Carbon-13 NMR Studies of Native and Modified Ovine Submaxillary Mucin†

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ABSTRACT: Natural abundance ^{13}C NMR spectroscopy has been used to study the solution structure and dynamics of the ovine submaxillary mucin (OSM). Results at both 45.3 and 67.9 MHz show the extremely viscous mucin to possess sufficient internal segmental flexibility to allow high-resolution ^{13}C NMR studies. Essentially all of the resonances in the spectra have been assigned to individual carbons of the carbohydrate disaccharide side chain α-NeuNAc2→6α-GalNAc-Ser/Thr and to the protonated carbons of the major peptide residues. Spin–lattice relaxation times and nuclear Overhauser enhancements reveal that the internal mobility of the mucin is unaffected by large changes in molecular weight and hence bulk viscosity. On the basis of the relaxation measurements the peptide and carbohydrate side chain mobilities increase stepwise from the glycosylated peptide residue α-carbons to the terminal sialic acid (NeuNAc) side-chain C9 carbon. Removal of the terminal sialic acid C8 and C9 side-chain carbons as well as the complete removal of the NeuNAc residue does not alter the dynamics of the peptide core. However, the removal of carbons C8 and C9 from the NeuNAc residue produces an increase in its ring mobility or conformational flexibility. Complete removal of sialic acid produces an increase in the mobility or flexibility of the GalNAc ring and reduces the chemical shift sensitivity of the GalNAc ring carbons to the different serine and threonine linkages. The pK_a value for the sialic acid carboxyl group in the intact mucin is 2.0, while it increases to 2.4 after the removal of the NeuNAc C8 and C9 side-chain carbons. This change in pK_a confirms the intramolecular hydrogen bond interaction of the C8 hydroxyl with the C2 carboxyl group in the α-NeuNAc residue as previously suggested by Jennings and Bhattacharjee [Jennings, H. J., & Bhattacharjee, A. K. (1977) *Carbohydr. Res. 55*, 105–112]. The relaxation time values and temperature dependence of the chemical shift of the NeuNAc C7 carbon suggest that this group is also involved in an intramolecular interaction. Overall the ^{13}C NMR results indicate that the relatively simple mucous glycoprotein, OSM, is a highly extended and internally flexible molecule which in solution possesses little secondary structure.

Mucous glycoproteins, mucins, are the major protein constituents of the mucous secretions lining the epithelium of the gastrointestinal, urogenital, and respiratory tracts of higher organisms. These large (10^5–10^7 daltons) polyionic glycoproteins are 50% or more by weight carbohydrate and are primarily responsible for the protection-rendering viscoelastic properties of mucous secretions. The carbohydrate side chains are commonly branched and consist of 1–20 (or more) sugar residues which are O-glycosidically linked to the serine and threonine residues of the peptide core through carbon 1 of α-*N*-acetylgalactosamine (GalNAc).[1] Although the actual role of the carbohydrate side chains is not well understood, they are thought to surround the peptide core with an environment of charged and highly hydrated moieties which produce an overall highly expanded and solution-filling structure (Harding et al., 1983). The high content of glycine and proline, found in all mucins, also suggests that the peptide core may be designed to exist in a highly flexible and randomly extended state. These properties, combined with the mucin's high molecular weight and its reported ability to self-aggregate (Hill et al., 1977a), may be the major sources of the physical properties of this class of protein.

Mucins are polydisperse with respect to molecular size and oligosaccharide composition and sequence. This, in addition to their high molecular weights and viscoelastic properties, has made it difficult to study mucins to the same extent as has been achieved with globular proteins. Most studies of mucins have dealt with their bulk physical properties, the chemical elucidation of their oligosaccharide side-chain structures, and to a limited extent studies of the peptide core. No detailed studies of the intramolecular solution structure and interactions of intact or native mucins have been reported. Thus, little is known of the actual solution environment and dynamics of either the mucin peptide or its attached carbohydrate side chains.

Carbon-13 NMR spectroscopy has been extensively used for the elucidation of the structure, conformation, interactions, and dynamics of a wide variety of macromolecules of biological significance. Several ^{13}C NMR studies of glycoproteins have been published (Dill & Allerhand, 1979a; Berman et al., 1980, 1981), but only recently have spectra of the more highly viscous mucous glycoproteins been reported (Barrett-Bee et al., 1982a,b). The latter studies with the hog gastric and dog trachea mucins confirmed that ^{13}C NMR can be useful for the study of the complex mucous glycoproteins. Unfortunately few specific resonances were assigned in these studies, and neither relaxation time nor NOE measurements were reported.

This paper presents the results of a detailed carbon-13 NMR study of the solution structure and dynamics of the relatively

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[1] Abbreviations: GalNAc, *N*-acetylgalactosamine; NeuNAc, *N*-acetylneuraminic acid (sialic acid); Gal, galactose; OSM, ovine submaxillary mucin; des-C8,C9-OSM, OSM with the C8 and C9 carbons of NeuNAc removed, forming the 5-acetamido-3,5-dideoxy-L-*arabino*-2-heptulosonic acid derivative; Hyp-OSM, OSM treated with hydroxylapatite; asialo-OSM, OSM with NeuNAc removed; Pro-OSM, OSM treated with Pronase; FPDG, freezing point depression glycoprotein; T_1, spin–lattice relaxation time; NT_1, spin–lattice relaxation time multiplied by the number of directly attached protons, *N*; NOE, nuclear Overhauser enhancement; τ_c, rotational correlation time; ω_c, ^{13}C NMR resonance frequency; Me_4Si (TMS in figures), tetramethylsilane.

0006-2960/84/0423-1485$01.50/0

simple mucin obtained from the sheep submaxillary gland. Ovine submaxillary mucin, OSM, is attractive for initial study since it is a well-characterized mucin whose carbohydrate side chains consist exclusively of the disaccharide α-NeuNAc2→6α-GalNAc (Gottshalk & Bhargava, 1972). Its peptide core contains predominantly glycine, serine, threonine, alanine, and proline with reportedly 80–95% of the serine and threonine residues being glycosylated (Tettamanti & Pigman, 1968; Hill et al., 1977a). While OSM forms very viscous solutions it does not form a gel as do some gastric, tracheobronchial, and cervical mucins. This study shows that the highly viscous mucin possesses a great deal of internal segmental flexibility, sufficient to allow high-resolution ^{13}C NMR studies. Essentially all of the resonances in the ^{13}C NMR spectra of OSM can be assigned to the major carbohydrate side-chain and peptide core residues. With these assignments and by use of covalent and noncovalent perturbations of the mucin structure, specific intramolecular interactions involving the carbohydrate residues have been detected.

Materials and Methods

Materials. Frozen ovine submaxillary glands were obtained from Pelfreeze, neuraminidase type VIII from Sigma Chemical Co., and Pronase-B from Calbiochem. All other chemicals and materials were from commercial sources and of the highest purity available.

Analytical Methods. Sialic acid was measured by the resorcinol methods of Svennerholm (1958) and *N*-acetylgalactosamine by the method of Morgan and Elson as modified by Reissig et al. (1955). Amino acid analyses were performed on a Beckman 119CL amino acid analyzer using conditions established to resolve amino sugars (Fauconet & Rochemont, 1978). Mucin was hydrolyzed in vacuo in 6 M HCl at 110 °C for 24, 48, and 72 h with the amino acid compositions for serine and threonine obtained by extrapolations to zero time (Hill et al., 1977A).

Mucin Isolation. Ovine submaxillary mucin was obtained from frozen sheep submaxillary glands following the first four steps of the procedures of Hill et al. (1977a). These steps include (1) gland homogenization and extraction, (2) precipitation of non-mucin protein at pH 4.7 and treatment with cation-exchange resin (SP-Sephadex or CM-cellulose), (3) clotting of mucin with cetyltrimethylammonium bromide, and (4) 75% ethanol precipitation of mucin. The precipitate was dissolved, exhaustively dialyzed against distilled water and Chelex cation-exchange resin, and lyophilized. Lyophilized OSM was stored at −20 °C. The lyophilized OSM required one to several days to redissolve (depending on the final concentration) and produced very viscous solutions. This mucin produced slightly cloudy solutions which could only be partially clarified by centrifugation at 30000*g*.

Following the procedures of Hill et al. (1977a) (i.e., their step 5) portions of the dialyzed mucin from step 4 were applied to 2.5 × 10–20 cm columns of hydroxylapatite (Bio-Gel HTP) in phosphate buffer. The sialic acid containing eluate was pooled and dialyzed against H_2O and Chelex. This mucin as isolated was nonviscous. After lyophilization it dissolved immediately (even at several hundred mg/mL) after the addition of water. Both the viscous mucin, from step 4, and the nonviscous mucin, after step 5 (to be called Hyp-OSM), were used for subsequent amino acid, gel filtration, and ^{13}C NMR comparisons. A final purification step proposed by Hill and co-workers to remove trace non-mucin protein, dansylation followed by gel filtration on Sepharose 4B, was not attempted due to the limited amounts of mucin that could be easily obtained after this step. A comparison of these two mucin preparations will be presented in the results.

Asialomucin. Sialic acid was enzymatically removed from OSM (step 4) by using procedures similar to that of Hill et al. (1977a) by an overnight incubation of mucin (100–400 mg, 2–10 mg/mL) at 37 °C with neuraminidase (5–10 units) in 1 M NaCl, 0.1 M sodium acetate, pH 5.0, and 0.03% sodium azide. Treated mucin was dialyzed exhaustively against water and lyophilized prior to the determination of its sialic acid content. Usually 75–95% of the sialic acid could be removed in a single treatment with neuraminidase as determined chemically and by ^{13}C NMR.

Sialic Acid Modification. The glycerol side chain of the sialic acid in OSM (step 4) was shortened to the C7 derivative by periodate oxidation followed by borohydride reduction using the method of Suttajit & Winzler (1971) as modified by Aplin et al. (1979). Potassium periodate was added to OSM (200 mg/100 mL in 0.5 M NaCl) to give a 5-fold molar excess sialic acid. After 1.5 h at 0 °C in the dark the periodate was destroyed by the addition of a solution of potassium iodide and sodium thiosulfate in sodium bicarbonate buffer. The resultant solution was dialyzed against several changes of water and then brought to pH 7.5 with 1 M Na_2HPO_4. The C7 aldehyde was reduced by the addition of ca. 5 mL of a 2 M solution of $NaBH_4$ in 1 M Na_2HPO_4 while stirring and maintaining the pH below 9.2 by the addition of 1 M NaH_2PO_4. After 1 h unreacted $NaBH_4$ was destroyed by adjusting the pH to 6.0 with 1 M acetic acid. The modified mucin was exhaustively dialyzed and lyophilized. The ^{13}C NMR spectra show complete removal of the NeuNAc 8- and 9-carbons and show no evidence of GalNAc oxidation following the above procedure. This modified mucin will be called des-C8,C9-OSM.

