Polymeric structure of a high-molecular-weight glycoprotein from bovine cervical mucus

Frank A. MEYER*

Polymer Research Department, The Weizmann Institute of Science, Rehovot 76100, Israel†

(Received 9 August 1983/Accepted 12 October 1983)

A 16×10^6 - M_r glycoprotein isolated from bovine oestrus cervical mucus when reduced under conditions where disulphide-bond cleavage is essentially quantitative produces chains whose M_r from light-scattering and from sedimentation and diffusion data is some 4×10^6 – 5×10^6 . Pronase digestion of the chains indicates that glycosylated sequences of M_r 0.3×10^6 – 0.5×10^6 are interspersed with enzyme-susceptible non-glycosylated peptide sequences.

The structure of mucus glycoproteins is of considerable interest, since these are mainly responsible for the gel character and biological functions of mucus secretions (Gibbons & Sellwood, 1973; King *et al.*, 1974; Wolf *et al.*, 1978). Mucus glycoproteins are generally considered to involve disulphide-linked chains composed of glycosylated and non-glycosylated ('naked' peptide) sequences, which differ in amino acid composition and in susceptibility to proteinases (Donald, 1973; Gibbons & Sellwood, 1973; Roberts, 1976; Bhushana Rao & Masson, 1977; Scawen & Allen, 1977; Marshall & Allen, 1978; Mantle *et al.*, 1981). The glycosylated regions comprise most of the glycoprotein, whereas the 'naked' peptide regions account for only a few per cent of the mass.

With bovine oestrus cervical mucus as source, it has been shown (Meyer & Paradossi, 1983) that a glycoprotein of high M_r can be isolated when the mucus gel is brought just beyond the point of dissolution by either mild mechanical stirring or limited thermal treatment. Studies on the mechanically solubilized material, termed GP-S, suggest that it is a covalently linked entity with M_r 16×10^6 (Meyer & Paradossi, 1983). The polymeric structure of GP-S in terms of the size of the chain obtained on reduction of disulphide bonds and the size of the glycosylated regions obtained by proteolytic digestion with Pronase has now been in-

vestigated. Since the extent of disulphide-bond cleavage in mucus glycoproteins depends on the conditions of reduction (Mantle *et al.*, 1981), the reaction has to be monitored for completeness. Also, in view of the inherent polydispersity and high M_r of these materials, the reliability of the M_r estimates was checked by two independent methods.

Materials and methods

Materials

Bovine oestrus cervical-mucus samples were collected and pooled as previously described (Meyer & Paradossi, 1983). All chemical reagents were of analytical grade. Pronase (B grade) was from Calbiochem, San Diego, CA, U.S.A.

Isolation of GP-S

GP-S was isolated at 4°C by the procedure of Meyer & Paradossi (1983). In brief, mucus was brought just beyond the point of dissolution by gentle stirring on a magnetic stirrer, and the glycoprotein was purified by an initial centrifugation in a CsCl-density gradient to remove unbound protein, followed by a subsequent centrifugation to band the glycoprotein. The banded material, found in the density range 1.43–1.64 g/ml, was collected, dialysed extensively against PBS and stored frozen until used.

Reduction and alkylation

Samples (approx. 1 mg/ml) in 6 M-guanidine hydrochloride/0.135 M-Tris/HCl buffer, pH 8.0, were reduced under an atmosphere of N_2 with 0.1 M-dithiothreitol for 48 h at 4°C. Thiol groups were then alkylated in the dark at room temperature with 0.5 M-iodoacetamide for 20 min and the solu-

Abbreviation used: PBS, phosphate-buffered saline (0.127 M-NaCl/0.017 M- Na_2HPO_4 /0.02% NaN_3 , adjusted to pH 7.3).

* Present address: Department of Tumor Biology, The University of Texas–M. D. Anderson Hospital and Tumor Institute, 6723 Bertner Avenue, Houston, TX 77030, U.S.A.

† Reprint requests to this address.

tions were dialysed in the dark against several changes of PBS at 4°C. Reduction was also performed in 0.135 M-Tris/HCl buffer, pH 8.0, with 2 mM-dithiothreitol at 37°C for 1 h under an atmosphere of N₂, and alkylation was performed as above with 5 mM-iodoacetamide. Thiol groups in unreduced materials were alkylated with 5 mM-iodoacetamide.

Amino acid analyses were performed (Meyer & Paradossi, 1983) and the extent of reduction was obtained by comparing the *S*-carboxymethylcysteine content with the total half-cystine content of the sample. The latter was determined as cysteic acid on samples that had been oxidized (Spencer & Wold, 1969).

