A Novel Approach for Chemically Deglycosylating O-Linked Glycoproteins. The Deglycosylation of Submaxillary and Respiratory Mucins†

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ABSTRACT: A new approach for removing O-glycosidically linked carbohydrate side chains from glycoproteins is described. Periodate oxidation of the C3 and C4 carbons in peptide-linked *N*-acetylgalactosamine (GalNAc) residues generates a dialdehyde product which, under mild alkaline conditions, undergoes a β-elimination which releases carbohydrate and leaves an intact peptide core. The pH and time dependence, and intermediates of the elimination, have been extensively followed by carbon-13 NMR spectroscopy and amino acid analysis using ovine submaxillary mucin (OSM) as the substrate. The deglycosylation of OSM is complete and provides apomucin in high yield with an amino acid composition identical to the starting material. Carboxymethylated OSM when deglycosylated by this method gives an apomucin with an apparent molecular weight of ca. 700×10^3. The molecular weight is the same as that calculated for the peptide core of the starting mucin, demonstrating the absence of peptide core cleavage. This contrasts with the use of trifluoromethanesulfonic acid (TFMSA), which generates apomucin products of lower molecular weights. Oligosaccharide side chains substituted at C3 of the peptide-linked GalNAc residue are resistant to the oxidation and elimination. Glycoproteins containing these more complex side chains can be deglycosylated by pretreatment with TFMSA under mild (0 °C) conditions, which removes peripheral sugars (while leaving the peptide-linked GalNAc residue intact), followed by oxidation and β-elimination. Studies on the deglycosylation of porcine submaxillary mucin and human tracheobronchial mucin indicate that this approach provides more efficient removal of carbohydrate and less peptide core degradation than a more vigorous (25 °C) treatment with TFMSA alone. ^{13}C NMR spectroscopic studies and carbohydrate analysis of the deglycosylation intermediates of the human mucin indicate that certain sialic acid containing and *N*-acetylglucosamine-containing oligosaccharides have elevated resistance to TFMSA treatment at 0 °C. By the use of neuraminidase, repeated mild TFMSA treatments, and multiple oxidations and β-eliminations, the human mucin can be nearly completely deglycosylated. It is expected that all mucins and most glycoproteins containing O-glycosidic linkages can be readily and nearly completely deglycosylated using this combined approach.

The carbohydrate contents of mucous glycoproteins typically range from about 55 to 85% by weight. The carbohydrate is distributed along the peptide core in the form of hundreds of short, heterogeneous oligoscaccharide side chains (≲20 residues) that are attached to serine and threonine residues via *N*-acetylgalactosamine (GalNAc).[1] These oligosaccharide side chains alter the conformation of the mucin peptide core (Shogren et al., 1989) and dominate the chemical and physical properties of mucins. The true molecular weight and subunit structure of native mucins and the mucin peptide core remain controversial [see Carlstedt et al. (1985) and Hill et al. (1977)]. This is primarily due to their very large and heterogeneous size and high content of carbohydrate which greatly complicate the interpretation of most biochemical and biophysical approaches applicable to the study of mucin structure. Alternatively, one can study mucins that have their carbohydrate side chains removed. Such deglycosylated (apo-) mucins, by lacking carbohydrate and associated structural heterogeneity, are more amenable to study by conventional biochemical techniques to yield information on the size and subunit nature of the mucin peptide core. Unfortunately, most methods for deglycosylating mucins lead to incomplete removal of carbohydrate and extensive degradation of the peptide core, limiting their usefulness.

Mucins can be deglycosylated either by chemical or enzymatic methods. Sequential enzymatic deglycosylations have been successfully used on submaxillary mucins which have simple carbohydrate side-chain structures (Eckhardt et al., 1987; Hill et al., 1977). However, unlike the N-linked oligosaccharides, it is unlikely that this approach can be used easily with mucins of more complex carbohydrate structures or longer side chains, such as the intestinal or tracheobronchial mucins. Enzymatic methods can also be complicated by the presence of trace contaminating proteases. Since native and deglycosylated mucins possess open random-coil structures (Shogren et al., 1989), they are likely to be more susceptible to proteolytic degradation than globular proteins.

Chemical methods for deglycosylating glycoproteins with anhydrous HF (Mort & Lamport, 1971) or trifluoro-

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[1] Abbreviations: GalNAc, *N*-acetylgalactosamine; Gal, galactose; GlcNAc, *N*-acetylglucosamine; NeuNAc, *N*-acetylneuraminic acid or sialic acid; NeuNGl, *N*-glycoloylneuraminic acid or sialic acid; Fuc, fucose; TFMSA, trifluoromethanesulfonic acid; OSM, ovine submaxillary mucin; PSM, porcine submaxillary mucin; HTBM, human tracheobronchial mucin; OxE, periodate oxidation–β-elimination; RCM, reduced and S-carboxymethylated; TRIS, tris(hydroxymethyl)aminomethane; PAS, periodic acid Schiff.

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methanesulfonic acid (TFMSA) (Edge et al., 1981) effectively remove peripheral sugars from the oligosaccharide side chains of mucins, and when they are used at 0 °C, they give only partial cleavage of the peptide-linked GalNAc residues (Edge et al., 1981; Gerken, 1986; Woodward et al., 1987; Bhavanandan & Hegarty, 1987; Byrd et al., 1989). At higher temperatures, the methods more efficiently remove the linkage GalNAc residues at the cost of peptide core cleavage (Marianne et al., 1986; Bhattacharyya et al., 1990a,b; Shogren et al., 1989). Other approaches for removing the remaining carbohydrate after mild TFMSA treatment include HF treatment (Perini et al., 1989) and exo-α-*N*-acetylgalactosaminidase treatments (Woodward et al., 1987) but are technically demanding and expensive and do not guarantee a nondegraded product. Since the true benefits of deglycosylation are only realized when all carbohydrate is removed, improved methods for deglycosylating mucins are required.

Recently, we have investigated how carbohydrate structure influences mucin conformation using intact mucins and mucins with modified carbohydrate side chains. NMR studies of one derivative, periodate-oxidized ovine submaxillary mucin (OSM), demonstrated an unexpected loss of carbohydrate. The expected structure of the periodate-oxidized product indicated the potential for a β-elimination reaction in which the leaving group was the intact hydroxyamino acid rather than the oligosaccharide found for the more common alkaline–borohydride β-elimination of O-linked oligosaccharides (Carlson, 1968; Spiro, 1972). We report further studies on this oxidation–elimination reaction and on its utility in combination with pretreatments with TFMSA for the deglycosylation of mucins possessing a range of oligosaccharide side-chain structures.

Materials and Methods

Materials. Trifluoromethanesulfonic acid (TFMSA) (10-g ampules) and anhydrous anisole were obtained from Aldrich Chemical Co. Ovine submaxillary glands were obtained frozen from Pel-Freeze while porcine submaxillary glands were obtained from a slaughterhouse and frozen. Sputum samples were collected from cystic fibrosis patients and frozen prior to mucin purification. Neuraminidase type X was obtained from Sigma Chemical Co.

Mucin Isolation and Modification. Ovine and porcine submaxillary mucins were isolated as previously described (Gerken & Dearborn, 1984; Gerken & Jentoft, 1987). Human tracheobronchial mucin (HTBM) was isolated and purified as described by Gupta et al. (1990) except that CsCl density gradient centrifugation was omitted. Single pools of purified OSM and PSM and five different pools of HTBM were used in these studies. Asialo-HTBM was obtained by treating mucin overnight at 37 °C with neuraminidase (Gerken & Dearborn, 1984) using 3 units/100 mg of mucin. OSM was reduced and S-carboxymethylated with iodoacetamide as described by Gupta and Jentoft (1989).

Analytical Methods. Carbohydrate analyses were performed using the method of Jentoft (1985) and amino acid analyses by the method of Gupta and Jentoft (1989). Mannose and α-aminobutyric acid were used as standards. Carbon-13 NMR spectroscopy was also used to analyze the reaction products and to quantitate the loss of carbohydrate as described below.

S-Carboxymethylated OSM was separated into three different molecular weight fractions by gel filtration chromatography on Sephacryl S-1000 (Pharmacia) in 5 M guanidine hydrochloride (Shogren et al., 1987, 1989). The molecular weights of these fractions were estimated from their elution positions after rechromatography on S-1000 as described previously (Shogren et al., 1987). Column void and total volumes were determined using T4 phage DNA and adenosine 5′-monophosphate as markers. The protein content of OSM was taken as 44% on the basis of its amino acid composition, having oligosaccharides on 75% of the Ser and Thr residues (Gerken & Dearborn 1984).

