Stability of Belladonna Mottle Virus Particles: the Role of Polyamines and Calcium

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SUMMARY

The stability of belladonna mottle virus (BDMV) has been studied with respect to elevated pH and to freezing and thawing. BDMV, purified by a modified procedure, was stable at alkaline pH, in contradiction to earlier reports. This difference in the stability could be attributed to the presence of 90 to 140 molecules of spermidine, 20 to 50 molecules of putrescine and 500 to 900 calcium ions in each virus particle. The polyamines could be easily exchanged with other cations such as potassium or caesium and this resulted in a loss of particle stability. These cations may therefore play a role in maintaining the integrity of particle structure. The formation of empty protein shells as a result of freezing and thawing BDMV particles parallels earlier observations on turnip yellow mosaic virus particles.

INTRODUCTION

Belladonna mottle virus (BDMV, Moline & Fries, 1974) belongs to the tymovirus group of monopartite RNA plant viruses. The nucleic acid in these viruses is encapsidated in a T = 3 icosahedral shell of protein subunits with an approximate $M_r$ of 20 000. The type member of this group, turnip yellow mosaic virus (TYMV) has been extensively studied in terms of the forces that stabilize the particle structure. The virions of TYMV are stabilized by strong hydrophobic interactions between the protein subunits (Kaper, 1971, 1975). Neutron scattering experiments revealed that there is little or no penetration of nucleic acid into the densely packed protein coat of TYMV (Jacrot et al., 1977). A variety of conditions, such as pH above 11.5, freezing and subsequent thawing, elevated temperatures around 45°C or exposure to certain chemical agents, result in the release of nucleic acid and formation of empty shells from the virions of TYMV (Kaper, 1960, 1971, 1975; Katouzian-Safadi et al., 1980; Katouzian-Safadi & Berthet-Colominas, 1983; Keeling & Matthews, 1982). While TYMV has been the subject of many detailed studies, other members of this group of viruses have been examined in less detail. Recently, it has been shown that RNA is released from BDMV at alkaline pH but that the addition of polyamines and cations prevents this release (Virudachalam et al., 1983a, b). However, BDMV purified in our laboratory by a modified procedure was stable at alkaline pH. This observation prompted a detailed examination of the stability of BDMV particles. The results presented in this paper highlight the role of polyamines and other cations in stabilizing the particle structure and provide an explanation for the instability of BDMV isolated earlier (Virudachalam et al., 1983a).

METHODS

Isolation of BDMV. BDMV was propagated in either Nicotiana glutinosa or N. clevelandii or the hybrid N. clevelandii × N. glutinosa (Christie, 1969) plants. Infected leaves were harvested 10 to 14 days after inoculation and homogenized in either 0.05 M-sodium citrate or 0.05 M-sodium acetate buffer at pH 5.5. The virus was purified by
differential centrifugation. Empty capsids formed in vitro were separated from intact particles by centrifugation in a Beckman model L-8 ultracentrifuge for 4 h at 25000 r.p.m. in an SW27 rotor using a 15 to 40% (w/v) sucrose gradient. Subsequently the virus was processed through one more cycle of differential centrifugation and resuspended in 0·05 M-sodium citrate (or acetate) buffer pH 5·5. Only trace amounts of empty capsids were detected when the purified virus was examined using a Beckman model E analytical ultracentrifuge equipped with Schlieren optics (Fig. 3a, top trace). This virus sample was found to be serologically identical with purified BDMV kindly supplied by Dr P. Argos, Purdue University, West Lafayette, Ind., U.S.A.

Stability of BDMV at alkaline pH. BDMV (2 to 10 mg/ml) was dialysed against 500 ml of 0·05 M-sodium citrate pH 5·5 for 18 h at 4°C for equilibration. The virus solution was then dialysed separately against 500 ml of 0·01 M-sodium acetate pH 5·0 or 0·01 M-Tris-HCl, at either pH 7·0 or pH 9·0 for another 48 h with four changes of 500 ml of the corresponding buffers. The virus samples (400 μg in 400 μl) were layered on 10 to 45% (w/v) sucrose density gradients in the respective buffers prepared for a Beckman SW50·1 rotor and centrifuged at 45000 r.p.m. for 1 h. The fractions (nine drops collected manually) were analysed by estimating the protein concentration by the Lowry method (Lowry et al., 1951), using bovine serum albumin as standard, and by centrifugation in either a Beckman model E analytical ultracentrifuge equipped with Schlieren optics or a Beckman model L8-70 ultracentrifuge with a u.v. preparation scanner.

