthesis)—A simpler strategy was also used to synthesize diverse glycopeptides as described in Scheme 3. Cellobiose (32), lactose (49), GlcNAc (50), and CTB (39) were directly converted to the corresponding glycosylamines (31–33) by dissolving in a saturated NH4HCO3 solution and keeping at room temperature for 10 days, followed by repeated lyophilization to remove excess NH4HCO3. The peptide portion was synthesized by stepwise elongation as shown in Scheme 3. The β-O-Bzl of Asp was removed by hydrogenation to expose the β-carboxyl group for subsequent coupling with the glycosylamine. The condensation of peptide with glycosylamine using 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline as a condensing agent gave a >60% yield. The glycopeptides thus obtained were purified by gel filtration on a Sephadex G-10 column (1 x 60 cm) eluted with 20 mM ammonium acetate buffer (pH 6.0), followed by lyophilization to yield white powder.

**Identification of Synthetic Glycopeptides**

Glycopeptides with O-acetylated sugars were analyzed by 1H NMR analysis in Me2SO-d6. The signals were assigned by decoupling. Table II shows the chemical shifts of some of the glycopeptides. All H-1 signals were doublet (J1,2 ~ 8 Hz), sup-

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**Scheme 2.** Synthesis of CTB glycopeptides. Unless otherwise specified, GN and GN9 denote β-GlcNAc and β-GlcNAc(OAc)3, respectively. i: Ac2O, pyridine, 4 h; ii: trimethylsilyltrifluoromethanesulfonate, CH2Cl2, 50 °C, 5 h; iii: trimethylsilyl-N3, SnCl4, CH2Cl2, 24 h; iv: H2, palladium/carbon, CHCl3/ Ethanol (1:3), 6 h; v: 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline, CHCl3/Ethanol (10:1), 18 h; vi: a) H2, palladium/carbon, CHCl3/Ethanol (1:3), 4 h; b) DCC, HOBT, Et3N, DMF, 18 h; vii: NaOMe, MeOH, 2 h; viii: a) 4 M HCl/dioxane, dioxane, 0.5 h; and b) DCC, HOBT, Et3N, DMF, 18 h. Bn, benzyl.

**Scheme 3.** En bloc synthesis of glycopeptides. i: DCC, HOBT, Et3N, DMF, 18 h; ii: a) 4 M HCl/dioxane, dioxane, 0.5 h; b) DCC, HOBT, Et3N, DMF, 18 h; and iii: b) H2, palladium/carbon, 95% EtOH and 60% AcOH (2:1), 2 h. satn., saturation; r. t., room temperature; Cel, cellobiose; Lac, lactose; Bn, benzyl; EEDQ, 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline.

**Substrate Requirements of Glycoamidases**
porting the \( \beta \)-anomeric configuration of GlcNAc linked to Asn or Gln. The variation in the peptide portion did not significantly affect the chemical shifts of the carbohydrates. The de-O-acetylated glycopeptides were subjected to amino acid analysis after acid hydrolysis, and the results agreed well with the expected values.

**Action of Glycoamidases A and F on Synthetic Glycopeptides**

All the glycopeptides synthesized were tested as substrates under the conditions described under “Experimental Procedures” and also in Footnote a to Table III. Results are summarized in Table III. The following conclusions can be made from Table III.

1) Neither glycoamidase A nor F can hydrolyze any of the lactose and cellobiose glycopeptides. 2) When the carbohydrate is linked to a single amino acid (Asn or Gln), neither enzyme can hydrolyze such glycopeptides, even when both \( \alpha \)-NH\(_2\) and \( \alpha \)-COOH groups are masked. A natural glycopeptide containing only Asn (i.e., Man\(_9\)-GlcNAc\(_2\)-Asn) could not be hydrolyzed either. 3) Glycopeptides containing only a single GlcNAc can be cleaved by both enzymes, albeit not as effectively as CTB peptides. 4) The length and the nature of the peptide affect the hydrolysis by both enzymes. The CTB pentapeptide was totally hydrolyzed by both enzymes under the conditions described for Table III. However, the rate of hydrolysis of CTB dipeptides by glycoamidase A varied considerably, depending on the position and the type of amino acids. CTB dipeptides with Asn at the COOH terminus (17 and 18) were much poorer substrates than the dipeptides with Asn at the NH\(_2\) terminus (16 and 19). The contrast between 17 and 18 is especially striking; the reversal in the order of Asn and Phe caused the extent of hydrolysis to drop from 100% (Asn-Phe) to 9.2% (Phe-Asn). Among the glycotripeptides, the presence of Gly at the COOH terminus seemed to lower the hydrolysis rate. This is true for both enzymes and for both the GlcNAc peptide (12) and the CTB tripeptide (22). Both enzymes could hydrolyze Glc glycopeptides (Fig. 1C), but at diminished rates (compare 27 versus 31 and 20 versus 30).

