A Salt-stable Mutant of Cowpea Chlorotic Mottle Virus

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SUMMARY

The properties of a mutant of cowpea chlorotic mottle virus which, although infective, cannot be disassembled in 1 M-NaCl, pH 7·5, are described. The mutant coat protein contains a lysyl to arginyl replacement which affects the hydrodynamic and titration characteristics of the virus which are discussed in regard to the anomalous ionization of a carboxyl-carboxylate pair and a lysyl residue.

INTRODUCTION

The amino acids, other than a cysteiny] residue (Bancroft et al. 1971) and a carboxyl-carboxylate pair, critical in the swelling and disassembly of cowpea chlorotic mottle virus (CCMV) are unknown although titration studies (Johnson, Wagner & Bancroft, 1973) have implicated basic residues. In this paper, we describe and discuss effects of a lysyl to arginyl replacement on the behaviour of the virus.

METHODS

Mutagenesis of wild-type CCMV RNA with nitrous acid was carried out as before (Bancroft et al. 1971; Bancroft et al. 1972) and the mutant was passed through three serial single-lesion transfers.

The mutant and type CCMV were grown, purified, phenol extracted, examined in the analytical centrifuge, assayed and their amino acid composition determined as previously described (Bancroft et al. 1971; Bancroft et al. 1972). Titrations were carried out as for Johnson et al. (1973) except that pH 2·5 rather than pH 4·2 was used as the starting point in the critical acid-base titrations.

RESULTS

Stability and hydrodynamics

The mutant is more stable than wild-type CCMV. The latter disassembles in 1 M-NaCl, pH 7·5, whereas the mutant does not (Fig. 1 A) even if it is extracted from tissue under reducing conditions (Bancroft et al. 1971). When wild-type virus at about 10 mg/ml is subjected to disassembly conditions, the protein yield is usually about 50 % of the theoretical. The residual nucleoprotein which sediments is not soluble at pH 5 or 7 and is not infective. In contrast, the mutant nucleoprotein obtained after dialysis against 1 M-NaCl, pH 7·5, followed by ultracentrifuging is recovered quantitatively in solution and is infective (Table 1).

The mutant and wild-type viruses both sediment at 88 S, at pH 5, in 0·1 M-NaCl. At pH 8·0, 0·13 M-NaCl, wild-type CCMV partly degrades and partly precipitates, the re-
Fig. 1. Schlieren diagrams of mutant and wild-type CCMV at 2 mg/ml after various treatments. (A) Upper, wild-type; lower, mutant; after 48 h dialysis against 1 M-NaCl, pH 7.4 (0.03 M-tris) containing 1 × 10⁻³ M-dithiothreitol. (B) Wild-type virus after; upper, 48 h dialysis against 0.13 M-NaCl, 0.03 M-tris, pH 8.0; lower, 48 h dialysis against 0.1 M-NaCl, 0.03 M-tris, pH 8.0, 0.01 M-MgCl₂. (C) Mutant virus after: upper, 48 h dialysis against 0.13 M-NaCl, 0.03 M-tris, pH 8.0; lower, 48 h dialysis against 0.1 M-NaCl, 0.03 M-tris pH 8.0, 0.01 M-MgCl₂. Pictures taken 10 to 13 min after reaching a speed of 40000 rev/min.

Table 1. The specific infectivities of mutant and wild-type CCMV before and after dialysis against 1 M-NaCl, pH 7.5, and of their RNA's after different storage periods