Pronase-Treated Mucin. Native mucin (500 mg/20 mL of H_2O, pH 7) was proteolytically degraded with 20 mg of Pronase-B at 37 °C. The solution was maintained at pH 7 during the first 2 h of reaction with dilute NH_4OH. The digestion was continued for 2 days after which it was Millipore filtered. High and low molecular weight fractions were separated by dialysis using M_r 1000 cut-off membrane tubing (Spectrapor) and lyophilized. The mucin retained by the dialysis tubing will be called Pro-OSM.

Viscosity Estimates. Relative viscosities were estimated by passing dilute (~0.1 mg/mL) mucin solutions through an Ostwald type viscometer. Passage times through the viscometer under these conditions were linear with mucin concentration.

NMR Methods. Natural abundance proton decoupled ^{13}C NMR spectra were obtained on a Bruker WH 180/270 pulsed Fourier transform spectrometer. Carbon-13 spectra were obtained at 45.3 MHz by using a 20-mm diameter sample tube and at 67.9 MHz by using a 10-mm sample tube. NMR samples were prepared by gently packing the weighed lyophilized mucin to the bottom of an NMR tube, adding a measured volume of solvent (100 mM KCl, 20% D_2O, 0.04% NaN_3, and ca. 1% acetonitrile), and allowing the mucin to dissolve over a period of several days. The consistency of these solutions ranged from being water-like (i.e., Hyp-OSM and Pro-OSM) to being very viscous and nearly gellike (i.e., asialo-OSM and especially the mucin from step 4). The methyl resonance of acetonitrile taken as 2.134 ppm from tetramethylsilane at 21 °C was used as a secondary internal chemical shift reference. From our temperature studies, we observed that this resonance was temperature dependent, shifting downfield 0.006 ppm/°C between 21 and 65 °C. Spectra were obtained by using 8K or 16K data points and Fourier transformed with zero filling using 32K points.

Base-line corrections, resolution enhancements, and resonance integrations were performed by using the NTCFT program running on the spectrometer's NTC-1180 computer (Nicolet Technology Corp.).

Spin–lattice relaxation time experiments were performed following the fast inversion recovery method of Canet et al. (1975) using 10–12 different τ values. To optimize instrument time usage, T_1 determinations were only attempted for the protonated carbons ($T_1 < 0.5$ s, maximum τ value $\sim$ 1.5 s, and sequence delay time $\sim$ 0.75 s). The 90° and 180° radio-frequency excitation pulses were determined prior to each experiment. A typical T_1 experiment under these conditions required 30–60 h of instrument time. The three-parameter nonlinear least-squares fitting method of Kowaleski et al. (1977) was used to calculate T_1 values. The three parameters obtained from this fit typically fit the experimental data with less than 5% standard error. Nuclear Overhauser enhancements (NOE's) were obtained by using the methods of Opella et al. (1976). To minimize instrument time, decoupler on (or off) delay times of 3–6 s were used to determine most of the protonated carbon NOE's. Reported NOE values are estimated to be correct to ±15%.

Hydrogen ion concentrations were determined on a Radiometer PHM 64 or 26 pH meter before and after spectral accumulation; no adjustments were made for the 20% D_2O of the solvent. The pH-dependent ^{13}C NMR shifts were fit to theoretical pH titration curves (Dwek, 1973). Most pK_a values were determined to the nearest 0.05 pH unit by using a program that minimized the errors between the experimental titration curve and the calculated theoretical curve.

Results

Mucin Characterization. The analytical gel filtration profiles of the mucin preparations used in our studies are shown in Figure 1. The viscous mucin from step 4 gives gel filtration profiles having a sharp high molecular weight peak and shoulder at the excluded volume and a broader low molecular weight peak extending across the included volume, as shown in Figure 1A. The nonviscous Hyp-OSM mucin obtained after chromatography on hydroxylapatite (step 5) shows a much lower molecular weight distribution as shown in Figure 1D. It is obvious that the two mucins have greatly different molecular weights and absorption profiles. The sheer forces produced in the process of passing mucin through hydroxylapatite are apparently sufficient to irreversibly reduce its molecular weight, or alternatively the high molecular weight fractions do not elute from the column. Our low yield through this step may suggest the latter. Batch treatment of OSM with hydroxylapatite is also reported to reduce the viscosity of the resultant mucin [see Tettamanti & Pigman (1968)]. The high molecular weight mucin that elutes in the excluded volume (Figure 1A) shows an increased absorption at 280 nm compared to mucin that is included in the gel. This high molecular weight fraction may be concentrated and rechromatographed giving the high molecular weight peaks with elevated absorbance at 280 nm and little or none of the broad included peak. This mucin also shows a higher viscosity than the unfractionated mucin from step 4 (data not shown). A correlation appears to exist between the viscosity of the mucin with the size of the excluded volume peak and the degree of noncentrifugable turbidity. This is consistent with the observation that the broad included fraction is much less viscous than the mucin prior to fractionation (data not shown) and that this fraction does not give the high molecular weight excluded peak after rechromatography. Thus, the high molecular weight peaks may represent aggregated mucin and intact mucin

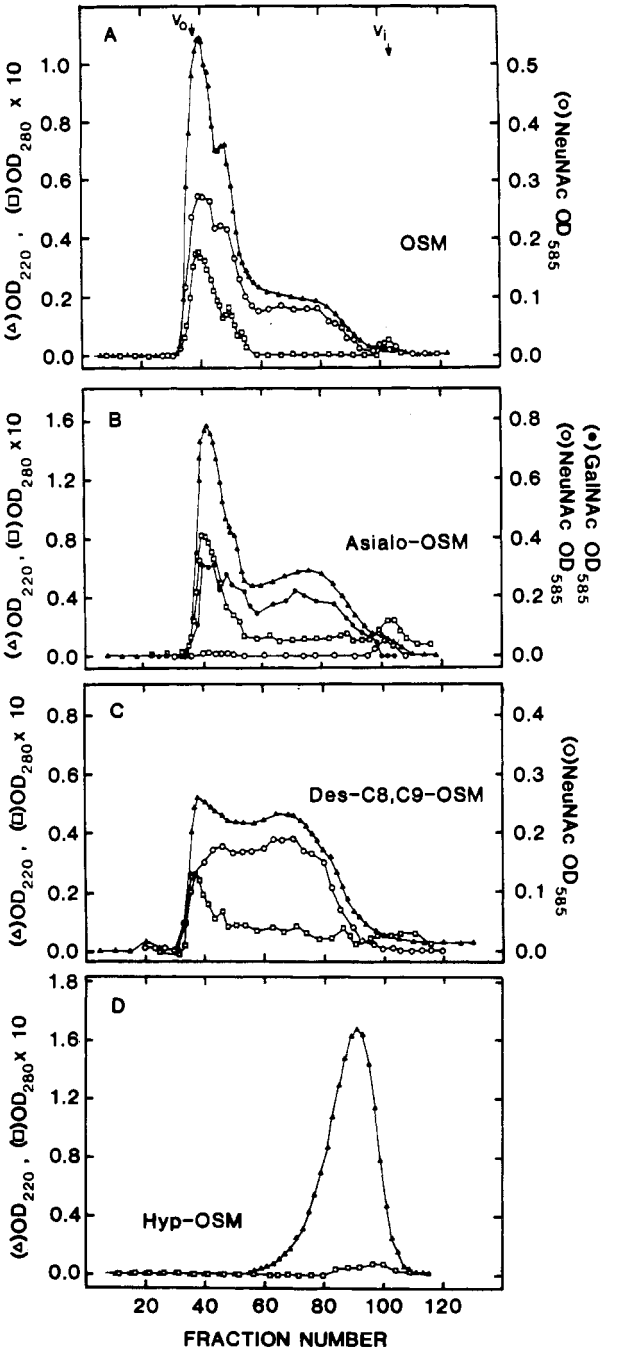

FIGURE 1: Gel filtration chromatograms of native and modified OSM on Bio-Rad A-50-m. Five to seven milliliters of mucin (1–3 mg/mL) was applied to the columns (2.5 × 31 cm) and eluted with 0.5 M NaCl and 0.01 M sodium cacodylate, pH 6.0 (Hill et al., 1977a). Fractions were collected every 1.5 mL. V_0 and V_i are the excluded and included column volumes corresponding to the approximate molecular weight range of 5×10^7 to 1×10^5 for globular proteins. (A) Native OSM, step 4; (B) asialo-OSM; (C) des-C8,C9-OSM; (D) Hyp-OSM.

monomer while the broad low molecular weight peak represents irreversibly degraded mucin.

