Studies on the Phenomenon of Turnip Yellow Mosaic Virus RNA Release by Freezing and Thawing

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SUMMARY

In particles of turnip yellow mosaic virus (TYMV), the interactions between proteins are particularly strong when compared to those between proteins in some other icosahedral viruses. Intact RNA is released from TYMV particles on freezing and this process has been studied by examining several parameters that influence cryodenaturation such as dehydration, pressure, aggregation and the presence of protective agents (glycerol and ammonium sulphate). Pressure of $1.5 \times 10^8$ Pa had no effect on virus particles whereas dehydration of a virus suspension had a drastic effect. Aggregate formation resulting from freezing of solutions containing high virus concentrations seems to be a prerequisite for RNA release; cryoprotective agents hindered RNA release.

INTRODUCTION

Turnip yellow mosaic virus (TYMV), the type member of the tymovirus group, is a small icosahedral plant virus composed of 180 identical coat protein subunits of 20000 mol. wt. (Klug et al., 1966) packaging a single-stranded RNA of $2 \times 10^6$ mol. wt.; the RNA makes up 33% of the total weight of the particle. TYMV is considered the best example of viruses in which protein–protein interactions largely predominate over protein–RNA interactions in ensuring stability of the virion (Kaper, 1975). Indeed, neutron scattering studies showed that the interpenetration of the RNA within the protein capsid is extremely limited (Jacrot et al., 1977). Another feature of TYMV and of viruses stabilized mainly by protein–protein interactions is their ability to produce natural empty capsids in vivo (Markham, 1951; Matthews, 1981).

Different methods (Kaper, 1975) allow separation of the TYMV capsid from its RNA in vitro, such as alkaline treatment (Keeling et al., 1979) and freezing and thawing of the virus (Kaper & Alting Siberg, 1969; Katouzian-Safadi et al., 1980a). In these two methods, RNA release is accompanied by the loss of five to nine coat protein subunits from the capsid (Keeling & Matthews, 1982; Katouzian-Safadi et al., 1983; Matthews & Witz, 1985). The RNA recovered after alkaline treatment is degraded (Keeling et al., 1979) whereas after the freeze–thaw treatment it retains all its biochemical properties (Katouzian-Safadi et al., 1980b). Cryodenaturation is thus one of the mildest methods available for study of TYMV RNA release in vitro and has been utilized in the present study.

A number of perturbations probably act in concert during cryodenaturation of macromolecules. These include dehydration (Lin et al., 1976), pressure exerted by shearing (Van Venrooij et al., 1975) and aggregation of foreign particles (Van Venrooij et al., 1975). The effects of these perturbations on TYMV RNA release and the influence of certain protective agents such as glycerol or various ions have been investigated. The behaviour upon freezing and thawing of brome mosaic virus (BMV), another icosahedral virus, has also been examined.

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METHODS

Viruses and viral RNA. TYMV was extracted from infected Chinese cabbage leaves (kindly provided by S. Astier-Manifacier & P. Cornuet, INRA, Versailles, France), prepared as described by Leberman (1966) and stored in 10 mM-Tris-HCl pH 7.5 and 0.1 mM-sodium azide at 4 °C. The viral RNA was extracted by using phenol as described by Porter et al. (1974), suspended in 10 mM-Tris-HCl pH 7.5 and stored at −80 °C. TYMV (Kaper & Litjens, 1966) and TYMV RNA (Hirth et al., 1965) concentrations were estimated using the coefficients $A_{260}^{\text{EtdBr}} = 8.6/\text{cm}$ and $21.8/\text{cm}$ respectively. Natural and artificial empty capsids were prepared and stored as described (Pleij et al., 1977; Katouzian-Safadi et al., 1983).

BMV in 100 mm-potassium acetate pH 5.5, a kind gift of M. Cuillel (EMBL, Grenoble, France) was stored at 4 °C. The BMV concentration was estimated using the coefficient $A_{260}^{\text{EtdBr}} = 5.08/\text{cm}$ (Chauvin et al., 1978).

Freezing and thawing of the virus. Unless otherwise indicated, the virus solution (50 to 100 µl in 1 ml Eppendorf tubes) in 10 mM-Tris-HCl pH 7.5 was immersed in liquid nitrogen for 2 min and left to thaw at room temperature.

Fluorescence measurements. The assay of RNA liberation is based on the increase in fluorescence intensity of ethidium bromide (EtdBr) when intercalated into double-stranded regions of nucleic acids (Le Pecq, 1971; Favre et al., 1975). A Jobin-Yvon-Bearn spectrofluorimeter maintained at 22 °C by thermostatic control was used for the fluorescence measurements (arbitrary units). The conditions were essentially those described by Katouzian-Safadi et al. (1980b). Samples (600 µl) contained 12 µM-EtdBr in 10 mM-Tris-HCl pH 7.5 and 100 mM-NaCl, and a constant final RNA concentration of 0.5 $A_{260}^{\text{EtdBr}} \text{unit/ml}$; this corresponds to 0.59 $A_{260}^{\text{EtdBr}} \text{units/ml}$ of TYMV (Katouzian-Safadi et al., 1980b).

