Characterization of a Disassembly Deficient Mutant of Cowpea Chlorotic Mottle Virus

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An understanding of virus disassembly requires a detailed understanding of the protein–protein and protein–nucleic acid interactions which stabilize the virion. We have characterized a mutant of cowpea chlorotic mottle virus [cpR26C (coat protein R26C)] that displays increased virion stability and is abnormal in virion disassembly when purified under nonreducing conditions. Reduced virions are infectious, whereas nonreduced virions are noninfectious. The cpR26C mutant virions purified under nonreducing conditions resist disassembly in 0.5 M CaCl₂, pH 7.5. The nonreduced cpR26C mutant virions swell in neutral pH conditions (pH 7.5) but do not disassociate when the ionic strength is increased. In contrast, wild-type virions or cpR26C mutant virions isolated under reducing conditions completely disassociate into the RNA and capsid protein components at pH 7.5 and high ionic strength (i > 1.0). Sequence analysis of the cpR26C mutant identified a single C to U nucleotide change at position 1435 of RNA 3 (position 86 of RNA 4), which results in an arginine to cysteine change at position 26 of the coat protein. The cpR26C mutant provides an ideal chemical switch for examining virion assembly and disassembly.

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The bromoviruses, and cowpea chlorotic mottle virus (CCMV) in particular, provide a model system for examining the protein–protein and protein–RNA interactions dictatingicosahedral virus assembly, stability, and disassembly (1, 2). Bromoviruses are members of the Bromoviridae virus family (alphavirus-like superfamily). The 28-nm icosahedral virus particles encapsidate four (+) single-stranded viral RNA's (vRNAs) (for reviews see (3–5)). The RNA genome is packaged separately into three virions, all with similar or identical capsid structures. RNA 1 and RNA 2, which encode for proteins involved in RNA-dependent RNA replication, are each packaged in separate virions. RNA 3 (a mRNA for the 32-kDa viral movement protein) and RNA 4 (a subgenomic RNA expressed from RNA 3 which serves as a mRNA for the 20-kDa coat protein) are copackaged into a third virion in an approximate 1:1 molar ratio (6).

In vitro assembly studies of CCMV have established that changes in ionic strength and pH alter the stability of the virions and influence virion morphology, presumably by altering RNA–protein and protein–protein interactions (7–13). CCMV virions are stable at pH < 6.0 and low ionic strength (i < 0.2). Wild-type virions swell approximately 10% in diameter when the pH is raised to >7.0 and the ionic strength remains low (i < 0.2). Virion swelling is the result of an expansion at the quasi-three-fold axis of the virion and is thought to require the removal of Ca²⁺ at these axes (14). The swelling of the virion has been suggested to be required for the cotranslational disassembly of the virion (15, 16). Virions can be disassociated into their coat protein and RNA components by further increasing the pH (>7.4) and ionic strength (i > 1.0). Virions can be assembled in vitro from the purified coat protein and vRNA that are infectious and morphologically indistinguishable from native virions purified from plants (2).

CCMV disassembly mutants provide a valuable resource for understanding the chemical basis for virion stability and disassembly (17, 18, 15, 19). The ability to isolate viable mutants with increased virion stability which alter the viral assembly and disassembly processes is one of the advantages of the CCMV system. Previous work in our laboratory characterized a salt-stable CCMV mutant which, in comparison to wild-type CCMV, was resistant to disassembly in high pH and ionic strength conditions (20). The salt stable mutant virions are incapable of swelling when incubated under neutral pH conditions. In the early 1970's Bancroft et al. (17) reported the isolation of a CCMV coat protein mutant (originally termed the cysteiny1 mutant) that lost infectivity unless it was purified in the presence of cysteine. We report here the further characterization of the cysteiny1 mutant (now termed the cpR26C mutant). The cpR26C CCMV mutant is resistant to disassembly under condi-
tions that disassemble wild-type virions, but is capable of swelling. This mutant provides a valuable chemical switch for examining virion assembly and disassembly.

