Effects of Deglycosylation on the Architecture of Ovine Submaxillary Mucin Glycoprotein*

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The structural features of native and deglycosylated ovine submaxillary mucin (OSM) were determined by electron microscopy of platinum unidirectionally shadowed preparations and by ultracentrifugation. Thin filamentous molecules, of which 90% were 100–230 nm in length with estimated diameters of 1.0–1.4 nm, were observed with dilute samples of OSM in high ionic strength solvents (5–30 µg/ml in 0.8 M NaCl or NH₄Ac). Ultracentrifugation studies indicated that these filamentous structures were monomers and/or dimers. At higher mucin concentrations or in lower ionic strength solvents, OSM molecules were oligomers that appeared as long rope-like strands. Removal of sialic acid residues by incubation with Clostridium perfringens neuraminidase yielded filamentous structures similar to those observed with OSM and some smaller less extended structures. Subsequent removal of the GalNAc residues of asialo-OSM with C. perfringens neuraminidase resulted in a dramatic change in appearance, from an extended filament to a globular form. The frictional ratios of OSM and deglycosylated OSM were consistent with the marked structural differences of these molecules. Native OSM had a frictional ratio of 3.09, comparable to that of highly asymmetric tropomyosin (3.22); deglycosylated OSM had a frictional ratio of 1.11, comparable to that of globular ovalbumin (1.08).

The biological consequences of glycosylation of the polypeptide core of glycoproteins are not fully understood and little is known about the effect of covalently linked carbohydrate units on the properties of glycoproteins. Current hypotheses based on studies of lectin interactions, biogenesis of lysosomal glycosidases, and the structure of IgG suggest that carbohydrate units may function as recognition markers and/or as one of the determinants of polypeptide conformation (1–3). Carbohydrate units have also been implicated in the aggregation behavior of OSM (6).

The relatively simple structure of the oligosaccharide chains² of OSM (NeuAcα2–6)GalNAc and the availability of purified glycosidases that permit the stepwise removal of each carbohydrate component (6, 7) have prompted the use of OSM as a model system in studying the characteristics of mucin-type glycoproteins. The saccharide units of OSM are attached via O-glycosidic linkages to serine and threonine residues of the polypeptide core, with approximately 290 disaccharide units/molecule or polysaccharide chain of 650 amino acids (6). The monomer (M₉ = 154,000) self-associates to yield tetramers (M₉ = 557,000–640,000) and at high mucin concentrations forms large aggregates (6). Gottschalk and McKenzie (8) studied the architecture of OSM by hydrodynamic techniques and concluded that this mucin is slightly elongated and resembles a random chain polymer. In contrast, the results of the present studies employing electron microscopy of platinum-shadowed specimens and sedimentation techniques suggest that OSM is a very elongated structure resembling an extended filament. The mucin assumes a globular conformation on enzymatic removal of the saccharide units, and the carbohydrate residues are required for maintenance of the filamentous conformation of OSM and may play a role in mucin aggregation.

EXPERIMENTAL PROCEDURES

Materials—The following materials were obtained from commercial sources: sheep submaxillary glands, Pel-Freeze; azocoll and casein yellow, Calbiochem-Behring; azocasein and NeuAc, Sigma; denatured Hb, ICN; GalNH₂ and GalNAc, Phansteihl Laboratories; dansyl chloride and amino acid standards, Pierce Chemical Co. Ovine submaxillary gland mucin was isolated and purified by the procedure of Tettamanti and Pigman (9) as modified by Hill et al. (6). Clostridium perfringens neuraminidase was prepared by a modification of the procedure of Cassidy et al. (10). α-N-Acetyl-D-galactosaminidase from C. perfringens (6), was a generous gift from Dr. L. Glasgow, Department of Medicine, Duke University. Absence of protease activity in the OSM and glycosidase preparations was confirmed colorimetrically after incubation of 0.1 unit of either glycosidase or 1 mg of OSM with each of the following substrates: azocoll, casein yellow, denatured Hb, and azocasein in 1 ml of 0.5 M NaAc, pH 5.5, at 37°C for 24 h.