Pronase digestion

This was performed as previously described, with a digestion time of 9 h at 37°C (Meyer & Paradossi, 1983). The glycopeptide material produced was isolated between densities of 1.47 and 1.64 g/ml of a CsCl gradient run at 4°C (Meyer & Paradossi, 1983).

Analytical methods

The concentrations of glycoprotein solutions were routinely obtained from sialic acid determinations (Warren, 1959). Dry-weight determinations of stock solutions dialysed against distilled water and dried at 70°C to constant weight indicated a sialic acid content of 9.0% (w/w), and of 9.6% (w/w) for material after Pronase digestion.

Physical methods

Samples studied by these methods were all dialysed against PBS. Light-scattering measure-

ments with polarized laser light of wavelength 632.8 nm were performed at 23°C (Meyer & Paradossi, 1983). M_r values were calculated by using the specific-refractive-index increment (0.158 ml/g) found at 632.8 nm in a Hilger-Rayleigh interferometer (Jolly & Eisenberg, 1976) for a solution of GP-S (approx. 3 mg/ml) that had been reduced with 2 mM-dithiothreitol and alkylated. Sedimentation-velocity runs and viscosity measurements were performed at 20°C as described previously (Meyer & Paradossi, 1983). Height-area diffusion coefficients were determined at 20°C from the spreading of boundaries formed between solution and solvent in a double-sector synthetic-boundary cell run at 4800 rev./min in a Beckman model E analytical ultracentrifuge equipped with schlieren optics (Chervenka, 1973). D^0 was obtained from a plot of $1/D$ against concentration or taken as the D value at a single concentration, since variation with concentration (up to 6 mg/ml) was not found to be significant. A viscosity of 1.013 relative to water and a density of 1.007 g/ml were used for PBS to calculate $s_{20,w}^0$ and $D_{20,w}^0$.

Results and discussion

Sedimentation-velocity runs on the various preparations indicated that each sedimented essentially as a single unimodal peak. The schlieren profiles of GP-S and the reduced glycoproteins showed self-sharpening peaks consistent with these materials exhibiting non-ideal behaviour. At low concentrations (0.5–2.0 mg/ml), however, polydispersity was evident, and the profiles tended to be broad and asymmetric. Polydispersity was also indicated in the schlieren profiles of materials treated with Pronase.

Table 1. Physical and chemical data on the glycoprotein materials obtained by reduction and/or by Pronase digestion of GP-S

All values are averages of determinations on at least two different preparations. Physical measurements were performed in PBS buffer. M_r values from sedimentation and diffusion data(*) were calculated from the Svedberg equation by using a partial specific volume of 0.64 ml/g (Scawen & Allen, 1977). R_g is the radius of gyration obtained from light-scattering. Reduction followed by alkylation(†) was performed either with (i) 2 mM-dithiothreitol or with (ii) 0.1 M-dithiothreitol in 6 M-guanidine hydrochloride as described in the Materials and methods section.

	$10^{-6} \times M_r$		$s_{20,w}^0$ (S)	$10^7 \times D_{20,w}^0$ (cm ² ·s ⁻¹)	R_g (nm)	Half-cystine (residues/ 100 residues)	Percentage of half- cystine undergoing alkylation
	Light-scattering	(S + D)*					
GP-S	16.4	—	65.8	—	210	2.9	3 ± 3
Reduction†							
(i) 2 mM-dithiothreitol	5.2	4.6	24.7	0.36	139	—	70 ± 5
(ii) 0.1 M-dithiothreitol + 6 M-guanidine hydrochloride	—	4.2	39.6	0.63	—	—	97 ± 5
Pronase digestion	0.84	0.50	11.7	1.57	54	0.60	3 ± 3
Pronase digestion followed by reduction (ii)†	0.47	0.26	7.8	2.03	47	0.60	92 ± 10
Reduction (ii)† followed by Pronase digestion	—	0.29	7.5	1.72	—	0.82	100 ± 5

Table 1 shows physical data for the various preparations, together with total half-cystine contents and the fraction of the half-cystine residues alkylated with iodoacetamide. The spread of the M_r values and the reliability of their determination are indicated by the extent of agreement between the results based on light-scattering measurements and on sedimentation and diffusion data respectively. Since the samples are polydisperse, a higher average M_r is to be expected from light-scattering measurements than from sedimentation and diffusion data.

