Transfer of Glycerol by Endo-β-N-acetylgalcosaminidase F to Oligosaccharides during Chitobiose Core Cleavage*

(Received for publication, April 25, 1986)

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N-Linked oligosaccharides, when hydrolyzed by glycosidase-containing preparations of endo-β-N-acetylgalcosaminidase (Endo F) from Flavobacterium meningosepticum were found to have glycerol attached to their reducing ends. The absence of a reducing end was confirmed by high-field 1H NMR spectroscopy, and the incorporated glycerol was verified through mass spectrometry and collisionally activated decomposition fast atom bombardment mass spectrometry/mass spectrometry techniques. Periodate oxidation of [1(3)-14C]glycerol-labeled oligosaccharides indicated glycerol was glycosidically linked via its 1(3) carbon to the C1 of the reducing end N-acetylgalcosamine. In a second, less favored reaction, the glycerol glycoside was hydrolyzed by Endo F using water as the terminal nucleophile, thus regenerating the N-acetylgalcosamine reducing end. Glycerol could be removed from Endo F preparations without affecting enzyme stability, and chitobiol core hydrolysis in its absence provided intact oligosaccharides with normal N-acetylgalcosamine reducing ends. The incorporation of labeled glycerol may provide a useful method for monitoring of Endo F release of oligosaccharides.

Endo F§ prepared from cultural filtrates of Flavobacterium meningosepticum by the method of Elder and Alexander (1) is a mixture of two endoglycosidases: Endo F and peptide-N'-N-(N-acetyl-β-glucosaminyl) asparagine amidase (EC 3.5.1.52) (PNGase F) or N-GLYCANASE (2, 3). Endo F hydrolyzes the di-N-acetylated core of high mannose and biantennary complex oligosaccharides leaving one N-acetylgalcosamine attached to asparagine and one to the reducing end of the released oligosaccharide. In contrast, PNGase F hydrolyzes the amide bond between asparagine and the di-N-acetylated core of both high mannose and complex oligosaccharides to yield a peptide-bound aspartic acid and a liberated 1-aminooligosaccharide. The subsequent nonenzymatic release of NH2 yields N-acetylgalcosamine at the reducing end of the intact oligosaccharide.

During studies on the substrate specificity of Endo F preparations, we observed that, depending on the reaction conditions and the glycopeptide hydrolyzed, some or all of the released oligosaccharides failed to incorporate tritium on reduction with alkali NaBH4. The work reported in this paper provides an explanation for this effect by demonstrating that glycerol, a stabilizing component added to some commercial Endo F preparations, becomes attached to the C1 carbon of the reducing end GlcNAc during oligosaccharide hydrolysis. These results appear to explain the observation by other investigators (4) that Endo F-released oligosaccharides from a variety of sources have no reducing end. This finding may prove useful for the single-step labeling of oligosaccharides during endoglycosidic release from glycopeptides and glycoproteins.

EXPERIMENTAL PROCEDURES

Materials

Endo F from F. meningosepticum (3) and cloned Streptomyces plicatus Endo H (5) were purified to homogeneity as described. A partially purified sample of the Endo F mixture in 50% glycerol was kindly provided by Drs. Elder and Alexander (1). [1(3)-14C]Glycerol (30 mCi/mmol) was purchased from Amersham, and [5-1H]dimethylamino-1-naphthalenesulfonfonyl (dansyl) chloride (21.3 Ci/mmol) and NaB3H4 (528 mCi/mmol) were obtained from New England Nuclear. Unlabeled dansyl chloride and NaBH4 were from Sigma. Ovalbumins asparagine-oligosaccharides with the composition AsnGlcNAc(5)Man3 (peak E) and AsnGlcNAcMan4 (peak C) were purified as described (6). D2O was from Sigma (99.8% and 99.96% low in paramagnetics) and Stohler Isotope Chemicals, Waltham, MA (99.99%). NMR tubes (5 mm inside diameter, #528FP) were from Wilmet Glass Co., Buena, NJ.

