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The disassembly, reassembly and stability of CCMV protein capsids

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Abstract

Efficient procedures are described for the disassembly of Cowpea Chlorotic Mottle Virus (CCMV) into its viral-RNA and capsid-protein components, the separation of the RNA and protein, and the reassembly of the purified protein into higher order nanoscale structures. These straightforward biochemical techniques result in high yield quantities of protein suitable for further biophysical studies (AFM, X-ray scattering, NMR, osmotic stress experiments, protein phase-diagram) and nanotechnology applications (protein enclosed nanoparticles, protein-lipid nanoemulsion droplets). Also discussed are solution conditions that affect the stability of the self-assembled protein structure and explicitly show that divalent cation is not required to obtain stable protein structures, while the presence of even small amounts of Ba^{2+} have a significant impact on protein self-assembly. However, since high ionic strength solution conditions result in good yields of CCMV-like protein capsids, it is suggested that the highly charged cationic protein N-terminus could act as an electrostatic switch for protein self-assembly and therefore be modulated by ionic strength and salt type. It was also found that $CaCl_2$/RNA precipitation methods do not yield sufficiently pure protein samples.

Keywords: Bromovirus; Viral protein purification; Virus disassembly; Virus reassembly; Protein nanoparticle assembly; Virus stability; Protein UV spectrum

1. Introduction

Cowpea Chlorotic Mottle Virus (CCMV) belongs to the Bromoviridae family (alphavirus-like superfamily; Universal Virus Database, 2004) which includes two other members, Broad Bean Mottle Virus (BBMV) and Brome Mosaic Virus (BMV). Bromoviruses have icosahedral ($T = 3$) nucleocapsids which are made of 180 identical capsid protein subunits (molecular weight $\sim 2 \times 10^4$ g mol^{-1}) clustered into 12 pentamers and 20 hexamers (Speir et al., 1995 and references therein). These 28 nm icosahedral virus particles encapsulate four, positive sense, single-stranded viral RNA molecules into three different virions of similar structure (Bancroft and Horne, 1977; Ahlquist, 1992; Speir et al., 1995). Another particularly interesting feature of wild-type CCMV assembly properties is the wide variety of polymorphic shapes that CCMV capsids can adopt depending on the chemical and physical conditions (Bancroft and Hiebert, 1967; Hiebert and Bancroft, 1969; Bancroft et al., 1968, 1969; Hiebert et al., 1968; Johnson and Speir, 1997). Thus, changes in ionic strength, pH or temperature can alter the virion morphology and its stability by presumably modifying the protein–protein and protein–RNA interactions when the genome is encapsulated inside the viral shell.

CCMV constitutes a model system for viral assembly studies since it was the first spherical virus to be reassembled *in vitro* into an infectious form from its wild-type purified components, i.e., the capsid protein dimers and the RNA genome (Bancroft and Hiebert, 1967; Hiebert and Bancroft, 1969; Bancroft et al., 1968, 1969; Hiebert et al., 1968). More recently, CCMV has been studied *in vitro* as a model system to gain a better understanding of the protein–protein and protein–RNA interactions governing the disassembly, reassembly and stability of the virus (Zhao et al., 1995; Fox et al., 1998; Douglas and Young, 1998).

Wild-type CCMV virions, that is with their viral RNA, are known to be stable between pH 3 and 6 and under low ionic strength ($I \sim 0.1$ M). They disassemble under high ionic strength ($I \sim 1$ M) and pH (above 7). CCMV virions are also known to radially swell around 10% when the ionic strength is low ($I < 0.1$ M) and the pH is raised above 7. Previous studies have shown that the swelling process is due to the radial expansion of the virion at the quasi-threefold axes. Ca^{2+} ions bound at these axes contribute to virion stability at low pH and ionic strength,

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and need to be removed for the transition to the swollen state to occur (Speir et al., 1995). Also, by lowering the pH below 5 and the ionic strength to 0.1–0.2 M, disassembled wild-type CCMV capsid proteins can reassemble into $T = 3$ empty particles of similar structure which are devoid of RNA (Zhao et al., 1995; Fox et al., 1998).

The aim of this paper is to describe efficient procedures leading to reproducible formation of protein-only capsids from disassembled CCMV virions, and to determine the stability of these reassembled capsids under different pH and ionic strength conditions. Sample preparations based on this work are being used to further characterize the physical properties of CCMV by AFM, X-ray scattering, NMR, and osmotic stress experiments; to fully determine the capsid-protein phase-diagram; for various nano-application studies to develop novel protein-only nanoparticles that have the potential to carry internal payloads; and to create CCMV capsid protein-lipid nanoemulsion droplets. The contents of this paper should therefore be of particular interest to non-virology laboratories in need of Bromovirus samples for biophysical studies and nanotechnology applications.

