Carboxylate Interactions Involved in the Disassembly of Tobacco Mosaic Tobamovirus

BINGU,* GERALD STUBBS,† and JAMES N. CULVER*‡,1

*Department of Plant Biology, University of Maryland, College Park, Maryland 20742; †Department of Molecular Biology, Vanderbilt University, Nashville, Tennessee 37235; and ‡Center for Agricultural Biotechnology, University of Maryland Biotechnology Institute, College Park, Maryland 20742

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Structural studies of tobacco mosaic tobamovirus (TMV) have identified two coat protein (CP) intersubunit carboxyl–carboxylate interactions and one CP carboxylate–RNA phosphate interaction whose electrostatic repulsion is believed to drive virion disassembly. In this study, the involvement of each interaction in the disassembly process was examined. Site-directed mutagenesis was used to replace selected negatively charged CP residues, E or D, with neutral residues, Q or N, respectively. Purified mutant CPs were assayed for their ability to inhibit wild-type TMV disassembly both in vitro and in vivo. Results indicate that the lateral carboxylate interaction made by residue E106 is much more complex than previously thought, involving three residues, E95, E97, and D109, from an adjacent subunit. Mutations at all three residues are required to inhibit disassembly significantly. Different mutant coat proteins inhibited disassembly of the wild-type virus to varying degrees. Mutant E50Q, which modified the axial intersubunit interaction, had the greatest ability to inhibit disassembly followed by mutants E95Q/E97Q/D109N and D116N, which modified the lateral and CP–RNA interactions, respectively. Within each set of interacting carboxylate groups, mutations in the face opposite the disassembling surface of the TMV virion conferred the greatest ability to inhibit disassembly. This observation is consistent with the polar nature of TMV disassembly and confirms that repulsive intersubunit interactions derived from the 5′ terminal subunits provide the key controlling mechanisms for virion disassembly.

INTRODUCTION

Simple RNA viruses depend upon macromolecular assemblies of structural proteins to protect their genomes from degradation. The paradox of the assembled virion is that it must remain stable within the extracellular environment, yet upon entry into a host cell, it must destabilize and disassemble in order to initiate virus translation and replication. This shift from stability to instability requires a molecular mechanism capable of sensing subtle changes in the virion's environment. Structural analysis of several RNA plant viruses reveals the presence of intersubunit clusters of carboxylate groups, whose electrostatic interactions could provide a sensitive switching mechanism with which to control virion stability (Casper, 1963; Bancroft, 1970; Stubbs, 1989). Under stabilizing conditions, negatively charged carboxylate groups could bind calcium ions or protons to offset the repulsive charge interactions. However, the loss of calcium ions or protons would lead to repulsion between the juxtaposed carboxylate groups, and thus between coat protein subunits, destabilizing the virion structure. Unfortunately, the precise effect of carboxylate groups on virion stability has remained unclear.

The tobacco mosaic tobamovirus (TMV) virion is a rigid rod, consisting of approximately 2130 identical coat protein (CP) subunits of molecular weight 17.5 kDa assembled in a right-handed helix around a single strand of viral RNA (reviewed by Bloomer and Butler, 1986; Stubbs, 1990). The intact viral structure has been determined at 2.9 Å resolution by X-ray diffraction methods (Namba et al., 1989; Fig. 1). Two intersubunit electrostatic interactions between CP subunits and one electrostatic interaction between the CP and RNA molecules were identified in the structure. Carboxylate groups from residues E50 and D77 on adjacent subunits were predicted to interact in the axial (longitudinal) direction, while carboxylate groups from residues E95 and E106 were predicted to interact in the lateral direction. The CP–nucleic acid interaction was predicted to occur between residue D116 of each subunit and a phosphate group from the viral RNA. All interactions were thought to contribute to the anomalous titration behavior of TMV (Casper, 1963; Namba et al., 1989). It was suggested that the E106–E95 interaction and the D116–phosphate interaction may also act as calcium binding sites, accounting for the two sites in TMV for which calcium ions and protons compete (Gallagher and Lauffer, 1983; Namba et al., 1989). Thus, calcium ions and protons may stabilize these interactions under extracellular conditions.

