Mucus glycoproteins from cystic fibrotic sputum

Macromolecular properties and structural ‘architecture’

David J. THORNTON,*† John K. SHEEHAN,* Helén LINDGREN† and Ingemar CARLSTEDT†

*Department of Biochemistry and Molecular Biology, University of Manchester, Manchester M13 9PT, U.K., and †Department of Physiological Chemistry 2, University of Lund, P.O. Box 94, S-221 00 Lund, Sweden

Mucus glycoproteins (mucins) were isolated from sputum of patients with cystic fibrosis (CF) after separation into sol and gel phases. The mucus gel was solubilized with gentle stirring in 6 M-guanidinium chloride supplemented with proteinase inhibitors, and purification of mucins was subsequently achieved by isopycnic density-gradient centrifugation in CsCl/guanidinium chloride. Density-gradient centrifugation also revealed a heterogeneity of the macromolecules, the pattern of which varied between individuals, and mucins from the gel phase was pooled as ‘heavy’ and ‘light’ fractions. Gel chromatography on Sepharose CL-2B showed that the heavy fraction contained a larger proportion of smaller species than the ‘light’ fraction and that the gel phase mucins were much larger than those from the sol. An apparently homogeneous high- M_r mucin population from one individual contained approx. 70% (w/w) carbohydrate, the major sugars being *N*-acetylglucosamine (17.8%), *N*-acetylgalactosamine (6.7%), galactose (20.7%), fucose (13.2%) and sialic acid (11.4%). These mucins had an $s_{20,w}^0$ of 47 S, and an M_r of 15×10^6 – 20×10^6 , and rate-zonal centrifugation revealed a polydisperse size distribution [range $(5\text{--}30) \times 10^6$] with a weight-average M_r of 17×10^6 . The whole mucins were visualized with electron microscopy as linear and apparently flexible threads, disperse in size. Reduction produced subunits which were included on Sepharose CL-2B, and subsequent trypsin digestion yielded high- M_r glycopeptides which were further retarded. The size distributions and fragmentation patterns of mucins from two other CF patients were the same, as studied by gel chromatography, rate-zonal centrifugation and electron microscopy. We conclude that CF mucins are heterogeneous in both size and buoyant density and that the various populations, though differing in buoyant density, share the same architecture and macromolecular properties and are, in this respect, similar to mucins from normal respiratory secretions [Thornton, Davies, Kraayenbrink, Richardson, Sheehan & Carlstedt (1990) *Biochem. J.* **265**, 179–186] and human cervical mucus [Carlstedt & Sheehan (1989) *SEB Symp.* **XLIII**, 289–316].

INTRODUCTION

A thin layer of a mucous secretion covers the surface of the healthy large airways and participates in removing foreign particles through mucociliary transport (Sleigh *et al.*, 1988). Mucus glycoproteins (mucins) are a major constituent of ‘normal’ respiratory secretions (Thornton *et al.*, 1990), although other studies suggest that proteoglycans are the most prominent macromolecular component of the unstimulated healthy airways (Bhaskar *et al.*, 1985, 1986). Mucins are the large [M_r $(5\text{--}25) \times 10^6$] glycoproteins which provide the polymer matrix of mucus. Cervical mucins are linear and flexible chains which are composed of subunits (M_r approx. 2×10^6), each subunit containing alternating highly glycosylated and sparsely substituted regions of the protein core (Carlstedt & Sheehan, 1984a, 1989), and mucins from ‘normal’ respiratory secretions (Thornton *et al.*, 1990) as well as cystic fibrosis (CF) respiratory mucins appear to conform to this model (Gupta *et al.*, 1990). Histochemical studies suggest that the secretory cells of the respiratory tract, i.e. the mucous and serous cells of the submucosal glands, as well as the mucus-producing cells of the surface epithelium, may produce different mucins (Jeffrey & Reid, 1977; Mazzuca *et al.*, 1982; Spicer *et al.*, 1983).

The basic defect in CF has been identified as a mutation in a putative membrane-bound protein involved in chloride ion transport (Rommens *et al.*, 1989; Riordan *et al.*, 1989; Kerem *et al.*, 1989), but it is not clear how this derangement causes the progressive lung disease which is the major cause of morbidity and mortality in CF. Sputum from patients suffering from CF

has similar physical properties as sputum obtained from patients with other respiratory diseases (e.g. asthma, chronic bronchitis, bronchiectasis), provided that samples with the same degree of purulence and a similar amount of gel phase are compared (Lopez-Vidriero & Reid, 1977). No CF-specific mucin has been described, but an increase in the sulphate content of CF bronchial mucins has been reported (Boat *et al.*, 1976; Lamblin *et al.*, 1977), a finding supported by histochemical evidence (Lamb & Reid, 1972).