Methods

Endoglycosidase Digestions—Hydrolysis of Asn-oligosaccharides was carried out at 25 °C or 37 °C as indicated using Endo F or H at concentrations listed in the legends to the figures and tables. The buffered was 50 mM sodium acetate, pH 5.5. One milliunit of Endo F or H is defined as that amount of enzyme which will hydrolyze 0.5 mm ManGlcNAcAsn-dansyl at 1 mmol/min at 27 °C and pH 5.5.

Compositional Analyses—The content of mannose in oligosaccharides was determined by a scaled-down version (7) of the phenolsulfuric acid assay (8) using mannose as a standard. N-Acetylgalcosamine was determined as glucosamine on the amino acid analyzer (9). Mass spectrometry of glycopeptides was carried out on a Micromass ZAB-2F at the Department of Biochemistry, University of Nebraska, Lincoln, and at the Midwest Center for Mass Spectrometry, University of Nebraska, Lincoln.

* Some abbreviations used are: Endo, endo-β-N-acetylgalcosaminidase, dansyl, 5-dimethylamino-1-naphthalenesulfonyl; dimedon, 5,5-dimethyl-1,3-cyclohexanedione; MS-MS, tandem mass spectrometry.

† This work was supported in part by United States Public Health Service-National Institutes of Health Grants GM23900 (to R.B.T.), CA13402 (to P.H.A.), GM22816 (to F.M.), GM30471 (to A.L.T. and R.B.T.).

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This enzyme was originally given the trivial name peptide/N-glycosidase F. The IUB nomenclature is now being used with the abbreviation PNGase F.

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present as an internal standard. Asparagine was determined on the glucosamine and aspartic acid was greater than 96%.

**Chromatography—**Oligosaccharide products of endoglycosidase digestions were resolved on a calibrated Bio-Gel P-4 (~400 mesh) column (90 × 195 cm) at room temperature with an eluant of 0.1 N acetic acid in 1% 1-butanol; 0.5-ml fractions were collected at a flow rate of 7 ml/h. Paper chromatography for the resolution of cleavage products from dansylated oligosaccharides was performed ascendingly on Whatman No. 3MM paper using 1-butanol:ethanol:water (2:1:1) as the solvent.

**Detection Procedures—**Ann-oligosaccharides were dansylated as described (10) and desalted on a Sephadex G-10 column (0.9 × 100 cm) using a UV lamp (360 nm). Mannose was located in column profiles by paper chromatograms with a UV lamp (360 nm). Asparagine moieties of Asn-oligosaccharides was located in column profiles as the fluorescent product after reacting with fluorescamine (11). Radioactivity was eluted from excised paper chromatography spots with 1 ml of H2O for 1 h at 37 °C in 20-ml scintillation vials, after which 10 ml of Beckman HP/B scintillation fluid was added. Radioactivity (disintegrations/min) was quantitated on a Beckman LS7500 spectrometer equipped with a dual label (3H/14C) data reduction program.

**'H NMR—**Oligosaccharides were exchanged twice by rotary evaporation with 99.8% D2O, once by lyophilization from 99.96% D2O, and stored over P2O5 for several days. Samples were dissolved at a concentration of 1–2 mM in 99.996% D2O, and spectra were recorded at 298 K using the 500 MHz spectrometer at the Northeast Regional NSF-NMR Facility at Yale University. Chemical shifts were compared to equimolar acetone added as an internal marker (2.225 ppm compared to 4.4-dimethyl-4-silapentanesulfonate). Parameters were as described previously (7).

