VIROLOGY 244, 13–19 (1998)
ARTICLE NO. VY989099

RAPID COMMUNICATION

Intersubunit Interactions Allowing a Carboxylate Mutant Coat Protein to Inhibit Tobamovirus Disassembly

Bin Lu,* Zenobia F. Taraporewala,† Gerald Stubbs,‡ and James N. Culver*,†,§[1]

*Department of Plant Biology and †Molecular and Cell Biology Program, 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

Received December 5, 1997; returned to author for revision January 30, 1998; accepted February 16, 1998

Tobacco mosaic tobamovirus (TMV) coat protein (CP) mutant E50Q lacks a repulsive intersubunit carboxylate group and can effectively inhibit the disassembly of wild-type TMV (Culver *et al.,* 1995, *Virology* 206,724). To investigate the ability of this mutant CP to block disassembly, a series of second-site amino acid substitutions were added to the E50Q CP. These second-site mutations were designed to disrupt specific intersubunit stabilizing interactions involving hydrophobic or polar residues, salt bridges, and CP–RNA contacts. Results showed substitutions disrupting intersubunit interactions that face the disassembling surface of the virion dramatically reduced the ability of CP E50Q to inhibit TMV disassembly. Substitutions that disrupted the CP inner loop, RNA binding capabilities, or intersubunit interactions that faced away from the disassembling surface did not dramatically interfere with CP E50Q's ability to inhibit disassembly. Taken together, these findings suggest that intersubunit interactions made by 5′ terminal E50Q subunits, not associated with RNA, provide the stabilizing forces that prevent virion disassembly. The role of these stabilizing interactions in TMV disassembly and their potential use for creating disassembly inhibiting CPs are discussed.

Virus particles alter their stability in response to changing environmental conditions. This feature protects the virus genome from degradation while insuring its release upon entry into a host cell. Crystallographic studies of several plant RNA viruses have indicated a range of intersubunit protein–protein or protein–nucleic acid interactions that potentially contribute to virion stability and/or instability (*1*). One such interaction involves the clustering of intersubunit carboxylate groups whose electrostatic repulsion could destabilize subunit to subunit interactions (*1–3*). Under conditions favoring virion stability these repulsive interactions could be neutralized by protons or calcium ions. However, changes in pH and calcium concentrations associated with host cell entry may displace the stabilizing protons and calcium ions, allowing the repulsive carboxylate groups to interact and thus initiate disassembly. In this fashion, carboxylate interactions could provide a sensitive switching mechanism for controlling virion stability.

The involvement of carboxylate groups in the disassembly of tobacco mosaic tobamovirus (TMV) has been investigated (*4, 5*). The TMV virion is a rigid rod composed of approximately 2130 identical 17.5-kDa coat protein (CP) subunits assembled to form a right-handed helix around a single strand of plus-sense RNA (*1, 6*; Fig. 1). The structure of TMV at 2.9 Å resolution reveals three intersubunit carboxylate interactions: one axially between residues E50 and D77, one laterally between residues E106 and E95, and one between residue D116 and an RNA phosphate group (*7*). All three charge pairs are thought to contribute to the anomalous titration behavior of TMV, with the E95/E106 and D116/phosphate pairs acting as calcium binding sites (*7*). Site-directed mutagenesis was used to change these negatively charged E or D residues to neutral amide Q or N residues, respectively (*4, 5*). These substitutions alter only the repulsive charge and do not affect hydrogen bonding or hydrophobic interactions. The effects of amide mutations on TMV disassembly were determined by measuring the ability of purified mutant CPs to inhibit the disassembly of wild-type TMV virions in both plant infectivity and *in vitro* cotranslational assays. Results showed that wild-type TMV virions partially reconstituted with specific amide mutant CPs, such as CP E50Q, were significantly inhibited in cotranslational activity and infectivity. These findings demonstrated the importance of repulsive intersubunit interactions in driving disassembly.

In contrast to repulsive carboxylate interactions, the majority of TMV CP–CP interactions act to stabilize the

[1] To whom correspondence and reprint requests should be addressed at Center for Agricultural Biotechnology, University of Maryland, College Park, MD 20742-4450. E-mail jc216@umail.umd.edu.