In this study, we have isolated mucins from the sol and gel phases of sputum from five individuals with CF. The macromolecules were heterogeneous in buoyant density and polydisperse in size, the mucins in the sol being smaller than those in the gel. The major populations from the gel phase encompassed species with values of M_r between 5×10^6 and 30×10^6 which were visualized as linear chains by electron microscopy. The molecules were fragmented into ‘subunits’ and high- M_r glycopeptides, as previously observed for mucins from normal human tracheobronchial secretions and cervical mucus. Though CF mucins are heterogeneous, the various populations have the same macromolecular properties and architecture.

EXPERIMENTAL

Materials

Guanidinium chloride was obtained from Sigma Chemical Co. (practical grade) and from Bethesda Research Laboratories (Ultrapure grade). Stock solutions (approx. 8 M) of the former were treated with charcoal before use. Di-isopropyl phosphoro-

Abbreviations used: CF, cystic fibrosis; PAS, periodic acid–Schiff.

† To whom correspondence should be addressed.

Fig. 1. Density-gradient centrifugation in CsCl/4 M-guanidinium chloride (a-e) and in CsCl/0.2 M-guanidinium chloride (f-j) of mucins from the gel phase

(a-e) After centrifugation (approx. 90 h, 84000 $g_{av.}$, Beckman L65 Ultracentrifuge, 42.1 rotor, 15 °C), fractions were collected from the bottom of the tubes and analysed for sialic acid (●), A_{280} (○) and density (△). Fractions were pooled as shown by the horizontal bars. (f-j) The mucin fractions, twice purified by density-gradient centrifugation in CsCl/4 M-guanidinium chloride, were subjected to density-gradient centrifugation in CsCl/0.2 M-guanidinium chloride and analysed as described above. The mucins were pooled as heavy and light fractions, as indicated by the letters H and L. The non-mucin protein (P) and DNA were recovered where indicated.

fluoridate was purchased from Fluka and was stored as a 0.1 M solution in propanol at -20 °C. Trypsin (EC 3.4.21.4; type XI, diphenylcarbonyl chloride-treated) was from Sigma, and Sepharose CL-2B was from Pharmacia.

Analytical methods

Hexose was determined as described by Goa (1955), and sialic acid was determined using an automated procedure (Lohmander *et al.*, 1980) based on the method described by Jourdain *et al.*, (1971). Quantification of mucins was also achieved by slot-blotting followed by periodic acid-Schiff (PAS) staining (Thornton *et al.*, 1989). Briefly, samples were diluted to 0.1 M, or less, of guanidinium chloride, blotted on to 0.2 μ m-pore-size nitrocellulose membranes (Anderman, Kingston, Surrey, U.K.) pre-treated with poly(L-lysine), stained with PAS and the colour yield measured. Solution densities were determined using a 500 μ l Hamilton syringe or a Carlsberg pipette as a pycnometer.

Isolation of mucins

Sputum samples were collected from patients with CF during sessions of physiotherapy, frozen immediately and stored at -20 °C until used. The diagnosis was based on typical pulmonary and gastrointestinal manifestations and was confirmed by a sweat test. The five patients (age 9-21 years) are referred to below as CF-I to CF-V. All sputa were infected, but no attempt was made to grade the degree of purulence.

The sputum samples were thawed into an approximately equal weight of 0.2 M-NaCl/5 mM- Na_2EDTA /5 mM-*N*-ethylmaleimide/5 mM-di-isopropylphosphorofluoridate/10 mM-sodium phosphate buffer, pH 6.5. The sol and gel phases were separated by centrifugation (149000 $g_{av.}$ for 45 min; Beckman 70 Ti rotor) and the sol was mixed with approx. 2 vol. of 6 M-guanidinium chloride/5 mM- Na_2EDTA /5 mM-*N*-ethylmaleimide/10 mM-sodium phosphate buffer, pH 6.5 (extraction