The molecular weights of OSM deglycosylated by various approaches were compared by electrophoresis on 1% agarose gels containing 0.1% sodium dodecyl sulfate in TRIS (40 mM)/acetic acid (30 mM)/EDTA (2 mM) buffer, pH 7.8. Gels were first stained with Schiff's reagent after treatment with 0.5% periodic acid for 2 h (Fairbanks et al., 1971) followed by staining with Coomassie brilliant blue.

NMR Methods. Natural abundance proton-decoupled carbon-13 NMR spectra were obtained at 67.9 MHz on a Bruker WH 270 or Bruker AC-270 spectrometer as described previously (Gerken & Jentoft, 1987). All spectra were acquired (0.77-s recycle time) and processed (5-Hz line broadening) under identical conditions to allow direct comparisons between spectra.

Mucin Deglycosylation by Trifluoromethanesulfonic Acid Treatment. Mucins were partially deglycosylated by TFMSA using the basic methods of Edge et al. (1981) (with added anisole) or of Sohar and Bahl (1987a,b) (in the absence of anisole). Care was taken to prevent sample heating at all steps of the procedure. Briefly, Teflon screw-top tubes containing lyophilized mucin (30–300 mg), dried under P_2O_5, were cooled in an ethanol/dry ice bath while being purged with dry N_2. Ice-cold TFMSA/anisole reagent, combined immediately before use, was then added. The cold mucin/reagent mixture was allowed to warm to 0 °C with shaking. After the appropriate incubation time, the reaction mixture was cooled on dry ice and 2 volumes of precooled anhydrous diethyl ether was added. This mixture was added slowly to 25 mL of a frozen slush of 60% pyridine (per 10 g of TFMSA used). The solution was warmed to room temperature and extracted several times with ether, and the aqueous phase was dialyzed and lyophilized. Treatments of mucin with TFMSA in the absence of anisole (Sojar & Bahl, 1987a,b) were performed identically as above with the omission of anisole. The incubation temperatures, time, and ratios of TFMSA to mucin varied as described in the text and figure legends.

Mucin Deglycosylation by Oxidation–Elimination. The oxidation–elimination reaction is performed in our laboratory as follows. OSM (or other mucin-containing unsubstituted C3 GalNAc), 10 mg/mL (or less), is solubilized overnight in 0.33 M NaCl at 4 °C. Acetic acid is added to 0.1 M, and the pH is adjusted to 4.5 with 1 M NaOH. The oxidation is begun by adding ice-cold 200 mM $NaIO_4$ to a final concentration of 100 mM $NaIO_4$ and allowing the solution to stand in the dark at 0–4 °C for either 5 h or overnight. Following the oxidation, unreacted periodate is destroyed by adding $^1/_2$ volume of 400 mM $Na_2S_2O_3$/100 mM NaI/100 mM $NaHCO_3$ (final concentration 133 mM $Na_2S_2O_3$). The elimination is begun by adding 1 M NaOH to pH 10.5. After standing for 1 h in the cold while the pH is maintained, the solution is dialyzed overnight at 4 °C against cold 2–5 mM $NaHCO_3$ buffer, pH ca. 10.5. After being dialyzed against deionized water, the sample is lyophilized.

pH Dependence of Elimination Reaction. For these experiments OSM (100–150 mg) was oxidized and excess periodate was destroyed as described above except that during and following the quenching reaction the pH was maintained

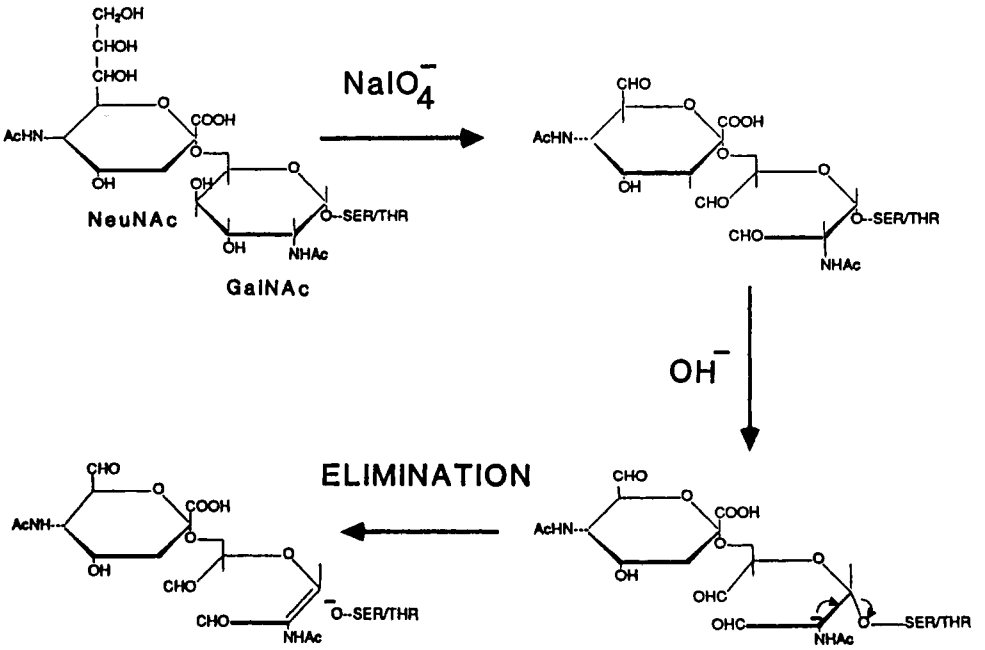

FIGURE 1: Proposed reaction scheme for the oxidation–elimination reaction using the O-linked disaccharide side chain of OSM as a model. Note that the GalNAc C3 aldehyde is required for the β-elimination to proceed.

at values in the range of 8–11 by manual titration with 1 M HCl and/or 1 M NaOH. After the pH was monitored for 1–2 h, the incubation mixtures were stored overnight at 4 °C. The next morning, after the pH was measured, any remaining oxidized carbohydrate side chains were stabilized against further elimination by reducing the free aldehydes to the alcohol with the rapid addition of 0.5 g of $NaBH_4$ in 5 mL of 1 M Na_2HPO_4 at 0 °C. After 1 h, excess borohydride was destroyed by titrating with dilute acetic acid (to pH 7), and the samples were dialyzed, lyophilized, and analyzed by ^{13}C NMR spectroscopy.

Time Course of Elimination Reaction. Periodate-oxidized OSM was prepared as described above, and excess periodate was destroyed. The mucin was incubated for 0, 5, 20, and 60 min and overnight at pH 10.4. After the indicated times (except for the overnight incubation) the elimination was stopped by reducing the remaining aldehydes with $NaBH_4$ as described above. The modified mucins were dialyzed, lyophilized, and analyzed by ^{13}C NMR spectroscopy.

NMR Analysis of pH and Time Dependence. The loss of the OSM disaccharide side chain was quantitated from the peak heights of the periodate-oxidized NeuNAc residue after normalizing to the height of the invariant Gly α-carbon resonance. These NeuNAc ring carbon resonances shift little upon borohydride reduction and show no intermediate chemical shift species after partial elimination, as found for the oxidized GalNAc residue (see Figure 2 and Results). On this basis, the sialic acid resonances are the most reliable monitors of the OSM carbohydrate content. The heights of the NeuNAc resonances in an immediately reduced, noneliminated preparation (Figure 2B) were taken as 100% glycosylation.

Results

Deglycosylation of OSM

OSM is chosen as a model mucin for preliminary studies of the oxidation–elimination reaction because it has a relatively simple and defined carbohydrate structure, in which 95% of the side chains are the disaccharide α-NeuNAc(2–6)α-GalNAc, with the remaining side chains GalNAc alone. In both of these side chains the GalNAc is unsubstituted at both C3 and C4 and thus susceptible to periodate oxidation and subsequent elimination. The reaction scheme for the elimination reaction for OSM is shown in Figure 1. The first step in the reaction sequence is the generation of the C3,C4 dialdehyde by periodate oxidation of the linkage GalNAc. Subsequently, under alkaline conditions, this derivative undergoes a β-elimination leaving an unsaturated oxidized carbohydrate moiety and an unmodified peptide core.