The early stages of TMV disassembly may be described as follows (Namba et al., 1989). When a virion first enters a cell, the high pH and low calcium concentra-
FIG. 1. Structure of the TMV virion and CP. (A) Computer representation looking at the virion 5' end. Three individual subunits are numbered. (B) Alpha carbon backbone of three CP subunits corresponding to the subunits labeled in A, but viewed from the side. Interacting carboxylate groups are shown in space-filling form.

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and N, respectively, affecting the electrostatic repulsion between the residues but having no effect on the hydrogen bonding patterns or hydrophobic interactions. Virions extracted from leaf tissue infected by either CP mutant appeared by electron microscopy to be morphologically normal. However, gel electrophoresis and spectroscopy showed that mutant virion preparations contained very little RNA, indicating that removal of the repulsive forces between E50 and D77 stabilizes a helical protein aggregate at physiological pH. Wild-type TMV virions treated with excess E50Q CP were greatly inhibited in their ability to disassemble both in vitro and in vivo. In contrast, wild-type TMV virions treated with excess D77N CP did not show a significantly reduced ability to disassemble. The different degrees of inhibition displayed by E50Q and D77N CPs are likely the result of the polar nature of TMV disassembly and suggest that the positions of these residues relative to the direction of disassembly are an important factor in disassembly.

In this study, the involvement of all three carboxylate interactions in the disassembly of TMV was investigated. Mutagenesis of amino acids in both the lateral E106–E95 interaction and the D116–RNA phosphate interaction confirmed the involvement of these residues in disassembly. Interestingly, however, E106 was found to participate in a much more complex interaction, involving not only E95 but also E97 and D109. The mutant coat proteins showed considerable variation in their ability to inhibit disassembly of wild-type virus, with E50Q CP having the greatest effect. Proteins having mutations in carboxylate groups that would face the exposed disassembling surface in TMV were found to inhibit disassembly much more than proteins having mutations on the opposite surface, while the protein D116N, in which the carboxylate–phosphate interaction was modified, had an intermediate effect. Taken together with the known three-dimensional structure of TMV, these observations are shown to be consistent with the polar nature of TMV disassembly.

**MATERIALS AND METHODS**

**Construction of TMV CP carboxylate mutants**

Full-length infectious cDNA clones of the U1 strain of TMV, joined to the phage SP6 promoter, pBGC150, or to the T7 promoter, pSNC004, were used as the parental constructs for all described mutations (Dawson et al., 1986; Kumagai et al., 1993; Turpen et al., 1995). A subclone, pDL3, containing the 3' end of TMV RNA, including the coat protein open reading frame (ORF), (nucleotides 5081 to 6406) served as the template for mutagenesis (Culver and Dawson, 1989). TMV RNA numbering is from Goelet et al. (1982).

Amino acid substitutions were carried out using site-directed oligonucleotide mutagenesis as described by Geisselsoder et al. (1987). A uracil-containing single-stranded pDL3 template was obtained by coinfection with phage M13KO7 in Escherichia coli CJ236 (Vieira and Messing, 1987). Second-strand synthesis was performed using synthetic oligomers, 18 nucleotides in length, designed to alter a specific CP amino acid codon. Plasmid DNA from bacterial colonies transformed with second-strand products was sequenced to confirm the presence of the desired mutations. In addition, the entire CP ORF was sequenced to ensure that no other mutations had occurred during mutagenesis. Once sequenced, Ncol (nucleotide 5460) to BsiWI (nucleotide 6245) fragments containing the entire CP ORF were ligated into similarly cut full-length TMV constructs. CP ORFs from full-length constructs were also sequenced to confirm the presence of the desired mutation. Each CP substitution is identified by the single-letter code for the wild-type residue in front of the residue number and followed by the code of the substituting amino acid.

Infectious virus RNA was generated in vitro and used to mechanically inoculate leaves of Nicotiana tabacum L. cv. Xanthi (systemic host) and Xanthi-nc (local lesion host) (Dawson et al., 1986; Kumagai et al., 1993; Turpen et al., 1995). Inoculated plants were maintained in environmental growth chambers at 25° under a 12-hr photoperiod.

