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Deglycosylation of Asparagine-Linked Glycans by Peptide:*N*-Glycosidase F†

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Received December 26, 1984

ABSTRACT: Endo-β-*N*-acetylglucosaminidase F (Endo F) and peptide:*N*-glycosidase F (PNGase F) were purified from cultures of *Flavobacterium meningosepticum* by ammonium sulfate precipitation followed by gel filtration on TSK HW-55(S). This system separated the two enzymes and provided PNGase F in a high state of purity, but the basis for the resolution appeared to be hydrophobic interaction and not molecular size. Studies using purified Endo F and PNGase F with defined glycopeptides demonstrated that Endo F was somewhat similar to Endo H in that it hydrolyzed many, but not all, high-mannose and hybrid oligosaccharides, as well as complex biantennary oligosaccharides. PNGase F, in contrast, hydrolyzed all classes of asparagine-linked glycans examined, provided both the α-amino and carboxyl groups of the asparagine residue were in peptide linkage. Deglycosylation studies with PNGase F revealed that many proteins in their native conformation were susceptible to this enzyme but that prior denaturation in sodium dodecyl sulfate greatly decreased the amount of enzyme required for complete carbohydrate removal.

Recently (Plummer et al., 1984), we reported that cultures of *Flavobacterium meningosepticum* contain two oligosaccharide chain cleaving enzymes active on asparagine-linked glycans: an endo-β-*N*-acetylglucosaminidase F (Endo F),[1] first reported by Elder & Alexander (1982), and a new peptide:*N*-glycosidase F, designated PNGase F (Plummer et al., 1984) in accordance with our previous nomenclature (Plummer & Tarentino, 1981). Endo F cleaves the oligosaccharide chain between the di-*N*-acetylchitobiose moiety of some asparagine-linked glycans, primarily those of the high-mannose type. PNGase F, a potent enzyme of broader substrate specificity, hydrolyzes at the glycosylamine linkage and generates a carbohydrate-free peptide and an intact oligosaccharide with the di-*N*-acetylchitobiose unit at the reducing end.

Partially purified preparations of Endo F, including the commercially available material, have enjoyed widespread

† This work was supported by National Institutes of Health Research Grant GM30471, awarded from the National Institute of General Medical Sciences, USPHS/DHHS.

[1] Abbreviations: Endo F, endo-β-*N*-acetylglucosaminidase F; PNGase F, peptide:*N*-glycosidase F; Dns, 5-(dimethylamino)naphthalene-1-sulfonyl; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; RNase B, ribonuclease B; HTF, human transferrin; α_1GP, α_1-acid glycoprotein; OVB, ovalbumin; IgM, immunoglobulin M; Fet, fetuin; PGP, pentaglycopeptide; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; HPLC, high-pressure liquid chromatography; Con A, concanavalin A.

0006-2960/85/0424-4665$01.50/0

usage because of the ability to deglycosylate a wide variety of high-mannose and complex glycoproteins. However, it is now apparent that such preparations are in fact a mixture of Endo F and PNGase F and that the outcome of an enzyme reaction depends on two factors: (i) the pH of the reaction, which selects for a particular enzyme cleavage on the basis of stability and pH optimum, and (ii) the nature of the peptide chain and oligosaccharide class, which determines the course of the reaction on the basis of specificity (Plummer et al., 1984). Investigators, using an Endo F mixture to release oligosaccharides for analysis, introduce heterogeneity in their products because Endo F will produce secondary cleavages on susceptible substrates generated by PNGase F, even at pH 9.3 (Plummer et al., 1984).

In this report, we demonstrate a very simple and reproducible procedure for obtaining PNGase F in good yield (51%) and a degree of purity (>90%), nearly free of Endo F. Studies on the substrate specificity of the separated enzymes with defined glycopeptides and intact glycoproteins demonstrate that PNGase F is in fact the enzyme in the Endo F mixture responsible for the deglycosylation of most glycoproteins with complex oligosaccharides.

Experimental Procedures

Materials. *Flavobacterium meningosepticum* (ATCC 33958) was obtained from the American Type Culture Collection, and Endo F was from New England Nuclear. TSK HW-55(S) (lot 2090), a gel filtration medium with a polyvinyl matrix, was obtained from Merck. For maximum resolution, the column was poured at a constant flow rate of 100 mL/h. The high-mannose asparagine oligosaccharide Asn-$(GlcNAc)_2(Man)_5$ was prepared by Pronase digestion of ovalbumin and fractionated according to Huang et al. (1970). Ovalbumin octaglycopeptide and IgM biantennary hexaglycopeptide were prepared as previously described (Plummer & Tarentino, 1981). The triantennary glycopeptide Leu-Ala-Asn(CHO)-Cys-Ser was prepared from fetuin (Spiro method, Gibco Laboratories) as reported previously (Plummer et al., 1984). Bovine pancreatic ribonuclease B (RNase B; Sigma) was purified on concanavalin A–Sepharose (Baynes & Wold, 1976). Human α_1-acid glycoprotein (α_1GP) was purified as previously indicated (Tarentino & Galivan, 1980). A tetraantennary nonaglycopeptide was isolated from α_1-acid glycoprotein previously reduced and alkylated with iodoacetic acid, followed by thermolytic digestion and chromatography on columns of Sephadex G-50, Dowex 50-X2, and DE-52. Amino acid analyses were consistent with the structure Phe-Thr-Pro-Asn(CHO)-Lys-Thr-Glu-Asp-Thr, previously reported by Schmid et al. (1973). Human transferrin (HTF) was a gift of Dr. G. A. Jamieson, American National Red Cross. Because of known microheterogeneity of glycoproteins, all purified glycopeptides were subjected to compositional analyses to verify that the isolated form was representative of the anticipated class of oligosaccharide chains.