buffer), and dialysed in the cold against 10 vol. of this solution. The gel was treated with di-isopropyl phosphorofluoridate (approx. 2 ml of a 0.1 M solution in propanol) for 5 min before the addition of ice-cooled extraction buffer. After dispersion (three strokes with a Dounce homogenizer; loose pestle), the gel was stirred for approx. 15 h at 4 °C. Insoluble material was removed by centrifugation (42000 $g_{max.}$ for 30 min; Beckman 20.1 rotor) and re-extracted twice with extraction buffer. A small insoluble residue was discarded. Caesium chloride and 5 mM- Na_2EDTA /10 mM-sodium phosphate buffer, pH 6.5, were added to an initial density of 1.39 g/ml in 4 M-guanidinium chloride, and isopycnic density-gradient centrifugation was performed as described in the legends to Figs. 1 and 2. This purification step was repeated once for the major mucin fractions from the gel phase to ensure the complete removal of non-mucin proteins. The mucins were then dialysed against 0.2 M-guanidinium chloride/5 mM- Na_2EDTA /10 mM-sodium phosphate buffer, pH 6.5, CsCl and buffer were added to an initial density of 1.50 g/ml in 0.2 M-guanidinium chloride, and isopycnic density-gradient centrifugation was performed as described above. The final mucin fractions were pooled as indicated in Figs. 1 and 2. Side fractions containing DNA and protein were recovered by freeze-drying after extensive dialysis against water.

Mucin fractions were finally subjected to preparative gel chromatography on Sepharose CL-2B as described in the legend to Fig. 3. Material eluting with the void volume of the column was concentrated over a PM-10 ultrafilter (Amicon) and stored in 4 M-guanidinium chloride.

Preparations of subunits were obtained after reduction of 'whole' mucins and T-domains after subsequent trypsin digestion (Carlstedt *et al.*, 1983b).

Carbohydrate analysis

Neutral sugars were determined as their corresponding alditol acetates by g.l.c. with L-arabinose as internal standard (Lindahl,

Fig. 2. Density-gradient centrifugation in CsCl/4 M-guanidinium chloride (a-e) and in CsCl/0.2 M-guanidinium chloride (f-j) of mucins from the sol phase

(a-e) After centrifugation (approx. 90 h, 84 000 g_{av} , Beckman L65 ultracentrifuge, 42.1 rotor, 15 °C) fractions were collected from the bottom of the tubes and analysed for sialic acid (●), A_{280} (○) and density (△). Fractions were pooled as shown by the horizontal bar. (f-j) The major mucin fractions purified by density-gradient centrifugation in CsCl/4 M-guanidinium chloride were subjected to isopycnic density-gradient centrifugation in CsCl/0.2 M-guanidinium chloride and analysed as described above. The mucins were pooled as indicated by the horizontal bars. The non-mucin protein (P) and DNA were recovered as indicated.

1970). Samples were hydrolysed in 2 M-trifluoroacetic acid at 100 °C for 4 h in sealed tubes under N_2 . Hexosamines were quantified with a Durrum automatic amino acid analyser with D-glucosaminitol as internal standard after hydrolysis in sealed tubes under argon with 4 M-HCl (AristaR) for 10 h at 100 °C.

Analytical ultracentrifugation

Sedimentation-velocity and analytical isopycnic density-gradient centrifugations were performed using an MSE 75 Centriscan analytical ultracentrifuge. Samples for isopycnic centrifugation were dialysed into CsCl/4 M-guanidinium chloride (Ultrapure grade), initial density 1.46 g/ml. Centrifugation was performed at 145 000 g_{av} for 45 h at 25 °C and the absorbance optics were used at 280 nm. Sedimentation-velocity centrifugation of the mucins was performed at 28 000 g_{av} at 20 °C, and the schlieren optics were used to monitor the sedimentation rate. Samples were dialysed into 6 M-guanidinium chloride (Ultrapure)/1 mM- Na_2EDTA /1 mM-sodium phosphate buffer, pH 6.5. The apparent sedimentation coefficients (s) were determined from the peak positions, and $1/s$ was extrapolated to zero concentration and corrected to standard conditions (water, 20 °C). A value of 0.65 ml/g was used for the partial specific volume (Carlstedt *et al.*, 1983a).

Light scattering

A Malvern Molecular Analyser System 4300 was used with a 30 mW He/Ne laser for quasi-elastic and total intensity measurements (Sheehan & Carlstedt, 1984). Readings were taken at several angles (θ) down to 20° and the values were extrapolated to zero. Measurements were performed at 25 °C and corrected to 20 °C. Solutions were clarified by centrifugation (1–2 h, 20 000 g , 20 °C; 10 × 10 ml angle rotor; MSE 50 centrifuge), and mucin

concentrations were assessed after the measurements by sialic acid determination using the cognate stock solution as a standard (Carlstedt *et al.*, 1983b).