**Virus purification, CP isolation, mutant conformation, and electron microscopy**

Mutant virions from infected N. tabacum cv. Xanthi tissue were isolated and purified as described by Gooding and Hiebert (1967). Virions were further purified by centrifugation at 25,000 rpm in a Beckman SW28 rotor for 2 hr in a 10 to 40% sucrose density gradient at 10°. Virus concentrations were determined by absorbance at 260 nm, corrected for light scattering at 320 nm, using an extinction coefficient of 3.01 mg⁻¹cm⁻³ (King and Perham, 1971). CP was isolated from purified virion samples by acetic acid degradation according to Fraenkel-Conrat (1957). Purified CP concentrations were determined by Bradford assay (Bradford, 1976).

RNA was extracted from purified mutant virions by protease K treatment in 10 mM NaCl, 10 mM MgCl₂, 20 mM Tris - HCl, pH 8.0, with 5% SDS for 1 hr at 65°. Solutions were extracted twice with equal volumes of phenol/chloroform and the RNA was precipitated with 95% ethanol. Reverse transcription and polymerase chain reaction amplification were used to amplify the CP open reading frame from purified RNA samples as described by Kawaski (1990). Amplified fragments were directly sequenced to confirm the maintenance of the original mutation.

Infected plant extracts, prepared by grinding 0.1 g of infected N. tabacum cv. Xanthi tissue in 300 μl of 0.01 M phosphate buffer, pH 7.3, at 4°, and the purified virion preparations were spotted (3 μl) onto formvar-coated electron microscope grids and allow to dry. Grids were
then negatively stained with 1% uranyl acetate for 2 min, wicked dry with filter paper, and examined by electron microscopy.

In vivo infectivity and in vitro translation assays

Mutant TMV CPs were tested for their ability to inhibit wild-type TMV disassembly both in vivo and in vitro. Purified wild-type TMV virions, 0.4 μg, were incubated for 15 min in 0.01 M Tris—HCl, pH 8.0, buffer, conditions that favor the removal of a small number of CP subunits from the RNA 5’ end (Wilson, 1984; Mundry et al., 1992; Wu et al., 1994). The pH of the buffer was then adjusted with an equal volume of 0.1 M Tris—HCl, pH 7.4, buffer containing an excess of purified mutant or wild-type CP, 4.0 μg, and incubated for an additional 30 min. These conditions favor virion reconstitution (Fraenkel-Conrat and Williams, 1955). Treated virions were purified by centrifugation at 65,000 rpm in a Beckman TLA100.3 rotor for 10 min at 4 °C. Partially reconstituted virion pellets were resuspended in water and used immediately to inoculate fully expanded leaves of N. tabacum cv. Xanthi-nc or charge an in vitro translation system.

Infectivity assays were performed by inoculating half-leaves of N. tabacum cv. Xanthi-nc (local lesion host) with treated virions. One half of each leaf received 50 μl containing 0.2 μg of wild-type virions treated with excess wild-type or mutant CPs while the other half-leaf received wild-type virions treated identically except with the omission of extra CP. The number of local lesions on each half-leaf was counted 4 days postinoculation and used to calculated the percentage infectivity relative to untreated wild-type TMV.

Translational assays followed the procedure outlined by Wilson (1984). Twenty-five micrograms of virions treated with excess wild-type or mutant CP, as described above, was added directly to an mRNA-dependent rabbit reticulocyte lysate system (Promega, Madison, WI), containing 250 μCi/ml L-[35S]methionine and incubated at 30° for 90 min. Translational reactions were terminated by mixing with an equal volume of 2× Laemmli buffer [125 mM Tris, pH 6.8, 10% (w/v) DTT, 20% (w/v) SDS, 0.01% (w/v) bromophenol blue] and incubating at 65° for 3 min. SDS–PAGE was carried out, as described by Laemmli (1970), using acrylamide concentrations of 4.8 and 10% for stacking and separation gels, respectively. 35S-labeled protein bands were visualized and quantified using a Phospholmager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Quantifying the effects of carboxylate mutations on TMV disassembly