2. Materials and methods

2.1. UV measurement

Virus concentration and purity were determined by measuring the UV-absorbance at 260 nm and 280 nm in a 1 cm quartz cell with a Hewlett-Packard 8453 spectrometer. The reference spectra of the corresponding buffers were subtracted from the UV-spectra for CCMV in this buffer.

2.2. Electron microscopy (EM)

Electron micrographs were obtained from CCMV samples in various aqueous buffers in the concentration range of 0.1–0.5 mg ml^{-1}. Carbon-coated, parlodion support films mounted on copper grids were made hydrophilic immediately before use by high-voltage, alternating current glow-discharge. 5 μl CCMV samples were applied directly onto grids and allowed to adhere for 2.5 min. Grids were rinsed with 3 drops of distilled water, negatively stained with 1% uranyl acetate for 30 s and air-dried. Specimens were examined in a Hitachi H-7000 electron microscope at an accelerating voltage of 75 kV. Images were recorded on Kodak electron microscope film 4489. All the scale bars in the electron micrograph figures represent 28 nm (which is the diameter for normal CCMV virions).

2.3. CCMV purification by ultrafiltration

The protocols for plant infection, leaf harvest, and CCMV purification by ultrafiltration are described in a previous paper (Michel et al., 2004). Purified CCMV was stored at −80 °C in *virus buffer* (0.1 M sodium acetate, 1 mM EDTA, pH 4.8). Fig. 1a is a typical electron micrograph showing intact, spherical and uniform size virions. Fig. 1b is the corresponding UV profile of the CCMV virion solution. The UV spectrum is dominated by the nucleic acid absorption maximum located at 260 nm, and

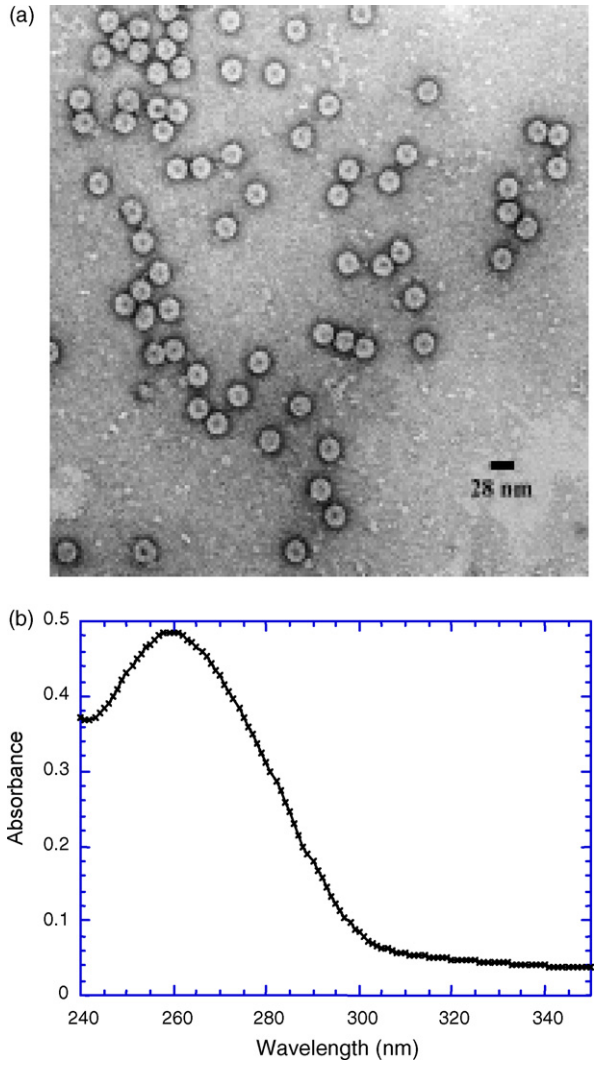

Fig. 1. (a) Electron micrograph and (b) UV profile of a sample of CCMV in virus buffer (0.1 M sodium acetate, 1 mM EDTA, pH 4.8).

the Abs_{260}/Abs_{280} ratio is equal to 1.6 indicating pure CCMV (Michel et al., 2004).