0042-6822/98 $25.00

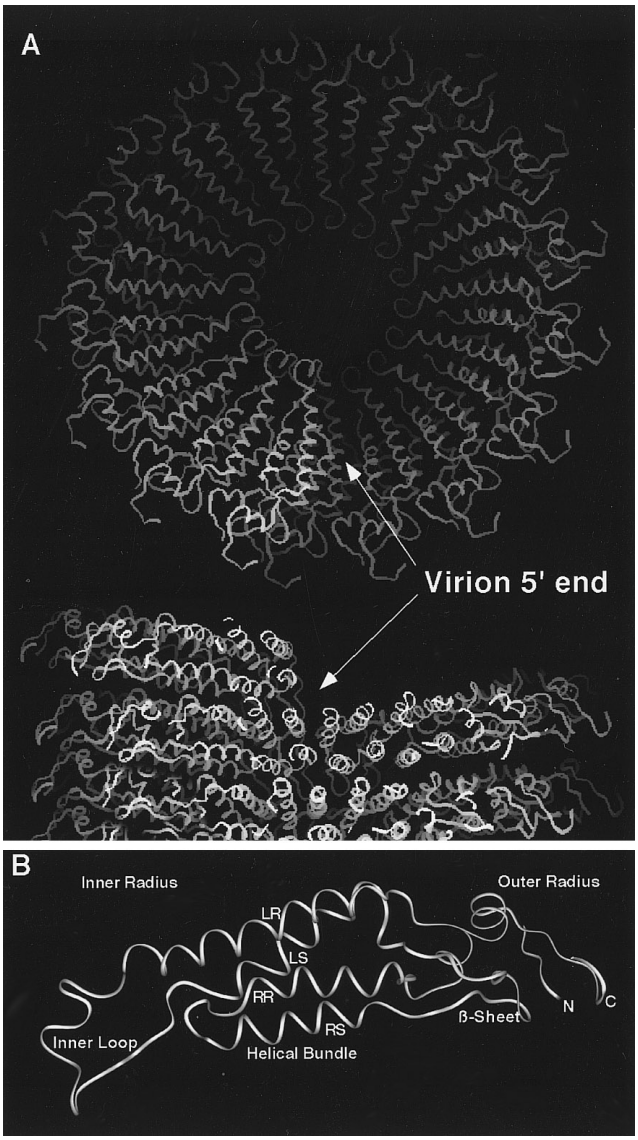

FIG. 1 Computer graphic representations of the 5′ end of the TMV virion and single coat protein subunit. (A) Virion 5′ end showing the disassembly surface viewed from the top, perpendicular to the viral axis, or from the side, parallel to the viral axis. (B) Ribbon representation of the α carbon backbone of a single TMV coat protein subunit. The four α-helices are labeled LS (left slewed), RS (right slewed), LR (left radial), and RR (right radial). The inner loop connects the LR and RR helices and a short loop connects LS and RS helices. The RNA binding site is formed by residues from both loops and the LR helix. The N- and C-termini are at the outer radius of the virus.

virion. These stabilizing interactions include patches of hydrophobic and polar residues, intersubunit salt bridges, and CP–RNA interactions (*7*). The ability of amide mutant CPs lacking repulsive carboxylate groups to inhibit TMV disassembly is likely the result of enhanced virion stability due to some or all of these stabilizing interactions. Understanding how these interactions stabilize the virion and prevent disassembly should expand our understanding of the disassembly process and may reveal novel approaches for the rationale design of additional CP mutants that inhibit virus disassembly.

To identify the molecular interactions that allow carboxylate CP mutants to inhibit TMV disassembly, a series of mutations designed to disrupt specific CP stabilizing features were added to the existing carboxylate mutant CP E50Q. Site-directed mutagenesis (*5, 8*) and *in vitro* transcriptions (*9, 10*) were performed as previously described. Infectious RNA transcripts for each new double mutant were mechanically inoculated onto leaves of *Nicotiana tabacum* cv. Xanthi. Twenty days after infection, virus particles were purified as described (*11*), using differential centrifugation steps or fractionation in a 10–40% sucrose gradient. The maintenance of each created CP mutation was confirmed by the sequencing of reverse transcription and polymerase chain reaction-amplified cDNA products obtained from viral RNA isolated from infected plant tissue (*12*). Coat protein from each double mutant was purified from isolated virus particles via acidic acid degradation (*13*) and concentrations were determined by Bradford assay (*14*). Polyacrylamide gel electrophoresis (*15*) was also used to compare CP concentrations and to insure the purity of each preparation.