The majority of purified carboxylate mutants displayed an OD260/280 ratio that was lower than wild-type TMV (Table 1). This indicated that the mutant virion preparations contain significantly less viral RNA. Mutant viruses also produced virions of greatly varying lengths (Table 1, Fig. 2). The combination of RNA-empty virions and virions of dramatically different lengths made it impossible to isolate a homogenous virus fraction for direct use in disassembly assays. Thus, disassembly assays based on quantifying the exposure of viral RNA or measuring changes in virion length could not be used. To circumvent this problem, an assay measuring the ability of purified mutant CPs to inhibit the disassembly of wild-type virions was utilized (Culver et al., 1995). TMV disassembly initiates from the RNA 5’ end and under appropriate buffer conditions is a reversible process (Fraenkel-Conrat and Williams, 1955; Wilson, 1984; Mundry et al., 1992; Wu et al., 1994). These two factors allow wild-type virions to be partially reconstituted at the virion 5’ end using purified mutant CP. Excess CP not associated with virions can be removed by centrifugation. Virions reconstituted in this manner can be easily inoculated onto a local lesion host, N. tabacum cv. Xanthi-nc, to assay for effects on infectivity or tested in vitro for effects on cotranslational activity. Comparisons between virions treated with excess wild-type or mutant CPs can be used to determine the relative ability of a mutant CP to inhibit TMV disassembly.

Effects of the lateral carboxylate interaction on disassembly

Residues E95 and E106, which were predicted to make a lateral carboxyl–carboxylate interaction are in the inner loop of CP within 5 Å of each other across the intersubunit interface (Fig. 1). Mutant E106Q produced virions that were significantly longer and contained approximately 80% less RNA than wild-type TMV (Table 1). Mutant E106Q also moved long distances in tobacco at rates

<table>
<thead>
<tr>
<th>Mutants</th>
<th>OD260/OD280</th>
<th>Rod length (nm)</th>
<th>Systemic symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>U1 Wild-type</td>
<td>1.18</td>
<td>300 ± 29</td>
<td>&lt;1 week</td>
</tr>
<tr>
<td>E50Q/D77N</td>
<td>0.77</td>
<td>940 ± 470</td>
<td>3–4 weeks</td>
</tr>
<tr>
<td>E106Q</td>
<td>0.88</td>
<td>422 ± 243</td>
<td>3–4 weeks</td>
</tr>
<tr>
<td>E95Q</td>
<td>1.18</td>
<td>323 ± 158</td>
<td>&lt;1 week</td>
</tr>
<tr>
<td>E97Q</td>
<td>0.96</td>
<td>1450 ± 640</td>
<td>1–2 weeks</td>
</tr>
<tr>
<td>D109N</td>
<td>0.87</td>
<td>770 ± 220</td>
<td>1–2 weeks</td>
</tr>
<tr>
<td>E95Q/E97Q</td>
<td>1.01</td>
<td>950 ± 420</td>
<td>1–2 weeks</td>
</tr>
<tr>
<td>E95Q/D109N</td>
<td>1.06</td>
<td>1000 ± 280</td>
<td>1–2 weeks</td>
</tr>
<tr>
<td>95Q/97Q/109N</td>
<td>1.13</td>
<td>660 ± 230</td>
<td>3–4 weeks</td>
</tr>
<tr>
<td>D116N</td>
<td>1.14</td>
<td>254 ± 108</td>
<td>3–4 weeks</td>
</tr>
</tbody>
</table>

* Lengths are the average length ± standard deviation of 100 rods measured from electron micrograph photography of purified virus.
* Systemic symptoms were observed as the appearance of mosaic/patchy mosaic symptoms on N. tabacum cv Xanthi-nc.

TABLE 1: Characterization of TMV Amide CP Mutants
FIG. 2. Electron micrographs of purified wild-type and mutant TMV virions/helical aggregates. (A) Wild-type; (B) D109N; (C) E95Q/D109N; (D) D116N. Bars represent 300 nm.
FIG. 3. Infectivity and cotranslational inhibition of wild-type TMV virions treated with excess mutant or wild-type CP. Infectivity data were averaged from eight independent half-leaf assays. Cotranslational data was averaged from three independent translation experiments. Bars represent the percentage of infectivity or translation activity in relation to that of untreated wild-type virions. Error bars represent standard deviation.

substantially slower than wild-type, producing patchy mosaic symptoms (Table 1). Purified E106Q CP inhibited the disassembly of wild-type TMV virions in infectivity and cotranslational assays by a combined average of 44% (Fig. 3). In contrast, mutant E95Q produced virions that were identical in size and RNA content to wild-type TMV (Table 1). Purified E95Q CP also had no inhibitory effects on the disassembly of wild-type TMV (Fig. 3).