Enzyme Assays. A highly sensitive radioactive dansyl (Dns) procedure (Tarentino & Maley, 1974) was used to assay oligosaccharide chain cleaving enzymes but was modified slightly (Plummer & Tarentino, 1981) to accommodate glycopeptide substrates. PNGase F was assayed with a [^{3}H]Dns-fetuin pentaglycopeptide ([^{3}H]Dns-Fet PGP) and Endo F with [^{3}H]Dns-ovalbumin Asn$(GlcNAc)_2(Man)_5$, ([^{3}H]Dns-OVB 1-2-5) as described previously (Plummer et al., 1984). Enzyme activity was measured as nanomoles of dansyl product formed per minute at 37 °C from the appropriate Dns substrate. However, to conform to IUB standards, the data are expressed as milliunits. Where indicated, a [^{3}H]Dns-IgM hexaglycopeptide (Plummer & Tarentino, 1981) was used to detect enzymatic cleavage of biantennary chains in column effluents. Oligosaccharide release from underivatized glycopeptides was quantitated by a difference assay on the amino acid analyzer (Plummer & Tarentino, 1981).

Commercial Endo F (New England Nuclear) contained 240 milliunits/mL PNGase F and 138 milliunits/mL Endo F as assayed with our substrate-specific probes.

Identification of Products. Aliquots of an IgM biantennary glycopeptide [1.25 μmol, Tyr-Lys-Asn(CHO)-Asn-Ser-Asp; Putnam et al., 1973] were incubated at 37 °C with either PNGase F (143 milliunits) or Endo F (142 milliunits) at pH 9.3 and 5.1, respectively. After 2.5 h, the reaction products were fractionated on a column (0.9 × 198 cm) of Fractogel TSK HW-40 (25–40 μm) developed in 0.1 N acetic acid. The flow rate was 7.2 mL/h, and 0.6-mL fractions were collected.

Hydrolysis of Glycoproteins. Glycoprotein stocks (5 mg/mL) were made in either 0.05 M NaCl (native) or 1% SDS and boiled 3 min (denatured). Endo F reactions were conducted in 0.25 M sodium acetate, pH 5.0, with 6 milliunits of purified enzymes. Reactions of 100 μL contained 50 μg of test protein and the following: (1) for native, 20 mM EDTA and 10 mM β-mercaptoethanol; (2) for denatured, 20 mM EDTA and 0.6% NP-40; PNGase F reactions were conducted similarly in 0.25 M sodium phosphate, pH 8.6, with 6 milliunits of enzyme in the absence of EDTA. Incubations were conducted at 37 °C for 18 h unless otherwise indicated.

These conditions were chosen to demonstrate complete deglycosylation of all susceptible asparagine-linked oligosaccharides. However, depending on the glycoprotein and whether it is in its native conformation or has been unfolded by denaturation, the actual amount of PNGase F required may be much lower (0.6–30 milliunits/mL). Because of this variability, the susceptibility of an unknown glycoprotein to a given PNGase F concentration must be determined empirically.

Other Methods. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli (1970). Glycoproteins were detected on acrylamide gels using ^{125}I-labeled lectins (Chu et al., 1981). Quantitation of amino acids, amino sugars (glucosamine/glucosaminitol), and neutral sugars was described previously (Plummer & Tarentino, 1981).

Cell Culture and Enzyme Production. *Flavobacterium meningosepticum* (ATCC 33958) was grown to mid-log phase at 25 °C in 50 mL of 1% Tryptone–0.5% NaCl–0.5% yeast extract. The cells were harvested by low-speed centrifugation, suspended in fresh media (12.5 mL) and 20% glycerol (12.5 mL), divided into 0.5-mL aliquots, and stored at −70 °C until needed. A starter culture was prepared by inoculating 0.5 mL of cells with 100 mL of rich medium in a 250-mL flask. Cultures were grown aerobically at 25 °C for about 6 h (early log phase). For enzyme production, a 2-L flask containing 1 L of M9 medium + 0.55% casamino acids (Elder & Alexander, 1982) was inoculated with 100 mL of starter culture and grown to stationary phase (40–48 h) at 25 °C in a New Brunswick metabolic shaker.

