Comparison of properties of particles of *Cucumber mosaic virus* and *Tomato aspermy virus* based on the analysis of molecular surfaces of capsids

Luis F. Pacios¹ and Fernando García-Arenal²

Departamento de Biotecnología, ETSI Montes¹ and Departamento de Biotecnología, ETSI Agrónomos², Universidad Politécnica de Madrid, 28040 Madrid, Spain

The plant RNA viruses *Cucumber mosaic virus* (CMV) and *Tomato aspermy virus* (TAV) (genus *Cucumovirus*) have similar icosahedral particles, the crystal structures of which have been reported recently. Similarity in particle structure agrees with reports of stable capsids assembled from their capsid proteins and of viable recombinant viruses with chimeric capsid proteins derived from CMV and TAV. However, differences between the cucumoviruses have been reported for physicochemical properties. Here, structural and electrostatic features of the molecular surfaces are studied to investigate their relationship with these observations. Two coat-protein recombinants with structures modelled by taking CMV and TAV as templates were also included in the analysis. Results show that there exists an external region of negative electrostatic potential that has arisen from strictly conserved charged residues situated near the external HI loop of the subunits in the capsomers. This negative domain surrounds the fivefold and quasi-sixfold axes and locates above regions of positive potential that extend to cover, nearly homogeneously, the inner surface of capsids, where interaction with encapsidated RNA occurs. Differences between the outer electrostatic distributions in CMV and TAV explain the distinct response of both viruses to variations in physicochemical conditions required for particle stability and are essential to rationalize the biological activity of the coat-protein recombinants, in spite of their seemingly distinct electrostatic characteristics.

INTRODUCTION

The genus *Cucumovirus* (family *Bromoviridae*) is characterized by a tripartite, plus-sense, single-stranded RNA genome that is encapsidated into icosahedral particles. The coat protein (CP) is the only protein associated with cucumovirus particles and, in the type species of the genus, *Cucumber mosaic virus* (CMV), it is also necessary for cell-to-cell movement, systemic movement and aphid transmission. Systemic movement and aphid transmission of CMV are thought to occur as virus particles. The structure and physicochemical properties of cucumovirus particles have been analysed in detail (Palukaitis & García-Arenal, 2003).

Cucumoviral particles are 29 nm in diameter, built of 180 CP subunits with $T=3$ quasisymmetry and have an RNA content of about 18% (Habili & Francki, 1974a, b). Biologically active particles can be reassembled readily from RNA and soluble CP by lowering salt content or removing SDS (Kaper, 1969). In contrast to closely related bromoviruses, cucumovirus particles are stable at pH 9–0 and do not swell at pH 7–0, although circular-dichroism and UV-absorption spectra of CMV indicate a transition as pH rises from 5 to 7 (Savithri et al., 1984). Unlike bromoviruses, cucumoviruses do not form empty particles *in vitro* (Kaper & Geleen, 1971). Heterologous assembly between CP and RNAs of CMV and a second cucumovirus, *Tomato aspermy virus* (TAV), has been shown *in vitro* and *in vivo* (Chen & Francki, 1990; Perry & Francki, 1992) and CP subunits of CMV and TAV can assemble *in vitro* into mixed-subunit capsids, emphasizing particle-structure similarity (Chen et al., 1995). Also, hybrid viruses that are CMV/TAV recombinants at the CP gene have been shown to be infectious and to form stable particles (Salánki et al., 2004; Llamas et al., 2006).

The physicochemical properties of CMV and TAV were analysed in detail in the 1970s. Particles are stabilized by RNA–protein interactions and disrupt easily at high concentrations of neutral chloride salts or low concentrations of SDS (Kaper, 1975). Particles are stabilized with EDTA and aggregate in the presence of Mg$^{2+}$ (Habili & Francki, 1974b). Ribonuclease can penetrate intact particles and degrade encapsidated RNA (Kaper & Geleen, 1971; Habili & Francki, 1974b). In spite of their similarities, differences between CMV and TAV in sensitivity to sodium salts, SDS, EDTA and Mg$^{2+}$, and ribonuclease digestion of the encapsidated RNA were noticed (Habili & Francki, 1974a, b).
Recently, the structures of TAV and CMV particles have been solved by X-ray crystallography, with an effective resolution of 3-2 Å for CMV (Smith et al., 2000) and 4-0 Å for TAV (Lucas et al., 2002). The capsid structures offer the opportunity to address existing information about physico-chemical properties of both viruses in the light of structural features, which will help to rationalize experimental data. We focus here on a property essential to investigate interactions involving molecular surfaces: the Poisson–Boltzmann (PB) electrostatic potential. The analysis is presented first for CMV and TAV and then extended to two CMV/TAV CP recombinants. Our results allow us to propose interaction sites and essential surface features relevant to explain the viability of these recombinants.