<table>
<thead>
<tr>
<th>Days storage of virus‡</th>
<th>Virus*</th>
<th>RNA§</th>
<th>Virus</th>
<th>RNA§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 days</td>
<td></td>
<td>40 days</td>
<td></td>
</tr>
<tr>
<td>Mutant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Previous treatment†</td>
<td>Virus</td>
<td>RNA</td>
<td>Virus</td>
<td>RNA</td>
</tr>
<tr>
<td>Mutant</td>
<td>—</td>
<td>221</td>
<td>65</td>
<td>112</td>
</tr>
<tr>
<td>Mutant 1 M-NaCl</td>
<td>1 M-NaCl</td>
<td>241</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>—</td>
<td>315</td>
<td>223</td>
<td>125</td>
</tr>
<tr>
<td>Wild-type 1 M-NaCl</td>
<td>—</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Virus obtained from sister groups of cowpea infected for 13 days.
† A dash signifies that virus was kept in 0.1 M, pH 5, acetate buffer. 1 M-NaCl signifies that the virus (8 mg/ml) was dialysed for 2 days against 1 M-NaCl, pH 7.5, and was then pelleted in the ultracentrifuge.
‡ Storage of virus was in 0.1 M, pH 5, acetate buffer at 4 °C. The RNA was extracted from virus stored either for 15 or 40 days.
§ Local lesions on 10, half-leaves of soybean. Assays were made at E₆₀₀ = 0.1. The denatured product in the wild-type 1 M-NaCl, pH 7.5, treated CCMV was applied at about 3 mg/ml.

remaining virus sedimenting at 78 S (Fig. 1 B). Under the same conditions, the mutant remains largely intact although a shoulder is apparent (Fig. 1 C) and the virus sediments at 83 S. The heterogeneity of the mutant occurs regardless of whether or not the virus has first been dialysed against 1 M-NaCl, pH 7.5, and recentrifuged, a treatment which removes all wild-type CCMV. Both the wild-type and mutant virus respond to Mg²⁺ at pH 8 (Fig. 1 B, C) but to different extents in terms of sedimentation rates. The wild-type sediments about 5 S faster at pH 8.0 in the presence of Mg²⁺ than in its absence, whereas the increase in sedimentation rate of the mutant is only about 2 S under the same conditions in sister cells.
Salt-stable CCMV mutant

Fig. 2. Mutant (left) and wild-type CCMV symptoms on cowpea 3 weeks after inoculation.

Infectivity

The specific infectivity of the mutant, which produces very mild symptoms in comparison to those of wild-type in cowpea (Fig. 2), is about 70% of that of sister preparations of wild-type CCMV (Table 1). The infectivity of mutant virus decreased in comparison to that of wild-type during storage, whereas the infectivities of the RNA’s remained about the same (Table 1), suggesting that the infectivity loss was coat associated. The RNA from the mutant gave identical patterns on acrylamide gels to those of wild-type. The length of infection time in cowpea also affected relative specific infectivities. Mutant virus from plants infected for 6 days and assayed 3 days after purification had a specific infectivity of 68% of the wild-type virus, whereas mutant virus from the same series of plants infected for 26 days was only 25% as infective as wild-type virus from the same aged infection assayed 3 days after purification.

The infectivity of the mutant in 1 M-NaCl, at pH 7.5 (Table 1) and at pH 8.0 in 0.13 M-NaCl (Table 2), in comparison to that of the wild-type, reflects the differences in the physical stabilities of the two isolates. Mg^{2+} had a marked effect on the infectivity of wild-type CCMV at pH 8, whereas the effect was relatively slight on the mutant which is unable to assume the 78 S form in the absence of Mg^{2+} at pH 8.
Table 2. The percentage specific infectivities of mutant and wild-type CCMV before and after dialyses against 0.1 M-NaCl with and without Mg$^{2+}$ at pH 5 and 8

<table>
<thead>
<tr>
<th>Virus*</th>
<th>Previous treatment†</th>
<th>Specific infectivities (%)</th>
<th>§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant</td>
<td>pH 5</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>Mutant</td>
<td>pH 8†</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>Mutant</td>
<td>pH 8 + Mg$^{2+}$†</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Mutant</td>
<td>1 M-NaCl, pH 8†</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>pH 5</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>pH 8</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>pH 8 + Mg$^{2+}$</td>
<td>76</td>
<td></td>
</tr>
</tbody>
</table>

* Virus obtained from sister groups of cowpea infected for 13 days.
† Dialysis was for 2 days against 0.13 M-NaCl buffered with 0.02 M-acetate or tris at pH 5 or 8, respectively or against 0.1 M-NaCl, pH 8 containing 0.01 M-MgCl$_2$.
‡ Virus first sedimented from 1 M-NaCl, pH 7.5, as for Table 1, and then dialysed at pH 8.0 as above.
§ Local lesions on 10 half-leaves of soybean. Assays made at pH 5, at $E_{260} = 0.1$.

