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Electrophoretic Mobilities of a Viral Capsid, Its Capsid Protein, and Their Relation to Viral Assembly

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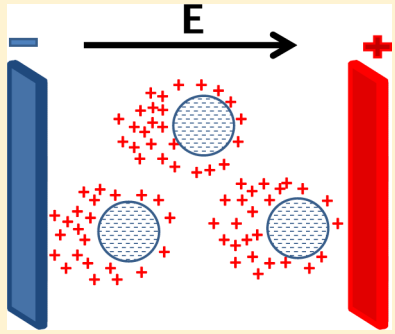
Supporting Information

ABSTRACT: The self-assembly of many viral capsids is dominated by protein−protein electrostatic interactions. To have a better understanding of this process, it is important to know how the protein and the capsid surface charges vary as a function of the pH and ionic strength. In this work, using phase analysis light scattering, we measured the electrophoretic mobility (EM) of the cowpea chlorotic mottle virus (CCMV), its capsid protein (CP), and a cleaved CP that lacks its basic terminus, as a function of pH and ionic strength. The EM measurements of the CP are difficult to carry out due to its tendency to self-assemble into capsids; we show how to circumvent this problem by appropriately changing the CP concentration. We found that the isoelectric points (pIs) of the virion and of the CP are insensitive to ionic strength. The onset of multishell structures in the phase diagram of the CCMV CP as a function of ionic strength and pH (and its absence in the brome mosaic virus (BMV) CP phase diagram) can be related to the pI of the capsid. We propose that the transition from multiwall shells to nanotube structures is due to a change in the spontaneous curvature of the CP at its pI. A nonzero limit of the EM at high ionic strength is characteristic of a *soft colloid*, but a near identity of the EMs of empty capsids and those containing RNA indicates that the EM reflects only the charge distribution in the CP. The Henry equation has been used to provide approximate values of the capsid surface charge as a function of pH and *I*.



INTRODUCTION

The importance of electrostatic interactions in the assembly and stability of viruses has been established by numerous experimental and theoretical studies. A prime example is cowpea chlorotic mottle virus (CCMV), which can self-assemble *in vitro* from its pure components, allowing the effect of charge to be examined in experiments in which the electrostatic interactions can be changed by altering pH and ionic strength.[1,2] The CCMV capsid protein (CP) has a basic N-terminus, which interacts electrostatically with the single-stranded (ss) RNA phosphate backbone during virus self-assembly. Interactions *between* CPs are also of importance, as evidenced by the self-assembly of the CP alone into a variety of structures—empty capsids, multishells, and tubes—whose stability is controlled by the ionic strength and pH.[1,3−5]

While estimates of the charge on the CP can be made from a knowledge of the primary structure and the pK_as of the individual residues, it is difficult to account for the collective interactions within individual proteins and the distributions of ions that control screening. Some insight into these effects and how they are influenced by ionic strength and pH can be obtained from measurements of the solution electrophoretic mobility, EM, and such studies have been carried out for a number of viruses.[6−13] But unlike rigid charged colloids, for which the relation between EM and surface charge is relatively straightforward, viruses are "soft diffuse particles"[14] for which the EM is thought to depend on both the distribution of charge and the permeability with respect to ions and solvent. For example, the total charges of "solvent-accessible" residues computed at pH 7 from the crystal structures of viruses such as MS2, cucumber mosaic virus, and turnip crinkle virus are positive,[15] but their EMs at this pH are negative, suggesting a contribution from the encapsidated RNA to the effective virion charge. On the other hand, the EMs of empty CCMV capsids and those containing the 3000-nucleotide RNA are essentially identical,[6] the negative value consistent with the computed surface-accessible charge, which is negative.[15]

We show how EM studies offer opportunities for enhancing our understanding of the electrostatic interactions that control viral assembly. We focus on CCMV because it is one of the few viruses for which it is possible to examine not only the wild-type (wt) virion but also empty capsids, free CP, and CP from

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which the positively charged N-terminus has been cleaved, as functions of pH and ionic strength, allowing their contributions to the EM to be determined independently. But, as we show in discussing Brome mosaic virus (BMV), the principles are more generally applicable.

