Deglycosylation Studies on Tracheal Mucin Glycoproteins†

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ABSTRACT: Following several model experiments, conditions were developed for optimal deglycosylation of tracheal mucin glycoproteins. Exposure of rigorously dried material to trifluoromethanesulfonic acid at 0 °C for up to 8 h results in cleavage of essentially all fucose, galactose, and *N*-acetylglucosamine, about 80% of the *N*-acetylneuraminic acid (NeuNAc), and a variable amount of *N*-acetylgalactosamine (GalNAc), the sugar involved in linkage to protein. Residual *N*-acetylneuraminic acid is sialidase susceptible and apparently in disaccharide units, presumably NeuNAc2 → GalNAc. The remaining *N*-acetylgalactosamine is mostly present as monosaccharides, and a few Galβ1 → 3GalNAcα units are also present; both are cleaved by appropriate enzymatic treatment. The saccharide-free proteins obtained from either human or canine mucin glycoproteins have molecular weights of about 100 000 and require chaotropic agents or detergents for effective solubilization.

Tracheobronchial mucin glycoproteins are polyanionic macromolecules involved in the maintenance of mucociliary transport in the respiratory tract. Analytical equilibrium sedimentation analysis has indicated molecular weights ranging from 1.8×10^6 to 10×10^6 for native human mucin glycoproteins (Feldhoff et al., 1979). The precise contribution of the carbohydrate and protein portions of the molecule to the molecular weight and aggregation behavior of the mucin is not known. Since respiratory mucins are, in general, 70–80% carbohydrate by weight (Yeager, 1971), the polypeptide backbone may be in the range of 10^5 or less. Enzymatic deglycosylation studies on ovine submaxillary mucin (OSM)[1] (Hill et al., 1977), which contains 70% carbohydrate by weight and has a native molecular weight of over 500 000, indicated that the polypeptide molecular weight is 58 300 ± 3000. The core protein of BSM was shown to have a molecular weight of about 60 000 by cell-free translation studies (Bhavanandan & Hegarty, 1987). These studies demonstrated that the native ovine and bovine submaxillary mucin structure was maintained through aggregation of mucin monomers with each monomer containing a polypeptide backbone of molecular weight in the range of 58 000–60 000.

In contrast to the disaccharides (NeuNAc → GalNAc) present in OSM, the saccharides of the tracheobronchial mucins are very heterogeneous and complex. Further, the exact structures of these saccharides, some of which are presumably sulfated, are not yet known. Thus, the enzymatic deglycosylation of these tracheobronchial mucins, requiring a battery of highly purified glycosidases and sulfatases, is not feasible at present. The reports of chemical deglycosylating agents, HF and trifluoromethanesulfonic acid (TFMS), which cleave both N- and O-linked saccharide structures in glycoproteins without degrading the polypeptide backbone (Mort & Lamport, 1977; Edge et al., 1981) suggested that these reagents might be useful in studying the size and organization of the polypeptide backbone of mucin glycoproteins. This paper reports the results of studies on the chemical and enzymatic removal of carbohydrate from human and canine tracheobronchial mucin glycoproteins.

Materials and Methods

Human tracheobronchial mucin was isolated and purified (Woodward et al., 1982) from bronchial aspirates of a patient who had undergone surgery for nonpulmonary illness. Delipidated high-density HLM was solubilized by incubating overnight at 4 °C at a concentration of 1.0–2.0 mg/mL in 16.7 mM sodium phosphate, pH 6.8, containing 4 M guanidinium chloride, 33 mM NaCl, 0.02% NaN_3, 20 mM β-mercaptoethanol, and 42% (w/w) cesium bromide. Density gradient centrifugation (Creeth et al., 1977) of the solubilized mucin was performed at 42 000 rpm for 72 h in a Beckman 60 Ti rotor. Following centrifugation, 1-mL fractions were collected and assayed for neutral sugar by the phenol–sulfuric acid method (Dubois et al., 1956) and for protein by the absorbance at 280 nm. The high buoyant density, carbohydrate-rich fraction (βF_3) was used for subsequent deglycosylation studies.

Canine mucus secretions were collected from two beagle dogs having surgically constructed tracheal pouches (Wardell et al., 1970) and provided to us by Dr. Mitchell Litt of the University of Pennsylvania, Philadelphia, PA. The mucin glycoprotein was isolated from the mucus secretion by gel filtration on Sepharose CL-4B and purified by density gradient centrifugation followed by extraction with chloroform–methanol to remove lipids. Delipidated high-density CTM was either reduced with β-mercaptoethanol or subjected to oxidation by performic acid and fractionated by density gradient centrifugation in the presence or absence of β-mercaptoethanol.

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[1] Abbreviations: GalNAc(OH), *N*-acetylgalactosaminitol; NeuNAc, *N*-acetylneuraminic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFMS, trifluoromethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; TSF, α-toluenesulfonyl fluoride; VCN, *Vibrio cholerae* neuraminidase; CTM, canine tracheal mucin; HLM, human lung mucin; OSM, ovine submaxillary mucin; BSM, bovine submaxillary mucin; BSA, bovine serum albumin; TCA, trichloroacetic acid; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine.

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The high buoyant density fractions, either βF_3 (isolated after reduction of disulfide bonds) or PF_3 (isolated after oxidation with performic acid), were used for subsequent studies. For additional details regarding the purification of the canine mucin, see the following paper in this issue (Ringler et al., 1987).

Homogeneity of the mucin preparations was assessed by amino-terminal analysis (Zanetta et al., 1976) and SDS–PAGE. All preparations characteristically lacked detectable free amino termini and showed no low molecular weight material on SDS–PAGE that was detectable by Coomassie blue or periodate–Schiff staining (Fairbanks et al., 1971).