All E50Q second-site CP mutants assembled into virion-like helical rods of various lengths *in planta* (Table 1). Purified rods displayed $OD_{260/280}$ ratios that were similar to the parent mutant E50Q, but lower than wild-type TMV (Table 1). This indicated that the double mutant rods, like their E50Q parent, contained less than wild-type levels of RNA. The E50Q mutation is known to increase the stability of the protein helix, resulting in the formation of greater than wild-type length helical rods (*5*; Table 1). This stabilization results in fewer of the 20S CP aggregates required for RNA assembly and, therefore, fewer virions (*16*). The ability to form virion-like rods indicates

TABLE 1

Characterization of E50Q Double Mutants

Coat protein substitutions combined with E50Q	Position	Rod length[a] (nm)	OD_{260}/OD_{280}
F10Y	N terminus	151 ± 23	1.02
T281	LS helix	106 ± 41	0.87
T42R	RS helix	168 ± 52	1.06
F67A	β sheet	318 ± 159	0.90
D88N	RR helix	476 ± 126	0.82
R90Q/R92E	Long loop	336 ± 140	0.83
P102L	Long loop	213 ± 93	1.06
D116A	LR helix	484 ± 190	0.84
I129A	LR helix	265 ± 185	1.14
I133L	LR helix	131 ± 80	0.99
Wild-type	NA	300 ± 20	1.18
E50Q	RS helix	1042 ± 246	0.87

[a] Rod lengths are the average length ± standard deviation of 40–60 rods measured from electron micrographs of virus particles purified from infected *N. tabacum* tissues.

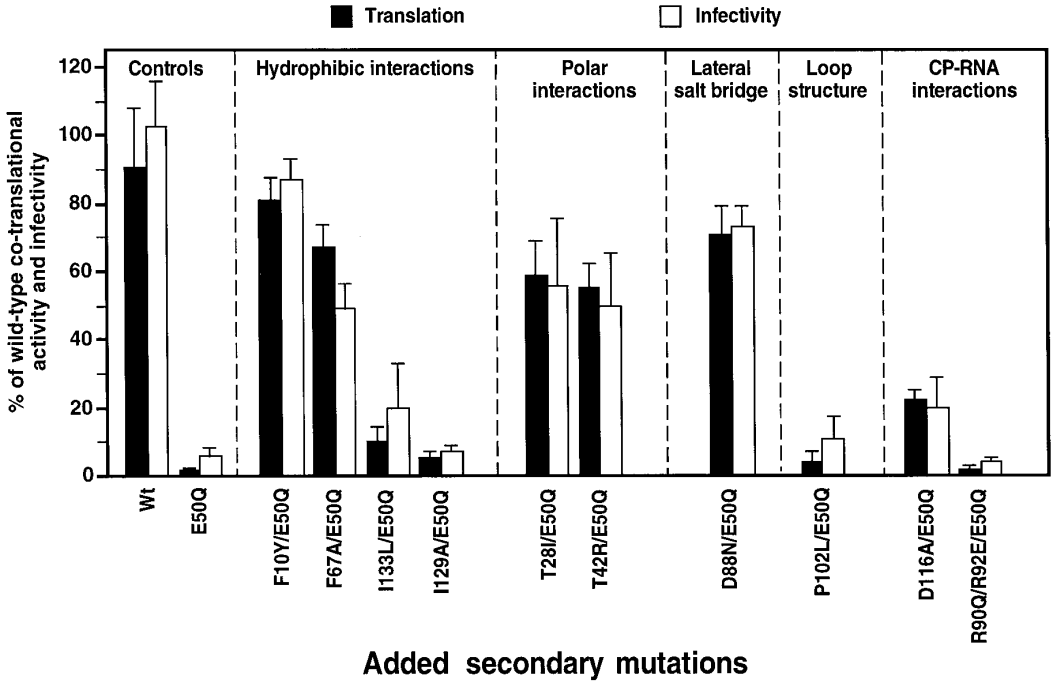

FIG. 2. Infectivity and translational inhibition of wild-type TMV virions treated with a 10,000 fold excesses of mutant or wild-type CP. Infectivity assays were conducted by inoculating one half of each leaf with 0.2 μg of wild-type virions partially reassembled with excess wild-type or mutant CPs, while the other half-leaf received identically treated wild-type virions except with the omission of added extra CP. Numbers of local lesions appearing on each half leaf were used to calculate the percentage of infectivity relative to untreated wild-type TMV. Infectivity data was averaged from eight independent half-leaf assays. Translational assays were done by charging a rabbit reticulocyte lysate system containing 250 μCi/ml of L-[^{35}S]methionine with 25 μg of virions partially reassembled with excess wild-type or mutant CPs. Translational products were resolved by SDS–PAGE and the viral 126-kDa protein was quantified using a PhosphoImager Storm 680 (Molecular Dynamics, Sunnyvale, CA). Translational data were averaged from three independent experiments. Bars represent the percentage of infectivity or translation activity in relation to that of untreated wild-type virions. Error bars represent standard deviation.

that these mutant CPs maintained their overall three-dimensional structures and were competent to self associate. However, the virion-like rods produced by the double mutants were significantly shorter then those produced by the parent E50Q mutant (Table 1). The presence of shorter rods suggests that each of the second-site substitutions interfered enough with the self-association of CP E50Q to disrupt its ability to form long helical rods.