MATERIALS AND METHODS

CCMV was extracted from California black eye pea (*Vigna unguiculata*) plants and purified according to the methods described by Rao et al.[16] to a purity exceeding 99.5%; it was kept in virus buffer (0.1 M sodium acetate, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 4.8) until used. The CP, which was obtained by disassembly of the virus, was also purified according to Rao et al.[16] and was kept in a protein buffer (1 M NaCl, 20 mM Tris pH 7.2, 1 mM EDTA, 1 mM dithiothreitol (DTT), and 1 mM PMSF) in which it is stable and does not assemble. Chidlow and Tremaine[17] demonstrated that the N-terminal residues could be cleaved from CCMV CP by digestion with chymotrypsin. CP in protein buffer was treated with the enzyme at a 500:1 CP:chymotrypsin ratio for 24 h at 4 °C. MALDI-TOF mass spectrometery showed that 22 terminal residues were cleaved, which corresponds to a loss of all but one of the terminal basic residues (see Figure S1 in the Supporting Information).

EM measurements were carried out with a Zetasizer NanoZS (Malvern, U.K.), which uses phase analysis light scattering for EM determination. Samples were prepared by taking aliquots from virus or CP stock solutions and then equilibrating them by dialysis against a buffer at a given pH and ionic strength for more than 12 h, using a membrane cutoff of 8−12 kDa. For both the virion and the CP each EM data point is an average of 10 measurements.

The pH was varied from 3.2 to 7.4 using appropriate buffers (citrate buffer from pH 3.2 to 5.0, acetate buffer from pH 4.5 to 6.5, and phosphate buffer from pH 7 to 7.4), and the ionic strength was varied with NaCl. The buffers were prepared with autoclaved Milli-Q water and passed through a 0.1 μm filter. Prior to each measurement the Zetasizer cells were washed with water filtered through a 0.02 μm filter, until no particles could be detected in the light-scattering mode, assuring cleanliness of the cell. Virion EM measurements were made at I = 0.02, 0.1, 0.2, 0.4, and 1 M NaCl. The EM of the protein was measured only at ionic strengths of 0.02 and 0.2 M. The protein samples for EM measurements were used at concentrations as low as 0.04 mg/mL (1 μM CP dimers) at pH $\leq$ 5 and about 2−2.5 μM at higher pHs. The concentration of positive ions is negligible, ca. 10^{-5} M, due to Mg^{2+} associated to the virus itself. However, due to the presence of EDTA in the buffer before EM measurements, this concentration becomes even smaller; afterward, EDTA was diluted to about 1 μM by dialysis for EM measurements.

RESULTS AND DISCUSSION

The measured EMs of the virus over the pH range 3.2−7.5 for ionic strengths 0.02, 0.2, and 1.0 M are shown in Figure 1a. [To avoid clutter, measurements made at I = 0.1 and 0.4 M are shown only in the Supporting Information (Figure S2) along with the Bancroft et al. measurement at I = 0.1 M.[18]] The EMs are in good agreement with those determined by Johnson et al.[6] at I = 0.2 M, which are also shown in the figure. The EM decreases in magnitude with increasing ionic strength, but the isoelectric point, pI = 3.7, is independent of I.