On the basis of the above, we decided to concentrate our ^{13}C NMR studies on the most viscous (i.e., most native) mucin obtained from step 4 even though this mucin may contain trace contaminating globular proteins (Hill et al., 1977a) in addition to partially degraded mucin. Since the ^{13}C NMR spectra both of the viscous mucin from step 4 and of the nonviscous Hyp-OSM from step 5 are identical, their amino acid compositions essentially the same, and in agreement with published analyses for OSM (Tettamanti & Pigman, 1968; Hill et al., 1977a), we conclude that the more viscous mucin is not significantly

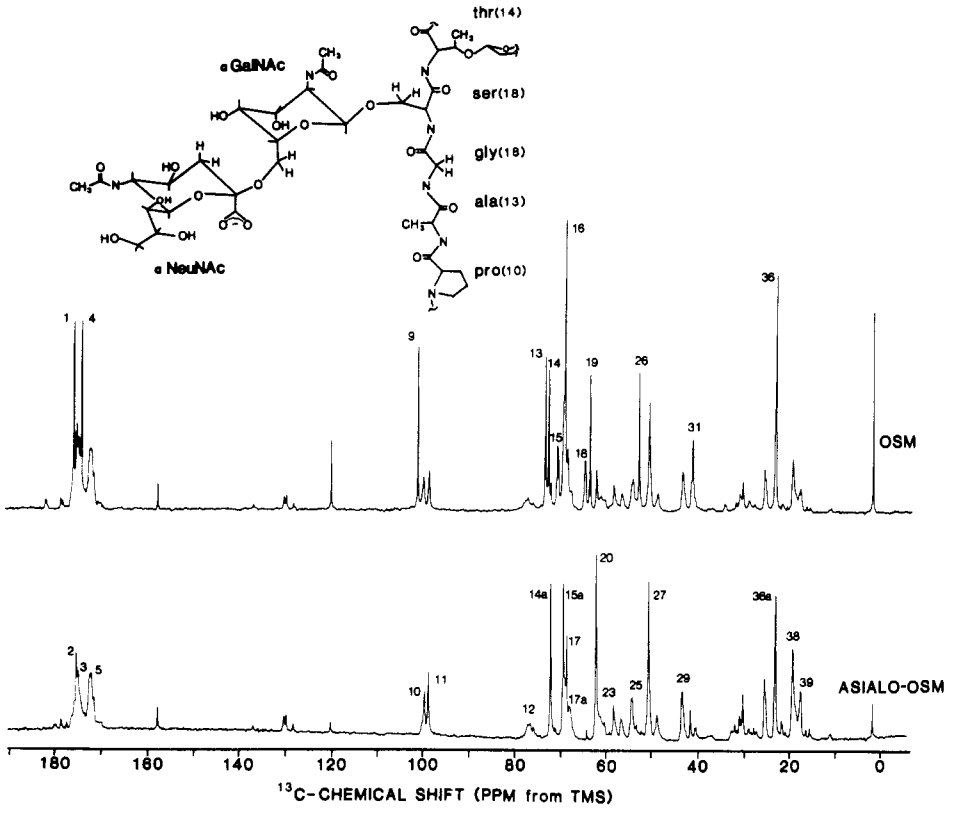

FIGURE 2: 67.9-MHz ^{13}C NMR spectra of native OSM and asialo-OSM. (Top) NOE-suppressed proton decoupled spectrum of native OSM (100 mg/mL in 0.1 M KCl, 20% D_2O, and 0.04% NaN_3, pH 4.6) requiring 49 436 scans with a repetition rate of 6.275 s. (Bottom) Proton-decoupled spectrum of asialo-OSM (100 mg/mL in 0.1 M KCl, 20% D_2O, and 0.04% NaN_3, pH 4.7) requiring 16 872 scans with a repetition rate of 2.775 s. Numbered resonances refer to carbons assigned in Tables I–III. Resonances at 2 and 120 ppm are from CH_3CN internal reference. A representative structure for OSM showing the disaccharide side chains and the major peptide amino acids is shown. Numbers in parentheses represent the number of residues/100 residues for the indicated amino acid obtained from amino acid analysis.

contaminated with extraneous proteins. In addition the T_1 values for the degraded Hyp-OSM were found to be identical, within experimental error, to the T_1 values of the more native mucin from step 4 (see Table IV), thus making it unnecessary in our current study to remove the lower molecular weight fractions from the mucins obtained from step 4.

The gel filtration chromatograms of asialo- and des-C8,C9-OSM on A-50m (Figure 1B,C) show that these modifications alter the original molecular weight distribution, although for both modified mucins the high molecular weight fractions are still present. Trace proteases in neuraminidase and the chemical procedures of oxidation and reduction, respectively, are probable sources of this apparent degradation. As expected from the molecular weight distribution, the asialomucin is significantly more viscous than the des-C8,C9-mucin. The nonviscous, Pronase-treated mucin has the lowest molecular weight distribution of the mucins studied (data not shown).

Carbon-13 NMR. The complete ^{13}C NMR spectra of "native" (step 4) and asialo-OSM are shown in Figure 2. A schematic structure for OSM is also given in the figure showing the carbohydrate side-chain structure and the most abundant amino acids of the peptide core. The spectra appear simple and well resolved considering that both mucins were predominantly high in molecular weight and that the NMR samples were highly viscous. The most pronounced and sharpest resonances in the spectra of intact OSM are due to the terminal sialic acid residues as demonstrated by the loss of these resonances in the spectra of the asialomucin. The majority of these resonances can be assigned to individual sialic acid carbons solely on the basis of previously published ^{13}C NMR data of sialic acid and its derivatives (Jennings & Bhattacharjee, 1977; Jacques et al., 1977; Bhattacharjee et al., 1975; Eschenfelder et al., 1975) and are listed in Table I. For those resonances that could not be unambiguously assigned by inspection to specific sialic acid carbons (i.e., resonances, 13, 14, and 16 representing C8, C6, C7, and C4) the assignments were made by comparative pH titration and temperature dependence studies of native OSM and des-C8,C9-OSM as discussed below. The individual resonances of the modified sialic acid residue in des-C8,C9-OSM can be uniquely assigned on the basis of their chemical shift alone. Expanded spectra comparing the native OSM and des-C8,C9-OSM are shown in Figure 3A,B with the resonance assignments listed in Table I.

Assignments for the α-GalNAc carbons are also based on previously reported chemical shift values (Prohaska et al., 1981; Jacques et al., 1980; Bundle et al., 1973) with unambiguous assignments to C6 and C5 being afforded by the comparison between native OSM and asialo-OSM. Upon the removal of sialic acid the resonance for GalNAc C6 (resonance 18) shifts upfield 2.4 ppm (resonance 20) while the GalNAc C5 (resonance 15) shifts downfield 1.5 ppm (resonance 14a). These shifts upon deglycosylation are in agreement to those reported by Prohaska et al. (1981) and Jacques et al. (1980) for α-NeuNAc2→6α-GalNAc. The assignments for the GalNAc carbons of native and modified OSM are listed in Table II. Since GalNAc is attached to the peptide via both serine and threonine residues, in essentially equal proportions, many of the GalNAc resonances appear as "doublets". This is most pronounced for the anomeric carbon which appears as two resonances (10 and 11 in Figure 2). On the basis of our unpublished previous observations with the freezing point depression glycoprotein containing the glycopeptide β-

Table I: Effects of pH, Temperature, and Chemical Modification on the Chemical Shifts (δ) of the Sialic Acid Residues in OSM

native OSM						des-C8,C9-OSM					
carbon no.	peak no.[a]	δ[b]	Δδ (pH)[c]	pK_a[c]	Δδ (temp)[d]	peak no.[a]	δ[b]	Δδ (mod)[e]	Δδ (pH)[c]	pK_a[c]	Δδ (temp)[f]
1	4	174.48	−2.33	(2.00)	−0.71	4	174.55	+0.07	−2.40	(2.40)	−0.56
2	9	101.48	−1.58	(2.05)	+0.60	9	101.92	+0.44	−1.85	(2.35)	+0.32
3	31	41.41	−1.07	(2.05)	0	31	41.44	0	−1.62	(2.45)	−0.49
4	16	69.53	−0.96	(2.00) (b)[g]	0 (b)[g]	16b	69.00	−0.51	−0.64	(2.35)	+0.19
5	26	53.17	−0.15	(1.90)	+0.50	26	53.27	+0.10	−0.14	(2.50)	+0.84
6	13	73.71	+0.26	(2.30)	+0.20	12a	75.82	+2.09	+0.68	(2.50)	+0.36
7	16	69.54	+0.11	(2.0) (a)[g]	+0.85 (a)[g]	20a	62.28	−7.26	0		+1.23
8	14	73.05	−0.94	(2.00)	0						
9	19	63.97	+0.31	(2.25)	+0.69						
10	1	176.21	0		0	1	176.03	−0.18	0		−0.51
11	36	23.39	0		0	36	23.41	0	0		0

[a] Peaks labeled in Figures 2 and 3. [b] Chemical shift value, from Me_4Si. Average of values obtained between pH 4.5 and pH 8.5, 21 °C. [c] Δδ (pH): calculated chemical shift difference, between the nonprotonated (high pH) and protonated (low pH) ionization states of sialic acid C1. A minus shift indicates a high-field shift in the protonated state. pK_a: calculated ionization constant for the above ionization. Both Δδ (pH) and pK_a values were calculated to fit the titration data in Figure 5. [d] The temperature dependence of the chemical shift in ppm/°C × 100. Obtained from a linear fit of the data between 5 and 65 °C at pH 5-7. A minus shift indicates a shift to high field at elevated temperatures. [e] Chemical shift change upon modification, δ des-C8,C9-OSM − δ native OSM. Differences less than ~0.05 ppm are listed as 0. [f] The temperature dependence of the chemical shift in ppm/°C × 100 obtained from a linear fit of the data between 21 and 55 °C at pH 0.5. A minus shift indicates a shift to high field at elevated temperatures. [g] Resonance 16 is resolved at low pH and/or elevated temperature. (a) Downfield resonance; (b) upfield resonance.

Table II: Effects of Temperature and Chemical Modification on the Chemical Shifts (δ) of the *N*-Acetylgalactosamine Residues in OSM

native OSM				des-C8,C9-OSM				asialo-OSM			
carbon no.	peak no.[a]	δ[b]	Δδ (temp)[c]	peak no.[a]	δ[b]	Δδ (mod)[d]	Δδ (temp)[e]	peak no.[a]	δ[b]	Δδ (mod)[f]	Δδ (temp)[e]
1: Ser	11	98.97	0	11	99.0	0	−0.16	11	99.06	+0.09	0
Thr	10	100.12	−0.33	10	100.14	0	−0.25	10	99.90	−0.22	−0.36
2	27	50.94	+0.22	27	50.97	0	+0.27	27	51.00	0	+0.24
3	17	68.90	+0.60	17	68.97	0	+0.19	17	69.05	+0.15	+0.47
4	15a	69.90	+0.23	15a	69.90	0	+0.13	15a	69.79	0	+0.43
		69.75			69.74						
5	15	71.17	0	15	71.21	0	0	14a	72.59	+1.51	−0.12
		70.99			70.99						
6	18	65.06	−0.85	18	64.92	−0.22	−0.80	20	62.55	−2.43	−0.20
		64.89			64.56						
7: Ser	3	175.21	+0.42	3	175.24	0	−0.20	3	175.29	0	0
Thr	2	175.58	−0.18	2	175.62	0	−0.16	2	175.61	0	−0.24
8	36a	23.6	0	36a	23.6	0	0	36a	23.6	0	0
		23.4			23.5				23.4		

[a] Peaks labeled in Figures 2 and 3. [b] Chemical shift values, from Me_4Si. Average of values obtained between pH 4.5 and 8.5, 21 °C. Multiple entries given where serine and threonine linkages split the peak into doublets. [c] The temperature dependence of the chemical shift in ppm/°C × 100. Obtained from a linear fit of the data between 5 and 65 °C at pH 5-7. A minus shift indicates a shift to high field at elevated temperatures. [d] Chemical shift change upon modification, δ des-C8,C9-OSM − δ native OSM. Differences less than ~0.05 ppm are listed as 0. [e] The temperature dependence of the chemical shift in ppm/°C × 100. Obtained from a linear fit of the data between 21 and 55 °C at pH 5.5. A minus shift indicates a shift to high field at elevated temperatures. [f] Chemical shift change upon modification, δ asialo OSM − δ native OSM. Differences less than ~0.05 ppm are listed as 0.