Rate-zonal centrifugation

Rate-zonal centrifugation was performed on a guanidinium chloride gradient as described by Sheehan & Carlstedt (1987). Samples (100 μ l in 4 M-guanidinium chloride) were layered on to the pre-formed gradient with a Hamilton syringe and spun at 160 000 g_{av} for 2.5 h and 20 °C in an MSE 6 × 4.2 ml Ti swing-out rotor. The tubes were emptied from the top directly into light-scattering cells. The concentration of mucins was determined by PAS staining after slot-blotting on to nitrocellulose membranes (using the unfractionated mucins as a standard), and the concentration of guanidinium chloride was determined by measuring the refractive index with an Abbé refractometer. Total-intensity and quasi-elastic light-scattering measurements were then performed at an angle (θ) of 20° and a temperature of 25 °C on a Malvern 4700c system equipped with a Siemens 30 mW He/Ne laser (Thornton *et al.*, 1990). Values were corrected to 4 M-guanidinium chloride and 20 °C.

Electron microscopy

Electron microscopy was performed as described by Sheehan *et al.* (1986). Whole mucins, subunits and T-domains were spread in a monolayer of benzyldimethylalkylammonium chloride from a hypophase of either 6 M-guanidinium chloride (whole mucins) or 50 mM-magnesium acetate (subunits and T-domains). The latter molecules were diluted directly into the hypophase from a solution in 4 M-guanidinium chloride. Molecules were visualized with a Phillips 400 electron microscope at 100 kV, and contour lengths were measured with a Planix digital planimeter (Hall &

Fig. 3. Gel chromatography on Sepharose CL-2B of mucins purified by density-gradient centrifugation

Chromatography was performed on a column (22 mm $\times$ 1400 mm) eluted with 4 M-guanidinium chloride, adjusted to pH 7, at a flow rate of approx. 40 ml/h. Fractions (approx. 7.3 ml) were analysed for sialic acid and pooled as indicated by the horizontal bars.

Watts, Links Trading Estate, Yeovil, U.K.). Measurements were performed on 150–300 molecules and the number-average (l_n) and weight-average (l_w) lengths were calculated.

Analytical gel chromatography

Gel chromatography of whole mucins, subunits and T-domains (200–300 μ g in 0.5 ml) was performed on a column (0.9 cm $\times$ 60 cm) of Sepharose CL-2B eluted with 4 M-guanidinium chloride, pH 7.4, at a flow rate of 2 ml/h. Fractions (0.5 ml) were analysed by PAS staining with slot-blotting on to nitrocellulose membranes. The void and totally included volumes were determined with Dextran Blue and *N*-acetylneuraminic acid respectively.

Inhibition e.i.s.a.

Subunits prepared from CF-I-light and heavy, CF-III-light and CF-V-light were studied in an inhibition e.i.s.a. using a polyclonal antiserum raised against human cervical mucin subunits as described by Sheehan *et al.* (1991).

RESULTS

Isolation and purification of CF mucins

The amount of gel phase after centrifugation of the sputum samples at 149000 g_{av} varied between 14 and 36 % (g/100 g of

sputum). The gel readily dissolved in 6 M-guanidinium chloride, and after two re-extractions only a tiny residue was left, which was discarded.

The result of the first isopycnic density-gradient centrifugation in CsCl/4 M-guanidinium chloride of the mucins from the gel is shown in Figs. 1(a)–(e). The major mucin fraction banded between the densities 1.38 g/ml and 1.50 g/ml. Hexose (anthrone method) co-distributed with sialic acid (results not shown). Most of the u.v.-absorbing material was found at the top of the gradient and as a relatively narrow peak at the high-density part of the mucin distribution. The former comprises proteins and lipids and the latter peak appears at the position expected for DNA (Carlstedt *et al.*, 1983a). The mucin fractions were subjected to a second isopycnic density-gradient centrifugation step in CsCl/4 M-guanidinium chloride to ensure complete removal of non-mucin proteins (results not shown), and then to a third density-gradient step in CsCl/0.2 M-guanidinium chloride (Figs. 1f–1j). Under these conditions the mucins appeared at a much higher buoyant density than in CsCl/4 M-guanidinium chloride, consistent with previous observations (Carlstedt *et al.*, 1983a). In three of the samples (CF-II, CF-IV and CF-V) a prominent shoulder was evident at the high-density part of the mucin distribution (Figs. 1g, 1i, and 1j). In sample CF-III (Fig. 1h) only a small shoulder was present, whereas sample CF-I (Fig. 1f) displayed a split peak, suggesting the presence of two different components. The mucins were pooled as heavy and light fractions

Fig. 4. Concentration-dependence of (a) the reciprocal sedimentation coefficient and (b) the translational diffusion coefficient of CF-V-light