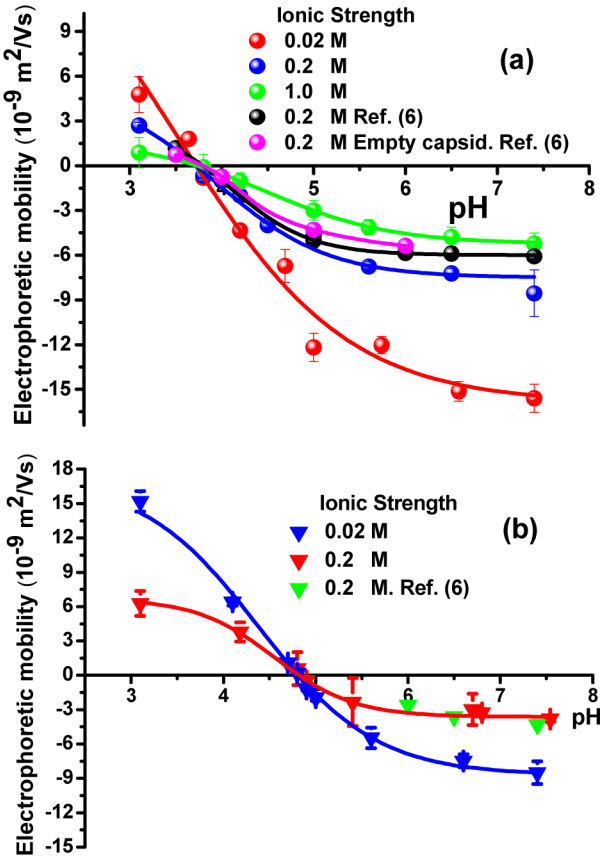

Figure 1. (a) EM for the wild-type (wt) CCMV virion at three different ionic strengths as a function of pH. We include data at I = 0.2 M from Johnson, et al.[6] The measured pI is 3.7. (b) CCMV capsid protein EM for I = 0.02 and 0.2. Johnson et al.[6] results are also in good agreement with our results for the same value of I. The curves are guides to the eye.

The EM of the CP was also examined by Johnson et al.,[6] but only at three values of pH at a single ionic strength. To extend the pH range, it is necessary to first determine the conditions under which the CP does not self-assemble. Adolph and Butler[19] examined the effect of total CCMV CP concentration on the fraction of assembly into single-wall capsids for three pHs at an ionic strength of 1.0 M and showed that the behavior was similar to that in micelle formation (what they termed quasi-crystallization), in which at a critical composition the protein dimer concentration plateaus while that of capsids rises (Figure 2a−c). Similar behavior was found by Johnson et al.;[20] they delineated the boundary between isolated dimers and the assembly of capsids in terms of a pseudocritical concentration $K_{D,apparent}$, the total protein concentration at which the concentrations of CP dimers and capsids are equal. The plot of ln $K_{D,apparent}$ against pH in Figure 2d shows the constants reported by them as well as values calculated from the measurements by Adolph and Butler. The line through the points can be represented well by

$$\ln K_{D,apparent} = -17.8 + 3.86\text{pH} \quad (1)$$

We have used these results as a guide to determining conditions for measuring the EM of the CP, e.g., identifying a dimer concentration of 1 μM below pH 5 for ionic strengths of 0.02 and 0.2 M. The data are shown in Figure 1b along with the three points measured by Johnson et al.[6] for the CP at I = 0.2

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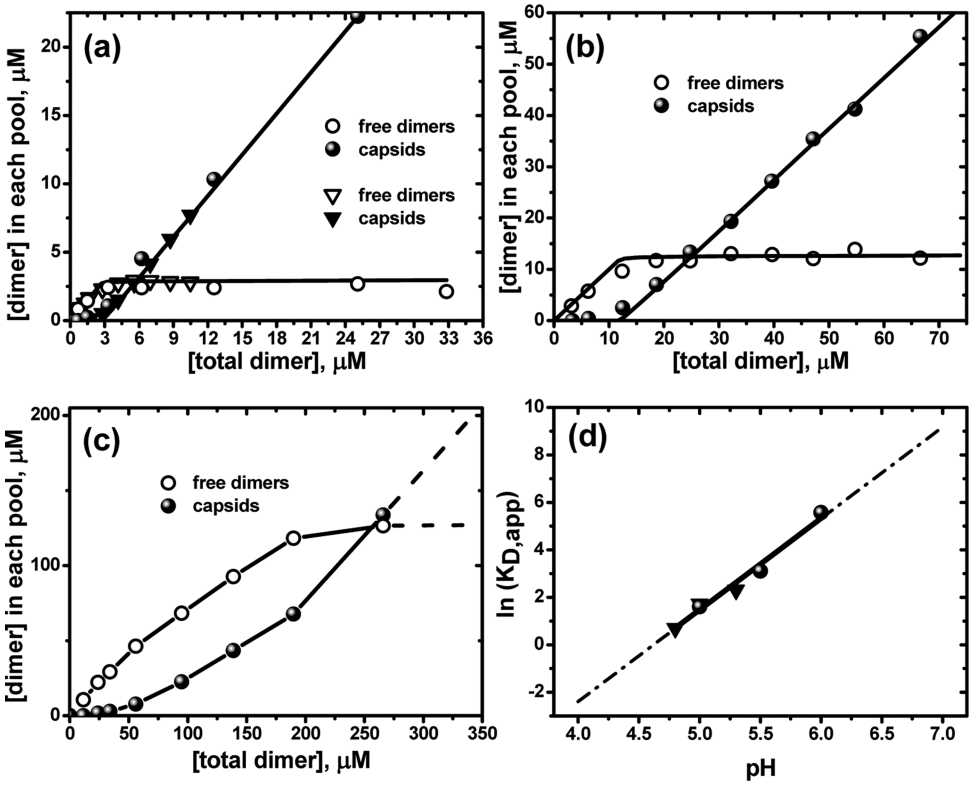

