Conformational Changes Facilitate the in vitro Translation of Intact Virions of Robinia Mosaic Virus

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SUMMARY

Intact virions of robinia mosaic virus (RoMV) which were inactive in a rabbit reticulocyte in vitro translation system when suspended in 10 mM-phosphate buffer pH 6.0, exhibited an efficient translation activity after treatment with 10 mM-phosphate buffer pH 8.0. The estimated Mr values of the translation products of the intact virions in vitro were 130K, 93K, 77K, 60K, 43K, 34K and 31K by SDS-PAGE. In comparison with the treated intact virions, the total viral genomic RNA produced only three polypeptides, of Mr approx. 31K, 25K and 22K. Circular dichroism (CD) measurements of the virions showed that the α-helical structure of the viral coat protein subunits might undergo some conformational changes after treatment under mildly alkaline conditions. Fluorescence emission and quenching analysis indicated that the microenvironment of chromophores in the virions suspended in 10 mM-phosphate buffer pH 6.0 might be different from that of the virions in 10 mM-phosphate buffer pH 8.0. Both CD and fluorescence evidence suggested that as a result of conformational changes in the viral coat protein subunits, the fine structure of the virions might be altered after short exposure to the mildly alkaline buffer, although such alteration was undetectable by electron microscopy. The conformational changes of the viral coat protein subunits and consequently the alteration of the fine structure of the virions could facilitate the gene expression and uncoating of RoMV in vitro.

INTRODUCTION

In contrast to DNA, dsRNA or minus-sense RNA viruses, plant viruses with positive-sense RNA have the advantage of being able to initiate virus-specific protein synthesis in infected cells as soon as the nucleocapsids have disassembled sufficiently to release all or part of the genetic material (Wilson, 1985). However, the components involved in the dissociation of the genomic RNA of positive-sense RNA plant viruses from the coat protein subunits in vivo are unknown. There are several theories on how viruses might be uncoated after making contact with the host (Caspar, 1963; Kiho et al., 1979; Gaard & De Zoeten, 1979; Durham et al., 1977; Roenhorst et al., 1988). The most interesting evidence for the uncoating of positive-sense RNA plant viruses has come from recent investigations by Wilson et al., who suggested that eukaryotic ribosomes contained in cell-free translation systems were able to uncoat tobacco mosaic virus (TMV) and also to initiate the release of viral RNA from suitably swollen or pretreated isometric virus particles (Wilson, 1984a, b; Brisco et al., 1985). This leaves, however, the problem of how the coat protein subunits located at the 5' end of viral RNA might be removed to expose the ribosome-binding site, the presupposition on which Wilson's model could work. It was speculated that low Ca$^{2+}$ concentrations or hydrophobic interactions might be involved in removing the coat protein subunits from the 5' end of the RNA (Wilson & Shaw, 1985), though both should be open to experimental test.

Robinia mosaic virus (RoMV), an isometric plant virus, was first described by Schmelzer in 1971. From leaves of diseased locust trees in north China, a Chinese isolate of RoMV was
identified and purified. The $M_i$ of the virus coat protein was estimated to be 31K by SDS-PAGE, and the sizes of the four RNA fragments of the viral genome were found to be approx. $2.5 \times 10^6$, $2.2 \times 10^6$, $1.8 \times 10^6$ and $0.6 \times 10^6$ by 1% agarose gel electrophoresis (Tong et al., 1981; Zheng et al., 1984). It was observed that if the pH level of the extracting buffer was greater than pH 7.0, virus particles gradually disappeared under electron microscopy and the u.v. absorption spectrum characteristic of the virus disappeared correspondingly. It appears, therefore, that the structure of RoMV is very unstable, and that a neutral pH transition may occur in RoMV, much similar to those of brome mosaic virus, cowpea chlorotic mottle virus and broad bean mottle virus. Furthermore the reassembly of the coat protein of isometric RoMV into rod-shaped structures was observed under certain conditions in vitro [Kung (Gong) & Tsao, 1985]. This gives us a hint that the pH of the microenvironment in infected cells might be one of the factors affecting the early steps of RoMV uncoating in vivo. In the present paper, we report the effect of pH in vitro on the fine structure of RoMV, and on the expression of viral genes.

