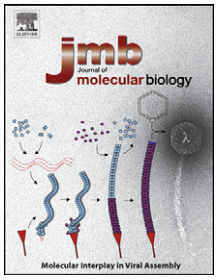

The Assembly Pathway of an Icosahedral Single-Stranded RNA Virus Depends on the Strength of Inter-Subunit Attractions

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Abstract

The strength of attraction between capsid proteins (CPs) of cowpea chlorotic mottle virus (CCMV) is controlled by the solution pH. Additionally, the strength of attraction between CP and the single-stranded RNA viral genome is controlled by ionic strength. By exploiting these properties, we are able to control and monitor the *in vitro* co-assembly of CCMV CP and single-stranded RNA as a function of the strength of CP–CP and CP–RNA attractions. Using the techniques of velocity sedimentation and electron microscopy, we find that the successful assembly of nuclease-resistant virus-like particles (VLPs) depends delicately on the strength of CP–CP attraction relative to CP–RNA attraction. If the attractions are too weak, the capsid cannot form; if they are too strong, the assembly suffers from kinetic traps. Separating the process into two steps—by first turning on CP–RNA attraction and then turning on CP–CP attraction—allows for the assembly of well-formed VLPs under a wide range of attraction strengths. These observations establish a protocol for the efficient *in vitro* assembly of CCMV VLPs and suggest potential strategies that the virus may employ *in vivo*.

Introduction

The architecture of viruses is beautifully simple. For many single-stranded RNA (ssRNA) genome plant viruses, the virion consists of only two structural elements: the RNA carrying the genetic information, and the capsid protein (CP), many copies of which form an icosahedrally symmetric protective shell (the capsid) around the genetic cargo. During its life cycle, the virus particle must be both stable enough to protect its genome from degradation along the treacherous journey between hosts and labile enough to disassemble inside the host cell and release its genetic contents. Additionally (and remarkably), many viruses are also capable of spontaneous self-assembly. These characteristics are made possible by a delicate balance of attractive and repulsive forces that has evolved among the viral structural units. The primary interactions within the virus particle are those between neighboring protein molecules forming the capsid (CP–CP) and those between CP and nucleic acid (CP–RNA). The present work focuses on the interplay between these key interactions and their effect on the *in vitro* assembly pathway of the plant RNA virus, cowpea chlorotic mottle virus (CCMV).

The CCMV virion consists of ~3000 nt of ssRNA packaged within a 28-nm-diameter capsid made up of 180 chemically identical copies of a 190-residue CP. We chose to study CCMV assembly for three reasons. First, CCMV is among the most robust viral self-assembly systems—it was the first icosahedral virus to be reconstituted *in vitro* from purified components [1], and CCMV CP has since been shown to package a wide range of heterologous RNAs [2], in addition to being capable of assembling into empty capsids [3,4]. Second, high-resolution reconstructions of the virion obtained by X-ray crystallography and cryo-electron microscopy (cryo-EM) [5], in conjunction with computational simulations [6], have yielded great insight into the specific molecular interactions that govern the structure, stability, and dynamic properties

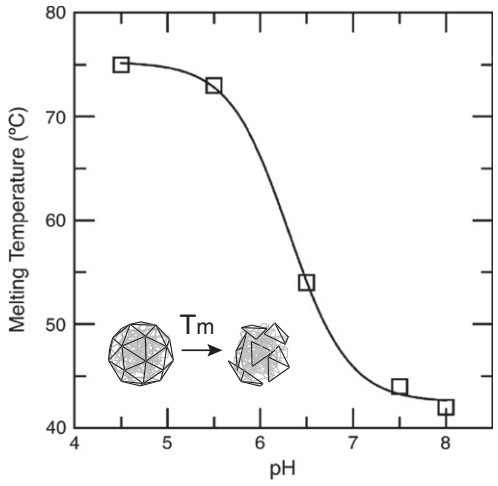

Fig. 1. Strength of CP–CP attraction is pH dependent. The apparent melting temperature of wt CCMV decreases sharply as a function of pH at $I = 0.1$ M.

of the virion. Third, and most importantly, the strength of the CP–CP and CP–RNA interactions can be controlled by adjusting the pH and ionic strength (I), respectively [7,8]. This last point, which we exploit heavily in the present work, is described in detail in the following sections.