Figure 2. Equilibrium concentration of capsids and free CP dimers as a function of the total dimer concentration at pH (a) 5.0, (b) 5.5, and (c) 6.0. Figures adapted from Adolph and Butler[19] (circles) and Johnson et al.[20] (triangles) in panel a were obtained at I = 1.0 and 0.5 M, respectively. Panels b and c were obtained at I = 1.0. In panel d, ln $K_{D,apparent}$ vs pH was obtained from panels a–c and two additional data points at pH 4.8 and 5.3 from Johnson, et al.[20]

M; they agree well with our values. We speculate that no measurements were reported by them for values lower than pH 6 because the studies were carried out at relatively high concentrations for which the CP self-assembles.

As in the case of the virion, the EM of the CP decreases with increasing ionic strength. The pI is 4.8 for the protein, and, as in the case of the virus, it is insensitive to ionic strength. The basic residues at the N-terminus have high pK_as so that they remain charged throughout the pH range that we have studied. As a result, the EM of the cleaved CP lies below that of the intact molecule at the same I and the pI is shifted to lower pH, 4.1, consistent with the loss of the positively charged basic residues, as shown in Figure 3.

Relation between the EM and the Phase Behavior of the CP. The CCMV CP is able to self-assemble into empty capsids and a variety of other structures;[1−3,5,19] it can package by self-assembly its homologous RNAs, heterologous RNAs of a wide range of lengths,[21] and a wide range of other anionic species such as poly(styrene sulfonate).[22] The key factors controlling assembly are the CP−CP and CP−cargo interactions,[2,23] which in turn depend on the pH and ionic strength. This can be seen, for example, in the pH−I phase diagram for the CP.[1−3]

The variation of the EMs of the capsid and the CP with pH and I can provide insight into the protein phase diagram;[1,3,4] see Figure 4. At high pH, the electrostatic interactions between the negatively charged CPs overpower the largely hydrophobic interactions between CPs that stabilize the capsid. Hence, assembly of the CP into empty capsids is favored by lowering the pH and raising the ionic strength, and indeed, Tang et al.[24] observed no assembly of empty capsids at pH > 5.75; assembly

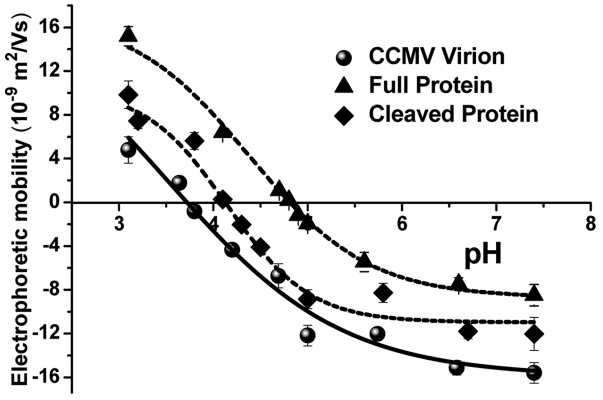

Figure 3. Comparison of the electrophoretic mobilities of the CCMV virion, the CP, and the cleaved CP as a function of pH at I = 0.02. The lines are guides to the eye.

does take place at pH 5.25, but at pH 4.75 it is very rapid, resulting in kinetic traps. The EM measurements show that pH = 4.75 corresponds to the CP pI, the point at which the repulsions are minimal and at which protein aggregates.[25]

