Macromolecular properties and polymeric structure of canine tracheal mucins

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Two high- M_r mucus glycoproteins (mucins), CTM-A and CTM-B, were highly purified from canine tracheal pouch secretions, and their macromolecular properties as well as polymeric structure were investigated. On SDS/composite-gel electrophoresis, a diffuse band was observed for each mucin. Polyacrylamide-gel electrophoresis using 6% gels also showed the absence of low- M_r contaminants in the mucins. Comparison of chemical and amino acid compositions revealed significant differences between the two mucins. Using a static-laser-light-scattering technique, CTM-A and CTM-B were found to have weight-average M_r values of about 11.0×10^6 and 1.4×10^6 respectively. Both mucins showed concentration-dependent aggregation in buffer containing 6 M-guanidine hydrochloride. Under similar experimental conditions, reduced-alkylated CTM-A had an M_r of 5.48×10^6 and showed no concentration-dependent aggregation. Hydrophobic properties of the mucins, investigated by the fluorescent probe technique using dansylphenylalanine as the probe, showed the presence of a large number of low-affinity (K_D approx. 10^{-5} M) binding sites. These sites appeared to be located on the non-glycosylated regions of the protein core, since Pronase digestion of the mucins almost completely eliminated probe binding. Reduction of disulphide bonds of CTM-A and CTM-B did not significantly alter the probe-binding properties. Also, addition of increasing NaCl concentrations (0.03–1.0 M) to the buffer caused only a small change in the hydrophobic properties of native and reduced-alkylated mucins. CTM-A was deglycosylated, without notable degradation, using a combination of chemical and enzymic methods. On SDS/PAGE the protein core was estimated to have an M_r of approx. 60000. On the basis of the protein and carbohydrate contents of the major mucin CTM-A, the mucin monomer was calculated to have an M_r of approx. 140000. The high M_r (11×10^6) observed by physical methods is therefore due to self-association of the mucin monomer subunits.

INTRODUCTION

Tracheobronchial mucus secretions play an important role in the normal functioning of airways. Mucins present in tracheobronchial secretions are responsible, to a large extent, for the viscoelastic properties of the secretions (Matthews *et al.*, 1963; Litt *et al.*, 1974). The mucins are high- M_r macromolecules with oligosaccharide chains covalently linked to a protein core (Carlstedt *et al.*, 1985). They generally contain 50–80% carbohydrate by weight (Bhavanandan & Hegarty, 1987) and have sulphate moieties. Although considerable progress has been made towards understanding the structure of respiratory mucin oligosaccharides, the total polymeric structure of mucin and its exact role in the formation of the mucus gel are poorly understood (Allen, 1983; Roussel *et al.*, 1983; Carlstedt *et al.*, 1985). Mucins exist as macromolecular aggregates in tracheobronchial and other mucus secretions as a result of both covalent and non-covalent interactions. These interactions may be between the mucin monomers themselves (Hill *et al.*, 1977; Houdret *et al.*, 1983) or may involve other (glyco)protein components (Woodward *et al.*, 1982; Allen, 1983). In order to understand, at a molecular level, how mucus is formed and how its physical properties are regulated physiologically or deranged as a result of disease, it is essential to have basic information regarding the size, configuration and polymeric structure of the native mucin molecules.

The canine tracheal pouch model is an ideal system, as it provides aseptic mucus secretions and, in addition, canine pulmonary anatomy closely resembles that of the human. Also, it has been reported previously that these secretions show similarities to human respiratory secretions (Ringler *et al.*, 1987). In our previous studies (Sachdev *et al.*, 1978), the isolation of canine tracheal mucin glycoproteins involved treatment of the

mucus secretions with reducing agents, followed by gel filtration and ion-exchange chromatography. The reduction, however, may affect the aggregation properties as well as structure of the mucin molecules, and thus precludes the investigation of macromolecular properties of the native mucin molecules. Isolation of mucins from non-reduced mucus secretions is therefore essential in order to study the physicochemical properties of the intact mucin molecules. The present paper describes the macromolecular properties and polymeric structure of two highly purified native (non-reduced) mucins, CTM-A and CTM-B, isolated from canine tracheal pouch secretions.

