The effect of guanidinium chloride on the behaviour of human cervical-mucus glycoproteins

Evidence for unfolding regions of ordered structure in 6M-guanidinium chloride

John K. SHEEHAN* and Ingemar CARLSTEDT††

*Department of Biological Sciences, University of Lancaster, Lancaster LA1 4YQ, U.K., and †Department of Physiological Chemistry, University of Lund, P.O. Box 750, S-220 07 Lund 7, Sweden

(Received 14 February 1984/Accepted 16 March 1984)

The macromolecular properties of cervical-mucus glycoproteins (mucins) were studied as a function of the concentration of guanidinium chloride with conventional light-scattering, photon-correlation spectroscopy and sedimentation-velocity centrifugation. No evidence for an association of the mucins in 0.2M-NaCl as compared with 6M-guanidinium chloride was found at mucin concentrations below approx. 0.5 mg/ml. However, an increase in the frictional coefficient and in the radius of gyration occurred with increasing concentrations of guanidinium chloride, in particular between 4M and 6M, suggesting an expansion of the individual macromolecule. The change in the particle-scattering function is consistent with a transition from a 'stiff' random coil in 0.2M-NaCl into a more flexible one in 6M-guanidinium chloride. We suggest that the mucins contain regions of 'ordered' structure which can undergo a reversible 'unfolding' analogous to the behaviour of a conventional globular protein exposed to a denaturing solvent. Such regions might carry sites for specific interactions between mucins and/or be decisive for their conformation and macromolecular properties in physiological solvents.

Biological characteristics, such as spermatozoal penetrability, are correlated with changes in the rheology of cervical mucus during the ovulatory cycle (Wolf *et al.*, 1978). The physical properties of the gel are determined by the mucus glycoproteins (mucins). Previous work suggests that these are long, linear and flexible macromolecules with M_r in the order of 10×10^6 – 15×10^6 (Lee *et al.*, 1977a,b; Silberberg & Meyer, 1982; Carlstedt *et al.*, 1983a,b). Hydrodynamic data show that the individual macromolecule occupies a spheroidal domain in dilute solution, behaving as a random coil (Sheehan & Carlstedt, 1984; see also Harding *et al.*, 1983). Although entanglement of the mucins might suffice to account for the formation of mucus (Lee *et al.*, 1977a,b), a contribution by secondary bonds between the glycoproteins in the gel seems likely (Bell & Allen, 1982; Harding & Creeth, 1982; Silberberg & Meyer, 1982).

We have previously proposed a model for the macromolecular architecture of cervical mucins (Carlstedt *et al.*, 1983b) and studied their hydro-

dynamic properties in 6M-guanidinium chloride (Sheehan & Carlstedt, 1984). In the present work the possible effects of guanidinium chloride on the macromolecular behaviour of cervical mucins were investigated. The frictional coefficient, the radius of gyration and M_r were measured as a function of the concentration of guanidinium chloride in order to decide whether the macromolecules contain regions of ordered conformation and/or are prone to self-associate in a physiological solvent at low concentration.

Experimental procedures

Materials

Guanidinium chloride (practical grade) was obtained from Sigma Chemical Co. Stock solutions (approx. 8M) were treated with charcoal before use. Human cervical pregnancy mucus was solubilized with the use of 6M-guanidinium chloride in the presence of proteinase inhibitors, and mucins were purified from DNA and non-mucin proteins by isopycnic density-gradient centrifugations in

† To whom requests for reprints should be addressed.

CsCl as described in detail elsewhere (Carlstedt *et al.*, 1983a). The purified mucins were stored at 4°C in 4M-guanidinium chloride, and the concentration of the stock solutions was determined gravimetrically by freeze-drying a sample after extensive dialysis against water and correction for moisture. The concentrations of solutions subjected to physical measurements were assessed by sialic acid determinations (Jourdan *et al.*, 1971), with the cognate stock solution as a standard.

Light-scattering

Absolute-intensity measurements were performed with a Sofica model 42000 photo-goni-diffusometer at 436nm as described previously (Carlstedt *et al.*, 1983a). Measurements were obtained at angles between 30° and 150°, and the data were analysed, including corrections for back-scattering (Tomimatsu *et al.*, 1968), and plotted in accordance with Zimm (1948). The angular dependence of the intensity of the scattered light was used to determine the radius of gyration (R_G) and the shape-dependent particle scattering function [$P(\theta)$] by standard procedures (see, e.g., Tanford, 1961; Evans, 1972; Kratochvil, 1972).