Enzyme Purification. (*A*) *Step I: Ammonium Sulfate Precipitation.* All operations were conducted at 4 °C. The medium was centrifuged at 8000*g* for 20 min, and the cultural filtrate (1950 mL) was adjusted to 10 mM EDTA to protect the enzymes from proteolysis. The extract was concentrated to 400 mL by hollow-fiber ultrafiltration (DC-2 with H1P10 cartridge, Amicon Corp.) and adjusted with constant stirring to 90% ammonium sulfate (600 g/L of extract). After 24 h,

Table I: Purification of *Flavobacterium meningosepticum* Oligosaccharide Chain Cleaving Enzymes

purification step	volume (mL)	total protein[a] (mg)	total milliunits[b]	sp act. (milliunits/mg of protein)	yield (%)
Purification of Peptide:*N*-Glycosidase F					
concd cultural filtrate[c]	400	ND[d]	13260		100
$(NH_4)_2SO_4$ pptn	4.1	43.2	11300	262	85
TSK HW-55(S)[e]	3.0	2.6	5860	2253	44
second TSK HW-55(S)	2.5	1.05	4860	4600	37
Purification of Endo-β-*N*-acetylglucosaminidase F					
concd cultural filtrate[c]	400	ND[d]	6240		100
$(NH_4)_2SO_4$ pptn	4.1	43.2	5429	126	87
TSK HW-55(S)[e]	2.5	6.3	2368	378	38
sulfopropyl-Sephadex C-25	3.0	0.95	1776	1868	28

[a] Protein was estimated spectrophotometrically by the method of Warburg & Christian (1941). [b] 1 milliunit equals 1 nmol of Dns substrate hydrolyzed per minute (see Experimental Procedures). [c] 1950 mL of cultural filtrate concentrated to 400 mL by ultrafiltration. [d] ND, not determined. [e] These values represent only the main pools of peptide:*N*-glycosidase F and endo-β-*N*-acetylglucosaminidase F following TSK HW-55(S) chromatography.

the extract was centrifuged at 8000*g* for 45 min. Occasionally, the supernatant was still turbid and required a second centrifugation to pellet all of the precipitated proteins. The ammonium sulfate pellet in each of the large tubes was resuspended in a small volume of 90% ammonium sulfate containing 10 mM EDTA and combined into a single 40-mL tube and centrifuged (12000*g* for 15 min). Material could be stored at −70 °C at this stage. The precipitate was resuspended in about 4 mL of 20 mM Tris-HCl, pH 7.1, containing 100 mM NaCl and 5 mM EDTA (TSK column buffer), and the insoluble material was removed by centrifugation at 12000*g* for 15 min. From the cultural filtrate, 85% of the Endo F and PNGase F activity was recovered in the solubilized ammonium sulfate fraction.

(*B*) *Step II*: *TSK HW-55(S)*. The viscous extract obtained from step I (total A_{280nm} = 42; 4.1 mL) was applied to a TSK HW-55(S) column (2.0 × 165 cm) equilibrated in TSK column buffer. The column was developed at 17 mL/h (7 psi), and fractions of 2.2 mL were collected.

(*C*) *Step III*: *Rechromatography of PNGase F on TSK HW-55(S)*. The main PNGase F peak from step II (tubes 231–245) was concentrated by ultrafiltration (YM-10 membrane, Amicon Corp.) to 3.0 mL. The retentate (total A_{280nm} = 2.6), which contained all of the PNGase F activity, was chromatographed on TSK HW-55(S) as indicated in step II. The active fractions were pooled, concentrated by ultrafiltration to 2.5 mL, and used for the specificity studies detailed later.

(*D*) *Step IV*. The major Endo F pool from the first TSK HW-55(S) column was dialyzed against 10 mM sodium acetate, pH 5.7, and applied to a 1.0 × 9.0 cm column of sulfopropyl-Sephadex C-25, equlibrated in the same buffer, at a flow rate of 23 mL/h. More than 80% of the protein eluted in the nonretarded fractions of the column, and Endo F was subsequently eluted by addition of 0.20 M NaCl to the starting buffer. Fractions containing endo F activity were concentrated to 3.0 mL by ultrafiltration.

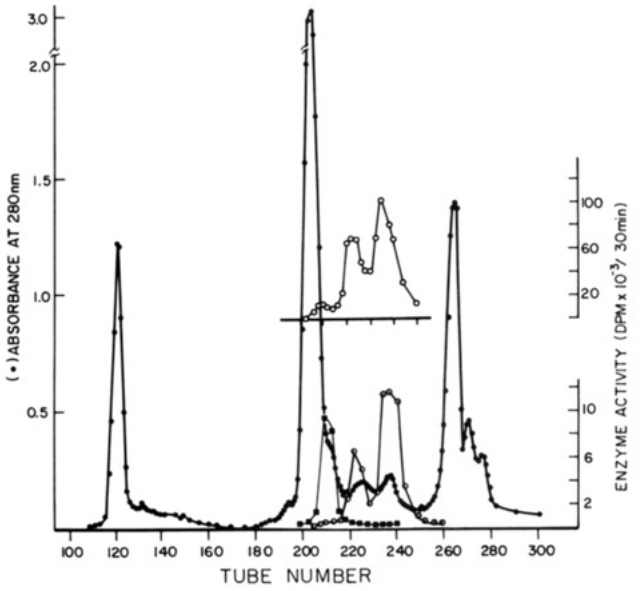


FIGURE 1: Separation of Endo F and PNGase F on TSK HW-55(S). Fractions were assayed with [^{3}H]Dns-OVB 1-2-5 for Endo F (■), with [^{3}H]Dns-Fet PGP for PNGase F (⊙), and [inset (○)] with [^{3}H]Dns-IgM hexaglycopeptide for both Endo F and PNGase F. Chromatography conditions are described under Experimental Procedures.

