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PRIMARY STRUCTURE OF *O*- AND *N*-GLYCOSYLIC CARBOHYDRATE CHAINS DERIVED FROM MURINE SUBMANDIBULAR MUCIN (MSM)*

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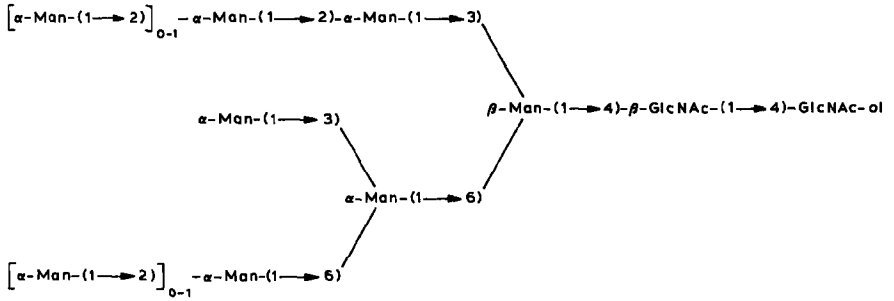
ABSTRACT

The carbohydrate moiety of mouse submandibular mucin (MSM) contains mainly D-mannose and 2-acetamido-2-deoxy-D-glucose together with sialic acid, D-galactose, and 2-acetamido-2-deoxy-D-galactose. *O*-Glycosylically bound saccharides, obtained by treatment of MSM with alkaline borohydride, were shown by methylation analysis to have the structure: α-NeuAc-(2→3)-β-Gal-(1→3)-GalNAc-ol. *N*-Glycosylically bound saccharides obtained from MSM by hydrazinolysis, and analysed by 500-MHz ^{1}H-n.m.r. spectroscopy, were shown to have the following comprehensive structures.



*Dedicated to the memory of Karl Freudenberg on the centenary of his birth.
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INTRODUCTION

The seromucous acinar cells of the submandibular salivary glands (MSM) of the mouse secrete, as a major component, a mucin, designated[1] MSM. This mucin is not related chemically and immunologically to the mucin of the sublingual glands of the mouse[1], nor to that of the submandibular glands of the rat[2,3]. MSM can be isolated easily by boiling the glandular water-extract, followed by passage[4] over Biogel P-300 or centrifugation at 100,000*g* overnight[5].

Roukema *et al.*[4] reported the following characteristics, which were recently confirmed by others[6]: MSM (*a*) migrates into a 7.5% polyacrylamide gel, indicating a mol. wt. of <300,000, in accordance with its sedimentation velocity ($S_{20,w} = 5.5$); (*b*) is not composed of different types of sub-units; (*c*) contains substantial proportions of D-mannose and 2-acetamido-2-deoxy-D-glucose in its carbohydrate moiety.

Since D-mannose is present in the MSM preparations, it has been suggested that *N*-glycosylically bound carbohydrate chains were present, in addition to *O*-glycosylic ones[4]. This supposition has been substantiated recently[7,8]. We now report the primary structure of the *O*- and *N*-glycosylic carbohydrate chains.

EXPERIMENTAL

Preparation of O- *and* N-*glycosylically bound carbohydrate chains.* — MSM was isolated as described[4]. In order to isolate the *O*-glycosylically bound carbohydrate chains, a sample of MSM, containing 1500 μg of sialic acid, was incubated[9] with 0.1M KOH and M KBH_4 at 45° for 16 h in order to effect β-elimination. After chromatography on a column of Dowex 50W-X2, two carbohydrate-containing fractions were obtained, I eluted with 10mM formic acid, and II eluted with M ammonium acetate[8]. Fraction I was separated on a column of Biogel P-4 into Fractions Ia and Ib. Fraction Ia was too small to permit further analysis. Fractions Ib and II were subjected to methylation analysis as described below.

N-Glycosylic carbohydrate chains were split off by treatment of a sample of MSM, containing 455 μg of sialic acid, with anhydrous hydrazine (200 μL) at 100° for 10 h. After evaporation of the hydrazine, the residue was *N*-acetylated and reduced with $NaBH_4$ as described by Tahasaki *et al.*[10]. High-voltage paper electrophoresis then gave acidic and neutral fractions which were studied by 500-MHz ^{1}H-n.m.r. spectroscopy.

Methylation analysis. — Fractions Ib and II were methylated by the Hakomori[11] method as modified by Björndal *et al.*[12]. The chloroform-soluble products were treated with methanolic 0.5M HCl at 80° for 24 h. The resulting methyl glycosides were acetylated[13] (pyridine–acetic anhydride) and analysed by g.l.c.–m.s.