**Fast Atom Bombardment/MS—**All mass spectra were obtained on a Kratos (Manchester, UK; MS-50 Triple Analyzer (12), which consists of a high resolution MS-I, with Nier-Johnson forward geometry, and an electrostatic analyzer as MS-II. The fast atom bombardment source is of standard Kratos design equipped with an Ion Tech saddle field atom gun (Teddington, UK). Samples (1–5 μg) in methanol (1 μl) were added to 1 μl of the matrix (dithiothreitol/dithioerythritol for positive ions and triethanolamine for negative ions) on the copper probe tip of a direct insertion probe. Bombardment of the sample with 6–8-keV xenon atoms desorbs the preformed ions. The collisionally activated decomposition MS-MS spectra were obtained using standard Kratos software. The accurate mass measurements were provided by the National Institute of Standards and Technology source is of standard Kratos design equipped with an Ion Tech saddle field atom gun (Teddington, UK). Samples (1–5 μg) in methanol (1 μl) were added to 1 μl of the matrix (dithiothreitol/dithioerythritol for positive ions and triethanolamine for negative ions) on the copper probe tip of a direct insertion probe. Bombardment of the sample with 6–8-keV xenon atoms desorbs the preformed ions. The collisionally activated decomposition MS-MS spectra were obtained using standard Kratos software. The accurate mass measurements were determined by peak matching with glycerol standard at a resolution of 10,000.

**Periodate Oxidation and CH2O Precipitation—**Approximately 0.6 nmol of oligosaccharide containing 4305 dpm of [1,3-14C]glycerol was oxidized in the dark overnight at 4 °C with 10 μmol of sodium periodate in a final volume of 0.25 ml. A comparable sample was included which did not contain periodate. Following the oxidation, 0.3 ml of 50 mM sodium acetate, pH 4.5, was added to each sample plus 0.2 ml of 0.1 M formaldehyde and 0.3 ml of 0.4 M dimedone. The tubes were boiled for 5 min and cooled on ice. After centrifugation, the resulting precipitates were extracted into 1 ml of toluene, and duplicate 0.5-ml aliquots were taken for radioactivity measurements.

**RESULTS AND DISCUSSION**

During preliminary experiments, we observed that oligosaccharides released from Man3GlcNAc2Asn by Endo F were not reducible with NaBH4. Based on our knowledge of the activity of Endo H (10, 13), this was a highly unexpected result and suggested, at first, a possible difference in the mechanism of hydrolysis by the two endoglycosidases. Detailed comparative studies were conducted using the homogeneous asparagine-oligosaccharide, Man4GlcNAc2Asn, in order to simplify product identification.

As expected (10), Endo H quantitatively hydrolyzed Man4GlcNAc2Asn to Man3GlcNAc-Asn (peak Ia) and AsnGlcNAc (peak IIa) (Fig. 1A). However, the profile of the reaction products from hydrolysis of Man3GlcNAc2Asn by Endo F was clearly different. As shown in Fig. 1B, it contained about 30% unhydrolyzed Man3GlcNAc2Asn (peak Ib), the expected products Man3GlcNAc-Asn (peak IIIB) and AsnGlcNAc (peak IVB), and, unexpectedly, a new peak (peak IIb) in a position where oligosaccharides with the composition of Man6GlcNAc and Man4GlcNAc would elute. However, since the parent oligosaccharide could not yield Man6GlcNAc and Man4GlcNAc cannot be formed by the PNGase F activity in Endo F (2, 3), we concluded that a new oligosaccharide of larger mass had been generated during the initial cleavage.

To investigate this species further, the content of Asn, GlcNAc, and Man in the four peaks in Fig. 1B was determined (Table I). Peaks Ib, IIb, and IVb provided the expected molar ratios of Asn:GlcNAc:Man, while peak IIb contained GlcNAc:Man in a 1.5 ratio. The sum of Asn in peaks Ib and IVb, of mannose in peaks Ib–IIb, and of glucosamine in peaks Ib–IVb accounted for all of the starting material. Peaks Ib and IIb, which exhibited the same Man/GlcNAc ratio, but...
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| Table I | Molar ratios of asparagine, GlcNAc, and Man in peaks Ib–IVb from Fig. 1 |
|---------|---------------------------------------------------------------------|
| Peak    | Asn:GlcNAc:Man             |
| Ib      | 1.0   | 2.0   | 4.9   |
| IIb     | 1.0   | 5.1   |
| IIIb    | 1.0   | 5.0   |
| IVb     | 1.0   | 1.0   |