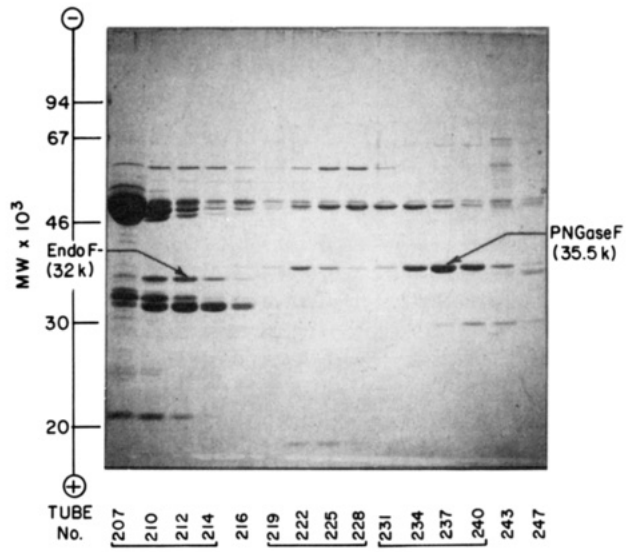


FIGURE 2: SDS–PAGE of column fractions from TSK HW-55(S) in Figure 1. Aliquots (20 μL) from the indicated fractions were added to 10 μL of a 2-fold-concentrated sample buffer, boiled 3 min, and loaded (20 μL) on an SDS gel (4% stacking, 12.5% resolving). Molecular weights were estimated from a standard semi-log plot of molecular weight vs. migration distance of known proteins.

Results

PNGase F and Endo F were secreted into the medium during growth of *Flavobacterium meningosepticum* and approached levels of 7 and 3.5 milliunits/mL, respectively, at stationary phase. The purification of these enzymes is summarized in Table I. The very dilute cultural filtrate was first concentrated about 5-fold by ultrafiltration so that the enzymes could be precipitated with ammonium sulfate in good yield (>85%).

Attempts to separate PNGase F and Endo F on standard gel filtration media (Sephadex G-75, Ultrogel AcA44) were unsuccessful, and the enzymes essentially coeluted with an estimated molecular weight of 30 000. On TSK HW-55(S), however, Endo F and PNGase F were almost completely re-

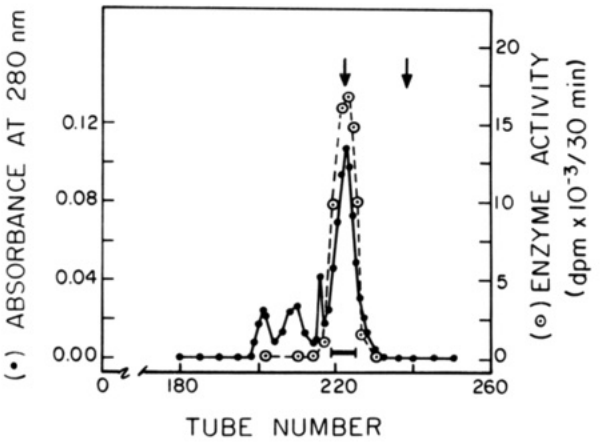


FIGURE 3: Rechromatography of the major PNGase F pool depicted in Figure 1. Tubes 231–240 (Figure 1) were combined, concentrated by ultrafiltration, and reapplied to TSK HW-55(S). Symbols and conditions as in Figure 1. The arrow at the far right indicates the prior elution position on TSK, and the arrow above the enzyme peak is the shift during rechromatography.

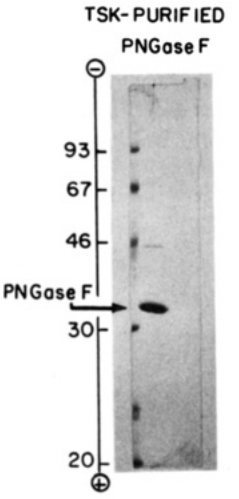


FIGURE 4: SDS–PAGE of PNGase F after rechromatography on TSK HW-55(S). Conditions as in Figure 2 except that a 10% resolving gel was used. Protein load was approximately 3.5 μg as estimated from absorbance (A_{280nm}).

solved (Figure 1), but the basis for this resolution is not size, and the mechanism will be discussed below. Endo F chromatographed normally in this system and corresponded to a 32000-dalton species present in fractions 207–214 (Figure 2). PNGase F, however, was retarded on the column and eluted in two fractions: (1) a minor pool (tubes 219–228) which contained 15% of the applied PNGase F (9% Endo F) at a relative specific activity of 660 milliunits/mg of protein and (2) a major pool (tubes 231–240) with 52% of the applied enzyme (0.4% Endo F) at a purity of 1969 milliunits/mg of protein. PNGase F corresponded to the 35 500-dalton species apparent in polyacrylamide gels of the column fractions (Figure 2). Although PNGase F has a larger apparent molecular weight (35 500) than Endo F (32 000), it elutes much later, suggesting a strong hydrophobic interaction with other proteins and the column matrix. Similar effects have been observed on TSK HW-40(S), which strongly retards hydrophobic peptides and glycopeptides and provides unexpectedly sharp peak resolution (Plummer et al., 1984) of these compounds. The hydrophobicity of this matrix results in pronounced retention of aromatic amino acids, with tryptophan interacting the most strongly.[2]