Dehydration by agarose. Agarose (1 % in 10 mM-Tris-HCl pH 7.5) was poured into Petri dishes, and wells (0.5 cm diam.) were made by puncturing essentially as described by Augier de Montgremier (1964). Samples of TYMV in solution (100 µl) were deposited into the wells and the Petri dishes covered to limit evaporation. After incubation at room temperature for 48 h, the gels were soaked for 20 min in EtdBr (1 µg/ml) and photographed using u.v. light.

Freeze-fracture. The method developed by Moor & Mühlethaler (1973) was adapted to TYMV. Samples (1 µl) containing TYMV at different concentrations were deposited on gold planchettes, brought to −160 °C by immersion into Freon 22 and transferred to liquid nitrogen. Freeze-fracture was achieved in a Balzers 360 apparatus under a vacuum of 133.3 × 10⁻⁶ Pa. Immediately after fracture, replicas were obtained by evaporation of platinum–carbon at an angle of 45°, and subsequently reinforced by a perpendicular evaporation of carbon. After thorough washing with sodium hypochlorite solutions and distilled water, the replicas were examined in a Philips 400 electron microscope.

RESULTS

During freezing of an aqueous suspension, the orientation and the physical state of the water molecules surrounding the foreign particles are modified (Bachmann & Schmitt-Fumian, 1973). This has two main effects: it dehydrates the macromolecules (Lin et al., 1976), and creates a mechanical stress (Van Venrooij et al., 1975).

Dehydration

To dissociate the dehydration phenomenon from freezing, two methods were used to examine whether dehydration alone could lead to TYMV RNA release.

In the first experiment, a virus solution in water was dehydrated under vacuum in a rotating evaporator maintained at 30 °C. After rehydration the accessibility of the viral RNA to EtdBr was examined (not shown). The RNA was completely released from such a sample, and as observed by electron microscopy the capsid had lost its icosahedral structure (not shown); as discussed by Bello (1985) this could result from a refolding of the polypeptide chain in the dry state.

In the second experiment, a milder procedure was used to dehydrate the virus based on the ability of agarose to absorb water. TYMV solutions at 2 or 20 mg/ml were deposited into the wells of an agarose gel (Augier de Montgremier, 1964); a third well contained pure TYMV RNA. After incubation at room temperature for 48 h, the gel was stained with EtdBr and photographed. The purified RNA diffused freely into the gel and formed a halo around the well (Fig. 1 a). Encapsidated RNA is inaccessible to EtdBr but the RNA originally enclosed in the viral capsid also formed a halo (Fig. 1 b, c), indicating that the RNA had been released from the capsid. This phenomenon most likely resulted from the capture by the agarose of water molecules in the solution in the wells. However, at similar RNA concentrations (compare a and
Fig. 1. Visualization by EtdBr of RNA migration in agarose gels. Well (a) contained 0.7 mg/ml of purified TYMV RNA [corresponding to the RNA contained in the particles of TYMV in well (b)]. Wells (b) and (c) contained 2 mg/ml and 20 mg/ml of TYMV respectively.

Fig. 2. Comparison of the behaviour of TYMV frozen in liquid nitrogen (−196 °C) or in Freon 22 (−150 °C). TYMV at different concentrations was frozen in liquid nitrogen (●) as indicated in Methods. When they were to be frozen in Freon 22 (△), the virus solutions (2 µl aliquots) were deposited on gold planchettes, frozen for 2 min and thawed at room temperature. The fluorescence intensity of unfrozen TYMV (▲), of viral RNA (■) or of buffer (▼) in the presence of EtdBr is indicated close to the ordinate.

b) the halos were of different sizes. This could be due to a slow rate of RNA release from the particles under the slow dehydration conditions used.

Effect of pressure

Hydrophobic interactions in macromolecules have been reported to become weaker as the pressure increases (Douzou, 1979). It was reported that a pressure of $4 \times 10^7$ Pa has no effect on TYMV (Fahey et al., 1969), and that a pressure of $10^8$ Pa releases the RNA from the rod-shaped particles of tobacco mosaic virus (Hirth et al., 1957). Propagation of the ice network during freezing produces a local increase in pressure due to shearing. However, this pressure does not exceed $2 \times 10^8$ Pa if no attempt is made to maintain a constant volume of solution (Franks, 1972). To study the pressure effect of freezing, pressure was exerted on a virus solution. Using a hand-driven hydraulic press, a pressure of $1.5 \times 10^8$ Pa was exerted for 30 min at room temperature to a virus solution (15 mg/ml). No effect of the pressure applied was detected (not shown) either on the virus (fluorescence measurements) or on natural or artificial empty capsids (2 mg/ml; measured by analytical centrifugation). Thus, pressure alone produces no alteration of TYMV; however, shearing in association with other parameters could participate in virus RNA release.