METHODS

**Viruses and viral RNA.** Virus was purified by the method described by Zheng et al. (1984) with slight modifications. Briefly, 100 g diseased leaves were minced in 300 ml of 10 mM-phosphate buffer pH 6.0, and filtered through a piece of nylon cloth. The extract was stored at 4°C for more than 5 h after addition of 2% NaCl (w/v) and 6% PEG (w/v), the pellets were collected by low-speed centrifugation, then resuspended in an appropriate amount of 10 mM-phosphate buffer pH 6.0 and subjected to chromatography through a Sepharose 2B column (2 x 85 cm). Peak II, the fraction containing the virus, was pooled, centrifuged at 135600 g for 2 h at 4°C and virus was further purified by 15 to 45% sucrose density gradient centrifugation at 111100 g for 2.5 h at 4°C. The virus was precipitated by ultracentrifugation, dissolved in 10 mM-phosphate buffer pH 6.0, and stored at 4°C.

The phenol–chloroform method (Maniatis et al., 1982) was used to extract RNA from the purified RoMV preparation.

**Alkaline treatment of RoMV.** Prior to in vitro translation, the virus stored at pH 6.0 was sedimented by centrifugation at 135600 g for 2 h at 4°C, and resuspended in a suitable amount of 10 mM-phosphate buffer pH 8.0. After being kept in an ice-bath for 2 h, the concentration of the virus preparation was adjusted to $A_{260}$ = 2.2 x 10$^{-6}$, 1.8 x 10$^{-6}$ and 0.6 x 10$^{-6}$ by 1% agarose gel electrophoresis (Tong et al., 1984). It was observed that if the pH level of the extracting buffer was greater than pH 7.0, virus particles gradually disappeared under electron microscopy and the u.v. absorption spectrum characteristic of the virus disappeared correspondingly. It appears, therefore, that the structure of RoMV is very unstable, and that a neutral pH transition may occur in RoMV, much similar to those of brome mosaic virus, cowpea chlorotic mottle virus and broad bean mottle virus. Furthermore the reassembly of the coat protein of isometric RoMV into rod-shaped structures was observed under certain conditions in vitro [Kung (Gong) & Tsao, 1985]. This gives us a hint that the pH of the microenvironment in infected cells might be one of the factors affecting the early steps of RoMV uncoating in vivo. In the present paper, we report the effect of pH in vitro on the fine structure of RoMV, and on the expression of viral genes.

**RESULTS**

Translation of genomic RNA and intact virions of RoMV in the in vitro translation system

The total genomic RNA of RoMV was able to direct the synthesis of virus-specific proteins in the rabbit reticulocyte lysate, in vitro translation system. The main translation product was a 31K protein which could be precipitated by antiserum against the virus and which comigrated with the viral coat protein. Besides the 31K protein, two other bands of $M_i$ about 25K and 22K could be enhanced by immunoprecipitation. Products larger than the 31K protein could not be observed in SDS-PAGE.

RoMV remained stable at pH levels ranging from 5.2 to 6.4 [Kung (Gong) et al., 1985]. Virions suspended in 10 mM-phosphate buffer pH 6.0 were inactive in the in vitro translation system.

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Fig. 1. Analysis of translation products on SDS–10% polyacrylamide gel. The products were produced in rabbit reticulocyte lysates by the genomic RNA and the intact virions of RoMV. (a) products of the genomic RNA (lane 2) and those immunoprecipitated by the antiserum to RoMV (lane 1). (b) products produced by intact virions suspended in 10 mM-phosphate buffer pH 6·0 (lane 3) and by intact virions treated with 10 mM-phosphate buffer pH 8·0 (lane 6). Lanes 3 and 4 represent controls (no RNA).