RNA empty rods of differing lengths preclude the use of disassembly assays based on quantifying the exposure of RNA or measuring changes in virion length. To circumvent these problems, an assay measuring the ability of purified mutant CPs to inhibit the disassembly of wild-type TMV virions was used (*5*). This assay takes advantage of the fact that under the appropriate buffer conditions TMV disassembly initiates from the RNA 5′ end and is a partially reversible process (*17–20*). For this procedure, purified wild-type TMV virions (0.4 μg) were incubated for 15 min under mild alkaline conditions (0.01 M Tris–HCl, pH 8.0) that favor the removal of a few CP subunits from the RNA 5′ end (*18, 19*). However, TMV virions treated in this fashion remain resistant to micrococcal nuclease treatment (*18*), indicating that the viral RNA is not sufficiently exposed to allow degradation. Alkaline-buffered virions were subsequently treated by the addition of an equal volume of 0.1 M Tris–HCl, pH 7.4 buffer containing excess purified mutant CPs (4.0 μg) and incubated for an additional 30 min. These conditions favor virion reconstitution (*17*) and presumably allow the mutant CPs to bind to the exposed surface of the virion 5′ end. The partially reassembled virions were pelleted by centrifugation (65,000 rpm in a Beckman TLA100.3 rotor for 10 min at 4°C) to remove any unassociated CP. Virions reconstituted in this manner were then used to inoculate a TMV local lesion host (*N. tabacum* cv. Xanthi-nc) to test for effects on infectivity or assayed in a rabbit reticulocyte system for effects on cotranslational activity. Comparisons in the infectivity (numbers of local lesions) and cotranslational abilities (levels of viral 126-kDa protein) of TMV virions partially reassembled with either excess wild-type, E50Q, or E50Q double mutant CPs were used as measures of the ability of second-site substitutions to interfere with E50Q's inhibition of TMV disassembly. Results from both assays were well correlated, indicating that the observed effects occurred both *in vitro* and *in vivo* (Fig. 2).

Intersubunit hydrophobic interactions play an important role in stabilizing the TMV virion (*7*). At the outer radius of the virion residues P20 and F67 from one

