Electron microscopy of cervical, gastric and bronchial mucus glycoproteins

John K. SHEEHAN,*† Ken OATES* and Ingemar CARLSTEDT†

*Department of Biological Sciences, University of Lancaster, Lancaster LA1 4YQ, U.K., and †Department of Physiological Chemistry 2, University of Lund, P.O. Box 94, S-221 00 Lund, Sweden

Mucus glycoproteins (mucins) were obtained from human cervical and pig gastric mucus as well as from chronic-bronchitic sputum after low-shear extraction. The mucus gel was solubilized in guanidinium chloride supplemented with proteinase inhibitors, and the macromolecules were purified by using isopycnic density-gradient centrifugation. The macromolecules were spread in monolayers of benzyldimethylalkylammonium chloride and studied with electron microscopy after staining with uranyl acetate and/or shadowing with platinum/carbon. The mucins appeared as flexible linear threads with lengths varying from approx. 200 nm to about 4000 nm. No regularly branched or star-shaped structures were observed. The macromolecular architecture of cervical, respiratory and gastric mucins is thus similar.

INTRODUCTION

Mucus glycoproteins (mucins) are the macromolecules responsible for the characteristic features of mucus. Before we can describe the molecular mechanisms by which the gel is formed and how its properties are regulated, the macromolecular behaviour and polymeric structure of these glycoproteins must be well known. Several investigators have used electron microscopy to examine respiratory mucins (Jenssen *et al.*, 1980; Harding *et al.*, 1983; Rose *et al.*, 1984; Slayter *et al.*, 1984; Mikkelsen *et al.*, 1985), but no such investigations on gastric and cervical mucins are known to us. The general picture emerging from these studies is that respiratory mucins are flexible filamentous structures, polydisperse in length and with the occasional appearance of very complex 'aggregate' structures.

We have isolated cervical, bronchial and gastric mucins by a procedure designed to minimize physical and enzymic degradation of the macromolecules (Carlstedt *et al.*, 1983). On the basis of hydrodynamic data we have proposed that mucins obtained from these sources have a similar macromolecular architecture (Carlstedt & Sheehan, 1984a,b). The molecules were described as long linear threads ($M_r = 10 \times 10^6$ – 40×10^6), polydisperse in size and adopting a highly hydrated spheroidal configuration in dilute solution. By using electron microscopy on mucins spread in a monolayer of benzyldimethylalkylammonium chloride, as employed in the study of nucleic acids, we present evidence supporting our original description.

EXPERIMENTAL

Isolation of mucus glycoproteins

Mucins from human cervical and pig gastric mucus as well as chronic-bronchitic sputum were purified as described in detail by Carlstedt *et al.* (1983). The final preparation was stored as a solution in 4 M-guanidinium chloride at 4 °C, the concentration of which was assessed by dialysing and freeze-drying a known volume.

Electron microscopy

Carbon films (approximate thickness 2 nm) were made by evaporation of carbon on to freshly cleaved mica. The films were floated off on water and deposited on 600-mesh grids (Gilder Grids, Grantham, Lincs., U.K.). Specimens for electron microscopy were prepared with a modification of spreading methods used in the study of DNA (Kleinschmidt & Zahn, 1959; Koller *et al.*, 1969; Lang & Mitani, 1970). Solutions of mucins (0.02–0.05 µg/ml) in 50 mM-magnesium acetate or 6 M-guanidinium chloride were put into a Teflon or plastic Petri dish. A small amount of fine graphite powder was sprinkled on the surface, which was then touched with the tip of a plastic pipette containing a solution (1 g/100 ml) of benzyldimethylalkylammonium chloride in water so that approx. 1 µl was spread over the surface, displacing the graphite to the edge of the dish. After 10–15 min, carbon-coated grids were touched to the surface, stained for 1–2 s in a solution of uranyl acetate [1 mM in 95% (v/v) ethanol] and finally washed for 1–2 s in ethanol (95%, v/v). When the molecules were spread on a hypophase of 6 M-guanidinium chloride the grids were washed in 50 mM-magnesium acetate before staining. After drying in air the grids were subjected to either rotary or unidirectional shadowing at 7–12° with platinum/carbon. Electron microscopy was performed with a JEOL 100CX scanning transmission instrument at 60 or 80 keV. Contour lengths were measured by tracing over photographic enlargements placed on a digital drawing pad (British Micro, Watford, Herts., U.K.) connected to a BBC microcomputer.