METHODS

Viruses. The capsid structures reported previously (Smith et al., 2000; Lucas et al., 2002) correspond to strains Fny of CMV (Fny-CMV) and Blencowe of TAV (B-TAV). CP recombinants were obtained between Fny-CMV and strain 1 of TAV (1-TAV, with the same CP amino acid sequence as B-TAV; GenBank accession numbers A)277269 and S72468, respectively) by using the Nhel and Eagl restriction sites at nt 1429 and 1694, respectively, of 1-TAV RNA3. Recombinant CNheT had CP aa 1–59 from Fny-CMV and the remainder (160 aa) from 1-TAV. Recombinant TNheCEagT had aa 1–59 and 149–219 from 1-TAV and aa 60–148 from Fny-CMV. Both recombinants were infectious to tobacco plants and formed stable particles (Llamas et al., 2006).

Structures. The coordinates of crystal structures were downloaded from the Protein Database (PDB), entries 1F15 (CMV, 3-2 Å resolution; Smith et al., 2000) and 1LAI (TAV, 4-0 Å resolution; Lucas et al., 2002). Pentameric and hexameric capsomers were generated by using transformation matrices with Swiss-PdbViewer 3.7 (Guex & Peitsch, 1997). Both CMV and TAV geometries were taken as templates to model target structures for CPs of CMCV and TNheCEagT. Target sequences aligned with template sequences were submitted to the SWISS-MODEL service (Peitsch, 1995; Guex & Peitsch, 1997). Both fragments 1–28 in CMV and 1–33. Structures for these segments were modelled as follows. In electron-density maps, from disordered N termini of the three CPs of CMV, the model peptide 28–61 was predicted with SWISS-MODEL by using transformation matrices with Swiss-PdbViewer 3.7 (Guex & Peitsch, 1997). Both CMV and TAV geometries were taken as templates for CPs of CMCV and TNheCEagT. Target sequences aligned with template sequences were submitted to the SWISS-MODEL service (Peitsch, 1995; Guex & Peitsch, 1997; Schwede et al., 2003) on ‘Project (optimize)’ mode to model two structures for each recombinant from templates CMV and TAV. For ease of notation, we shall hereafter refer to the structures of CNheT modelled from CMV and TAV as v1-CMV and v1-TAV, respectively, and those of TNheCEagT as v2-CMV and v2-TAV. Levels of sequence sharing between templates and models are v1-CMV, 63 %; v2-CMV, 67 %; v1-TAV, 80 %; v2-TAV, 76 %. As these values are well above the 50 % stability threshold, mean square deviations smaller than 1 Å are expected (Guex & Peitsch, 1997).

Crystal structures of CMV and TAV miss residues that are not visible in electron-density maps, from disordered N termini of the three CP subunits. In CMV, segments 1–61, 1–28 and 1–27 are missing from subunits A, B and C, respectively, whilst in TAV, these are 1–43, 1–35 and 1–33. Structures for these segments were modelled as follows. In CMV, the model peptide 28–61 was predicted with SWISS-MODEL by using subunits B and C in 1F15 as templates, whilst segment 1–28 was predicted ab initio by using the Rosetta server (Simons et al., 1997). As Rosetta provides backbones only, non-hydrogen atoms of side chains were rebuilt with PROGEN (Mandal & Linticum, 1993). The resulting structure was corrected for steric hindrances after adding hydrogens with PDB2PQR (Dolinsky et al., 2004) and then reoptimized with GROMOS96 (van Gunsteren et al., 1996). Both fragments 1–28 and 28–61 were then added together upon matching coordinates of pivoting Ala28 and the resulting geometry was reoptimized with GROMOS96, fixing the conformation of segment 28–61. As in TAV there is no information available for homology modelling, an ab initio structure was predicted with the same multi-step procedure of peptide 1–28 in CMV.