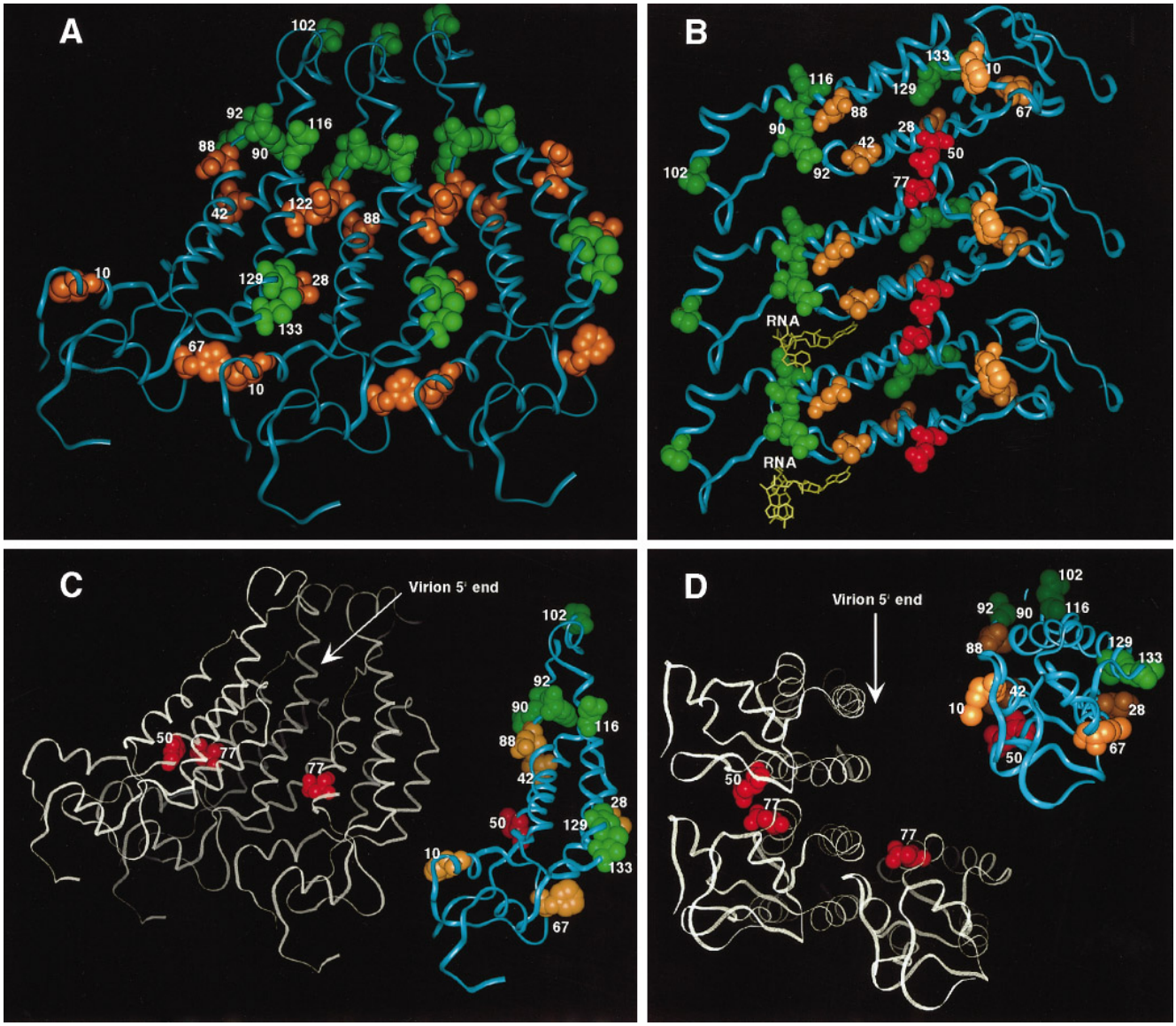

FIG. 3. Spatial location of substituted amino acid residues in the structure of the TMV CP. (A) Lateral interactions between three subunits within the same virion layer, viewed perpendicular to the viral axis. (B) Axial CP–CP and CP–RNA interactions between three subunits located in different layers of the virion, viewed parallel to the viral axis. (C and D) Three subunits at the virion 5′ end and one subunit representing the added CP, viewed either perpendicular or parallel to the viral axis, respectively. Red, carboxylate residues E50 and D77; orange, substituted residues that greatly affected E50Q's ability to inhibit disassembly; green, substituted residues that had less than a 20% effect on E50Q's ability to inhibit disassembly.

subunit along with residues F10 and P7 of the laterally adjacent subunit make up part of a hydrophobic girdle that encircles each CP layer in the virion. At the virion mid-radius the side chains of residues I129 and I133 from one subunit reside in close proximity to residue Y72 of the laterally adjacent subunit. To determine the effects of these hydrophobic interactions on TMV disassembly, disrupting substitutions F10Y, F67A, I129A, and I133L were added to CP E50Q (Fig. 3). Translational and infectivity assays using purified double mutant CPs indicated that substitutions F10Y and F67A reduced E50Q's inhibition of wild-type TMV by an average of 80 and 54%, respectively, while substitutions I133L and I129A did not greatly affect E50Q's ability to inhibit disassembly (Fig. 2).

Surface-exposed polar residues also act to stabilize CP–CP aggregation. Polar residues T28 and T42, located on opposite interfaces, were mutated to I and R, respectively (Fig. 3). These substitutions were designed to disrupt patches of polar residues located along the lateral intersubunit interface. Substitutions T28I and T42R both interfered with the ability of E50Q to inhibit disassembly by more than 52% in translational assays and 43% in infectivity assays (Fig. 2). This indicates that lateral polar interactions disrupted by these mutations contribute to CP E50Q's inhibition of TMV disassembly.

Within the TMV virion there are several intersubunit salt bridges that act to stabilize CP–CP interactions. One such salt bridge is composed of residue D88 from one

subunit interacting laterally with residue R122 of an adjacent subunit (Fig. 3). To disrupt this interaction, D88 was mutated to N. This substitution should not affect CP secondary or tertiary structure but should prevent the attractive negative–positive interaction between D88 and R122. Breaking this salt bridge by mutation D88N caused a 65% reduction in the ability of E50Q to inhibit disassembly (Fig. 2), suggesting that this salt bridge provides an important CP–CP stabilizing factor.