Chemical deglycosylation with TFMS (Edge et al., 1981) and with anhydrous liquid HF (Mort & Lamport, 1977) was performed according to published procedures. The samples and glassware used were dried in a vacuum oven at 80 °C over anhydrous $CaSO_4$ for 16 h or in high vacuum using P_2O_5 and a liquid nitrogen trap (Parekh et al., 1985). The TFMS deglycosylation reaction was initiated by the addition of 1 mL of ice-cold TFMS–anisole (2:1) to 10 mg of sample in a Teflon-lined screw-capped tube. The tube was flushed with N_2, capped tightly, and then stirred at 0 °C (ice bath) or 20 °C for the indicated time, usually 3–5 h. At the end of the reaction period, the sample was cooled in an ice bath, and 2 volumes of diethyl ether precooled in dry ice–acetone was added followed by an equal volume of ice-cold 50% aqueous pyridine. Samples were carefully mixed by vortexing, and the upper, ether, layer was removed. The lower, aqueous, phase was reextracted twice with precooled diethyl ether, dialyzed exhaustively against deionized H_2O at 4 °C, and lyophilized.

Treatment with anhydrous HF was performed in a Kel-F reaction vessel using a Teflon manifold. After addition of 100 μL of anisole to the dried sample, 2 mL of HF was distilled into the reaction vessel and the mixture stirred at 0 or 20 °C for 3 h. At the end of the reaction, HF was removed by vacuum distillation into a trap filled with calcium oxide. The residue in the reaction vessel was dissolved in 50% aqueous pyridine, dialyzed against deionized water, and lyophilized.

Preliminary deglycosylation experiments were performed on the following: (1) bovine serum albumin, from Sigma (St. Louis, MO); (2) α_1-acid glycoprotein, a gift from Dr. Yoshio Hatae, The M. S. Hershey Medical Center, isolated by isoelectric focusing of normal human serum; and (3) fetuin, obtained from Gibco (Grand Island, NY) and used without further purification. The lyophilized, deglycosylated products were not readily soluble in aqueous buffers, particularly those obtained by the HF method. Solubility appeared to be inversely dependent on the degree of deglycosylation. Briefly, samples which had been deglycosylated by TFMS treatment at 0 °C were stirred overnight in 50 mM Tris-HCl, pH 8.0, and centrifuged at 2000 rpm for 15 min. The supernatant was assayed for protein by the absorbance at 280 nm. The pellet was dissolved by stirring overnight in 50 mM Tris-HCl, pH 8.0, containing 8 M urea and assayed for protein (A_{280}). Protein solubility was estimated by the ratio of A_{280} in the supernatant and solubilized pellet. Approximately 70% of the TFMS 0 °C treated materials were soluble in 50 mM Tris-HCl, pH 8.0, after extensive stirring. Mucins deglycosylated with HF or TFMS at 20 °C were soluble only in buffers containing 4 M guanidine hydrochloride, 8 M urea, 0.1% Triton X-100, 1% SDS, or 0.1 N acetic acid. In general, the recovery of protein [as measured by A_{280} or by the Lowry assay (Lowry et al., 1951)] after TFMS treatment of the model compounds was greater than 80%. For CTM and HLM, the recovery of protein was usually about 90%.

Galactose Oxidase–NaB^3H_4 Labeling of Deglycosylated Mucin Glycoproteins. TFMS-treated, reduced, delipidated human mucin glycoprotein (βF_3) was radiolabeled by treatment with galactose oxidase and NaB^3H_4 (Morell & Ashwell, 1972). The active enzyme solution was prepared by dissolving 1.6 mg of horseradish peroxidase (638 units/mg; Worthington) in 1 mL of 0.02 M sodium phosphate, pH 7.0, containing 0.15 M NaCl and transferring 300 μL of this solution to a tube containing 150 units (84 units/mg; Worthington) of galactose oxidase in 700 μL of the above buffer. The active enzyme solution (300 μL) and 50 μL of toluene (a bacteriostat) were added to the glycoproteins, and the mixture was incubated at room temperature in the dark for 85 h. The reaction was terminated by lyophilization of the incubation mixture.

To label the oxidized glycoprotein, 200 μL of a NaB^3H_4 solution (2.5 mCi of NaB^3H_4/200 μL of 0.1 N NaOH; New England Nuclear; specific activity 40 mCi/mmol) was added to the lyophilized sample and the reaction mixture incubated at room temperature for 45 min. Sodium borohydride (200 μL of a 10 mg/mL solution in 0.1 N NaOH) was then added, and the mixture was incubated on ice for 1 h. The sample was neutralized by the careful addition of 4 N acetic acid. The labeled glycoprotein was desalted, either by dialysis against deionized H_2O at 4 °C or by chromatography on a Sephadex G-25 column equilibrated in deionized H_2O, and then lyophilized.

To localize the label, an aliquot of the radiolabeled glycoprotein was hydrolyzed in 2 N HCl at 100 °C for 4 h or in 4 N HCl at 100 °C for 8 h. After removal of the HCl in vacuo, the radiolabeled sample was dissolved in H_2O and analyzed by descending paper chromatography in 1-butanol–pyridine–H_2O (6:4:3) for 24 h. Galactosamine (Sigma) and galactose (Pfanstiehl) standards were detected by alkaline $AgNO_3$ (Trevelyan et al., 1950). The paper was cut into 1 × 2 cm sections and extracted with water, and the radioactivity was measured by liquid scintillation spectrometry.

Equilibrium Sedimentation Analysis. Deglycosylated mucin was examined by analytical equilibrium ultracentrifugation using the meniscus depletion method (Yphantis, 1964). Prior to centrifugation, samples (0.5–2.0 mg/mL) were equilibrated in the solvent by dialysis at room temperature for 24 h. Centrifugation was performed at 8000–20 000 rpm at 20 °C; equilibrium was usually attained after 72 h.

Density gradient centrifugation was performed in CsBr as described previously. Fractions were analyzed for protein by the absorbance at 280 nm, by the phenol–sulfuric acid procedure for saccharide content (Dubois et al., 1956), and for density by weighing 100-μL aliquots of every fifth fraction.