Aggregation

RNA release from TYMV during freezing is highly dependent on the virus concentration. In standard conditions, 50% of RNA release is reached at 2 to 3.8 mg/ml of TYMV; it is complete at 6 mg/ml (Katouzian-Safadi et al., 1980b). The sigmoidal nature of the curve of RNA release as a function of TYMV concentration led us to postulate that the formation of aggregates might be responsible for this release (Katouzian-Safadi et al., 1980b).

As determined either by elastic or by quasielastic light scattering, before freezing TYMV in
solution had no noticeable tendency to aggregate even at high virus concentration (not shown). (i) Experiments in which the amount of scattered light (550 nm) was measured at 90° as a function of virus concentration (0.2 to 18 mg/ml) revealed essentially a straight line with a slight curvature corresponding to excluded volume effects (Ashcroft & Lekner, 1966). (ii) Experiments
using quasielastic light scattering (Cuilliel et al., 1983) clearly revealed single-exponential behaviour with $D_{20, w} = 1.44 \pm 0.04 \times 10^{-7}$ and $1.47 \pm 0.02 \times 10^{-7}$ cm$^2$/s at 2 and 50 mg/ml of TYMV respectively; these values of the diffusion coefficient correspond to hydrodynamic radii of $14.7 \pm 4$ and $14.4 \pm 2$ nm respectively, in good agreement with published values (Harvey, 1973). However, our results are somewhat at variance with those of Harvey (1973) who reported that TYMV exhibits appreciable electrostatic interaction at low ionic strength. This discrepancy could be due to the state of the virus. Indeed, we have previously observed (Katouzian-Safadi et al., 1983) that virus samples prepared according to the procedure of Matthews (1960) which entails a step at pH 4.8 [the method adopted by Harvey (1973)] tend to
aggregate, whereas those prepared by the method of Leberman (1966) which was used here, do not.

The possible formation of virus aggregates in the frozen state was examined using the freeze-fracture technique, since during the freezing process the solute becomes increasingly concentrated (Bachmann & Schmitt-Fumian, 1973). As a prelude, RNA release upon freezing in Freon 22, the cooling agent used in freeze-fracture, was measured as a function of TYMV concentration: much higher virus concentrations are required with Freon 22 than with liquid nitrogen to reach total RNA release (Fig. 2). Based on the Freon 22 curve, two extreme TYMV concentrations were chosen for the freeze-fracture experiments: 1 mg/ml at which the virus remains intact after thawing, and 50 mg/ml which produces >80% RNA release. The freeze-fracture replicas revealed that at both virus concentrations, aggregates were formed (Fig. 3); however, the size of the aggregates was very different. At low virus concentration (Fig. 3a) aggregates were small, the surface/volume ratio of the aggregates was high and consequently, the majority of the virus particles were still in contact with the surrounding water molecules. Conversely, at high virus concentration (Fig. 3b), the aggregates were large and the surface/volume ratio was low. Here the environment of the virus particles depended on their position: the inner particles were probably more dehydrated than the outer particles. It can therefore be postulated that the inner particles are the most likely to release their RNA upon thawing and rehydration. The addition to native virions of virus-related proteins such as coat protein subunits or natural empty capsids, but not of unrelated proteins such as bovine serum albumin or concanavalin A, favours RNA release upon freezing and thawing (Katouzian-Safadi et al., 1980b). It thus appears that the inter-particle interaction in the aggregates was specific.

This model based on aggregate formation could explain the sigmoidal nature of virus uncoating as a function of TYMV concentration during freezing (Katouzian-Safadi et al., 1980b).

Effect of protective agents

Glycerol

In solutions of glycerol and water, a layer of water free of glycerol remains in close contact with protein molecules (Lehmann & Zaccai, 1984). Moreover, upon freezing, glycerol binds to water molecules and thereby hinders the normal course of ice crystal formation (Riehle & Hoechli, 1973).

The effect of increasing glycerol concentration on TYMV RNA release during freezing was studied in conditions that led to 100% uncoating (i.e. 10 mg TYMV/ml) in the absence of glycerol. At 100 mM-glycerol (corresponding to about 1% glycerol) the virion was >95% protected (not shown). This latter glycerol concentration is much lower than that commonly used to abolish the formation of the ice network. Indeed, 75% glycerol is required to reach minimal structural alteration of serum low density lipoproteins by preventing crystallization of the aqueous solution (Aggerbeck & Gulik-Krzywicki, 1982).

