Biochimica et Biophysica Acta, 746 (1983) 114–119
Elsevier

BBA 31654

POLYELECTROLYTE BEHAVIOUR IN MUCUS GLYCOPROTEINS

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(Received February 7th, 1983)

Key words: Glycoprotein; Polyelectrolyte effect; Viscosity; Conformational change; (Human ovarian cyst and sputum)

Mucus glycoproteins isolated from a human ovarian cyst and the sputum of a cystic fibrotic exhibit a significant decrease in reduced viscosity with increase in ionic strength, *I*. The molecular weights of the glycoproteins showed little variation with *I*, implying that the change is conformational rather than a dissociation. This change is ascribed to a polyelectrolyte-type contraction rather than to a reduction in particle asymmetry. Guanidine hydrochloride acts as a classical electrolyte in the reversible suppression of charge effects, and not as a denaturing or dissociation agent. These observations help to resolve some discrepancies in earlier studies. The occurrence of polyelectrolyte effects in these glycoproteins is ascribed to flexibility of structure and to their content of *N*-acetylneuraminic acid. The ionic strength values necessary for different types of physical measurement are discussed.

Introduction

Although high concentrations of electrolytes or protein denaturants have often been employed to solubilize gelatinous mucous secretions (see, e.g., refs. 1–4), conflicting claims have been made [4,5–8] as to whether these substances have a deleterious effect on the glycoprotein components of the secretions. The main interest lies in the occurrence of any conformational change, or dissociation, and the extent of reversibility of such phenomena, if found. Therefore, we have studied the effect of guanidinium hydrochloride up to 6 M, and sodium chloride up to 1 M, on three representative glycoproteins. The techniques chosen were viscometry and molecular weight measurement, these being the most informative in respect of the changes anticipated. Because the apparent molecular weights of macro-ionic species at very low ionic strengths are difficult to interpret [9], we have restricted the observations to ionic strengths greater than 0.10 M. The results are useful in showing the minimum salt concentrations necessary for various types of physico-chemical measurement.

Materials and Methods

Solvents. The primary solvent used throughout was a phosphate/chloride buffer, pH 6.8, *I* 0.10, of the following composition: 0.033 M NaCl, 0.0167 M Na_2HPO_4, 0.0167 M NaH_2PO_4, 0.002 M NaN_3, 0.001 M EDTA. GuHCl and NaCl solutions were prepared in this solvent. For the density-gradient experiments, Cs_2SO_4 to a weight fraction of 0.315 was added.

Glycoproteins. Two of the glycoproteins were prepared from human ovarian cysts by the phenol-extraction, ethanol fractionation, method of Morgan [10]. One of these (603 AmS) was soluble in hot saturated ammonium sulphate, was the most carbohydrate-rich and had the lowest molecular weight of the six main fractions isolated from this cyst [11]. The other cyst glycoprotein

(603/43-50, from the same cyst) was insoluble in ammonium sulphate, and was precipitated from aqueous solution by ethanol in the range 43%–50% v/v. It had a composition and molecular weight [11] more typical of these materials [12].

Both these glycoprotein fractions had been prepared some years earlier [11], and were found to yield appreciably lower molecular weights than when first prepared. Accordingly, a more recently prepared glycoprotein (CF/Phi), from the sputum of a cystic fibrosis patient, was also employed. This substance was prepared by a slight modification of the density-gradient procedure [4]: protease inhibitors [13] were incorporated in the initial dispersing solution, one centrifugation was made in CsBr, and two in CsCl. Trace amounts of a nucleic acid impurity were removed by treatment with ribonuclease and deoxyribonuclease (bovine pancreas, Sigma (London), London U.K.) prior to the second CsCl centrifugation, which served to remove the enzymes from the glycoprotein. Its composition and physical properties were very similar to those other bronchial glycoproteins previously characterized [4]. No trace of lipid, protein or DNA contaminant was detected in any of the three glycoprotein preparations by the techniques of analytical density-gradient centrifugation or polyacrylamide gel electrophoresis.