The retardation of PNGase F on TSK HW-55(S) depends on two factors: (1) the length of the column and (2) the state of purity of the applied enzyme. On shorter columns (100 cm) of TSK HW-55(S) (data not shown), PNGase F eluted as a single peak that corresponded to the minor fraction resulting from chromatography on the 165-cm column (Figure 2). In addition, when the highly purified major PNGase F pool (tubes 231–240) was rechromatographed (Figure 3), it eluted as a single species, but the elution position shifted to where the minor pool would normally be. Rechromatography of PNGase F increased its specific activity to 4600 milliunits/mg of protein with a 83% recovery of applied enzyme. On SDS–PAGE, a minor contaminant (43 000 daltons) was still apparent (Figure 4), but the preparation contained less than 0.1% Endo F and was essentially protease free. Confirmation of the 35 500-dalton species as having PNGase F activity was accomplished by HPLC gel permeation chromatography, which also reaffirmed our estimation of purity greater than 90%.

Endo F was recovered in fractions 207–214 (Figure 1) in 50% yield with a PNGase F contamination of 1.6%. Another 15% was recovered in fractions 219–228, the minor PNGase F pool. The relative specific activity of the major pool of Endo F was 378 milliunits/mg of protein. Since this fraction contained considerable proteolytic activity, it was purified further by sulfopropyl-Sephadex C-25 chromatography (data not shown) so that a better comparative study could be made with PNGase F. This step increased the purity of Endo F to 1868 milliunits/mg of protein, mainly by removal of the 46 000-dalton cluster shown in Figure 2. Much of the protease activity was eliminated at this step, but EDTA (20 mM) was still necessary to control proteolysis of some glycoproteins.

Table II: Carbohydrate Composition of an IgM Hexaglycopeptide

sugar residue	after PNGase F treatment		after Endo F treatment	
	peptide[a]	released CHO[b]	peptide	released CHO[b]
glucosamine	0.00	3.97	0.88	2.75
mannose	0.00	3.00		3.00
galactose	0.00	1.93		2.04
fucose	0.00	1.02	1.06	0.00

[a] Peptide sequence is Tyr-Lys-Asn(CHO)-Asn-Ser-Asp; molar ratios are based on Tyr = 1.00. [b] Oligosaccharide molar ratios are based on mannose = 3.00.

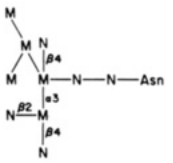
When TSK HW-55(S) column fractions were monitored with a [^{3}H]Dns-IgM hexaglycopeptide, most of the cleavage activity was associated with PNGase F (Figure 1). However, a bona fide cleavage was associated with the active Endo F peak (Figure 1, insert), suggesting that this enzyme could hydrolyze biantennary chains, albeit at a much slower rate than PNGase F. A preparative reaction with Endo F and PNGase F was conducted to verify the sites of enzymatic cleavage of the biantennary chain. A large amount of Endo F was used in order to obtain complete hydrolysis, since our preliminary studies with the corresponding dansyl glycopeptide indicated that the rate with Endo F was at least 30-fold slower than with PNGase F. The products were separated on TSK HW-40(S) into the peptide and oligosaccharide fractions and analyzed (Table II). PNGase F, as expected, yielded a carbohydrate-free peptide, with all the sugars accounted for in the released oligosaccharide. Endo F hydrolyzed the biantennary chain at the di-*N*-acetylchitobiose core, distributing the *N*-acetylglucosamine residues and neutral sugars in the expected fashion, i.e., the proximal *N*-acetylglucosamine with its linked fucose remained with the peptide.

[2] T. H. Plummer, Jr., unpublished observations.

Table III: Hydrolysis of Glycopeptide Classes by Peptide:*N*-Glycosidases and Endo-β-*N*-acetylglucosaminidases[a]

oligosaccharide structure	peptide: *N*-glycosidase[b] A	peptide: *N*-glycosidase[b] F	Endo[c] F	Endo[c] H
OVB–high mannose[d]	+	+	+	+
OVB–hybrid containing GlcNAcβ1,4Man[e]	+	+	–	+
OVB–hybrid without GlcNAcβ1,4Man[e]	+	+	+	+
ovomucoid hybrids[f]	+	+	–	–
biantennary complex[g]	+	+	+	–
triantennary complex[h]	+	+	–[i]	–
tetraantennary complex[j]	+	+	–	–