MATERIALS AND METHODS

Mucus collection and solubilization

Tracheobronchial secretions were collected from pure-bred beagle dogs fitted with tracheal pouches by the surgical procedure of Wardell *et al.* (1970). A detailed description of the procedure has been reported by Sachdev *et al.* (1978). On collection, the specimens were chilled on ice, diluted at least 5-fold with distilled water containing gentamicin sulphate (100 µg/ml) and dialysed exhaustively against deionized water containing 0.02% Na₂S₂O₃ for 16 h, freeze-dried and stored at –20 °C. Freeze-dried mucus secretions were then solubilized (10 mg/ml) in 0.1 M-Tris/HCl buffer, pH 7.5, containing 4 M-guanidine hydrochloride, 0.22 M-potassium thiocyanate and 0.02% Na₂S₂O₃ and gently stirred for 96 h at 4 °C. The solution was centrifuged at 12000 *g* for 1 h and the supernatant was used to purify mucins.

Gel-filtration chromatography

Solubilized mucus was initially fractionated on a Sepharose CL-4B column (5 cm × 90 cm) pre-equilibrated with 0.1 M-Tris/

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HCl buffer, pH 7.5, containing 4 M-guanidine hydrochloride, 0.22 M-potassium thiocyanate and 0.02 % NaN_3 . The elution was carried out with the same buffer. The major fractions A and B were rechromatographed individually on the Sepharose CL-4B column under similar experimental conditions. The glycoprotein fractions (A and B) were further chromatographed on a Superose 6 (f.p.l.c., Pharmacia) preparative column (1.6 cm $\times$ 50 cm) to obtain highly purified mucins, CTM-A and CTM-B. Native (non-reduced), reduced-alkylated and Pronase-treated mucins (2 mg/ml) were chromatographed separately on an analytical Superose 6 column (1.0 cm $\times$ 30 cm) and eluted with the above buffer. Gel-filtration chromatography of deglycosylated CTM-A was carried out on an analytical Superose 6 column equilibrated with 0.01 M-sodium cacodylate buffer, pH 6.0, containing 0.5 M-NaCl and 0.02 % NaN_3 and eluted with the same buffer at 4 °C as reported previously (Hill *et al.*, 1977; Bhavanandan & Hegarty, 1987).

Reduction-alkylation and Pronase digestion of purified native CTM-A and CTM-B

Reduction-alkylation and Pronase digestion of the purified mucins were performed as described by Chace *et al.* (1985) and Shankar *et al.* (1990) respectively.

Deglycosylation of purified CTM-A

The purified native CTM-A was first desialylated as follows. Purified mucin solubilized (7 mg/ml) in 50 mM-sodium acetate buffer, pH 5.5, containing 1 mM- CaCl_2 was treated with a pre-determined (based on mmol of sialic acid present in the native mucins) quantity of neuraminidase (*Vibrio cholerae*; Calbiochem) for 24 h at 37 °C, dialysed exhaustively against deionized water at 4 °C and freeze-dried. Further deglycosylation of the desialylated mucin was then performed by a slight modification of the method of Woodward *et al.* (1987) as described by Desai *et al.* (1991).

Chemical characterization of purified mucins

Chemical and amino acid analyses of purified mucins were performed as described previously (Chace *et al.*, 1985; Shankar *et al.*, 1990). N-Terminal amino acid(s) analysis of deglycosylated CTM-A was carried out with an automated gas-phase sequenator (Applied Biosystems, Inc.) employing Edman chemistry (Walsh *et al.*, 1981). Lipid analyses of purified native mucins were performed as described previously (Chace *et al.*, 1989; Shankar *et al.*, 1990).

Gel electrophoresis and electroblotting

The purity of mucins was examined by SDS/PAGE using composite vertical slab gels [2 % (w/v) polyacrylamide + 0.5 % (w/v) agarose containing 0.1 % (w/v) SDS] (Holden *et al.*, 1971; Sachdev *et al.*, 1978) and 6 % (w/v) polyacrylamide gels (Laemmli, 1970). Duplicate gels were run for each sample, one of which was stained with the periodic acid/Schiff (PAS) reagent to stain for carbohydrate and the other with Coomassie Brilliant Blue for proteins as described by Segrest & Jackson (1972). The gels were also stained by the silver-stain method of Heukeshoven & Dernick (1985).