These data confirm that the negative charge of E106 is involved in TMV disassembly. Unexpectedly, however, the data do not demonstrate the involvement of E95 in disassembly, despite the proximity of E95 and E106 in the structure of TMV. One possible explanation for this finding is that other carboxylate residues interact with E106, either in addition to or instead of E95. Two such residues are potentially close enough to E106 to make such interactions: E97 and D109. CP substitutions E97Q and D109N were therefore made. Both of these mutants produced virions that were longer and contained between 30 and 70% less RNA than wild-type TMV. They also moved long distances in tobacco at reduced rates (Table 1). However, purified E97Q CP showed no significant ability to inhibit wild-type TMV disassembly, while purified D109N CP reduced wild-type translation and infectivity by only an average of 20% (Fig. 3). Thus, no single carboxylate group from E95, E97, or D109 could be solely responsible for the interaction with E106 that affects virion disassembly. To investigate the possibility that E106 interacts with two or more of the adjacent carboxylate groups, several combination mutants were created. Combination mutants E95Q/E97Q and E95Q/D109N both produced virions that contained 50% less RNA and were significantly longer than wild-type TMV (Table 1). In disassembly assays both E95Q/E97Q and E95Q/D109N CPs inhibited translation and infection by wild-type TMV by as much as 50%, levels comparable to E106Q (Fig. 3). Combination mutant E95Q/E97Q/D109N also produced longer virions containing 70% less RNA than wild-type TMV (Table 1). Purified CP from this triple mutant had the greatest inhibiting effect on wild-type TMV disassembly, reducing infectivity by 77% and inhibiting cotranslation activity by 93%. Thus, all three carboxylate members, E95, E97, and D109, appear to be necessary to make the full repulsive intersubunit interaction with E106.

Effects of the carboxylate–RNA interaction on disassembly

A carboxylate–phosphate interaction is predicted from the TMV structure to occur between residue D116 of each subunit and an RNA phosphate group (Fig. 1). Mutant D116N was made to determine the importance of this potential interaction in virion disassembly. Purified virions of mutant D116N showed a near normal RNA content (Table 1). However, electron microscopy showed that the virions were slightly shorter and more variable in length than wild-type TMV (Table 1). Purified mutant CP D116N inhibited wild-type TMV infectivity by over 70% and translational activity by over 60%. Thus, the repulsive interaction between D116 and viral RNA also contributes to efficient virion disassembly.

Combining carboxylate mutations in the axial interaction

The axial carboxylate interaction is made by residues E50 and D77, located in the middle radius of the CP
molecule (Fig. 1). Culver et al. (1995) demonstrated that CP E50Q was capable of reducing the infectivity of wild-type TMV by as much as 95%, while CP D77N showed no significant reduction in infectivity. In the present study, purified E50Q CP was shown to reduce the cotranslational activity of wild-type TMV by over 90%, whereas purified D77N CP produced no significant reduction in the cotranslational activity of wild-type TMV. D77N virus was, however, significantly more stable than wild-type virus under alkaline conditions (Culver et al., 1995). In order to confirm the mechanism of interaction between E50 and D77, a combination mutant, E50Q/D77N, was created. If the properties of the mutant virions are due to electrostatic interactions between E50 and D77, the E50Q/D77N mutant should have similar properties to E50Q. If the two residues have independent effects on the virus, however, the double mutant properties should be enhanced. This mutant moved long distances in tobacco at a substantially reduced rate (Table 1) and inhibited translation and infectivity of wild-type virus by as much as 90% (Fig. 3). This inhibition is not significantly different from the inhibition conferred by the E50Q mutation alone.