In Vitro Radiolabeling by Reductive Methylation. The radiolabeling procedure used was that of Jentoft and Dearborn (1979). Reduced, delipidated HLM or CTM was weighed into a screw-capped tube and dissolved by stirring at room temperature in 0.2 M sodium borate, pH 7.8; the concentration was usually less than 5 mg of mucin/mL; 1 mCi of [^{3}H]HCHO (100 μL; New England Nuclear; specific activity 100.0 mCi/mmol) was added and the mixture stirred at room temperature for 10 min. Two hundred microliters of $NaCNBH_3$ (10 mg/mL in H_2O) was then added to the mixture, and stirring was continued at room temperature. Three more equivalent additions of $NaCNBH_3$ were made every 10 min. One hour after the addition of [^{3}H]HCHO, 37% formaldehyde (5 μL) was added, and the solution was mixed. The reaction tube was flushed with N_2, and the mixture was allowed to stand at room temperature for 15 min. Radiolabeled samples were recovered either by exhaustive dialysis against deionized

H_2O or by gel filtration on a Sephadex G-25 column (PD-10 column; Pharmacia) equilibrated in H_2O.

Protein labeling with ^{125}I was carried out according to the Chloramine-T procedure (Hunter & Greenwood, 1962). To the protein (20 μg) in 50 μL of phosphate-buffered saline, pH 7.5, 1 mCi of $Na^{125}I$ was added, followed by 20 μg of Chloramine-T. The reaction was allowed to proceed for 3–4 min and then terminated by addition of 25 μL of 0.3 mg/mL tyrosine. Protein was isolated by gel filtration on Sephadex G-25 followed by exhaustive dialysis.

Glycosidase Treatments of Deglycosylated Mucin Glycoprotein. Galactose oxidase–NaB^3H_4-labeled, TFMS-treated, reduced delipidated HLM was treated with 10 milliunits of α-*N*-acetylgalactosaminidase from *limpet* (a gift from Dr. Y.-T. Li, Tulane University, New Orleans, LA) in 0.05 M sodium citrate, pH 4.0, containing 0.1% Triton X-100 and 0.1 mM TSF for 24 h at 37 °C. The incubation mixture was heated in a boiling H_2O bath for 3 min to inactivate the enzyme and then chromatographed on a Bio-Gel P-4 (200–400 mesh; 1.5 × 97 cm) column in 0.1 N pyridinium acetate, pH 5.0. Fractions were assayed by liquid scintillation spectrometry. The released tritium-labeled product was recovered, hydrolyzed with 2 N HCl at 100 °C for 4 h or in 4 N HCl at 100 °C for 8 h, and examined by descending paper chromatography in 6:4:3 1-butanol–pyridine–H_2O for 24 h.

Galactose oxidase–NaB^3H_4-labeled, TFMS-treated mucin was also treated with β-*N*-acetylglucosaminidase (Sigma; 12.5 units/mg) at 37 °C for 24 h in 0.05 M sodium citrate buffer, pH 5.0. After heat inactivation of the enzyme, the reaction mixture was lyophilized and then treated with α-*N*-acetylgalactosaminidase as described above. Labeled mucin was also treated with endo-α-*N*-acetylgalactosaminidase from *Diplococcus pneumoniae* (Umemoto et al., 1977) at 37 °C for 24 h in 100 mM Tris–maleate, pH 7.0, containing 0.1 mM TSF.

Neuraminidase treatment of native or deglycosylated glycoprotein was performed with *Vibrio cholerae* enzyme (Behring Diagnostics). Incubations were for 24 h at 37 °C in 200 mM sodium acetate, pH 5.6, containing 2 mM Ca^{2+}.

All glycosidases were assayed for proteolytic activity by incubation with [^{14}C]methemoglobin (Williams & Lin, 1971). Briefly, 5 μL of methyl-^{14}C-labeled methylated methemoglobin was added to 495 μL of the glycosidase solution. After incubation at 37 °C for 6 h, 1 mg of BSA followed by 500 μL of cold 10% TCA was added and precipitation allowed to occur for 1 h at 4 °C. Samples were then centrifuged for 15 min at 4 °C in a Beckman microfuge, and the supernatant was assayed for radioactivity by liquid scintillation spectrometry. Positive controls were run in parallel with protease solutions. Only glycosidase preparations free of detectable proteases were used.

Other Methods. SDS–PAGE was performed according to Weber and Osborn (1969), and the radiolabeled samples were detected by fluorography (Bonner & Laskey, 1974). Amino-terminal analysis was carried out as described previously (Zanetta et al., 1976). Amino acid analysis was performed on a Dionex MBF/SS amino acid analyzer after hydrolysis in vacuo in 6 N HCl at 110 °C for 24 h. Glucosamine and galactosamine were determined on the same analyzer following hydrolysis for 8 h at 100 °C in 4 N HCl. Sialic acid was determined by the thiobarbituric acid assay (Warren, 1959), or HPLC (Silver & Karim, 1981), after hydrolysis in 0.1 N H_2SO_4 for 1 h at 80 °C.

Quantitative analysis for carbohydrate was performed by gas–liquid chromatography of the alditol acetates (Sawardekar et al., 1965; Lehnhardt & Winzler, 1968) or trimethylsilyl ethers (Clamp et al., 1971); derivatives were prepared following hydrolysis of samples with trifluoroacetic acid or methanolic HCl, respectively.

Results

Preliminary studies with bovine serum albumin, α_1-acid glycoprotein, and fetuin indicated that suitable conditions for deglycosylation using TFMS were 3 h at 0 °C. Under these conditions, no polypeptide breakdown could be detected by SDS–PAGE or end group analysis (data not shown). In addition, over 80% of the total carbohydrate and 50% of the glucosamine were removed from α_1-acid glycoprotein, and about 65% of the total carbohydrate, 50% of the glucosamine, and 5% of the galactosamine were removed from fetuin. In contrast, Edge et al. (1981) were able to remove 74% of the glucosamine and 23% of the galactosamine by treating fetuin with TFMS at 0 °C for 2.5 h. The resons for this discrepancy are not clear. In our SDS–PAGE system, the molecular weight of deglycosylated α_1-acid glycoprotein was estimated to be 27 000, and the native glycoprotein had a molecular weight of 46 000. Similarly, the deglycosylated and native fetuins had apparent molecular weights of 48 000–50 000, and 66 000, respectively. The calculated molecular weights for completely deglycosylated α_1-acid glycoprotein and fetuin are 27 100 and 51 500, respectively.