While much is known about the structure and intermolecular forces within the CCMV virion, the pathway of assembly remains poorly understood. In an influential paper by Zlotnick *et al.*, empty capsids of CCMV CP were shown to assemble, under acidic conditions and in the absence of RNA, by first generating a pentamer of CP dimers (the nucleation phase) followed by the stepwise association of additional CP dimers (the growth phase) until capsid completion [9]. To provide insight into the assembly pathway of RNA-containing CCMV virus-like particles (VLPs), Zlotnick *et al.* carried out further studies, involving RNA and a limiting amount of CP under physiological (neutral) pH conditions, in which they demonstrated that CP initially binds RNA with low cooperativity due to the relatively weak lateral attraction between CPs [10].

Expanding on this work, we previously investigated the co-assembly of ssRNAs of varying lengths and an excess of CCMV CP [2,11]. We reported evidence that the assembly of CCMV CP around ssRNA followed a two-step, pH-dependent, mechanism in which CP reversibly binds RNA at neutral pH to form a pre-capsid complex that, upon acidification, undergoes an irreversible reorganization into the final icosahedral capsid [11].

In the present work, we study the pathway of assembly of CCMV CP and brome mosaic virus (BMV) RNA 1, a 3200-nt heterologous RNA shown to be efficiently packaged by CCMV CP [2,11]. We choose to work with heterologous RNA in order to focus on generic features of viral assembly and avoid specific interactions that may exist between CCMV CP and CCMV RNA—specific interactions cannot be completely ruled out, however, due to the high degree of sequence homology between CCMV and BMV RNA. We monitor assembly by a combination of electron microscopy (EM) and velocity sedimentation. By controlling the relative strength of the CP–CP and CP–RNA interactions through the pH and ionic strength of the assembly buffer, we are able to characterize the structures of disordered intermediates and kinetically trapped assembly products, as well as elucidate key features of the final capsid reorganization.

Results and Discussion

CP–CP interactions

Knowledge on CP–CP interactions in CCMV has come from decades of biophysical theory and experiment [8,12,13] and, more recently, from a high-resolution structural determination of the CCMV virion [5] and subsequent computational studies [6].

The CP exists in solution as a dimer [8] stabilized by a strong non-covalent clamp between the amino- and carboxy-terminal arms of each monomer [5]. The dimer serves as the fundamental assembling unit [8]. Lateral interactions between dimers within the capsid arise from a combination of hydrophobic, electrostatic, and divalent metal-mediated forces between amino acid residues that line the dimer–dimer interfaces. The average strength of attraction between CP dimers has been shown to be pH dependent, decreasing sharply with increasing pH [13]. This is primarily due to electrostatic repulsions that are generated by the deprotonation of adjacent acidic residues at neutral pH, in the absence of divalent metal ions [6]. This effective decrease in lateral attraction is illustrated by the decrease in thermal stability of wild-type (wt) CCMV at elevated pH values (Fig. 1). Here we see that the virion is stable to 75 °C at pH 4.5 and $I = 0.1$ M, but a sharp drop of 30 °C in melting temperature occurs upon raising the pH above 6.

CP–RNA interaction

CP interacts with RNA primarily through a disordered, highly basic, N-terminal arginine-rich motif (ARM). Studies exploiting truncated CP lacking this highly basic region have shown that RNA packaging requires the ARM [14,15]. While sequence-specific interactions have been shown to play a role in the binding of CP to RNA—both for CCMV [16] and other icosahedral plant viruses [17–20]—the predominant interaction in the case of CCMV is non-specific