Fig. 2. 500 MHz $^1$H NMR spectrum of peak IIIb compared to that of the unknown peak IIb from Fig. 1B. About 0.8 $\mu$mol of each oligosaccharide was exchanged with D$_2$O and its 500 MHz $^1$H NMR spectrum was recorded at 25 °C. Panel A displays the C1-H anomeric proton resonance signature characteristic of the Man$_3$GlcNAc structure shown (13). Each proton is keyed by number to its respective chemical shift signal. Panel B shows C1-H proton chemical shifts identical with those of Man$_5$GlcNAc, with the exception that the $\alpha/\beta$ conformation of the reducing end GlcNAc C1-H (resonances 1 and 2) are missing. A new proton resonance appears at 4.539 ppm, which may represent the C1-H of the glucosamine. A second difference in the two spectra relates to the absence in Panel B of the split resonance at 5.098/5.077 ppm seen in Panel A, which is indicative of mannose 7 folding back and interacting with the GlcNAc reducing end (7, 13).

eluted with different sizes on the Bio-Gel P-4 column (Fig. 1), were treated with NaB$^3$H, to test for reducing ends. Peak IIb failed to incorporate label, while peak IIIb (Man$_5$GlcNAc) was quantitatively reduced to Man$_5$GlcNAc-[$^3$H]ol as anticipated (data not shown).

$^1$H NMR Studies on Peak IIb and IIIb Oligosaccharides—Peak IIb and IIIb oligosaccharides were exchanged with D$_2$O and examined by 500 MHz $^1$H NMR spectroscopy to identify peak IIb through the resonance signatures of the C1 anomeric protons. Fig. 2A shows the anomeric proton region of the spectrum for peak IIb, which reveals the C1-H chemical shifts characteristic of the Man$_5$GlcNAc structure depicted (14). Fig. 2B shows the spectrum of the oligosaccharide in peak IIb, which is lacking resonances 1 and 2 seen in Fig. 2A. These are the reporter resonances for the $\alpha/\beta$ conformation of the reducing end N-acetylglucosamine C1-H which is normally formed on hydrolysis of the core GlcNAc$\beta$1,4GlcNAc linkage by either Endo F or Endo H. This proton appears in Fig. 2B at 4.539 ppm, which represents an upfield shift of 0.176 ppm from its usual position at 4.715 ppm in unhydrolyzed Man$_5$GlcNAcAsn (14). This suggests that the GlcNAc on the Endo F-released oligosaccharide is still in a glycosidic linkage.

Further NMR evidence for an aberrant C1 terminus on the peak IIb oligosaccharide is the failure of the upper arm $\alpha$-linked mannose (residue 7) to fold back and interact with the core GlcNAc in a manner producing the split ($\alpha/\beta$) resonance normally seen for the C1-H at 5.098/5.077 ppm (7, 14). This is reflected in the C2-H of residue 7 as well, by the loss in anomericity at 4.061 ppm. The C2-H on the $\beta$-linked core mannose (residue 3) at 4.257 ppm also shows a loss of anomericity on comparing the Endo H and Endo F products (Figs. 2, A and B). MS Studies on Peaks IIb and IIIb—The larger size on Bio-Gel P-4 of the oligosaccharide in peak IIb relative to that in peak IIIb (Fig. 1), coupled with its failure to display a reducing end either by NaB$^3$H$_4$ reduction or by $^1$H NMR (Fig. 2B), suggested that a glycosidically linked blocking group had been introduced during enzyme cleavage. To analyze for the presence of a blocking group, peak IIb and IIIb oligosaccharides were compared by mass spectrometric techniques. Fig. 3 shows a portion of the spectra for Man$_5$GlcNAc (peak IIIb) which gives the expected parent $m/z$ ions at (M + $^2$H)$^+$ = 1032 (Panel A) and (M − $^2$H)$^-$ = 1030 (Panel B). Panels C and D show comparable spectra for the peak IIb oligosaccharide where the parent ions are seen at $m/z$ of (M + $^2$H)$^+$ = 1106 and (M − $^2$H)$^-$ = 1104, indicating that the peak IIb oligosaccharide is larger than Man$_5$GlcNAc by C$_3$H$_6$O$_2$ (74 Da).