The inner loop structure is located inside the TMV virion and contains many of the residues involved in CP–RNA binding (Figs. 1 and 2). Second-site substitutions made in this CP region were designed to either disrupt the structure of the loop or directly prevent RNA binding. Residue P102 is located at the innermost radius of the loop (Fig. 3). Substitution P102L was created to disrupt the local secondary structure of this region with the intent of obstructing the ability of CP to bind RNA and/or associate with neighboring subunits. However, substitution P102L produced no significant effect on the ability of E50Q to inhibit TMV disassembly (Fig. 2). Within the inner loop, positively charged residues R90 and R92 are directly involved in binding negatively charged RNA phosphate groups (Fig. 3). Substitutions of R90Q and R92E were designed to block or repulse these phosphate groups and prevent RNA from binding beneath the added 5′ terminal subunits. Substitutions R90Q and R92E did not, however, affect the ability of E50Q to inhibit TMV disassembly (Fig. 2). Finally, the side chain of D116 extends upward from the 5′ end of the virion and interacts with the RNA from the subunit surface opposite R90 and R92 (Fig. 3). Within the virion, D116 hydrogen bonds with a ribose hydroxyl group and forms part of a calcium binding site with an RNA phosphate group. Disruption of this CP–RNA interaction by substitution D116A resulted in an average reduction of only 17% in the ability of E50Q to inhibit disassembly (Fig. 2). This reduction in the ability of E50Q to inhibit disassembly may be the indirect result of the D116A mutation altering the position of neighboring residues, including R122, which forms a salt bridge with D88 as described above.

Of the seven second-site mutations made to disrupt intersubunit CP–CP interactions five significantly reduced E50Q's ability to inhibit disassembly by more than 50%. These substitutions were F10Y and F67A, disrupting the outer radius hydrophobic girdle; T28I and T42R, disrupting mid-radius polar interactions; and D88N, disrupting an intersubunit salt bridge. Each of these substitutions differ in the way they affect intersubunit interactions. However, all share the ability to disrupt CP–CP interactions that are oriented toward the virion 5′ end (Fig. 2). Moreover, substitutions I129A and I133L, designed to disrupt interactions oriented away from the virion 5′ end, did not greatly affect E50Q's inhibition of disassembly (Fig. 2). This difference in the effects of second-site substitutions on the ability of E50Q to inhibit TMV disassembly are a consequence of the polar nature of disassembly and the presumed mechanism of inhibition, that is, the exchange of wild-type subunits for mutant subunits at the virion 5′ end. Thus, second-site mutations, F10Y, F67A, T28I, T42R, and D88N, that face the virion 5′ end would interfere directly with the ability of an added CP subunit to bind to the virion 5′ end surface. In contrast, second-site mutations I129A and I133L, which face away from the virion 5′ end, would not directly affect the ability of an added CP to bind to the virion 5′ end. However, already bound subunits of either double mutant I129A or I133L CPs could interfere with the binding of additional CPs, either laterally or axially (Fig. 2). This polar effect on disassembly is consistent with the finding that repulsive carboxylate pair members that face the virion 5′ end, such as E50, have a greater affect on TMV disassembly than pair members, such as D77, that face away from the virion 5′ end (*4*). Thus, the intersubunit interfaces between the exposed 5′ end virion surface and the added terminal CP subunits provide the stabilizing factors that allow E50Q CP to inhibit disassembly.

Second-site substitutions P102L, R90Q/R92E, and D116A were designed to disrupt the RNA binding capabilities of CP E50Q. These mutations failed to significantly interfere with E50Q's inhibition of TMV disassembly. This indicates that the ability of E50Q CP to block virus disassembly is independent of the stabilizing forces conferred from binding viral RNA. This also suggests that the addition of E50Q CP to the 5′ end of a fully assembled virion would be sufficient to inhibit disassembly. To test this possibility, wild-type TMV virions (3 μg) that had not been partially disassembled by exposure to pH 8.0 buffer were treated with (30 μg) either purified E50Q CP or, as a control, purified wild-type CP. Wild-type virions treated in this fashion were subsequently centrifuged as before to remove any unbound CP and inoculated onto a TMV local lesion host to test for infectivity (see Fig. 3 legend for description of infectivity assay). Results demonstrated that the addition of E50Q CP to TMV virions that had not been partially disassembled inhibited infectivity by approximately 67% (averaged from four independent half-leaf assays) compared to an inhibition of approximately 95% for TMV virions that had been partially disassembled prior to the addition of E50Q CP (*4*). In contrast, the addition of wild-type CP had no affect on the infectivity of fully assembled TMV virions. Thus, treatment to partially disassemble TMV virions prior to the addition of E50Q CP is not required to obtain significant reductions in virus disassembly. However, the disassembly treatment does enhance levels of inhibition, possibly by increasing E50Q CP's access to terminal subunit positions at virion 5′ end. Taken together, these findings indicate that E50Q CP does not require binding to exposed 5′ end viral RNA to inhibit disassembly.