At pH 3.7 at low ionic strength, concentric multishell structures—in which multiple CP layers form around the empty capsid—begin to form. Prinsen et al.[5] showed theoretically that this threshold behavior was associated with the electrostatic interaction between the positively charged protein tail and the negatively charged capsid surface. At low ionic strength, the multishell region ends at about pH 4.8 and tube structures become dominant. This pH corresponds to the isoelectric point of the CP, suggesting that the corresponding

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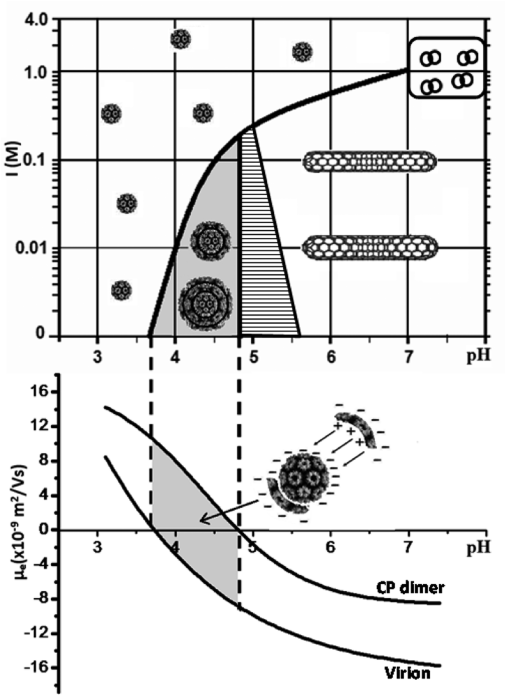

Figure 4. Schematic representation of the phase diagram of the CP of CCMV (upper panel), showing the different regions associated with the dominant polymorphs observed—such as single-wall capsids, multiwall capsids, tubes, and free protein—and its relation to the electrophoretic mobilities (or surface charges) of both the CCMV capsid and the CP dimer (lower panel).

increase in charge asymmetry in the CP leads to an increase in its spontaneous curvature[26] and a destabilization of pentamers in favor of hexamers with formation of tubes composed of hexamers, as shown schematically in Figure 4. However, the transition from spherical shells to tubes might be gradual and a coexistence region may exist (hatched area in Figure 4) containing single-shell and multishell structures with tubes and multiwall tubes, as has been shown experimentally.[1−4]

It is instructive to compare the electrophoretic mobilities of BMV and CCMV whose CPs are quite similar. Like CCMV, BMV CP has a basic N-terminus but with one fewer positive charge. The proteins are essentially identical in length and have a 70% sequence homology. Yet the phase diagram of BMV CP[27] is markedly less rich than that of CCMV CP; the only well-defined species are empty capsids and native protein dimers. The charge on the solvent-accessible residues of BMV specified in VIPER is +1020, compared to −120 for CCMV;[15] similarly, measurements of the EM of BMV by Johnson et al.[6] showed that it is more basic than CCMV, with a pI of about 4.5, and that the EMs of the virion and empty capsids were closely similar. An even higher pI of 7.9 was reported for the virion by Bockstahler and Kaesberg in earlier work.[28] The situation can be clarified easily by agarose gel electrophoresis because the sizes of BMV and CCMV capsids are closely similar; hence, their relative displacements in gels are proportional only to their charges. We find (Supporting Information, Figure S3) that, at pH 5.5 and I = 50 mM, CCMV moves toward the positive electrode and BMV moves half as far but toward the *negative* electrode; i.e., for BMV this pH lies below the pI. At pH 7, BMV is also positively charged but has a smaller displacement relative to CCMV, consistent with results reported by Bockstahler and Kaesberg. The absence of multishell structures in BMV is then understandable in the light of the EMs. While the N-termini remain positively charged over a wide pH range, the positive surface charge of the capsid precludes the stabilization of additional shells by electrostatic interactions.