(a) Sedimentation rate measurements were performed on solutions with concentrations of 1.59, 1.19, 0.79, 0.59 and 0.40 mg/ml. The reciprocal sedimentation rate is plotted against concentration, and extrapolation to zero concentration was performed by linear regression with the use of least-squares analyses. The angular-dependence of the diffusion coefficient D_t was performed on solutions (0.30, 0.22, 0.12, 0.06 and 0.03 mg/ml), and extrapolation of D_t to zero angle was performed using values obtained at $\theta = 20$ – 40° . The concentration-dependence of the diffusion coefficient at zero angle (b) is extrapolated to zero concentration by linear regression with the use of least-squares analyses.

when appropriate. A sharp u.v.-absorbing peak was evident at a density > 1.6 g/ml and showed a u.v. spectrum typical of DNA. In the gel phase most of the proteins (92–95%) were removed from the mucins by the first density-gradient centrifugation step, and less than 1% was removed by the third one.

When the mucins from the sol phase were subjected to isopycnic density-gradient centrifugation in CsCl/4 M-guanidinium chloride, the results shown in Figs. 2(a)–2(e) were obtained. The glycoproteins were distributed over a broader density range (approx. 1.34–1.50 g/ml) than were the major population from the gel phase. More sialic-acid-positive material was found at a lower buoyant density than was observed for the mucins in the gel phase, whereas less u.v.-absorbing material was present at the expected position for DNA. The results of the second isopycnic density-gradient centrifugation in CsCl/0.2 M-guanidinium chloride (Figs. 2f–2j) showed that the mucins had a higher buoyant density in this solvent than in CsCl/4 M-guanidinium chloride, as did the mucins from the gel phase. Much less u.v.-absorbing material at the position of DNA was noted compared with the gel phase samples, whereas more u.v.-absorbing material (proteins) was present at the top of the gradient.

By recovering the major side-fractions from the density gradients, it was possible to estimate the amount of non-dialysable solids in the sputa and how this material is distributed between protein (top fractions), mucins and DNA. The amount of non-dialysable material varied from 2.3 g/100 g to 5.4 g/100 g of sputum, most of which was confined to the gel phase (73–88%). The major protein fractions accounted for 54–66% by wt. of the total non-dialysable solids (73–82% of this was found in the gel phase), whereas 10–18% were recovered as the major mucin

Table 1. Carbohydrate composition of CF-V-light

Amino sugars were determined with an amino acid analyser and neutral sugars by g.l.c. Sialic acid is expressed as *N*-acetylneuraminic acid. All values are corrected for moisture (9%).

	Content (g/100 g of mucin)
<i>N</i> -Acetylgalactosamine	6.7
<i>N</i> -Acetylglucosamine	17.8
Galactose	20.7
Fucose	13.2
Sialic acid	11.4

Fig. 5. Zimm plot of light-scattering data for CF-V-light

Readings were taken at θ values of 20° , 30° , 40° , 50° , 60° , 75° and 90° , and extrapolation to $\theta = 0$ was performed from 20° to 40° . The concentrations used were 0.06, 0.12, 0.22 and 0.30 mg/ml. The optical constant was $9.32 \times 10^{-8} \text{ cm}^2 \text{ g}^{-2}$. C , concentration; R_θ , the absolute scattering at angle θ .

fractions. Most of the mucins (53–68%) were obtained from the gel phase. DNA comprised 6–10%, more than 90% of which was found in the gel phase.

The majority of the mucins from the light fractions (gel phase) chromatographed with the void volume of Sepharose CL-2B (Figs. 3a–3e), whereas a significant proportion of the heavy fractions were included (Figs. 3f–3j). The molecules from the sol phase were largely included, and only a small amount of material chromatographed with the void volume (Figs. 3k–3o). The light fraction (gel-phase mucins) from CF-V that chromatographed with the void volume of Sepharose CL-2B (Fig. 3e) accounted for approx. 35% of the mucins from this sample. This population was subjected to detailed analysis and is referred to below as CF-V-light.

Carbohydrate composition and macromolecular properties

Analytical isopycnic density-gradient centrifugation in CsCl/4 M-guanidinium chloride of CF-V-light yielded a single unimodal peak with no evidence of heterogeneity (result not shown). A tiny amount of DNA was present, but no protein was found at the expected position at the meniscus. It was estimated from the u.v. scan with the use of standard DNA (results not shown) that the weight contribution of DNA to the mucins must be less than 0.1%. The carbohydrate composition is given in Table 1.