The collisionally activated decomposition spectrum of the (M + $^2$H)$^+$ ions from each oligosaccharide was obtained. In Fig. 4A, the collisionally activated decomposition spectrum of Man$_5$GlcNAc (peak IIIb) reveals a series of sequence ions showing the loss of all 5 mannose residues. In contrast, the collisionally activated decomposition spectrum of peak IIb oligosaccharide reveals an intense loss of 92 Da, then two series of sequence ions: one equivalent to those from Man$_5$GlcNAc (Panel A) and one 92 Da higher. Peak matching determines the accurate mass to be 1106.4006 (C$_{58}$H$_{82}$O$_{30}$), calculated = 1106.3987. These results are consistent with glycerol (C$_3$H$_6$O$_3$) replacing the OH moiety on the reducing end of the peak IIb oligosaccharide.

The presence of glycerol on the oligosaccharide's reducing end was surprising, but was consistent with the available data: a glycerol residue would add mass and would be liberated during acid hydrolysis, but it would be detected neither by amino acid analysis nor by the phenol-sulfuric acid assay (Table I). The available glycerol appeared to be that added to the Endo F mixture as a stabilizing agent.

$^{13}$C/Glycerol Incorporation Studies—To show that glycerol was being incorporated into the oligosaccharide from the reaction mixture, [1(3)-$^{13}$C]glycerol was added to a comparable Endo F incubation and the radiolabeled peak IIb oligosaccharide was isolated on Bio-Gel P-4 as in Fig. 1 (data not shown). The specific activity of the oligosaccharide, based on 5 mannoses, was equal to that of the input labeled glycerol (6000 dpm/nmol) indicating that each oligosaccharide had incorporated one glycerol. Since glycerol could be incorporated via a C1(3) or a C2 linkage, periodate oxidation was used to distinguish between the two possible linkages depicted in Scheme I.
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**Fig. 3.** Positive and negative ion mass spectra of peaks IIIb and IIb from Fig. 1B. Positive ion spectra were generated using a dithiothreitol/dithioerythritol matrix (panels A and C), while negative ion spectra were generated with triethanolamine as the matrix (panels B and D). The spectra show that the peak IIb oligosaccharide is Man5GlcNAc with an added mass of 74 Da (C3H6O3), which is consistent with glycerol replacing OH on the GlcNAc reducing end.

**Scheme I**

In case A, periodate oxidation between C1(3) and C2 of the glycerol should release 1 mol of labeled formaldehyde, while none should result on oxidation of the glycerol in case B because of the absence of vicinal hydroxyl radicals. Labeled peak IIb-oligosaccharide was treated overnight in the dark at 4 °C with periodate, and unlabeled CH2O was added to the reaction as carrier. Precipitation of the formaldehyde with dimedon revealed 48% of the label to be in the complex, indicating that glycerol was forming a glycosidic linkage with N-acetylglucosamine via its C1(3) hydroxyl (Scheme I, A).

Because the Endo F was known not to be pure, it was important to show that glycerol incorporation occurred during Endo F cleavage of the oligosaccharide's chitobiosyl core, and not by subsequent addition to free Man5GlcNAc by a contaminating enzyme activity distinct from Endo F. That this was not the case was shown by incubating the partially purified Endo F mixture with Man5GlcNAc and [1(3)-14C]glycerol under the same hydrolysis conditions described in Fig. 1. Chromatography of the products revealed no glycerol incorporation and that the Man5GlcNAc was unaltered.