Viscometry. Reduced viscosities were determined using extended Ostwald viscometers timed automatically [14]. Since the glycoproteins were completely soluble, the concentrations for use in evaluating the reduced viscosities were measured by weight, and then by serial dilution. The concentrations were chosen so that the flow times for the three samples were similar (see legends to Figs. 1 and 2).

Sedimentation equilibrium. The intermediate speed method described earlier [11,15] was used. In this method, the speed is sufficiently low to ensure complete resolution of the fringes near the base of the cell, so that high-molecular-weight components are not lost. Because of the low speed, the meniscus concentration at equilibrium remains finite, and is obtained by mathematical manipulations of the data [16]. The results of the data processing emerge as various point-average molecular weights, but comparisons are most simply made in terms of the appropriate whole-cell averages, denoted M°_n and M°_w respectively. The latter average is of greatest value, since it is affected only slightly by errors made in the determination of the meniscus concentration. It should be emphasized that the method does not depend on an independent estimate of the initial concentration, the quantity M°_w emerging as the limiting value of a particular directly determinable point-average [15]. All determinations were made at the lowest possible concentration (approximately 0.2 mg/ml), using 3-mm columns in cells of 30-mm optical path.

Density-gradient ultracentrifugation

Experiments were carried out following standard procedures [4,17] using a Centriscan 75 (M.S.E. Ltd., Crawley, U.K.) ultracentrifuge, operating in the absorption mode at a wavelength of 280 nm. Beckman 12 mm, 4° Kel F cells were used, with adaptors. Cs_2SO_4 was chosen as the gradient medium because of its particular suitability for density-disperse materials [18]. The glycoprotein distributions recorded were simulated in terms of two components using the equations for broad distributions formulated by Creeth and Horton [18].

It should be pointed out that this procedure was developed for single macromolecular solutes of fixed buoyant density, so that the greater spreading produced by solutes which are polydisperse in both molecular weight and density is observed as an apparent molecular weight much lower than the true value [17,18]. This characteristic has no disadvantage for comparative interpretations.

Results

Viscosity. On increasing the salt concentration (i.e., in addition to the primary solvent contribution, $I = 0.10$) a significant reduction (10–40%) occurs in the reduced viscosity for all three glycoproteins examined (Figs 1 and 2). This effect is evident with both GuHCl and NaCl. With GuHCl, further increase in the concentration to protein-denaturing levels induced a small increase in the reduced viscosity, possibly indicative of a minor unfolding of the molecules. However, the effect is small, at the limit of significance, and will not be further discussed.

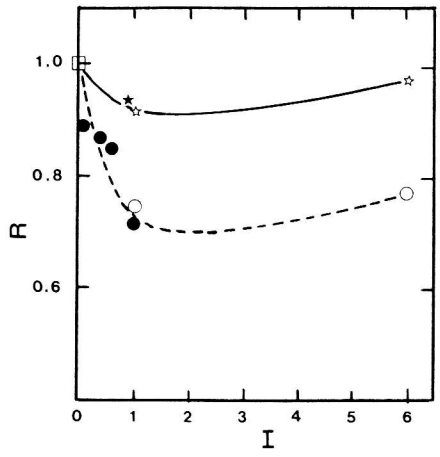

Fig. 1. Effect of high salt concentrations on viscosities of glycoproteins 603/AmS and 603/43-50. Results are expressed in terms of the ratio η_{red} $(I)/\eta_{red}$ (0.1), denoted R. I is the ionic strength (molar concentration) due to the added salt (NaCl or GuHCl), and the reference viscosity η_{red} (0.1) is the value for the glycoprotein in the standard reference solvent (see text) of $I = 0.1$ M. Reduced viscosities were measured at a glycoprotein concentration of 2.0 mg/ml for 603/AmS and 1.4 mg/ml for 603/43-50. ★, 603/AmS in NaCl; ☆, 603/AmS in GuHCl. η_{red} (0.1) = 34.0 ml·g^{-1}. ●, 603/43-50 in NaCl, η_{red} (0.1) = 92.1 ml·g^{-1}; ○, 603/43-50 in GuHCl, η_{red} (0.1) = 100.9 ml·g^{-1}; □, origin in all cases.