The courses of the oxidation and elimination reaction sequences were studied by natural abundance carbon-13 NMR spectroscopy (Gerken & Dearborn, 1984; Gerken et al., 1989). Figure 2A shows the NMR spectrum of intact mucin while Figure 2B shows the spectrum of mucin treated with periodate and then immediately reduced with borohydride, conditions under which minimal carbohydrate is eliminated. This derivative shows the loss of the C6 and C7 NeuNAc resonances and altered chemical shifts for the C1–C5 resonances of GalNAc demonstrating the complete periodate oxidation (and reduction) of both NeuNAc and GalNAc. The spectrum of fully deglycosylated apo-OSM obtained after periodate oxidation and β-elimination of native OSM is given in Figure 2D. The complete absence of carbohydrate is apparent from the loss of the anomeric carbon resonances (ca. 100 ppm), the carbohydrate ring carbon resonances (80–40 ppm), the NeuNAc C3 resonance (41 ppm), and the carbohydrate *N*-acetyl, carbonyl, and methyl carbon resonances (175 and 24 ppm, respectively). The loss of O-linked carbohydrate is confirmed by the upfield shifts in the α- and β-carbon resonances of Ser and Thr which are sensitive to their glycosylation state (Gerken et al. 1989) and typical of intact undegraded Ser and Thr residues. In addition, no new resonances appear in the spectral region where unsaturated Ser/Thr carbon resonances would appear (ca. 115 and 136 ppm, data not shown) if the more common β-elimination reaction were to occur (Carlson, 1968; Plantner & Carlson, 1972). This is confirmed by the amino acid analysis (Table I) of fully deglycosylated OSM which reveals no change in the amino acid composition of the peptide after oxidation and β-elimination.

pH Dependence of Elimination. After overnight incubation at pH 8.4, more than 40% of the carbohydrate side chains remain compared to 12% at pH 10.0 and 4% at pH 10.8. Dialyzing the sample against pH 10.5 buffer during the initial

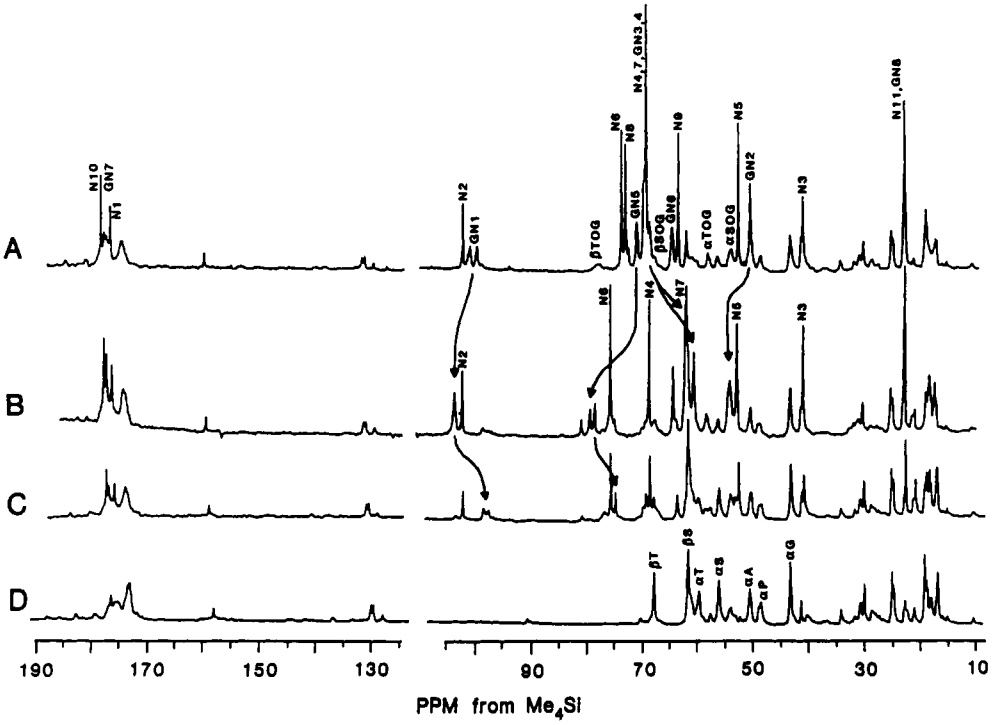

FIGURE 2: Natural abundance carbon-13 NMR spectra of native and modified OSM demonstrating the loss of carbohydrate following alkaline treatment of periodate oxidized OSM. (A) Native OSM; (B) oxidized OSM followed by immediate borohydride reduction; (C) oxidized OSM followed by a 5-min incubation at pH 10.4 and subsequent borohydride reduction; (D) oxidized OSM incubated at pH 10.4 for 1 h and exhaustively dialyzed (giving apo-OSM). Carbon resonances assigned to the GalNAc and NeuNAc residues are prefaced by GN and N, respectively (Gerken & Dearborn, 1984). Selected peptide resonances are also labeled while glycosylated Ser/Thr residues are indicated as SOG and TOG, respectively. Arrows between spectra indicate the proposed resonance shifts for the GalNAc residue as a function of mucin modification (Gerken et al., 1989). The spectra are plotted with nearly constant α-Gly carbon resonance intensities (44 ppm) to demonstrate the loss of carbohydrate.

Table I: Amino Acid Analyses of Mucins Deglycosylated by Trifluoromethanesulfonic Acid (TFMSA) and Oxidation–Elimination (OxE)

	residues/100								
	OSM[a,g]		PSM[a]				HTBM[b]		
amino acid	native	OxE	native[c]	TFMSA,[d] O °C	TFMSA,[e] 25 °C	TFMSA,[f] OxE	native	TFMSA,[d] O °C	TFMSA,[f] OxE
Asx	2.1	1.9	2.8	1.9	0.8	0.7	3.8	3.0	3.5
Glx	4.8	4.6	7.1	5.3	4.9	5.4	5.9	5.0	5.3
Ser	17.6	17.6	20.3	20.1	21.9	22.8	12.8	15.7	15.4
Gly	20.2	20.5	16.6	18.1	20.7	21.0	8.6	7.2	6.9
His	0.2	0.2	0.7	0.6	0.1	0.1	1.0	1.9	1.3
Thr	13.0	13.1	13.5	14.2	13.8	15.2	24.3	29.1	30.3
Ala	12.4	12.4	14.7	14.4	16.7	15.5	10.2	9.8	9.8
Arg	3.8	3.8	3.8	2.8	2.9	2.3	3.2	2.7	2.4
Pro	9.7	9.9	7.3	6.5	6.6	6.5	10.9	11.2	11.9
Tyr	0.8	0.7	0.4	0.7	0.6	0.8	4.0	1.1	0.7
Val	5.8	5.8	9.0	7.6	7.2	6.2	5.2	4.7	4.8
Met	0	0	nr	0.1	0	1.1	0.2	0.4	0.4
Ile	1.3	1.3	2.3	2.9	2.5	2.1	2.3	2.2	2.0
Leu	3.5	3.4	1.6	2.6	0.6	0.4	4.1	3.4	3.3
Phe	1.5	1.5	0.8	0.9	0.5	0.2	2.0	1.4	1.2
Lys	3.3	3.2	1.2	1.6	0.6	0.1	1.6	1.3	1.1

[a] Average of duplicate determinations; nr, not reported [b] Average of duplicate determinations from two or more different HTBM pools. [c] Values from Gupta and Jentoft (1989). [d] TFMSA reaction time was ca. 4 h at 0 °C. [e] TFMSA reaction time was ca. 7 h at 25 °C. [f] The treatments were 4-h TFMSA or 2 × 4-h TFMSA treatments (0 °C) followed by oxidation–elimination. [g] Samples were Sephacryl S-1000 fractionated, reduced, and carboxymethylated OSM.

overnight incubation, followed by an exhaustive dialysis, maximized the loss of carbohydrate. Typically 2% or less carbohydrate remains after such a treatment.

Time Course and Reaction Intermediate of Elimination. The time course of the elimination reaction was monitored by ^{13}C NMR; spectra of the 0- and 5-min time points are given in Figure 2B,C. At 5 and 60 min (data not shown), 42 and 15% of the carbohydrate remain as determined from NeuNAc carbon resonances. Surprisingly, the resonances assigned to the GalNAc residue in the derivative which was reduced immediately (Figure 2B) were nearly absent in the samples incubated at pH 10.4 for 5 min or longer (Figure 2C and data not shown). Instead, a new set of resonances appeared which were also weakly observed in the rapidly reduced sample. The chemical shift patterns suggest that these new resonances represent an intermediate GalNAc species formed after short incubation at elevated pH. This intermediate most likely represents the intramolecular condensation product of the GalNAc C3,C4 dialdehyde giving a relatively stable cyclic acetal derivative (Goldstein et al., 1965). This 7-member ring acetal, in equilibrium with the free dialdehyde, would be expected to be somewhat less stable than the 6-member ring

acetals readily obtained after periodate oxidation of unsubstituted pyranosides (Goldstein et al., 1965). The formation of the acetal, which would not be expected to undergo β-elimination, is consistent with the observed biphasic nature of the rate of carbohydrate loss (data not shown). Similar intermediate and elimination kinetics are also observed when asialo-OSM is deglycosylated (data not shown). Thus, in spite of the relatively rapid initial loss of carbohydrate, the formation of the acetal and subsequent low equilibrium concentration of the free dialdehyde make an overnight incubation necessary for maximal removal of carbohydrate.

