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An analysis of the heterogeneity of mucins

No evidence for a self-association

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There are two possible contributions to the heterogeneity of mucus glycoproteins as observed in the analytical ultracentrifuge: firstly, from associative phenomena, and, secondly, from a polydisperse distribution of non-interacting species of different molecular masses. It is shown from the non-superposability of plots of point-weight-average relative molecular masses against concentration for differing initial cell-loading concentrations that polydispersity must be significant. It is further shown, by attempting to block any associative phenomena by competitive inhibition of potential sites for hydrophobic inter-particle interaction, that the observed heterogeneity is primarily a result of polydispersity and not a self-association.

It is evident from physical studies such as analytical equilibrium ultracentrifugation and light-scattering that mucus glycoproteins are heterogeneous substances. [For convenience I use the term 'heterogeneity' in its widest sense to describe any system where the solute species do not have a single value for the relative molecular mass, no matter what the origin of the variation may be (Creeth & Harding, 1982*a*).] It has been suggested (Creeth, 1980; Harding & Creeth, 1982) that the observed heterogeneity might be due to a property of mucins to self-associate, and, if so, possibly isodesmically (i.e. with constant free-energy increments). On the other hand, it might be due to a polydisperse distribution of non-interacting species of different molecular masses.

Is therefore the observed heterogeneity due to association, polydispersity or significant contributions from both? A further complication to the interpretation of physical measurements is that mucins are highly non-ideal in the thermodynamic sense. This arises from the very high affinity of these substances for water, with resulting high excluded volumes (corresponding to a molecular expansion of about 100-fold in solution; Creeth & Knight, 1967, 1968; Harding *et al.*, 1983*b*).

In the present study the problem has been re-examined for two well-characterized bronchial glycoproteins: one from the sputum of a cystic-fibrosis patient 'CF PHI' ($M_w \sim 2 \times 10^6$) and the other 'BM GRE' ($M_w \sim 6 \times 10^6$) from the sputum of a chronic bronchitic, where M_w is the weight-average relative molecular mass. It is firstly shown that the effects of polydispersity cannot be ignored for these substances, and, finally, by competitive inhibition of possible sites for self-association, that such an association cannot be verified for these substances in the native state.

Materials and methods

Solvents

The primary solvent used throughout was a phosphate/chloride buffer, pH 6.8 and I 0.10, of the following composition: 0.33M-NaCl, 16.5mM-Na_2HPO_4, 167mM-NaH_2PO_4, 2mM-NaN_3 and 1mM-EDTA. CsCl, NaCl, guanidinium chloride, fucose and *N*-acetylglucosamine solutions were prepared in this solvent. Before analysis by sedimentation equilibrium all solutions had been exhaustively dialysed against the relevant solvent (>48h at 4°C; >72h for guanidinium chloride).

Glycoproteins

The preparation of the cystic-fibrosis glycoprotein CF PHI, involving several density separations in caesium salts, has been described previously (Harding & Creeth, 1983); the purified glycoprotein was found to be free of lipid, protein or DNA contaminants, as determined by the techniques of analytical density-gradient ultracentrifugation or polyacrylamide-gel electrophoresis.

The chronic-bronchitic glycoprotein BM GRE has been separated and purified by using similar procedures (see also Creeth *et al.*, 1977). In this case, however, the glycoprotein had been subfractionated on the basis of density in the final steps.

Sedimentation equilibrium

A Beckman model E analytical ultracentrifuge was used, and solute distributions were recorded by using Rayleigh interference optics. In this work 3mm solution columns were used in both conventional double-sector and also Yphantis-style (Yphantis, 1964; Teller, 1973) multi-channel cells (Fig. 1). In all experiments 30mm-path-length cells were used, except for the CF PHI high-concentration experiment (12mm). The intermediate-speed method (Creeth *et al.*, 1974; Creeth & Harding, 1982*b*) was used. The speed is sufficiently low in this method so as to ensure complete resolution of the fringes near the base of the cell, so that large-molecular-mass components are not lost. At equilibrium the concentration at the air/solution meniscus remains finite, and is obtained by mathematical manipulation of the data (see, e.g., Creeth & Harding, 1982*b*). Point-weight-average relative molecular masses, M_w, were obtained by employing sliding-strip quadratic fits to the observed fringe data. Whole-cell weight-average relative molecular masses, M_w^0, were extracted by using the limiting value of a particularly directly determinable point average (Creeth & Harding, 1982*b*): an independent estimate for the initial concentration was not required.