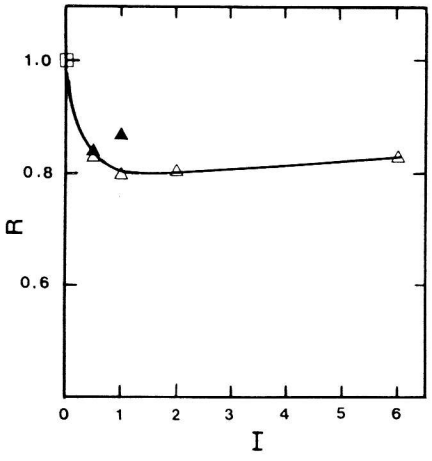

Fig.2. Effect of high salt concentrations on the viscosity of glycoprotein CF/Phi. Results are expressed as in Fig. 1. Reduced viscosities were measured at a glycoprotein concentration of 1.0 mg/ml. ▲, CF PHI in NaCl, η_{red}, (0.1) = 118.7 ml·g^{-1}; △, CF PHI in GuHCl, η_{red}, (0.10) = 120.6 ml·g^{-1}; □, Origin for both salts.

The main salt effect was tested for reversibility as follows. The glycoprotein CF/Phi was dissolved in the primary solvent described. One half was then dialysed against 6 M GuHCl for approximately 36 h, then against the primary solvent for 24 h and finally against distilled water for 72 h. The other half was merely dialysed against the primary solvent for 100 h and then against distilled water. Both samples were then freeze-dried and re-dissolved to make up solutions of concentration 1.0 mg/ml in the primary solvent. Their reduced viscosities were determined, giving the values 153 ml/g for the control and 142 ml/g for the GuHCl-treated material.

Bearing in mind the errors inherent in attempting to prepare salt-free polyelectrolyte solutions by dialysis against water, and the effect of the substitution of a small proportion of guanidinium for sodium counter-ions, the difference between these values is probably not significant. In any event, the figures show clearly that no major irreversible change in viscosity is induced by exposure to 6 M GuHCl.

Molecular weight determination. The number- and weight-cell-average molecular weigts, M°_n and M°_w, for the three glycoproteins are shown in Table I as a function of the salt concentration. Although the values in high concentrations of NaCl or GuHCl are slightly lower than those at $I = 0.1$, in two cases the differences are not significant. Thus, the changes in viscosity which occur as the ionic strength is raised are not paralleled by the molecular weight.

Density-gradient distributions. The distribution curves of the glycoprotein CF/Phi before and after treatment with GuHCl are shown in Fig. 3. The same samples as were used for the test of reversibility of viscosity were employed. This glycoprotein gives a bimodal distribution, which is a fairly common feature of bronchial glycoproteins which have not been sub-fractionated on the basis of density [4]. The distribution pattern cannot be accurately simulated in terms of the modified Gaussian distributions [18] expected for two discrete components differing in M, ρ_0 and c°, but the simulations shown are sufficiently close to the observed curve to demonstrate that the same values of the molecular parameters are applicable to both native and GuHCl-treated glycoproteins,

TABLE I

WEIGHT AND NUMBER CELL-AVERAGE MOLECULAR WEIGHTS ($M°_w$ AND $M°_n$, RESPECTIVELY) OF GLYCOPROTEINS 603/AmSsol, 603/43-50 AND CF/Phi AS A FUNCTION OF ADDED SALT CONCENTRATION

The composition of the phosphate buffer is described in text.