These preliminary data suggested that substantial deglycosylation with minimal, if any, polypeptide degradation was possible. Therefore, a sample of canine tracheal mucin was deglycosylated with TFMS at 0 °C for 3 h. Approximately 60% loss by weight occurred. Comparison of the amino acid composition of deglycosylated CTM (βF_3, 0 °C for 3 h) with native CTM (βF_3) indicated essentially no change upon TFMS treatment (Table I, columns 2 and 3); however, after a second treatment for 5 h, minor changes in some amino acids (Val, Met, and Tyr) are noted. Amino-terminal analysis of deglycosylated CTM indicated that no NH_2 termini were generated after treatment, suggesting no polypeptide breakdown. Further, low molecular weight components were not detected after SDS–PAGE of CTM and TFMS–CTM which had been subsequently radiolabeled by reductive methylation (Figure 1). Carbohydrate analysis showed substantial reduction of fucose, galactose, and *N*-acetylglucosamine; there was minimal change in *N*-acetylgalactosamine and sialic acid content under these reaction conditions. Other studies (Edge et al., 1981) have also reported that the *N*-acetylgalactosaminyl linkage to the protein is resistant to hydrolysis by TFMS. Equilibrium sedimentation analysis indicated a molecular weight of 110 000 for deglycosylated CTM (βF_3, 0 °C, 3 h) compared to a molecular weight of about 500 000 for native, reduced CTM (βF_3).

Additional deglycosylation of CTM was examined by use of more stringent conditions. Following treatment with TFMS as above, the resultant product was further reacted for 3–5 h at 0 °C. Alternately, a sample of desialylated PF_3 (VCN–PF_3) was treated with TFMS at 20 °C for 5 h. The data in Table II show that the low-temperature sample (PF_3, 0 °C, 3–5 h) is essentially free of fucose and *N*-acetylglucosamine and has lost about 70% of the galactose. The apparent retention of some sialic acid was unexpected in view of the known acid lability of the ketosidic linkage of this sugar. The identity of the residual sialic acid was examined by treatment with *Vibrio cholerae* neuraminidase followed by exclusion chromatography and HPLC. All of the remaining sialic acid was susceptibile to the neuraminidase and had an elution time identical with that of NeuNAc on HPLC and TLC (Figure 2). Mild alkaline borohydride treatment followed by HPLC

Table I: Amino Acid Composition of Canine Tracheal Mucin Glycoprotein Samples before and after TFMS Treatment[a]

amino acid	βF_3	βF_3, 0 °C, 3 h	βF_3, 0 °C, 3–5 h	PF_3	PF_3, 0 °C, 5 h	PF_3, 20 °C, 5 h	PF_3–VCN, 20 °C, 5 h[b]
Asp	25	27	24	38	15	66	76
Thr	263	250	222	264	275	186	75
Ser	238	240	254	217	194	164	143
Glu	111	110	127	98	103	153	165
Gly	106	108	122	97	116	111	199
Ala	46	43	51	60	56	34	93
Val	57	59	42	44	55	47	40
Met	3	4	1	14	11	25	3
Ile	29	29	26	24	32	21	18
Leu	33	30	24	45	50	66	53
Tyr	11	12	9	2	6	19	16
Phe	15	14	12	14	22	28	29
His	15	16	16	20	20	20	30
Lys	31	30	46	20	20	21	35
Arg	31	28	22	43	24	40	25

[a] Data are in residues per 1000 residues excluding proline. βF_3 and PF_3 refer to high-density material isolated after exposure to β-mecaptoethanol or performic acid oxidation and reisolation by density gradient centrifugation, respectively. Temperatures and reaction times are indicatd for TFMS-treated samples. [b] PF_3 was incubated with VCN and reisolated by chromatography on Sephacryl S-200 prior to TFMS treatment.

Table II: Carbohydrate Composition of Canine Tracheal Mucin Glycoprotein Samples before and after TFMS Treatment[a]

sugar	PF_3	PF_3, 0 °C, 3 h	PF_3, 0 °C, 3–5 h	PF_3–VCN, 20 °C, 5 h[b]
fucose	16.3	6.0	2.0	0.5
galactose	22.9	7.0	6.5	0.5
GlcNAc	9.8	0.7	0.1	0
GalNAc	6.0	5.0	3.4	0.5
NeuNAc	8.8	6.2	2.2	0.7

[a] Data are in weight percent. PF_3 refers to high-density material after performic acid oxidation and reisolation by density gradient centrifugation. Temperatures and reaction times are indicated for TFMS-treated samples. [b] PF_3 was incubated with VCN and reisolated by chromatography on Sephacryl S-200 prior to TFMS treatment.

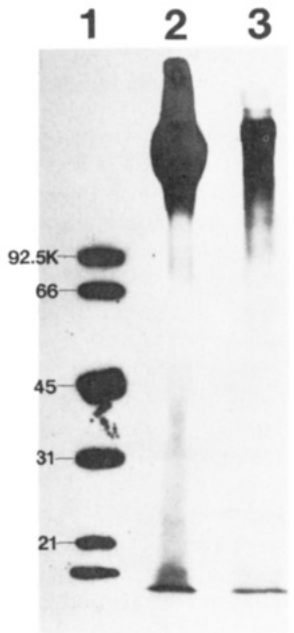

FIGURE 1: SDS–PAGE of CTM and TFMS–CTM radiolabeled by reductive methylation with [^{3}H]HCHO. Lane 1, Bio-Rad low molecular weight standards; lane 2, ^{3}H-labeled CTM; lane 3, ^{3}H-labeled CTM treated with TFMS for 3 h at 0 °C.