Table 1 shows that GP-S contains some 2.9 half-cystine residues per 100 amino acids and that these are almost exclusively present as disulphide-bonded cystine residues. The latter can be reduced, essentially quantitatively, when conditions of 0.1 M-dithiothreitol and 6 M-guanidine hydrochloride are employed. The M_r of the material that results is approx. 4×10^6 – 5×10^6 . Although M_r determinations were performed in PBS, aggregates involving self-association are unlikely to be present, since the reduced viscosity of the preparation is unaffected in PBS containing 1% sodium dodecyl sulphate or 6 M-guanidine hydrochloride. Reduction is only some 70% complete when conditions of 2 mM-dithiothreitol are used. Despite this, a material of similar M_r is produced. This suggests that no further interchain bonds are disrupted when reduction is completed. On reduction from 70% to essentially 100%, there is an increase in the sedimentation and diffusion coefficients of the material (Table 1). Since there is essentially no change in the M_r , a change to a more compact structure is suggested. This is not an irreversible effect of the 6 M-guanidine hydrochloride environment, since exposure of the 70%-reduced glycoprotein to this solvent, followed by dialysis against PBS, does not produce a conformational change, as judged by viscosity measurements before and after. Presumably, therefore, intrachain disulphide bonds are in some way involved in maintaining the partially reduced glycoprotein in a more extended configuration.

Performic acid oxidation can also cause quantitative cleavage of disulphide bonds in glycoprotein preparations from bovine cervical mucus (Meyer, 1977). The material obtained by performic acid oxidation has a sedimentation coefficient corresponding to an M_r of approx. 0.6×10^6 (Meyer, 1977), which is considerably smaller than that of the material obtained by essentially 100% reductive cleavage of disulphide bonds. Presumably, therefore, other bonds have been disrupted by performic acid treatment, which in general cannot be assumed to be specific for disulphide bonds (Hirs, 1956).

The M_r of approx. 4×10^6 – 5×10^6 found for GP-S after reduction is considerably higher than that of approx. 0.5×10^6 found by Gibbons *et al.* (1973) and by Bhushana Rao & Masson (1977) from

ultracentrifuge studies on preparations from bovine oestrus cervical mucus that had been subjected to reduction under conditions not too dissimilar to those used in the present study. The reason for the difference is not clear. It may, however, have to do with the polydispersity of reduced preparations, since the lower values are minimum estimates of the M_r (Bhushama Rao & Masson, 1977) and are obtained after high- M_r components have been removed by extensive centrifugation (Gibbons *et al.*, 1973). Since an M_r of approx. 0.5×10^6 is close to that of material that has been reduced and digested with Pronase (Table 1), it is possible that a subpopulation of molecules may exist in reduced preparations that have undergone proteolysis by proteinases that are believed to be present in native mucus (Gibbons & Sellwood, 1973; Schumacher, 1973; Carlstedt *et al.*, 1983a). The possibility, on the other hand, that some disulphide bonds have remained intact in our reduced preparation seems unlikely, in view of the close to 100% reduction that is obtained.

Digestion of GP-S with Pronase produces a glycosylated entity having M_r approx. 0.5×10^6 – 0.8×10^6 (Table 1). The material still contains some 20% of the half-cystine residues, though most have been removed as part of the 'naked' peptide region, as previously found by Roberts (1978). The Pronase-resistant disulphide bonds associated with the glycosylated entity can be reduced quantitatively with 0.2 M-dithiothreitol/6 M-guanidine hydrochloride to give material with M_r approx. 0.3×10^6 – 0.5×10^6 (Table 1). Essentially the same material is obtained if the order of the treatments is reversed and reduction is followed by Pronase digestion (Table 1).

The present study suggests that the molecule of GP-S consists of three to four chains each of M_r approx. 4×10^6 – 5×10^6 linked by disulphide bonds, with each peptide chain consisting of glycosylated regions of M_r approx. 0.3×10^6 – 0.5×10^6 interspersed with Pronase-sensitive 'naked' peptide regions. Since the M_r values of GP-S and the chains are considerably greater than that of the material subjected to Pronase digestion, it is unlikely that the former preparations have undergone much degradation by endogenous proteinases. The latter, however, may have contributed to some of the polydispersity seen in these preparations.