Glycoprotein	*N*-acetylneuraminic acid	Solvent	$M°_w$ ($\times 10^{-6}$)	$M°_n$ ($\times 10^{-6}$)
603/AmSsol	6.1	Phosphate buffer ($I = 0.10$)	0.56	0.40
		+ 1 M NaCl	0.57	0.44
		+ 6 M GuHCl	0.55	0.39
603/43-50	7.9	Phosphate buffer ($I = 0.10$)	0.90	0.78
		+ 1 M NaCl	0.84	0.75
		+ 6 M GuHCl	0.85	0.79
CF/Phi	10.3	Phosphate buffer ($I = 0.10$)	2.41	2.28
		+ 1 M NaCl	2.25	2.15
		+ 6 M GuHCl	2.18	2.19

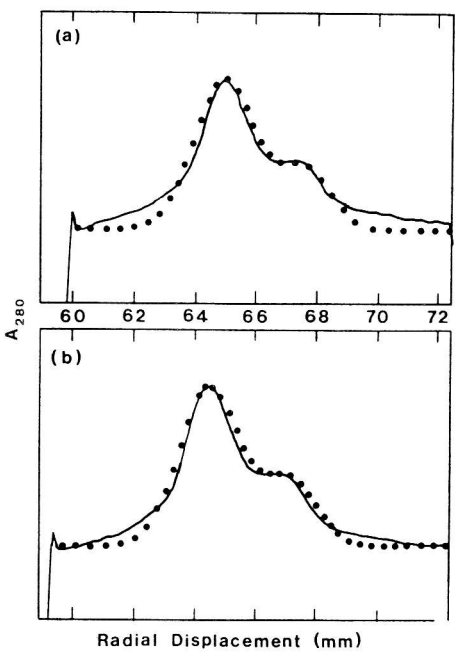

Fig. 3. Density-gradient patterns and simulations for native and GuHCl-treated glycoprotein. Solutions of glycoprotein CF/Phi before (a) and after (b) treatment with 6 M GuHCl (see text), or original concentration 1.25 mg/ml, were centrifuged to equilibrium in phosphate-buffered Cs_2SO_4 solution, initial density 1.337 g/ml, at 40000 rev./min and 20°C. Simulations (●) were each calculated on the basis of two macromolecular components (1 and 2) of buoyant densities (1) 1.3110 g/ml and (2) 1.3525 g/ml; apparent molecular weights: (1) 202000 and (2) 200000. In (a) (native), the initial concentrations were (1) 0.882 mg/ml and (2) 0.365 g/ml; in (b) (treated) the corresponding values were (1) 0.900 and (2) 0.372 mg/ml. There were small differences in the values denoting the positions of the menisci (the absorbance scale is arbitrary, and numerical values have therefore been omitted).

within the limitations of optical registration. Thus, by this criterion, the effect of GuHCl is fully reversible.

Discussion

The glycoproteins used in this study range from the simplest type — a glycopolypeptide with little naked segment [19] — through a medium-sized fraction [19], to one with comparatively high molecular weight [4]; in terms of conventionally accepted structure, they are, respectively, monomeric, dimeric and pentameric. It is interesting, therefore, that all three behave in a qualitatively identical fashion, implying that the results are probably valid for mucus glycoproteins generally, at least up to the maximum size stated.

The results are chiefly important for the demonstration of a significant conformational change in the glycoproteins brought about by NaCl and GuHCl. The effects with NaCl largely parallel the extensive observations of Snary et al. [5] on pig gastric glycoprotein in KCl solution, where a reduction in viscosity was recorded over a similar ionic strength range, but only partial reversibility was found. Subsequently, it was shown [6] that the extraction procedure had not eliminated all the non-combined protein from this material. The results of Snary et al. [6] on CsCl and GuHCl were obtained with material freed from extraneous protein by density-gradient centrifugation [17]: its behaviour in 4 M GuHCl was broadly similar to

that observed here, but removal of the salt caused aggregation, the molecular weight increasing from $2.3 \cdot 10^6$ to $8.2 \cdot 10^6$.