Lyophilized plant tissue infected with the original Bancroft cpR26C mutant was obtained from the John Innes Institute (Norwich, England). We were unable to recover an infectious cpR26C mutant from the lyophilized tissue using both standard inoculation techniques and inoculation in the presence of reducing agents. Therefore, it was necessary to use deoxyoligonucleotide-directed reverse transcription-polymerase chain reaction amplification (RT/PCR) of the coat protein gene from the total RNA extracted from the lyophilized tissue as described previously for the salt stable mutant (20). The entire coat protein gene was amplified by RT/PCR and cloned into the Sal I site of pBluescript SK +, resulting in pBSKL3 essentially as described previously (20). DNA sequence analysis of pBSKL3 revealed a single C to U nucleotide transition at position 1435 of CCMV RNA 3 (86 of CCMV RNA 4) in the cpR26C mutant as compared to wild type. This mutation corresponds to an R26C amino acid mutation in the capsid protein (Fig. 1).

The clone pBSKL3 was used to subclone the coat protein cDNA of the putative mutant into the corresponding position in the wild-type CCMV RNA3 cDNA. This clone (pCC3L3) was used for in vitro transcription of RNA and cowpea (Vigna unguiculata (L.) var. California Blackeye) plant inoculations (20). The resulting virions, when purified under nonreducing conditions (2), were not infectious and demonstrated a disassembly deficient phenotype when dialyzed in high pH, ionic strength conditions (7.5, 0.5, respectively). The wild-type virion controls were infectious and completely disassembled under similar conditions of high pH and ionic strength (Fig. 2A). Likewise, the cpR26C mutant virions that were purified under reducing conditions (in the presence of 5 mM dithiothreitol) remained infectious and disassembled like wild-type virions under the high pH, ionic strength conditions (Fig. 2B). The cpR26C virions appear structurally similar to wild-type virions as determined by electron microscopy and RNA to protein ratios ($A_{260}/A_{280} = 1.70$). When reduced cpR26C virions or cpR26C RNA are used as inoculum the virus replicates and accumulates to similar levels as wild-type CCMV in vivo (data not shown). Although the nonreduced cpR26C virions did not disassemble they did swell in the pH 7.5 conditions as determined by altered mobility in the sucrose gradients (78S for swollen vs 83S for native virions; Fig. 2A).

Analysis of the nonreduced cpR26C mutant virions by SDS–PAGE indicated that the coat protein was covalently linked in multimeric forms (Fig. 3). In contrast, when either wild-type virions or cpR26C mutant virions purified under reducing conditions were analyzed by SDS–PAGE the coat protein existed primarily in the monomer form (Fig. 3). Pretreating the nonreduced mutant virions with reducing agent prior to loading on the gel resulted in conversion of the larger multimeric forms to the monomeric coat protein. The dimer form of the coat protein was isolated from SDS–PAGE gels and subjected to cleavage by CNBr. SDS–PAGE analysis of the CNBr cleavage products indicated that the intersubunit cross-linking occurs in the N-terminal half of the coat protein (data not shown).

Our identification of the R26C mutation is consistent with earlier data of Bancroft and coworkers (17). Using total amino acid composition analysis they identified a single arginine to cysteine replacement in the mutant coat protein. Because of the limited tools available at the time, Bancroft was unable to further characterize the mutant, identify the site of the mutation, or provide a structural basis for the phenotype.

The structure of CCMV has been determined to 3.2 Å resolution (14). The CCMV virion is made up of 180 copies of the coat protein subunit arranged with a $T = 3$ quasi-symmetry and organized in 20 hexameric and 12 pentameric capsomers. A striking feature of the coat protein subunit structure is the presence of N- and C-terminal arms that extend away from the central, eight-stranded, anti-parallel $\beta$-barrel core (Fig. 1). Each coat protein subunit consists of a canonical $\beta$-barrel fold (formed by amino acids 52–176) from which long N-terminal (residues 1–51; residues 1–27 are not seen in the crystal structure) and C-terminal arms (residues 176–190) extend in opposite directions. The N- and C-terminal arms may provide an intricate network of ropes which tie subunits together. The extended C-terminal arms facilitate the intercapssomer contacts. This arm in each subunit points away from its capsomer of origin and interacts with the adjacent twofold related subunit. The N-terminal arms provide additional stability for the interpenetrating

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**Fig. 1.** (A) A single CCMV coat protein subunit. The CCMV coat protein is composed of a central eight-stranded anti-parallel $\beta$-sheet ($\beta$-barrel) with the N- and C-terminal ends of the protein extending away from the $\beta$-barrel in opposite directions. The location of the R26C mutation is highlighted with an arrow. The first 26 amino acids of the coat protein were not resolved in the X-ray crystal structure determination. (B) The arrangement of coat protein subunit around the icosahedral threefold axis (pseudosixfold) and the fivefold axis. Six N-termini interact around the threefold axis to form the $\beta$-hexamer structure. An equivalent structure is not formed around the fivefold axis. (C) The linear representation of the CCMV RNA 3. CCMV RNA 3 codes for two genes, the movement protein gene and the coat protein gene. The coat protein is functionally expressed in vivo (data not shown). Although the nonreduced cpR26C virions did not disassemble they did swell in the pH 7.5 conditions as determined by altered mobility in the sucrose gradients (78S for swollen vs 83S for native virions; Fig. 2A).