The chemical shifts of the modified NeuNAc resonances remain constant throughout the course of the elimination (Figure 2B,C and data not shown), indicating that the oxidized NeuNAc residues are unaffected and remain linked to the peptide core through the oxidized GalNAc residues. These features are most clearly observed in the anomeric carbon region (100 ppm) and in the carbohydrate ring carbon resonances (40–80 ppm).

Effects of Deglycosylation Procedures on Yield and Molecular Weight. OSM was used to compare the oxidation–elimination reaction with the separate and combined use of TFMSA/anisole for deglycosylating mucins with substituents at GalNAc C3. OSM is well suited for this comparison since it can be completely deglycosylated by (1) a single-step oxidation–elimination reaction; (2) a two-step procedure using TFMSA/anisole at 0 °C [leaving intact GalNAc residues (Gerken, 1986)], followed by oxidation–elimination; or (3) using TFMSA/anisole at 25 °C (Gerken, 1986; Shogren et al., 1989). Partial deglycosylations using TFMSA in the absence of anisole were also included in these comparisons (Sojar & Bahl, 1987a,b).

To reduce the inherently large molecular weight heterogeneity of standard preparations of OSM and to eliminate the possibility of cleaving disulfide bonds by periodate (Clamp & Hough, 1965), the mucin used in these studies was reduced, carboxymethylated, and molecular weight fractionated by Sephacryl S-1000 gel filtration chromatography prior to deglycosylation. The highest molecular weight fraction was selected and chromatographed on Sephacryl S-1000 (Figure 3A). This fraction displays a broad molecular weight distribution, typical for mucins, centered at approximately 1700 $\times 10^3$ on the basis of the Sephacryl S-1000 calibrations of Shogren et al. (1987). Light-scattering studies of similarly derived fractions gave identical molecular weights (R. Gupta, unpublished data). This fraction is expected to give an apomucin with a molecular weight distribution centered around 730 $\times 10^3$, calculated on the basis of a native mucin protein content of 44%.

The yields of partially or fully deglycosylated products (i.e., asialo- or apomucin), obtained by subjecting OSM to various deglycosylation procedures, are listed in Table II. Treatments of OSM with TFMSA alone or in combination with anisole at 0 °C resulted in 80–90% recovery of asialo-OSM product.[2] However, treatment with TFMSA at 25 °C, conditions required for removing 90% of the GalNAc (Gerken, 1986; Shogren et al., 1989), provided only a 24% yield. In contrast to these two approaches, the oxidation–elimination procedures alone or following mild TFMSA treatment gave nearly 100% yields, while completely removing nearly all carbohydrate.

[2] The peptide-linked GalNAc residues of asialo-OSM are resistant to cleavage by TFMSA/anisole even after overnight incubations at 0 °C (Gerken, 1986). Our studies with PSM (data not shown) also show that at 0 °C TFMSA in the absence of anisole removes very little of the peptide-linked GalNAc.

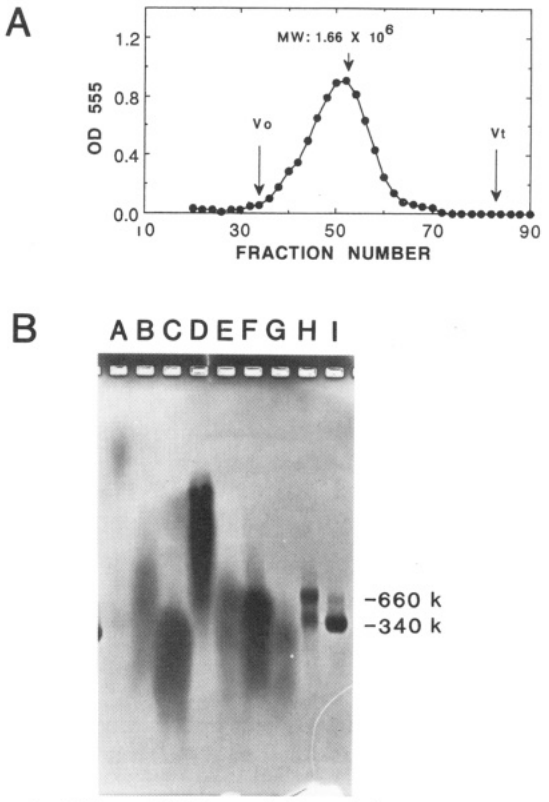

FIGURE 3: Effects of different deglycosylation treatments on the apparent molecular weight of fractionated S-carboxymethylated-OSM. (A) Sephacryl S-1000 gel filtration chromatograph of the fraction prior to deglycosylation. Mucin was monitored by periodic acid–Schiff at 555 nm (Mantle & Allen, 1978). (B) Analysis of the relative sizes of the deglycosylation products by sodium dodecyl sulfate agarose gel electrophoresis after the following treatments: lane A, initial untreated OSM; lane B, apo-OSM obtained after oxidation–elimination; lane C, apo-OSM obtained after treatment with TFMSA (10 g) and anisole (3 mL), 7 h at 25 °C; lane D, asialo-OSM obtained after treatment with TFMSA (10 g) and anisole (3 mL), 4 h at 0 °C; lane E, asialo-OSM obtained after treatment with TFMSA (10 g), 2 h at 0 °C; lane F, apo-OSM obtained after oxidation–elimination of the asialo-OSM in lane D; lane G, apo-OSM obtained after oxidation–elimination of the asialo-OSM in lane E. Lanes H and I are molecular weight markers of 660 $\times 10^3$ (thyroglobulin) and 340 $\times 10^3$ (fibrinogen), respectively. Gels were stained by both periodic acid–Schiff and Coomassie blue. See the text, footnotes 3 and 4, and Table II for further details.

Table II: Comparative Yields of Various Deglycosylation Procedures on OSM[a]

deglycosylation method	percent of theoretical yield[b]
Native → Apomucin	
(1) oxidation–elimination	127
(2) TFMSA + anisole, 25 °C, 7 h	24
Native → Asialomucin	
(3) TFMSA + anisole, 0 °C, 4 h	86
(4) TFMSA, 0 °C, 2 h	90
Asialomucin → Apomucin	
(5) oxidation–elimination of 3	72
(6) oxidation–elimination of 4	110
Native → Apomucin	
(7) combined steps 3 and 5	62
(8) combined steps 4 and 6	99

[a] OSM was fractionated on Sephacryl S-1000 after reduction and S-carboxymethylation as shown in Figure 3A. A 40-mg aliquot of fractionated mucin was used for each initial deglycosylation step. [b] Calculated for the indicated reaction based on the amino acid composition and 75% glycosylation of the Ser and Thr residues with the disaccharide (Gerken & Dearborn, 1984).

Table III: Carbohydrate Analyses of Sequentially Deglycosylated PSM

PSM treatment	% CHO[a]	GalNAc/protein[b]	mole ratio			
			GalNAc	Fuc	NeuNGl	Gal
native (A)[c]	67	0.73	1.00	0.43	0.62	0.73
TFMSA/anisole, 0 °C, 4.5 h (B)	31	0.44	1.00	0	0	0.05
TFMSA/anisole, 25 °C, 7 h (C)	8	0.085	1.00	0	0	0.08
OxE of B (D)	0.7	0.007	1.00	0	0	0

[a] Weight percent of carbohydrate based on quantitative amino acid and carbohydrate analyses. [b] Milligrams of GalNAc per milligram of protein. [c] The letter in parentheses refers to the PSM spectra in Figure 4.

