Structure and Mucoadhesion of Mussel Glue Protein in Dilute Solution†

Matt P. Deacon,‡ Stanley S. Davis,§ J. Herbert Waite,|| and Stephen E. Harding*,‡

NCMH Unit, School of Biological Sciences, University of Nottingham, Sutton Bonington, LE12 5RD, U.K., School of Pharmacy, University of Nottingham, Nottingham, NG7 2RD, U.K., and Department of Chemistry & Biochemistry, University of Delaware, Newark, Delaware 19716

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ABSTRACT: Purified mussel adhesive protein *mefp-1* (*Mytilus edulis* foot protein 1) has been studied regarding its state of oligomerization and gross conformation in dilute solution. Sedimentation equilibrium in the analytical ultracentrifuge of a dilute solution of protein (0.4 mg/mL) in acetate buffer at pH 4.5 and $I = 0.10$ M yielded an apparent molecular weight (whole distribution weight average, $M_{w,app}$) of 114 000 ± 5000 via the "*M**" procedure, a value in almost exact agreement with the monomeric molecular weight obtained by MALDI mass spectrometry. At this low concentration, it is reasonable to assume thermodynamic ideality, i.e., $M_{w,app} \sim M_w$. This result, together with plots of point weight average apparent molecular weight versus concentration for three different loading concentrations (0.4, 0.8, 1.0 mg/mL), clearly demonstrates that this protein is essentially monomeric in dilute solution. Sedimentation velocity experiments yielded an estimate of the sedimentation coefficient $s^0_{20,w} = 2.34 \pm 0.17$ S, which for $M =$ 110 000 gives a frictional ratio $f/f_0 = 3.2 \pm 0.3$. The interpretation of this, in terms of an extended rather than globular conformation for the structure of *mefp-1* in dilute solution, is considered, within plausible limits of molecular hydration, and models for the structure in solution are considered, in light of the thermodynamic nonideality behavior of these molecules and previously published circular dichroism data. The significance of these observations in terms of the bioadhesive properties of *mefp-1* is described, and the very strong interaction in dilute solution with a mucin glycoprotein is demonstrated.

The protein *mefp-1* is one of the major adhesive proteins used by marine mussels to bind strongly to underwater surfaces. This has been related to its strong surface-active and adsorptive behavior (*1*−*3*). This and related mussel adhesive proteins are characterized by having high lysine contents and hydroxylated amino acids: *mefp-1*, for example, consists of tandemly repeated decapeptides each containing two residues of lysine, one to two residues of Dopa (*4*, *5*), one or two residues of *trans*-4-hydroxyproline, and a single residue of *trans*-2,3-*cis*-3,4-dihydroxyproline (*6*).

Several attempts are being made to make biomedical and commercial use of the adhesive properties of these substances (*7*): for example, in experimental epikeroplasty and for cellular attachment (*8*, *9*), and in the attachment of osteoblasts and epiphyseal cartilage cells to substrata (*10*). The strong adhesive properties have recently inspired a proposed use for these proteins as mucoadhesives for drug delivery (*11*), and these substances may offer a strong alternative to substances such as chitosans (*12*). Applications, however, have been hampered by the lack of knowledge on the solution structure and adhesive nature of the purified protein (*7*), and very little is known of the oligomeric state or overall morphology of these molecules in solution (dilute or otherwise). In this study, we address this for the case of dilute solution conditions (1.0 mg/mL and less) by using a combination of low-speed sedimentation equilibrium and sedimentation velocity experiments in the analytical ultracentrifuge, together with previously published information on the monomeric molecular mass from MALDI (matrix-assisted laser desorption/ionization) mass spectrometry (*13*). Models for the structure in solution are considered, in light of the thermodynamic nonideality behavior of these molecules and previously published circular dichroism data (*14*). The significance of these observations in terms of the bioadhesive properties of *mefp-1* is considered, and the very strong interaction in dilute solution with a mucin glycoprotein is demonstrated using sedimentation velocity in the analytical ultracentrifuge.