Results and discussion

Fig. 2(*a*) shows a typical solute equilibrium distribution for a mucus glycoprotein recorded by using Rayleigh interference optics. The solution fringes are for the chronic-bronchitis glycoprotein BM GRE, at a low initial loading concentration (approx. 0.4mg/ml): the steep rise of the fringes near the cell base without depletion of the meniscus of solute is indicative of heterogeneity. Fig. 3 gives the corresponding plot of $\ln J$ versus ξ, where J is the fringe concentration and ξ is a function of the square of the radial displacement, r^2:

$$\xi = \frac{r^2 - a^2}{b^2 - a^2} \quad (1)$$

where a and b are the radial positions of the meniscus and base respectively. It was shown in earlier communications (Creeth, 1980; Harding & Creeth, 1982) that such plots could be represented

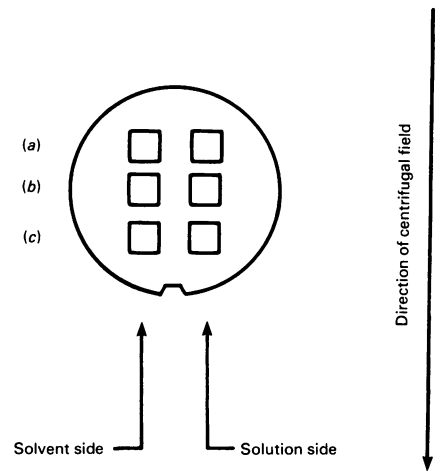

Fig. 1. *Contents of multi-channel cells corresponding to Fig. 2*

Solvent for (*a*), phosphate/chloride buffer containing 0.4M-CsCl; solvent for (*b*), phosphate/chloride buffer containing fucose (5mg/ml); solvent for (*c*), phosphate/chloride buffer containing *N*-acetylglucosamine (5mg/ml). The solution channels contained in addition inert fluorocarbon oil to provide a sector-shaped bottom. Channels were filled to give 3mm columns. The initial glycoprotein loading concentrations for all three solution channels were approx. 0.4mg/ml.

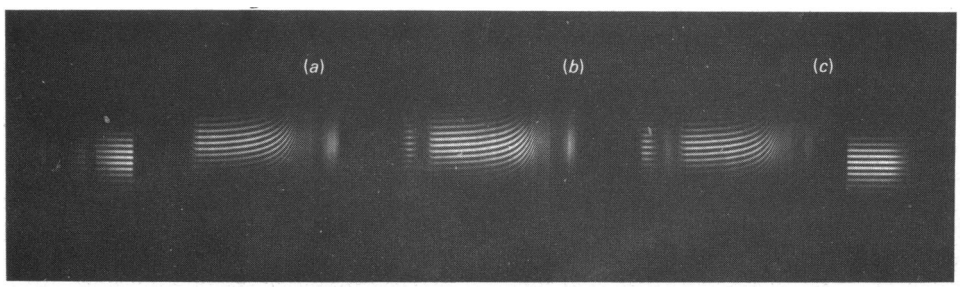

Fig. 2. *Rayleigh equilibrium interference patterns for the three glycoprotein solutions corresponding to the cell-channel contents as shown in Fig. 1*

(*a*) BM GRE in phosphate/chloride buffer containing 0.4M-CsCl; (*b*) BM GRE in phosphate/chloride buffer containing fucose (5mg/ml); (*c*) BM GRE in phosphate/chloride buffer containing *N*-acetylglucosamine (5mg/ml). The initial glycoprotein cell-loading concentration in each case was approx. 0.4mg/ml. The rotor speed was 1967 rev./min, and the temperature 20.31°C.

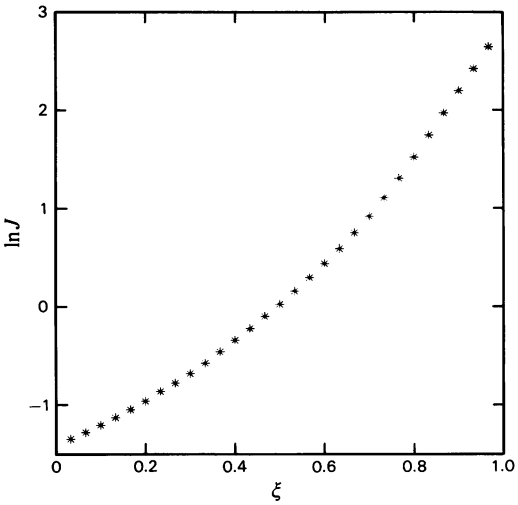

Fig. 3. *Plots of the natural logarithm of the fringe concentration (J) versus the square of radial displacement function ξ (see eqn. 1) corresponding to the Rayleigh equilibrium pattern (a) of Fig. 2 (namely glycoprotein BM GRE in phosphate/chloride buffer containing 0.4M-CsCl)*

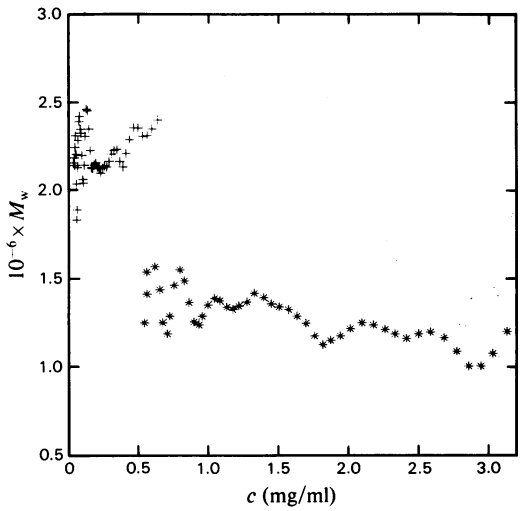

Fig. 4. *Plots of point-average relative molecular masses against concentration for two different initial cell-loading concentrations, c^0, of glycoprotein*
CF PHI: $+c^0 \sim 0.2$mg/ml; *, $c^0 \sim 2.0$mg/ml. Rotor speeds, 1967rev./min. Solvent: phosphate/chloride buffer containing 1M-NaCl.