Deglycosylation of OSM—Asialo-OSM prepared by incubating OSM with neuraminidase as described by Hill et al. (6), was dialyzed versus 0.15 M NH₄Ac for 12 h at 4°C and purified by gel filtration on a column (2 × 73 cm) of Sepharose 4B using 0.15 M NH₄Ac as the eluent buffer. Asialo-OSM eluted near the void volume; its amino acid and hexosamine composition corresponded to that of OSM and no sialic acid was detected (0.5% w/w). Deglycosylated OSM was prepared by incubating asialo OSM with α-N-acetyl-D-galactosaminidase (6). The amount of free GalNAc in the incubation sample was equivalent to that present in the bound state prior to incubation. The sample was dialyzed versus 0.15 M NH₄Ac and subjected to gel filtration on Sepharose 4B as described above. Deglycosylated OSM eluted in the included volume; its amino acid composition was the same as found for OSM and asialo-OSM. All analyses were in agreement with values reported by Hill et al. (6).

Analytical Procedures—Amino acid and hexosamine analyses were

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1The abbreviations used are: OSM, ovine submaxillary mucin; DG-OSM, deglycosylated OSM; GalNAc, N-acetylgalactosamine; NeuAc, N-acetylneuraminic acid; dansyl, 5-dimethylaminonaphthalene-1-sulfonil.

2All monosaccharides mentioned in this paper are assumed to be in the pyranose form and to have a β configuration.
performed as described earlier (11) except that a Glenco custom equipped with a photoelectric scanner. Molecular weights and sedimentation coefficients were determined in 0.8 and 2 M NaCl, in the presence and absence of 0.02 M sodium cacodylate buffer, pH 0.0, and in 5 M guanidine HCl. Weight average molecular weights and frictional ratios were calculated according to standard equations (15). The partial specific volumes of OSM (0.663) and DG-OSM (0.712) were calculated from the amino acid and carbohydrate compositions.

**Hydrodynamic Measurements**—Sedimentation equilibrium and velocity analyses were carried out in a Spinco model E ultracentrifuge equipped with a photoelectric scanner. Molecular weights and sedimentation coefficients were determined in 0.8 and 2 M NaCl, in the presence and absence of 0.02 M sodium cacodylate buffer, pH 0.0, and in 5 M guanidine HCl. Weight average molecular weights and frictional ratios were calculated according to standard equations (15). The partial specific volumes of OSM and DG-OSM were determined in 0.8 and 2 M NaCl. Molecular weights were calculated from the amino acid and carbohydrate compositions.

**Electron Microscopy**—Shadowed specimens were prepared for electron microscopy by procedures utilized previously for visualizing fibronectin (16) and microtubule-associated protein-2 (17). Mucin samples were diluted into buffers containing 50% glycerol and NH₄Ac and/or NaCl to final mucin concentrations of 5–100 µg/ml. Approximately 20 µl of each solution were sprayed onto a freshly cleaved mica surface, dried in vacuo at room temperature in a Balzers BAE 120 high vacuum coating unit (Lichtenstein), and unidirectionally shadowed with platinum-carbon at an angle of 5–5.5°. Electron microscopy and photography were performed as previously described (16). Filament diameters were calculated by triangulation from the tangent of the shadowing angle and the length of the shadow using calf thymus DNA as a calibration standard. The determination of diameters of highly elongated molecules can, however, only be considered an estimate as the methodology employed frequently yields low values because of the uncertainty of the shadow length relative to the narrow diameter (18, 19).

**RESULTS**

Ovine submaxillary mucin appeared as thin, extended filaments (Fig. 1a) when the shadowed specimens were prepared from dilute solutions of mucin (5–30 µg/ml) at high ionic strength. These filaments had a number average length of 156 ± 42 nm, 90% measurement of contour lengths were performed as previously described (16). The estimated diameter of the filaments ranged from 1 to 1.4 nm with a mean diameter of 1.2 nm. No correlation was evident between those particles varying in length and those varying in diameter. Thus, the ratio of length/diameter of the predominant species ranged from 83 to 192; a value of 117 was calculated for filaments clustered at 140 nm.