Concentration of mutant CPs required to inhibit disassembly

The relative ability of mutants in each carboxylate cluster to inhibit the wild-type disassembly process was investigated by measuring cotranslational activity as a function of mutant CP concentration (Fig. 4). Purified CPs of E50Q/D77N and E95Q/E97Q/D109N at molar ratios of only 1:1 (CP:virion) showed reductions in cotranslational activity of approximately 35 and 22%, respectively, whereas D116N CP had no significant effect on disassembly at the 1:1 ratio. Increasing the concentrations of mutant CPs resulted in a corresponding increase in the inhibition of wild-type virus cotranslational activity (Fig. 4). The greatest degree of inhibition, over 90%, was reached at a molar concentration of 1000:1 for mutant CP E50Q/D77N. CP E95Q/E97Q/D109N reached a similar level of inhibition at a concentration of 10,000:1, whereas the highest level of inhibition observed for CP D116N, 70%, occurred at the 20,000:1 concentration.

Effect of wild-type CP on disassembly inhibition by mutant CPs

The ability of excess wild-type CP to compete for virion interactions with mutant E50Q/D77N CP was examined by adding increasing molar ratios of wild-type CP to a fixed molar concentration of mutant CP (Fig. 5). Wild-type virions treated with various mixtures of mutant and wild-type CP were analyzed for cotranslational activity. At a molar ratio of 1000:1 (E50Q/D77N CP to wild-type virus), the ability of the mutant CP to diminish cotranslational activity was unaffected even by the presence of 100-fold excess of wild-type CP (Fig. 5). These data provide additional evidence that the mutant CP has a significantly stronger affinity than wild-type CP for virions.

DISCUSSION

The amide substitutions made in this study altered only residue charge and had no significant effect on overall CP structure, as demonstrated by the ability of all amide mutants to form virions or virion-like particles. Variations in virion length and RNA content had precluded direct assays of amide mutant virion disassembly, but the effects of the CP mutations on disassembly could be measured by means of wild-type TMV virions partially reconstituted with purified amide mutant CPs. Two assays were used: infectivity of the reconstituted particles and in vitro translation of the RNA from the disas-
would be abolished completely, inhibited infection and translation by more than 80%.

The relative strengths of the inhibitory effects of the different mutants are consistent with the polar nature of TMV disassembly, and with the presumed mechanism of inhibition, that is, by exchange between free mutant CP subunits and the wild-type CP subunits nearest the 5' end of the virion. The highest levels of inhibition were observed in experiments with E50Q and E95Q/E97Q/D109N. E50Q inhibited both alkaline disassembly and infectivity by as much as 95% (Culver et al., 1995), while E95Q/E97Q/D109N inhibited infectivity by almost 80% and translation by over 90% (Fig. 3). E50Q/D77N (which appears to be structurally equivalent to E50Q) and E95Q/E97Q/D109N inhibited cotranslational disassembly at molar ratios as low as 1:1 and reached their highest levels of inhibition at ratios of 1000:1 and 10,000:1 respectively (Fig. 4). In contrast, D77N inhibited alkaline disassembly only to a relatively small extent and did not significantly inhibit infectivity at all (Culver et al., 1995);

FIG. 5. Effect of added wild-type CP on the ability of E50Q/D77N CP to inhibit the cotranslational disassembly of wild-type TMV. Each lane shows the results obtained from adding increasing molar concentrations of wild-type CP to a fixed concentration of E50Q/D77N CP. Concentration mixtures are listed at the top. (A) Representative gel showing TMV 183- and 126-kDa translational products. (B) Bars represent the average and standard deviation of translational products from three independent assays.

All of these observations can be understood in terms of the positions of the mutated residues on the CP subunit and the relative orientation of the exchanging subunit and the disassembling virus. E50 and the negatively charged patch consisting of E95, E97, and D109 are on the surface of the CP subunit facing the disassembling virion surface, so that an incoming subunit of either E50Q or E95Q/E97Q/D109N will bind strongly to the exposed face, forming intersubunit hydrogen bonds but making no repulsive interaction with the other member (D77 or E106, respectively) of the carboxylate cluster (Fig. 6). This complex will therefore be much more stable than the corresponding pure wild-type complex. An incoming subunit of D77N or E106Q, like D77N and E106Q, make wild-type interactions with the wild-type virion, but a stabilizing effect would occur if the disassembling RNA re-bound to the virion at the D116N subunit. While the strength of this effect is difficult to
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FIG. 6. Electrostatic surface potential of a single CP subunit and a single turn of the TMV virion viewed from its 5' side. Red, negatively charged areas; blue, positively charged areas; and white, neutral areas. To show the interacting CP surfaces, the single subunit was positioned as if it were removed from the virion in a fashion similar to opening a book, with the spine represented by the two-headed arrow.

predict, such a mechanism is consistent with the intermediate strength of inhibition by D116N.