Estimation of calcium and polyamines. A suspension of virus (50 mg/ml) in 0·05 M-sodium acetate pH 5·5 was dialysed at 4°C for 48 h against 50 ml of the same buffer with four changes. The virus concentration was re-estimated after dialysis by the Lowry procedure (Lowry et al., 1951). The calcium content of these samples was estimated by the fluorometric method of Kepner & Hercules (1963). Protein was precipitated by the addition of trichloroacetic acid to 10% (w/v) and the mixture was centrifuged for 1 min in an Eppendorf centrifuge. To a 20 μl sample of this clear supernatant fraction, 1 μl of calcein (Sigma) (16 mg/ml) and 5 μl of 0·4 M-KOH were added. The fluorescence intensity at 520 nm, when the excitation wavelength was set to 470 nm was recorded immediately after the addition of the dye. The calcium content was estimated from the observed intensity data by comparison with that obtained from known amounts of calcium.

The polyamine content of samples of BDMV (1·5 mg) was estimated essentially by the method of Nickerson & Lane (1977). The dansylated polyamine derivatives were spotted on to a silica gel 60 F254 plate (E. Merck) and developed for 3 h using cyclohexane: ethyl acetate (3:2, v/v) as the solvent. The intensity of fluorescence was enhanced by spraying the plates with 10 μl of triethanolamine: isopropanol (1:4, v/v) and subsequently drying the plates overnight in a vacuum. The plates were viewed and photographed under a u.v. lamp. Dansylated spermidine, spermine, putrescine and cadaverine were used as standards. The photographs were digitized using a Joyce-Loebl film scanner attached to a PDP 11/44 computer. The peaks were integrated after subtracting the average intensity in the background. The polyamines were estimated by comparison with the integrated intensities for a set of standards of authentic spermine, spermidine and putrescine.

Exchangeability of viral polyamines. Exchangeability of viral polyamines with other cations was tested by dialysing BDMV (5 mg) in 0·05 M-sodium citrate pH 5·5 against 100 ml of 0·05 M-sodium citrate containing 3 M-CsCl, 3 M-KCl or 3 M-NaCl for 48 h at 4°C with two changes of the same buffer (100 ml) followed by dialysis against 0·05 M-sodium citrate pH 5·5, to decrease the concentration of CsCl, KCl or NaCl. Similarly, the exchangeability of polyamines at alkaline pH was tested by dialysing BDMV (5 mg) against 100 ml of 0·01 M-Tris-HCl pH 9·0 containing 3 M-CsCl with two changes of the same buffer followed by dialysis against 0·01 M-Tris-HCl pH 9·0. This was followed by dialysis against 0·01 M-sodium citrate pH 5·5 to remove Tris-HCl which otherwise gave additional spots on thin layer chromatography (t.l.c.) plates. The stability of BDMV particles after removal of polyamines was examined by sucrose density gradient centrifugation.

Effect of freezing and thawing. BDMV (500 μg) in 10 to 200 μl of 0·01 M-Tris–HCl pH 7·5 was immersed in liquid nitrogen for 2 min and subsequently thawed at room temperature (approx. 20°C). The samples were diluted to 400 μg in 400 μl and analysed by centrifugation in 10 to 45% (w/v) sucrose density gradients. The amount of empty capsid formed was calculated as a percentage of the total area under the peaks corresponding to intact particles and empty shells. The estimates had a root mean square error of approximately 5%.

Electron microscopy. Virus samples were negatively stained with 2% uranyl acetate and examined in a Philips 301 electron microscope operating at 60 to 80 kV using formvar-coated grids.

RESULTS

Calcium and polyamine content of BDMV

The calcium contents of particles in five different preparations of the virus were in the range 500 to 900 molecules per virus particle. Approximately 90 to 140 molecules of spermidine and 20 to 50 molecules of putrescine were present in each virus particle (Fig. 1). The amount of spermine could not be reliably estimated as it was present at a relatively low concentration. The
Fig. 1. Estimation of polyamines. Samples of 1 to 3 μl of dansylated polyamines obtained from 1.5 mg of virus were spotted on silica gel plates along with 0.75 nmol of standard dansylated polyamines. Lane 1, spermine (A); lane 2, spermidine (B); lane 3, putrescine (C); lane 4, cadaverine (D); lanes 5, 6 and 7, samples of viral polyamine derivatives (1, 2, 3 μl respectively) of BDMV isolated from *N. glutinosa*; lanes 8 and 9, polyamine derivatives of BDMV purified from the *Nicotiana* hybrid and *N. clevelandii* respectively (3 μl). The plates were developed and photographed as described in the text. E, Dansyl derivative of NH₃; F and G byproducts; H, dansoic acid. O, origin.