In addition to the CP−CP interaction, the CP−RNA interaction comes into play in assembly around RNA. The electrostatic attraction between the phosphate backbone and the basic N-terminus of the CP is essentially pH-independent because of the high pK_as of the basic residues: hence, CP−RNA binding is favored by low ionic strength. The canonical assembly conditions[2,21] are first equilibration at low ionic strength of the CP and RNA at neutral pH, followed by dialysis to pH 4.8. As seen from the drop in the EM, lowering the pH to 4.8 reduces the electrostatic repulsions between the CPs and allows the hydrophobic interaction between them become effective in assembling the capsid; the binding to the RNA inhibits possible isoelectric aggregation of the CP.

Relation between EM and Charge Distribution. The close similarity between the EM of the empty shells and the virion was noted by Johnson et al.,[6] who took it as evidence that the mobility was controlled solely by the surface charge density, uninfluenced by the presence of the RNA. They also surmised that the interior of the capsid contained a higher ratio of basic to acidic residues, as is now known to be the case from structural studies.[29] This conclusion is also supported by a comparison between the EMs of the cleaved protein and that of the virion at the same ionic strength; Figure 3 shows that they are close, with the somewhat higher pI for the cleaved protein consistent with the fact that one basic terminal residue remains.

A clear understanding of the relative contributions to the EM of the internal to the external charge requires a consideration of what have been termed "diffuse soft particles", in which the charge is distributed over a permeable polymeric layer.[30] A key parameter is $1/\lambda$, the hydrodynamic penetration length. When $\lambda \rightarrow \infty$, the EM is that of a rigid (hard) particle with a fixed surface charge, the situation considered the treatment by Henry,[31] and for which the charge density can be determined. A test of the appropriateness of this limit is to examine the dependence of the EM as a function of the ionic strength. In the high-λ limit, i.e., for a hard colloid, the limiting EM at high ionic strength goes to zero; a nonzero limit indicates hydrodynamic permeability. A plot against I of the measured EM values for CCMV at pH 7.4, Figure 5, shows that in the limit of high I there is a nonzero plateau, suggesting "soft colloid" behavior.

The only detailed analysis of the EM of a virus as a soft colloid was undertaken by Duval and collaborators for MS2.[10,11] Their electrohydrodynamic treatment includes a three-shell modeling of the distribution of the viral protein and RNA based on the structure as determined by cryoelectron microscopy and X-ray diffraction. It is further complicated by the need to go beyond the linearized Poisson−Boltzmann model to describe nonlinear ionic concentration jumps in regions close to the CP, which has been shown to be necessary by rigorous analyses of the electrostatic properties of viruses.[32,33] A comparison between measurements of the EM of MS2 as a function of pH and theoretical values obtained using parameters derived from the ionic strength dependence at pH 7[10] shows fair to good agreement only for pHs greater than 5. It is suggested that the failure is a result of the tendency of MS2 to aggregate at low pH. In contrast to CCMV, Dika et al.[11] found that the EM of nominally empty MS2 capsids is

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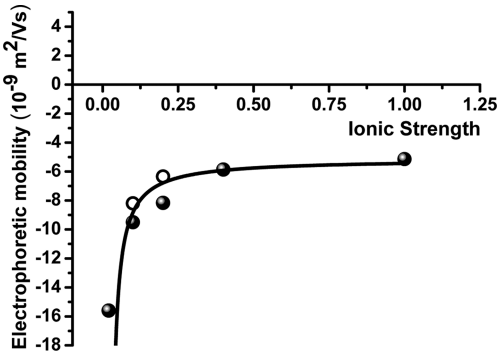

Figure 5. Electrophoretic mobility at pH 7.4 plotted against ionic strength. Included in the figure are two points (open circles) interpolated from the data of Johnson et al.[6] and Bancroft et al.[18] In the case of a charged hard colloid, the plateau at high ionic strength would occur at a mobility of zero.