EXPERIMENTAL SECTION

Preparation of Solutions. *Mytilus edulis* foot protein 1 was prepared according to the method of Waite (*4*) from material supplied by Sigma Chemical Company (St. Louis, MO) and was recovered as a freeze-dried mat. This mat was dissolved for 2 h in buffer before use. Pig-gastric mucin glycoprotein for the mucoadhesive study was isolated and purified as described before (*15*). All solution measurements were performed in an acetate buffer, pH 4.6 and $I = 0.10$ M (*16*).

Sedimentation Equilibrium in the Analytical Ultracentrifuge. An Optima XL-A ultracentrifuge (Beckman Instru-

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* Corresponding author: NCMH Unit, Nottingham University, School of Biological Sciences, Sutton Bonington, LE12 5RD, U.K. Telephone: 0044-115-951-6148. Fax: 0044-115-951-6142. Email: Steve.Harding@nottingham.ac.uk.

‡ School of Biological Sciences, University of Nottingham.

§ School of Pharmacy, University of Nottingham.

|| University of Delaware.

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ments, Palo Alto, CA) was used at a rotor speed of 14 000 rpm, temperature 20.0 °C. Standard 12 mm optical path length aluminum-filled epoxy double sector cells were employed, filled to 0.100 mL (yielding ~2.8 mm solution and solvent columns). An initial cell loading concentration of 0.4 mg/mL was used so as to minimize the effects of nonideality. Equilibrium was established within 48 h and confirmed by successive overlay of scans separated by 10 h. A partial specific volume, $\bar{v}$, of 0.753 mL/g was calculated from the amino acid sequence, according to the consensus formula of Perkins (*17*).

Equilibrium solute distributions were captured as an ASCII data set of concentration (expressed as ultraviolet absorbance at a wavelength of 265 nm) versus radial displacement from the rotor center, r (cm), using the M^* procedure *(18)* incorporated into the PC routine MSTARA (*19*). The M^* procedure yields the apparent weight average molecular weight (for the whole distribution of macromolecular solute in the ultracentrifuge cell), $M_{w,app}$, from the identity $M_{w,app} = M^*(r = \text{cell base})$. MSTARA also, among other things, produces plots of point average molecular weight data sets of $M_{w,app}(r)$ versus local concentrations, $c(r)$ [expressed in absorbance units, $A(r)$], at different radial positions, r, in the ultracentrifuge cell, and these were also obtained for the initial loading concentration of 0.4 mg/mL. Further data sets of $M_{w,app(r)}$ versus $c(r)$ were also obtained for cell loading concentrations of 0.8 and 1.0 mg/mL (to check for the presence of any associative phenomena).

Sedimentation Velocity. The Optima XL-A ultracentrifuge was also used for sedimentation velocity experiments at a rotor speed of 40 000 rpm and temperature of 20 °C, using the 12 mm optical path length cells. Loading concentrations of 0.2, 0.3, 0.4, 0.6, 0.66, and 0.8 mg/mL were corrected for radial dilution. Sedimentation coefficients $s_{T,b}$ (where T,b means at temperature T and in buffer b) were evaluated using the routine XLA-VEL (*20*). All sedimentation coefficient measurements were performed in triplicate in order to minimize errors and then corrected to standard conditions of solvent density (ρ) and viscosity (η), namely, those of water at 20 °C, by means of the usual expression (*21*):

$$s_{20,w} = \left\{\frac{(1 - \bar{v}\rho)_{20,w}}{(1 - \bar{v}\rho)_{T,b}}\right\}\left\{\frac{\eta_{T,b}}{\eta_{20,w}}\right\}s_{T,b} \qquad (1)$$