^{1}H-N.m.r. spectroscopy (500 MHz). — A Bruker WM-500 spectrometer (Department of Biophysics, Nijmegen, The Netherlands), operating in the Fourier-

transform mode and equipped with an Aspect-2000 computer, was used. The probe temperature was 27°[14,15]. Before analysis, each sample was treated repeatedly with D_2O with intermediate lyophilisation, finally using 99.96% D_2O. The chemical shifts (δ) are expressed in p.p.m. downfield from the signal for internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), but were actually measured by reference to internal acetone (δ 2.225) with an accuracy of $\pm$0.002 p.p.m.

Analytical assay. — Sialic acid was measured, after hydrolysis of a sample in 0.05M H_2SO_4 at 80° for 1 h, by the thiobarbituric acid assay[16] and expressed as *N*-acetylneuraminic acid.

RESULTS

Structure of the O-*glycosylically linked oligosaccharides.* — Fraction Ib, obtained from MSM by β-elimination[8], behaved as a trisaccharide on Biogel P-4. Methylation analysis gave 2,4,6-tri-*O*-methylgalactose, 4,7,8,9-tetra-*O*-methylneuraminic acid, and 2-amino-2-deoxy-1,4,5,6-tetra-*O*-methylgalactitol, indicating Fraction Ib to have the structure: α-NeuAc-(2→3)-β-Gal-(1→3)-GalNAc-ol.

The largely undegraded material obtained after β-elimination, designated[8] Fraction II, when subjected to methylation analysis, gave 3,4,6-tri-*O*-methylmannose, 2,4,6-tri-*O*-methylgalactose, 2,4-di-*O*-methylmannose, 2-amino-2-deoxy-3,6-di-*O*-methylglucose, and 4,7,8,9-tetra-*O*-methylneuraminic acid, indicative of either complex chains or a mixture of *O*-glycosylic and oligomannoside-type chains.

Structure of the N-*glycosylically linked oligosaccharides.* — The neutral and acidic fractions, obtained after hydrazinolysis of MSM (Table I), were studied by 500-MHz ^{1}H-n.m.r. spectroscopy. The spectrum of the neutral fraction indicated a mixture of manno-oligosaccharides, as reflected by several signals in the region (δ 4.8–5.4) for anomeric protons attributable to α-Man residues and *N*-acetyl signals at δ 2.0–2.1. In detail, β-GlcNAc-*2*, (1→4)-linked to reduced GlcNAc-*1* (see annexed structure) is characterised[17] by the signals for H-1 at δ 4.614 (d, $J_{1,2}$ ~8 Hz), and NAc at δ 2.051.

The presence of β-Man-*3* is indicated[14] by the typical doublet for H-2 at δ

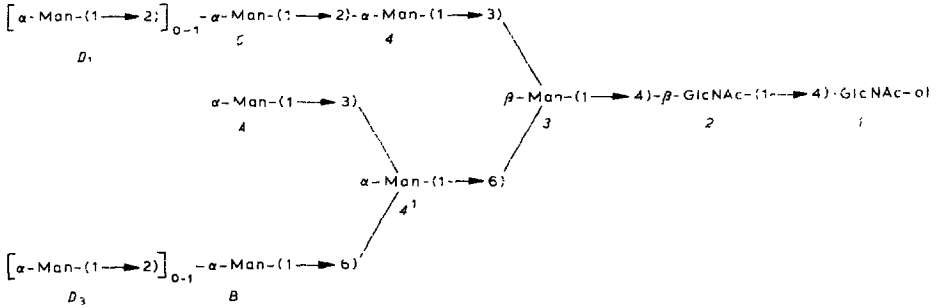
TABLE I

METHYLATION ANALYSIS OF THE OLIGOSACCHARIDES OF MSM AFTER HYDRAZINOLYSIS

Sugar derivatives	*Molar ratio*
2,3,4,6-Tetra-*O*-methylmannose	1.2
3,4,6-Tri-*O*-methylmannose	0.5
2,4-Di-*O*-methylmannose	1.0
2,4,6-Tri-*O*-methylgalactose	3.0
3,6-Di-*O*-methylglucosamine	3.3
4,7,8,9-Tetra-*O*-methylNeuAc	2.9