The filamentous morphology observed in Fig. 1a was also found in samples in which NaCl was replaced with 2 M NH₄Ac. Thus, the replicas of samples prepared by the glycerol drying technique were not obscured by high concentrations of volatile or nonvolatile salts. These findings are in agreement with the observations of Tyler and Branton (20). However, at lower ionic strength (0.15 M NaCl or NH₄Ac) or at higher solute concentrations (50–100 µg/ml), OSM appeared as long rope-like strands; considerable variation in length and width was observed (Fig. 1b).

In order to determine if the observed variation in filament lengths reflects aggregation of OSM monomers, sedimentation equilibrium analyses were undertaken. The molecular weight of OSM could not be determined by sedimentation analyses at the same concentrations used for the microscopy studies in Fig. 1a, since the low absorbance of OSM even at 230 nm did not permit accurate measurements of concentrations lower than 40 µg/ml. However, reliable measurements were obtained at concentrations of 90 µg/ml in 0.8 M NaCl (Fig. 3a); addition of 0.02 M cacodylate buffer did not influence the sedimentation equilibrium results. The In A versus square of the radial distance plots were concave, results consistent with either an associating or nonassociating system of monomers and oligomers. Within the concentration range of this experiment (40–200 µg/ml), limiting slopes near the meniscus and rear of the solution column (Fig. 3a, lines a and b) yielded weight average molecular weights (Mr) of 320,000 and 600,000, respectively. These values are similar to those reported for the dimeric and tetrameric forms of OSM (6).

The ultracentrifugation analyses (Fig. 3) showed that (a) the higher the OSM concentration and (b) the lower the salt concentration, the higher the Mr limits at the front and rear of the solution column and the higher the Mr value averaged over the whole solution column. For example, in 2 M NaCl the Mr values ranged from 220,000 to 670,000 over a concentration range of 40–180 µg/ml (Fig. 3b) and in 5 M guanidine HCl Mr values of 230,000 and 340,000 were obtained for the lower and upper limiting slopes (Fig. 3c). This latter result indicates also that guanidine HCl, a protein denaturant, did not dissociate OSM to its monomeric form. The above results are consistent with a system of reversibly associating monomers and oligomers held together by interactions which are moderately altered by salt concentration. Since the concentration of OSM used in the shadowing studies was 8-fold lower than the lowest concentration employed in the hydrodynamic studies, we conclude that monomeric and/or dimeric species of OSM predominate at high salt concentrations (Fig. 1a) with lesser amounts of trimeric and/or tetrameric species, and that the rope-like strands of OSM (Fig. 1b) are polymeric aggregates formed by reversible association at low salt and high mucin concentrations.

In contrast to OSM, asialo-OSM showed a variety of molecular structures, ranging from long strands to short filaments with a few small nonfilamentous structures (Fig. 1c).

Enzymatic removal of the GalNAc residues of asialo-OSM yielded a deglycosylated protein (DG-OSM) which, by electron microscopy, contained only compact globular structures (Fig. 4) of a size similar to known globular proteins of the same approximate size (~60,000). The observed change in morphology was not caused by proteolysis of asialo-OSM or DG-OSM, since no proteolytic activity was detected in either the OSM or the glycosidase preparations. Furthermore, the Mr of DG-OSM determined by sedimentation equilibrium was 57,000 (Fig. 3d) in close agreement with that reported by Hill et al. (6) and the molecular weight of DG-OSM plus the weight of the released oligosaccharides added up to the molecular weight of the OSM subunit (154,000). In control incubations that lacked DG-OSM or asialo-OSM, no globular or filamentous structures were observed.