Deglycosylated mucin sample was also electrophoresed on 12.5 % (w/v) polyacrylamide resolving gels as described above and the gels were equilibrated in blotting buffer {0.01 M-Caps [3-(cyclohexylamino)propanesulphonic acid], pH 11.0, in 10 % (v/v) methanol} (Matsudaira, 1987). Blotting was performed on a Hoefer Semiphor blotting apparatus at a constant current of 100 mA for 30 min. Immunostaining of membrane blots was

carried out with rabbit polyclonal antibodies to deglycosylated mucin as described by Sambrook *et al.* (1989).

Antibody production

Polyclonal antibodies were raised against native and deglycosylated CTM-A in New Zealand white rabbits. A suspension containing antigen (100 μg) emulsified in 250 μl of 0.9 % (w/v) NaCl and 250 μl of Freund's complete adjuvant was injected subcutaneously at multiple sites in the animal. A booster dose (400 μg of antigen in an emulsion containing 200 μl of 0.9 % NaCl and 200 μl of Freund's incomplete adjuvant) was given after 3 weeks. The animal was bled at day 0 (pre-immune serum) and biweekly 3 weeks after immunization. The serum was monitored for antibody activity using an e.i.s.a. The sera were stored at -20 °C.

Fluorescence studies

Mansylphenylalanine (Mns-Phe) was prepared essentially as described by Sachdev *et al.* (1973). For these studies a 200 μM stock solution of the probe was used. All measurements were carried out with a Shimadzu RF-5000 fluorescence spectrophotometer as described by Shankar *et al.* (1990). The native, reduced-alkylated and Pronase-digested mucins were dissolved at a concentration of 2.5 mg/ml by stirring for 48 h at 4 °C. To remove dust particles, the solutions were centrifuged at 12000 g for 20 min.

The binding of the probe with CTMs was studied by measuring mucin-induced alterations in the emission spectrum of the fluorescent ligand. The excitation and emission spectra of Mns-Phe (12.5 μM) were measured alone and in the presence of mucin (500 $\mu\text{g}/\text{ml}$). The affinity of Mns-Phe binding to the canine tracheal mucins was determined by Scatchard-plot analysis (Scatchard, 1949) as described previously (Azzi, 1973; Shankar *et al.*, 1990).

Mucin solutions (0.5 mg/ml) in 20 mM-Tris/HCl buffer, pH 7.4, containing 0.02 % NaN_3 plus the desired NaCl concentration were used to study the effect of salt on the hydrophobic binding properties of the mucins. The probe concentration was kept constant at 12.5 μM and fluorescence titrations were performed with increasing mucin concentrations.

Light-scattering studies

Static-light-scattering studies were carried out with the Photol DLS-700 laser-light-scattering spectrophotometer (Otsuka Electronics, Osaka, Japan), equipped with a He/Ne laser (632.8 nm) light source. The DLS-700 instrument is equipped with Zimm, Berry and Debye plots and one-concentration-method software for the analysis of static-light-scattering data. For these experiments, mucin stock solutions (1–2 mg/ml) were prepared in 0.02 M-Tris/HCl buffer, pH 7.4, containing 0.02 % NaN_3 and 6 M-guanidine hydrochloride. Buffer solutions were filtered through a 0.22 μm -pore filter to remove dust particles. The mucin solutions were centrifuged at 5000 g for 1 h to sediment dust particles. The supernatant was diluted to give the desired concentrations. Two different concentration ranges, 0.002–0.010 mg/ml and 0.02–0.10 mg/ml, were used for these studies. A minimum of five different mucin concentrations and seven different angles were used for calculation of the data. Calculations of the radii of gyration and weight-average M_r were based on the method of Tanford (1961) as outlined by Chace *et al.* (1989). The refractive-index increment (dn/dc) was obtained by using a Waters R401 differential refractometer. By the method of Zimm (1948), the Kc/R_θ values were plotted against $\sin^2(\theta/2)$ and extrapolated to zero degree angle and zero concentration for determination of M_r . Second virial coefficients (A_2) and radii of gyration (R_g) were also determined from the Zimm plots.

RESULTS AND DISCUSSION

Purification of mucins

The canine tracheal pouch model has been used (Wardell *et al.*, 1970) for collecting large volumes of non-purulent respiratory mucus secretions, and thus allows the isolation of intact mucins (i.e. with no degradation *in situ* by bacterial proteinases). Although solubilization of native human mucus secretions by using potassium thiocyanate has been reported previously (Khan *et al.*, 1976; Brown *et al.*, 1981; Chace *et al.*, 1985), a major portion of the canine mucus secretions was soluble only on prolonged stirring in buffer containing 4 M-guanidine hydrochloride and 0.22 M-potassium thiocyanate. Since the aim of the present study was to ascertain the properties of intact mucin molecules, we omitted the use of SDS (Liao *et al.*, 1979) or reduction-alkylation (Sachdev *et al.*, 1978) to solubilize the secretions.