Electrostatic potentials and surfaces. Electrostatic PB potentials were obtained for pentamers and hexamers of CPs of CMV, TAV, v1-CMV, v1-TAV, v2-CMV and v2-TAV with the APBS (Adaptive Poisson–Boltzmann Solver) software (Baker et al., 2001). Input PQR files were prepared with PDB2PQR, assigning AMBER99 (Wang et al., 2000) charges and radii to all of the atoms (including hydrogens). Fine grid spacings of approximately 0.5 Å were used to solve the linearized PB equation in automatically configured, sequential-focusing multigrid calculations in meshes of 193–257 points per dimension. Counterion salt concentration was 0·150 M for both negative and positive charges, dielectric constants were 2 for coat proteins and 78–54 for water and temperature was 298–15 K. Accessibility surfaces (Richards, 1977; Connolly, 1983) were computed with APBS and represented graphically with PB potentials mapped onto them. Molecular graphics and images of PB potentials and surfaces were prepared with VMD 1·8·4 (Humphrey et al., 1996) and rendered with POV-Ray 3·6 (POV-Ray, 2004). Values of PB potentials are given in units of kT/e (k, Boltzmann’s constant; T, absolute temperature; e, unit electron charge).

RESULTS AND DISCUSSION

The structures of CMV and TAV have been discussed in depth (Smith et al., 2000; Lucas et al., 2002), so we recall here just some details relevant to our analyses. Particles share an icosahedral shape displaying a T = 3 lattice, with capsids composed of 60 copies of three subunits designated A, B and C that form trimers with quasi-threefold symmetry. There are 20 hexameric capsomers of B and C subunits with quasi-sixfold symmetry and 12 pentameric capsomers of A subunits with fivefold symmetry. We first present how structural differences between CMV and TAV affect features related to surface interactions. The differences in conditions required for particle integrity are then discussed, relating observations with structural and surface characteristics. Finally, the modelled structures of CMV/TAV recombinant variants v1 and v2 are addressed, applying the same qualitative analysis to gain insight into the molecular details of their infectious nature.

Effect of structural differences between CMV and TAV particles on surfaces

Fig. 1 illustrates the structures of individual CPs and trimers of A, B and C subunits, identifying regions of interest to discuss surface properties. Subunit chains have 218 aa in CMV and 217 aa in TAV because of a single deletion (Ser148 in CMV), so residue numberings differ by 1 from position 148. Lucas et al. (2002) provided a thorough comparison of CMV and TAV structures; hence, we discuss here only details that either cast further light on this comparison or affect surface properties. Fig. 1(a) shows some minor differences in local conformations of subunit proteins. The segment containing the EF helix is slightly shifted outwards from the β-barrel in TAV, whilst charged residues making subunit contacts have a wider distribution and greater exposition in TAV. Also, in TAV there is a disulfide bond...
Comparison of surface properties of CMV and TAV capsids

(Cys64–Cys106) linking an external side of the $\beta$-barrel and the coil preceding N-terminal (N-term) helices, whereas CMV has Arg106. The few differences among charged residues are important regarding the electrostatic nature of surfaces. In the region of ionic contacts between subunits, TAV has no acidic residue equivalent to Asp100 in CMV (mutated to Pro100). In the exterior of the $\beta$-barrel, there is a concentration of exposed charged residues, with five acidic amino acids and one basic amino acid in the HI loop that are conserved, but nearby residues differ: four minor mutations (Lys76, Lys79, Asp81 and Arg82 in CMV are Arg76, Arg79, Glu81 and Lys82 in TAV), the substitution Asp118 (CMV) to Asn118 (TAV) (omitted in Fig. 1) and the Asp83–Lys84 acid–base pair in TAV is absent in CMV, which has Gly83 and Ser84.

Regarding trimer arrangements, Fig. 1(b) reveals two major differences that are analysed below in connection with electrostatic potentials. The first is that, in CMV, the A chain is oriented away from B and C, bending toward the fivefold axis, where marked electrostatic differences with TAV are noticed (see pentamers in Figs 4 and 6). The second difference regards the N-term helices protruding into the RNA interior. The shape of these parts in the X-ray structures is somewhat misleading because the missing residues preceding N termini in the A chain include a helix (partly visible in TAV) whereas, in the B and C chains, they form presumably non-helical random-coil segments (Fig. 3). However, CMV and TAV trimers (Fig. 1b) show a clear difference: whilst the N-term helices in the B and C chains are very close and have their helical axes nearly parallel in CMV, they make a large angle, giving the trimer a tripod-like shape, in TAV. As analysed below, the bundles of these helices in the B and C chains cause distinct pore sizes in surfaces around the quasi-sixfold axis in hexamers (Figs 5–7).