Sedimentation-velocity ultracentrifugation revealed a single unimodal but polydisperse peak, and extrapolation of the reciprocal sedimentation rate (Fig. 4a) to zero concentration yielded a value for the sedimentation coefficient ($s_{20,w}^0$) of 47.1 S. The z-average translational diffusion coefficient (D_t), determined by using photon correlation spectroscopy, was obtained after extrapolation to zero angle and concentration (Fig. 4b) and used to

Table 2. Summary of physical data for CF-V-light

All measurements were performed in 6 M-guanidinium chloride/1 mM- Na_2EDTA /1 mM-sodium phosphate buffer, pH 6.5. The sedimentation coefficient was determined by analytical ultracentrifugation, and the diffusion coefficient by photon correlation spectroscopy, and the M_r value was calculated from sedimentation and diffusion data by using the Svedberg equation. The M_r and the radius of gyration (R_G) were determined from a Zimm plot of light-scattering data from total intensity measurements.

Parameter	Value
$s_{20,w}^0$ (S)	47.1
$10^8 \times D_{20,w}^0$ (cm^2/s)	1.8
R_G (nm)	262
$10^{-6} \times M_r$	
Light-scattering	14.7
Sedimentation-diffusion	20.3

Fig. 6. Rate-zonal centrifugation of CF-V-light

(a) Mucins were fractionated on a guanidinium chloride gradient as described in the text; ○, concentration of guanidinium chloride; ●, concentration of mucins. (b) The values of D_t (□) were determined as described in the text, and values of M_r (■) were determined from the expression $1/D_t = 10.5 \times 10^3 M_r^{0.53}$.

determine the weight-average M_r with the Svedberg equation. An independent estimate of the weight-average M_r and a value of the radius of gyration (R_G) were obtained by absolute intensity light-scattering (Fig. 5). For a summary, see Table 2.

The size polydispersity of the macromolecules was investigated by rate-zonal centrifugation in a guanidinium chloride gradient (Fig. 6). The polydispersity is not due to interactions between the molecules, as fractions from the top, middle and bottom of the

Fig. 7. Analytical Sepharose CL-2B chromatography of CF-V-light

Whole mucins (—) as well as subunits (---) and T-domains (○) thereof were chromatographed on a column of Sepharose CL-2B as described in the text. Fractions were analysed using the PAS procedure after slot-blotting on to nitrocellulose membranes.

gradient migrated to the same relative position when re-run under identical conditions (result not shown). The translational diffusion coefficient was determined for each fraction and the values of M_r were calculated using the relationship $1/D_t = 10.5 \times 10^3 M_r^{0.53}$, obtained from studies on cervical mucins (Sheehan & Carlstedt, 1987). The distribution of M_r (Fig. 6b) lay within the range $(5-30) \times 10^6$, and the weight-average value of M_r (17×10^6) obtained by integration across the distribution is in good agreement with those obtained for the unfractionated mucins (Table 2). Rate-zonal centrifugation of mucins from two other patients (CF-I, heavy and light; CF-III, light) yielded size distributions similar to that in Fig. 6 (results not shown).

Polymeric structure

The CF-V-light mucins were subjected to reduction alone as well as to reduction followed by trypsin digestion, and they were then chromatographed on Sepharose CL-2B (Fig. 7). After reduction, the molecules were included on the gel, showing that fragmentation into subunits had occurred. The high- M_r glycopeptides (T-domains) obtained after subsequent trypsin digestion were significantly smaller than the subunits. Similar fragmentation patterns were obtained (results not shown) with the mucins from two other patients (CF-I, light and heavy; CF-III light).

Electron microscopy of the CF-V-light mucins spread on 6 M-guanidinium chloride (Fig. 8a) shows the molecules as long, linear and apparently flexible threads. The subunits and T-domains were also examined by electron microscopy. Subunits were visualized as polydisperse filaments, often with a thickened or beaded appearance (Fig. 8b), with a length distribution within the range 100–400 nm (Fig. 9a). T-domains were complex in appearance, some being 'tadpole-like', and others consisting of individual head or tail structures (Fig. 8c). The length of the latter varied from 30 to 180 nm (Fig. 9b). The whole mucins from CF-I-light and CF-I-heavy, as well as from CF-III-light, were similar in appearance to those from CF-V-light (results not shown).

Inhibition e.i.s.a.