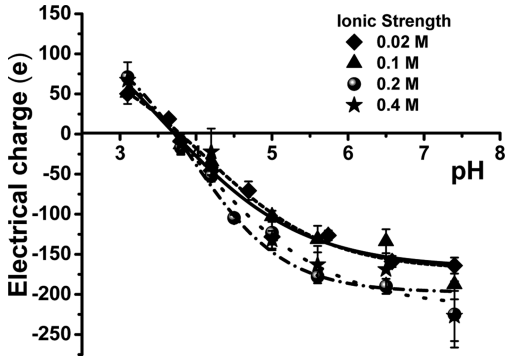

Figure 6. Electrical charges per virion calculated from eq 2 using the EM data from Figure 1a and Supporting Information Figure S2 (at I = 0.02−0.4 M). The lines are guides to the eye.

markedly different from that of the virion, consistent with the evidence that the capsid is permeable to electroosmotic flow. The analysis is complicated, however, by the tendency of MS2 to aggregate at low pH and the likely presence of host-derived RNAs in the "empty" capsids; further another comparison of the EMs of full and empty MS2 capsids[12] showed no difference over the pH range 3−10 for a broad range of ionic strengths.

The ambiguity in the measurements for MS2 makes it difficult to evaluate the validity of the extensive theoretical analysis. The dependence of the EM of CCMV on the charge distribution in the capsid protein layer, as evidenced by the close similarity of the EMs of the virion and empty capsids, suggests that its pH and ionic strength dependence might be described to first order by the Henry equation,[31] which is appropriate for a charged spherical colloid:

$$\mu_e = \frac{eq_o f(\kappa\alpha)}{4\pi\alpha\eta(\kappa\alpha + 1)} \quad (2)$$

Here μ_e is the electrophoretic mobility, e is the fundamental charge, q_o is the number of charges per particle, η is the viscosity of the medium, α is the particle radius, κ is the inverse Debye screening length, and $f(\kappa\alpha)$ is the Henry function, which can be approximated by

$$f(\kappa\alpha) = 1 - \frac{3}{\kappa\alpha} + \frac{25}{(\kappa\alpha)^2} - \frac{220}{(\kappa\alpha)^3} + \ldots \quad (3)$$

We can use eq 2 to estimate the capsid surface charge as a function of the pH for I = 0.02, 0.1, 0.2, and 0.4 M, as shown in Figure 6. At pH 7 there are about 170 negative charges per capsid, essentially one per protein. The binding of divalent ions to the capsid would be expected to reduce the charge, as observed by Bancroft et al.,[18] who reported that at pH 7 the EM of wild-type (wt) CCMV was increased from −7.1 to −6.0 in the presence of 0.01 Mg^{2+}. Note that at the conditions of our experiments there should be no residual binding of divalent ions to the capsid. Basu et al.[34] found that the dissociation constant, K_d, for the dissociation of Ca^{2+} from the CCMV capsid at pH 6.5 is 1.97 mM. In contrast, the concentration of the virion under the conditions of our experiments is of the order of 0.01 mM, ensuring essentially complete dissociation.

The value of charge estimated from the Henry equation can be compared with the 120 surface charges for CCMV at neutral pH in the VIPER database[13] based on the surface-exposed residues determined from the crystal structure. Another specification of the charged residues, made by Prinsen et al.,[5] led to an estimate of two charges per protein. Both of these estimates relied on the isolated pK_a values which can differ significantly from those in the protein environment. These different estimates point up the arbitrariness in relating capsid charge to crystallographically determined surface residues.

CONCLUSION

The ability to perform electrophoretic mobility measurements of wt CCMV, empty capsids, capsid protein, and capsid protein lacking its positively charged N-terminus provides detailed insight into the role that electrostatic interactions play in the pure protein phase diagram and the canonical conditions for assembly of the capsid protein around its genome and other cargo. While CCMV is unusual in the richness of its phase behavior, we can expect that the insights apply as well to closely related viruses such as hepatitis B, for which the path of capsid self-assembly also exhibits a micelle-like behavior[35] and for which a relation such as eq 1 applies, elucidating the conditions under which the isolated protein can be studied.

ASSOCIATED CONTENT

Supporting Information

Figure S1 displaying comparison of the MALDI-TOF spectrum of the wild-type CCMV CP and that of the N-terminal-cleaved CCMV CP, Figure S2 showing EM for the wild-type CCMV virion at I = 0.1 and 0.4 M, and Figure S3 displaying gel electrophoresis measurements of CCMV and BMV at pH 5.5 and 7.0. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Notes

The authors declare no competing financial interest.

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