[a] Hydrolysis of glycopeptides was followed by a difference assay on the amino acid analyzer (Plummer & Tarentino, 1981). [b] Asn oligosaccharide required to be in peptide linkage for efficient *N*-glycosidase cleavage. [c] Asn oligosaccharide could be free or in peptide linkage. [d] Cyanogen bromide released, tryptic-cleaved, Con A retarded ovalbumin octaglycopeptide (Plummer & Tarentino, 1981). [e] Ovalbumin–Con A nonretarded glycopeptides. Hybrid fractions containing an *N*-acetylglucosamine residue linked β1,4 to the trimannosyl core (α1,3 branch) were resistant to cleavage by Endo F. Structures were analyzed by 500-MHz ^{1}H NMR spectroscopy (unpublished collaborative study with Drs. Robert Trimble and Paul Atkinson). Hybrids are of the type



where M is mannose and N is acetylglucosamine. [f] Thermolytic glycopeptide mixture partially purified on Sephadex G-50 and Dowex 50-X8. [g] Tryptic glycopeptide from IgM (Plummer & Tarentino, 1981). [h] Thermolytic glycopeptide from fetuin (Plummer et al., 1984). [i] Slight cleavage on 24-h hydrolysis, but most, if not all, could be accounted for by the trace PNGase F contamination. [j] Thermolytic glycopeptide from human α_1-acid glycoprotein (T. H. Plummer and A. L. Tarentino, unpublished experiments).

Specificity studies of the purified *Flavobacterium* glycosidases were conducted using defined glycopeptides or glycopeptide fractions, and the results are illustrated diagrammatically in Table III. For comparison, the data obtained with Endo H and PNGase A are also included. PNGase A and PNGase F hydrolyzed all asparagine-linked glycans tested. Endo F appeared similar in specificity to Endo H, but with two exceptions. Endo F cleaved the biantennary chain of an IgM glycopeptide, albeit slowly, but was unable to release the oligosaccharide from an ovalbumin hybrid glycopeptide (e.g., peak A and peak C ovalbumin oligosaccharides; Huang et al., 1970) containing an external *N*-acetylglucosamine residue linked β1,4 to the α1,3 branch of the trimannosyl core (Table III, footnote *e*).

The results of deglycosylation experiments with Endo F and PNGase F on intact glycoproteins (Figure 5) generally paralleled the specificity studies determined on defined glycopeptides; both PNGase F and Endo F hydrolyzed high-mannose chains, but only the former was effective on all types of complex oligosaccharides. An additional parameter not relevant to studies with defined glycopeptides, enzyme accessibility to glycosylation sites, was studied by comparing carbohydrate removal from native and SDS-denatured proteins (Figure 5).

Endo F removed the single high-mannose chain from native (lane 12) and denatured (lane 13) RNase B as shown by an increase in migration on SDS–PAGE and corresponding loss of ^{125}I-Con A binding (not shown). Complete deglycosylation

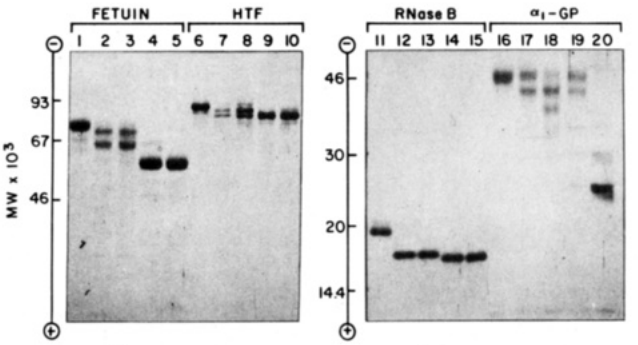


FIGURE 5: Comparison of the deglycosylation efficiency of Endo F and PNGase F on some asparagine-linked glycoproteins. Reactions (see Experimental Procedure) contained 60 milliunits/mL Endo F or PNGase F. Controls (no enzyme), lanes 1, 6, 11, and 16; Endo F (native), lanes 2, 7, 12, and 17; Endo F (denatured), lanes 3, 8, 13, and 18; PNGase F (native), lanes 4, 9, 14, and 19; PNGase F (denatured), lanes 5, 10, 15, and 20. Fetuin and human transferrin (HTF), 10% resolving gel; ribonuclease B (RNase B) and α_1-acid glycoprotein (α_1-GP), 12.5% resolving gel. Approximately 2 μg of protein per slot.

was also achieved with PNGase F (lane 14 vs. 15). The small difference in mobility between Endo F and PNGase F treated RNase B reflects the contribution of the *N*-acetylglucosamine residue which remains with the former after cleavage of the di-*N*-acetylchitobiosyl moiety. This migration difference can be magnified by employing higher cross-linked gels (15%) and the system used as a simple method for distinguishing the two enzymes.

The complex asparagine-linked oligosaccharides of HTF (mostly biantennary chains), fetuin (mostly triantennary chains), and α_1GP (mostly tetraantennary chains) (Montreuil, 1980) were susceptible to hydrolysis by PNGase F (Figure 5). As observed with RNase B, HTF (lane 9 vs. 10) and fetuin (lane 4 vs. 5) could be deglycosylated easily in their native conformation. Only α_1GP (lane 19 vs. 20) required prior denaturation to render its oligosaccharides susceptible to PNGase F. In all cases, ^{125}I-lectin overlays (concanavalin A and wheat germ agglutinin) were performed (data not shown) to verify that deglycosylation and not proteolysis was responsible for the observed increases in electrophoretic mobility after PNGase F treatment (Tarentino & Plummer, 1982).