The consensus sequence for N-glycosylation is not required by either enzyme (Fig. 1D). 6) Glycoamidase F tends to work more sluggishly than glycoamidase A (Fig. 2, compare A and B).

**Substrate Requirements of Glycoamidases**

| Residue | 36 | 38 | Boc-(Lac)-Asn-OMe | 44 | 46 |
|---------|----|----|------------------|----|----|
| H-1     | 5.14 | 5.15 | 5.16 | 4.85 | 4.84 |
| 2"      | 4.81 | 4.84 | 4.80 | 3.94 | 3.94 |
| 3       | 5.27 | 5.25 | 5.29 | 5.21 | 5.21 |
| 4       | 3.74 | 3.71 | 3.87 | 3.69 | 3.70 |
| 5       | 3.67 | 3.64 | 3.70 | 3.63 | 3.64 |
| 6a      | 4.45 | 4.43 | 4.42 | 4.39 | 4.39 |
| 6b      | 4.03 | 4.02 | 4.12 | 4.02 | 4.02 |
| H-1'    | 4.49 | 4.49 | 4.45 | 4.54 | 4.54 |
| 2"      | 4.93 | 4.84 | 5.10 | 3.86 | 3.86 |
| 3       | 5.14 | 5.14 | 4.94 | 5.02 | 5.03 |
| 4"      | 5.07 | 5.07 | 5.36 | 4.97 | 4.99 |
| 5"      | 3.69 | 3.69 | 3.87 | 3.64 | 3.65 |
| 6a"     | 4.38 | 4.38 | 4.17 | 4.39 | 4.39 |
| 6b"     | 4.11 | 4.11 | 4.06 | 4.28 | 4.28 |
| Asn\(_b\) | 4.56 | 4.45 | 4.57 | 4.57 | 4.46 |
| Ala\(_b\) | 4.38 | 4.38 | 4.39 |
| Ser\(_b\) | 4.62 | 4.65 |

*a* Lac, lactose.  
*b* \( \alpha \)-Proton of the amino acid.

**Activities of glycoamidases A and F on synthetic substrates**

| Substrate | Glycoamidase A | Glycoamidase F |
|-----------|----------------|----------------|
| 1–9       | ND*            | ND             |
| 10        | 39             | ND             |
| 11        | 38             | 4.2            |
| 12        | 31             | 3.3            |
| 13        | 100            | 3.3            |
| 14        | 48             | 1.8            |
| 15        | 6.4            | 0.9            |
| 16        | 8.2            | ND             |
| 17        | 100            | 34             |
| 18        | 100            | 34             |
| 19        | 100            | 34             |
| 20        | 100            | 83             |
| 21        | 3.8            | 0.4            |
| 22        | 43             | 17             |
| 23        | 76             | 16             |
| 24        | 88             | 20             |
| 25        | 100            | 76             |
| 26        | 98             | 76             |
| 27        | 100            | 57             |
| 28        | 44             | 1.1            |
| 29        | 21             | 1.5            |
| 30        | ND             | ND             |
| 31        | Man\(_\alpha\)GlcNAc\(_2\)-Asn-Phe | 100 |
| 32        | Man\(_\alpha\)GlcNAc\(_2\)-Asn-Ala-Ser-NH\(_2\) | 100 |
| 33        | Tyr-Ile-Man\(_\alpha\)GlcNAc\(_2\)-Asn-Ala-Ser-NH\(_2\) | 100 |

\( ^a \) The enzymatic reaction was carried out with 10 nmol of substrate and 0.2 milliunit of glycoamidase A or 0.5 milliunit of glycoamidase F in 15 \( \mu \)l of 0.1 m \( \alpha \)-methylmannoside 0.5 \( \ell \) buffer (pH 5.0 for glycoamidase A and pH 8.0 for glycoamidase F). The mixture was incubated at 37 °C for 18 h and analyzed by HPAEC.

\( ^b \) ND, not detectable.

**Kinetic Constants of Glycoamidase A Digestion of Boc-(CTB)-Asn-Ala-Ser-OMe (20) as Substrate**

Boc-(CTB)-Asn-Ala-Ser-OMe (20) was used as substrate for the kinetic study of glycoamidase A. The initial rate (\( v \)) was determined by HPAEC analysis. The \( K_m \) and \( V_{max} \) values of the enzyme were 2.1 ms and 0.66 \( \mu \)mol/min/mg, respectively (from the Lineweaver-Burk plot shown in Fig. 3).