Fig. 1. Sepharose CL-4B chromatography of canine tracheal mucus secretions

The mucus secretions (400 mg) were solubilized (10 mg/ml) in 0.1 M-Tris/HCl buffer containing 4 M-guanidine hydrochloride, 0.22 M-KCNS and 0.02% NaN₃. The supernatant after centrifugation was loaded on a Sepharose CL-4B column (5 cm × 90 cm) and eluted with the above buffer. Flow rate was 0.5 ml/min, peaks were monitored at 280 nm and 10 ml fractions were collected.

Fig. 2. Superose 6 chromatography of purified canine tracheal mucins

Purified mucins CTM-A (●) and CTM-B (○) (500 µg each) were loaded on a pre-equilibrated Superose 6 column (1 cm × 30 cm) and eluted with 0.1 M-Tris/HCl buffer containing 4 M-guanidine hydrochloride, 0.22 M-KCNS and 0.02% NaN₃. Peaks were monitored at 280 nm.

Fig. 3. SDS/composite-gel electrophoresis of purified mucins CTM-A and CTM-B

Purified mucins A and B were dissolved in sample buffer and electrophoresed as described in Materials and Methods section. Lane 1, phosphorylase B markers; lane 2, 100 µg of CTM-A; lane 3, 50 µg of CTM-B. Gel was stained by Coomassie Brilliant Blue.

Initial fractionation of solubilized canine tracheal mucus secretions on a Sepharose CL-4B column yielded four major fractions (Fig. 1). Fraction A was eluted close to the void volume of the column, whereas fractions B, C and D were included. Fractions A and B contained major mucin components of the secretions. In order to purify the mucins further, the fractions were rechromatographed separately on the Sepharose CL-4B column under similar experimental conditions. Rechromatography yielded partially purified mucin fractions which were purified further by f.p.l.c. on a Superose 6 preparative column. The purified mucins gave a single peak on chromatography on a Superose 6 analytical column (Fig. 2). Also, SDS/composite-gel electrophoresis gave a diffuse band for both mucins (Fig. 3). SDS/PAGE analyses using 6% gels, followed by staining with Coomassie Brilliant Blue and silver-staining protocols, further confirmed the absence of low- M_r protein contaminants in the purified mucins.

Chemical characterization of CTM-A and CTM-B

Chemical compositions of CTM-A and CTM-B are shown in Table 1. In both mucins carbohydrate analyses revealed the presence of fucose, galactose, *N*-acetylgalactosamine and *N*-acetylglucosamine. In addition, the mucins contained sulphate and sialic acid. Both CTM-A and CTM-B were found to have a lower content of sulphate and sialic acid than that observed for human respiratory mucins (Chace *et al.*, 1985; Shankar *et al.*, 1990). Total carbohydrate content, expressed as percentage dry weight of mucin, was about 57% for CTM-A and 30% for CTM-B. Absence of mannose, deoxyribose and uronic acid showed that both mucins were free of serum glycoprotein(s), DNA and proteoglycans respectively. Only trace amounts of triacylglycerols, cholesterol and cholesterol esters were detected upon lipid analyses of the purified mucins. Although both mucins had less than 1% of non-covalently associated lipids, no covalently bound lipids were detected after deacylation of the mucins with hydroxylamine. In contrast, Woodward *et al.* (1982)

Table 1. Compositional analyses of purified canine tracheal mucins

Amino acid composition data are presented as residues per 1000 residues; carbohydrate and sulphate data are presented as weight per cent.