Fig. 2. Effect of pH on the stability of BDMV. BDMV (2 to 10 mg/ml) was dialysed against 0.01 M-sodium acetate pH 5.0 or 0.01 M-Tris-HCl pH 7 or pH 9 for 48 h and analysed on 10 to 45 % (w/v) sucrose density gradients prepared in the respective buffers. The arrow indicates the anticipated position of empty capsids. Sedimentation was from right to left. O, pH 5; △, pH 7; □, pH 9.

polyamines extracted from virus samples purified using different species of *Nicotiana* exhibited identical patterns on t.l.c. plates (Fig. 1) indicating that the nature and content of polyamines in BDMV is independent of the propagation host.

**Effect of pH on the stability of BDMV**

BDMV was dialysed against 0.01 M-sodium citrate pH 5.0, or 0.01 M-Tris–HCl pH 7 or 9, and analysed by sucrose gradient centrifugation (Fig. 2). Only 5 to 10% of a slower sedimenting component was found at pH 9 in contrast to the formation of 100% empty capsid reported by Virudachalam *et al.* (1983a). Similar results were obtained when the samples at pH 5, 7 or 9 were centrifuged in a Model E analytical ultracentrifuge equipped with Schlieren optics (Fig. 3, top trace) indicating that there was little or no effect of pH on the stability of the virus particles.
Exchangeability of polyamines with cations

Dialysis of virus samples against 3 M-CsCl at pH 5.5 or pH 9.0 resulted in a complete loss of polyamines (Fig. 4). Similar results were obtained when the samples were dialysed against 3 M-KCl or 3 M-NaCl, although, 3 M-NaCl was less effective in exchanging with polyamines (data not shown). Dialysis of these BDMV samples devoid of polyamines against 0.01 M-Tris-HCl pH 9.0 resulted in the formation of empty protein shells (Fig. 5) clearly demonstrating that the exchange of polyamines with monovalent cations results in the loss of stability at alkaline pH.

In a separate experiment, BDMV samples were dialysed against 0.1 M-CsCl at different pH values. At this concentration of CsCl, there was no observable loss of polyamines. However, the sedimentation coefficient of the virus increased by about 20% above pH 7, while no such increase was observed for empty capsids (Fig. 3).

Formation of empty capsids by freezing and thawing

Although significant amounts of the slower sedimenting components were not observed by dialysis against pH 5, 7 or 9 (Fig. 2), they were readily formed by freezing and subsequent thawing of the virus solution at each of the three pH values (Fig. 6). Electron microscopy of the intact virus (Fig. 7a) and the slowly sedimenting component obtained by freezing and thawing indicated that the latter consisted predominantly of empty shells (particles stained at centre; Fig. 7b). Preliminary results indicate that the empty capsids formed by freezing and thawing have approximately the same sedimentation coefficient (54S) as the empty capsids formed in vivo (data not shown). However, the empty capsids produced upon freezing and thawing TYMV lack between five and seven coat protein molecules (Katouzian-Safadi & Berthet-Colominas, 1983). A similar loss of some coat protein could occur in BDMV. The formation of empty capsids upon freezing and thawing was found to be dependent on virus concentration, buffer molarity, and presence or absence of different anions or cations.
To examine the effect of virus concentration on the formation of empty capsids by freezing and thawing, BDMV was frozen and thawed at concentrations indicated in Fig. 8 and then diluted to the same final concentration prior to analysis on sucrose density gradients. It was apparent from Fig. 8 that 50% of empty capsids were formed at approximately 2.5 to 3 mg/ml in 0.01 M-Tris-HCl pH 7.5. However, complete formation of empty shells could be observed even at 0.5 mg/ml in 5 mM buffer, indicating that the ionic strength of the buffer plays a crucial role in the release of RNA. Hence, at a fixed concentration of virus (9.3 mg/ml), the effect of ionic strength on the formation of empty shells was monitored. Increase in ionic strength resulted in the protection of virus against release of RNA (Table 1).

In addition to the protection observed by an increase in buffer ionic strength, certain anions and cations also prevented the release of RNA. Table 2 shows the effect of chloride salts on the formation of empty capsids. The order of effectiveness in preventing the formation of empty shells was Mg\(^{2+}\) > Na\(^+\) > Ca\(^{2+}\) ≈ Li\(^+\) ≈ Cs\(^+\) > K\(^+\). As is apparent from Table 3, none of the K\(^+\) salts tested was able to protect the virus particles. Even with sodium salts, there was no correlation between the degree of protection and the Hofmeister series of electrolytes. Similar results have been obtained with TYMV and with chicory yellow mottle virus (Katouzian-Safadi et al., 1980; Quacquarelli et al., 1972).
Fig. 5. Stability of BDMV particles after removal of polyamines. BDMV (5 mg/ml) was dialysed against 0.01 M-sodium citrate pH 5.5 containing 3 M-CsCl for 48 h and then transferred to 0.01 M-sodium citrate pH 5.5 (○) or 0.01 M-Tris-HCl pH 9.0 (□). ■, BDMV dialysed against 0.01 M-Tris-HCl pH 9.0 containing 3 M-CsCl for 48 h followed by dialysis against buffer not containing CsCl. ●, Undialysed BDMV. The samples were analysed as described for Fig. 2.