The precise 5′ end structure of the TMV virion remains

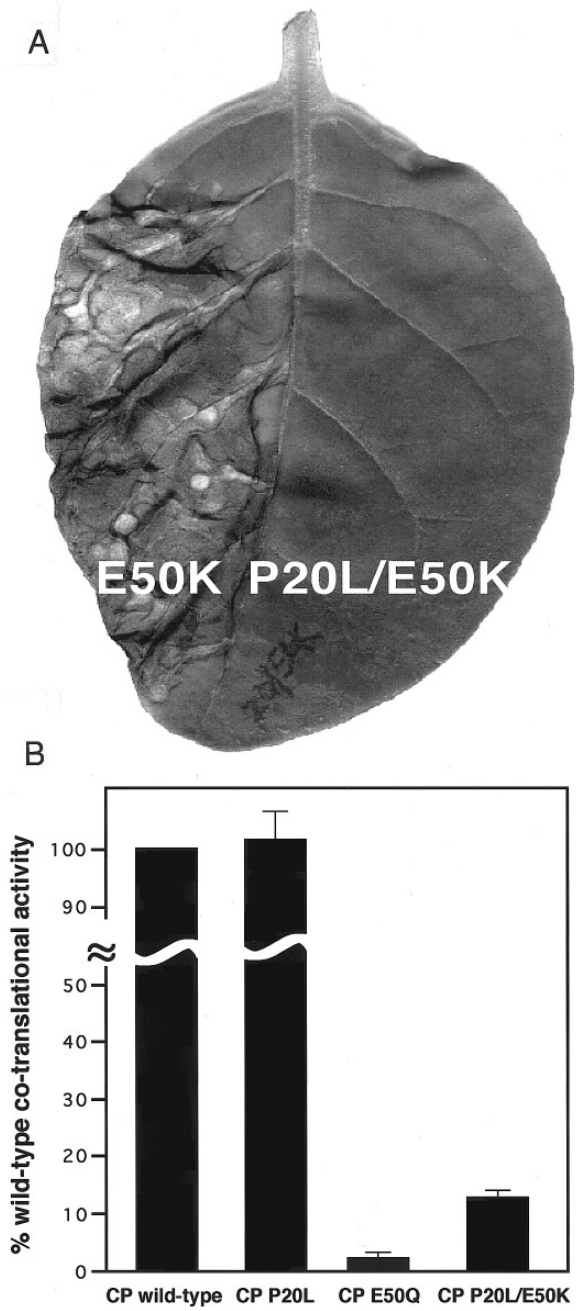

FIG. 4. Introduction of a novel intersubunit salt bridge. (A) Phenotypes of CP mutant E50K and P20L/E50K produced on an inoculated leaf of *N. tabacum* cv. Xanthi; photograph taken 10 days postinoculation. (B) Effects of added CP E50Q and CP P20L/E50K on the ability of wild-type TMV to disassemble in translational assays. Translation of TMV virions with added wild-type, P20L, E50Q, or P20L/E50K CPs. Bars represent the average ± standard deviation of the viral 126-kDa translational product from three independent experiments.

unknown. TMV virions treated under mild alkaline conditions, similar to those used in this study, have been shown to release a few CP subunits from the 5′ end of the RNA (*18, 19*). However, the RNA remains largely protected from nuclease attack (*18*). Wilson (*18*) has suggested that during assembly the 5′ end of the TMV RNA may be completely covered by a final helical turn of CP. These terminal CPs would be inherently more unstable than preceding turns because they would make protein–protein interactions on only one surface, having their other surface exposed to the aqueous environment. This instability would make these terminal subunits more susceptible to the effects of repulsive carboxylate interactions. The loss of these terminal subunits would expose RNA-bound CP subunits that could continue to protect the genome from degradation. Further virion disassembly would require the striping of these CPs from the RNA 5′ end. Factors involved in the removal of these subunits potentially include destabilization by repulsive intersubunit carboxylate groups, aberrant CP–RNA interactions due to the structure of the 7-methylguanosine RNA 5′ end cap (*21, 22*), and weaker CP–RNA interactions in the 5′ RNA leader due to a lack of guanine residues, to which CP binds the most strongly (*23, 24*). Together, these factors may lead to sufficient viral RNA exposure to allow the formation of a translation initiation complex followed by cotranslational disassembly. The ability of CP E50Q to inhibit TMV disassembly through stabilizing interactions that contact the disassembling virion surface but not the RNA suggest that failure to release 5′ terminal CP subunits, which are not bound to viral RNA, blocks subsequent TMV disassembly steps. This finding also indicates that blocking the removal of terminal CP subunits is an effective strategy for preventing disassembly.