For long rod-like macromolecules (length = L ; radius = r) the mass per unit length can be estimated from an asymptotic form of the particle scattering function (see, e.g. Sadron, 1960; Eisenberg, 1971). For $hL \gg 1$ and $rh \ll 1$, $Kc/R_\theta = 2/\pi^2 M_r + (L/M_r)(h/\pi)$, where h is the scattering vector ($4\pi n_0/\lambda_0 \cdot \sin \theta/2$) and all other symbols have their usual meaning (see, e.g. Tanford, 1961). The mass per unit length (M_r/L) is obtained from the slope of a plot of Kc/R_θ versus h at large values of h .

The refractive-index increment ($\partial n/\partial c$) was determined at 20°C with a Waters R401 Differential Refractometer as described previously (Carlstedt *et al.*, 1983b). Samples were dialysed extensively at 20°C to ensure constant chemical potential of all diffusible components (Casassa & Eisenberg, 1964).

A Malvern Molecular Analyser System 4300 (Precision Devices and Systems, Malvern, Worcs., U.K.) equipped with a 5mW He/Ne laser (Coherent Radiation, Cambridge, U.K.) and a 48-channel K7023 correlator was used to determine the translational diffusion constant (D_T) by quasi-elastic light-scattering as described previously (Sheehan & Carlstedt, 1984). The logarithm of the experimentally observed correlation function was fitted to a second-order polynomial by using least-squares analysis, and the diffusion coefficients were calculated from the linear term of the quadratic fit (see, e.g., Pusey, 1974). Measurements were performed at 25°C and corrected to standard conditions (water, 20°C).

Analytical ultracentrifugation

Sedimentation-velocity experiments were performed with an MSE Centriscan 75 analytical ultracentrifuge at 31000 rev./min and 20°C. The mucins sedimented as single peaks (schlieren optics) in all solvents, and the apparent sedimentation coefficients were determined from the peak positions. Sedimentation coefficients were obtained from plots of $1/s_{app}$ versus concentration (approx. 0.5–3mg/ml) with linear least-squares regression and corrected to standard conditions (water; 20°C).

The partial specific volume was determined at 20°C with a Kratky densitometer as described previously (Carlstedt *et al.*, 1983b). Samples were subjected to extensive dialysis at 20°C to ensure constant chemical potential of all diffusible components (Casassa & Eisenberg, 1964).

Results and discussion

The partial specific volume and the refractive-index increment as a function of the solvent conditions are shown in Figs. 1(a) and 1(b) respectively. The accuracy of the measurements is limited by the maximum concentration of the mucin solutions that can be handled, but the numbers are estimated

Fig. 1. Apparent specific volume (a) and apparent refractive-index increment (b) as a function of the solvent conditions

Samples were dialysed into 0.2M-NaCl (○) and guanidinium chloride (●). All solvents contain, in addition, 1 mM- Na_2EDTA /1 mM-sodium phosphate buffer, pH 6.5. The values for the apparent specific volumes are means for three different mucin preparations, whereas the measurements of the refractive-index increments were performed on a single preparation.

to be within $\pm 5\%$ of the true ones. The values obtained from a linear least-squares regression line of the data were used for all calculations.

The radius of gyration and M_r as a function of the solvent are shown in Figs. 2(a) and 2(b) respectively. The radius of gyration increases with the concentration of guanidinium chloride, suggesting an expansion of the macromolecules. This behaviour was fully reversible and was observed when mucins from six different preparations were dialysed from 6M-guanidinium chloride into 0.2M-NaCl (or 4M-guanidinium chloride) and back again. No change in M_r was observed within the probable errors of the measurements.

A change in the macromolecular conformation of the mucins is expected to influence the particle scattering function $[P(\theta)]$, which, in 6M-guanidinium chloride, is close to that expected for a random coil (Fig. 3). The data suggest that in 0.2M-NaCl the mucins either become somewhat 'stiffer' and/or participate in a partial aggregation leading to an increased degree of polydispersity. As there is

no change in M_r , a conformational change is the more likely interpretation.