Ammonium sulphate

This salt strongly protects TYMV from cryodenaturation. At 40 mM, it left >85% of the virus particles intact (Fig. 4), whereas sodium sulphate, ammonium nitrate or ammonium chloride did not; a five- or 15-fold increase in sodium sulphate or ammonium chloride concentration respectively was still unable to protect the virus (not shown).

This cryoprotective effect of ammonium sulphate which is stronger than that of other salts (Kaper & Alting Siberg, 1969; Katouzian-Safadi et al., 1980b) could result from the different interactions of this salt with the water molecules surrounding the sample. Indeed, in solution ammonium sulphate maintains the stability of certain protein–nucleic acid complexes such as the tRNA\textsuperscript{asp}–aspartyl-tRNA complex (Giégé et al., 1980) and the ternary aminoacyl-tRNA·EF-Tu·GTP complex (Antonsson & Leberman, 1982).
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Fig. 4. Influence of ammonium sulphate on the effects of freezing and thawing of TYMV. The virus solution (10 mg/ml in 10 mM-Tris-HCl pH 7.5) was frozen and thawed in the presence of increasing concentrations of ammonium sulphate. 100% corresponds to the fluorescence intensity (=312) of the viral RNA in solution. The blank value (=30) of EtBr in buffer has been subtracted.

Table 1. Behaviour of BMV upon freezing and thawing*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Without RNase A</th>
<th>With RNase A</th>
</tr>
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<tbody>
<tr>
<td>Unfrozen BMV pH 7.5</td>
<td>100</td>
<td>55</td>
</tr>
<tr>
<td>Frozen BMV pH 7.5</td>
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<tr>
<td>Unfrozen BMV pH 5.5</td>
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<td>100</td>
</tr>
<tr>
<td>Frozen BMV pH 5.5</td>
<td>100</td>
<td>71</td>
</tr>
</tbody>
</table>

* BMV was frozen at 10 mg/ml. After thawing, KCl (40 mM final concentration) was added. The samples to be further treated received RNase A (EC 3.1.27.5, from Sigma; 5% by weight of RNA) and were incubated for 30 min at room temperature before measuring the fluorescence intensity. Based on the coefficient $A_{260}^\text{RNA} = 23$/cm for BMV RNA (Bockstahler & Kaesberg, 1965), samples of 0.58 $A_{260}$ unit/ml of BMV corresponding to 0.5 $A_{260}$ unit/ml of BMV RNA were used. The results are expressed as percentage of fluorescence intensity, the value obtained with BMV RNA (=310) corresponding to 100%.

Comparison with other icosahedral viruses

The icosahedral particles of BMV contain 21.8% RNA (Lane, 1974) and their overall structure (Chauvin et al., 1978) depends on the pH: at pH 5.5 the particle is compact and inaccessible to RNase A, whereas at pH 7 it swells allowing access of the RNase (Icardona & Kaesberg, 1964). At both pH values the virion was totally accessible to EtBr (Table 1). Upon freezing and thawing, the RNA was not released from the capsid protein as determined by sucrose gradient centrifugation (not shown). However (Table 1), the freeze-thaw treatment altered the permeability of BMV for RNase A at pH 5.5, but not at pH 7.5.

Southern bean mosaic virus (SBMV) has been reported to behave similarly to BMV upon freezing (Sehgal & Das, 1975) since its permeability is also modified by this treatment. Conversely, chicory yellow mottle virus (CYMV) liberates its RNA upon freezing but retains the structural arrangement of its capsid as does TYMV (Quacquarelli et al., 1972). Thus, viruses with strong protein–protein and protein–RNA interactions such as BMV and SBMV undergo changes in permeability upon freezing but do not liberate their RNA. Conversely, viruses such as TYMV and CYMV in which protein–protein interactions predominate, lose their RNA upon
freezing and thawing but retain their icosahedral architecture: such viruses can give rise to empty capsids in vivo.

**DISCUSSION**

Unlike all other destabilizing conditions (increasing the pH, the temperature, or addition of urea, etc.) that require an ionic strength of about 1 to maintain the architecture of the capsid after TYMV RNA release (Kaper, 1975), during cryodenaturation no salt is required for capsid stability.

We show here that among the perturbations induced by freezing of TYMV, aggregation is an important factor. Moreover, our results suggest that dehydration also causes drastic structural perturbations to TYMV even though the dehydration conditions used here (in the absence of freezing) only mimic the complex phenomena that occur during freezing and thawing.

Thus, TYMV can serve as a model system for investigating the phenomenon of freezing of biological macromolecules. It is a simple and well-studied biological system, cryodenaturation of the virion is an all-or-nothing phenomenon, and alterations to the virus induced by freezing can be precisely quantified using the fluorimetric test. Nevertheless, understanding the complex phenomenon of freezing requires understanding of the interactions that exist between the macromolecules, water and all other molecules or ions present in the solution.

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