in terms of an isodesmic self-association, the latter paper (Harding & Creeth, 1982) taking into account thermodynamic non-ideality. For such simulations, however, the effects of polydispersity could not be taken into account. This, in fact, is not a reasonable assumption. Mucus glycoproteins are inherently polydisperse by virtue of the process of their biosynthesis (see, e.g., Silberberg & Meyer, 1982). Two types of polydispersity are to be expected: firstly, 'primary' polydispersity arising from a quasi-continuous distribution of carbohydrate side-chain lengths (Roussel *et al.*, 1975); secondly, 'secondary' polydispersity arising from a discrete variability of the numbers of fundamental 'basic units' (Silberberg & Meyer, 1982; Harding *et al.*, 1983*a*).

In Fig. 4 I have demonstrated the presence of polydispersity for CF PHI by using the diagnostic technique of non-overlap of M_w-versus-c plots (Roark & Yphantis, 1969) for two solute distributions corresponding to two different initial cell-loading concentrations. The fall-off in the M_w values with increase in concentration for the higher cell-loading concentration (approx. 2.0mg/ml) is a result of thermodynamic non-ideality, corresponding to a value for the second virial coefficient, B, of at least 1.5×10^{-4}ml·mol·g^{-2} (Harding & Creeth, 1982).

With the added complication of such non-ideality for these substances, it is a non-trivial problem to evaluate the precise nature of polydispersity or self-association phenomena. However, it is possible at least to establish whether there is a self-association present or not, just as we have established that polydispersity is present. There are two possible sites for self-association on a mucin molecule: first of all, there may be hydrophobic patches on the glycosylated regions arising from possible localized groups of fucose or *N*-acetylglucosamine. This does not at first sight appear unreasonable, since fucose is always present as a terminal residue of a carbohydrate side chain, and *N*-acetylglucosamine is normally the most abundant carbohydrate residue (see, e.g., Silberberg & Meyer, 1982). If these residues do provide sites for self-association, then it is reasonable to assume that they may be blocked competitively by having a swamping concentration of fucose or *N*-acetylglucosamine in free solution.

Fig. 2 illustrates the results of such an experiment involving the chronic-bronchitis glycoprotein BM GRE. An Yphantis-type multi-channel cell was used (Yphantis, 1964), loaded as indicated in Fig. 1 legend. The inner solution fringes (*a*) correspond to the solute equilibrium distribution of glycoprotein, at an initial loading concentration of approx. 0.4mg/ml ($\equiv 0.2\,\mu$M) in a non-dissociating solvent (phosphate/chloride buffer, as described above, containing 0.4M-CsCl). The middle fringes (*b*) correspond to the same glycoprotein loading concentration in phosphate/chloride buffer containing fucose (5mg/ml, $\equiv$ 30mM) and the outer fringes (*c*) to that in phosphate/chloride buffer

containing *N*-acetylglucosamine (5mg/ml, ≡ 30mM).

No visible differences are apparent between the three solution fringe patterns. Indeed, there is no significant difference between the whole solute distribution weight-average relative molecular masses: for (*a*), $M_w^0 = 6.2 \times 10^6$; for (*b*), $M_w^0 = 6.0 \times 10^6$; for (*c*), $M_w^0 = 6.2 \times 10^6$. Simulations showed that the concentrations of fucose and *N*-acetylglucosamine remained virtually constant throughout the solute redistribution: that is, the redistribution of fucose or *N*-acetylglucosamine caused by the centrifugal field was negligible. It is therefore unlikely that fucose or *N*-acetylglucosamine residues are sites for self-association phenomena.

The other possible sites on a mucin for self-association phenomena are regions of naked peptide, devoid of carbohydrate, that are known to be present in mucins (see, e.g., Allen *et al.*, 1982). A similar experiment with the same initial loading glycoprotein concentration on a similar fraction of BM GRE in phosphate/chloride buffer but with 6M-guanidinium chloride present (to block competitively any hydrophobic free peptide sites) again yields a similar relative-molecular-mass average for the distribution (M_w^0 5.5×10^6).

It has to be concluded therefore that there is no detectable self-association for this mucin. This conclusion is supported by data (for experiments in 6M-guanidinium chloride and non-dissociating solvents) for other mucins of smaller molecular mass (Harding & Creeth, 1983). This conclusion is also supported by observations done in parallel on BM GRE and also pig gastric mucin by Creeth & Cooper (1984). The observed heterogeneity of mucins is evidently a manifestation of polydispersity and not of self-association phenomena. In order, however, to establish the nature of the polydisperse distributions, for example, whether they are log–normal or otherwise, will involve curve-fitting with the use of the Rinde type of equations (Rinde, 1928; see also Creeth & Harding, 1982*b*) but with due allowance for thermodynamic non-ideality, which is a formidable problem.

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