RESULTS AND DISCUSSION

Oligomeric Structure. The weight average molecular weight, $M_{w,app}$, was determined from extrapolation of the "M^*" function to the cell base for the data-set for the lowest loading concentration (0.4 mg/mL), where it is reasonable to assume $M_w \sim M_{w,app}$. Using this procedure, $M_w = 114\,000 \pm 5000$. Since the molecular weight of a monomer is known from MALDI mass spectrometry to be 110 000 (*14*), we can reasonably infer that the *mefp1* protein is also monomeric in dilute solution, at least under these conditions (pH 4.6, $I = 0.10$ M). This view is strengthened when we consider plots of point apparent weight average molecular weight, $M_{w,app}(r)$, as a function of local concentration [expressed as absorbance units $A(r)$ at radial positions r from the rotor center]. Figure 1 shows clearly that for loading concentrations of 0.4, 0.8, and 1.0 mg/mL there is no evidence of associative behavior, and this view is strengthened by the single symmetric nature of the boundaries from sedimentation velocity experiments (Figure 2).

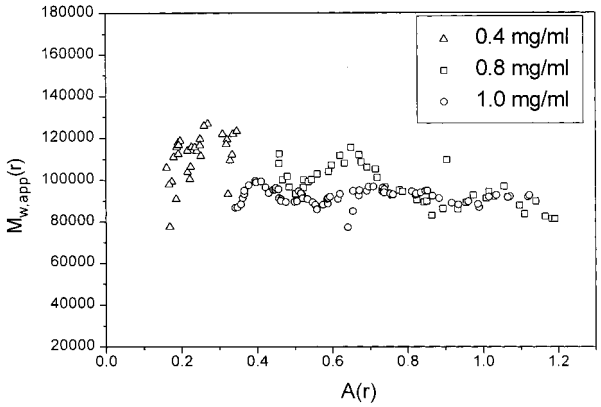

FIGURE 1: Plot of point weight average molecular weights, $M_{w,app}(r)$, versus local concentration [expressed as absorbance units at 265 nm, $A(r)$] at various radial positions r in the ultracentrifuge cell for different loading concentrations (0.4, 0.8, 1.0 mg/mL).

Gross Conformation in Dilute Solution. From the plot of $s_{20,w}$ versus sedimenting concentration (i.e., corrected for radial dilution), c (Figure 3), a value of $s^0_{20,w}$ of 2.34 ± 0.17 S is obtained by linear regression analysis. This is quite low for a protein of $M = 110\,000$ and suggests an asymmetric and/or highly hydrated form for the macromolecule in solution. We can investigate this further by calculating the frictional ratio f/f_0 and the corresponding Perrin function P for the hydrodynamically equivalent prolate ellipsoid of revolution. f/f_0 [see, *for example*, (*21*)] is the ratio of the translational frictional coefficient of a macromolecule to the corresponding coefficient for a spherical particle of the same mass and (anhydrous) volume, and is given by

$$\frac{f}{f_0} = \left[\frac{M(1 - \bar{v}\rho_0)}{N_A(6\pi\eta_0 s^0_{20,w})}\right]\left(\frac{4\pi N_A}{3\bar{v}M}\right)^{1/3} \qquad (2)$$

where N_A is Avogadro's number and ρ_0 and η_0 are the density and viscosity of water at 20 °C. Using values of $M = 110\,000$, $s^0_{20,w} = 2.34 \pm 0.17$ S, $\bar{v} = 0.753$ mL/g, $\rho_0 = 0.9982$ g/mL, and $\eta_0 = 0.01$ P, we estimate f/f_0 to be ~3.2 ± 0.3.

The frictional ratio is related to two molecular parameters describing the molecule in solution: shape and the molecular expansion of the molecule in solution through (aqueous) solvent association. The shape contribution is represented by the Perrin (*22*) function P, and the molecular expansion through solvent association is popularly represented by the "apparent hydration" δ (the mass of aqueous solvent chemically or physically associated with the protein per unit dry mass of protein). P is given by [see, for example, (*23*)]:

$$P = \left(\frac{f}{f_0}\right)\left(\frac{\delta}{\bar{v}\rho_0} + 1\right)^{-1/3} \qquad (3)$$

We consider two cases:

(1) Calculation of the Axial Ratio of the Hydrodynamically Equivalent Prolate Ellipsoid of Revolution for a "Typical" Value of δ. From the shape function P, the overall asymmetry of the protein can be represented in terms of the axial ratio a/b ($a > b$) of the hydrodynamically equivalent