However, when the virions were suspended in 10 mM-phosphate buffer pH 8·0, and kept in an ice-bath for 2 h, they became active in protein synthesis in vitro. However, morphological differences between the virions suspended in pH 6·0 and pH 8·0 buffers could not be observed by electron microscopy. The translation products produced by mild alkali-treated virions could be separated into seven polypeptide bands by SDS–PAGE. The $M_r$ values of these bands were estimated to be 130K, 93K, 77K, 60K, 43K, 34K and 31K (Fig. 1).

CD spectra of the virions at pH 6·0 and pH 8·0

As shown in Fig. 2, virions suspended in 10 mM-phosphate buffer pH 6·0, which were inactive in protein synthesis in vitro, showed three negative peaks at 208, 213 and 222 nm in the far u.v. region (200 to 250 nm), and two positive peaks at 264 and 268 nm, with three positive shoulders at 274, 282 and 287 nm in the near u.v. region (250 to 300 nm). Virions in 10 mM-phosphate buffer pH 8·0, which were active in protein synthesis in vitro, showed three negative peaks at 208, 211 and 222 nm in the far u.v. region; the 208 and 222 nm negative peaks were reduced significantly compared to those of the virus at pH 6. In the near u.v. region, the peaks at 264 nm and 268 nm were broadened and the shoulders at 274 and 282 nm had disappeared.
Fig. 2. CD spectra of intact virions of RoMV suspended in 10 mM-phosphate buffer pH 6.0 (solid line) and in 10 mM-phosphate buffer pH 8.0 (dashed line). The concentration of the virus preparation was adjusted to $A_{260} = 0.54$.

Fig. 3. Fluorescence emission spectra of intact virions of RoMV suspended in 10 mM-phosphate buffer pH 6.0 (solid line) and in 10 mM-phosphate buffer pH 8.0 (dashed line) excited at 280 nm. The concentration of the virus preparation was adjusted to $A_{260} = 0.29$.

Fig. 4. Quenching effects of acrylamide on the intrinsic fluorescence intensity of virions in 10 mM-phosphate buffer pH 6.0 (a) and in 10 mM-phosphate buffer pH 8.0 (b). Eighteen µl of 8 M-acrylamide was added to 3 ml of the virus suspension. $\Delta F$ represents the reduction of fluorescence intensity after the addition of acrylamide.
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Comparison of the intrinsic fluorescence of pH 6.0-buffered virions with those of mild alkali-treated virions

RoMV had an emission peak in the region of 300 to 400 nm with a maximum excitation wavelength at 280 nm. The virions stored in 10 mM-phosphate buffer pH 6.0 showed a maximum peak in the emission spectrum at 328 nm, whereas those in 10 mM-phosphate buffer pH 8.0 had a maximum peak at 331 nm, i.e. the maximum emission peak red-shifted by 3 nm when the pH value of the dissolving buffer changed from 6.0 to 8.0 (Fig. 3). Furthermore, after addition of 18 μl of 8 M-acrylamide to 3 ml of the virus suspension, the emission intensity of the virions at pH 8.0 decreased more than that of the virions at pH 6.0 (Fig. 4a, b).

DISCUSSION

Uncoating of positive-sense RNA plant viruses in infected cells is an intricate but interesting process to investigate. Efforts have been made to elucidate this problem, among which, the 'cotranslational disassembly' model proposed by Wilson (1984a, b) might bear some resemblance to uncoating in vivo. Brisco et al. (1986) indicated that swelling of isometric particles of plant viruses was necessary for viral protein synthesis in vitro. Wilson & Shaw (1985) suggested that this swelling was required to remove a small number of protein subunits from the region near the 5' end of the viral RNA genome to expose the specific binding site for ribosomes, and that Ca2+ or hydrophobic interactions might have an effect on this process.