**Hydrolysis of a Natural Glycodepide, Man\(_\alpha\)GlcNAc\(_2\)-Asn-Phe, by Glycoamidase A**

Glycoamidase A could totally hydrolyze a glycopeptide, Man\(_\alpha\)GlcNAc\(_2\)-Asn-Phe, derived from soybean agglutinin. As shown in Fig. 4, the substrate disappeared completely after incubation of the enzyme with substrate at 37 °C for 18 h, and a new peak eluted at the position corresponding to Man\(_\alpha\)GlcNAc\(_2\), clearly indicating that the substrate was digested by glycoamidase A. However, glycoamidase F failed to hydrolyze the same substrate (data not shown).

**DISCUSSION**

**Synthesis of Glycopeptides**—We have chemically synthesized a number of glycopeptides to systematically study the substrate requirement of glycoamidases A and F. The chemical construction of the glycosylamine–\( \beta \)-l-aspartyl bond has been achieved with protected sugars and protected Asp by various methods (34–37), and the Asp was further extended to glycopeptides (38, 39). When we, as well as others (40, 41), followed methods (34–37), and the Asp was further extended to glycoamidases A and F, respectively. Hydrolysis of the GlcNAc peptide (13) by glycoamidase F was considerably slower (3.3%) than that of the corresponding CTB peptide (27) (98%). 4) The length and the nature of the peptide affect the hydrolysis by both enzymes. The CTB pentapeptide (27) was totally hydrolyzed by both enzymes under the conditions described for Table III. However, the rate of hydrolysis of CTB dipeptides by glycoamidase A varied considerably, depending on the position and the type of amino acids. CTB dipeptides with Asn at the COOH terminus (17 and 18) were much poorer substrates than the dipeptides with Asn at the NH\(_2\) terminus (16 and 19). The contrast between 17 and 18 is especially striking; the reversal in the order of Asn and Phe caused the extent of hydrolysis to drop from 100% (Asn-Phe) to 9.2% (Phe-Asn). Among the glycotripeptides, the presence of Gly at the COOH terminus seems to lower the hydrolysis rate. This is true for both enzymes and for both the GlcNAc peptide (12) and the CTB tripeptide (22). Both enzymes could hydrolyze Glc glycopeptides (Fig. 1C), but at diminished rates (compare 27 versus 31 and 20 versus 30).

5) The consensus sequence for N-glycosylation is not required by either enzyme (Fig. 1D). 6) Glycoamidase F tends to work more sluggishly than glycoamidase A (Fig. 2, compare A and B).
acetamido group is required by the enzymes. Neither glycoamidase A nor F could act on any cellobiose and lactose glycopeptides. Since both enzymes released GlcNAc from GlcNAc-containing glycopeptides (e.g. 13), the resistance of cellobiose and lactose glycopeptides to the glycoamidases clearly indicates that at least the 2-acetamido group of the GlcNAc linked to Asn is involved in the recognition by the enzymes. Our data agree well with the results of a recent crystallographic analysis (44), which showed that the N-acetyl group of the Asn-linked GlcNAc is wrapped into the glycoamidase F molecule and makes contact with Asp-60, the purported primary catalytic residue of glycoamidase F.

The glycopeptides with carbohydrate linked to the γ-amide nitrogen of Gln instead of the β-amide nitrogen of Asn could also be hydrolyzed by glycoamidase A, with a slower hydrolytic rate. This indicates that glycoamidase A is not sensitive to the distance between the carbohydrate and the peptide backbone.

Oligosaccharide Chain Length—It has been reported that glycoamidase A is capable of releasing GlcNAc from peptides containing a single GlcNAc (17, 24), although at 15–3000 times slower rates than the corresponding glycopeptides with larger glycans, but glycoamidase F requires at least the di-N,N9-acetylchitobiosyl core unit because the enzyme is unable to cleave the GlcNAc–Asn bond in ribonuclease B or external invertase treated with endo-β-N-acetylglucosaminidase H (45). In agreement with the published results, we found that glycoamidase A could hydrolyze all of the synthetic peptides containing only GlcNAc if the peptide was larger than a tripeptide, although at rates slower than those of glycoamidase F. Interestingly, glycoamidase F failed to act on Cbz-(GlcNAc)-Asn-Ala-Thr-OMe (10), which was a fair
Substrate Requirements of Glycoamidases

A Thr at the C2+ position of Asn (21) improved the glycoamidase F activity compared with that of the Ser-containing glycotripeptide (20). Likewise, Cbz-(GlcNAc)-Asn-Ala-Thr-OMe (11) was cleaved by glycoamidase F (Fig. 1B), but Cbz-(GlcNAc)-Asn-Ala-Ser-OMe (10) was not. Interestingly, this difference was not observed with glycoamidase A. The glycotripeptide with a glycosyl-Asn at the COOH terminus (e.g. 23) is a poorer substrate for both enzymes than those in which the glycoside chain is located at the NH2 terminus (e.g. 20 and 21). Extending one amino acid at the COOH terminus (25) did not yield significant improvement in glycoamidase F activity, but additional sequence resulted in a significant increase in the hydrolytic rate. Thus, having a dipeptide sequence at the COOH-terminal side of Asn seems to be important for glycoamidase F activity.