Altering molecular interactions that stabilize the virion 5′ end could provide a novel method to prevent disassembly and improve transgenic strategies for disease resistance. Results from this study indicate that stabilizing CP–CP interactions that face the virion 5′ end have the greatest potential to block disassembly. One such interaction found to significantly contribute to 5′ end stability was the intersubunit salt bridge between residues D88 and R122. The D88N substitution created to prevent this interaction should not have disrupted CP secondary or tertiary structure. However, second-site mutant D88N did counteract E50Q's inhibition of disassembly by more than 67%, demonstrating the contribution of this salt bridge in virion stability. Using this information, we attempted to engineer a novel salt bridge into the CP–CP interface. Substitution E50K was designed to have two effects on virion stability. First, E50K removes a repulsive carboxylate group responsible for destabilizing the virion and second, E50K introduces into the intersubunit space a positively charged residue directly opposite the negatively charged D77 residue. Interestingly, the E50K substitution resulted in the induction of generalized necrosis on inoculated plant tissue, an undesirable trait for use in disease resistance strategies. However, combining E50K with substitution P20L mitigated this necrosis (Fig. 4). The mechanism responsible for this varied host response remains unknown, but may be the result

of different CP–host interactions being either enhanced or blocked as a result of the structural changes conferred by the different mutations. The P20L/E50K CP was found to be approximately 20% less effective at inhibiting TMV disassembly than the E50Q substitution (Fig. 4). The reduced inhibition of disassembly by CP E50K could be the result of differences between the side chain volumes of residues E and K, resulting in a suboptimal salt bridge with residue D77 of the axially adjacent subunit. However, the ability of P20L/E50K to inhibit disassembly by more than 80% in comparison to wild-type CP indicates the potential of this strategy to engineer novel CPs that inhibit virus disassembly.

ACKNOWLEDGMENT

This work was supported in part by USDA-NRI Grant 9601102.

REFERENCES

1. Stubbs, G., *In* "Prediction of Protein Structure and Principle of Protein Conformation" (G. Fasman, Ed.), pp. 117–148. Plenum, New York, 1989.
2. Casper, D. L. D., *Adv. Protein Chem.* 18, 37–123 (1963).
3. Bancroft, J. B. (1970). *Adv. Virus Res.* 16, 99–134.
4. Culver, J. N., Dawson, W. O., Plonk, K., and Stubbs, G., *Virology* 206, 724–730 (1995).
5. Lu, B., Stubbs, G., and Culver, J. N., *Virology* 225, 11–20 (1996).
6. Bloomer, A. C., and Butler, P. J. G., *In* "The Plant Viruses" (M. H. V. van Regenmortel and H. Fraenkel-Conrat, Eds.), pp. 19–57, Plenum, New York, (1986).
7. Namba, K., Pattanayek, R., and Stubbs, G., *J. Mol. Biol.* 208, 307–25 (1989).
8. Geisselsoder, J., Witney, F., and Yuckerberg, P., *Bio/Techniques* 5, 786–791 (1987).
9. Dawson, W. O., Dawson, W. O., Beck, D. L., Knorr, D. A., and Grantham, G. L., *Proc. Natl. Acad. Sci. USA* 83, 1832–1836 (1986).
10. Turpen, T. H., Reini, S. J., Charoenvit, Y., Hoffman, S. L., Fallarme, V., and Grill, L. K., *Bio/Technology* 13, 53–57 (1995).
11. Gooding, G. V., and Hebert, T. T., *Phytopathology* 57, 1285 (1967).
12. Kawasaki, E. S., *In* "PCR Protocols: A Guide to Methods and Applications" (M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, Eds.), pp. 21–27. Academic Press, San Diego, CA, (1990).
13. Fraenkel-Conrat, H., *Virology* 4, 1–4 (1957).
14. Bradford, M. M., *Anal. Biochem.* 72, 248–254 (1976).
15. Laemmli, U. K., *Nature (London)* 227, 680–685 (1970).
16. Fraenkel-Conrat, H., and Williams, R. C., *Proc. Natl. Acad. Sci. USA* 41, 690–698 (1955).
17. Bulter, P. J. G., and Klug, A., *Nature New Biol.* 229, 47–50 (1971).
18. Wilson, T. M. A., *Virology* 137, 255–265 (1984).
19. Mundry, K. W., Watkins, P. A., Ashfield, T., Plaskitt, K. A., Eisele-Walter, S., and Wilson, T. M. (1991). *J. Gen. Virol.* 72, 769–77 (1991).
20. Wu, X., Xu, Z., and Shaw, J. (1994). *Virology* 200, 256–262.
21. Keith, J., and Fraenkel-Conrat, H. (1975). *FEBS Lett.* 57, 31–33.
22. Zimmern, D. (1975). *Nucleic Acids Res.* 2, 1189–1201.
23. Steckert, J. J., and Schuster, T. M. (1982). *Nature (London)* 299, 32–36 .
24. Richards, K., Guilley, H., Jonard, G., and Keith, G. (1977). *Nature (London)* 267, 548–550.