With our glycoproteins, the effects of NaCl and Gu HCl are indistinguishable, where comparison is possible, and accordingly the conformational change can be no more than a modest contraction with increase of ionic strength in the range 0.1–1.0 M, a typical polyelectrolyte effect (see, for example, Ref. 20). Although the charge density is much lower than with a true polyelectrolyte, the observed effect implies a degree of flexibility in the macro-structure, which is in agreement with expectations from other physical properties of these glycoproteins: a loose, highly-solvated, greatly expanded structure in solution provides the most acceptable model [19]. Complete reversibility of the effect would, of course, be expected, in conformity with the results of the viscosity, molecular weight and density-gradient distributions observed. It should be emphasised that the latter procedure, in particular, is highly sensitive to both the possible liberation of protein or peptide by GuHCl, and any conformational change in the glycoprotein resulting in a change of molar volume or solvation — the buoyant density implies a selective solvation of approximately 0.5 g H_2O per g glycoprotein [17].

The finding that GuHCl at 6.0 M produces only a minor contraction conflicts at first sight with the observations of List et al. [21] on highly viscous pig gastric mucin, where 7.0 M urea increased the viscosity by about 30%. However, these experiments were carried out at glycoprotein concentrations much greater than we employed, so that molecular overlap [22] will become significant. Assuming an unchanged conformation, overlap would be significantly increased by a reduction in the mol fraction of water from unity to 0.85, so that interpretation of the viscosity increase as a molecular expansion is open to criticism.

The absence of any corresponding decrease in molecular weight shows that the viscosity changes we observe cannot be due to reversible dissociation, confirming the conclusions of Snary et al. [5,6]. This, incidentally, makes the point that a relatively low ionic strength (e.g., 0.10) is sufficient to suppress the primary salt effects with these glycoproteins, so that the correct molecular weight is obtained at $I = 0.1$. This is not unexpected, for the conformational change that is manifested by the viscosity behaviour would influence the virial coefficient, and hence the concentration-dependence, rather than the extrapolated value at $c = 0$: the molecular weight measurements were purposely done at very low concentrations, where non-ideality should essentially vanish. However, other physical measurements which are especially sensitive to excluded volume should be performed at ionic strengths not less than 0.5 M.

The glycoproteins chosen for this study had not previously been dissolved in strongly chaotropic solvents, although the freeze-dried cyst solids had been extracted with 95% phenol; the glycoproteins are insoluble in this solvent. Ammonium sulphate and ethanol were used to fractionate the glycoprotein fraction after solution in water. The density gradient-prepared glycoprotein had been exposed to high concentrations of CsBr, which is a mild chaotrope [23], and CsCl, which is virtually inert. The fact that all three glycoproteins behaved similarly is strong presumptive evidence, therefore, that their initial conformation was essentially native, but that this term does not connote a uniquely organised structure. Therefore, the use of denaturing solvents in the preparation of mucus glycoproteins is justified, subject to adequate precautions against disulphide bridging to proteins.

The polyelectrolyte nature of sialic acid-rich glycoproteins, particularly when in the gelatinous complex found in some mucous secretions, has been emphasised by Tam and Verdugo [24]. Such an entangled matrix, with its necessary accompaniment of counter-ions, has long been recognised [25] to provide the framework for a Donnan equilibrium; the fact that gelatinous luteal-phase cervical mucus swelled more extensively when exposed to low concentrations of NaCl than to high was interpreted [24] in these terms. However, the extent of swelling was found to decrease sharply with pH over the range 6.5–8.0, and indeed vanished at the higher value. Over this range, the sialate ion remains fully charged ($pK_a = 2.6$) [26] and the only groups in the glycoprotein which could conceivably titrate are the histidine residues in the peptide backbone, whose effect must be negligible when compared with the approx. 10-fold excess of sialic acid. (The figures apply to a glycoprotein

containing 15% sialic acid and 20% peptide, of which His accounts for three of every 100 residues: such values are typical for cervical mucus glycoprotein.) An alternative explanation is called for: the protein content of the secretion probably cannot be ignored.

Acknowledgements

S.E.H. gratefully acknowledges the receipt of a Post-Doctoral Award from the Lister Institute of Preventive Medicine. Dr. J.L. Bridge assisted in the preparation and initial characterisation of glycoprotein CF/Phi.

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