Fig. 2 shows the arrangement of trimers into pentameric capsomers about the fivefold axis. The network of ionic intersubunit contacts in the internal space (Fig. 2b) concentrates at the quasi-threefold axes of trimers in the directions indicated by the arrows in Fig. 2(a). Three intersubunit acid–base contacts, Asp100–Lys127, Asp100–Lys182 and Lys101–Asp179 (Fig. 1a), were identified in CMV (Smith et al., 2000), whereas the mutation Asp100Pro prevents two of these contacts in TAV. In fact, Fig. 2(a) shows that TAV has fewer internal contacts than CMV, although the presence of the pair Glu98–Arg127 (Fig. 1a) may compensate partially for that loss (Lucas et al., 2002). As for the charged residues at the external parts of loops, their distances on separate chains seems so large (Fig. 2a) that they probably would not act as direct ionic linkages between subunits (see also Fig. 5a). However, their stabilizing role on the virions is crucial, yet it is played essentially through electrostatic interactions with the exterior of capsids (see below).

**Fig. 1.** (a) C subunit and (b) trimers of A (yellow), B (blue) and C (green) subunits in CMV and TAV. Sticks represent charged, exposed residues (acidic, red; basic, blue) or residues involved in acid (orange)–base (cyan) intersubunit contacts. The N-term helix is directed toward the RNA interior, the EF helix makes intersubunit contact, the FG loop locates at wall surfaces of central pores in pentamers and hexamers and the HI loop (aa 191–198 in CMV, 188–198 in TAV) is mostly exposed. Purple balls in TAV represent disulfide bonds. The nomenclature for secondary elements is that used by Smith et al. (2000).

**Missing residues from crystal structures**

Fig. 3 displays modelled missing structures. In CMV, the helix 31–47 (Fig. 3a) can be located safely at the N terminus of chain A as far as the prediction for residues 28–61 is homology-based (see Methods). The $ab$ initio model of residues 1–28 predicts a tightly packed random-coil backbone that includes nine basic (eight arginines and one...
lysine) and two acidic (aspartate and glutamate) residues. In TAV, the fully \textit{ab initio} model (Fig. 3a) suggests a ‘loose’ helix 27–41, in fair agreement with two independent secondary-structure predictions: \textsc{jufo} (Meiler \textit{et al.}, 2002) predicts a helix at residues 27–41 and \textsc{nnpredict} (Kneller \textit{et al.}, 1990) does the same for residues 28–40. As the X-ray structure of TAV includes N-term helices 44–50 (chain A), 36–49 (B) and 38–49 (C) (Fig. 1b), the model suggests that these should extend to residue 27, thus becoming as long as in CMV. Two nearly parallel coils are predicted for segment 1–27 in TAV, with six exposed arginines in the loop formed by residues 11–18 (bottom of Fig. 3a), no acidic residues and six contiguous asparagines (aa 19—24).

Given the predominance of basic (13/61 in CMV, 9/41 in TAV) over acidic (three in CMV, one in TAV) residues in these fragments, the electrostatic potential is clearly positive, as seen in the greater extension of the +1 isosurface vs the −1 isosurface (Fig. 3b). This strongly electropositive character is also noticed at the extended (−2, +2) scale (Fig. 3c), with electrostatic features rather similar to those of the inner capsid surfaces of CMV and TAV (Figs 4, 5 and 7). However, we do not include these models in the crystal structure of either virus because of their different reliability. When a similar approach was followed to add missing N-term residues in \textit{Cowpea chlorotic mottle virus} (CCMV), nuclear magnetic resonance experimental data were available for a peptide representing them (Zhang \textit{et al.}, 2004). In our case, the inclusion of these models could introduce

Fig. 2. Upper (a) and side (b) views of pentameric arrangements of A, B and C subunits in CMV and TAV. Charged residues are drawn as balls and sticks with the same colours as in Fig. 1. Larger red balls at the quasi-threefold axis in TAV are Glu175.

Fig. 3. Two views rotated vertically by 180° of modelled fragments missing from crystal structures of CMV (residues 1–61) and TAV (1–43). (a) Backbones; (b) +1 (blue) and −1 (red) isosurfaces of PB potential; (c) PB potential mapped onto solvent-accessible surfaces.
shape distortions at the N termini without adding essential information to the electrostatic nature of inner capsid surfaces (see below).