To show that the glycerol-oligosaccharide was due to Endo F, a highly purified preparation of enzyme, which provided a single band on SDS gels and was free of detectable PNGase F activity, was used to hydrolyze both Man5GlcNAc5Asn and
Additional experiments have been performed in an effort to better understand the parameters governing the Endo F-directed incorporation of glycerol at the reducing end of released oligosaccharides. The range of glycerol concentrations tested (1% v/v to 15% v/v) has little effect on the proportion of blocked ends, although at higher levels of glycerol (10–15% v/v) the hydrolysis reaction is slowed somewhat (20%). In the absence of glycerol, hydrolysis produces oligosaccharides with normal reducing C1 termini. Since some commercial Endo F preparations are packaged in 50% (v/v) glycerol, a 1:50 dilution (=1% v/v = 1.26% w/v = 0.14 M) is sufficient to produce a high proportion of blocked ends. In fact, the experiment in Fig. 1 contained only 1% (v/v) glycerol.

Clearly, there are two potential problems introduced by the addition of glycerol to the oligosaccharides. The first is the obvious loss of a reducing end. The second is the aberrant increase in apparent size of the oligosaccharide by an amount equal to two mannoses or one N-acetylglucosamine, which would affect the proper evaluation and characterization of metabolically labeled products. Endo F does not require glycerol for stability, and preparations stored for several months at 4 °C retain full activity in its absence. Thus, glycerol may be removed from commercial preparations of Endo F mixtures without affecting the enzyme.

The two most significant factors in generating glycerol-blocked ends appear to be the level of Endo F added and the structure of the oligosaccharide being used as substrate. Oligosaccharides which are poorer substrates for Endo F (i.e., Man6GlcNAcAsn relative to Man5GlcNAcAsn) tend to retain a higher level of glycerol-blocked ends. Lowering the amount of Endo F greatly favors the retention of glycerol by the oligosaccharides. It is not clear yet what the reaction mechanism is, but one possibility is that Endo F incorporates glycerol to the oligosaccharide in a transglycosidase type of
reaction during displacement of the AsnGlcNAc. Subsequently, in a less favored reaction, Endo F acts as a hydrolase to remove the glycosidically linked glycerol from the oligosaccharide employing water as a terminal nucleophile. Since the rate of regeneration of the reducing end depends on the level of Endo F and the oligosaccharide, the kinetics of glycerol removal would have to be determined empirically for each set of conditions.

The off-rate for glycerol implied in the experiment in Fig. 5 could be an underestimate because Endo F may continually recycle glycerol onto the oligosaccharide ends in competition with its hydrolysis by water. To test this possibility, 40 nmol of Man$_6$GlcNAc$_2$Asn was hydrolyzed with purified Endo F in the presence of [3H]glycerol as in Fig. 5, and the Man$_6$GlcNAc$_2$[3H]glycerol was isolated on Bio-Gel P-4.

Parallel reactions containing labeled oligosaccharide and Endo F, ±1.1 M glycerol, were monitored by paper chromatography for [3H]glycerol excision. Under these conditions, unlabeled glycerol did not impede Endo F hydrolysis of the [3H]glycerol from the oligosaccharides. Both reactions had identical time courses, revealing loss of 75% of the label by 30 min and 90% by 1 h. Thus, the slow loss of glycerol from oligosaccharides in a complete reaction (Fig. 5) may not be due to cycling of glycerol, but rather to the formation of an enzyme-substrate activation complex which stabilizes the glycerol-oligosaccharide. Alternatively, the released AsnGlcNAc may be a competitive inhibitor of glycerol hydrolysis. Future experiments will address more thoroughly the mechanism of this reaction.

Finally, experiments show that Endo H will also incorporate labeled glycerol into the oligosaccharides, although the efficiency appears much less than observed with Endo F. So far, no more than 65% of the reducing ends have been found to be blocked by Endo H, and this occurs very early in the reaction. Nevertheless, the utilization of glycerol in preference to water during primary cleavage of the GlcNAc-GlcNAc glycosidic bond by both Endo F and Endo H suggests that these enzymes recognize a common structural feature of the original substrate for which glycerol can partially substitute. Such a determinant might be the three carbon (C3-C4-C5 or C4-C5-C6) glycerol sequence of the asparagine-proximal GlcNAc residue.

Acknowledgments—We thank Don U. Guarino (New York State Dept. of Health) for assistance with the amino acid and GlcNAc analyses and Drs. Elder and Alexander for a generous supply of Endo F mixture.

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