Conformational states of cowpea chlorotic mottle virus ribonucleic acid components

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ABSTRACT

The conditions determining conformational changes in the four ribonucleic acid components of cowpea chlorotic mottle virus have been studied. All four components have at least two electrophoretically separable conformers, the occurrence of which can be regulated by both monovalent and polyvalent cations. This phenomenon also occurs, in a much less striking way, in the ribonucleic acids of the two other members of the bromovirus group, brome mosaic virus and broad bean mottle virus. Although specific in some respects, these changes have much in common with effects which have been observed in tRNAs, 5S RNAs and rRNAs. A provisional interpretation of the conformational behaviour of the viral RNAs is given in terms which have been proposed for certain tRNAs which have been studied in great detail.

INTRODUCTION

The genome of cowpea chlorotic mottle virus (CCMV), like that of the other two members of the bromovirus group, brome mosaic virus (BMV) and broad bean mottle virus (BBMV) is composed of four separate single-stranded ribonucleic acid (RNA) molecules with molecular weights of approximately 1×10^6 (two components) 0.8×10^6 and 0.3×10^6 (1). We have observed that, when separated from the virion, each component can occur in more than one conformation with structural differences large enough to be detected by polyacrylamide gel electrophoresis (PAGE). This tendency is found in the RNAs of all three of the bromoviruses but is the most marked in the RNAs of CCMV. Each of these can exist in at least two forms which are sufficiently different in mobility to be resolved when the complete genome is run on 7 cm x 0.5 cm diameter 2.6% polyacrylamide disc gels for 2.5 hr at 4 mA/gel.

It has been found that these conformation changes, some of which, at least, must be large, are mediated by cations, apparently by a mechanism similar to that whereby they cause comparable changes in tRNAs (2), 5S RNA (3) and ribosomal RNA (4). Detailed studies of the conformational forms of certain tRNAs (5) and their relation to biological activity have been made. These can be used as a model for the investigation of conformation in viral RNAs and its role in their biological activity.

MATERIALS AND METHODS

Preparation of viruses. Type CCMV was prepared from cowpea (Vigna unguiculata (L) Walp. var. Blackeye) inoculated seven to ten days after germination and the leaves harvested after ten to fifteen days further growth. Virus was prepared by the method of Bancroft *et al.* (6)

Type BMV was grown in barley (Hordeum vulgare var. The Sultan) under the same conditions as CCMV in cowpea and the virus was prepared from the leaves by the same method as CCMV.

BBMV, John Innes Strain, was obtained by inoculation of broad bean (Vicia faba L. var. The Midget). Growth and harvesting of the plants and conditions of preparation of the virus were the same as for BMV and CCMV.

Preparation of RNA. The RNA was prepared from the freshly isolated viruses by a phenol method (7) and stored as an ethanol precipitate at about -20° .

Conditions for testing the effect of cations. Nucleic acid solutions 1 mM with respect to nucleotide phosphate were incubated with the cation in unbuffered, neutral solution for 30 min at the stated temperature.

Polyacrylamide gel electrophoresis. Tests were usually done by applying 10 μ g of RNA as a 1 mM solution in 15% w/v sucrose (25 μ l) to 2.6% (w/v) polyacrylamide disc gels (7 cm x 0.5 cm diameter), and running for 2.5 hr at 4 mA per tube using Loening's (8) phosphate buffer pH 7.6. The gels were stained with toluidine blue and photographed. To study the mobility of the RNAs in a highly denatured state they were run on 4% (w/v) polyacrylamide gels in 98% formamide (9) (equivalent to 2.6% aqueous gels).

Separation of RNA components by zonal centrifugation. A 10 mg sample of viral RNA dissolved in 5 ml 7% (w/v) sucrose con-

taining salt (usually 0.4 M-KCl) and 1 mM EDTA adjusted to pH 5-6 with KOH was applied to a 15-40% (w/v) sucrose gradient containing the same buffered salts overlaid with 50 ml 1 mM-EDTA in the same salt concentration at pH 5-6 in a Beckman Ti14 zonal rotor and centrifuged for 18 hr at 40K-rev/min at 5° using a Beckman L2-65B ultracentrifuge. The rotor was unloaded by displacement with 50% (w/v) sucrose. The first 50 ml were discarded then 120 x 5 ml samples collected by hand and the E_{260} measured against water in a Pye-Unicam SP800 spectrophotometer. The RNA was precipitated by adding 2-3 vol. ethanol to each fraction and 40% (w/v) potassium acetate solution to give a final concentration of 2% (w/v).