	CTM-A	Deglycosylated CTM-A	CTM-B
Amino acid			
Aspartic acid	82	85	98
Threonine	136	112	88
Serine	120	110	113
Glutamic acid	100	110	107
Proline	93	87	86
Glycine	98	102	100
Alanine	67	70	73
Valine	63	69	64
Methionine	7	6	10
Isoleucine	27	30	27
Leucine	67	72	93
Tyrosine	20	25	12
Phenylalanine	26	29	12
Histidine	20	18	24
Lysine	30	28	46
Arginine	44	47	40
Carbohydrate			
Fucose	11.17		5.45
Galactose	17.08		10.34
Sialic acid	4.37		2.73
N-Acetylglucosamine	10.27		5.47
N-Acetylgalactosamine	11.33		3.89
Sulphate	2.82		2.29

reported large amounts of non-covalently bound lipids in the purified human respiratory mucin, as have Slomiany *et al.* (1981, 1983) in purified gastrointestinal mucins. On the other hand, Mantle & Forstner (1986) have reported less than 5% by weight of non-covalently bound lipids in purified intestinal mucin. Purified bovine gall-bladder mucin was found to have only trace levels of lipid (Smith & LaMont, 1984). The reason for the very low levels of lipids in the mucins purified by us is perhaps the different purification protocol used in this work.

Amino acid analyses of CTM-A and CTM-B (Table 1) revealed significant differences in their compositions. For example, CTM-A had notably higher content of threonine and lower amounts of aspartic acid, leucine and lysine than those observed for CTM-B. For CTM-A and CTM-B, the combined content of threonine, serine, proline, glycine and alanine was about 51% and 46% respectively of the total amino acids. Interestingly, chemical and amino acid compositions of CTM-A were similar to those reported for the highly purified mucin fraction (F3) isolated from canine tracheal pouch secretions by Ringler *et al.* (1987), but differed from the partially purified native mucin isolated by Liao *et al.* (1979), which contained 70% carbohydrate by weight. The carbohydrate compositions of CTM-A and the F3 fraction were 57% and 56% by weight, respectively, whereas the combined content of serine, threonine, proline, glycine and alanine was 51.4% and 52.7% for the two mucins respectively. Also, both CTM-A and mucin fraction F3 have apparent $M_r > 10^6$. Thus this major canine mucin also differed from the human tracheo-bronchial mucins, which are known to have approx. 80% carbohydrate.

Determination of M_r

M_r estimations on the Superose 6 analytical column in the presence of buffer containing 4 M-guanidine hydrochloride indi-

Fig. 4. Zimm plot of light-scattering data of CTM-A in 0.02 M-Tris/HCl buffer, pH 7.4, containing 6 M-guanidine hydrochloride and 0.02% Na₂S₂O₃.

Mucin concentrations used were (a) 2, 4, 6, 8 and 10 µg/ml and (b) 20, 40, 60, 80 and 100 µg/ml. Scattering angles were 30°, 40°, 50°, 60°, 70°, 80° and 90°. K is the optical constant, c the mucin concentration (mg/ml) and R_θ the Rayleigh ratio of the mucin solution = $r^2 i_\theta / I_0 (1 + \cos^2 \theta)$, where i_θ is the intensity of the scattered light at the scattering angle θ , I_0 is the intensity of the incident light and r is the distance of the photomultiplier tube from the scattering solution.

cated an $M_r > 1 \times 10^6$ for CTM-A and approx. 650 000 for CTM-B. Electrophoresis on composite gels indicated an M_r of approx. 550 000 for CTM-B, whereas CTM-A barely entered the gel matrix. Since mucins are known to show a concentration-dependent aggregation (Carlstedt *et al.*, 1985), M_r values were determined at two concentration ranges. At the lower concentration range (0.002–0.010 mg/ml) and higher concentration range (0.02–0.10 mg/ml), the weight-average M_r values for CTM-A were determined to be 11.0×10^6 and 25.9×10^6 respectively (Figs. 4a and 4b, Table 2). For CTM-A, under these experimental conditions, the radii of gyration were 169 and 271 nm respectively. The M_r of CTM-B in the concentration range 0.02–0.10 mg/ml was determined to be 1.37×10^6 , with radius of gyration of 85 nm (Fig. 5). By the same technique, reduced-alkylated CTM-A in the concentration range 0.02–0.10 mg/ml was estimated to have an M_r of 5.48×10^6 , with radius of gyration of 211 nm (Fig. 6).

Deglycosylation of purified CTM-A

The combination of chemical and enzymic deglycosylation procedures used in this work yielded a protein core with no detectable sialic acid, neutral sugar or N-acetylglucosamine and less than 1% N-acetylgalactosamine. Amino acid compositions

Table 2. Light-scattering data of purified CTM-A, CTM-B and reduced-alkylated CTM-A

The concentration range is that used for extrapolating the data to $c = 0$. Mucins were dissolved in 0.02 M-Tris/HCl buffer, pH 7.4, containing 6 M-guanidine hydrochloride and 0.02 % NaN_3 . M_r and radii of gyration (R_G) were obtained by extrapolating data to zero concentration and zero scattering angle. A_2 is the second virial coefficient.