RESULTS AND DISCUSSION

Electron microscopy of DNA has provided much information on the structure, configuration and behaviour of these flexible linear macromolecules. The appearance of the molecules is, however, largely dependent on how well they adhere to the supporting 'substrate'. A weak attachment may give rise to artifacts referred to as

† To whom correspondence should be addressed.

Fig. 1. Electron micrographs of pig gastric mucins spread in benzyltrimethylammonium chloride monolayers

For full experimental details see the text. (a) Mucins spread on a hypophase of 50 mM-magnesium acetate, stained with uranyl acetate and rotary-shadowed with platinum/carbon. The macromolecules appear as 'relaxed' linear structures with even contrast along the length. (b) Mucins spread on a hypophase of 6 M-guanidinium chloride, stained with uranyl acetate and rotary-shadowed with platinum/carbon. The macromolecule(s) appear as (a) linear structure(s) with a tendency for segments to align and form hairpin loops that are 'open' or 'closed' to various extents. Completely 'closed' hairpin structures are indistinguishable from branch points. (c) Mucins showing 'clumping' and 'streaking', possibly owing to poor adhesion to the carbon film; prepared as in (a). (d) Mucins showing apparent branch points at irregular intervals; prepared as in (a). (e). At higher magnifications an 'eye of the needle' was sometimes noticed at the end of a hairpin fold (see also b). (f) Mucins spread on 6 M-guanidinium chloride and subjected to unidirectional shadowing. A completely extended macromolecule with no folds, kinks or branch points is shown. (g) A 'relaxed' structure with kinks and 'spurs' along its length; prepared as in (a).

Fig. 2. Electron micrographs of human bronchial mucins spread in benzyldimethylalkylammonium chloride monolayers

For full experimental details see the text. (a) Mucins spread on a hypophase of 50 mM-magnesium acetate, stained with uranyl acetate and rotary-shadowed with platinum/carbon. The macromolecules appear in general as linear structures, some extended and some with kinks, folds and apparent branch points. A large range of sizes is present. (b) Complex structures, possibly formed by interpenetrating and aggregating threads, were occasionally seen; prepared as in (a). (c) At higher magnifications the staining of the particles appeared 'lumpy' and uneven, maybe owing to the formation of intramolecular folds along the chain; prepared as in (a). (d) and (e) Mucins spread on 6 M-guanidinium chloride, stained with uranyl acetate and subjected to unidirectional shadowing. Extended macromolecules (d) appear as smooth threads with no 'spurs', whereas relaxed ones (e) show hairpin loops and apparent branch points.

Fig. 3. Electron micrographs of human cervical mucins spread in benzyltrimethylammonium chloride monolayers

For full experimental details see the text. (a) Mucins spread on a hypophase of 50 mM-magnesium acetate, stained with uranyl acetate and rotary-shadowed with platinum/carbon. The macromolecules appear as threads with occasional kinks, 'spurs' and apparent branch points. (b) In some experiments the macromolecules are 'decorated' with globular structures, as highlighted by using scanning transmission electron microscopy; prepared as in (a). (c) At higher concentrations many of the threads intertwine, forming structures possibly related to an incipient mucin 'fibre'; prepared as in (a).

'streaking' and 'lateral clumping' when the molecules move under the influence of a retreating solvent front either in the initial transfer to the grid or in subsequent washing and staining steps (Fisher & Robley, 1979).

The resulting entanglement and apparent branching effects would interfere with measurements of contour lengths, which are best performed if the molecules are uniformly spread, untangled and 'relaxed', i.e. having a gently meandering contour with no kinks and folds.

Applying the same electron-microscopy techniques to

mucins, we have encountered similar effects. However, the combination of benzyltrimethylammonium chloride (rather than cytochrome *c*) as the spreading agent together with magnesium acetate as the solvent provides conditions for reproducible studies. 'Relaxed' pig gastric mucins spread on a hypophase of 50 mM-magnesium acetate are shown in Fig. 1(a). The macromolecules appear as predominantly linear structures about 5 nm in diameter, and in numerous preparations this appearance was representative of all the particles.