Determination of metals in CCMV RNA. The determinations of these were kindly made by Mr. A.M.C. Davies of the Food Research Institute, Norwich, using atomic absorption spectroscopy (A.A.S.) with flame (F.A.) or carbon rod atomisation (C.R.A.). Samples of RNA for analysis were charred with concentrated H_2SO_4 and ashed at 500°. The ash was heated with 0.1 M HCl, evaporated to dryness and dissolved in a known volume of 0.1 M HCl. Mg^{2+} was measured using an air/acetylene flame and Ca^{2+} using a nitrous acids/acetylene flame. KCl was added, at a level of 2,000 $\mu g/ml$, to all solutions of Ca^{2+} to suppress its ionisation. Absorption readings were converted to concentrations using calibration curves calculated from known solutions under the same conditions. Co^{2+} , Cu^{2+} and Ni^{2+} were measured by C.R.A. and the results calibrated by the method of standard addition using B.D.H. standard solutions for A.A.S.

RESULTS

RNAs prepared from the bromoviruses by the method described above were examined for the presence of degradation products by running them on 2.6% (w/v) polyacrylamide gels. The preparations of CCMV RNA, which were highly infective in a local lesion host, (7) (11) appeared to be only slightly degraded (< 5%). When run on 95% formamide gels they were found to be free from hidden breaks. Among the many preparations which were examined some were found to contain additional bands when run on 2.6% aqueous acrylamide gels. The occurrence of these bands was not regular but when they appeared they fitted the pattern shown in Fig. 1a

Figure 1. Conformers of CCMV RNAs revealed by P.A.G.E.

which displays the maximum number of conformers which have been found in any preparation made from CCMV. The same preparation run on 4% gels in 98% formamide showed a single band corresponding to each component (Fig. 1b).

The three conformers of component 1, 1_1 , 1_2 and 1_3 , numbered in the order of their increasing electrophoretic mobility, were observed in many different preparations and seemed, by eye-judgment of the stained gels, to vary in relative amounts. Component 2 appeared to exist in two forms the slower of which, component 2_1 , was only present in very low proportions. This and its close proximity to component 1_3 raised doubts as to its authenticity but these were removed by showing that it could be generated from component 2_2 but not from component 1, using samples of components 1 and 2 separated by zonal centrifugation (7). Components 3 and 4 were frequently found as doublets 3_1 and 3_2 and 4_1 and 4_2 respectively. Table 1 lists the increase in apparent molecular weight of each conformer relative to its fastest moving variant.

When it was found that metal ions were likely to be involved in these conformational changes subsequent preparations

Table 1 Relative molecular weights of CCMV conformers.
(calculated from Fig. 1a)

<u>Component</u>	<u>Approximate molecular weight x 10⁻⁶</u>	<u>Conformer</u>	<u>% increase of apparent molecular weight relative to fastest conformer</u>
1	1.1	1	22.6
		2	8.7
		3	-
2	0.98	1	32.4
		2	-
3	0.78	1	8.6
		2	-
4	0.30	1	7.2
		2	-

of the virus and viral RNAs were made using freshly distilled, deionised water. The patterns of RNA components shown by P.A.G.E. were then reduced to the simplest form with each component appearing as a single band, (Fig. 1c), since, as will be shown, it was in the "denatured" state (12) (13). Component 1 was exceptional in containing a small proportion of a slower moving conformer which could not be removed.

If certain cations were added to CCMV RNA preparations which had minimal conformational heterogeneity according to P.A.G.E. (Fig. 1c) an increase in heterogeneity occurred according to a definite pattern. The effective cations (effectors) and the conditions under which they act are comparable to those which induce similar changes in tRNAs, 5S RNA and rRNA and some of which are frequently found to stimulate the activity of a wide variety of biological systems (14). They include monovalent metal ions, divalent alkaline earth and transition group metal ions and polyamines. In the systems studied here monovalent cations are optimally active when the molecular ratio of added cation to nucleic acid phosphate is 100 : 1 or higher. The nucleic acids used would be expected to be in the form of potassium salts. Other cations will be present but in very low proportions. Comparable, but not necessarily identical changes, occur in the presence of divalent metal cations and polyamines. However with

these the molecular ratio of cation to nucleotide phosphate is very much lower 0.1 - 1.0 : 1.0.

The monovalent cations Li^+ , Na^+ and K^+ were tested by incubating the RNAs at 4° or at 37° in 1, 10 and 100 mM salt. They responded in a similar way to all three cations, but there were quantitative differences, disregarded in this survey, which appeared to depend on the nature of the cation. After incubation at 4° no conformational changes could be detected in components 2, 3 and 4. However a change was observed in component 1 when the salt concentration was raised to 100 mM. This caused a transition to a higher proportion of component 1_1 (Fig. 1d). After incubation of 10 mM salt at 37° a similar transition also confined to component 1 (Fig. 1e) was observed when, under these conditions the salt concentration was raised to 100 mM, the transition of component 1 to 1_1 was complete and partial transitions were observed in the other three components (Fig. 1f).