To examine the relationship between viral structure and gene expression, and the factors involved in the early stages of uncoating, the in vitro translation activities of RoMV RNA and of virions suspended in pH 6.0 and 8.0 phosphate buffers were compared. The results indicated that the unencapsidated genomic RNA of RoMV produced three polypeptides of approx. 31K, 25K and 22K. The virions suspended in 10 mM-phosphate buffer pH 6.0 were inactive in the in vitro translation system, but those treated with 10 mM-phosphate buffer pH 8.0 produced seven polypeptide bands of about 130K, 93K, 77K, 60K, 43K, 34K and 31K. The 31K peptide produced by both the pH 8.0-treated virions and the viral RNA comigrated with the viral coat protein in SDS-PAGE and could be precipitated by virus antiserum, and was therefore presumed to be encoded by the viral coat protein gene. It is proposed that plant viruses often have genes located close to the 5' end of their RNA which encode large proteins, and that these large proteins are involved in virus replication. It was interesting that the treated, intact RoMV virions produced a series of large polypeptides which did not exist in the products produced by the unencapsidated viral RNA alone; this implied that using intact virions as translation templates might facilitate the expression of some large proteins.

The differences between the protein synthesis in rabbit reticulocyte lysate of virions at pH 6.0 and pH 8.0 suggested that the fine structure of the virus might be different at different pH levels. Wilson (1984a) has proposed that pH 8.0 treatment causes alteration of the 5'-terminal nucleoprotein structure of TMV in such a way as to make a portion of the RNA leader sequence available for interaction with ribosomes to initiate translation, but such a structural difference or alteration could not be distinguished under electron microscopy for isometric viruses. CD and fluorescence spectroscopy were used to investigate the structural changes of the virions between pH 6.0 and pH 8.0. It was observed that the CD spectra of the virions changed if the virions had been treated with the mildly alkaline buffer. It is interesting to note that CD peaks at 208 and 222 nm, which are characteristic of the α-helical structure in protein subunits, reduced significantly if the virions were suspended in pH 8.0 buffer. Such changes in far u.v. CD spectra indicated that the pH 8.0 treatment of virions caused conformational alterations in the peptide chain of the coat protein subunits, particularly in the α-helical portion and consequently affected non-covalent interaction between coat protein subunits, as well as between RNA and protein. In the near u.v. region, where the CD peak of the nucleic acid predominates, differences in ellipticity of virions at pH 6.0 and 8.0 might reflect differences in the conformation of the viral RNA and of the side chains of the coat protein subunits or the protein–RNA interactions within the virus particle (Homer & Goodman, 1975). Our results were consistent with CD studies on turnip rosette virus (Denloye et al., 1978). These observations lead to the conclusion that...
swelling of the virus particles by raising the pH could cause slight changes in the conformation of the RNA and some changes in the protein conformation.

The fluorescence emission spectra of RoMV in 10 mM-phosphate buffer pH 6.0 showed a broad peak at 328 nm, whereas the major peak of the virions in 10 mM-phosphate buffer pH 8.0 was at 331 nm. This red shift indicated that the chromophores of the virus were exposed to a more hydrophobic microenvironment after being treated with the mildly alkaline buffer (Homer & Goodman, 1975). The quenching effects of acrylamide on the intensity of fluorescence of the virions in pH 8.0 buffer were greater than on that of the virions in pH 6.0 buffer. This suggested that the chromophores in virions treated with pH 8.0 buffer were located near the surface of the virus particles, and therefore they were more easily quenched by dynamic collision with acrylamide molecules (Lakowicz, 1983). Results from fluorescence suggest that the fine structure of the virions had changed when the virions were suspended in the mildly alkaline buffer.

Our results from in vitro translation, CD analysis and fluorescence analysis of the virions suggest that as a result of conformational changes in the viral coat protein subunits, there is an alteration in the fine structure of the virions. The alteration is required for the cotranslational disassembly of RoMV, and the pH level of the microenvironment is an important factor initiating the structural alteration and uncoating of the virus in vitro.

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REFERENCES


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