4.23. The signals for H-1 and H-2 at δ 4.868 and 4.15 belong to α-Man-*4*[1], which is (1→6)-linked to Man-*3*. The precise values of these chemical shifts indicate that Man-*4*[1] is substituted by Man-*A* and -*B* at positions 3 and 6, respectively. Man-*A* occurs in a non-reducing terminal position, because of the presence of its H-1 signal at δ 5.091 and the absence of a Man H-1 doublet at δ ~5.4. Heterogeneity exists at Man-*B* with regard to the presence or absence of Man-D_3. Most of the Man-*B* residues in the oligosaccharide samples are bearing (1→2)-linked α-ManD_3, because of the relatively intense H-1 signal for Man-*B* at δ 5.143, in conjunction with that for Man-D_3 at δ 5.041. However, some of the Man-*B* residues may occur in terminal positions (see below). The arm that is α-(1→3)-linked to the branching Man-*3* comprises mainly a disaccharide, namely, α-Man-*4* that is substituted at position 2 by Man-*C*. The H-1 signal for Man-*4* is found at δ 5.341, whereas that for Man-*C* is observed at δ 5.050. There is some evidence that 10–15% of Man-*C* may carry an additional Man-D_1 residue (H-1 of substituted Man-*C* at δ 5.302, H-1 of Man-D_1 at δ 5.041)[14].

Thus, the oligomannosidic *N*-glycosylically linked chains can be represented by the structure:



The 500-MHz ^{1}H-n.m.r. spectrum of the acidic fraction showed α-NeuAc (2→3)-linked to Gal. This structure results from *O*-glycosylic chains, partly degraded under hydrazinolysis conditions[10,18]. This is evident from the signals for H-3*a* (δ 1.800, t) and H-3*e* (δ 2.758, dd) (together with the NAc signal at δ 2.031), whereas the Gal H-1 doublet at δ 4.549 confirms the structural element mentioned above[19].

DISCUSSION

It is generally accepted that mucins carry many *O*-glycosylically linked carbohydrate chains. However, evidence has now been reported for the presence of both *O*- and *N*-glycosylically linked carbohydrate on the polypeptide core of a mucin. Earlier chemical analyses of MSM pointed in this direction[4,6], and subsequently the separation of both types of carbohydrate chains was described[7,8]. We now propose structures for these carbohydrate chains as determined by methylation analysis and 500-MHz ^{1}H-n.m.r. spectroscopy.

Whereas MSM contains both *O*- and *N*-glycosylically bound carbohydrate chains, Fleming *et al.*[2] have reported that the rat counterpart (RSM) does not contain D-mannose although other authors have reported[20] the presence of substantial proportions of this sugar. On the other hand, for the submandibular mucin (GSM) of the goat, the substantial proportion of D-mannose is possibly linked in unique *O*-glycosylic carbohydrate chains[21,22]. We did not obtain any evidence for the existence of D-mannose-containing *O*-glycosylically linked chains in MSM. A mucin of low molecular weight (200,000–250,000) isolated from human SM-SL saliva contains, *inter alia*, 28 D-mannose residues per 1000 amino acid residues[23]. Although the greater part of the oligosaccharides were alkali-labile, the presence of Asn-linked carbohydrate units could not be excluded. Other submandibular mucins have not been reported to contain substantial proportions of D-mannose. The sublingual mucins of the mouse[4] and of the rat[24,25] contain only small proportions of D-mannose.

From the results of the methylation analysis, the existence of an *O*-glycosylically bound trisaccharide is now proposed for MSM. Recently, two tetrasaccharides of the *O*-glycosylic type each containing L-fucose have been reported[26] for RSM. These structures are different from that of MSM and resemble those of porcine SM mucin[27] and canine SM mucin[28].

500-MHz ^{1}H-N.m.r. spectroscopy demonstrated that the *N*-linked chains of MSM are of the well-known manno-oligosaccharide type containing 6–8 D-mannose residues. No indications were obtained for the presence of complex chains. Denny and Denny[6] have reported that MSM contains both *O*- and *N*-glycosylically linked carbohydrate moieties.

Methylation analysis of Fraction II, obtained after β-elimination, also pointed to the possible presence of complex-type chains (indicated by the formation of methylated galactose and *N*-acetylneuraminic acid). However, separation of Fraction II into an acidic and neutral components by high-voltage paper electrophoresis, followed by ^{1}H-n.m.r. spectroscopic analysis, showed that the galactose and *N*-acetylneuraminic acid are located in the *O*-linked chains.

Previously, we reported[4] that MSM was composed of monomeric units with a molecular weight of not more than 300,000. Although several other submandibular mucins, *e.g.*, of sheep and pig[29], are composed of a major and a minor component, Denny and Denny[6] demonstrated that this is not so for MSM. Since MSM is not easily hydrolysed by pronase and papain[8] and since there are 4–5 carbohydrate chains per 20 amino acids, it seems likely that the resistance of MSM to proteolysis is due to a dense packing of carbohydrate chains along the polypeptide core. The effect of salivary proteinases on MSM is being investigated.

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