Subunits from CF-I-light and -heavy, CF-III-light and CF-V-light caused similar inhibition of binding of a protein-directed polyclonal antiserum raised against human cervical mucin sub-

Fig. 8. Electron microscopy of CF-V-light and fragments thereof

The macromolecules were spread and shadowed as described in the text. (a) Whole mucins (spread on a hypophase of 6 M-guanidinium chloride) are visualized as flexible filaments polydisperse in length and heterogeneous in appearance. (b) Subunits (fractions 23–35 from Sepharose CL-2B chromatography; Fig. 7) appear irregular in length and shape, having thickened and blob-like regions along their length. (c) T-domains (fractions 43–70 from Sepharose CL-2B chromatography; Fig. 7) are visualized as filaments, flattened blobs or combinations of the two. The bar represents in (a) 500 nm and in (b) and (c) 200 nm.

units in an inhibition e.l.i.s.a. However, the inhibition produced was lower than that observed with an equivalent concentration of cervical mucin subunits (results not shown). The data suggest that these different mucin fractions all share epitopes on the naked protein region that are exposed after reduction of whole mucins.

DISCUSSION

The object of this work was to isolate mucins from CF sputum with macromolecular properties representative of those expressed in the mucus gel. Great care was thus exercised to minimize the risk of degradation by proteinases present in sputum, or by shear forces. The CF mucus gel is purulent and the removal of DNA and non-mucin proteins became the major objective of the

purification protocol adopted. The procedure used for the solubilization of the mucus gel and the subsequent purification of the mucins has been applied previously to cervical, respiratory and gastric mucins, with consistent results (Carlstedt *et al.*, 1983a; Carlstedt & Sheehan, 1984a,b; Thornton *et al.*, 1990). Sputum was separated into the gel and sol phases prior to isolation of the mucins, as the sol and gel have been shown to contain mucins of different sizes (Bhaskar & Reid, 1980).

The mucus gel dissolved well when subjected to slow stirring in 6 M-guanidinium chloride, and the molecules banded at similar densities in the isopycnic gradients to the tracheobronchial mucins isolated from healthy human airways (Thornton *et al.*, 1990). However, the CF mucins exhibited a more heterogeneous distribution, as observed by other investigators for respiratory

Fig. 9. Length distributions of subunits and T-domains

Subunits and T-domains were studied with electron microscopy as described in the Experimental section. The length distributions of (a) the subunits gave l_n 220 nm and l_w 260 nm and (b) the T-domains give l_n 82 nm and l_w 95 nm ($\square$, l_n bars; $\boxtimes$, l_w).

mucins from CF, chronic bronchitic and asthmatic sputa by using analytical density-gradient centrifugation (Creeth *et al.*, 1977; Bhaskar & Reid, 1980).

Most of the mucins from the sol phase were included on Sepharose CL-2B and are thus smaller than those in the gel, in agreement with previous observations (Bhaskar & Reid, 1980). There is much evidence for an increase in proteolytic activity in CF sputum (Hastie *et al.*, 1983; Jackson *et al.*, 1984; Suter *et al.*, 1984), and it is thus likely that the mucins in the sol, at least in part, are degradation products of the larger species in the gel. Tissue explant studies suggest that CF tracheobronchial mucins are not inherently smaller than their normal counterparts (Rose *et al.*, 1987).

The major mucin population studied here (CF-V-light) contained the expected monosaccharides, and the analyses agree well with those obtained by other investigators for CF respiratory mucins (see e.g. Roussel *et al.*, 1975; Chace *et al.*, 1985; Houdret *et al.*, 1989; Gupta *et al.*, 1990). Galactose was the major sugar found, mannose could not be detected, and the ratio of glucosamine to galactosamine was relatively high.

Sedimentation-velocity studies on CF-V-light revealed a unimodal distribution. The high value (approx. 47 S) for $s_{20,w}^0$ indicated that the macromolecules are very large, and this was confirmed by laser light-scattering data. An M_r value of approx. 15×10^6 was obtained with total intensity measurements, whereas sedimentation-diffusion studies gave an M_r of approx. 20×10^6 . The size distributions for all the mucin populations studied here (CF-I-light and -heavy, CF-III-light and CF-V-light) are similar to that of cervical mucins (Sheehan & Carlstedt, 1987) and mucins from normal respiratory secretions (Thornton *et al.*, 1990).