Gal1→3α-GalNAc-Thr [see also Berman et al. (1980)] and on the basis of model glycopeptide studies (Dill et al., 1981), we can assign the upfield resonance (11) to the α-GalNAc-Ser anomeric carbon and the downfield resonance (10) to the α-GalNAc-Thr anomeric carbon. The splitting and broadening of the other GalNAc resonances, presumably due to the different Ser/Thr linkages, are best observed in the resolution-enhanced spectra shown in Figure 3. The enhanced spectra also resolve the GalNAc C4 and C3 resonances (15a and 17) from the sialic acid C4 and C7 resonances (16). A comparison of the spectra of the modified OSM (Figure 3A–D) shows the GalNAc ring carbons C3 to C6 to be broadened or split in all derivatives except asialo-OSM. This is most clearly observed by comparing resonances 15, 15a, and 18 in the sialylated mucins (Figure 3A,B,D) with resonances 14a, 15, and 20 (carbons 5, 4, and 6, respectively) in the asialomucin (Figure 3C). The anomeric carbon resonances (10 and 11) also shift closer together after the removal of sialic acid (see Table II). Thus, it appears that the sensitivity of the GalNAc ring carbons to the serine or threonine linkage is lost or reduced after the removal of sialic acid.

The remaining generally broader and less intense resonances can be assigned to the amino acid peptide α-, carbonyl, and side-chain carbons on the basis of their chemical shifts by comparison to model peptides (Wuthrich, 1976) and glycopeptides (Berman et al., 1980; Dill et al., 1981, 1982, 1983; Prohaska et al., 1981). The assignments to specific amino acid residues can be corroborated by comparing the approximate resonance areas of the NOE-suppressed spectrum to the amino acid composition (see Table III). Thus, the α-carbon of glycine, the most prevelant amino acid residue, is readily assigned to the broad and slightly asymmetric resonance at 43.7 ppm (peak 29, Figure 3A). The α-carbons of glycosylated threonine and serine appear at 58.7 and 54.4 ppm, respectively (peaks 23 and 25, Figure 3A). On the basis of its chemical shift peak 25 must also contain the α-carbon resonances of

Table III: ^{13}C NMR Resonance Assignments for the Major Amino Acid Residues in OSM

residue	residues/100 AAA[a]	residues/100 NMR[b]	chemical shift (resonance no.)[c] α	β	γ	δ	ζ
Gly	18	18	43.72 (29)*				
Ser-O-GalNAc-	18	11[d]	54.42 (25)[l,a,g]	68.2 (17a)[G]			
Ser-OH		8	56.80 (24)	62.48 (20)			
Thr-O-GalNAc-	14	12[d]	58.67 (23)*	77.6 (12)*	19.65 (38)[v]		
Thr-OH		~2	60.69 (22)[v]	67-68	19.65 (38)[v]		
Ala	13	13	50.94 (29)[G]	17.98 (39)*			
Pro	10	9	61.72 (21)*	29-31	25-26	49.00 (28)*	
Val	7	~5	60.69 (22)[t]	29-31	19.65 (38)[t]		
					19.65 (38)[t]		
Glu/Gln	6	~3	54.42 (25)[l,s,a]	28-29	32-35	179-183	
Leu	3	~3	54.42 (25)[s,a,g]	41[N]	25-26	23.0[N]	
						21.97	
Arg	4	2	54.42 (25)[s,l,g]	29-31	25-26	41[N]	158.00*
GalNAc		23[d]					
NeuNAc		23[d]					

[a] By amino acid analysis of native OSM. [b] Approximate values from integrated ^{13}C NMR NOE suppressed spectrum, Figure 2A, based on Gly = 18. "~" indicates values not necessarily unique. [c] Resonances numbers assigned in Figure 3A. Asterisks indicate unique resonance. Overlapping resonances denotated as the following: a, Arg; g, Glu; s, Ser; t, Thr, l, Leu, v, Val; G, GalNAc; N, NeuNAc. [d] Obtained from integrations of GalNAc-Cl-Ser/Thr resonances 11 and 10 and resolved GalNAc and NeuNAc resonances 13-15.

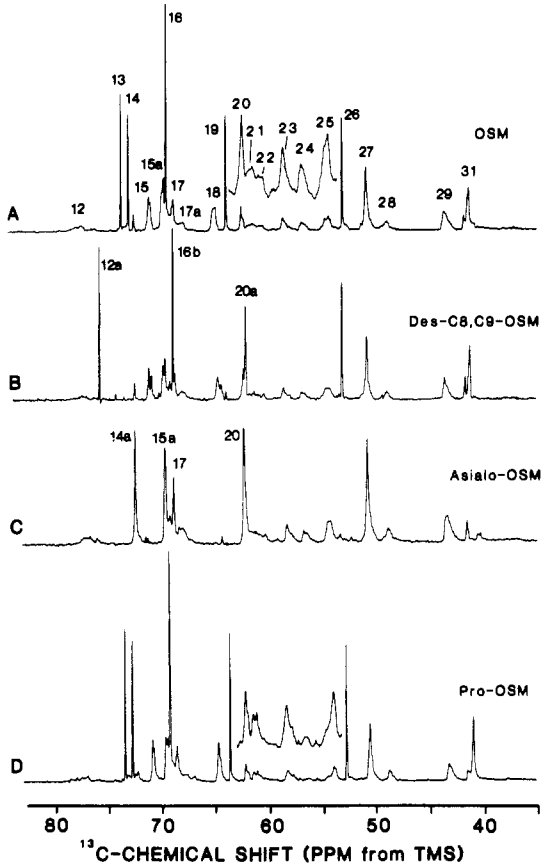

FIGURE 3: Resolution-enhanced ^{13}C NMR spectra (40–80 ppm region) of native and modified OSM. (A) Native OSM (same data set as Figure 2, top); (B) des-C8,C9-OSM (100 mg/mL; 20% D_2O, 0.1 M KCl, and 0.04% NaN_3, pH 7.8) requiring 26 352 scans with a repetition rate of 2.5 s; (C) asialo-OSM (same data set as Figure 2, bottom); (D) Pro-OSM (100 mg/mL; 20% D_2O, 0.1 M KCl, and 0.04% NaN_3) requiring 45 274 scans with a repetition rate of 1.77 s. Expanded vertical scale portions in (A) and (D) are not resolution enhanced.

glutamic acid, glutamine, leucine, and arginine. No other mucin peptide resonances are expected to interfere with resonance 23. Comparing the areas of these resonances to the areas of the C1 carbons of GalNAc bound to either threonine or serine (resonances 10 and 11, respectively, Figure 2) reveals that approximately one-third of the area of peak 25 is due to non-serine amino acid residues while the area of peak 23 is solely due to glycosylated threonine. From the amino acid analyses both native OSM and Hyp-OSM contain a greater proportion of serine than threonine while the areas of the C1 GalNAc carbons indicate an equal number of glycosylated serine and threonine residues (Table III). This suggests that a larger proportion of the serine residues may be nonglycosylated. This is confirmed by the observation of a peak at 56.8 ppm (resonance 24) which is identical with the shift reported for the α-carbons of nonglycosylated serine residues in peptides (Prohaska et al., 1981). For OSM the only other resonances expected at this chemical shift are the α-carbons of phenylalanine and the α-carbons of glycosylated threonine which precede proline in the peptide sequence (Torchia et al., 1975; Berman et al., 1980). On the basis of the areas of the phenylalanine ring carbons (137–127 ppm) only a small proportion of peak 24 can be attributed to phenylalanine. Likewise, the large decrease in this peak in Pro-OSM (Figure 3D) with relatively no change in the proline resonances (see below) suggests this resonance contains only a small proportion of the Pro-Thr sequence. The β-carbons of the nonglycosylated serine residues resonate at 62.5 ppm (resonance 20, Figure 3A). The area of this peak in native mucin is in good agreement with the area of the α-carbon of nonglycosylated serine, taking into account the contribution of the overlapping C6 carbon from the nonsialylated GalNAc residues. Likewise a small peak for the α-carbon of nonglycosylated threonine may be identified at 60.7 ppm (resonance 22) which must contain in addition the α-carbon of valine [see Prohaska et al. (1981) and Dill et al. (1983)]. The nonglycosylated β-carbons of threonine are buried under the carbohydrate carbons at ~69 ppm (Wuthrich, 1976) and may perhaps be the small resonances observed between peaks 15a and 16 or 17 in asialo-OSM or des-C8, C9-OSM (Figure 3B,C).[2] With glycosylation the β-carbons of both serine and threonine shift 8–10 ppm downfield (Prohaska et al., 1981; Dill et al., 1983; Berman et al., 1980). Thus, the broad resonances at 68.1 (resonance 17a) and 77.6 ppm (resonance 12) in the spectra of native and modified OSM are assigned to the β-carbons of glycosylated serine and threonine residues. The breadth of these resonances, we believe, arises from the sensitivity of the

[2] The resonance assignments of the carbons of the unglycosylated serine and threonine residues have recently been confirmed by our preparation of the carbohydrate-free apo-OSM.

chemical shifts of these carbons to amino acid sequence, as there is very little sequence homology around the serine or threonine residues in OSM (Hill et al., 1977b). The γ-methyl carbon of threonine in model compounds and peptides appears at ca. 20 ppm and is less sensitive to glycosylation (Dill et al., 1983). This peak at 19.6 ppm (resonance 38) also overlaps the methyl groups of valine.