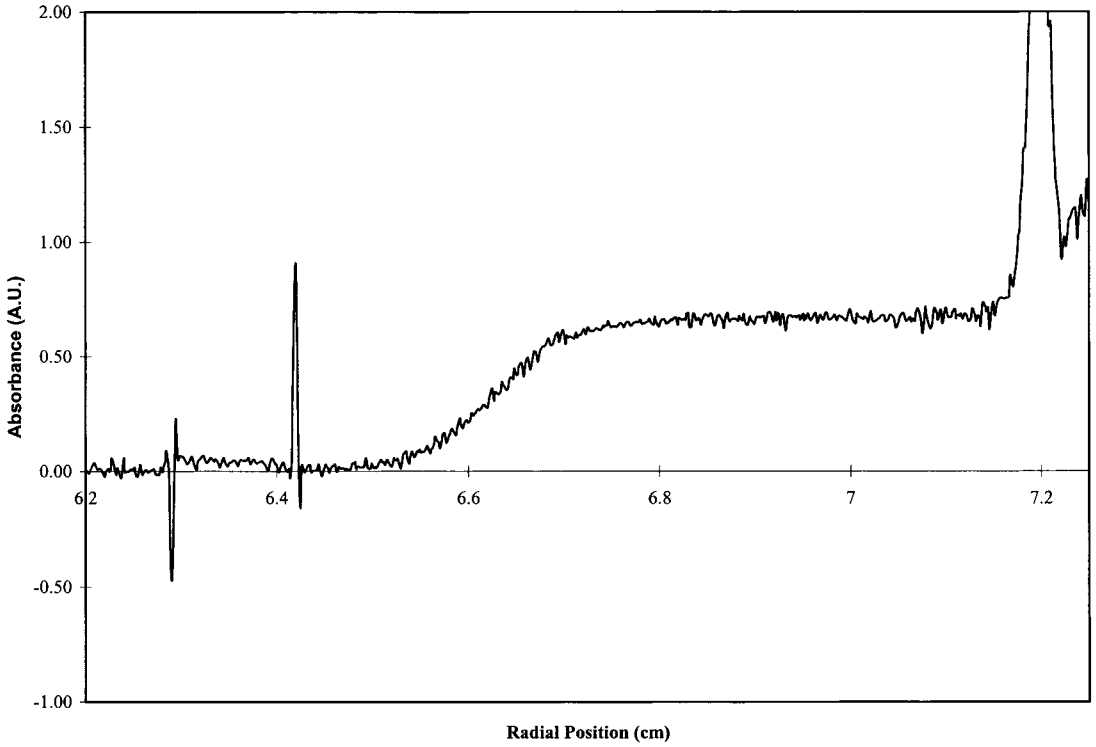

FIGURE 2: Sedimenting boundary for *mefp-1*. Rotor speed = 40 000 rpm, temperature = 20.0 °C, loading concentration = 0.8 mg/mL.

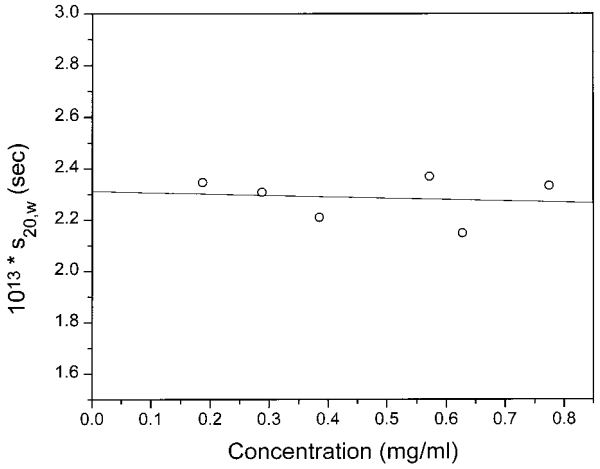

FIGURE 3: Concentration dependence of the sedimentation coefficient, $s_{20,w}$, for *mefp1* in acetate buffer (pH = I = 0.10) at 20 °C.