The frictional coefficients (f/f0) of OSM and DG-OSM, calculated from molecular weights and sedimentation coefficients measured at the same concentrations and solvent conditions, were consistent with the structures observed by heavy metal shadowing. The S value for DG-OSM at 100 µg/ml in 0.8 M NaCl was 4.7. When combined with a Mr value of 57,000, a value of f/f0 = 1.11 was obtained, which is comparable to that of 1.08 for the globular protein ovalbumin (15).

\[ f/f_0 \]

\[ f/f_0 = \frac{f}{f_0} \]

\[ \text{f/f0} = 1.11 \]

\[ \text{f/f0} = 1.08 \]

\[ \text{f/f0} = 1.1 \]

\[ \text{M} = \frac{f}{f_0} \]

\[ \text{M} = 1.1 \]

\[ \text{M} = 1.08 \]

\[ \text{M} = 1.11 \]

\[ \text{M} = 1.08 \]

\[ \text{M} = 1.11 \]

\[ \text{M} = 1.08 \]

\[ \text{M} = 1.11 \]

\[ \text{M} = 1.08 \]
several different concentrations of OSM (80–160 µg/ml) in 0.8 M NaCl and at a single concentration (180 µg/ml) in 5 M guanidine HCl a single broad protein-solvent boundary was seen, again suggesting a reversibly associating system for OSM (as opposed to a mixture of covalently bonded oligomers). Assuming that the measured S value of 6.5 represents the major OSM species present under these conditions a value of \( f/f_0 = 3.09 \) is calculated for the dimer, which is comparable to that of 3.22 for tropomyosin, a rod-shaped protein (15). The high \( f/f_0 \) values obtained are consistent with either highly asymmetric or very extensively hydrated symmetrical ones. Although the possibility that OSM falls into the latter class is not ruled out by the limited hydrodynamic studies reported above, the electron microscopy and hydrodynamic data taken together suggest that OSM monomers and dimers are highly asymmetric.

**DISCUSSION**

Heavy metal shadowing techniques have proven useful for determining the architecture of large extended macromolecules. Several types of structures have been observed, including a “cross” typical of basement membrane laminin (21), tangled filamentous structures of human tracheobronchial mucin,\(^4\) trinodular structures of fibrinogen (18), and long flexible strands of fibronectin (16) and spectrin (19). The structure of OSM appears to be a relatively rigid elongated filament, similar to that of several other glycoproteins, including epiglycanin from TA3-Ha mouse mammary adenocarcinoma ascites cells (22) and lubricin, the boundary lubricant for bovine articular cartilage (23). Epiglycanin and lubricin, like OSM, contain numerous short oligosaccharide chains in O-glycosidic linkages, and although their oligosaccharide chains are not identical,\(^5\) these molecules share similar characteristics with OSM and can be considered mucin-type glycoproteins. OSM is the only deglycosylated glycoprotein examined so far by electron microscopy, as far as we are aware. If, however, lubricin and epiglycanin, like OSM, are globular after removal of their carbohydrate chains, it would suggest that newly synthesized mucin and mucin-like polypeptide chains may undergo a conformational change in secondary structure during or after the initial glycosylation event, i.e. the addition of GalNAc to serine and threonine residues.

We propose, on the basis of microscopy and hydrodynamic studies, an extended filamentous structure for OSM. This structure differs from that proposed by Gottschalk and McKenzie (8), who suggest that OSM resembles a random-chain polymer with some rigidity and is slightly elongated and highly hydrated. Their analysis utilized sedimentation velocity measurements at high mucin concentrations (1–8 mg/ml) from which the solute parameters, \( J \) (the ellipticity) and \( V' \) (the effective hydrodynamic volume), were estimated. Since OSM self-aggregates to tetramers and higher oligomers