To compare the extent of mucin peptide core degradation caused by the various deglycosylation approaches, the products described in Table II were analyzed by SDS agarose gel electrophoresis (Figure 3B). Since the partially deglycosylated mucins will likely exhibit altered migration behavior (depending on their carbohydrate contents), we will discuss only the fully deglycosylated derivatives and those with similar carbohydrate side-chain structures.[3]

The electrophoretic patterns of fully deglycosylated apo-OSM derivatives are shown in lanes B, C, E, and F of Figure 3B.[4] The highest molecular weight apomucin was obtained after a single oxidation–elimination of OSM (lane B), while the lowest molecular weight apomucin was obtained after treatment with TFMSA/anisole at 25 °C (lane C). We estimate that these differently prepared apomucins have broad molecular weight distributions centered around 700×10^3 and 300×10^3, respectively. In the absence of peptide cleavage, broad ranges in apomucin molecular weight are expected since the gel filtration profile of the initial mucin preparation shows significant molecular weight polydispersity (Figure 3A). Apomucins derived from OSM previously treated at 0 °C with TFMSA in the presence or absence of anisole (lanes F and G) gave molecular weights intermediate to the above values, with OSM treated with TFMSA in the presence of anisole showing less peptide degradation (lane F).

The estimated size, 700×10^3 Da, of the apomucin obtained after a single oxidation–elimination (lane B) was nearly identical to the calculated size of the protein core of the starting mucin (730×10^3 Da). It appears, therefore, that minimal to no peptide core cleavage results from the use of the oxidation–elimination reaction. TFMSA appears to degrade the peptide core even at 0 °C, although the degradation is reduced in the presence of anisole. This was shown by the migration behavior of both the asialo-OSM and apomucin derivatives obtained directly after TFMSA treatment (lanes D and E) or after complete deglycosylation by oxidation–elimination (lanes F and G), respectively. On the basis of these results and our observations that the products isolated after treatment with TFMSA in the absence of anisole are yellow to brown in color, we recommend that anisole be included when partially deglycosylating mucins with TFMSA.

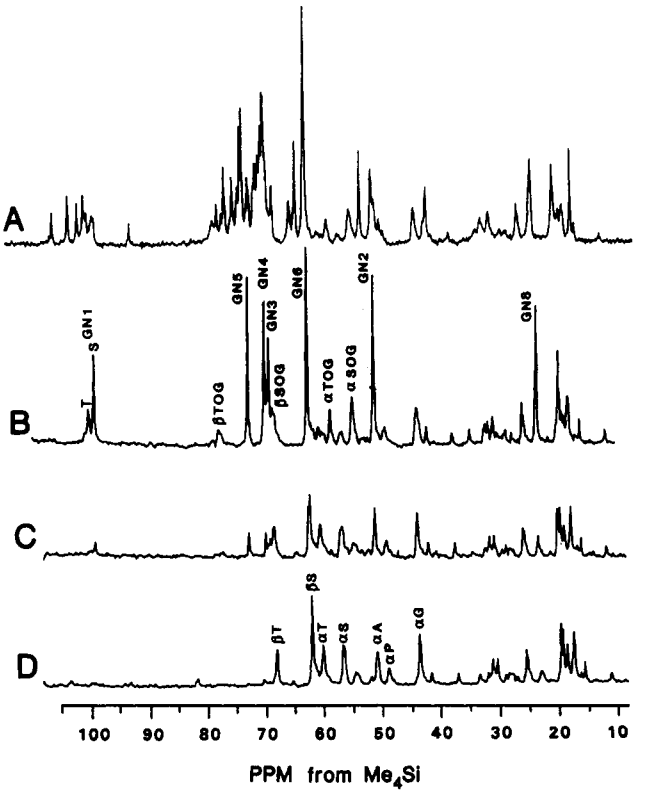

FIGURE 4: Partial carbon-13 NMR spectra of native and sequentially deglycosylated PSM. (A) Initial native PSM; (B) PSM partially deglycosylated with TFMSA/anisole, 0 °C, 4.5 h (PSM 300 mg, TFMA 10 g, anisole 3 mL); (C) PSM deglycosylated with TFMSA/anisole, 25 °C, 7 h (PSM 300 mg, TFMSA 10 g, anisole 3 mL); (D) PSM from part B fully deglycosylated by oxidation–elimination. Resonances are labeled as described in Figure 2. The PSM used for these experiments was estimated to have, on the basis of its ^{13}C NMR spectrum (Gerken & Jentoft, 1987), an oligosaccharide side-chain composition of 25% α-GalNAc; 28% β-Gal(1–3)α-GalNAc; 32% α-Fuc(1–2)β-Gal(1–3)α-GalNAc; and 15% α-Fuc(1–2)[α-GalNAc(1–3)]β-Gal(1–3)α-GalNAc, with 79% of the side chains containing *N*-glycoloylneuraminic acid (NeuNGl). From this distribution, an average side-chain length of 3.1 residues is obtained.

Deglycosylation of PSM

Porcine submaxillary mucin (PSM) was used as a model to test the combined approach of TFMSA/anisole followed by oxidation–elimination for deglycosylating mucins containing substituents at C3 of the peptide-linked GalNAc residue. PSM contains a range of carbohydrate side-chain structures which are derived from the incomplete biosynthesis of the blood group

[3] It should be noted that the mucin carbohydrate side chains have been found to significantly alter the conformation of the mucin peptide core (Shogren et al., 1989). These sterically based conformational changes (due to the first one or two carbohydrate residues of each side chain) may significantly affect the molecular weight determinations for native and partially deglycosylated mucins, by electrophoretic and chromatographic methods. Interactions with SDS can also be altered by the differences in carbohydrate content. Molecular weight estimates are likely to be valid only for completely deglycosylated apomucins since the gels were calibrated using nonglycosylated proteins. For native and partially deglycosylated OSM, valid comparisons of relative molecular sizes can only be made when those species are compared with similar carbohydrate contents or conformations [see Shogren et al. (1989) for further discussion].

[4] Some aspects of gel staining behavior (Figure 3B) are worth noting. First, native mucin (lane A) is strongly stained by periodic acid Schiff (PAS) but not by Coomassie blue. Second, asialomucin is not stained with PAS but is stained with Coomassie blue. This difference in PAS staining behavior probably reflects the formation of the nonreactive GalNAc acetal derivative. Finally, we note that the Coomassie blue staining appears to be strongest for those products where anisole was included in the deglycosylation procedure, perhaps due to trace amounts of anisole that were not removed from the deglycosylated mucin by dialysis.

A oligosaccharide α-GalNAc(1–3)[α-Fuc(1–2)]β-Gal(1–3)[α-NeuNGl(2–6)]α-GalNAc-O-Ser/Thr (Carlson, 1968). The carbon-13 NMR spectrum of native PSM and its oligosaccharide side-chain composition (Gerken & Jentoft, 1987) are given in Figure 4. The chemically derived carbohydrate composition, weight percent of carbohydrate, and ratio of GalNAc to protein are given in Table III.

As found for OSM, the treatment of PSM with TFMSA with anisole for 4.5 h at 0 °C gives a product with all carbohydrate removed except for the peptide-linked GalNAc residues (Table III). The NMR spectrum of this derivative (Figure 4B) is almost indistinguishable from asialo-OSM (Gerken & Dearborn 1984; Gerken et al., 1989), with no loss of the peptide-linked GalNAc. PSM treated with TFMSA and anisole for 7 h at room temperature results in more complete removal of the carbohydrate with the loss of approximately 80% of the peptide-linked GalNAc residues on the basis of the GalNAc/protein ratios (Table III) and its NMR spectrum (Figure 4C).

Fully deglycosylated PSM is obtained after oxidation–elimination of the product obtained following incubation with TFMSA/anisole for 4.5 h at 0 °C. Essentially complete removal of carbohydrate is shown from its ^{13}C NMR spectrum (Figure 4D) and percent carbohydrate and GalNAc/protein ratio (Table III).

The amino acid compositions of deglycosylated PSM derivatives are given in Table I. The amino acid compositions of these derivatives are nearly identical, although variable losses in the Asx, Glx, His, Leu, and Lys content are noted after the treatments with TFMSA/anisole. Decreases in the contents of Asx, His, Leu, and Lys are also detected after the oxidation–elimination reaction. These differences may arise because the PSM pool was not purified by Sephacryl S-1000 chromatography and hence contains trace non-mucin contaminating proteins. Nevertheless, the results demonstrate the feasibility of using a mild, 0 °C, TFMSA/anisole treatment followed by oxidation–elimination for deglycosylating O-linked oligosaccharides with C3 substituents at the peptide-linked GalNAc.