**Electrostatic potentials and surfaces of capsids**

The arrangement of A subunits about the fivefold axis in pentamers (Fig. 4a) shows a large, central hollow surrounded by an upper ring of charged residues that is slightly closer in CMV than in TAV because, as noted above, subunit A is bent slightly toward the fivefold axis in CMV with respect to TAV. This feature, plus the presence in CMV of one extra acidic residue in this region (Asp118), results in differences in outer +1 isosurfaces (Fig. 4b): CMV shows a starry, pentagonal, continuously negative area, whereas TAV has a ring of negative potential, leaving a central hollow connecting the positive potential arising from the lower bundle of N-term helices. The absence of these helices in CMV is the only distinctive feature at inner +1 isosurfaces (Fig. 4b): in TAV, a small protuberance is seen at the

---

**Fig. 4.** (a) Upper (left) and side (right) views of pentamers of A subunits in CMV and TAV. Charged residues are drawn as balls and sticks with the same colours as in Fig. 1. (b) +1 (blue) and −1 (red) isosurfaces of PB potential viewed from the outside (left) and rotated horizontally by 110° (right).

**Fig. 5.** Hexamers of B and C subunits as in Fig. 4.
bottom, whereas CMV exhibits a slight depression. The presence of many basic residues at inner sides of capsids renders positive potentials that dominate the whole internal surface, without suggesting specific sites for RNA interaction.

Hexameric capsomers (Fig. 5a) also show a ring of negative potential about the quasi-sixfold axis, although differences between CMV and TAV are less marked than for pentamers. The outer $-1$ isosurface is connected in CMV, but leaves small clefts in TAV. Below this outermost isosurface, CMV shows a smooth, positive surface that covers also the central hole, whereas TAV has several levels of alternating $-1$ and $+1$ isosurfaces covering the hole’s internal walls. This difference probably arises from the distinct arrangement of N-term helices in the B and C chains noticed when comparing trimers: they are nearly parallel in CMV and at a bent angle in TAV (compare upper views in Fig. 5a). The inner $+1$ isosurface also differs: although the N-term helices retained in X-ray structures are much longer in CMV (compare side views in Fig. 5a), the positive potential is more protruding in TAV. The presence of Asp29 at the innermost end of these helices in CMV, but not in TAV, produces the small, negative hole at the bottom of the inner positive potential in CMV. However, one can reasonably conjecture that, if the missing residues were added, this difference would disappear, leaving a strongly positive potential at the whole internal surface of both capsids.

It should be stressed that charged residues in the HI-loop region play an essential role in configuring the electrostatic nature of surfaces. The external HI loop, whose sequence is conserved among cucumoviruses, is highly antigenic and has a role in aphid transmission, which suggests that it is probably involved in ionic interactions. The ring arising from that region is similar in both capsomers of TAV, but shows marked differences in CMV, which is interesting to relate to differences in quasi-equivalence between subunits reported for the HI loop in CMV: a mAb specific to this loop was found to bind pentamers, but not hexamers (Bowman et al., 2002).

If PB isosurfaces provide information about electrostatic effects originated by the capsomers, accessibility surfaces represent the physical shape of the protein aggregates themselves. Figs 6 and 7 display these surfaces with the PB potential mapped onto them for outer and inner sides.
respectively, of pentamers and hexamers. The existence of large, electronegative rings covering great areas around the centre is the most outstanding feature of external surfaces (Fig. 6). CMV shows a greater contribution of electro-positive regions, especially in hexamers at the external perimeter and at the central hole, these regions being much less positive or even neutral in TAV. As for the internal surfaces (Fig. 7), there is no doubt about their electrostatic role. The strongly positive potential covers the totality of surfaces almost uniformly, except at the outer perimeter. On the whole capsid, however, the internal surfaces of capsomers should merge and the apparent connection with the exterior conveyed by these plots would disappear. The lack of particular features that could highlight the role of specific sites suggests that the interaction with negative phosphate groups of encapsidated RNA must be highly non-specific, as suggested by Smith et al. (2000) for CMV and shown by Zhang et al. (2004) for CCMV. The presence of Asp29 at the innermost end of the N-term helices in the B and C chains of CMV results in the triangular, negative area at the quasi-sixfold axis of hexamer inner surfaces (Fig. 7), although this area would probably vanish if the missing residues were added. The contribution of missing fragments could also change the size of central pores exterior to N-term helices at both fivefold and quasi-sixfold axes reported for CMV and TAV. As no N-term helical segment in the A chain is present in the X-ray structure of CMV, a large pore connects the outer (Fig. 6) and inner (Fig. 7) surfaces in pentamers. The crystal structure of TAV keeps a short, helical segment and TAV pentamers have a small pore (scarcely visible in Figs 6 and 7). However, it is unlikely that missing residues in the B and C chains would change the pore in hexamers, as the N-term helices in these chains are nearly complete in X-ray structures.