of the released products revealed that almost all of the residual NeuNAc is present in disaccharides. The disaccharide had an identical elution time on HPLC and mobility on TLC as NeuNAc2 → 6GalNAc(OH) isolated from OSM (Figure 2). Insufficient material was available for further characterization. There was still significant GalNAc (>50%) remaining after the two TFMS treatments at 0 °C. The reaction carried out

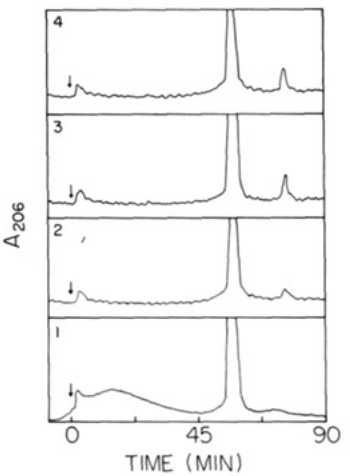

FIGURE 2: HPLC of TFMS-treated canine tracheal mucin. Chromatography on a 7.8 × 300 mm HPX-87H Aminex column operated at a flow rate of 0.1 mL/min with 0.006 N H_2SO_4 as eluting solvent. Panel 1, TFMS-treated PF_3; panel 2, TFMS–PF_3 after treatment with VCN; panel 3, ovine submaxillary mucin following treatment with alkaline borohydride; panel 4, TFMS–PF_3 following treatment with alkaline borohydride. The peaks eluting at approximately 75 min in panels 3 and 4 and the peak in panel 2 were isolated and identified by TLC as the disaccharide NeuNAc → GalNAc(OH) and NeuNAc, respectively.

at 20 °C resulted in an 80–90% loss by weight and in more extensive sugar removal, although some GalNAc still remained (Table II, last two columns). However, there is also an indication of peptide cleavage as evident from the significant alterations in the amino acid composition, particularly Thr, Ser, Asp, and Glu (Table I). Cleavage of NeuNAc by treatment of the mucin glycoprotein with *Vibrio cholerae* neuraminidase prior to reaction with TFMS at 0 °C yielded a product with residual carbohydrate content, analogous to that obtained for the 20 °C sample (data not shown).

The reduced, delipidated HLM was reacted with TFMS at 0 °C for 3–4 h and subsequently for 5 additional h also at 0 °C. The initial 3–4-h treatment resulted in approximately 60% loss by weight; the subsequent 5-h treatment resulted in a further loss of 14%. Changes in the amino acid composition after either treatment were minimal (Table III). Amino-terminal analysis showed no NH_2 termini. Carbohydrate analysis of the twice TFMS-treated HLM indicated a 99% loss of neutral suger, 85% loss of sialic acid, and 98% loss of glucosamine but only a 58% loss of galactosamine (Table IV, last column). Prior enzymatic desialylation of the mucin with

Table III: Amino Acid Composition of Human Tracheal Mucin Glycoprotein Samples before and after TFMS Treatment[a]

amino acid	βF_3	βF_3, 4 h	βF_3, 4–5 h
Asp	42	44	41
Thr	260	249	264
Ser	180	203	196
Glu	57	82	81
Gly	141	118	111
Ala	118	105	108
Val	33	29	30
Met	6	3	3
Ile	12	14	15
Leu	41	42	42
Tyr	6	10	9
Phe	14	14	14
His	35	27	29
Lys	31	36	33
Arg	24	24	24

[a] Data are in residues per 1000 residues excluding proline. βF_3 refers to high-density material isolated after exposure to β-mercaptoethanol. TFMS treatment was performed at 0 °C; reaction times are indicated.

Table IV: Carbohydrate Composition of Human Tracheal Mucin Glycoprotein Samples before and after TFMS Treatment[a]

sugar	βF_3	βF_3–VCN, 4 h[b]	βF_3, 4 h	βF_3, 4–5 h
fucose	9	2.8	1.2	0 (100)
galactose	29	5.4	6.0	1.0 (99)
GlcNAc	32	9.3	13.6	2.3 (98)
GalNAc	9	2.9	24.2	14.0 (58)
NeuNAc	8	0	3.5	3.9 (85)

[a] Data are in weight percent. Values in parentheses refer to overall percent sugar loss based on the amount present in the starting material, βF_3. TFMS treatment was performed at 0 °C; reaction times are indicated. [b] This sample was pretreated with VCN and reisolated by chromatography on Sephacryl S-200 prior to exposure to TFMS.

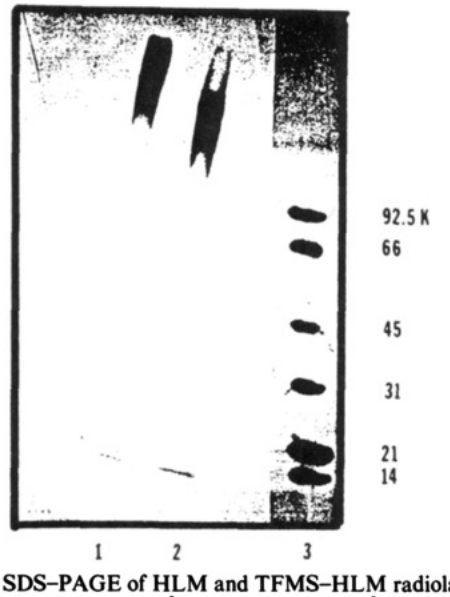

FIGURE 3: SDS–PAGE of HLM and TFMS–HLM radiolabeled by reductive methylation with [^{3}H]HCHO. Lane 1, ^{3}H-labeled HLM; lane 2, ^{3}H-labeled HLM treated with TFMS for 3 h, at 0 °C; lane 3, Bio-Rad low molecular weight standards.