Deglycosylation of Human Tracheobronchial Mucin

The carbohydrate side chains of the mucins from the respiratory and digestive tracts are significantly larger and more complex than the side chains of either porcine or ovine submaxillary mucins. Therefore, human tracheobronchial mucin (HTBM) isolated from cystic fibrosis patients was used to assess the utility of the TFMSA/oxidation–elimination approach for deglycosylating more complex mucins. Several attempts to deglycosylate tracheobronchial mucins via TFMSA alone have been reported (Marianne et al., 1986; Woodward et al., 1987; Ringler et al., 1988; Perini et al., 1989; Bhattacharyya et al., 1990b), with the retention of significant amounts of GalNAc and GlcNAc in these products. As shown below, similar results are obtained in our hands with TFMSA-treated HTBM. We have characterized the TFMSA-resistant oligosaccharide side chains and employed additional approaches for maximizing the removal of the carbohydrate from HTBM.

The carbohydrate analysis and carbon-13 NMR spectrum of native HTBM are given in Table IV and Figure 5A. Consistent with its approximately 85% carbohydrate content, its NMR spectrum is dominated by the carbohydrate carbon resonances with few distinguishable peptide core α- or β-carbon resonances.

A single treatment of HTBM with TFMSA and anisole at 0 °C results in surprisingly little carbohydrate removal (Table

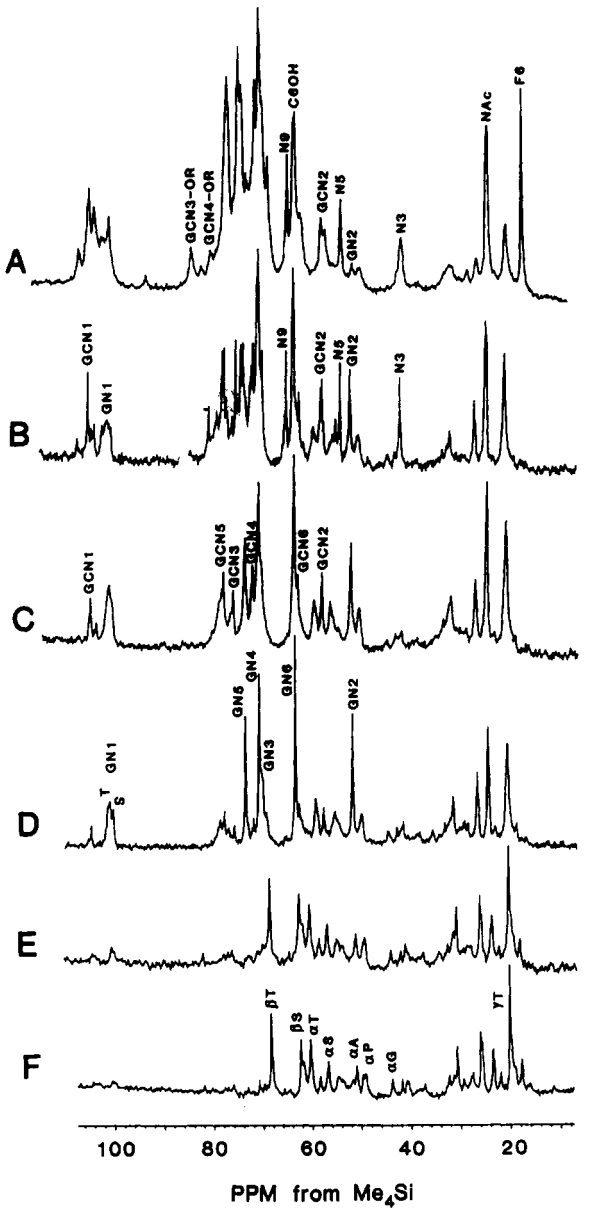

FIGURE 5: Partial carbon-13 NMR spectra of native and sequentially deglycosylated human tracheobronchial mucin (HTBM). (A) Native HTBM; (B) native HTBM partially deglycosylated with TFMSA/anisole (HTBM 170 mg, TFMSA 10 g, anisole 3 mL, 4 h at 0 °C); (C) asialo-HTBM partially deglycosylated with TFMSA/anisole (HTBM 130 mg, TFMSA 20 g, anisole 6 mL, 4 h at 0 °C); (D) asialo-HTBM partially deglycosylated with two consecutive TFMSA/anisole treatments (first, HTBM 84 mg, TFMSA 20 g, anisole 6 mL, 4 h at 0 °C; second, HTBM 27 mg, TFMSA 10 g, anisole 3 mL, 4 h at 0 °C); (E) oxidation–elimination of sample D; (F) similar to E after a second oxidation–elimination. Note that the vertical scales are approximately 2-fold attenuated in spectrum A compared to the remaining spectra. Abbreviations used to label the resonances in spectra A through E: N, NeuNAc; GN, GalNAc; GCN, GlcNAc; G, Gal; F, Fuc. In spectrum F, standard single-letter amino acid abbreviations are used. Readily identified resonances in native mucin are the NeuNAc C9, C5, and C3 resonances (63, 52, and 40 ppm) which are absent in asialomucin (spectrum not shown). Other resonances are the GlcNAc C2 (57 ppm, with fine structure reflecting substitutions at C3 and C4), the GalNAc C2 (51 ppm), the carbohydrate *N*-acetyl methyl and C6 hydroxymethylene carbons (25 and 62 ppm), and the Fuc C6 methyl group (16 ppm). Tentative assignments of the GlcNAc C3 and C4 aglycon carbons, involved in glycosidic linkages, are also indicated [83 and 80 ppm, respectively (Sabesan & Paulson, 1986)] while the resonance at 93 ppm is assigned to the C1 of the terminal GalNAc residue in the A blood group determinant, as in PSM (Gerken & Jentoft, 1987).

Table IV: Carbohydrate Analyses of Sequentially Deglycosylated HTBM

HTBM treatment	% CHO[a]	GalNAc/ protein[b]	mole ratio				
			GalNAc	GlcNAc	Fuc	NeuNAc	Gal
native HTBM (A)[c]	85	0.72	1.00	2.33	1.67	0.67	2.58
asialo-HTBM	nd	nd	1.00	2.00	1.13	0.07	3.00
TFMSA/anisole (native HTBM), 0 °C, 4 h (B)	63	0.77	1.00	0.55	0	0.25	0.50
TFMSA/anisole (asialo-HTBM), 0 °C, 4 h (C)	52	0.72	1.00	0.46	0	0	0.08
TFMSA/anisole (asialo-HTBM), 0 °C, 8 h	nd	nd	1.00	0.24	0	0	0.09
2X TFMSA/anisole (asialo-HTBM), 0 °C, 4 h X 2 (D)	36	0.47	1.00	0.13	0	0.02	0.04
OxE (2X TFMSA/anisole asialo-HTBM) (E)	11	0.10	1.00	0.30	0	0	0
2X OxE (2X TFMSA/anisole asialo-HTBM) (F)	5	0.04	1.00	0.18	0	0	0.17

[a] Weight percent of carbohydrate based on quantitative amino acid and carbohydrate analyses; nd, not determined. [b] Milligrams of GalNAc per milligram of protein. [c] The letter in parentheses refers to the HTBM spectra in Figure 5.

IV and Figure 5B). Significant amounts of NeuNAc remain. This unusual resistance of the typically acid-labile sialic acid residue with TFMSA was also reported for canine and human tracheobronchial mucins (Woodward et al., 1987; Ringler et al., 1988) and bovine submaxillary mucin (Bhavanandan & Hegarty, 1987). The carbohydrate analysis and ^{13}C NMR chemical shifts of TFMSA-treated native mucin indicate that α-NeuNAc(2–3)β-Gal disaccharide units are present, which are linked directly either via 1–3 linkages to α-GalNAc or via 1–4 linkages to β-GlcNAc. On the basis of NMR spectral simulations (data not shown), an oligosaccharide distribution consisting of both linear and branched tetrasaccharides [α-NeuNAc(2–3)β-Gal(1–4)β-GlcNAc(1–3 or –6)α-GalNAc and α-NeuNAc(2–3)β-Gal(1–3)[β-GlcNAc(1–6)]α-GalNAc, respectively] and the GalNAc monosaccharide is consistent with the observed NMR spectrum (Sabesan & Paulson, 1986; Bradbury & Jenkins, 1984; Berman, 1984; Gerken & Jentoft, 1987). These oligosaccharide structures are readily derived from the sialylated core structures reported for HTBM (Lamblin et al., 1984; Breg et al., 1987) after sequential cleavage of terminal Fuc, Gal, and GlcNAc residues. Oligosaccharides with 1–3 linkages to β-GlcNAc are lost after the TFMSA treatment since the resonance for the C3 carbon (83 ppm) of C3 substituted β-GlcNAc residues is absent from the spectrum (compare (A) and (B) of Figure 5).