**Fig. 7. Internal surfaces as in Fig. 6.**

**Comparison of surface properties of CMV and TAV capsids**

**Relation between observed physicochemical properties and surfaces**

**Effect of SDS on particle stability.** CMV and TAV are known to dissociate in the presence of SDS (Habili & Francki, 1974b), as expected from the strong effect of SDS in breaking protein aggregates, but TAV dissociates more readily than CMV and both viruses are more stable in SDS at pH 9 than at pH 7 (Habili & Francki, 1974b). Although trimers have equal net charge (+2e) for CMV (76 basic/56 acidic residues) and TAV (74 basic/54 acidic residues), its distribution differs, so the electrostatic nature of surfaces may provide a rationalization for the different response to SDS. Dissociation by SDS is ultimately produced by strong, non-electrostatic interactions between hydrophobic amino acids and hydrophobic tails of the...
detergent, but when SDS acts, its negative sulfate heads interact favourably with basic amino acids and unfavourably with acidic amino acids. The predominance of positive or negative charges in proteins will therefore favour or disfavour, respectively, SDS action. As for pH effects, a greater quantity of hydroxyl ions that neutralize positive basic residues is present in basic environments, resulting in less-favourable interactions with SDS and higher stability.

**Effect of Mg\(^{2+}\) and EDTA on particle stability.** Another difference between CMV and TAV is particle stability in the presence of divalent cations. Unlike for CMV, the integrity of the TAV capsid depends on the presence of Mg\(^{2+}\) (Habili & Francki, 1974b; Savithri et al., 1984). At low MgCl\(_2\) concentrations, CMV capsids aggregate and precipitate, whereas those of TAV remain stable. This is consistent with results obtained from EDTA treatment: TAV is degraded rapidly and irreversibly in the presence of EDTA at pH values above 7-5, whereas CMV apparently stabilizes (Habili & Francki, 1974b). It is noted that, although no magnesium was added intentionally during purification and crystallization of TAV particles, the crystal structure included a putative Mg\(^{2+}\) ion to match the electron density around the quasi-threefold axis (Lucas et al., 2002). The side chains of three Glu175 in every subunit are directed toward this axis (see TAV, Fig. 2a). There are no basic amino acids close enough to permit acid–base contacts with Glu175 in an internal region where two acid–base pairs (Glu98–Arg127 and Lys101–Arg178; see TAV, Fig. 1a) help protein aggregation in TAV. We conjecture that repulsions among close negative carboxylate groups at the quasi-threefold axis in TAV (Fig. 2a) could destabilize the aggregation if Mg\(^{2+}\) (or Ca\(^{2+}\)) should not locate within them. This conjecture is further supported by the observed pH dependence of EDTA treatment. At pH values lower than 7, the chelate is protonated and Mg\(^{2+}\) is free, but, at pH values higher than 7-5, EDTA strongly binds divalent cations that should be impeded to counteract the repulsion among glutamates, leading to TAV degradation, as observed.

Although no metals were included in the crystal structure of CMV, there remained an unassigned portion of electron density located above the cluster of acidic residues near the HI loop (Asp118, Asp192 and Glu198) that could represent a bound cation (Smith et al., 2000). Although cations are not necessary for CMV stability, our findings on potentials and surfaces (Figs 4–6) permit the possibility that HI-loop residues could form a metal-chelating site, as conjectured by Smith et al. (2000). Nevertheless, the observed effects of Mg\(^{2+}\) and EDTA on the stability of CMV should indicate that binding divalent cations could disfavour the (implicitly stabilizing) role played by these residues in untreated CMV virions. The observation that CMV apparently stabilizes with EDTA at pH values higher than 7-5 is consistent with this remark.