Mucin sample	Concentration range (mg/ml)				
		$10^{-6} \times M_r$	R_G (nm)	$10^{-4} \times A_2$	$M_r^{1/2}/R_G$
CTM-A	0.002–0.010	11.0	169	–27.3	19.62
CTM-A	0.020–0.100	25.9	271	–1.33	18.78
CTM-B	0.020–0.100	1.37	85	–6.96	13.77
Reduced-alkylated CTM-A	0.020–0.100	5.48	211	6.28	11.09

Fig. 5. Zimm plot of light-scattering data of CTM-B in 0.02 M-Tris/HCl buffer, pH 7.4, containing 6 M-guanidine hydrochloride and 0.02 % NaN_3

Mucin concentrations used were 20, 40, 60, 80 and 100 $\mu\text{g}/\text{ml}$, and scattering angles were 30°, 40°, 50°, 60°, 70°, 80° and 90°. K , c and R_θ are defined in Fig. 4.

Fig. 6. Zimm plot of light-scattering data of reduced-alkylated CTM-A in 0.02 M-Tris/HCl buffer, pH 7.4, containing 6 M-guanidine hydrochloride and 0.02 % NaN_3

Mucin concentrations used were 20, 40, 60, 80 and 100 $\mu\text{g}/\text{ml}$, and scattering angles were 30°, 40°, 50°, 60°, 70°, 80° and 90°. K , c and R_θ are defined in Fig. 4.

Fig. 7. Gel-filtration chromatography of 100 μg each of deglycosylated CTM-A (●) and BSA (○) on Superose 6 (f.p.l.c.) column (1 cm $\times$ 30 cm) in 10 mM-sodium cacodylate buffer, pH 6.0, containing 0.5 M-NaCl

Fractions 13–19 of deglycosylated CTM-A were pooled and concentrated.

is possibly the different rates of destruction during hydrolysis of the hydroxy amino acids. Similar observations have been reported for deglycosylated bovine submaxillary mucin (Bhavanandan & Hegarty, 1987). Also, *N*-terminal amino acid analysis of the deglycosylated mucin showed the absence of free *N*-terminal amino acid(s), indicating that the *N*-terminus was blocked and that no peptide-bond cleavage had occurred during deglycosylation.

The deglycosylated mucin was subjected to SDS/PAGE in a 12.5 % resolving gel. No discrete band(s) were observed either on staining the gels with Coomassie Brilliant Blue or with silver-staining methods. Instead a smear was obtained throughout the length of the gel, with a densely stained region at the top of the separating gel. The appearance of deglycosylated mucins as broad smears on electrophoresis and protein staining has been observed in earlier studies (Marianne *et al.*, 1986; Bhavanandan & Hegarty, 1987). Since amino acid compositions and *N*-terminal analyses of native and deglycosylated CTM-A showed no degradation of the latter, it is likely that this anomalous migration behaviour is a characteristic property of the apomucin molecules.

On gel-filtration chromatography on Superose 6 and elution with 0.01 M-sodium cacodylate buffer, pH 6.0, containing 0.5 M-NaCl, the canine apomucin was eluted in the region of BSA (Fig. 7) with an estimated M_r of 60000. The eluate under the apomucin peak was collected and concentrated, and a sample was electrophoresed. A discrete band migrating in the region of M_r approx.

of the native and deglycosylated CTM-A (Table 1) showed no significant differences, except for slightly less threonine and serine in the latter. The reason for this decrease is not clear, but

Fig. 8. SDS/PAGE (12.5%) and immunoblotting of deglycosylated CTM-A

Lane 1, M_r markers; lane 2, 20 μ g of deglycosylated CTM-A; lane 3, 20 μ g of deglycosylated CTM-A collected and concentrated as in Fig. 7. Lanes 1–3 were stained with Coomassie Brilliant Blue. Lane 4, 20 μ g of deglycosylated CTM-A as in lane 3 transferred to a polyvinyl difluoride membrane and stained with rabbit polyclonal antibody (1:1000) to deglycosylated CTM-A as described in the Materials and methods section.