Amino acid composition

Eight overall analyses of this mutant showed that it contained one lysine residue less and one arginine residue more than the wild-type which was also analysed eight times in tandem with the mutant. Since there was no detectable difference in the two-dimensional tryptic peptide maps of the mutant and wild-type proteins, the amino acid replacement(s) must occur in the two large peptides which constitute the major part of the carboxyl end of the protein. These peptides, which consist of 16 and 46 amino acid residues, respectively, do not move from the point of application of the tryptic digest to the paper.

Titrations

The mutant virus was titrated and compared with controls consisting of wild-type CCMV and another mutant which we have been unable to disassemble after normal extraction procedures because of an arginyl to cysteinyl replacement (Bancroft et al. 1971). Sequential basic and acidic titrations of the lysyl to arginyl mutant of about 1.5 h each showed only very limited hysteresis as compared to that found for the wild-type virus (Johnson et al. 1971). If wild-type CCMV is titrated from pH 2.5 to 9.0, the portion from pH 4.2 to 8.5 corresponds well with that of the narrower range titration (Johnson et al. 1973). If the pH is lowered to 3 again, there is considerable irreversible precipitation. This effect is not observed with lysyl to arginyl mutant, nor is it observed with wild-type CCMV titrated in the presence of Mg$^{2+}$. A comparison of basic titration curves from pH 2.5 to 8.75 (Fig. 3A) shows that the mutant differs from the wild-type virus in two titratable groups. The first of these corresponds to the proton released from the carboxylic-carboxylate pair. The proton dissociates at a lower pH ($pK \sim 4.6$) from the mutant than from wild-type CCMV. This is not simply a consequence of the mutant being unable to swell normally because the basic titration of the control arginyl to cysteinyl replacement mutant is similar to that of the wild-type virus. Further, the Mg$^{2+}$ titration of the arginyl to cysteinyl mutant (Fig. 3B) is like that of the wild-type virus (Johnson et al. 1973), whereas no protons are released by Mg$^{2+}$ from the lysyl to arginyl mutant (Fig. 3C) at pH 6 because they have already been lost in contrast to their loss at a higher pH for the other mutant and the wild-
type virus. We ascribe, as will be discussed, the second difference to the neutralization by \([\text{OH}^-]\) of the abnormally ionizing \(\epsilon\text{-NH}_3^+\) of a lysyl residue in the wild-type virus which is replaced in the mutant by an arginyl residue.

**DISCUSSION**

The mutant differs from a previously described stable mutant with an arginyl to cysteiny1 replacement (Bancroft *et al.* 1970). The latter was stable only if it was not extracted under reducing conditions in which case it was non-infective because it could not be uncoated by the host probably because covalent bonds were involved. The present mutant does not ‘swell’ normally either and as a result is also more stable than type CCMV. In spite of this and our inability to disassemble it, the mutant is infective suggesting that conversion to the 78 S form of the virus is not a necessary step in the uncoating mechanism prior to infection.

The lysyl to arginyl replacement represents the only change in the coat protein observed...
Fig. 4. An illustration of the ionization of titration groups and their difference plots critical in the configuration of CCMV. The pK's are estimated from Fig. 3A. (a) Wild-type CCMV releases a proton from a carboxyl-carboxylate pair resulting in the exposure of a lysyl residue and the uptake of a hydroxyl ion. (b) Wild-type CCMV also releases a proton in the presence of Mg\(^{2+}\), but Mg\(^{2+}\) stops repulsion in the acidic pair and the virus is stabilized and does not swell completely. There is no hydroxyl ion uptake around pH 6.8 because the lysyl residue is not exposed. (c) The lysyl to arginyl mutant carboxyl-carboxylate pair ionizes at a lower pH than the corresponding pair in the wild-type and there is no hydroxyl ion uptake at pH 6.8 because of the original replacement. There is no difference between the ionization of this mutant and wild-type virus at pH 3.6.