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\(^5\) The major oligosaccharide chain in epiglycanin is the disaccharide Gal(β1→3)GalNAc (24) and in lubricin is the trisaccharide NeuAc(α2→3)Gal(β1→3)GalNAc (25).
that the latter were observing the hydrodynamic behavior of large aggregates of OSM rather than of the OSM subunit. The molecular model which best fits the observed filamentous structure of OSM (Fig. 1a) is an elongated cylinder. Assuming 650 amino acid residues for the OSM monomer, a length of 38 nm would be observed for a helical conformation while the same chain arranged in its most extended form would be 226 nm. The observed filaments are intermediate between these values (Fig. 2). The variation in length of the OSM molecules could be due both to different degrees of extension of these asymmetric molecules (as proposed for other extended macromolecules (16, 23)), and to a rapid and reversible associating system of monomers and oligomers (see “Results”). Since the concentration of OSM used in the shadowing studies was 8-fold lower than the lowest one employed in the hydrodynamic studies, we conclude that the predominant species of OSM in the sample shadowed in Fig. 1a are the monomer and/or dimer with lesser amounts of trimer and/or tetramer. If one assumes a cylindrical shape for the OSM filaments in Fig. 1a with the average dimensions of 156 nm length by 1.2 nm diameter, the resultant volume/particle is 176 nm³. Assuming a ρ of 0.663 cm³/g the calculated Mᵣ of 156,000, a value very close to that calculated for the monomeric form of OSM from the Mₛ of DG-OSM and the chemical composition of OSM. There are, however, two experimental complications which must be considered when assessing whether the filaments represent monomers or dimers. (a) The measurement of the diameter of filaments is considerably less accurate than that of the length and, because it is squared, errors in its measurement are compounded. Thus, for a dimer of OSM with an average length of 156 nm, the calculated diameter is 1.7 nm, a value significantly greater than the largest diameter observed (i.e., 14 nm). However, because of the uncertainty inherent in the diameter measurements, we cannot rule out the possibility that the filaments are dimers. (b) The variation in the length of the particles is considerably higher than that seen with rod-like molecules like myosin (19) but comparable to that of other mucin-like glycoproteins such as lubricin (23). This larger variation could reflect a compressibility of the cylinder in the lengthwise direction, or the possibility of dimer formation by staggered side to side associations of monomer units. Ultracentrifugation studies also suggest that the filamentous structures of OSM are monomers/dimers. A sedimentation coefficient of 6.5 S for solutions of 80–160 µg/ml of OSM in 0.8 M NaCl is obtained under conditions where the major species are the dimer and tetramer. For the dimer this results in a f/fₒ value of 3.09 and for the tetramer an even higher value. Similar calculations lead to the conclusion that the filaments are not the tetramer, as they would have diameters of at least 2.4 nm or be at least twice the length of the observed filaments.

In contrast, the massive rope-like strands observed at high mucin and low salt concentrations are thicker and much longer (Fig. 1b) than the monomer/dimer filaments (Fig. 1a). These forms presumably represent polymeric OSM structures formed by extensive overlap of OSM monomers, although end to end and side to side interactions can not be ruled out. For the elongated asialo-OSM particles, both dimensions were considerably more variable, with thick and thin sections observed in individual filaments (Fig. 1c). However, direct comparisons of OSM and asialo-OSM images obtained under identical conditions showed that the two molecules have essentially the same diameter, when only the straight sections of asialo-OSM filaments are considered. In summary, the present studies show that OSM is filamentous and undergoes a conformational change to a globular structure on deglycosylation. Therefore, we propose the following model of structural and functional interrelations for OSM based on the above-discussed microscopy and hydrodynamic studies. (a) In the absence of oligosaccharide chains, the polypeptide core of OSM has the conformation of a compact spherical molecule that does not aggregate. (b) When GalNAc is linked to the serine and threonine residues of the polypeptide chain, the molecule assumes a filamentous structure. Addition of sialic acid residues to yield fully glycosylated OSM results in no appreciable change in the filamentous morphology of the molecules. At high ionic strength and low solute concentration, monomers and dimers are the major components. (c) At low ionic strength aggregates are formed by staggered overlap and/or side to side or end to end interactions. These rope-like aggregates are presumably implicated in the viscous properties of OSM solutions, suggesting that carbohydrate units may be important determinants of the architecture and physicochemical properties of OSM. Whether this model is also applicable to human mucin glycoproteins, which contain more complicated oligosaccharide chains (26, 27) and a more complex polypeptide backbone (28–30), remains to be determined.

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