Neither enzyme requires a strict Asn-Xaa-(Ser/Thr) sequence needed for natural glycosylation because both enzymes could release CTB from Boc-Tyr-Ile-(CTB)-Asn-Ala-Gly-OMe (28). The weak activities of both enzymes for Boc-(CTB)-Asn-Ala-Gly-OMe (22), on the other hand, may suggest that Gly plays an inhibitory role because the glycoamidase A reactivity was reduced from 48% for Boc-(CTB)-Asn-Ala-OMe (16) to 3.8% by adding a Gly at the COOH terminus.

Kinetic Constants—The kinetic constants of glycoamidases A and F for various glycopeptides have been investigated by several groups (1, 3, 24, 46, 47). The $K_m$ value of glycoamidase A was reported to be 4 mM for a glycopentapeptide with a heptaoligosaccharide from pineapple stem bromelain (3) and 1.1 mM for the fetuin tryptic glycopeptide derived from the Asn-138 site (1). We found that the $K_m$ value of glycoamidase A for Boc-(CTB)-Asn-Ala-Ser-OMe is 2.1 mM, which is comparable with the literature values for naturally occurring glycopeptides, again indicating that CTB is as effective as larger oligosaccharides and that a glycotripeptide is as effective as larger glycopeptides for glycoamidase A.

Conclusion—Our results revealed finer structural requirements for glycoamidases A and F, especially for di- or tripeptides. A tripeptide or a tetrapeptide is required for full expression of the activity of glycoamidase or F. The Asn-Xaa-(Ser/Thr) sequence is not mandatory, and Asn can be replaced with Gln.

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A. Length of the Peptide Chain—The structural requirement on the oligosaccharide chains for the hydrolysis by glycoamidases was studied previously by trimming the oligosaccharide chain with various exoglycosidases (24, 45). Only small differences were noted between the di-$N$-$N'$.acyctethylchitobiose and a larger sugar chain, and it was concluded that the hydrolytic rates are controlled more by the peptide portion than by the carbohydrate portion. In this study, we tried to determine the minimum peptide requirement of the two enzymes by using a series of synthetic glycopeptides with di-$N$-$N'$.acyctethylchitobiose.

Neither enzyme could release sugars from GlcNAc (9), CTB (15 and 29), or Man$_9$GlcNAc$_2$ bound to Asn or Gln, even when both ends were protected, thus agreeing with published results (1). The minimum peptide length for glycoamidase A seems to be a dipeptide; Man$_9$GlcNAc$_2$-Asn-Phe as well as all end-protected CTB dipeptides tested (16, 17, 18, and 19) were hydrolyzed by the enzyme, although the rate of hydrolysis varied among the peptides of different sequences. Glycoamidase A seems to prefer those substrates with glycosides at the NH$_2$ terminus (16 and 19) to those with them at the COOH terminus (17 and 18). The fact that Man$_9$GlcNAc$_2$-Asn-Phe could be totally cleaved by glycoamidase A (Fig. 4) suggests that the protection of NH$_2$ and COOH termini was not necessary. This differs from the early claim that glycoamidase A can only act very slowly on the glycopeptide when the glycosylated Asn is at the NH$_2$ or COOH-terminal position (6, 17). In contrast, among the glycopeptides tested, only Boc-(CTB)-Asn-Ala-OMe (16) and Boc-Ile-(CTB)-Asn-OMe (17) could be hydrolyzed by glycoamidase F, but at extremely slow rates (1.8 and 0.9% with 16 and 17, respectively). The hydrolytic rates with glycotripeptides were significantly improved over those with glycopeptides, suggesting that glycoamidase F requires at least a tripeptide for the activity.

![Fig. 4. Release of Man$_9$GlcNAc$_2$ from Man$_9$GlcNAc$_2$-Asn-Phe with glycoamidase A. The enzymatic reaction was carried out with 3 nmol of Man$_9$GlcNAc$_2$-Asn-Phe and 0.2 milliunit of glycoamidase A in 25 mM NH$_4$OAc buffer (pH 5.0) at 37°C for 18 h. After evaporating the buffer with a SpeedVac, the sample was applied to a PA-1 column for HPAEC-PAD analysis. The eluent was 100 mM NaOH with a linear gradient of NaOAc (0–100 mM) developed in 30 min. A, substrate (Man$_9$GlcNAc$_2$-Asn-Phe) only; B, after the enzymatic reaction.](image-url)
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