2.4. Disassembly of purified CCMV, and the separation of capsid protein and viral RNA

The CCMV disassembly procedure is based on a previously described method (Bancroft et al., 1967) where CCMV disassembles into separate capsid protein dimers and RNA (under high salt conditions and above pH 7). However, the disassembly buffer used in this study also contains DDT (Dithiothreitol or Clealand's reagent) to prevent the formation of disulfide bonds and PMSF (phenylmethylsulfonylfluoride) to inhibit proteases. All procedures in this paper used sterile, filtered, double-distilled H_2O; clean, sterile glassware; and nuclease- and protease-free pipet tips, tubes and gloves. Unless stated otherwise, all salts were sodium salts (e.g., sodium EDTA).

Approximately 3 ml of a 2 mg ml^{-1} stock of CCMV particles (in virus buffer) was dialyzed overnight at 4 °C against 1 l of *disassembly buffer* (0.9 M NaCl, 0.02 M Tris–HCl pH 7.4,

1 mM DTT, 0.5 mM PMSF). A Slide-A-Lyser dialysis cassette (Pierce Biotechnology Inc., Rockford, IL) with a 3.5 kDa molecular weight cut-off was used, which effectively retains CCMV capsid protein monomers (20 kDa).

The RNA–protein mixture obtained was then diluted at least two times (using disassembly buffer) and centrifuged at 4 °C in a swinging bucket rotor (Beckman rotor SW41) at 99,000 × *g* for 20 h. The upper 3/4 of the supernatant, containing the purified protein, was then collected. The lower 1/4 volume was discarded, while the yellowish RNA pellet located at the bottom of the centrifuge tube was resuspended in a neutral pH buffer with no divalent cations (to avoid hydrolysis) and stored at −80 °C.

Concentration of the purified protein (∼7 ml) was done by centrifugation at 4 °C in a Centriplus YM-3 filter (Millipore Corp., Billerica, Massachusetts) for 2 h at 3000 × *g* (nominal molecular weight limit cut-off of 3.0 kDa). Centriplus filters are compatible with any bench-top centrifuge. Purified, disassembled capsid protein was stored at 4 °C in disassembly buffer.

2.5. Protein reassembly

Using disassembled, purified capsid protein, several protein-only reassembly reactions were performed under different solution conditions (reassembly buffers of different ionic strength, pH, and the presence or absence of divalent cations). All reassembly reactions were done by overnight dialysis at 4 °C of capsid protein solutions (concentrations of 0.1–0.5 mg ml^{-1}) against a reassembly buffer, in Slide-A-Lyser dialysis cassettes (3.5 kDa molecular weight cut-off) (Pierce Biotechnology Inc.). The resulting protein structures were then analyzed by EM, and are discussed in Section 3.2.

3. Results

3.1. Disassembly of purified CCMV, and the separation of capsid protein and viral RNA

Fig. 2 shows the UV spectrum of the purified and concentrated capsid protein with a maximum at 277 nm. The UV spectrum is characteristic of proteins containing tryptophan, tyrosine, and phenylalanine with combined maxima around 280 nm. The CCMV capsid protein has 190 amino acid residues of which 3 are tryptophan, 5 are tyrosine, and 4 are phenylalanine. The shoulder at 290 nm is characteristic of tryptophan (Schuler et al., 2002).

The ratio Abs_{280}/Abs_{260} was used to determine the amount of nucleic acid contamination, Bancroft (1970) and others have used 1.5 as representative of 'pure' CCMV capsid protein. Here, $Abs_{280}/Abs_{260} = 1.52$, with typical results between 1.5 (∼0.5% nucleic acid contamination) and 1.6 (∼0.25% nucleic acid contamination).

The concentration of the pure protein solution was determined using the Beer–Lambert relationship, $Abs_{280} = \varepsilon_{280} CL$, where C is the protein concentration in mol l^{-1}and L the pathlength of the cuvette (1 cm). ε_{280} is the calculated extinction coefficient for the CCMV protein *monomer* and is equal to 23,590 M^{-1} cm^{-1}. For the sample shown in Fig. 2, $C = 12.52$ μmol l^{-1}, or using the protein monomer molecular weight (∼20,000 g mol^{-1}) the protein concentration is 0.25 mg ml^{-1}.