The limiting expression for c/R_θ at high values of hL for a rod-like macromolecule is related to the mass per unit length (M_r/L) of the macromolecules, and the intercept on the c/R_θ axis for this asymptote is expected to be positive for a rod. In contrast, the theory predicts a negative intercept on the c/R_θ axis for a more flexible macromolecule such as a worm-like chain (see, e.g., Sadron, 1960), which is indeed observed for, e.g., DNA (see, Eisenberg, 1961). The change in the intercept on the c/R_θ axis from 0.2M-NaCl to 6M-guanidinium chloride (Fig. 4a) is thus consistent with a gradual increase in flexibility for a linear macromolecule. Although the calculation of M_r/L is strictly valid only for a rod-like molecule, very reasonable estimates were obtained for DNA by this approach (see, e.g., Eisenberg, 1961). The estimated length of the heavily glycosylated regions is in the order of 100nm, and the M_r is 400000 (Sheehan & Carlstedt, 1984). Hence the mass per unit length for this region of the mucins is estimated to be approx. 4000nm^{-1} . This is in agreement with the value of approx. 5000nm^{-1} obtained here by light-scattering (Fig. 4b). The glycosylated regions of the

Fig. 2. Radius of gyration ($\square$, $\circ$) and relative molecular mass ($\circ$, $\bullet$), as a function of the solvent conditions. Samples were dialysed into 0.2M-NaCl ($\square$, $\circ$) and guanidinium chloride ($\blacksquare$, $\bullet$), and the weight-average M_r and the radius of gyration were measured by the use of light-scattering as described in the text. All solvents contain, in addition, 1 mM- Na_2EDTA /1 mM-sodium phosphate buffer, pH 6.5. The values in 0.2M-NaCl and 6M-guanidinium chloride are the means for four and six different preparations respectively. The mean (broken line) of all values for M_r is 10.1×10^6 , with s.d. 1.2×10^6 .

Fig. 3. Plot of particle scattering function versus scattering vector in 0.2M-NaCl ($\circ$) and in 6M-guanidinium chloride ($\bullet$).

The reciprocal of the particle scattering function $[P(\theta)]$ was plotted against $h^2 R_G^2$, where h is the scattering vector $(4\pi n_0/\lambda_0) \sin \theta/2$ and R_G is the radius of gyration. Solvents contain, in addition, 1 mM- Na_2EDTA /1 mM-sodium phosphate buffer, pH 6.5. The continuous line shows the values expected for a random coil derived from the equation $P(\theta) = (2/w^2)(e^{-w} + w - 1)$, where $w = h^2 R_G^2$ (see, e.g., Tanford, 1961). The behaviour in 0.2M-NaCl is compatible with an increased stiffness and/or a higher degree of polydispersity for a flexible, coil-like, macromolecule as compared with that in 6M-guanidinium chloride.

Fig. 4. Plot of c/R_θ versus scattering angle (a) and the mass per unit length as a function of the solvent conditions (b). Data were obtained in 0.2M-NaCl (○) and in guanidinium chloride (●). Both solvents contain, in addition, 1mM- Na_2EDTA /1mM-sodium phosphate buffer, pH 6.5. (a) The mass per unit length is calculated from the slope (broken line) at high values of θ . (b) The mass per unit length (M_r/L ; expressed as nm⁻¹) was estimated from the asymptotic slope of the curves in (a) by using linear least-squares regression of data obtained at $\theta = 105^\circ, 120^\circ, 135^\circ, 142.5^\circ$ and 150° . Not all data in (b) were included in (a) for reasons of clarity.

mucins must have a higher mass per unit length than the 'naked' stretches of the protein core, owing to the laterally projecting oligosaccharide chains. A folding of the non-glycosylated stretches of the protein core in 0.2M-NaCl would shorten the distance between the heavily glycosylated regions, and a higher average mass per unit length is obtained (Fig. 4b).

The transition into a more expanded form of the mucins in 6M-guanidinium chloride, as suggested by the increase in the radius of gyration, should be accompanied by a rise in the frictional coefficient of the macromolecules and thus with a decrease in the sedimentation coefficient, provided that there is no concomitant change in M_r . As predicted, a

Fig. 5. Sedimentation coefficient (a), diffusion coefficient (b) and the relative molecular mass (c) as a function of the solvent conditions

Data were obtained in 0.2M-NaCl (○, □) and in guanidinium chloride (●, ■). All solutions contained, in addition, 1mM- Na_2EDTA /1mM-sodium phosphate buffer, pH 6.5. (a) Sedimentation coefficients (○, ●) were corrected to standard conditions (water; 20°C). (b) Diffusion coefficients (○, ●) were obtained as described in the text and were corrected to standard conditions (water, 20°C). The corresponding radii of the equivalent spheres (R_e) were calculated by using the Stokes equation (□, ■). (c) M_r values were calculated with the Svedberg equation, with values for the apparent specific volume from Fig. 1(b) and the s^0 and D^0 values from Figs. 5(a) and 5(b) respectively. The weight-average M_r is obtained, because the diffusion coefficients are the z-average values (Kinell, 1959). The mean (broken line) of all values for M_r is 15.1×10^6 , with s.d. 1.1×10^6 .