Contour-length measurements yielded values ranging from 100 to 5000 nm. As previous physical characterization of gastric mucins was performed in 6 M-guanidinium chloride (Carlstedt & Sheehan, 1984a) attempts were made to spread the molecules in this solvent (Fig. 1*b*). The adherence to the carbon film was apparently not so strong as in magnesium acetate, and the macromolecules were often distorted by streaking, i.e. movement around points of firm attachment generating large loops all concave in the same direction. Sometimes such loops had a sharp bend at the point of attachment, forming 'hairpins' which, when completely pinched off, would resemble a branch point. Various stages in the formation of such structures are seen in Fig. 1*b*). In Fig. 1*c*) a more pronounced streaking and clumping behaviour is demonstrated. The polarization of the particles from bottom right to top left in the Figure is typical of a distortion generated by flow, and the looping structures in the larger particles are all concave facing the bottom right-hand corner of the picture. In Fig. 1*d*) molecules exhibiting partial alignment are shown. The folds in the mucins have in most cases pinched off, forming hairpin folds resembling branch points. Contour-length measurements on such preparations gave the same distribution of values as obtained on preparations exemplified by Fig. 1*a*). Occasionally an opening at the end of a hairpin may be discerned (Fig. 1*e*). Distortion due to flow and poor adherence to the grid sometimes allowed the observation of completely extended macromolecules (Fig. 1*f*). In contrast, 'relaxed' molecules were sometimes decorated by numerous 'spurs' (Fig. 1*g*).

Similar but not identical images were obtained of the bronchial mucins. Though most of the macromolecules appeared as linear threads (Fig. 2*a*), the larger ones, when relaxed, often formed complex entangled structures, making tracing of the contour length impossible (Fig. 2*b*). The molecules stained with an uneven 'lumpy' appearance, with hairpins and folds along the chains (Fig. 2*c*), and were slightly thicker than the gastric mucins, being typically 6–10 nm in width. When spread on a hypophase of 6 M-guanidinium chloride bronchial mucins resembled the gastric ones under these conditions (compare Figs. 1*f* and 1*b* with Figs. 2*d* and 2*e*). Some molecules were fully extended, with a smooth and even contour (Fig. 2*d*), whereas others showed loops, kinks and apparent branch points (Fig. 2*e*).

Cervical mucins showed features similar to the gastric and bronchial ones, being linear structures with a broad range of lengths (Fig. 3*a*). Sometimes, in a 'relaxed' state, the molecules showed a 'beaded' appearance, as highlighted in a scanning transmission micrograph (Fig. 3*b*). Such structures were not typically found with gastric and bronchial mucins, and may be explained by the presence of a local folding of the main chain. The appearance of such structures was, however, not possible to control. When aligned and thus stretched the molecules showed an even contour without beads, as in Fig. 3*c*) showing cervical mucins at a higher concentration. The molecules appear as smooth entangled threads, two or more sometimes run together to form an incipient 'fibre'.

Contour-length measurements were made on micrographs of the three different mucins after spreading on a hypophase of 50 mM-magnesium acetate. Only molecules with a clearly identifiable contour were included, thus perhaps introducing a systematic error, as the very largest

Fig. 4. Histograms of the contour length of mucins from (a) pig gastric, (b) bronchial and (c) cervical mucus

For full experimental details see the text. Mucins were spread in monolayers of benzyldimethylalkylammonium chloride on a hypophase of 50 mM-magnesium acetate, stained with uranyl acetate and rotary-shadowed with platinum/carbon. (a) A total of 300 molecules were measured. The number-average length (l_n) and the weight-average length (l_w) are 1060 nm and 1670 nm respectively. (b) A total of 250 molecules were measured. The number-average length (l_n) and the weight-average length (l_w) are 820 nm and 1540 nm respectively. (c) A total of 250 molecules were measured. The number-average length (l_n) and the weight-average length (l_w) are 1240 nm and 1840 nm respectively.

particles with a highly convoluted configuration are more likely to be rejected from the analysis than the smaller ones. The number distributions are shown in Figs. 4(a)–4(c). Because the configuration and perhaps the conformation of the macromolecules may be sensitive to the spreading conditions, the concept of contour length should be treated with caution at present. The lengths

varied from approx. 200 nm to about 4000 nm, and the gastric, bronchial and cervical mucins had number-average lengths ($l_n = \sum N_i \cdot l_i / \sum N_i$) of 1060, 820 and 1240 nm respectively. The corresponding weight-average lengths ($l_w = \sum N_i \cdot l_i^2 / \sum N_i \cdot l_i$) are 1670, 1540 and 1840 nm. The weight-average M_r of these molecules has previously been assessed by a number of methods and are typically 30×10^6 – 40×10^6 , 16×10^6 and 11×10^6 – 15×10^6 for gastric, bronchial and cervical mucins respectively (Carlstedt & Sheehan, 1984a; Sheehan & Carlstedt, 1984a). However, the value of M_r , as measured by light-scattering, for the actual preparation of gastric mucins used here was 15.2×10^6 . Combining the values for the weight-average M_r with those for the weight-average contour length provides an estimate of mass/length, which was 9100, 10400 and 6000 nm⁻¹ for gastric, respiratory and cervical mucins respectively.