Vibrio cholerae neuraminidase enhanced the susceptibility of GalNAc residues (Table III, second column), although a second 0 °C treatment would still be necessary for maximal deglycosylation. Only minimal, if any, polypeptide breakdown of TFMS-treated HLM was detected as judged by SDS–PAGE (Figure 3) of the reductively ^{3}H-methylated native and TFMS-treated mucin glycoproteins. There appeared to be a reduction in molecular size of the mucin after TFMS treatment, since the deglycosylated mucin completely entered the

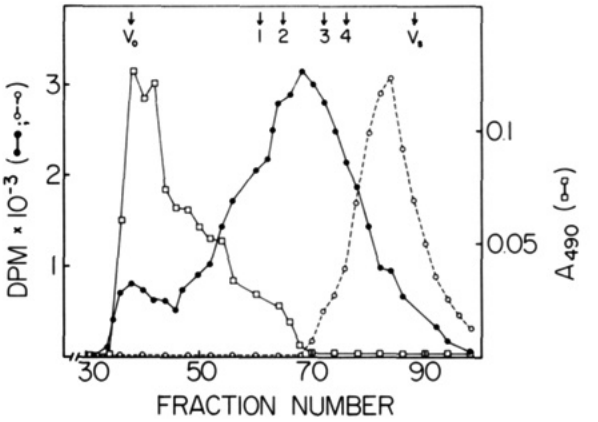

FIGURE 4: Sepharose CL-4B column (1.5 × 50 cm) chromatography of high-density human lung mucin glycoprotein before (□) and after [(●) 1×; (○) 2×] treatment with TFMS. The sample was applied in 50 mM Tris-HCl, pH 8.0, 0.02% NaN_3, and 0.1 mM TSF and the column eluted with the same buffer. The flow rate was 10 mL/h; 1-mL fractions were collected and analyzed for neutral suger or radioactivity as appropriate. Molecular weight markers were as follows: 1, thyroglobulin, M_r 669 000; 2, ferritin, M_r 440 000; 3, catalase, M_r 232 000; 4, aldolase, M_r 158 000.

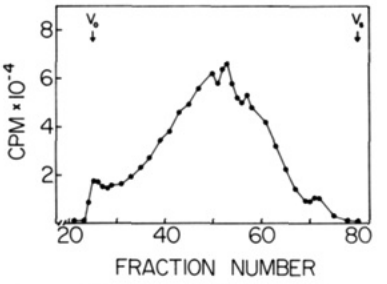

FIGURE 5: Sepharose CL-4B (1.5 × 45 cm) chromatography of ^{125}I-labeled human lung mucin glycoprotein following treatment with TFMS for 4 h at 0 °C. Conditions as for Figure 4.

Table V: Molecular Weights of Human Tracheal Mucin Glycoprotein Samples[a]

	exclusion	sedimentation
βF_3[b]		650 000
TFMS$_1$, βF_3[c]	360 000	160 000
TFMS$_2$, βF_3[d]	90 000	96 000
Pronase core[e]	224 000	160 000
TMFS$_1$, Pronase core	129 000	

[a] Molecular weights estimated by exclusion chromatography on Sepharose CL-4B or sedimentation equilibrium analysis. [b] βF_3 is high-density material after exposure to β-mercaptoethanol and reisolation by density gradient centrifugation. [c] TFMS$_1$ is material treated for 4 h at 0 °C. [d] TFMS$_2$ is material treated for 4–5 h at 0 °C. [e] Pronase core refers to the major carbohydrate-containing peak isolated by Sepharose CL-4B chromatography following Pronase digestion.

running gel, and no low molecular weight material was detectable (Figure 3). On the basis of Sepharose CL-4B chromatography (Figures 4 and 5) of ^{3}H-labeled TFMS-treated HLM, the molecular weights of the once- and twice-deglycosylated human mucin glycoprotein were estimated to be 360 000 and 90 000, respectively. Equlibrium sedimentation analysis of twice-deglycosylated mucin indicated a molecular weight of 96 000 after extrapolation to zero concentration (Table V). By density gradient centrifugation, the twice-deglycosylated mucin was found to have a density of 1.39 g/mL compared to the original (1.50 g/mL), confirming that the bulk of the carbohydrate had been removed (Figure 6).

Analysis of the once TFMS-treated human mucin after galactose oxidase–NaB^3H_4 labeling indicated that the ^{3}H label was primarily associated with galactosamine, with less than

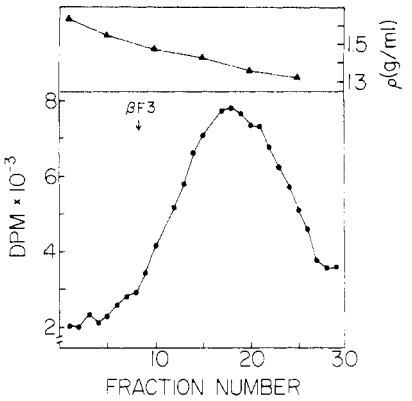

FIGURE 6: CsBr density gradient centrifugation of human lung mucin glycoprotein following treatment with TFMS for 4–5 h at 0 °C and radiolabeled with galactose oxidase–NaB^3H_4. Centrifugation was performed at 14 °C for 72 h at 42000 rpm in a Beckman 60 Ti rotor. 1.0-mL fractions were collected by aspiration from the bottom of the tube, and aliquots were assayed by liquid scintillation spectrometry. 100-μL aliquots of every fifth tube were weighed to determine the density. βF_3 represents the peak position of the human lung mucin glycoprotein prior to treatment with TFMS.

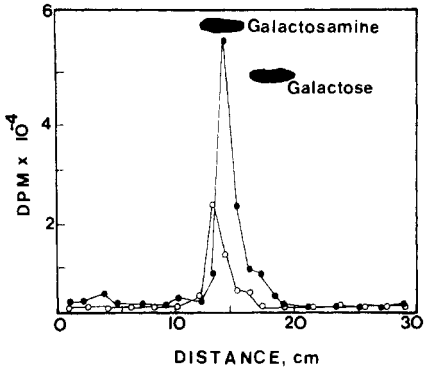

FIGURE 7: Descending paper chromatography in 1-butanol–pyridine–H_2O (6:4:3) for 24 h of galactose oxidase–NaB^3H_4-labeled, TFMS-treated HLM after hydrolysis in 2 N HCl at 100 °C for 4 h (●) and in 4 N HCl at 100 °C for 8 h (○). The positions of galactosamine and galactose were detected by alkaline $AgNO_3$.