decrease in $s_{20,w}^0$ from approx. 50S (0.2M-NaCl) to approx. 40S (6M-guanidinium chloride) occurred (Fig. 5a). The change in the frictional coefficient was also reflected as a decrease in the diffusion co-

efficient (Fig. 5b), and the corresponding increase in the radius of the equivalent hydrated sphere (the Stokes radius, R_s) was of approximately the same order as that for R_G (see Fig. 2a). Thus the ρ -ratio (Burchard *et al.*, 1980) was only slightly affected (1.81 in 0.2M-NaCl; 1.76 in 6M-guanidinium chloride). The M_r values obtained from sedimentation-diffusion by the Svedberg equation did not change (Fig. 5c), but were systematically somewhat higher than those obtained by light-scattering, as noted previously (Sheehan & Carlstedt, 1984).

General discussion

In a previous study (Carlstedt *et al.*, 1983a) it was suggested that an association to, on average, 'dimers' occurs when cervical mucins are dialysed from 6M-guanidinium chloride into 0.2M-NaCl. However, in a subsequent study we showed that M_r had been underestimated in 6M-guanidinium chloride, owing to an error in the refractive-index increment and that M_r in the latter solvent is close to that in 0.2M-NaCl (Carlstedt *et al.*, 1983b). In the present paper the M_r of the mucins has been determined at several concentrations of guanidinium chloride with two independent methods, light-scattering and sedimentation-diffusion. No change was detected within the probable errors of the measurements, and it is concluded that no pronounced association of the mucins occurs, at low concentrations, in 0.2M-NaCl. However, the mucins occupy a larger solvent domain in 6M-guanidinium chloride than in 0.2M-NaCl, as indicated by a higher radius of gyration and frictional coefficient in the former solvent. The transition was particularly pronounced between 4M- and 6M-guanidinium chloride, resembling the expected behaviour of a conventional globular protein exposed to a denaturing solvent. The conformational change was also evident from the particle scattering functions, which suggest that the mucins are somewhat 'stiffer' in 0.2M-NaCl than in 6M-guanidinium chloride. The increase in the frictional coefficient of the mucins in 6M-guanidinium chloride was expressed as a decrease in the sedimentation coefficient as well as in the diffusion coefficient. The polymeric structure suggested for the mucins (Carlstedt *et al.*, 1983b; Sheehan & Carlstedt, 1984) is that of a linear array of heavily glycosylated segments interspersed by less-glycosylated or 'naked' stretches of the protein core. A conformational change is likely to evolve from the 'naked' protein segments, and an unfolding of 'ordered' regions within these stretches would explain the expansion of the macromolecular domain in 6M-guanidinium chloride. However, the increase of the mass per unit length in 0.2M-NaCl to a value (approx.

8000nm^{-1}) twice that predicted for the heavily glycosylated regions (approx. 4000nm^{-1}) would also be consistent with a folding of the entire macromolecule into a hair-pin, followed by an intramolecular side-by-side association. At the much higher concentrations prevailing in mucus, such associations would be more likely to occur between the macromolecules and could contribute to the formation of the gel.

Pig gastric mucins apparently undergo an irreversible conformational change when exposed to high salt concentrations (Snary *et al.*, 1974). Exposure of the pig mucins to e.g., guanidinium chloride was followed by an expansion of the macromolecules, whereas no change occurred in the M_r value. However, the pig gastric mucins, in contrast with the cervical ones, aggregated into, on average, tetramers when dialysed from guanidinium chloride into 0.2M-KCl.

The soluble blood-group substances obtained from ovarian cysts have many structural features in common with the carbohydrate-rich stretches of the mucins (e.g. monosaccharide and amino acid content and composition). Such macromolecules have no secondary or tertiary structure and were proposed to have a flexible configuration approaching the behaviour of a random coil (Creeth & Knight, 1967).

Electron-microscopy data of ovarian-cyst glycoproteins and an intermediary-sized mucin from cystic-fibrotic sputum were interpreted to suggest that these macromolecules are coil-like (Harding *et al.*, 1983). A fall in the reduced viscosity was observed when the glycoproteins were transferred from 0.1M-NaCl into 1M-guanidinium chloride, but the effect was interpreted as a 'polyelectrolyte-type contraction' rather than a conformational change. No change in the macromolecular properties was noticed between 1M- and 6M-guanidinium chloride (Harding & Creeth, 1983).