Table 1: Summary of Solution Physical Properties of *mefp-1*

physical property	value
monomer molecular weight, M_1	110000
weight average molecular weight M_w	114000 ± 5000
sedimentation coefficient, $s^0_{20,w}$ (S)	2.34 ± 0.17
partial specific volume, $\bar{v}$ (mL/g)	0.753
translational frictional ratio, f/f_0	3.2 ± 0.3

Table 2: Perrin Function P and Axial Ratios a/b for *mefp-1* for Various Values of Molecular Hydration, δ

δ	P	a/b
0.2	3.0	50
0.35	2.8	45
0.5	2.7	40

prolate ellipsoid of revolution, for specified values of δ:

$$P = \frac{(1 - b^2/a^2)^{1/2}}{(b/a)^{2/3} \ln\left\{\frac{1 + (1 - b^2/a^2)^{1/2}}{b/a}\right\}} \quad (4)$$

a/b can be found for a specified P by simple numerical inversion of eq 4, using the PC routine ELLIPS1 (*23*). Considering a plausible range of values for δ (0.2−0.5) according to Squire and Himmel (*24*), we see from Table 2 that *mefp-1* is hydrodynamically equivalent to an extended rod shape in solution. Allowing for the experimental error in the frictional ratio, the limits of the axial ratio for *mefp-1* are within the approximate range 30:1 to 60:1. This would compare, for example, with a value of ∼80:1 for myosin and myosin rods [see, e.g., Harding (*25*)].

(2) Calculation of the Maximum Value the Apparent Hydration (δ) Can Have if the Overall Domain Occupied by the Molecule Is a Sphere. The axial ratio could of course be lower if the apparent hydration was unexpectedly higher (>0.5), and any molecular flexibility would increase the apparent hydration. For the case where molecular expansion is the sole contribution to the frictional ratio (i.e., P = 1), an f/f_0 of ∼3.2 corresponds to a δ of ∼25. This corresponds to a molecular expansion (volume occupied by hydrated molecule/volume of anhydrous molecule) of ∼35×, a value more typical for heavily glycosylated systems such as mucin glycoproteins which have more coil-like properties (*26*, *27*).

Both cases are consistent with our observation from sedimentation equilibrium that the effects of thermodynamic nonideality are relatively small (and that at the loading concentration the effects can be reasonably neglected). This can be shown using the routine COVOL (*28*, *23*), which, based on the Rallison−Harding (*29*) exclusion volume theory for general ellipsoids, predicts the second thermodynamic virial coefficient B for a macromolecule based on its shape

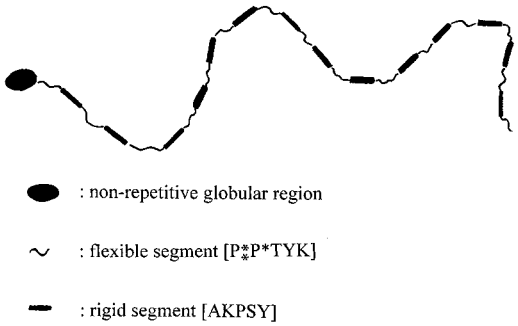

FIGURE 4: Consensus semi-flexible rod model for *mefp-1*. This model takes into account the linear flexible properties consistent with larger values for the hydration ($\delta > 0.5$), earlier CD studies (*5*), and the ability to adhere and hydrate at surfaces (*7*). The model consists of a globular region with a nonrepetitive amino acid sequence and an extended region consisting of repeat sequences of amino acids with alternating stiff and flexible segments. Except at high pH (>7) and ionic strength, the chain will be relatively stiff due to electrostatic repulsion of segments.