The α-carbon of alanine in model peptides and glycopeptides appears at 51 ppm which is identical with the chemical shift assigned to the α-GalNAc C2 carbon (resonance 27). The comparison of the areas of the GalNAc C1, C5, and C6 carbons to the area of resonance 27 suggests that approximately one-third of the area of this resonance is due to the α-carbon of alanine, in keeping with the composition of the mucin. The β-methyl of alanine assigned to the peak at 18.0 ppm (resonance 39) has an area that is in agreement with the above.

By use of the chemical shift values of Torchia et al. (1975) and London et al. (1978a) for the trans isomer of proline in peptides (61.7, 30.5, 25.5, and 49.1 ppm for the α-, β-, γ-, and γ-carbons, respectively) possible proline resonances can be identified in the spectra of native and modified OSM. Unfortunately due to the overlapping resonances of other less abundant amino acids [i.e., Val-C_β (31 ppm), Glu-C_β (29 ppm), Leu-C_γ (25 ppm), and Arg-C_γ (25 ppm)] only the resonance for the proline α- and δ- carbons at 61.7 (resonance 21) and 49.0 ppm (resonance 28), respectively can be specifically assigned to proline. The areas of these resonances are in good agreement with each other and the peptide composition.

The 80–40 ppm region of the ^{13}C spectra of OSM after exhaustive Pronase treatment is shown in Figure 3D. Several changes in the spectrum are observed, the most prominent being a large decrease in the areas of the peaks at 56.8, 60.7, and 62.5 ppm (resonances 24, 22, and 20). These peaks have been assigned in part to the nonglycosylated serine and threonine α- and β-carbons and are thus expected to be more susceptible to proteolysis than the glycosylated residues (Variyam & Hoskins, 1983). Our assignments are further supported by the amino acid analysis, which indicate an equal amount of serine and threonine present in the Pronase-treated mucin, in agreement with the areas of the GalNAc C1 carbons in the NMR spectrum.

Overall the resonance areas of the most prominent residues, in the NOE-suppressed spectrum of native OSM, are in reasonable agreement with the amino acid analysis (Table III). Differences may be due to residues having broad resonances because of sequence heterogeneity (i.e., neighboring proline) or less likely to regions of decreased mobility. The presence of contaminating globular proteins with long rotational correlation times may also be a source of these differences. Our results, however, do suggest that the proportion of nonglycosylated serine in both our native OSM and Hyp-OSM preparations is larger than the 5–20% reported by others (Hill et al., 1977a; Tettamanti & Pigman, 1968) and may approach up to 40% on the basis of the ^{13}C NMR results.

Temperature and pH Dependence of the ^{13}C Chemical Shifts. At elevated temperatures the single sharp peak representing the sialic acid C4 and C7 carbons (resonance 16) splits, giving two sharp equal intensity resonances. These and other temperature-dependent shifts for the sialic acid and GalNAc carbons in "native" OSM are plotted in Figure 4. Similar data for the modified mucins are listed in Tables I and II. The sialic acid carbons in both the native OSM and des-C8,C9-OSM show similar temperature shifts with the

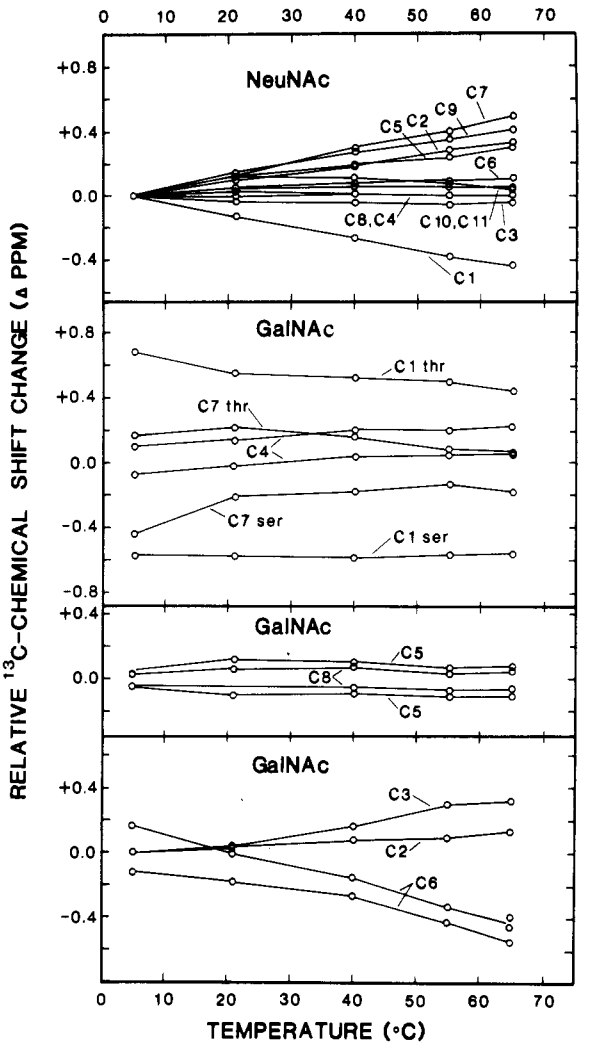

FIGURE 4: Temperature dependence of the ^{13}C chemical shifts of the carbohydrate residues in OSM. Data were obtained from both native (viscous) and Hyp-OSM (nonviscous) preparations and are plotted relative to the chemical shift values obtained at 5 °C. Spectra were obtained in the pH range of 4.6–7.0 with ca. 100 mg/mL mucin in 0.1 M KCl, 20% D_2O, and 0.04 NaN_3. Double resonances are shown for the GalNAc, C1, C4, C5, C6, C7, and C8 carbons due to the different serine or threonine linkages.

exception of C3 (Table I). A large temperature-dependent shift for C7 in both the intact and modified mucin (along with its unusual T_1 value) may suggest an interaction (see below). The temperature shifts for the individual GalNAc carbons in the three mucins listed in Table II, however, are less consistent when compared with each other. However, the GalNAc C6 carbon linked to intact or modified sialic acid shows a large shift while in the asialomucin this carbon's temperature-dependent shift is greatly reduced. This may indicate a new interaction or a change in the conformation of the disaccharide unit as a function of increased motion. Note also that the splittings of the GalNAc carbons due to the serine or threonine linkage are maintained between 5 and 65 °C (Figure 4). With the exception of the more hydrophobic carbon resonances (25–18 ppm) the peptide resonances show no detectable chemical shift or line-width changes with increased temperature, indicating the lack of any significant peptide conformational alterations within this temperature range, suggesting there is little if any peptide structure in the mucin.

The effect of the titration to low pH of the sialic acid carboxyl group on the chemical shifts of the sialic acid C1 through C9 and C1 through C7 resonances in both native OSM and des-C8,C9-OSM, respectively, are shown in Figure

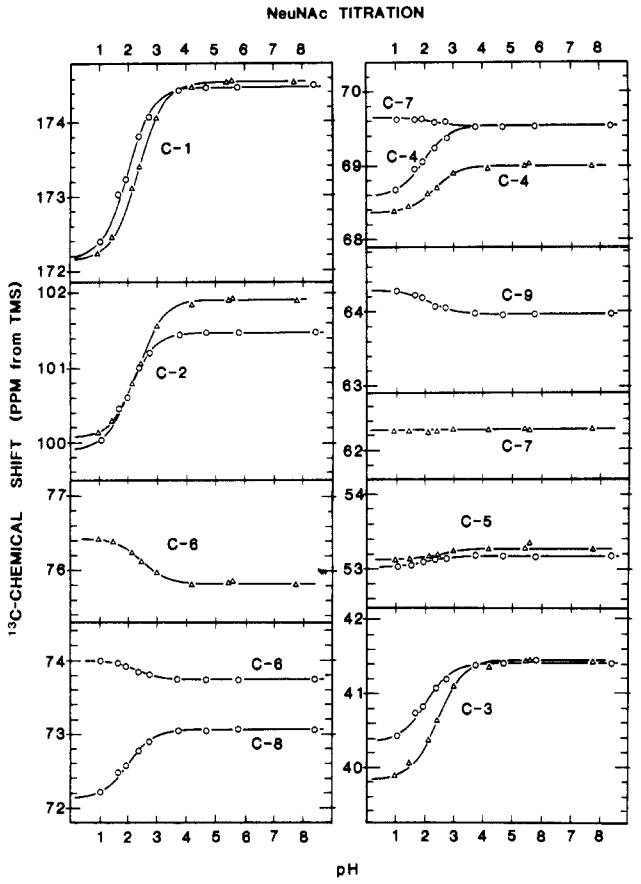

FIGURE 5: pH titration curves of the sialic acid ^{13}C NMR resonances in intact OSM and des-C8,C9-OSM. (○) Data from native (viscous) and Hyp-OSM (nonviscous); (△) data from des-C8,C9-OSM. All samples contained 100 mg/mL mucin, 20% D_2O, 0.1 M KCl, 0.04% NaN_3, and CH_3CN for an internal reference. ^{13}C NMR spectra required 4000–22 000 scans and were obtained at 21 °C. Solid curves represent the best theoretical fit to the data by using the pK_a and chemical shift limits listed in Table I.

5. The chemical shift limits and pK_a values obtained from the theoretically calculated best fit to the data (solid curves in Figure 5) are listed for each titrating carbon in Table I. As expected the sialic acid ring carbons C2 to C5, in both derivatives show induced shifts that decrease in magnitude as a function of the distance from the titrating carboxyl. However, an anomalously larger titration shift is observed for the resonance assigned to the C8 carbon in the intact sialic acid side chain (see below for the assignment of this carbon). A similar shift also has been reported by Jennings & Bhattacharjee (1977) for this carbon between the sodium salt and the free acid of methyl-α-D-*N*-acetylneuraminic acid. On the basis of this large shift these workers suggested that the C8 hydroxyl group forms an internal hydrogen bond with the carboxyl group. Daman & Dill (1982) have also shown that the esterification of 2-*O*-methyl-α-D-*N*-acetylneuraminic acid produces the same upfield shift on C8. The fact that we observe an increase of 0.4 pH unit in the pK_a value of the carboxyl group when the side-chain C8 and C9 carbons are removed further supports this interaction (Table I). The titration of the sialic acid carboxyl group has no effect on the chemical shifts of the GalNAc carbons (less than 0.05 ppm between pH 1 and pH 7), and the chemical shifts of the readily observed peptide carbons are also invariant with decreased pH.