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structure suggests a possible chemical basis for the increased virion stability phenotype. The first 27 N-terminal amino acids are not seen in the crystal structure due to the disordered nature in this region. However, we can still make some predictions based on the surrounding environment where amino acid 26 exists. The R26C mutation is located on the N-terminus of the coat protein involved in hexameric capsomer formation (β-hexamer). At each of the icosahedral threefold axes six N-terminal arms converge to form the β-hexamer. The β-hexamer is formed by amino acids 27-35. Thus, amino acid 26 would lie beneath the β-hexamer in the interior of the virion. Because of the sixfold related symmetry at these axes, six of the R26C mutations from separate coat protein molecules would be in close proximity to each other. We predict that disulfide linkages occur between these cysteine residues resulting in a cross-linking at the icosahedral threefold axes. In addition, even though a β-hexamer is not formed at the more mobile fivefold axis, we postulate that similar linkages could also occur at the fivefold axes between cysteine residues of separate fivefold-related subunits. Nonreduced mutant virions are noninfectious, but reduced mutants are as infectious as wild-type virions

**FIG. 2.** Sucrose gradient profiles of wild-type CCMV and cpR26C mutant CCMV under virion stabilizing and virion disrupting conditions. (A) Comparison of wild-type CCMV and cpR26C mutant CCMV purified under nonreducing conditions. The virus samples were dialyzed against either virus buffer (0.1 M NaOAc, pH 4.8) or disassembly buffer (0.5 M CaCl₂, 0.1 M Tris–HCl, pH 7.5) for 12 hr and loaded on linear 10-50% sucrose gradients. The gradients were centrifuged for 4 hr at 37,000 rpm in a SW41 rotor. The gradients were fractionated while monitoring the absorbance at 254 nm. The wild-type virions are stable in virus buffer (solid squares) but completely disassociate in the disassembly buffer (open squares). However, the cpR26C mutant virions are stable in both conditions (solid and open circles), although they do swell in the higher pH conditions of the disrupting buffer. (B) Comparison of wild-type CCMV and cpR26C mutant CCMV that was purified under reducing conditions. The virus samples were treated as described above. The cpR26C mutant virions have identical sedimentation rates as the wild-type virions in that they are stable in the virus buffer (wt-solid square, cys-solid circle) but completely disassociate in the disrupting buffer (wt, open square; cys, solid circle). Thus, the cpR26C mutant stability phenotype can be reversed by purifying the virus under reducing conditions.

C-terminal arms by clamping the arms between the β-barrel module and the extended N-terminal arm. The N-terminal extension also appears to stabilize hexameric capsomers. The six N-terminal arms of the hexameric capsomer intertwine at the icosahedral threefold axis to form a unique hexameric tubular structure (termed the β-hexamer). This structure is made up of six short parallel β-strands that are hydrogen bonded together (residues 29–33). The five N-terminal arms of the pentamer capsomer also approach the icosahedral fivefold axis but do not form an ordered β-pentamer.

Examination of amino acid position 26 in the crystal

**FIG. 3.** SDS–PAGE of wild-type CCMV, cpR26C mutant CCMV purified under reducing conditions, and cpR26C mutant CCMV purified under nonreducing conditions. Each virus sample was split in half and to one sample was boiled in SDS–PAGE loading buffer containing 5% β-mercaptoethanol while the other sample was boiled in the same buffer without the β-mercaptoethanol. The samples were then loaded on a 12% acrylamide gel, electrophoresed, and stained with Coomassie brilliant blue R-250. Lane order: molecular weight standards; (1) wild-type CCMV with β-ME; (2) wild-type CCMV without β-ME; (3) reduced cpR26C mutant CCMV with β-ME; (4) reduced cpR26C mutant CCMV without β-ME; (5) nonreduced cpR26C mutant CCMV with β-ME; and (6) nonreduced cpR26C mutant CCMV without β-ME.
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