TMV disassembly has been shown to initiate from the 5' end in vivo (Shaw, 1985; Wu et al., 1994). Furthermore, only virions with RNA exposed at the 5' end can serve as templates for cotranslational disassembly (Mundry et al., 1991). Mundry et al. (1991) demonstrated that under alkaline pretreatment conditions uncoating of the 5' terminal 70 to 200 nucleotides, corresponding to 25 to 65 CP subunits or 1 1/2 to 4 turns of the viral helix, occurs easily and rapidly. Amide mutant CPs were able to inhibit cotranslational disassembly only if added to wild-type virions before the initiation of translation; adding them to the rabbit reticulocyte system 5 to 15 min after the addition of pretreated wild-type virions had no effect on translational activity (data not shown). It appears that amide mutant CPs must bind to the virion prior to ribosome binding in order to have an inhibiting effect; the activation energies for removal of either amide mutant CP subunits or ribosomes are presumably both relatively large, and much larger than those for removal of wild-type CP subunits.

The repulsive electrostatic interactions due to the close approach of carboxylate groups are important not only in viral disassembly, but also in assembly. In general, the amide mutations stabilized the protein helix, producing virion-like rods that contained less than wild-type levels of RNA (Table 1, Fig. 2). In contrast, wild-type CP forms protein helices only at low pH, when carboxylate groups are in their un-ionized forms (Durham et al., 1971) and under physiological conditions aggregate into 20S structures that are required for both virion assembly initiation (Butler and Klug, 1971) and elongation (Shire et al., 1981). By stabilizing the protein helix, amide mutants form fewer 20S aggregates and thus fewer correctly assembled virions. The ability of a specific amide mutation to stabilize the protein helix is not necessarily correlated with its ability to inhibit disassembly; for example, E97Q produces long helical protein aggregates but appears to disassemble normally. Viruses with carboxylate mutations have reduced infectivity and slower systemic virus movement (Table 1) and are thus at an evolutionary disadvantage. The roles of the carboxylate groups in stabilizing the 20S aggregate, as well as their roles in viral disassembly, thus account for the evolutionary conservation of carboxyl-carboxylate interactions observed in tobamoviruses and many other simple plant viruses (Bancroft, 1970; Stubbs, 1989; Wang and Stubbs, 1994).

The ability of both E50Q/D77N and E95Q/E97Q/D109N to inhibit disassembly at molar ratios as low as 1:1 may have considerable potential significance. Under the conditions used in these assays, amide mutant CP must compete for reassembly against the wild-type CP removed by the alkaline treatment. The significant levels of inhibition observed for E50Q/D77N and E95Q/E97Q/D109N even at the 1:1 molar ratio (Fig. 4) demonstrate that mutant CP binding is very tight and suggests that the binding of as little as one mutant CP molecule per virion may be sufficient to inhibit disassembly. Amide CPs appear to compete for 5' end binding very effectively.
against wild-type CP, as was further demonstrated by the fact that 100-fold excesses of wild-type CP had no effect on the ability of E50Q/D77N CP to inhibit disassembly. Interestingly, the translational activity of wild-type virions treated with an equimolar concentration of E50Q/D77N CP was inhibited by as much as 35%, while virions treated with a 100,000-fold excess of wild-CP were inhibited by only 17% (Figs. 4 and 5; Wilson and Watkins, 1986). Amide mutant CPs clearly have a much stronger ability than wild-type CP to bind to the TMV virion and prevent disassembly. This ability may provide a useful method of improving transgene-derived CP-mediated protection (Powell-Abel et al., 1985; Fitchen and Beachy, 1993).

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