Since the assignment of the modified sialic acid carbons C4, C6, and C7 in des-C8,C9-OSM can be made unambiguously by their chemical shifts alone, the unique temperature and pH dependencies of these resonances could be used to make specific resonance assignments to the sialic acid C4, C7, C6, and C8 resonances in native OSM. The intact sialic acid C7 resonance is assigned (at elevated temperatures) to the downfield resonance, 16a, in part on the basis of the similar temperature shifts observed for this resonance and the resonance assigned to C7 in des-C8,C9-OSM (Table I). The lack of significant temperature shifts for the C4 resonances (resonance 16b, Figure 3B and Table I) in either native OSM or des-c8,C9-OSM further support these assignments. In addition by comparing the pH dependencies of these resonances in native and modified OSM, one can assign the downfield resonance at low pH to C7 (since it shows very little change with pH) and the upfield resonance to C4 (since it shows a large change with pH) as shown in Figure 5 and in Table I.

The resonances for the C6 and C8 carbons for a variety of sialic acid derivatives appear in the 70–75 ppm region of the ^{13}C NMR spectrum. Depending on the derivative and anomeric configuration, the order of these resonances may become reversed [see Jennings & Bhattacharjee (1977), Czarniecki & Thornton (1977a), and Jaques et al. (1977)]. We have assigned the lowest field resonance (13) in native OSM to the sialic acid C6 carbon because this resonance and the C6 resonance in des-C8,C9-OSM both show a moderate temperature dependence and both resonances shift to lower field at low pH. On the other hand, the C8 carbon is assigned to resonance 14 in native OSM because it shows no temperature dependence and shifts instead to higher field at low pH. These assignments for the C6 and C8 carbons of α-D-*N*-acetylneuraminic acid in native OSM are in agreement with the assignments, made by other means, for methyl-α-D-*N*-acetylneuraminic acid and its sodium salt (Jennings & Bhattacharjee, 1977; Czarniecki & Thornton, 1977a).

Carbon-13 Relaxation Studies. Carbon-13 NMR relaxation times, T_1, were determined at 45.3 MHz for native OSM and Hyp-OSM and subsequently determined at 67.9 MHz for the native mucin, des-C8,C9-mucin, and asialomucin. These measurements were focused on the protonated carbons, whose common ^{13}C–^{1}H dipolar relaxation mechanism will allow us to compare our results on the basis of NT_1 (where N is the number of directly attached protons) and the NOE (Allerhand et al., 1971). The NT_1 and NOE values will be interpreted here in a qualitative manner, utilizing the trends obtained from the simple model of a ^{13}C nucleus undergoing proton dipolar relaxation while isotropically rotating at an effective correlation time τ_c (Doddrell et al., 1972).[3] Experimental deviations (from the calculated values of T_1 and NOE) obtained from this model at a given τ_c and as a function of ^{13}C frequency (ω_c) will indicate the presence of more complex modes of motion, assuming complete proton dipolar relaxation and a constant C–H bond length (Dill & Allerhand, 1979b).

Values of NT_1 for the well-resolved protonated carbons of the peptide core and carbohydrate side chains in the differently modified mucins are listed in Table IV. For better comparison selected peptide and carbohydrate side-chain NT_1 values are plotted as a function of position in Figure 6. Since the line widths of these carbons are relatively narrow (~8 Hz for NeuNAc) and their NOE's are greater than one, the NT_1 values can be correlated with τ_c values short compared to $1/\omega_c$.

[3] With this model NT_1 values increase with decreasing τ_c for $\tau_c \lesssim 1/\omega_c$. Within this range the NOE values increase from ~1.2 (for $\tau_c \geq 1/\omega_c$) and plateau at a value of ~3.0 at shorter correlation times ($\tau_c \ll 1/\omega_c$). Likewise the ^{13}C resonance line widths decrease with decreasing τ_c. Increasing the ^{13}C observation frequency increases the NT_1 and decreases the NOE values for $\tau_c \gtrsim 1/\omega_c$, while the NT_1 and NOE values are invariant with changes in ω_c for $\tau_c \ll 1/\omega_c$.

Table IV: ^{13}C Relaxation Parameters for Native and Modified OSM

carbon	NT_1 (s), 45.3 MHz, 30 °C: native[a] OSM	Hyp-OSM[b]	av[c] (±SD)	NT_1 (s), 45.3 MHz, 40 °C: av[d] (±SD)	NT_1 (s) (NOE), 67.9 MHz, 30 °C: native[a] OSM	des-C8,C9-OSM[e]	asialo-OSM[f]
peptide							
Ser α-CH (O-GalNAc)[j]	0.107	0.115	0.114 (0.005)	0.140 (0.002)	0.155 (1.6)	0.155 (1.7)	0.156 (1.7)
Thr α-CH (O-GalNAc)	0.102	0.106	0.103 (0.008)	0.131 (0.018)	0.155 (1.7)	0.155 (1.5)	0.159 (1.5)
Ser α-CH (OH)	0.120	0.124	0.128 (0.009)	0.188 (0.020)	0.172 (1.7)	0.179 (1.8)	0.166 (1.4)
Gly α-CH_2	0.163	0.168	0.167 (0.013)	0.222 (0.007)	0.226 (1.6)	0.222 (1.8)	0.216 (1.8)
Pro δ-CH_2	0.180	0.148	0.173 (0.015)	0.218 (0.025)	0.235 (1.7)	0.254 (1.7)	0.242 (1.9)
Thr/Val γ^2-CH_3	0.76	0.81	0.79 (0.03)	1.26 (0.22)	0.89 (2.2)	0.84 (2.4)	0.92 (2.3)
Ala β-CH_3	0.78	0.95	0.91 (0.14)	1.34 (0.19)	0.90 (2.5)	1.03 (2.3)	1.00 (2.2)
GalNAc							
C1-Ser OCH	0.126	0.123	0.125 (0.005)	0.157 (0.007)	0.170 (1.8)	0.152 (1.7)	0.192 (1.8)
Cl-Thr OCH	0.123	0.114	0.114 (0.006)	0.128 (0.003)	0.170 (1.6)	0.163 (1.8)	0.182 (1.7)
2 NCH[j]	0.122	0.127	0.122 (0.004)	0.151 (0.002)	0.178 (1.7)	0.177 (1.7)	0.182 (1.5)
3 OCH	0.132	0.121	0.122 (0.011)	0.152 (0.019)	0.165 (1.8)	*h*	0.189 (1.6)
4 OCH	*h*	*h*	*h*	*h*	*h*	0.176 (1.7)	0.201 (1.5)
5 OCH	0.110	0.115	0.112 (0.003)	0.128 (0.002)	0.168 (1.5)	0.170 (1.6)	0.187 (1.5)
6 OCH_2	0.134	0.136	0.135 (0.014)	0.189 (0.11)	0.184 (1.7)	0.176 (1.8)	0.328 (2.3)
GalNAc/NeuNAc-CH_3	1.31	1.44	1.38 (0.09)[g]	3.03 (0.1)[g]	1.61 (1.7)	1.75 (1.8)	1.72 (1.9)
NeuNAc							
3 CH_2	0.153	0.162	0.156 (0.015)	0.203 (0.005)	0.210 (2.0)	0.234 (2.1)	
4 OCH	0.136[i]	0.138[i]	0.137 (0.004)[i]	0.183 (0.005)	0.185 (1.7)[i]	0.210 (2.0)	
5 NCH	0.142	0.153	0.143 (0.009)	0.192 (0.005)	0.188 (1.9)	0.218 (2.0)	
6 OCH	0.143	0.156	0.148 (0.006)	0.200 (0.008)	0.194 (1.9)	0.219 (2.0)	
7 OCH-(OCH_2)	0.136[i]	0.138[i]	0.137 (0.004)[i]	0.166 (0.006)	0.185 (1.7)[i]	0.256 (2.1)	
8 OCH	0.150	0.157	0.150 (0.004)	0.203 (0.007)	0.205 (1.9)		
9 OCH_2	0.220	0.228	0.218 (0.008)	0.330 (0.010)	0.278 (2.1)		

[a] Native OSM, 100 mg/mL in 0.1 M KCl, pH 4.6. [b] Hydroxylapatite-treated OSM, 100 mg/mL in 0.1 M KCl, pH 4.6. [c] Average NT_1 values from footnotes *a* and *b* plus Hyp-OSM at (1) 180 mg/mL, pH 4.3, (2) 180 mg/mL, 0.15 M NaCl, pH 4.3, and (3) 180 mg/mL, 0.15 M NaCl, pH 7.2. [d] Average NT_1 values from preparations 1-3 in footnote *c*. [e] Des-C8,C9-OSM, 100 mg/mL in 0.1 M KCl, pH 7.2. [f] Asialo-OSM, 100 mg/mL in 0.1 M KCl, pH 4.6. [g] Due to the long T_1 of this group and the different delay times and instrument conditions used, only two determinations can be directly compared. [h] Unresolved shoulder, T_1 not determined. [i] Overlapping resonances for NeuNAc C_4 and C_7 (resonance 16) at ambient temperatures. [j] Includes overlapping peptide resonance(s).

Thus, each carbon's NT_1 value can be taken to be roughly proportional to its relative mobility in the mucin. Confirmation that the NT_1 values are proportional to motion is found by the increase in NT_1 values at elevated temperature (Figure 6A and Table IV). A comparison of the NT_1 values obtained for the highly viscous, native, OSM and nonviscous Hyp-OSM is also shown in Table IV. It is apparent that the NT_1 values are unchanged between both mucins even though their molecular weights and macroscopic solution properties are quite different. The NOE values for these two mucins were also found to be the same within experimental error (data not shown). In addition limited changes in counterion (K^+ vs. Na^+), ionic strength (0–0.15 M), and pH (4.3–7.2) did not alter the relaxation times of the mucin (see average NT_1 column in Table IV). Thus, we concluded that the mucin possesses relatively rapid internal segmental mobility which is unaffected by minor changes in solution environment and by large changes in macroscopic viscosity. This is to be contrasted with the more rigid peptide backbone carbons in most globular proteins which usually have relaxation values that reflect the protein's overall rotational correlation time (Wilbur et al., 1976) and are therefore sensitive to the macroscopic solution viscosity. Our results indicate that these mucins are highly flexible presumably unstructured molecules whose internal dynamics can be probed by ^{13}C NMR.