The CF mucins isolated in the present study are larger than those (M_r 9.3×10^6) studied by Gupta *et al.* (1990), although of

the same order of magnitude. In contrast, the CF respiratory mucins analysed by Chace *et al.* (1985) were distinctly smaller (M_r 3.8×10^6). A possible explanation for this discrepancy is that the light-scattering data provided by Chace *et al.* (1985) were not obtained at low enough angles to allow an adequate extrapolation to zero angle. Respiratory mucins from patients with asthma and chronic bronchitis were extensively studied by Creeth *et al.* (1977). The apparent s values at 1 mg/ml for most of the samples were of the order of 20 S, with values for M_r of approx. $(5-7) \times 10^6$. Mucins isolated from asthmatic sputa (Feldhoff *et al.*, 1979; Woodward *et al.*, 1982) are much smaller than those studied here [approx. $(1.5-2.3) \times 10^6$], but the procedures used for the extraction of these mucins would not preclude proteolytic degradation of the mucins during handling.

The value of R_g (262 nm) in relation to M_r suggests that the CF-V light mucin has macromolecular properties similar to those of cervical, gastric and bronchitic mucins (Carlstedt & Sheehan, 1989), i.e. the molecules behave as random coil-like structures. In keeping with this observation, electron microscopy showed that the macromolecules were long, linear and flexible structures, as we have observed previously for mucins isolated from human normal tracheobronchial secretions, chronic bronchitic sputum, and cervical and pig gastric mucus (Sheehan *et al.*, 1986; Thornton *et al.*, 1990). These results are consistent with data obtained by using viscosity and birefringence relaxation measurements (Mikkelsen *et al.*, 1985), light scattering (Gupta *et al.*, 1990) and electron microscopy (Harding *et al.*, 1983; Rose *et al.*, 1984; Mikkelsen *et al.*, 1985; Marianne *et al.*, 1987).

The mucins from the various populations (CF-I-light and -heavy, CF-III-light and CF-V-light) were fragmented into subunits by reduction, and subsequent trypsin digestion showed that these fragments are composed of oligosaccharide clusters flanked by naked protein regions susceptible to proteolysis, as are human normal tracheobronchial (Thornton *et al.*, 1990) and cervical (Carlstedt *et al.*, 1983b) mucins. The subunit preparation contained a proportion of smaller fragments, indicating that some proteolytic degradation of the mucins had occurred. The length distribution obtained by electron microscopy of subunits showed that they were somewhat smaller than those from normal respiratory (Thornton *et al.*, 1990) and cervical (Sheehan & Carlstedt, 1990) mucins, in keeping with their behaviour on gel chromatography. The high- M_r glycopeptides (T-domains) corresponding to the oligosaccharide clusters in the mucins were not well stretched out on the grid surface, some appearing as tadpole-like structures. Those that were stretched out had a length distribution similar to that found for normal respiratory (Thornton *et al.*, 1990) and cervical (Sheehan & Carlstedt, 1990) mucins.

The presence of a subunit structure in respiratory mucins has been the subject of some controversy. No decrease in molecular size upon reduction of mucins was observed by Chace *et al.* (1985) by using light-scattering, although the molecules appeared smaller on gel chromatography. A possible explanation for this discrepancy is that the size of the whole mucins was underestimated with light scattering (see above). In the work by Creeth *et al.* (1977), a decrease in size was a regular finding when the whole mucins were subjected to reduction. The data of Gupta *et al.* (1990) are very similar to those presented here, and clearly point to a subunit structure in respiratory mucins. Finally, respiratory mucins isolated by using reduction (Rose *et al.*, 1987) showed a chromatographic behaviour similar to that of the subunits obtained here. In our opinion there is now ample evidence to support the existence of a subunit structure in respiratory mucins, which is significantly larger than the high- M_r glycopeptides obtained after proteolytic cleavage. The chromatographic behaviour on Sepharose CL-2B of the subunits from the mucins studied here is similar to that of subunits from cervical

mucins, which have M_r values of $(2-3) \times 10^6$ (Carlstedt *et al.*, 1983b; Sheehan & Carlstedt, 1984), and is in good agreement with the size (M_r 2.5×10^6) of the subunit structure obtained by Gupta *et al.* (1990) for CF respiratory mucins. However, subunits appear to be identified only when the procedures used to isolate the molecules are adequate in protecting the macromolecules from proteolysis.

In conclusion, mucins from CF sputum are heterogeneous with regard to both size and buoyant density. The largest species have similar size distributions and architecture, and the heterogeneity of these molecules thus must be due to differences in chemical composition. The heterogeneity could be caused by the presence of different secretory products, all polydisperse in size and only partially resolved in the density gradient. A comparison between mucins from normal respiratory secretions and sputum from patients with, for example, CF, asthma and chronic bronchitis, would be facilitated if these putative components could be identified and methods developed to isolate them for biochemical and physical studies. The identification of different mucin species in CF sputum will be addressed in the following paper (Thornton *et al.*, 1991).

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