3.2. Protein reassembly

3.2.1. Protein reassembly at pH 4.8 in high salt with Mg^{2+} present

Fig. 3 shows a sample of reassembled CCMV-like capsids (single-layer, spherical protein-capsids approximately 28 nm in diameter) which are devoid of RNA (dark, negative stained center). Analysis of several electron micrographs showed very few non-CCMV-like protein structures. This solution condition is very suitable for producing spherical protein-only nano-particles that have the potential to carry internal payloads of up to ∼10 nm in diameter.

3.2.2. Protein reassembly at pH 4.8 in high salt with no divalent cation present

Fig. 4 shows that CCMV capsid protein can reassemble in the explicit absence of divalent cation (extensive dialysis with a

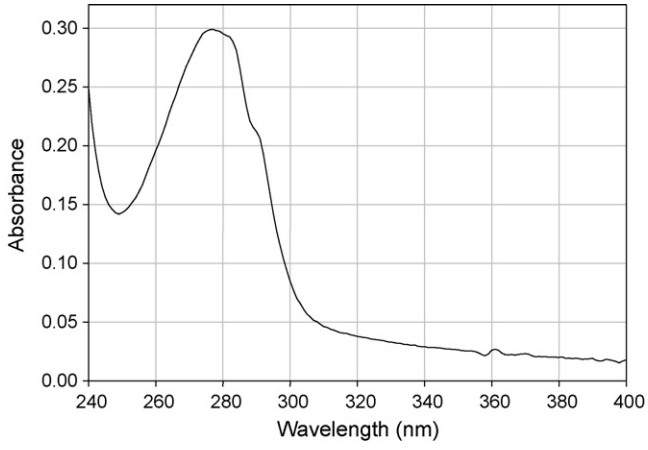

Fig. 2. Typical UV profile of purified and disassembled CCMV protein in disassembly buffer. The blank measurement was made with disassembly buffer.

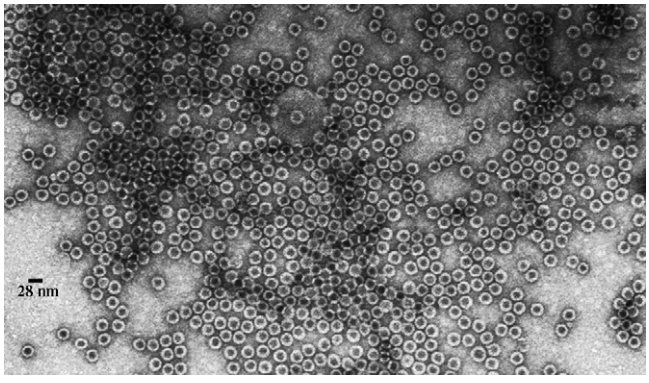

Fig. 3. Electron micrograph of reassembled CCMV capsids (spherical single-layer shells, intact and of uniform size (28 nm diameter)) which are devoid of RNA (dark negative stained center), under high salt conditions with Mg^{2+} present at pH 4.8 (0.9 M NaCl, 0.1 M sodium acetate pH 4.8, 10 mM $MgCl_2$, 0.5 mM PMSF).

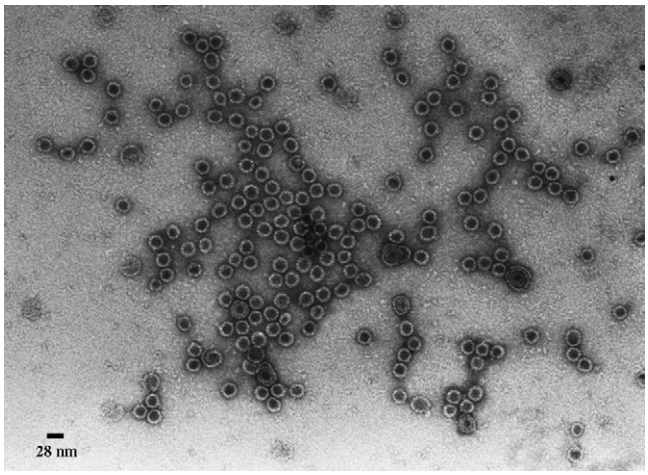

Fig. 4. Electron micrograph of protein reassembly done at pH 4.8 in high salt with no divalent cation present (0.9 M NaCl, 0.1 M sodium acetate pH 4.8, 10 mM EDTA, 0.5 mM PMSF).

buffer that has no divalent cation *and* the presence of EDTA to chelate any divalent cation that may be present). The majority of capsids are CCMV-like (spherical, single-layer shells, uniform in size and 28 nm in diameter).