60000 was observed after staining with Coomassie Brilliant Blue (lane 3, Fig. 8). A very broad band, observed upon immunostaining (lane 4, Fig. 8), is more probably due to minor aggregates that were recognized by polyclonal antibodies to deglycosylated CTM-A.

In recent experiments using a cell-free translation system (Sachdev *et al.*, 1990), we have examined the protein product of mucin-specific mRNA isolated from fresh canine tracheal epithelial cells. The identification of the mucin precursor among the translation products was achieved by immunoprecipitation with specific antiserum prepared against deglycosylated CTM-A. The results indicated that the primary translation product *in vitro* of the canine tracheal mucin gene was an M_r -72000 protein. This value agreed closely with the M_r observed for deglycosylated CTM-A (approx. 60000). These data are also comparable with the M_r values reported for the protein core(s) of bovine submaxillary mucin (approx. 60000; Bhavanandan & Hegarty, 1987) and ovine submaxillary mucin (approx. 58000; Hill *et al.*, 1977). On the basis of the apomucin M_r of approx. 60000, and considering approx. 43% protein content of CTM-A, it appears that the mucin monomer has an M_r of 140000.

Effect of mucin concentration on aggregation behaviour

The state-of-the-art laser-light-scattering spectrophotometer used in the present study gave highly reproducible data even at low mucin concentrations. The M_r values of CTM-A, obtained from the Zimm plots of the light-scattering data, at the lower and higher concentration ranges were estimated to be 11.0×10^6 and 25.9×10^6 respectively (Figs. 4a and 4b). At the concentration range 0.02–0.10 mg/ml, the M_r of native CTM-B was determined to be 1.37×10^6 (Table 2). The negative second virial coefficients obtained at these concentrations suggest aggregation of the native mucin molecules. Such concentration-dependent aggregation was observed previously for human respiratory mucins

(Chace *et al.*, 1989). However, the canine mucins appear to form larger aggregates than those observed for human respiratory mucins, which may explain why the canine mucins were more difficult to solubilize. Owing to the relatively low M_r of CTM-B, it was not possible to monitor reliably the scattering intensity from solutions of this mucin below concentrations of 0.02 mg/ml. Although the Zimm plot for CTM-B (Fig. 5) showed only a slight concentration-dependent aggregation, the M_r of 1.37×10^6 observed is perhaps much higher than the mucin monomer. Under similar experimental conditions the M_r of reduced-alkylated CTM-A was determined to be 5.48×10^6 , with a radius of gyration of 211 nm. It is noteworthy that the Zimm plot did not show concentration-dependent aggregation for the reduced mucin. It is possible that the reduced mucin did not aggregate to the same extent as native mucin, and hence a relatively lower M_r was observed.

Binding of fluorescent probe Mns-Phe with CTM-A and CTM-B

The excitation and emission spectra of unbound Mns-Phe (12.5 μ M) showed excitation and emission maxima at 325 nm and 465 nm respectively. On binding to either CTM-A or CTM-B (500 μ g/ml), the emission maximum shifted to 445 nm. The fluorescence intensity of the Mns-Phe–mucin complex was about 4-fold higher than that for Mns-Phe alone. On binding to reduced-alkylated mucins the fluorescence intensity was quite similar to that observed for native mucin–Mns-Phe complex. In contrast, on binding to Pronase-digested mucins, no enhancement of Mns-Phe fluorescence was observed.

Binding affinity and number of binding sites

The mansyl group has been shown previously (Cory *et al.*, 1968; Turner & Brand, 1968; Sachdev *et al.*, 1973) to be a sensitive fluorescent probe for the detection of hydrophobic domains in proteins. We therefore employed this probe to study the hydrophobic probe-binding properties of ovine submaxillary and reduced-alkylated canine tracheal mucins (Sachdev *et al.*, 1979) and more recently to investigate the hydrophobic domains in human respiratory mucins (Shankar *et al.*, 1990).