The longer oligosaccharide side chains remaining after mild (0 °C) TFMSA/anisole treatment terminate in the α-NeuNAc(2–3)β-Gal linkage. Since the NeuNAc residue apparently blocks the removal of these side chains, a pretreatment with neuraminidase is performed prior to the use of TFMSA/anisole. This yields an increased removal of carbohydrate with only GalNAc and smaller amounts of GlcNAc remaining (Figure 5C and Table IV). The NMR spectra of this derivative (and others not shown) reveals that the remaining carbohydrate side chains consist of the peptide-linked GalNAc residue and the disaccharide β-GlcNAc(1–3 and –6)α-GalNAc (with equal proportions of the 1–3 and 1–6 linkages). Table IV and the NMR spectra in Figure 5C,D illustrate that by increasing the length of time of the mild TFMSA/anisole treatment or by repeating the treatment a second time, the amount of β-GlcNAc remaining decreases. The percent of protein and GalNAc/protein ratio for the twice-TFMSA-treated derivative were similar to that of PSM after mild TFMSA/anisole treatment (Tables III and IV), further showing that the peptide-linked GalNAc residue is not easily removed by repeated mild TFMSA/anisole treatments.

HTBM is almost completely deglycosylated following oxidation–elimination of asialo-HTBM twice-treated with TFMSA/anisole at 0 °C (Figure 5E and Table IV). The carbon-13 NMR spectrum of this material is very similar to the spectra of apo-OSM and apo-PSM (Figures 2D and 4D) with the major differences due to relative amino acid contents. From the percent carbohydrate values (Table IV) only 2–3% of the original HTBM carbohydrate remains principally as β-Gal(1–3)α-GalNAc side chains that are resistant to oxidation. The equal molar ratio of GalNAc to GlcNAc expected for this disaccharide side chain, however, is not observed in the carbohydrate analysis or from the anomeric carbon resonances (ca. 100 and 103 ppm for α-GalNAc and β-GlcNAc, respectively). This is most likely due to partial destruction of GlcNAc by periodate and the β-elimination of GlcNAc from GalNAc. This would free additional peptide GalNAc residues for subsequent oxidation–elimination. This is confirmed since repeating the procedure gives an additional 60% decrease in GalNAc content (Table IV) and a further decrease in the area of the GalNAc anomeric carbon resonance (Figure 5F). After this treatment, less than 1% of the original HTBM carbohydrate remains.

The amino acid analyses of native HTBM and HTBM following TFMSA and oxidation–elimination treatments are given in Table I. Ser and Thr contents are elevated at the expense of Tyr and Phe after the TFMSA treatments while oxidation–elimination causes only small changes in composition. It is likely that the repeated TFMSA treatments may degrade the nonglycosylated portions of the HTBM peptide core, since similar elevations in Ser and Thr are observed after limited proteolytic digestions of native HTBM (Gupta & Jentoft, 1992).

Discussion

The most commonly used method for removing O-linked carbohydrate from glycoproteins, and mucins in particular, is that of Edge et al. (1981) utilizing anhydrous TFMSA in the presence of anisole. At 0 °C, this method provides minimal degradation of the peptide core but is not effective for removing the peptide-linked GalNAc residues. More vigorous reaction conditions give more complete removal of carbohydrate at the cost of extensive peptide core degradation. In earlier efforts to reduce peptide core degradation, less vigorous 0 °C TFMSA treatments were employed and the resistant GalNAc residues removed by anhydrous HF or exo-*N*-acetylgalactosaminidase (Perini et al., 1989; Woodward et al., 1987). These approaches are potentially difficult or very costly and are still capable of degrading the peptide core. The enzymatic approach is further hampered by the impracticality of deglycosylating milligram quantities of glycoprotein, making it is almost impossible to deglycosylate sufficient quantities of complex mucins for physical–chemical studies. Thus, new ways for deglycosylating mucins in high yield and with minimal peptide core degradation are desired. The oxidation–elimination reaction reported herein used in conjunction with TFMSA offers such an approach.

The oxidation–elimination reaction relies on the ability of the C3,C4 periodate-oxidized, peptide-linked GalNAc residue

to undergo β-elimination at elevated pH. The products of the elimination are the unsaturated oxidized oligosaccharide and the intact peptide core Ser or Thr residues (Figure 1). The reaction conditions for the periodate oxidation and elimination steps are relatively mild (pH 4.5 and 10.5, respectively), give product in high yield (Table II), and do not significantly alter the amino acid composition and peptide core molecular weight of highly purified mucins (Table I and Figure 3). Potential sources of peptide core modification are expected only during the periodate oxidation step where the side chains of Trp, Tyr, His, Met, and Cys may be oxidized (Clamp & Hough, 1965). These reactions are very slow and not expected to be significant under the reaction conditions used. Since free sulfhydryl groups and disulfide bonds are more readily oxidized by periodate (Clamp & Hough, 1965), it may be advantageous to S-carboxymethylate mucins prior to oxidation–elimination.

Most mucins and O-linked glycoproteins have more complex oligosaccharide side chains than OSM, having a substituent on C3 of the peptide-linked GalNAc residue. These side chains will be resistant to oxidation–elimination. To remove these more complex oligosaccharide side chains, TFMSA under mild conditions is used to first trim the oligosaccharide side chains of peripheral sugars followed by oxidation–elimination to remove the remaining peptide-linked GalNAc residues. Fully deglycosylated PSM and almost completely deglycosylated HTBM are obtained using this approach (Figures 4 and 5 and Tables III and IV). This procedure exploits a unique feature of TFMSA/anisole in that its use at 0 °C produces significantly less peptide core degradation than at ambient temperatures (Figure 3) while removing most carbohydrate except for the O-linked GalNAc (Figures 4 and 5 and Tables III and IV).

A few comments regarding the large size ($\sim 700 \times 10^3$ Da) and polydispersity of apo-OSM obtained after oxidation–elimination are warranted (Figure 3). The observed high molecular weight and polydispersity are consistent with recent molecular biology studies demonstrating that mucin peptide cores consist of a large and variable number of tandem repeats (Gendler et al., 1990; Gum et al., 1989; Eckhardt et al., 1991). These results contrast with earlier suggestions that mucins are aggregates of relatively small glycopeptides with peptide molecular weights of 58 300 for OSM (Hill et al., 1977), 60 000 for bovine submaxillary mucin (Bhavanandan & Hegarty, 1987), and 96 500 for PSM (Eckhardt et al., 1987). These discrepancies in molecular weights appear to be due to the methodologies employed in mucin purification. Purification strategies in which protease inhibitors are employed are known to give products of higher molecular weight (Carlstedt et al., 1985; Shogren et al., 1986; Gupta & Jentoft 1989) and the incorporation of protease inhibitors in the generation of apomucins similarly results in the generation of much larger peptides (Eckhardt et al., 1991). Thus, the observed size and molecular weight heterogeneity typical of mucins is not due to associative processes based on the interactions of carbohydrate as previously proposed (Hill et al., 1977).

To summarize, a method has been described for removing C3-unsubstituted peptide-linked GalNAc residues from mucins, with minimal peptide core degradation. The effectiveness of this method for deglycosylating the more complex mucins depends on the efficiency of TFMSA in removing C3 substituents from the peptide-linked GalNAc residues. Mucins with α-NeuNAc(2–3)β-Gal linkages may require prior neuraminidase treatment for optimal results since these linkages are relatively resistant to TFMSA (Figure 5 and Table IV). Mucins containing β-GlcNAc 1–3 or 1–6 linkages to the peptide GalNAc residue will also be more difficult to deglycosylate since the GlcNAc residue is only slowly removed by TFMSA at 0 °C. However, with repeated TFMSA treatments and multiple oxidation–elimination reactions a nearly fully deglycosylated mucin is possible. In spite of the problems encountered to free the peptide-linked GalNAc residue of substituents (a necessary step regardless of the deglycosylation approach), the oxidation–elimination reaction is possibly the only method that can completely remove this residue from mucins and other O-linked glycoproteins with virtually no peptide bond cleavage.

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References

Berman, E. (1984) *Biochemistry 23*, 3754–3759.

Bhattacharyya, S. N., Enriquez, J. I., & Manna, B. (1990a) *Inflammation 14*, 93–107.