However, also present are some non-uniform particles: single- and multi-layered spheres larger than 28 nm diameter and incompletely-closed, single-layer capsids. This suggests that the reassembly process for forming uniform CCMV-like capsids, at pH 4.8 in high salt, is more efficient in the presence of divalent cations.

3.2.3. Protein reassembly at pH 4.8 in high salt with Mg^{2+} and DTT present

This reassembly condition shows intact CCMV-like capsids and partially-assembled particles of similar size (Supplementary Fig. 1). No multi-shell or particles of different sizes were observed. Although the presence of the reducing agent DDT facilitates preservation of the disassembled protein stock solution, the efficiency of CCMV-like capsid reassembly is lowered in the presence of DTT (see Fig. 3 for comparison). This suggests that a reducing solution condition and/or a more complex reassembly condition (i.e., the presence of additional solutes) inhibits *in vitro* formation of intact CCMV-like capsids in the absence of its viral RNA.

3.2.4. Protein reassembly at pH 4.8 in low salt both with and without Mg^{2+}

Reassembly of intact CCMV-like capsids performed at pH 4.8 in low salt conditions, both with (Supplementary Fig. 2a) or without (Supplementary Fig. 2b) Mg^{2+}, forms a mixture of assembly products, particularly in the absence of Mg^{2+}. Many of the formed particles are aberrant with partially assembled shells, multi-layered spherical structures, and single-layer, spherical structures smaller than 28 nm in diameter. The number of intact CCMV-like protein capsids is much lower than that obtained in high salt conditions (more than one order of magnitude). This result highlights the inefficiency of protein reassembly in low salt conditions (in the absence of viral RNA), regardless of the presence of Mg^{2+}.

3.2.5. Protein reassembly at pH 4.8 in high salt with Ba^{2+} present

Ba^{2+} is a poor divalent cation substitute for Mg^{2+}, as the yield of intact CCMV-like capsids is lower (Supplementary Fig. 3) than the same reassembly conditions with Mg^{2+} (Fig. 3), and the number of partially-assembled capsids is higher.

3.3. pH stability dependence for pre-formed CCMV-like protein capsids

Since the stability and mechanical properties of CCMV-like protein capsids depend on ionic strength and pH, capsid samples at different ionic strengths and pH's were prepared for use in AFM, X-ray scattering, and NMR experiments. Supplementary Fig. 4 shows the effects of dialyzing a sample of intact, well-shaped, stable capsids (see Section 3.2.1 and Fig. 3, pH 4.8 in high salt with Mg^{2+} present) into various buffer conditions (electron micrographs in Supplementary Fig. 4 should be compared with Fig. 3).

Supplementary Fig. 4a shows mainly intact capsids and some partially disassembled capsids on lowering the Na^{+} concentration from 1 M to 0.1 M while keeping the pH constant at 4.8. The background in the electron micrograph suggests that protein debris is also present, which is consistent with disruption of some capsids. This shows that at pH 4.8 lowering the ionic strength by a factor of 10 reduces the stability of the empty capsids. This is also consistent with the low ionic strength reassembly results in Section 3.2.4.

Electron micrographs of protein capsids at higher pH's are shown in Supplementary Fig. 4b–e (at pH 5.5, 6.0, and 6.5, respectively, in 0.1 M sodium phosphate, 1 mM EDTA, 0.5 mM PMSF). As the pH is increased, less CCMV-like capsids are present, and in particular, as the pH is increased to 6.5, far fewer capsids are formed.

3.4. Native CCMV virions are stable at pH 7.5

Optimum stability of purified, RNA-containing CCMV virions is observed to be around pH 4.8. However, compared with the stability of the protein-only CCMV-like capsid-structures, RNA-containing CCMV is stable over a wider pH range. This is indicated by the presence of intact capsids at pH 7.5 (Supplementary Fig. 5), although the number of capsids is greatly reduced at pH 7.5, and the electron micrograph shows extensive debris from ruptured and disassembled CCMV.