The threshold of cation concentration relative to nucleic acid concentration above which major conformational changes were detected in the RNAs lies between 10 moles cation per 1 mole RNA phosphate and 100 moles cation per 1 mole RNA phosphate. Higher proportions of salt were without further detectable effects and as the concentration was raised again precipitation began to occur. K^+ was used to obtain the results shown in Fig. 1 d-f.

Divalent cations. Mg^{2+} was studied in detail as an example of this type of effector. It causes conformational changes, which, by the single criterion of electrophoretic mobility, are like those which can be produced by K^+ but which take place at considerably lower molecular proportions of Mg^{2+} relative to RNA phosphate. They also differ from the effects of K^+ in taking place at 4° as well as at 37° . Mg^{2+} was equally effective at all concentrations between 0.2 and 50 mM i.e. when the molecular ratio of cation to nucleotide phosphate was between 0.2 : 1 and 50 : 1. The results shown in the figure (1g) were obtained with 10 mM Mg^{2+} . All four RNA components were partially converted, to varying extents, to slower moving conformers. In the case of component 2 the amount of conversion is comparatively small. Fig. 1i-l shows that there is an antagonism between Na^+ and Mg^{2+} in which conformational changes induced by

100 mM - Na^+ (1i) are apparently reversed by 10 mM Mg^{2+} (1j) 1 mM Mg^{2+} (1k) and partially reversed by 0.1 mM Mg^{2+} (1l).

Ca^{2+} and Cu^{2+} induced changes similar to those caused by Mg^{2+} at molarities of 1, 10 and 50 mM added to RNA solutions containing 1 mM nucleotide phosphate. Ca^{2+} causes the production of a relatively high (cf the effect of Mg^{2+}) proportion of a slower moving conformer of component 2 (1m) which is not electrophoretically identical to the corresponding conformer produced by Mg^{2+} (1g).

The polyvalent organic cation spermidine. This cation caused the same conformational changes as Mg^{2+} over the same range of molecular ratios of cation to nucleotide phosphate (1h). In the presence of 0.1 M - Na^+ and 1.0 M Na^+ there were reversal effects similar to those obtained with combinations of Na^+ and Mg^{2+} .

Resolution of conformers by zonal centrifugation. Conformers of component 4 are each resolved (Fig. 2) by zonal centrifugation on a sucrose density gradient which makes it possible to obtain mg quantities of components 4_1 and 4_2 . This resolution was confirmed by P.A.G.E.

BMV and BBMV RNAs. These RNAs were tested using 10 mM Mg^{2+} and 10 mM spermidine, the effectors which induced the most pro-

Fig. 2. Separation of component 1+2, 3 and 4 of CCMV RNA on a sucrose density gradient (indicated by arrows); — gradient composition as in text; - - - - gradient contains 10 mM-tris and 0.65 M-KCl at pH 7.2. * Component 4_1 .

Fig. 3. BMV a-c, BBMV d-f: a and d untreated, b and c treated with 10 mM Mg^{2+} and c and f treated with 10 mM spermidine.

nounced changes in CCMV RNAs. With BMV RNA (Fig. 3 a-c) changes were detected only in components 1 and/or 2. Similarly in BBMV RNAs (Fig. 3 d-f) no clearcut changes were detected but components 1 and/or 2 were modified to same extent by both effectors.

DISCUSSION

For most purposes the components of the divided genomes of the bromoviruses, which are distributed between three different virions, are best separated by first preparing the whole genome and resolving it by P.A.G.E. or sucrose density gradient centrifugation. In each of these genomes certain of the RNA components have similar molecular weights and their optimal resolution depends on controlling the conformational changes which they readily undergo. This survey was an attempt to find the conditions for these changes and has shown that all the observed transitions are cation-specific and comparable to those which take place in some tRNAs (5), 5S RNAs (3) and ribosomal RNAs (4).

tRNAs have been taken as a model since they have been studied in most detail, mainly by Fresco and his collaborators (5). According to these investigators the tRNAs studied have two conformers in solution, D, the "denatured" form and N, the "native" form. The D form, which has a highly ordered secondary

structure, is switched without major changes in this structure, to a form which has a more highly ordered tertiary structure, the N form. Tertiary folding which takes place in these transitions is stabilised by monovalent cations and/or by multivalent cations for which the requirements are stringent. The folding brings a certain few, specifically located phosphate groups on distal loops of the molecule into very close proximity, thus creating a few locations with an exceptionally high affinity for cations. X-ray crystallographic studies (15) (16) complement the spectroscopic studies of tRNA solutions, on which these conclusions are very largely based, by demonstrating that many of the polyvalent cations involved are essential components in the crystalline structure of tRNAs and occupy well-defined sites.