GENERAL DISCUSSION

Mucins from the three sources studied here are linear in construction and very polydisperse in length. The fact that the macromolecules could appear as extended, almost rod-like, structures, with a smooth and even contour, or as threads with loops and kinks suggests that they are indeed very flexible. The presence of apparent branch points is explained by the formation of closed loops. Such structures varied in length and appeared at random along the chains, probably reflecting the fact that, owing to flexibility, the mucins have almost unlimited configurational possibilities. Bronchial mucins obtained from chronic-bronchitic and cystic-fibrotic sputa have been studied with electron microscopy by a number of investigators (Jenssen *et al.*, 1980; Harding *et al.*, 1983; Rose *et al.*, 1984; Slayter *et al.*, 1984; Mikkelsen *et al.*, 1985). Mucins purified by Jenssen *et al.* (1980) from sputum obtained from patients suffering from chronic bronchitis appeared as long flexible strands when negatively stained. Slayter *et al.* (1984) studied mucins from sputum of patients with cystic fibrosis as well as chronic bronchitis. By using a number of different preparation techniques they have also obtained bronchial mucus glycoproteins as polydisperse, linear and apparently flexible threads with lengths ranging from 200 to 1400 nm. The macromolecules are somewhat 'shorter' and with a correspondingly lower value of M_r than those studied here, presumably owing to differences in the preparative methods used. Their conclusions as to the architecture and behaviour of the macromolecules are, however, very similar to ours. The 'bush-like' aggregates observed by these authors are quite similar to the complex structure shown in Fig. 2(b). Rose *et al.* (1984) studied mucins from normal bronchial secretions as well as from sputum from patients with cystic fibrosis and asthma. By using a replica technique they obtained respiratory mucins as long filaments with estimated lengths of 550–1100 nm for the less-tangled structures. In addition, complex structures where filaments emanate from and return to a central 'core' were described. We do not see such core structures, though the compact convoluted conformation shown in Fig. 2(b) may be a related structure. Mikkelsen *et al.* (1985) describe mucins obtained from chronic-bronchitic sputum as unbranched flexible molecules with contour lengths ranging from 300 to 2500 nm.

No studies using electron microscopy on cervical

mucins are known to us. We and others have interpreted hydrodynamic data to favour a linear flexible structure (Gibbons & Glover, 1959; Sheehan & Carlstedt, 1984a). In addition, evidence that an expansion of the macromolecules takes place with increasing concentrations of guanidinium chloride have been presented (Sheehan & Carlstedt, 1984b). It was proposed that protein-rich regions could form 'globular' structures that unfold in a denaturing solvent, and in some micrographs (Fig. 2b) the chains do appear to have a 'beaded' structure. Alternatively, the expansion of the macromolecules could well be explained by unfolding of structures referred to above as apparent branch points and spurs. Neither features are, however, consistently found, and when extended the macromolecules have a smooth and even contour.

No electron-microscopy data has been presented for gastric mucins, and there is disagreement both as to the size and as to the appropriate model to fit the available hydrodynamic data. Extraction and purification methods using guanidinium chloride and/or proteinase inhibitors yield preparations with values of M_r in the order of 15×10^6 – 40×10^6 (Carlstedt & Sheehan, 1984a). It has been argued that such conditions would induce aggregation of the macromolecules and that the true weight-average M_r for pig gastric mucins is close to 2×10^6 – 3×10^6 (Allen *et al.*, 1984a,b). Furthermore, it has been proposed that gastric mucins are composed of four subunits (M_r 0.5×10^6) attached to a central 'cross-linking' protein, forming the so-called 'windmill' model (Allen, 1983). The vast degree of polydispersity as evident from the distribution of sedimentation coefficients is explained by polydispersity within the component subunits. The data presented here show no evidence of such a regular star structure. Furthermore, if the solvent conditions used by us during extraction and purification would induce aggregation, this process must be an extremely specific one, joining the mucins end-to-end only.