Similarity is evident between GP-S from bovine cervical mucus at oestrus and a glycoprotein derived from human cervical mucus at pregnancy, which has M_r 10.6×10^6 – 15.5×10^6 and which on reduction and proteolysis gives material of M_r 0.29×10^6 – 0.40×10^6 (Carlstedt *et al.*, 1983b; Sheehan & Carlstedt, 1983). The chains from the bovine cervical glycoprotein, however, are of higher M_r than those from the human glycoprotein, which have M_r

1.6×10^6 – 2.9×10^6 (Carlstedt *et al.*, 1983b; Sheehan & Carlstedt, 1983). The latter do not appear to have undergone degradation by endogenous proteinases, since isolation was performed in the presence of enzyme inhibitors and guanidine hydrochloride, which denatures proteinases (Houdret *et al.*, 1981, 1983). Rather, the difference in chain size suggests that GP-S involves fewer chains and is therefore less cross-linked with interchain disulphide bonds than the corresponding preparation from human pregnancy mucus. Whether this is related to the change in physical properties from oestrus or mid-cycle mucus to pregnancy mucus warrants further investigation.

Comparison of GP-S with glycoproteins from other mucus sources indicates some similarity with pig colonic glycoprotein, which has M_r 15×10^6 and which gives on reduction material of M_r 6×10^6 and on Pronase treatment and reduction material of M_r 0.7×10^6 (Marshall & Allen, 1978). Glycoproteins obtained from pig gastric (Scawen & Allen, 1977), human gastric (Pearson *et al.*, 1980) and pig small-intestine (Mantle *et al.*, 1981) mucus, on the other hand, are much smaller and consist of chains in the M_r range 0.24×10^6 – 0.50×10^6 , which involve a single glycosylated region of nearly the same M_r joined to a 'naked' peptide region(s).

This investigation was supported in part by Grant no. 2R01 NS 10048 from the National Institute of Neurological and Communicative Disorders and Stroke. Helpful discussions with Professor A. Silberberg and the technical assistance of Mrs. R. Finkel are gratefully acknowledged.

References

- Bhushana Rao, K. S. P. & Masson, P. L. (1977) *J. Biol. Chem.* **252**, 7788–7795
- 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
- Chervenka, C. H. (1969) *A Manual of Methods for the Analytical Ultracentrifuge*, pp. 83–86, Beckman Instruments, Palo Alto, CA
- Donald, A. S. R. (1973) *Biochim. Biophys. Acta* **317**, 420–436
- Gibbons, R. A. & Sellwood, R. (1973) in *The Biology of the Cervix* (Blandau, R. J. & Moghissi, K., eds.), pp. 251–265, University of Chicago Press, Chicago
- Gibbons, R. A., Boyd, L. J., Dixon, S. N., Parker, J., Sellwood, R. & Tasker, J. B. (1973) *J. Reprod. Fertil.* **35**, 469–476
- Hirs, C. H. W. (1956) *J. Biol. Chem.* **219**, 611–621
- Houdret, N., Le Treut, A., Lhermitte, M., Lamblin, G., Degand, P. & Roussel, P. (1981) *Biochim. Biophys. Acta* **668**, 413–419
- Houdret, N., Lamblin, A., Scharfman, A., Humbert, P. & Roussel, P. (1983) *Biochim. Biophys. Acta* **758**, 24–29
- Jolly, D. & Eisenberg, H. (1976) *Biopolymers* **15**, 61–95
- King, M., Gilboa, A., Meyer, F. A. & Silberberg, A. (1974) *Am. Rev. Respir. Dis.* **110**, 740–745
- Mantle, M., Mantle, D. & Allen, A. (1981) *Biochem. J.* **195**, 277–285
- Marshall, T. & Allen, A. (1978) *Biochem. J.* **173**, 569–578
- Meyer, F. A. (1977) *Biochim. Biophys. Acta* **493**, 272–282
- Meyer, F. A. & Paradossi, G. (1983) *Biochem. J.* **209**, 565–572
- Pearson, J. P., Allen, A. & Venables, C. W. (1980) *Gastroenterology* **78**, 709–715
- Roberts, G. P. (1976) *Arch. Biochem. Biophys.* **173**, 528–537
- Roberts, G. P. (1978) *Biochem. J.* **173**, 941–947
- Scawen, M. & Allen, A. (1977) *Biochem. J.* **163**, 363–368
- Schumacher, G. F. B. (1973) in *The Biology of the Cervix* (Blandau, R. J. & Moghissi, K., eds.), pp. 201–233, University of Chicago Press, Chicago
- Sheehan, J. K. & Carlstedt, I. (1983) *Glycoconjugates: Proc. Int. Symp. Glycoconjugates, 7th*, 601–602
- Spencer, R. L. & Wold, F. (1969) *Anal. Biochem.* **32**, 185–190
- Warren, L. (1959) *J. Biol. Chem.* **234**, 1971–1975
- Wolf, D. P., Blasco, L., Khan, M. A. & Litt, M. (1978) *Fertil. Steril.* **30**, 163–169