10% associated with galactose (Figure 7). Alkaline sodium borohydride treatment of ^3H-labeled, deglycosylated HLM, followed by chromatography of the released oligosaccharide alcohols on a column of Bio-Gel P-4, indicated that 69% of the total radioactivity eluted as a monosaccharide, presumably GalNAc(OH) (Figure 8).

The above results are consistent with a major portion of the remaining saccharide being present as *N*-acetylgalactosamine. Therefore, the galactose oxidase-NaB^3H_4-labeled, once- and twice-deglycosylated human mucin was treated with exo-α-*N*-acetylgalactosaminidase to remove additional saccharides. Of the radiolabel in the galactose oxidase–NaB^3H_4-labeled, once- and twice-deglycosylated mucin glycoproteins, 29% and 55%, respectively, were released as monosaccharide which eluted in the position of GalNAc on a Bio-Gel P-4 column (Figure 9A,B). This monosaccharide fraction was recovered and identified as galactosamine by acid hydrolysis followed by descending paper chromatography. In addition, 29% of the radiolabel was released as disaccharide (presumably Gal → GalNAc) by treatment with endo-α-*N*-acetylgalactosaminidase (Figure 9C). No change in the molecular size of the α-*N*-acetylgalactosaminidase-treated, deglycosylated mucin was detected by exclusion chromatography on Sepharose CL-4B. It was thought that if any GlcNAcβ → GalNAc linkages existed in the partially deglycosylated mucin glycoprotein, treatment with β-*N*-acetylglucosaminidase might

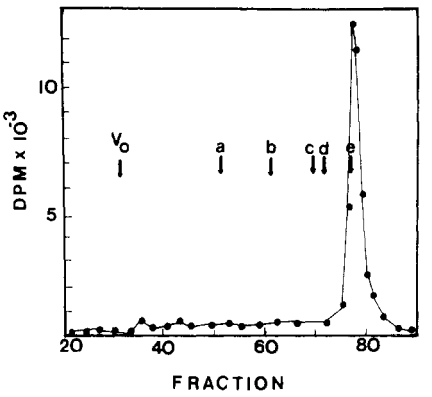

FIGURE 8: Bio-Gel P-4 (200–400 mesh; 1.5 × 97 cm) chromatography of galactose oxidase–NaB^3H_4-labeled, TFMS-treated HLM after alkaline $NaBH_4$ treatment. The column was equilibrated in 0.1 N pyridinium acetate, pH 5.0. The flow rate was 10–12 mL/h, and 1.9-mL fractions were collected and analyzed for radioactivity. Calibration standards were as follows: a, glycophorin tetrasaccharide; b, neuraminlactitol; c, maltotriitol; d, maltitol; e, glucose.

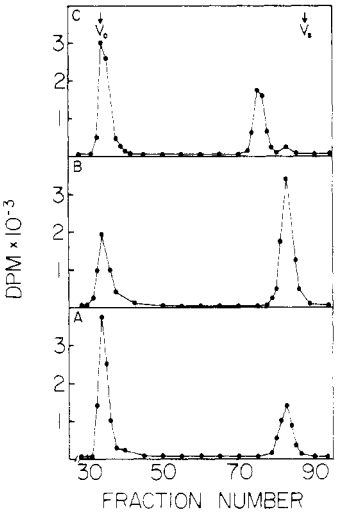

FIGURE 9: Bio-Gel P-4 column (0.9 × 108 cm) chromatography of enzymatically treated deglycosylated human lung mucin glycoprotein. (Panel A) Material exposed to TFMS for 3 h at 0 °C, radiolabeled with galactose oxidase–NaB^3H_4, and incubated with exo-α-*N*-acetylgalactosaminidase; (panel B) material exposed to TFMS for 4–5 h at 0 °C and then treated as panel A; (panel C) material exposed to TFMS for 4–5 h at 0 °C, radiolabeled, and incubated with endo-α-*N*-acetylgalactosaminidase. The elution buffer was 0.1 M pyridinium acetate, pH 5.0; the flow rate was 3.4 mL/h, and 1-mL fractions were collected and analyzed for radioactivity.

uncover more *N*-acetylgalactosamine that would be susceptible to α-*N*-acetylgalactosaminidase. However, incubation of galactose oxidase–NaB^3H_4-labeled, deglycosylated mucin glycoprotein with β-*N*-acetylglucosaminidase, prior to α-*N*-acetylgalactosaminidase treatment, did not result in an increase in the amount of *N*-acetylgalactosamine released.

Discussion

Successful deglycosylation of mucin without degradation of the protein would have many practical applications. First, it would allow one to determine the true size and the primary amino acid sequence of the core protein, as well as the distribution of disulfide bridges and possible glycosylation sites. Second, it would permit the investigation of the nature of the saccharide–saccharide or saccharide–protein interactions in terms of their contribution to the aggregation and/or polydispersity of the native mucin. Third, it would provide the natural substrate for investigating the biosynthesis of mucin, particularly the first reaction involving the transfer of GalNAc.

Fourth, it would be possible to raise antisera for the core protein for use in molecular biological experiments such as cloning of the mucin genes.

Initially, we carried out preliminary experiments of model compounds (bovine serum albumin, fetuin, and α_1-acid glycoprotein) using anhydrous HF (Mort & Lamport, 1977). The results were not encouraging since we noted substantial degradation of the polypeptide chain and encountered problems with solubility of the products. Similar studies on HF deglycosylation of transcortin, a plasma glycoprotein which binds corticosteroid hormones, have also indicated that this reagent results in peptide bond cleavage (Perini et al., 1985). The stringent anhydrous conditions required, the need of complex and expensive Teflon equipment, and the highly corrosive, toxic nature of liquid hydrogen fluoride are some disadvantages in using this procedure. However, others have used anhydrous HF to successfully deglycosylate carcinoembryonic antigen (Glassman et al., 1978) and human chorionic gonadotropin (Manjunath & Sairam, 1982). A modified procedure involving the use of HF–pyridine has also been successfully used to remove almost all chondroitin sulfate chains and about 80% of N- and O-glycosidically linked saccharides from hyaluronidase-treated rat chondrosarcoma proteoglycan (Olson et al., 1985). We have not tested this modified HF–pyridine procedure, which does not have some of the disadvantages mentioned above, for the deglycosylation of mucins.