As expected from the glycopeptide specificity studies shown in Table III, Endo F was unable to remove tri- and tetraantennary complex oligosaccharides from intact glycoproteins. Only the biantennary chains of HTF were hydrolyzed appreciably (lanes 7 and 8), and then only to a limited degree. Autoradiography following ^{125}I-Con A binding (data not shown) confirmed that the three species present in an Endo F digest of HTF (e.g., lane 8) represented unhydrolyzed HTF, HTF minus one chain, and HTF minus two chains (carbohydrate-free, Con A negative form). Even on prolonged incubation, not more than 60% complete deglycosylation was apparent, even though the enzyme was still active on [^{3}H]-Dns-OVB 1-2-5. Apparent cleavage of fetuin and α_1-acid glycoprotein could represent minor cleavage of biantennary oligosaccharide components or trace proteolysis still inherent in endo F.

The deglycosylation experiments shown in Figure 5 were overnight incubations (18 h) conducted at a PNGase F level of 60 milliunits/mL, conditions which ensure complete carbohydrates removal from most asparagine-linked glycans. However, glycoproteins differ widely in their susceptibility to deglycosylation, so that specific conditions of PNGase F and time of incubation must be established in each case. For example, denatured RNAse B was especially susceptible to

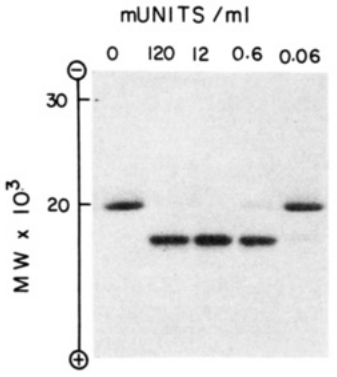


FIGURE 6: Deglycosylation of denatured RNase B as a function of added PNGase F. The reactions were conducted as described under Experimental Procedures. Serial dilutions of a stock PNGase F preparation (1200 milliunits/mL) were prepared in 0.1 M sodium phosphate, pH 8.6, and added as indicated. The incubation time was 1 h. Conditions for SDS–PAGE as in Figure 5.

carbohydrate removal by PNGase F. PNGase F at less than 12 milliunits/mL released 100% of the carbohydrate from this protein in 1 h; at 0.6 milliunits/mL, about 90% was removed in 1 h (Figure 6), and by 3 h (data not shown), complete removal had occurred. Deglycosylation of denatured RNase B was incomplete when PNGase F was used at less than 0.1 milliunits/mL, even after prolonged incubation. At this level, the enzyme appears to be unstable since enzyme activity cannot be detected with [^{3}H]Dns-Fet PGP.

α_1GP was more typical than RNase B of the deglycosylation potential of PNGase F. Complete removal of its five complex oligosaccharides (predominantly tetraantennary) occurred in 4 h at a PNGase F level of 60 milliunits/mL (Figure 4). With lower levels of PNGase F (data not shown), partially deglycosylated forms were apparent on SDS–PAGE, and extended incubation did not result in a progressive change to the carbohydrate-free species. For example, at 1 milliunits/mL, six bands were clearly evident in the SDS–PAGE profile. On the basis of ^{125}I-lectin-binding studies, these represent unhydrolyzed α_1GP and the "ladder" resulting from successive removal of individual oligosaccharides. Numerous observations have indicated that multiple species generated from one glycoprotein result from insufficient PNGase F.

Discussion

Flavobacterium meningosepticum is a relatively easy organism to cultivate, and large amounts of highly purified protease-free PNGase F (0.1% Endo F) can be obtained from only 1 or 2 L of cultural filtrate in less than 8 days, by simple column chromatography on TSK HW-55(S). The purified enzyme is stable at −70 °C and can be kept at 4 °C for months without loss of activity.

We assume PNGase F is a very hydrophobic protein or has some special structural feature which interacts with the TSK polyvinyl matrix and greatly retards its elution relative to Endo F. Whatever the explanation, the procedure is very reliable since four independent runs produced results identical with those shown in Figure 1. To date, we have found no other procedure which resolves these very similar enzymes.

On the basis of our earlier studies (Plummer et al., 1984) and the data in this report, some conclusions regarding the substrate specificities of Endo F and PNGase F have emerged. Endo F hydrolyzes primarily high-mannose oligosaccharides, although some structures containing peripheral *N*-acetylglucosamine substitutions (e.g., see Table III) are resistant. Endo F will slowly hydrolyze complex biantennary oligosaccharides, but large amounts of enzyme are required to obtain complete deglycosylation. Complex tri- and tetraantennary asparagine-linked glycans are resistant to Endo F. PNGase F is the all-purpose enzyme to hydrolyze high-mannose, hybrid, and bi-, tri-, and tetraantennary oligosaccharides.[3]

Enzymes which hydrolyze the glycosylamine linkage of asparagine-linked glycans have been described previously (Takahashi & Nishibe, 1978, 1981; Plummer & Tarentino, 1981; Tarentino & Plummer, 1982; Taga et al., 1984). We isolated and characterized the *N*-glycosidase from almonds (PNGase A) and showed that both high-mannose and complex glycopeptides were good substrates for this enzyme. PNGase A and PNGase F have a similar substrate specificity for the oligosaccharide moiety of asparagine-linked oligosaccharides, but PNGase F (35 500 daltons) is much more potent on glycoproteins than PNGase A (79 500 daltons), presumably due to differences in enzyme accessibility (Tarentino & Plummer, 1984).