and is consistent with the action of nitrous acid, but sequencing may show exchanges. It is perhaps remarkable that as a result of a putative point mutation on RNA species 3 which carries the coat protein gene, the mutant and type strains induce such different symptoms in cowpea. However, this has also been observed for other CCMV mutants (Bancroft et al. 1971; Bancroft et al. 1972) and, as discussed by Bancroft & Lane (1973), there is evidence based on various crosses and observations on mutation revertants that coat mutations can affect symptomatology.

The central structural questions with the present mutant are how a lysyl to arginyl replacement stops the virus from swelling completely (thereby restricting the hysteresis effect), stops disassembly and affects the ionization of a carboxyl-carboxylate pair. Two mutants with replacements which restrict complete swelling cannot be disassembled in vitro. The arginyl to cysteinyl mutant (Bancroft et al. 1971) is insensitive to 1 M-NaCl, pH 7.5, presumably because of intersubunit covalent bonds. The lysyl to arginyl replacement may also be concerned with an intersubunit linkage but electrostatically so that the ‘shared proton’ (Johnson et al. 1973) interaction is involved. That is, normally the ε-NH\(_3^+\) (with a lowered pK) of lysine is present but because the pK of the guanidino NH\(_3^+\) of arginine is higher, it does not deprotonate near neutrality and the subunits remain attracted either directly or through the RNA. Implicit in this statement is an alternative explanation to that, which invoked the loss of a proton from a carboxyl-carboxylate pair (Johnson et al. 1973), for the characteristic titration hump near neutrality such as found on curve a, Fig. 3A. That is, the
titration inflection with a $pK \sim 6.8$ of wild-type CCMV could result, not from the release of a proton, but rather from the uptake of a hydroxyl ion by a lysyl residue with an abnormally low $pK$ and the difference with the lysyl to arginyl mutant would result from the more basic nature of the guanidino group (Fig. 4). It is well established that the ionization of amino groups can be considerably reduced by, for example, the proximity of positively charged groups (Rometsch, Marxer & Miescher, 1951). The temperature independence noted by Johnson et al. (1973) as indicating that the titration hump reflected an acidic rather than a basic ionization could be a measure of the ionization of the carboxyl-carboxylate pair controlling the exposure of a basic residue. The hydroxyl ion uptake interpretation for the titration hump is consistent with the basic titration pattern of wild-type CCMV in the absence of $\text{Mg}^{2+}$ compared to that done in its presence (Fig. 4) as well as with the titration with $\text{Mg}^{2+}$ of CCMV (Johnson et al. 1973). Complete swelling of the virus from the 88 S to the 78 S form involves two principal reactions; the ionization of a carboxyl-carboxylate pair with a $pK \sim 6.2$ which controls the subsequent exposure and ionization of a lysyl residue with a $pK \sim 6.8$. The mutant allows us to observe that the virus may go through an intermediate 83 S stage, perhaps analogous to that reported for brome mosaic virus (Incardona, McKee & Flanegan, 1973), before reaching the fully swollen 78 S form found with wild-type CCMV when the amino groups become exposed giving the hysteresis effect (Johnson et al. 1973). We have estimated that there are four basic groups involved with CCMV (Johnson et al. 1973) as well as with brome mosaic virus (unpublished) and the exposure may occur in a ‘zipper’-like fashion, the lysyl to arginyl replacement representing the first of a series which is probably located near the C-terminus. The effect of the arginyl replacement on lowering the $pK$ of the carboxyl-carboxylate pair in the mutant may be steric in that the size of the guanidino side chain is such that it is not unreasonable to propose that the $pK$ of the carboxyl-carboxylate is lowered as observed. The essential electrostatic switch mechanism (Durham & Klug, 1972) of CCMV is thus conserved just as it is in diverse strains of tobacco mosaic virus (Butler & Durham, 1972).

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REFERENCES


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