**Salt effects on particle stability.** Cucumoviruses disassemble in high concentrations (1-5 M) of NaCl, reassembling into biologically active particles upon returning to physiological conditions, provided that RNA is present (Kaper, 1975). At lower salt concentrations, however, differences between TAV and CMV were observed: whilst over 50% of CMV particles precipitated on incubation in 0-14 M NaCl at 37 °C, this treatment had very little effect on TAV (Habili & Francki, 1974b). Unlike bromoviruses, particles of cucumoviruses do not swell at neutral pH and low ionic strength and remain stable at basic pH (Smith et al., 2000). These observations point to the existence of ion–acid–base interactions between subunits as a supporting effect to stabilize capsids. Figs 1, 2 and 5 show a number of acid–base pairs favouring attractions, whereas the equivalent interface in the bromovirus CCMV has a cluster of only acidic residues (Speir et al., 1995). However, to feel salt effects, the interacting side chains must be accessible to Na\(^{+}\) and Cl\(^{-}\) so that these ions are able to block carboxylate and ammonium groups, avoiding acid–base interactions that link subunits and thus causing disassembly of capsids. As PB isosurfaces (Figs 4 and 5) and molecular surfaces (Fig. 6) illustrate, acidic and basic residues below the HI loop are exposed enough in both CMV and TAV. The slight local predominance of positive and negative exposed sites in CMV relative to TAV could explain the lower sensitivity of TAV to low salt concentrations.

**Degradation by RNase.** In contrast to CCMV, which is RNase-resistant, both CMV and TAV are degraded in various concentrations of RNase A, although TAV is more resistant than CMV (Habili & Francki, 1974b; Kaper, 1975). It has been suggested that cucumovirus capsids must be sufficiently open to allow RNase A accessibility to encapsidated RNA (Jacrot et al., 1977; Smith et al., 2000; Lucas et al., 2002). In fact, whilst no pores are observed in the CCMV capsid (Zhang et al., 2004), CMV and TAV show open pores about the fivefold and quasi-sixfold axes (Figs 6, 7) that facilitate accessibility of the top of the N-term helices to particle exteriors. Assuming that the access of RNase must occur throughout these helices, larger pores should favour degradation of encapsidated RNA. However, whilst pentamers differ in showing a very small pore in TAV, hexamers have similar large pores in both viruses (Figs 6, 7). One therefore must consider whether only pentamers are involved in RNase sensitivity (thus explaining the greater resistance of TAV), as conjectured previously (Lucas et al., 2002). Cleavage of phosphate groups by RNase A involves an unusually reactive lysine that catalyses the process after electrostatic binding of negative phosphates of RNA to lysine and arginine in the enzyme (Metzler, 2001). RNase accessibility should therefore be hindered by the presence of positive potential near the pores, as occurs in the sixfold starry area around the hexamer pore in CMV (Fig. 6). As CMV is less resistant
to RNase than TAV, surfaces suggest that pore size is the only variable determining RNase accessibility, thus lending support to the conjecture that it occurs only at pentamers. The inclusion of missing residues should not change this, because it seems unlikely that the small pore in TAV pentamers would then become bigger and, besides, it is reasonable to assume that the effect of internal residues on the external surface is negligible.

**Variant viruses with CMV/TAV chimeric capsid proteins**

Notwithstanding the differences between CMV and TAV discussed so far, stable particles form from CP subunits of both viruses (Chen *et al.*, 1995) and recombinant viruses with chimeric CMV/TAV CP also form stable particles and are infectious (Salánki *et al.*, 2004), indicating that the similarities must be dominant for biological activity. We discuss the CMV/TAV chimeric variants *v1* and *v2* by comparing their surfaces and potentials with CMV and TAV in an attempt to identify properties essential to their activity. For this, one must analyse the mutations in the charged residues highlighted above (Fig. 1a), comparing both *x*-CMV variants with CMV and both *x*-TAV variants with TAV. Charged amino acids exposed near the HI loop and involved in subunit contacts are identical in *v1*-TAV and *v2*-CMV. All of the charged residues in the HI loop plus Lys116 are also conserved in the other variants. *v1*-CMV lacks Asp118 (substituted by Asn118), but adds the Asp83–Lys84 pair (Gly83 and Ser84 in CMV) plus the minor mutations Lys76Arg, Lys79Arg, Asp81Glu and Arg82Lys. Conversely, *v2*-TAV lacks the Asp83–Lys84 pair (having Gly83 and Ser84) and presents the minor mutations Arg76Lys, Arg79Lys, Glu81Asp and Lys82Arg. As for charged residues