As shown in Figure 6, the NT_1 values for the protonated carbons (excluding the methyl groups) increase nonlinearly from the glycosylated serine and threonine peptide residues to the terminal sialic acid side chain. This trend is observed for all OSM-derived mucins at either NMR frequency and at elevated temperatures. The NOE values follow a similar trend (Table IV). Thus, as one would expect the relative side-chain mobility increases as one proceeds away from the peptide core. The sharp resonances of the NeuNAc residue

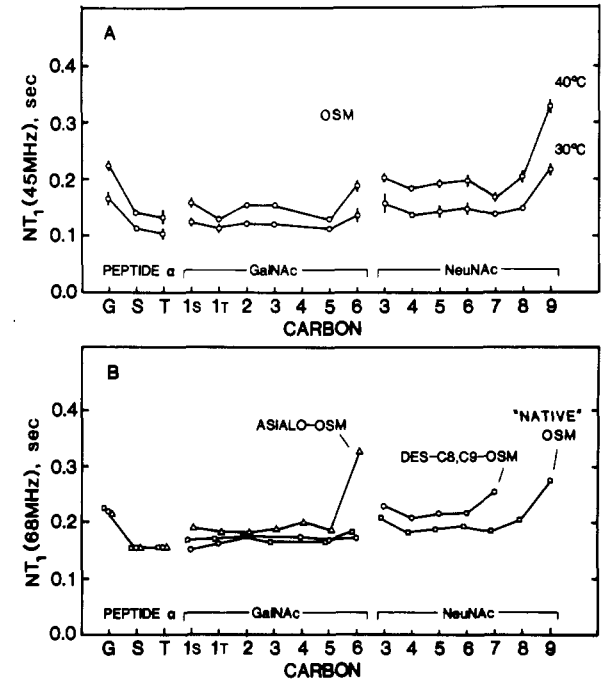

FIGURE 6: Plots of the ^{13}C NT_1 values as a function of carbon position for intact and modified OSM. (A) NT_1 values obtained at 45.3 MHz at 30 and 40 °C (data taken from Table IV). (B) NT_1 values obtained at 67.9 MHz at 30 °C for native OSM (□), des-C8,C9-OSM (○), and asialo-OSM (△) (data taken from Table IV). Peptide α-carbons: G, glycine; S, glycosylated serine; T, glycosylated threonine. GalNAc carbons: 1S, C1 serine linked; 1T, C1 threonine linked.

(and the GalNAc residue in asialomucin) further indicate substantial motional freedom for these terminal sugars. The broadening of the resonances of the remaining peptide and GalNAc carbons is most likely due to peptide sequence heterogeneity and serine or threonine glycosylation, respectively, and not due to highly restricted motion. The similar relaxation parameters obtained for the ring carbons within either the GalNAc or NeuNAc residues suggests that the conformational flexibility of the ring carbons is relatively uniform within each sugar residue. The higher NT_1 and NOE values of the methyl groups and the side-chain hydroxyl methylene carbons indicate these groups are spinning more rapidly than the residues to which they are attached. Because the observed NOE values are consistently lower than the values expected on the basis of the observed NT_1, it can be concluded that nonisotropic motions may contribute to the relaxation of a majority of the carbons in the mucin. Even the rapidly spinning *N*-acetylmethyls which have correspondingly long NT_1 values have NOE values much lower than the theoretical maximum of 3. Deviations from theory are also observed for most of the carbons when their NT_1 values obtained at the different NMR frequencies are compared. Thus, a significant contribution to the relaxation of these carbons is due to multiple and/or nonisotropic motions, presumably (on the basis of the low NOE) on a longer time scale than the apparent τ_c obtained from NT_1 alone. Thus, the peptide core and its attached carbohydrate side chains appear to be undergoing simultaneous motions ranging from rapid internal conformational reorientation to slower segmental bulk motions of the peptide and sugar residues. A comparison of the motional properties of the various constituents of the mucin and the extent to which their dynamics are altered by chemical modification follows.

Beginning with the peptide core the α-carbons of the glycosylated serine and threonine residues have identical NT_1 and NOE values at 67.9 MHz (Table IV) while the nonglycosylated serine α-, glycine α-, and proline δ-carbons have progressively higher NT_1 values and slightly larger NOE values. Thus, we conclude the mobilities of the glycosylated serine and threonine residues are similar, while the unsubstituted residues, as expected, have greater motional freedom. The high NT_1 values for the proline δ-carbons suggests that the proline ring as well is more flexible or mobile than the glycosylated amino acids. The relatively high NT_1 and NOE values obtained for the threonine C_γ, valine C_γ, and alanine C_β methyl groups indicate these groups are rapidly spinning. The small change in the NT_1 values of these groups between 45.3 and 67.8 MHz suggests isotropic rotation; however, the NOE values are low, indicating that the motion of these methyl groups are modulated by the slower movements of the peptide core. The relaxation values of the peptide carbons, at 67.9 MHz, are relatively unchanged between native OSM, des-C8,C9-OSM, and asialo-OSM, showing that the removal of sialic acid does not result in an increase in the mobilities of the peptide residues.

As discussed above the GalNAc ring carbons are more mobile than the serine or threonine α-carbons. The relatively constant NT_1 values obtained for the C1 through C5 carbons and the lower than expected NOE values are suggestive of the GalNAc ring carbons undergoing rapid but constrained internal motions (conformational flexibility) with hindered ring rotation. At 45.3 MHz but not at 67.9 MHz the NT_1 value of the GalNAc anomeric C1 carbon attached to a threonine residue is slightly but consistently lower than the NT_1 value of the C1 carbon linked to a serine residue. This may be caused by a slight reduction in mobility due to the steric effects of the threonine methyl group. The fact that these differences (and those not specifically discussed) were not observed at 67.9 MHz further points out the complex nature of the dynamics of these molecules in solution. Finally, the NT_1 values for the GalNAc C6 carbon are consistently higher than those of the GalNAc ring carbons, indicating a higher mobility for this pivotal linkage atom. As expected no significant changes in the mobility of the GalNAc ring are found when the C8 and C9 carbons of sialic acid are removed. However, in asialo-OSM the NT_1 values of the GalNAc ring increase with essentially no increase in the NOE values. Thus, the monosaccharide shows increased mobility although it may experience the slower segmental motions of the peptide core and/or hindered rotation. In the asialomucin the free GalNAc C6 methylene carbon shows a large increase in NT_1 and NOE, suggesting that this group is capable of rapid internal rotation. Its NOE, however, is somewhat lower than expected for simple isotropic rotation.

As shown in Figure 6 and Table IV the sialic acid ring carbons have higher NT_1 and NOE values than the GalNAc ring carbons. Interestingly, the C7 and C8 glycerol side-chain carbons of sialic acid have NT_1 and NOE values equivalent to the sialic acid ring carbons, C3 through C6. Only the terminal C9 carbon displays higher NT_1 and NOE values that suggest it may experience increased mobility. The same trend has also been observed in free and ganglioside-bound sialic acid residues by Czarniecki & Thornton (1977a,b) and Harris & Thornton (1978). These workers have proposed on the basis of the NT_1 values that the hydroxyl groups of C7 and C8 are hydrogen bonded to the sialic acid ring and are thus restricted in motion, while C9 is free to rotate. Our low NT_1 and NOE values suggest C9 undergoes rather restricted rotation especially when compared to the GalNAc C6 hydroxyl methylene in asialo-OSM. Thus, the C8 hydroxyl–C2 carboxyl group interaction proposed on the basis of the pH titration of C8 is further supported by the NT_1 values of C7, C8, and C9.

The removal of the C8 and C9 carbons of sialic acid results in an overall increase in the NT_1 values of the modified NeuNAc ring. These results suggest that the removal of carbons C8 and C9 causes an increase in the rotation or conformational flexibility of the sialic acid ring. The ca. 0.5 ppm change in chemical shift for the ring C2 and C4 carbons further suggests a change in ring conformation when the sialic acid side chain is modified. Unlike the GalNAc C6 hydroxyl methylene group in asialo-OSM, the C7 hydroxyl methylene group of the modified sialic acid shows only a moderate increase in NT_1 and NOE. In fact this C7 hydroxyl methylene group shows less mobility than the terminal C9 hydroxyl methylene group in the intact mucin. At elevated temperatures the resolved NeuNAc C7 carbon in the intact mucin is also found to have an anomalously low NT_1 (Table IV and Figure 6). These findings, in addition to the fact that the chemical shifts of C7, in both native OSM and des-C8,C9-OSM, show the largest temperature dependence (Table I), suggest that the hydroxyl group of this carbon is participating in an intramolecular interaction. Molecular models indicate that while the C8 hydroxyl can hydrogen bond to the C2 carboxyl group as discussed above, the C7 hydroxyl proton can interact either with the carbonyl of the *N*-acetyl group attached to C5 or with the NeuNAc ring oxygen.

The methyls of the *N*-acetyl groups on both GalNAc and NeuNAc display the largest NT_1 value found in the mucin. The NT_1 values of these overlapping resonances are relatively constant between the modified and unmodified mucins, in-

dicating that these groups may retain similar rates of motion. The relatively low NOE's obtained at 67.9 MHz, however, suggest these rapidly spinning methyl groups are also experiencing motions on a longer time scale, perhaps due to a slow rotation of the acetyl group itself.

Finally it is observed that the NeuNAc C3 methylene carbon has an increased NT_1 value compared to the remaining ring carbons in both the unmodified and the des-C8,C9-sialic acid residues at both NMR frequencies and at elevated temperatures. Similarly, increased NT_1 values have also been reported for the methylene C2′ carbon in the 2′-deoxyribose ring in 2′-deoxy-D-ribose, in its nucleotides, and in DNA (Levy et al., 1982). These results were attributed to a rapid ring puckering motion at the C2′ position due to the presence of the C2′ methylene. A similar rapid puckering motion may therefore be responsible for the higher NT_1 values for the C3 methylenes in the sialic acid residues.

Discussion

These studies were undertaken to ascertain the detailed solution properties of mucous glycoproteins by carbon-13 NMR spectroscopy. The submaxillary mucin from sheep was chosen for this initial study because of its simple structure and because of the extent of its characterization by others (Gottschalk & Bhargava, 1972; Tettamanti & Pigman, 1968; Hill et al., 1977a,b; Yamamoto & Yosizawa, 1978).