With few exceptions, oligosaccharide chain cleaving enzymes (Endo H, PNGase A) are generally inaccessible to glycosylation sites on proteins in their native state (Chu et al., 1981), and denaturation is necessary to promote complete access to these sites (Tarentino et al., 1978; Tarentino & Plummer, 1982). PNGase F was somewhat different in that deglycosylation could be achieved in many cases (Figures 5 and 6; also, e.g., lipase B, ovomucoid, and Fab fragment) in both the absence and presence of detergents. However, carbohydrate removal from native glycoproteins requires higher PNGase F levels than from detergent-denatured glycoproteins,[4] but the relative difference in the amount of PNGase F required varied considerably with the glycoprotein and must be determined empirically.

Deglycosylation of native proteins by PNGase F can provide a useful approach for investigating structure–function studies of biologically active glycoproteins. Studies are now in progress to test the ability of PNGase F to release oligosaccharides from the surface of cultured cells.[5]

Added in Proof

Recently we have observed the activity of a trace protease in highly purified PNGase F that degraded carbohydrate-free SDS-denatured ovalbumin. This proteolytic activity could be completely inhibited by 10 mM 1,10-*o*-phenanthroline but was not affected by high concentrations of EDTA (60 mM).

Acknowledgments

We thank Dr. F. Chu for helpful discussions during the course of this work.

Registry No. Endo F, 37278-88-9; PNGase, 83534-39-8; RNase B, 9001-99-4.

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NMR Structural Analysis of a Membrane Protein: Bacteriorhodopsin Peptide Backbone Orientation and Motion†

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Received January 7, 1985

ABSTRACT: In reconstituted vesicles above the lipid phase transition temperature, bacteriorhodopsin (BR) undergoes rotational diffusion about an axis perpendicular to the plane of the bilayer [Cherry, R. J., Muller, U., & Schneider, G. (1977) *FEBS Lett. 80*, 465]. This diffusion narrows the ^{13}C NMR powder line shape of the BR peptide carbonyls. In contrast, BR in native purple membrane is relatively immobile and exhibits a rigid-lattice powder line shape. By use of the principal values of the rigid-lattice chemical shift tensor and the motionally narrowed line shape from the reconstituted system, the range of Euler angles of the leucine peptide groups relative to the diffusion axis has been calculated. The experimentally observed line shape is inconsistent with those expected for structures which consist entirely of either α helix or β sheet perpendicular to the membrane or β sheet tilted at angles up to about 60° from the membrane normal. However, for two more complex structural models, the predicted line shapes agree well with the experimental one. These are, first, a structure consisting entirely of α_I helices tilted at 20° from the membrane normal and, second, a combination of 60% α_{II} helix perpendicular to the membrane plane and 40% antiparallel β sheet tilted at 10-20° from the membrane normal. The results also indicate that the peptide backbone of bacteriorhodopsin in native purple membrane is extremely rigid even at 40°. The experiments presented here demonstrate a new approach, using solid-state nuclear magnetic resonance (NMR) methods, for structural studies of transmembrane proteins in fluid membrane environments, either natural or reconstituted. Analysis of NMR powder line shapes which are narrowed by anisotropic rotational diffusion can provide information not only on secondary structure but also, in general, on the orientation of labeled groups relative to the axis of rotational diffusion. Such information on the orientation of membrane proteins in the bilayer plane is difficult to obtain by more conventional structural methods.

Membrane proteins have been notoriously difficult subjects for structural investigations. Because they rarely form well-ordered three-dimensional crystals, conventional high-resolution crystallographic methods have not been generally applicable. When two-dimensional crystals are available, the electron microscopic methods pioneered by Henderson & Unwin (1974) for three-dimensional reconstruction can be used, but to date, technical limitations have prevented the determination of such structures at atomic resolution. Another technique which has recently been applied to the structural analysis of proteins involves the use of two-dimensional solution nuclear magnetic resonance (NMR)[1] [see, for example, Wagner & Wuthrich (1982)]. However, most integral mem-

† This research was supported by the National Institutes of Health (GM-23289, GM-23316, and RR-00995) and the National Science Foundation through its support of the Francis Bitter National Magnet Laboratory (DMR-8211446). B.A.L. is the recipient of a U.S. Public Health Service postdoctoral fellowship (GM-09062).

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[1] Abbreviations: BR, bacteriorhodopsin; DMPC, 1,2-dimyristoylphosphatidylcholine; DPPC, 1,2-dipalmitoylphosphatidylcholine; MASS, magic-angle sample spinning; NMR, nuclear magnetic resonance; SDS, sodium dodecyl sulfate; NOE, nuclear Overhauser effect; Me_4Si, tetramethylsilane.

0006-2960/85/0424-4671$01.50/0