It is suggested that cervical mucins contain stretches of ordered structure which can participate in a reversible conformational change similar to that expected for a protein exposed to a denaturing solvent. This is an important feature, as mucins generally are regarded as hydrophilic space-filling polymers, with only time-average configurations which are determined by polymer-solvent and polymer-polymer interactions. Regions of ordered structure might provide for specific site-mediated interactions and could be decisive for the conformation and macromolecular behaviour of the mucins in a physiological solvent and contribute, in addition to entanglement, to the formation of the mucus gel.

This investigation was supported by grants from the Swedish Medical Research Council (567 and 5637;

Visiting Scientist Fellowship for J. K. S.), the Medical Faculty of Lund and Cystic Fibrosis Research Trust, U.K. (grant No. 198 to J. K. S.). Valuable discussions and criticism by Dr. Lars-Åke Fransson and Dr. Ian Nieduszynski are gratefully acknowledged.

References

- Bell, A. E. & Allen, A. (1982) in *Mucus in Health and Disease-II* (Chantler, E., Elder, J. & Elstein, M., eds.), pp. 97–99, Plenum Press, New York
- Burchard, W., Schmidt, M. & Stockmayer, W. H. (1980) *Macromolecules* **13**, 1265–1272
- Carlstedt, I., Lindgren, H., Sheehan, J. K., Ulmsten, U. & Wingerup, L. (1983a) *Biochem. J.* **211**, 13–22
- Carlstedt, I., Lindgren, H. & Sheehan, J. K. (1983b) *Biochem. J.* **213**, 427–435
- Casassa, E. F. & Eisenberg, H. (1964) *Adv. Protein Chem.* **19**, 287–395
- Creeth, J. M. & Knight, C. G. (1967) *Biochem. J.* **105**, 1135–1145
- Eisenberg, H. (1971) *Proced. Nucleic Acid Res.* **2**, 137–175
- Evans, J. M. (1972) in *Light Scattering from Polymer Solutions* (Huglin, M. B., ed.), pp. 89–164, Academic Press, London and New York
- Harding, S. E. & Creeth, J. M. (1982) *IRCS Med. Sci.* **10**, 474–475
- Harding, S. E. & Creeth, J. M. (1983) *Biochim. Biophys. Acta* **746**, 114–119
- Harding, S. E., Rowe, A. J. & Creeth, J. M. (1983) *Biochem. J.* **209**, 893–896
- Jouradian, G. W., Dean, L. & Roseman, S. (1971) *J. Biol. Chem.* **246**, 430–435
- Kinell, P.-O. (1959) *Arkiv Kemi* **14**, 327–336
- Kratochvil, P. (1972) in *Light Scattering from Polymer Solutions* (Huglin, M. B., ed.), pp. 333–384, Academic Press, London and New York
- Lee, W. I., Blandau, R. J. & Verdugo, P. (1977a) in *The Uterine Cervix in Reproduction* (Insler, V. & Bettendorf, G., eds.), pp. 68–77, Georg Thieme, Stuttgart
- Lee, W. I., Verdugo, P., Blandau, R. J. & Gaddum-Rosse, P. (1977b) *Gynecol. Invest.* **8**, 254–266
- Pusey, P. N. (1974) in *Photon Correlation and Light Beating Spectroscopy* (Cummins, H. S. & Pike, E. R., eds.), pp. 387–428, Plenum Press, New York
- Sadron, C. L. (1960) in *The Nucleic Acids* (Chargaff, E. & Davidson, J. N., eds.), pp. 1–37, Academic Press, New York and London
- Sheehan, J. K. & Carlstedt, I. (1984) *Biochem. J.* **217**, 93–101
- Silberberg, A. & Meyer, F. (1982) in *Mucus in Health and Disease-II* (Chantler, E., Elder, J. & Elstein, M., eds.), pp. 53–74, Plenum Press, New York
- Snary, D., Allen, A. & Pain, R. H. (1974) *Biochem. J.* **141**, 641–646
- Tanford, C. (1961) *Physical Chemistry of Macromolecules*, pp. 275–316, John Wiley and Sons, New York
- Tomimatsu, Y., Vitello, L. & Fong, K. (1968) *J. Colloid Interface Sci.* **27**, 573–580
- Wolf, D. P., Blasco, L., Khan, M. A. & Litt, M. (1978) *Fertil. Steril.* **30**, 163–169
- Zimm, B. H. (1948) *J. Chem. Phys.* **16**, 1099–1116