Bhattacharyya, S. N., Veit, B. C., Manna, B., Enriquez, J. I., Walker, M. P., Khorrami, A. M., & Kaufman, B. (1990b) *Inflammation 14*, 355–373.

Bhavanandan, V. P., & Hegarty, J. D. (1987) *J. Biol. Chem. 262*, 5913–5917.

Bradbury, J. H., & Lenkins, G. A. (1984) *Carbohydr. Res. 126*, 125–156.

Breg, J., Van Halbeek, H., Vliegenthart, J., Lamblin, G., Houvenaghel, M. C., & Roussel, P. (1987) *Eur. J. Biochem. 168*, 57–68.

Byrd, J. C., Lamport, D. T. A., Siddiqui, B., Kuan, S., Erickson, R., Itzkowitz, S. H., & Kim, Y. S. (1989) *Biochem. J. 261*, 617–625.

Carlson, D. M. (1968) *J. Biol. Chem. 243*, 616–626.

Carlstedt, I., Sheehan, J., Corfield, A., & Gallagher, J. (1985) *Essays Biochem. 20*, 40-76.

Clamp J., & Hough, L. (1965) *Biochem. J. 94*, 17–24.

Eckhardt, A. E., Timpte, C. S., Abernethy, J. L., Toumadje, A., Johnson, W. C., & Hill R. L. (1987) *J. Biol. Chem. 262*, 11339–11344.

Eckardt, A. E., Timpte, C. S., Abernethy, J. L., Zhao, Y., & Hill, R. L. (1991) *J. Biol. Chem. 226*, 9678–9686.

Edge, A. S. B., Faltynek, C. R., Hof, L., Reichert, L. E., & Weber, P. (1981) *Anal. Biochem. 118*, 131–137.

Fairbanks, G., Steek, T., & Wallach, D. (1971) *Biochemistry 10*, 2606–2616.

Gendler, S. J., Lancaster, C. A., Taylor-Papadimitriou, J., Duhig, T., Peat, N., Burchell, J., Pemberton, L., Lalami, E. N., & Wilson, D. (1990) *J. Biol. Chem. 265*, 15286–15293.

Gerken, T. A. (1986) *Arch. Biochem. Biophys. 247*, 239–253.

Gerken, T. A., & Dearborn, D. G. (1984) *Biochemistry 23*, 1485–1497.

Gerken, T. A., & Jentoft, N. (1987) *Biochemistry 26*, 4689–4699.

Gerken, T. A., Butenhof, K. J., & Shogren, R. (1989) *Biochemistry 28*, 5536–5543.

Goldstein, F., Hay, G., Lewis, B., & Smith, F. (1965) *Methods Carbohydr. Chem. 5*, 361–370.

Gum, J. R., Byrd, J. C., Hicks, J. W., Toribara, N., Lamport, D. T. A., & Kim, Y. S. (1989) *J. Biol. Chem. 264*, 6480–6487.

Gupta, R., & Jentoft, N. (1989) *J. Chromatogr. 474*, 411–417.

Gupta, R., & Jentoft, N. (1992) *J. Biol. Chem.* (in press).

Gupta, R., Jentoft, N., Jamieson, A., & Blackwell, J. (1990) *Biopolymers 29*, 347-355.
Hill, H. D., Reynolds, J. A., & Hill, R. L. (1977) *J. Biol. Chem. 252*, 3791-3798.
Jentoft, N. (1985) *Anal. Biochem. 148*, 424-433.
Lamblin, G., Boersma, A., Klein, A., Roussel, P., VanHalbeek, H., & Vliegenthart, J. F. G. (1984) *J. Biol. Chem. 259*, 9051-9058.
Mantle, M., & Allen, A. (1978) *Biochem. Soc. Trans. 6*, 607-609.
Marianne, T., Perini, J. M., Houvenaghel, M. C., Tramu, G., Lamblin, G., & Roussel, P. (1986) *Carbohydr. Res. 151*, 7-19.
Mort, A. J., & Lamport, D. T. (1971) *Anal. Biochem. 82*, 289-309.
Perini, J. M., Marianne, T., Lafitte, J. J., Lamblin, G., Roussel, P., & Mazzuca, M. (1989) *J. Histochem. Cytochem. 37*, 869-875.
Plantner, J. J., & Carlson, D. M. (1972) *Methods Enzymol. 28*, 46-48.
Ringler, N. J., Selvakumar, R., Woodward, H. D., Bhavanandan, V. P., & Davidson, E. A. (1988) *Biochemistry 27*, 8056-8063.
Sabesan, S., & Paulson, J. C. (1986) *J. Am. Chem. Soc. 108*, 2068-2080.
Shogren, R., Gerken, T. A., & Jentoft, N. (1989) *Biochemistry 28*, 5525-5536.
Shogren, R. L., Jamieson, A. M., Blackwell, J., & Jentoft, N. (1986) *Biopolymers 25*, 1505-1517.
Shogren, R. L., Jentoft, N., Gerken, T. A., Jamieson, A. M., & Blackwell, J. (1987) *Carbohydr. Res. 160*, 317-327.
Sojar, H. T., & Bahl, O. P. (1987a) *Methods Enzymol. 138*, 341-350.
Sojar, H. T., & Bahl, O. P. (1987b) *Arch. Biochem. Biophys. 295*, 52-57.
Spiro, R. G. (1972) *Methods Enzymol. 28*, 3-45.
Woodward, H. D., Ringler, N. J., Selvakumar, R., Simet, I. M., Bhavanandan, V. P., & Davidson, E. A. (1987) *Biochemistry 26*, 5315-5322.

Scyllatoxin, a Blocker of Ca^{2+}-Activated K^+ Channels: Structure-Function Relationships and Brain Localization of the Binding Sites†

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ABSTRACT: Chemical modifications of scyllatoxin (leiurustoxin I) have shown that two arginines in the sequence, Arg_6 and Arg_{13}, are essential both for binding to the Ca^{2+}-activated K^+ channel protein and for the functional effect of the toxin. His_{31} is important both for the binding activity of the toxin and for the induction of contractions on taenia coli. However, although its iodination drastically decreases the toxin activity, it does not abolish it. Chemical modification of lysine residues or of Glu_{27} does not significantly alter toxin binding, but it drastically decreases potency with respect to contraction of taenia coli. The same observation has been made after chemical modification of the lysine residues. The brain distribution of scyllatoxin binding sites has been analyzed by quantitative autoradiographic analysis. It indicates that apamin (a bee venom toxin) binding sites are colocalized with scyllatoxin binding sites. The results are consonant with the presence of apamin/scyllatoxin binding sites associated with Ca^{2+}-activated K^+ channels. High-affinity binding sites for apamin can be associated with very-high-affinity (<70 pM), high-affinity (~100-500 pM), or moderate-affinity (>800 pM) binding sites for scyllatoxin.

Polypeptide toxins are important tools for the study of ionic channels (Strong, 1990; Betz, 1990; Moczydlowski et al., 1988; Cook & Quast, 1990; Dreyer, 1990). The venom of the scorpion *Leiurus quinquestriatus hebraeus* contains two different types of polypeptide toxins capable of blocking two different types of Ca^{2+}-activated K^+ channels. The first one to be discovered has been called charybdotoxin (Miller et al., 1985; Gimenez-Gallego, 1988). It blocks large-conductance Ca^{2+}-activated K^+ (K_{Ca}) channels (Smith et al., 1986) and also several classes of voltage-sensitive K^+ channels (MacKinnon et al., 1988; Lewis et al., 1988; Schweitz et al., 1989; Stühmer et al., 1989). The second one has been initially called leiurotoxin I (Abia et al., 1986; Chicchi et al., 1988; Moczydlowski et al., 1988; Castle et al., 1989); it blocks a different class of small-conductance Ca^{2+}-activated K^+ channels which has also been shown to be the target of the bee venom toxin apamin (Hugues et al., 1982a,b,d; Blatz & Magleby, 1986; Castle & Strong, 1986; Bernardi et al., 1989; Auguste et al., 1990). Leiurotoxin I does not block charybdotoxin-sensitive K_{Ca} channels and vice versa. Because the name leiurotoxin I induces a possible confusion with classically called *Leiurus* toxins which block the voltage-sensitive Na^+ channel and also to mark clearly that charybdotoxin and leiurotoxin I block two different classes of K_{Ca} channels, we have previously proposed to rename leiurotoxin I scyllatoxin (ScyTx) (Charybdis and Scylla) (Auguste et al., 1990).

ScyTx is 31 amino acids long and has three disulfide bridges (Chicchi et al., 1988; Auguste et al., 1990). The main elements

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