Native canine mucins, CTM-A and CTM-B, contained a large number of hydrophobic binding sites for Mns-Phe. Scatchard-plot analyses of the binding of Mns-Phe with native mucins showed that in Tris/HCl buffer, the number of probe-binding sites for CTM-A and CTM-B were 46 and 35 respectively, with dissociation constant(s), K_D , approx. 10^{-5} M (Table 3). As observed previously for human respiratory mucins (Shankar *et al.*, 1990), these sites appeared to be localized on the non-glycosylated region of the protein core, since Pronase digestion of the native mucins almost completely eliminated probe binding. This observation is consistent with those reported previously for bovine gall-bladder mucin (Smith & LaMont, 1984), bovine cervical mucin (Bhushana Rao & Masson, 1977) and rat salivary mucin (Slomiany *et al.*, 1988). The involvement of associated and covalently bound lipids in the hydrophobicity of salivary mucins has been suggested by Slomiany *et al.* (1988). However, in our present study we believe that the contribution of lipids to the hydrophobic properties of the mucins may be very little, if at all, since mucins purified by our protocol contained less than 1% total lipids. The number of binding sites for CTM-A increased to 90 in buffer containing 0.1 M-NaCl and remained fairly constant in buffer containing up to 1.0 M-NaCl. However, in buffer containing 0.5 M- and 1.0 M-NaCl the K_D values were of the order 10^{-4} M. In the case of CTM-B there was an appreciable increase in the number of binding sites with increasing NaCl concentration (up to 1.0 M-NaCl) with K_D values approx. 10^{-5} M. Unlike the

Table 3. Binding of Mns-Phe with purified native and reduced-alkylated CTM-A and CTM-B in 0.1 M-Tris/HCl buffer, pH 7.4, containing different NaCl concentrations

Mucin sample	Tris buffer		[NaCl] (M)...		0.03		0.10		0.15		0.50		1.0	
	n*	K _D (M)	n	K _D (M)	n	K _D (M)	n	K _D (M)	n	K _D (M)	n	K _D (M)	n	K _D (M)

Native CTM-A	46	1.09 × 10 ⁻⁵	53	4.17 × 10 ⁻⁵	90	4.09 × 10 ⁻⁵	88	4.09 × 10 ⁻⁵	90	1.05 × 10 ⁻⁴	92	0.92 × 10 ⁻⁴
Native CTM-B	35	1.55 × 10 ⁻⁵	37	3.89 × 10 ⁻⁵	50	5.20 × 10 ⁻⁵	52	6.11 × 10 ⁻⁵	57	6.50 × 10 ⁻⁵	62	6.88 × 10 ⁻⁵
Reduced-alkylated CTM-A	60	1.60 × 10 ⁻⁵	63	5.04 × 10 ⁻⁵	63	3.21 × 10 ⁻⁵	57	3.15 × 10 ⁻⁵	62	5.84 × 10 ⁻⁵	64	1.20 × 10 ⁻⁴
Reduced-alkylated CTM-B	43	3.90 × 10 ⁻⁵	40	2.85 × 10 ⁻⁵	42	3.23 × 10 ⁻⁵	43	6.70 × 10 ⁻⁵	43	9.20 × 10 ⁻⁵	40	1.30 × 10 ⁻⁴

* n = number of binding sites per mg of mucin.

human mucins, the canine tracheal mucins did not show a marked alteration in hydrophobic binding properties in the presence of increasing NaCl concentrations (Shankar *et al.*, 1990). This is perhaps due to canine mucins having lower sialic acid and sulphate contents which are easily neutralized at a low salt concentration.

In our previous studies (Shankar *et al.*, 1990), we observed that reduction-alkylation of native human respiratory mucins caused a marked conformational change, as shown by an increase in the number of probe-binding sites and lower K_D values. Although the native human respiratory mucins exhibited only low-affinity binding sites, reduction-alkylation exposed high-affinity binding sites, presumably caused by unfolding of the native molecule. Also, the effects of increasing NaCl concentration on hydrophobicity were more marked in the reduced-alkylated human respiratory mucins than in the native mucins. In contrast, the canine mucins, on reduction-alkylation, did not expose any high-affinity binding sites, and there was no marked effect of increasing NaCl concentration on hydrophobic binding properties (Table 3). This suggests that the canine and human respiratory mucins may have different three-dimensional structures.

To the best of our knowledge, this is the first report describing the aggregation properties of the canine tracheal mucins. The major mucin component isolated from non-reduced mucus secretions exhibits a high M_r of approx. 11 × 10⁶, presumably due to self-association of mucin monomer subunits. On the basis of the apomucin M_r of approx. 60 000, the deduced M_r of the monomer subunit was approx. 140 000. The high degree of self-association may be due, in part, to the presence of hydrophobic domains as well as other non-covalent interactions between monomer subunits.

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