(as specified by its semi-axial dimensions *a,b,c* or axial ratios *a/b, b/c*), its molecular weight, and its molecular hydration or expansion. The routine also provides for calculating the polyelectrolyte contribution to *B*, where appropriate. To a first approximation, if we assume the ionic strength is sufficient to suppress this latter contribution, the predicted values of *B* are 5.97×10^{-4} mL·mol^{-1}·g^{-2} for case 1 above and 9.82×10^{-4} mL·mol^{-1}·g^{-2} for case 2. The apparent weight average molecular weight $M_{w,app}$ at a finite concentration c (g/mL) is, to first order in nonideality (*21*):

$$1/M_{w,app} = (1/M_w)(1 + 2BM_w c) \qquad (5)$$

For a concentration of 1 mg/mL, therefore, and M_w = 110 000, eq 5 predicts a value of 91 900 for case 1 and 83 100 for case 2, i.e., in both cases a drop in the apparent molecular weight of ~20% for case 1 and ~30% for case 2 as the concentration is varied from 0 to 1.5 mg/mL (corresponding to the concentration range of Figure 1).

Thus, both cases are consistent with the data. Unfortunately, it is not possible to compare this result with an X-ray crystallographic structure since as far as we are aware nobody has succeeded in crystallizing the protein or derived peptides. Also, the protein is too large to have its structure solved by NMR.

The solution conformations of *mefp-1* and a recombinant analogue with 20 repeats of the consensus decapeptide (AKPSYPPTYK) have, however, been studied using far-UV circular dichroism and enzyme-directed modification (*14*). Although the solution conditions differed (0.6 M NaCl with 0.1 M phosphate at pH 7.0) from those of the present study, it is intriguing to compare the results. The CD data suggested that the secondary structure in *mefp-1* and its recombinant analogue is limited to no more than 5% α-helix, 10% β-sheet, and 20% β-turns. The remainder was attributed to "random coil" as it was minimally perturbed by temperature or the addition of 6 M guanidine hydrochloride. This interpretation, however, was difficult to reconcile with the results of the tyrosinase-directed modification of tyrosines in the recombinant analogue: Tyr-9 of each consensus repeat was at least an order of magnitude more reactive than Tyr-5 (*14*). Taken together, the previous results suggested an overall extended and flexible *mefp-1* structure that is punctuated by regions of rigidity: i.e., a conformation between the extremes of case 1 and case 2, and is presented as the "consensus model" of Figure 4 for the structure of *mefp-1* in dilute solution.

Successful adhesion in nature is dependent on adsorptive and cohesive interactions that are difficult to unravel when they occur simultaneously. By defining conditions at which *mefp-1* is essentially a monomer, purely adsorptive interac-