Fig. 6. Effect of freezing and thawing on BDMV. Samples of BDMV (9.25 mg/ml) in 0.01 M-sodium acetate pH 5, or 0.01 M-Tris-HCl pH 7 or pH 9 were frozen for 2 min in liquid nitrogen and allowed to thaw at room temperature. The samples (400 μg in 400 μl) were layered on 10 to 45% sucrose gradients in 0.05 M-sodium phosphate buffer pH 6.2 and analysed as described in Fig. 2. Arrow indicates the anticipated position of intact particles. ○, pH 5; Δ, pH 7; □, pH 9.

Table 1. Effect of buffer ionic strength on freezing and thawing of BDMV*

<table>
<thead>
<tr>
<th>Buffer concentration</th>
<th>Empty capsids (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mM</td>
<td>100</td>
</tr>
<tr>
<td>10 mM</td>
<td>92</td>
</tr>
<tr>
<td>50 mM</td>
<td>56</td>
</tr>
<tr>
<td>100 mM</td>
<td>20</td>
</tr>
</tbody>
</table>

*BDMV (9.25 mg/ml) in Tris-HCl pH 7.5 at different molarities was frozen and thawed. Percentage of empty capsids was determined as described for Fig. 8.

Table 2. Protection of intact particles during freezing and thawing by the addition of different chloride salts*

<table>
<thead>
<tr>
<th>Salt</th>
<th>Empty capsids (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>NaCl</td>
<td>25</td>
</tr>
<tr>
<td>KCl</td>
<td>85</td>
</tr>
<tr>
<td>LiCl</td>
<td>40</td>
</tr>
<tr>
<td>CsCl</td>
<td>48</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>44</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>15</td>
</tr>
</tbody>
</table>

*Chloride salts (40 mM) were added to BDMV (8.75 mg/ml) in 0.01 M-Tris-HCl pH 7.5. The analysis was performed as described for Table 1 and Fig. 8.
Fig. 7. Electron micrograph of intact BDMV (a) and top component (b) obtained by freezing and thawing and subsequent separation as described in Fig. 6. The samples were stained with 2% uranyl acetate. Bar marker represents 50 nm.

Fig. 8. Effect of virus concentration on the formation of empty capsids obtained by freezing and thawing BDMV. Samples of BDMV at concentrations of 2 to 20 mg/ml in 0.01 M-Tris-HCl pH 7.5 were frozen and thawed. Analysis followed the procedure described for Fig. 2. The fraction of virus converted to empty shells was calculated as a percentage of the total area under the two peaks corresponding to intact particles and empty shells.
Table 3. Effect of different salts of sodium and potassium on the freezing and thawing of BDMV*

<table>
<thead>
<tr>
<th>Anion</th>
<th>Potassium salt</th>
<th>Sodium salt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloride</td>
<td>82</td>
<td>25</td>
</tr>
<tr>
<td>Nitrate</td>
<td>90</td>
<td>60</td>
</tr>
<tr>
<td>Iodide</td>
<td>100</td>
<td>ND†</td>
</tr>
<tr>
<td>Chlorate</td>
<td>ND</td>
<td>50</td>
</tr>
<tr>
<td>Thiocyanate</td>
<td>100</td>
<td>ND</td>
</tr>
<tr>
<td>Acetate</td>
<td>ND</td>
<td>17</td>
</tr>
</tbody>
</table>

* The concentrations of the virus and salts during freezing and thawing were 8.75 mg/ml in 0.01 M Tris-HCl pH 7.5 and 40 mM respectively.
† ND, Not determined.

DISCUSSION

BDMV has been studied recently using nuclear magnetic resonance (NMR) spectroscopy. These studies indicated that, unlike TYMV, it lacks polyamines (Virudachalam et al., 1983a, b). It has been proposed that this lack of polyamines results in the release of RNA at alkaline pH. However, BDMV particles purified in our laboratory by a modified procedure do not release RNA at pH 9 and, moreover, contain polyamines and Ca\(^{2+}\). There are about 500 to 900 Ca\(^{2+}\), approximately 90 to 140 spermidine, 20 to 50 putrescine molecules and traces of spermine per virion, amounts similar to those in TYMV except that TYMV particles also contain trace amounts of cadaverine (Cohen & Greenberg, 1981).