**Fig. 8.** −1 (red) and +1 (blue) isosurfaces of PB potential of CMV, TAV and the two variants studied. Images in every panel as shown for CMV: outside (a, b) and inside (c, d) views of pentamers of A subunits (a, c) and hexamers of B and C subunits (b, d).
making subunit contacts, positions 98, 101, 179 and 182 of CMV are conserved in v1-CMV, which lacks Asp100 (substituted by Pro100) and presents minor changes Lys127Arg and Asp176Glu. All of the positions in TAV are also conserved in v2-TAV, except the minor mutation Arg127Lys. Global net charges differ considerably. Although the number of acidic residues in every trimer of A, B and C subunits is the same as in the parent viruses (56 in CMV and x-CMV and 54 in TAV and x-TAV), the number of basic residues differs: 76 in CMV, 79 in v1-CMV and 70 in v2-CMV, 74 in TAV, 80 in v1-TAV and 68 in v2-TAV. This gives, for trimers, net charges of +23e in v1-CMV, +26e in v1-TAV and +14e in both v2-CMV and v2-TAV.

Fig. 6 compares external surfaces, whilst Fig. 8 shows external PB isosurfaces. According to the mutations introduced in chimeric CPs, overall similarities are noticed between CMV and v2-CMV and between TAV and v1-TAV. On the contrary, v1-CMV and CMV differ in the smaller negative potential, more so in hexamers than in pentamers, and v2-TAV exhibits greater negative external areas than TAV in both pentamers and hexamers, particularly in PB isosurfaces. The relative contribution of positive potential in less-outer exterior regions shows a different pattern: both v1 variants are more similar to their CMV and TAV counterparts, whereas both v2 variants exhibit smaller electropositive regions than their parent viruses (see especially Fig. 8). Another interesting difference regards the net charges, positive area located around hexameric pores; this distinctive feature of CMV occurs in both v1 variants and not in either v2 variant.

Fig. 7 compares internal surfaces, whilst Fig. 8 shows internal PB isosurfaces. Differences are now easier to summarize: the extension of positive potential dominating the capsid interiors is similar in both v1 variants and their CMV and TAV counterparts, but somewhat smaller in both v2 variants. It is worth noting that the v2 variants show small neutral or slightly negative regions in the internal surface (Fig. 7). Although Asp29, located at the end of the N termini in CMV, is conserved in both variants, its electrostatic contribution, seen as a triangular hole at the centre of inside views of +1 isosurfaces in hexamers (Fig. 8), is maintained in v1-CMV, but not in v2-CMV, which has a largely protruding, positive isosurface covering that centre completely.

As recombinants v1 and v2 are infectious and yield stable particles (Llamas et al., 2006), none of the differences should be determinant to their biological activity. Hence, one major conclusion is that an external surface with an outermost electrostatic negative ring protruding from an overall positive or neutral surface, plus an internal strongly positive surface, are two essential features needed for biological activity. Another issue is how differences might affect properties related to particle stability under distinct environments. From the above arguments and comparison between both variants, the following predictions can be made: (i) v1 particles should dissociate more readily in the presence of SDS than v2 particles, which have a more acidic exterior, (ii) v1 should be less sensitive to low salt concentrations than v2, which has marked ionic changes at side ends of external surfaces where intersubunit contacts occur, (iii) v2 should be more resistant to degradation by RNase than v1, which has positive potential at hexagonal pores, and (iv) v2 should present slightly less favourable interactions with RNA, permitting easier disruption of capsid–RNA association, as its internal positive potential is smaller than in v1. Finally, considering their rather different net charges, a slower electroforetic mobility can be predicted for v2.

More theoretical and experimental work is needed to reach a complete picture of molecular details explaining the biological activity of, and differences between, CMV and TAV, but the information and analyses presented permit some reasonable conjectures on the role of surface sites involved in physicochemical interactions underlying the biological activity of cucumoviruses. Moreover, this study could be the basis for future experiments: the availability of full-length, biologically active cDNA clones of the genome of several strains of CMV and TAV (Palukaitis & García-Arenal, 2003) would allow obtaining viral strains with mutant and chimeric capsids to test some hypotheses presented.

REFERENCES