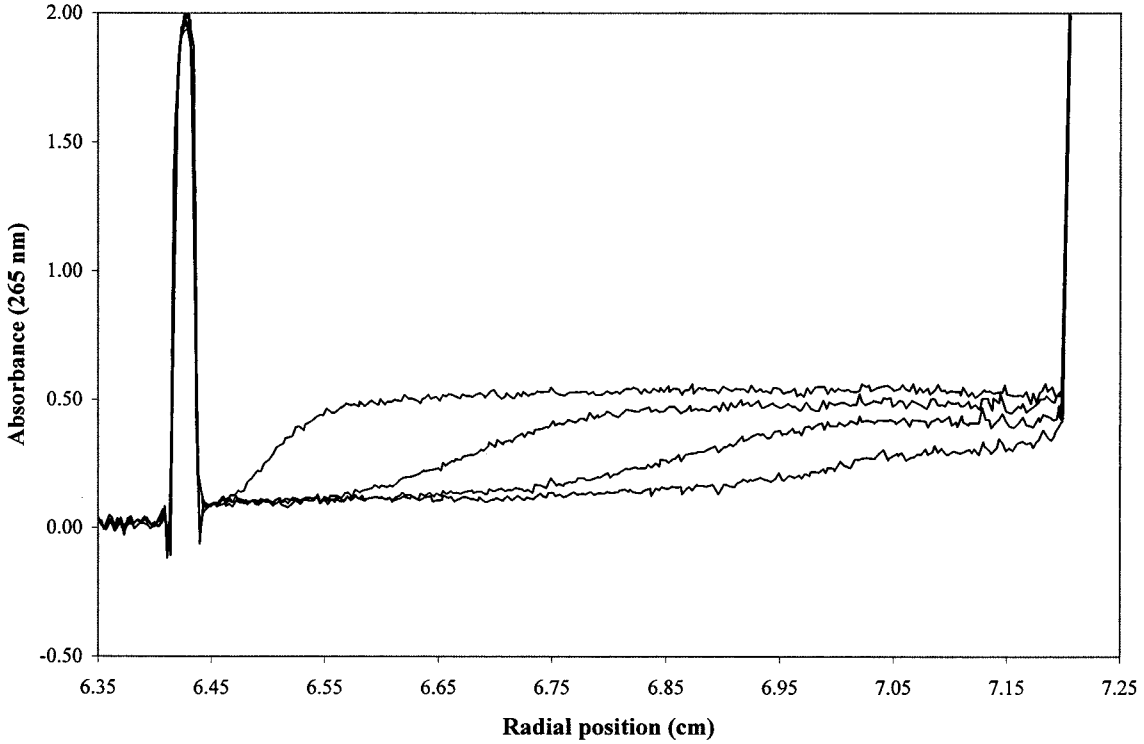

FIGURE 5: Sedimenting boundaries for the mucin−*mefp-1* complex. Rotor speed = 2000 rpm, temperature = 20.0 °C, scan interval = 10 min, concentration of mucin after mixing = 0.1 mg/mL, concentration of *mefp-1* after mixing = 0.4 mg/mL.

tions between it and other molecules can be studied. The use of lower pH conditions circumvents the cohesive behavior of *mefp-1*—the inevitable and irreversible aggregation at pH >7 when the large net positive charge on the molecule becomes suppressed (*5*). Aggregation phenomena at physiological pH are thought to be driven by primarily two nonconformational features of *mefp-1* structure (*5*): (1) autooxidation of Dopa residues to quinones and eventually cross-links; and (2) decreased repulsion within and between *mefp-1* molecules resulting from the dissociation of protons from lysine and phenolic side chains. However, by defining conditions at which *mefp-1* is essentially a monomer, as we have evidently shown, purely adsorptive interactions between it and other molecules can be studied. One such example, which illustrates this adhesive interaction well, and which is of potential significance in the field of drug delivery research for the development of mucoadhesives, is the interaction between *mefp-1* and a mucin glycoprotein of weight average molecular weight 9 million. Figure 5 shows the effect, in terms of increase of sedimentation rate, of adding pig gastric mucin glycoprotein at a concentration (after mixing with *mefp-1*) of 0.1 mg/mL (too small to give a significant UV absorption at 265 nm) to a solution of (after mixing with mucin) 0.4 mg/mL *mefp-1*. The increase is dramatic: native mucin by itself has an $s_{20,w}$ of 60 S, and the complex sediments at 7000 S with no residual *mefp-1* left. It is possible to estimate the size of the complex (too large to analyze by sedimentation equilibrium) by assuming a roughly spheroidal random coil conformation for both *mefp-1* and the mucin; we can use the Mark−Houwink−Kuhn−Sakurada relation [see (*30*)] of $s \sim M^{0.6}$ (0.6 chosen between the coil and sphere limits of 0.5 and 0.667, respectively) to estimate the size of the complex, and a molecular weight of $\sim 2.5 \times 10^{10}$ is predicted. It should be noted also, however, that the sedimenting boundaries are quite broad and strongly indicate considerable heterogeneity of the complexes formed.

The performance of *mefp*-1, a highly basic protein, as a mucoadhesive is thus broadly comparable to the polycationic polysaccharide chitosan which gave similar results (*12*). It is interesting to note that chitosan too appears to adopt a flexible rod conformation in solution (*31*) and this structure, together with their common cationic nature and high hydration, may be significant in the ability of these substances to adhere to surfaces, as considered in some detail for *mefp-1* by Baty et al. (*7*).

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