4. Discussion

For CCMV capsid protein purification (see Section 2.4) it is important to collect the upper 3/4 volume immediately after the 20 h centrifugation run is completed; otherwise, some of the pelleted RNA will redissolve into solution and contaminate the purified protein. Also, if RNase contamination has occurred, small fragments of RNA will not pellet, and the sample should

Fig. 5. Positively charged residues (in bold) for the first 26 residues of the N-terminus: K (lysine, pK_a 10.53); R (arginine, pK_a 12.48).

be discarded. If minor RNA contamination is a problem, indicated by $1.3 < Abs_{280}/Abs_{260} < 1.5$, then the centrifugation can be redone at higher forces ($120{,}000 \times g$). In addition, when taking the UV spectrum of the purified protein, unless the blank with disassembly buffer is the exact same buffer used for the sample, the Abs_{280}/Abs_{260} ratio can be affected by slightly different amounts of DDT and PMSF. If there is difficulty in obtaining $Abs_{280}/Abs_{260} \geq 1.5$, then the disassembly buffer can be made without DDT and PMSF. However, the resulting stock of purified protein should be used within a week. Or once the purity of the protein has been confirmed (in the absence of DDT and PMSF), a final dialysis against disassembly buffer is suggested to maintain sample integrity.

A protein purification method (see Method C, Verduin, 1974) was also used where $CaCl_2$ precipitates the RNA. Using two identical CCMV samples and applying Method C (Verduin, 1974) and the protocol described in Section 2.4, the respective Abs_{280}/Abs_{260} ratios obtained were 1.3 and 1.5. Several attempts using the simpler $CaCl_2$/RNA precipitation method yielded less pure CCMV capsid protein.

The observation that CCMV-like protein capsids can reassemble in the strict absence of divalent cations (reassembly buffer with no divalent cation and with the divalent cation chelator EDTA) (Fig. 4), shows that the formation of protein-protein contacts in CCMV does not require a divalent cation. This requirement has been implied in previous studies stating that magnesium or calcium ions bound at the protein capsid three-fold axes are required to stabilize the structure (e.g., Speir et al., 1995). Bancroft's original work on the role of divalent cations in the assembly process of CCMV proteins at pH 5, in the absence of RNA (Hiebert and Bancroft, 1969; Bancroft, 1970), also showed that Mg^{2+} is not required for protein-only self-assembly. However, these studies (Hiebert and Bancroft, 1969; Bancroft, 1970) did not use buffers containing divalent cation chelators to ensure the removal of divalent cations.

Ionic interactions, however, do play a role since high NaCl concentrations (in the absence of viral RNA) are needed for good yields of CCMV-like protein capsids. Also, the presence of Ba^{2+}, even with high salt conditions, results in lower yields of CCMV-like protein capsids. It is not clear how 10 mM Ba^{2+} in the presence of 1 M Na^+ destabilizes the protein capsid.

The need for high ionic strength conditions to screen the highly positively charged N-terminus of the capsid protein monomer (when no negatively charged, viral RNA is present) does make sense. In other words, since the N-terminal 26 residues of the capsid protein have a net +10 charge (Fig. 5) and are known to interact with the viral RNA in the inner volume of the capsid (Speir et al., 1995), there are, in the absence of RNA, 1800 positive charges (+10 times 180 capsid proteins) that need to be screened to allow for protein-protein close-packing in the CCMV-like capsid shell. This study, however, has demonstrated that pre-formed CCMV protein-only capsids are stable up to pH 6.5 under low salt conditions (Supplementary Fig. 4d and e).

Table 1
Calculated net charge of the first 26 residues of the CCMV capsid protein N-terminus as a function of pH

pH	Charge
3.0	10.2
4.0	10.0
5.0	10.0
6.0	10.0
7.0	10.0
8.0	10.0
8.5	9.9
9.0	9.7

Since the N-terminus has a net charge of +10 over the pH range 3–9 (Table 1), the CCMV structural changes that are known to occur over the pH range 5–7 (Bancroft et al., 1967; Speir et al., 1995) (more specifically over the pH range 6.5–7.5, Vriend et al., 1982), cannot be due to charge density changes of the protein N-terminus. Thus, the structural changes that occur in CCMV as a function of pH are not driven by the N-terminus. However, as a function of ionic strength and type of salt, the +10 N-terminus may have the potential to act as an electrostatic switch for CCMV's structural changes. Future studies on the pH dependence of CCMV's structural changes and CCMV's stability and the role of the N-terminus need to address solution conditions other than pH.

Given that CCMV's optimum stability is around pH 4.8, the observation in this study that CCMV is stable up to pH 7.5 (Supplementary Fig. 5) is a potentially useful result. It also demonstrates that encapsidated viral RNA imparts additional stability to virions.

Clearly, the range of solution conditions that allow for interesting protein nano-scale structures is wide. Samples from this study have already been used for physical characterization of reassembled CCMV protein capsids (Michel et al., 2006; Klug et al., 2006), and the phase diagram of CCMV's capsid protein is being studied currently.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jviromet.2007.07.020.

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