Experiments using a less vigorous deglycosylating agent, TFMS, proved to be valuable in partially deglycosylating both human and canine high-density mucin glycoprotein without detectable protein degradation. This reagent has proved useful in deglycosylating fetuin (Edge et al., 1981) and human chorionic gonadotropin (Thotakura & Bahl, 1982).

The overall loss of carbohydrate from CTM and HLM ranged from 60% to 90% by weight, depending on the conditions used. In the CTM samples, the apparent molecular weight of the protein product after one 0 °C treatment was approximately 110 000. This figure is consistent with the composition of the starting material and the native molecular weight of the high-density mucin. The human material after two 0 °C treatments had an apparent molecular weight of 96 000. This is also consistent with both compositional data and native molecular weight. In addition, the buoyant density (1.39 g/mL) of the TFMS product was that of a protein with only trace residual carbohydrate. Even though the original publication on this method (Edge et al., 1981) did not specify the need for anhydrous conditions, we found that drying of the samples and glassware as indicated under Materials and Methods yields reproducible and consistent results.

We conclude that deglycosylation of this class of glycoproteins is best accomplished by a combination of enzymatic and chemical steps. Maximum deglycosylation, with no notable protein degradation, was obtained by neuraminidase digestion, treatment of the asialomucin with TFMS at 0 °C for up to 8 h, and finally treatment with exo- and endo-α-*N*-acetylgalactosaminidases to remove additional GalNAc and Gal → GalNAc residues.

Hill et al. (1977), in a study on enzymatically deglycosylated ovine submaxillary mucin, demonstrated that, after complete removal of the carbohydrate (which, in OSM, is the disaccharide NeuNAcα2 → 6GalNAc), the molecular weight of the polypeptide was 58 300. By using this value as the molecular weight for the polypeptide backbone of OSM, and knowing how much carbohydrate was present in the mucin (62% by weight of the native mucin), they calculated a molecular weight for the mucin of 154 150. However, the molecular weight was experimentally determined by physical methods to be 559 000–640 000, suggesting that native OSM undergoes self-association through fully glycosylated subunits. Association was also demonstrated in asialo-OSM (i.e., when only the terminal GalNAc was present in the mucin). The cell-free translation product corresponding to the bovine submaxillary core protein was shown to have a molecular weight of about 60 000 in recent studies carried out by Bhavanandan and Hegarty (1987). This once again suggests that at least in the case of the submaxillary mucins, the high molecular weight ($>10^6$) previously reported is due to self-association of smaller subunits.

Since the human and canine tracheal mucin glycoproteins were not fully deglycosylated, it is difficult to establish the presence or absence of carbohydrate self-association. The decrease in aggregation and/or polydispersity after deglycosylation, however, suggests some contribution from the carbohydrate in mucin association.

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Structure of Canine Tracheobronchial Mucin Glycoprotein†

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ABSTRACT: Canine tracheal mucin glycoprotein was isolated from beagle dogs fitted with tracheal pouches. Following exclusion chromatography on Sepharose CL-4B, noncovalently associated proteins were further resolved by dissociative density gradient centrifugation in CsBr–guanidinium chloride, and the mucin was then extracted with chloroform–methanol. The delipidated high-density product obtained had a nominal molecular weight of about 10^6 and an overall composition characteristic for a mucin glycoprotein, viz., a high content of serine and threonine, about 80% carbohydrate by weight, the absence of mannose or uronic acid, measurable ester sulfate, and a Pronase-resistant domain of molecular weight $(1.75–3.0) \times 10^5$ which contains essentially all of the saccharide residues. Noncovalently bound lipid amounted to 6–10% by weight and was primarily cholesterol and cholesteryl esters. Cleavage of disulfide bonds by performic acid oxidation resulted in the release of a protein (M_r 65 000) not otherwise resolved by sodium dodecyl sulfate gel electrophoresis or the purification scheme.

The biological functions of upper respiratory tract secretions are critically dependent on their physical properties. For example, the effective functioning of the mucociliary transport system depends on the optimal viscosity and surface tension of the mucus secretion. The major component responsible for the desired properties of the mucus secretion is a single complex mucin glycoprotein. This macromolecule has binding domains for passive clearance of both protein and lipid and may also function as a ligand (via its saccharides) for cell surface receptors of microorganisms. Many previous studies have focused on the mucin isolated from the bronchial secretions of patients with cystic fibrosis or asthma (Chernick & Barbero, 1959; Feldhoff et al., 1979; Lamblin et al., 1977; Rose et al., 1979; Roussel et al., 1975) or normal individuals hospitalized for nonpulmonary problems (Sachdev et al., 1980; Lafitte et al., 1977; Boat et al., 1976; Woodward et al., 1982).

A model system used for collection of normal tracheobronchial secretions involves introduction of a subcutaneous tracheal pouch in dogs. This experimental procedure permits routine collection of mucus samples free of contaminants that might arise from salivary glycoproteins (Wardell et al., 1970). This method has been used for the isolation of canine tracheal mucus for investigating the rheological properties (Litt et al., 1974a,b; Khan et al., 1976) and for isolating and biochemically characterizing the glycoprotein components (Sachdev et al., 1978; Liao et al., 1979).

It has been indicated that mucus secretions from the canine tracheal pouch system show similarities to the secretions obtained from human sources (Liao et al., 1979). This report details chemical and physical studies on the mucin glycoprotein of canine tracheal secretions.

Materials and Methods

Crude Mucus Collection and Solubilization. Beagle dogs fitted with tracheal pouches following the general procedure

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