The mass/length parameter estimated here from the weight-average contour length and the weight-average M_r is 9100, 10400 and 6000 nm⁻¹ for gastric, respiratory and cervical mucins respectively. At present there are few data with which to compare these values. We have previously estimated the mass/length for cervical mucins by using high-angle light-scattering data (Sheehan & Carlstedt, 1984b), and values of approx. 7000 nm⁻¹ (0.2 M-NaCl) and 4500 nm⁻¹ (6 M-guanidinium chloride) were obtained, which is in reasonable agreement with the value of 6000 nm⁻¹ presented here. Furthermore, the distributions of contour lengths for the gastric and bronchial mucins (Figs. 4a and 4b) will, when transformed into the corresponding distributions of M_r , look quite similar to those presented by Creeth & Cooper (1984) obtained by using sedimentation-equilibrium data from the analytical ultracentrifuge. The higher values obtained by us for the weight-average M_r could be explained by a selective loss of the very largest species in the latter approach. Because polydispersity is likely to interfere with both the measurements of l_w and the measurements of M_r , further studies on material fractionated into narrow distributions are probably required before an accurate value of the mass/length parameter can be obtained.

The significance of the broad size distribution is not clear, but it may result from events occurring at bio-

synthesis and/or from extracellular modifications. A distinct possibility is that mucins are synthesized as very large species that are later modified by proteinases. An alternative explanation is that the broad distribution arises from a non-specific mechanical and/or enzymic degradation during collection of the samples or purification of macromolecules.

This investigation was supported by grants from the Medical Faculty of Lund, Sweden, the Swedish Tobacco Company, Greta och Johan Kocks Stiftelser, Trelleborg, Sweden, the National Swedish Board for Technical Development, the Sunnerdahls Handikappsfond, Sweden, the Swedish Society for Cystic Fibrosis and the Cystic Fibrosis Research Trust, U.K.

REFERENCES

- Allen, A. (1983) *Trends Biochem. Sci.* **169**–173
- Allen, A., Hutton, D. A., Mantle, D. & Pain, R. H. (1984a) *Biochem. Soc. Trans.* **12**, 612–614
- Allen, A., Hutton, D. A., Pearson, J. P. & Sellers, L. A. (1984b) *Ciba Found. Symp.* **109**, 137–156
- Carlstedt, I. & Sheehan, J. K. (1984a) *Biochem. Soc. Trans.* **12**, 615–617
- Carlstedt, I. & Sheehan, J. K. (1984b) *Ciba Found. Symp.* **109**, 157–166
- Carlstedt, I., Lindgren, H., Sheehan, J. K., Ulmsten, U. & Wingerup, L. (1983) *Biochem. J.* **211**, 13–22
- Creeth, J. M. & Cooper, B. (1984) *Biochem. Soc. Trans.* **12**, 618–621
- Fisher, H. W. & Robley, C. W. (1979) *Annu. Rev. Biochem.* **48**, 649–679
- Gibbons, R. A. & Glover, F. A. (1959) *Biochem. J.* **73**, 217–225
- Harding, S. E., Rowe, A. J. & Creeth, J. M. (1983) *Biochem. J.* **209**, 893–896
- Jenssen, A. O., Harbitz, O. & Smidsrød, O. (1980) *Eur. J. Respir. Dis.* **61**, 71–76
- Kleinschmidt, A. K. & Zahn, R. K. (1959) *Methods Enzymol.* **12B**, 361–371
- Koller, T., Harford, A. G., Lee, Y. K. & Beer, M. (1969) *Micron* **1**, 110–118
- Lang, D. & Mitani, M. (1970) *Biopolymers* **9**, 373–379
- Mikkelsen, A., Stokke, B. T., Christensen, B. E. & Elgsaeter, A. (1985) *Biopolymers* **24**, 1683–1704
- Rose, M. C., Voter, W. A., Brown, C. F. & Kaufman, B. (1984) *Biochem. J.* **222**, 371–377
- Sheehan, J. K. & Carlstedt, I. (1984a) *Biochem. J.* **217**, 93–101
- Sheehan, J. K. & Carlstedt, I. (1984b) *Biochem. J.* **221**, 499–504
- Slyter, H. S., Lamblin, G., LeTreut, A., Galabert, C., Houdret, N., Degand, P. & Roussel, P. (1984) *Eur. J. Biochem.* **142**, 209–218

Received 23 December 1985/24 April 1986; accepted 10 June 1986