The use of 40% (w/w) CsCl density gradient centrifugation in the purification of BDMV by earlier investigators (Virudachalam et al., 1983a) could have resulted in the replacement of polyamines by Cs\(^+\), an idea that is supported by the observation that polyamines in BDMV particles prepared in our laboratory are lost upon dialysis against 3 M-CsCl. The loss of polyamines during isolation would therefore reduce the stability of virus particles and result in consequent release of RNA at alkaline pH. On the other hand, purification of TYMV, a related virus, using caesium chloride density gradient centrifugation does not result in loss of polyamines as evidenced by \(^{13}\)C and \(^{1}H\) NMR spectroscopic studies (Virudachalam et al., 1983b). Also Ca\(^{2+}\) is not easily exchanged with protons when TYMV is titrated to neutral pH (Durham et al., 1977). These results suggest that the polyamines and calcium are intrinsic components of TYMV (Cohen & Greenberg, 1981) and are not easily exchanged for other cations. However, the data of Noort et al. (1982) indicate that the exchange between polyamines of TYMV and caesium ions occurs in more drastic conditions (3-6 M-CsCl, 37 °C for 30 min). In contrast, the bound cations can be easily exchanged in the capsid of BDMV. However, there are interesting differences between the exchangeabilities of polyamines with different cations. K\(^+\) and Cs\(^+\) ions replace the polyamines more readily than Na\(^+\). Also, the exchange of polyamines for caesium is concentration-dependent. These results suggest that the process of exchange of ions is probably not an all or none phenomenon. For TYMV, the exchange is slow and more difficult than for BDMV. The bottom component B\(_{1a}\) of TYMV is converted to a more dense component B\(_{2a}\) upon incubation of the virus with CsCl at elevated temperatures (Noort et al., 1982). This treatment results in partial degradation of RNA suggesting some conformational change and reduced stability of the virion (Noort et al., 1982). Further, the conversion of B\(_{1a}\) to B\(_{2a}\) is accompanied by a loss of polyamines and Mg\(^{2+}\) which might be responsible for the sensitivity of the B\(_{2a}\) component to ribonuclease. The nature of the viral capsids, the cation binding site or its access channel, could account for the observed differences in the exchangeability of polyamines by different cations, in TYMV and BDMV. These results suggest a correlation between the presence of polyamines and viral stability in tymoviruses.

Dialysis of BDMV against CsCl (0·1 M) above pH 7 results in an increase in the sedimentation coefficient by 20%. Although the sedimentation coefficient of BDMV does not increase upon dialysis against 0·1 M-CsCl at pH 5·5, the polyamines are exchanged with caesium ions when
particles are dialysed against 3 M-CsCl pH 5.5. Hence, apart from the caesium ions replacing polyamines, many more ions bind to the virus particle at alkaline pH values. This is understandable in terms of the recent laser Raman spectroscopic studies (Prescott et al., 1985) in which it was shown that the degree of protonation of RNA bases in BDMV is reduced by raising the pH from 5.0 to 8.0. The increased charge on RNA can then support a large number of caesium ions resulting in an increase in apparent sedimentation coefficient.

Although BDMV prepared by the present procedure does not form empty capsids at alkaline pH, it does form such capsids upon freezing and thawing. RNA was completely released from the virus at 0.5 mg/ml in 5 mM buffer suggesting that BDMV particles are more sensitive to freezing and thawing than those of TYMV. The effects of freezing and thawing in the presence of electrolytes are similar to those observed for TYMV (Katouzian-Safadi et al., 1980).

Freezing and thawing is a very complex phenomenon involving interactions between the water molecules, ions and virus particles (Katouzian-Safadi et al., 1980; Katouzian-Safadi & Haenni, 1986). Katouzian-Safadi et al. (1980) have discussed the concentration dependence of RNA release in terms of inter-particle interactions brought about during freezing and thawing. The protection by increased ionic strength of buffer and the electrolytes has been explained in terms of an increased dielectric constant of the medium resulting in decreased aggregation (Katouzian-Safadi et al., 1980). However, the lack of protection by potassium salts is difficult to explain on the basis of such a mechanism. It is also possible that incubation of virus with high concentrations of cations may release bound polyamines which might result in modified electrostatic interactions between particles.

The results presented in this paper clearly indicate that polyamines and calcium are inherent components of BDMV particles and their loss results in the instability of the virus.

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