Overall our results suggest that the mucin peptide exists in a flexibile extended conformation undergoing rapid segmental motion, with the attached disaccharide side chains undergoing progressively more rapid motions. Comparisons of the native, viscous mucin with a partially degraded (low molecular weight) nonviscous mucin, both of which give essentially identical ^{13}C NMR spectral and relaxation results, further illustrate the dominance of rapid internal segmental motions. This is in contrast to what would be observed for a more rigid peptide as in a globular protein where the relaxation parameters reflect the slower overall rotational diffusion of the protein or peptide and are sensitive to the solution macroscopic viscosity [see Wilber et al. (1976), Allerhand & Oldfield (1973), and Tancrede et al. (1978)]. The essentially identical spectra obtained for the mucin in structure-disrupting 8 M urea or 6 M guanidine hydrochloride (data not shown) and the temperature-independent NMR spectra of the peptide residues further indicate that the mucin peptide exists as a random coil with no long-lived secondary structure.

The only previously reported ^{13}C NMR relaxation study of a mucin-like glycoprotein is that reported for the Antarctic fish freezing point depression glycoprotein (FPDG) (Berman et al., 1980). This glycoprotein is composed predominantly of the repeat unit Ala-Ala-(β-Gal-(1→3)-α-GalNAc)Thr- and can be isolated in several fractions with molecular weights ranging from 2000 to 33 000 (DeVries et al., 1970). On the basis of ^{13}C NMR studies at 67.9 MHz, Berman et al. (1980) concluded the FPDG existed in solution as a flexible random coil. Essentially the same values of NT_1 and NOE (at 67.9 MHz) are reported for the peptide and GalNAc ring carbons in FPDG as we have obtained in high molecular weight native OSM and des-C8,C9-OSM (Table IV). The ring NT_1 values of the terminal β-Gal ring carbons (average of 0.22 s) in FPDG are identical with the values obtained for the modified sialic acid ring carbons in des-C8,C9-OSM while the values for the intact sialic acid ring carbons of the unmodified mucin are somewhat lower (~0.19 s; Table IV). Thus, the modified sialic acid residue without its side chain may have similar solution dynamics as the β-galactose residue which is attached to GalNAc via a different linkage and furthermore lacks the charged carboxyl group. Because the T_1 values of the sialic acid carbons in the 2→6 and 2→3 isomers of sialyl lactose [α-NeuNAc (2→6 or 3)β-Gal (1→4) Glc] are reported to be very similar (Jaques et al., 1980), differences in the T_1 values between the des-C8,C9-NeuNAc 2→6 and Gal 1→3 linkages are not necessarily expected on the basis of their different linkages alone. Our results suggest that the exocyclic side chain, and not necessarily the carboxyl group, is important for modifying the rotation and/or conformational dynamics of the sialic acid residue. Thus, the low T_1 values for the sialic acid ring carbons reported in the literature are most likely due to the bulk of the exocyclic side chain and not due to the hydration of the charged carboxyl group as originally proposed (Jaques et al 1980).

On the basis of their similar relaxation parameters, the GalNAc ring carbons in both FPDG and OSM appear to undergo similar molecular motion. Upon the removal of sialic acid, the GalNAc ring carbons show an increase in NT_1, indicating, as expected, an increase in rotation or ring flexibility. The NT_1 values of the peptide carbons listed in Table IV are largely unaltered in the asialomucin compared to those of the native mucin. Thus, for this simple mucin, the flexibility of the peptide core is unaffected by the removal of the bulk of its charge and the loss of half of its carbohydrate residues. However, as expected, the nonglycosylated serine residues are more mobile than when glycosylated. The similar relaxation values of the glycosylated threonine α- and γ-carbons in both FPDG and native and modified OSM further suggest the additional mucin peptide residues, i.e., glycine and proline, do not greatly alter the dynamics of the glycosylated residues in the mucin compared to FPDG. The mucin, however, differs in one respect with FPDG regarding the GalNAc *N*-acetyl group; i.e., the low ^{13}C–^{1}H coupling constant of the methyl group and the large chemical shift temperature dependence of the carbonyl group reported for FPDG are not observed for OSM. This may be the result of an altered or disrupted interaction with the peptide due to the sequence heterogeneity of OSM compared to the regular peptide repeat of FPDG.

An intramolecular hydrogen bond between the side-chain hydroxyl group at C8 and the axial carboxyl group of C2 has been proposed by Jennings & Bhattacharjee (1977) for 2-*O*-methyl-α-D-*N*-acetylneuraminic acid and its derivatives. We conclude the same interaction occurs in the sialic acid residues of OSM. This is shown clearly by the pH titration curves of intact mucin and by comparing the titration of the sialic acid residues in the intact mucin with the des-C8,C9-NeuNAc-mucin (Figure 5). In the intact mucin the pK_a value of the carboxyl group is 2.0 while in the mucin where C8 and C9 are removed its pK_a values is increased, as expected if a favorable interaction is destroyed, to a value of 2.4 (see Table I). This difference in pK_a values represents a stabilization of slightly more than 0.5 kcal/mol for this hydrogen bond interaction. It is interesting that a pK_a value of 2.6 is most frequently reported in the literature for the sialic acid residue in biological systems (Ledeen & Yu, 1976). This value originally reported by Svennerholm (1956) and later by Bettelheim (1963) is based on the pH titration of the free reducing sugar which in aqueous solution has been shown to be ~90% the β-anomer at pH 7 (Bhattacharjee et al., 1975; Jaques et al., 1977). These results suggest the C8 hydroxyl group may not interact with the carboxyl group in the β-anomer. Further evidence that the equatorial carboxyl group is unable to interact with the C8 hydroxyl in the β-anomer of sialic acid is found from (1) the X-ray crystal structure of β-D-*N*-acetylneuraminic acid dihydrate (Flippen, 1973), (2) the proton

NMR conformational analysis of the side chain of the β-anomer in aqueous solution (Brown et al., 1975), and (3) the comparisons of the ^{13}C NMR chemical shifts of C8 in the α- and β-anomers of both the free acid and sodium salt of 2-*O*-methyl-*N*-acetylneuraminic acid (Jennings & Bhattacharjee, 1977). Thus, it is very likely that the presence or absence of the hydrogen bond interaction is the source of the difference in pK_a values of free sialic acid and the sialic acid residues in native OSM. This interaction or lack of interaction may furthermore explain the multiple pK_a values (ranging from ca. 2 to 2.8) obtained presumably for the sialic acid residues in bovine submaxillary mucin by Scheinthal & Bettelheim (1968). The extent of O-acetylation of the sialic acid residue as suggested by these workers could easily affect the carboxyl group's interaction and alter its pK_a. As further support, Neuberger & Ratcliffe (1973) have suggested on the basis of the pH dependence of hydrolysis that the pK_a value of 2-*O*-methyl-*N*-acetyl-α-D-neuraminic acid is 2.15 (at 50 °C). The C1 carboxyl carbon shows a linear temperature dependence of its chemical shift while the NeuNAc C8 chemical shift shows no change in the temperature range studied. Perhaps the disruption of its hydrogen bond with the carboxyl group and the changes in conforomational flexibility with increased temperature produce compensating effects.

The intact side chain of sialic acid has been shown to be necessary for the full enzymatic activity of neuraminidases against sialic acid containing compounds (Suttijilt & Winzler, 1971), with the des-C8,C9 analogue being very resistant toward hydrolysis by neuraminidase. Thus, the unique conformation of the side chain, due to the C8-OH,C2-COO^- interaction, presumably plays an important role for the action of neuraminidase. Since the alteration in pK_a of the carboxyl group upon side-chain modification appears to decrease the stability of the sialic acid link toward acid (data not shown), the actual pK_a value of this group appears to be unimportant for the action of neuraminidase. The C8 and C9 carbons of sialic acid have also been demonstrated to be necessary for the binding of influenza virus and for the activity of neuraminic acid aldolase (Suttijiit & Winzler, 1971).

In the intact OSM and des-C8C9-OSM the sialic acid C7 carbon displays an unusually low NT_1 value and a relatively large chemical shift temperature dependence. These results suggest the hydroxyl group of this carbon may be involved in a hydrogen bond. Space-filling CPK models of the α-anomer of sialic acid (which include the C8-OH,C2-COO^- interaction) suggest that the C7 hydroxyl proton may hydrogen bond to the carbonyl oxygen of the *N*-acetyl group at C5 or the NeuNAc ring oxygen. The interaction with the acetamido carbonyl group, however, appears to be more favorable on the basis of model building. Similar to glucose the sialic acid residue contains no axial hydroxyl groups off its ring. Thus, there is a possibility that the NeuNAc C7 hydroxyl group may be capable of interacting with the pyranose ring oxygen, through a water molecule, in a similar manner proposed to explain the unusually short NT_1 value of the C6 hydroxyl methylene of glucose (Czarniecki & Thornton, 1977b). Simple steric hindrance due to the adjacent *N*-acetyl group may furthermore be the origin of the unusual behavior observed for the C7 carbons in the sialic acid derivatives.

The chemical shift temperature dependencies of the carbons of the carbohydrate side chains of intact and modified OSM appear to be linear functions of temperature within the range studied. Because of this we conclude that no significant concerted conformational change or large structural phase transition occurs within this temperature range. The observed shifts, therefore, are most likely due to local changes in average conformation as a result of changes in molecular dynamics. Those carbons that are involved in hydrogen bond interactions or in sterically hindered environments could be expected to display the largest temperature affects.

Our data suggest that the removal of sialic acid causes a change in the spatial position or conformation of the GalNAc carbohydrate unit directly attached to the peptide. This is based on the decreased chemical shift sensitivity of the GalNAc residue to the different serine or threonine linkages after the removal of sialic acid. Similar results have been observed for the GalNAc residues in several sialoglycopeptides from human glycophorin A from which an interaction between the GalNAc C6 oxygen and an adjacent peptide amine has been proposed (Prohaska et al., 1981). (These workers, however, propose the splitting of the GalNAc resonances is due to neighboring peptide residues and not the residue to which the sugar is directly attached). In the sialic acid containing mucin an interaction of the GalNAc C6 oxygen may further be suggested on the basis of the temperature dependence of its chemical shift, which appears to be reduced upon the removal of sialic acid.

Acknowledgments

We thank Drs. P. W. Cheng and N. Jentoft and C. Blackwell for their advice and assistance in the isolation and purification of mucins. We thank Drs. N. Jentoft and J. Jentoft for their valuable suggestions made during the preparation of the manuscript.

Registry No. NeuNAc, 131-48-6; hydroxylapatite, 1306-06-5.

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