The exact nature of the main cation-binding sites in the tRNAs which have been studied and the mechanism of the changes in tertiary structure which they undergo under the influence of polyvalent cations are not fully understood. However it has been proposed (17) that the conformers of such tRNAs are allosteric forms analogous to the allosteric forms of proteins (18). According to this view the D and N forms of tRNA^{Glu2} from Escherichia coli, which is "denaturable", exist together in an equilibrium in which cations are not directly involved. The N form differs from the D form in having, in addition to many relatively weak cation-binding sites, a small number of strong cation-binding sites. In the presence of a low concentration of a multivalent cation the equilibrium can be shifted in favour of the N form which is removed by the binding of cations to the strong sites to give a third form of the RNA stabilised by the bound cations. The energy change involved in shifting the equilibrium is considerable, that for the ion binding is negligible.

The transition from the D to the N form of certain tRNAs has been studied in great detail (19) (12) (20). They become biologically active after this transition in the sense that the N form and only this form can be recognised by tRNA specific enzymes. The transition involves a large change in the topography of the molecule which is reflected in a number of easily detected changes in its physical properties. Thus, for example, the D and

N forms of tRNA^{Glu2} from Escherichia coli have clearly different mobilities in P.A.G.E. (2). It has been proposed (12) that in such systems the N form is either a unique conformational form or an equilibrium mixture of several very similar forms. The D form is considered to be either unique or, alternatively, a mixture of many different conformers in a state of rapid equilibrium with each other.

These observations and interpretations suggest that other single-stranded RNAs will respond to cations in a similar way. This has proved to be so for 5S RNA (3), ribosomal RNA (4) and messenger RNAs (the present work). In all cases monovalent metal cations bring about the D→N transition at relatively high cation concentrations whereas alkaline earth metal ions, transition group metal ions and polyamines are maximally effective, other conditions being equal, at concentrations of about 100th that of the monovalent cations.

The experiments described here show that each of the components 2, 3 and 4 of CCMV can have two electrophoretically distinct forms and so can exist in at least two conformations. Component 1 of CCMV with three electrophoretically distinct forms, must be able to exist in at least three conformational states. It is significant that the different RNA components can respond in widely varying ways and to different degrees to a given cation effector. For example, component 2 from CCMV RNA differs from the other components in responding very little to monovalent cations as far as can be shown by P.A.G.E. (Fig. 1d-f). However it makes a marked response to Mg²⁺ (Fig. 1g) and Ca²⁺ (Fig. 1m) in which the D form is converted in part to a slower moving N form. Further, since the Mg²⁺ N form is relatively slower than the Ca²⁺ N form, it can be assumed that these two N forms differ in conformation.

It is known that tRNAs vary greatly in the ease with which the D→N transition can be effected. tRNA^{fMet} and tRNA^{Tyr}, both from Escherichia coli, assume that N form very readily (21) (22) and for this reason they have been described as non-denaturable. On the other hand tRNA^{Glu2} from Escherichia coli passes readily to the D form and is therefore described as denaturable (2) (17). The same considerations should apply to other groups of RNAs.

Consequently, while it is of interest, it should not be surprising that the different components of the CCMV genome respond more or less strongly to a given effector and that the components of the BMV and BBMV genome appear to be, in general, much less responsive than those from CCMV.

The striking effects of divalent cations on conformation in RNAs which can be observed in vitro raise two related questions.

1. Do such cations, normally present in living cells, have a similar effect on cell and viral RNAs in vivo and do they share such effects with cationic sites on proteins? 2. Do they and/or polyamines occur as components of native RNAs? Because of technical difficulties these questions remain open. However, in the case of CCMV RNAs analyses for Ca^{2+} , Mg^{2+} , Cu^{2+} , Co^{2+} and Ni^{2+} suggest that these cations were not present in the extracted viral RNAs in quantities which could influence their conformation in the manner observed when ions were added. Because of uncertainties in the measurements, they have not been quoted in detail but calculations of 95% confidence limits indicate that the metals occur, over-all, in molecular proportions which would not lead to the conversion of the RNAs to their N forms. Similarly, polyamine, shown to be present in CCMV RNA by the methods of Johnson and Markham (10), appears to be present in quite small amounts (Johnson, private communication).

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