THE PREPARATION OF CCMV-PROTEIN IN CONNECTION WITH ITS ASSOCIATION INTO A SPHERICAL PARTICLE

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Received 25 April 1974

1. Introduction

The self-assembly of a spherical plant virus was first described in 1967 for cowpea chlorotic mottle virus (CCMV) ([1]: for a recent review see [2]). CCMV consits of three nucleoprotein particles, each with a diameter of 25 nm and a protein coat constructed of 180 identical polypeptides. Four species of RNA of different size were found in those particles (fig 1A). The two smallest were associated with one particle; the other two were each associated with a different particle [3].

When the virus was dialyzed against 1 M NaCl at pH 7 it dissociated into RNA and protein subunits. Isolated protein subunits themselves associated into icosahedral particles (pseudo top component, PT) when they were subsequently dialyzed against 0.2 M NaCl at pH 5. The resulting protein particle had approximately the same geometric arrangement as in the native virus. However when a dialysis medium of pH 5.7 was used the protein particles which formed were a mixture of PT and PT surrounded by one or several layers of protein (pseudo top doubles and rosettes, PT-D) [4,5].

The virus protein association was influenced by nucleic acid; virus nucleic acid was found to cause the formation of an icosahedral particle *even* at low ionic strength and and neutral pH, where the protein

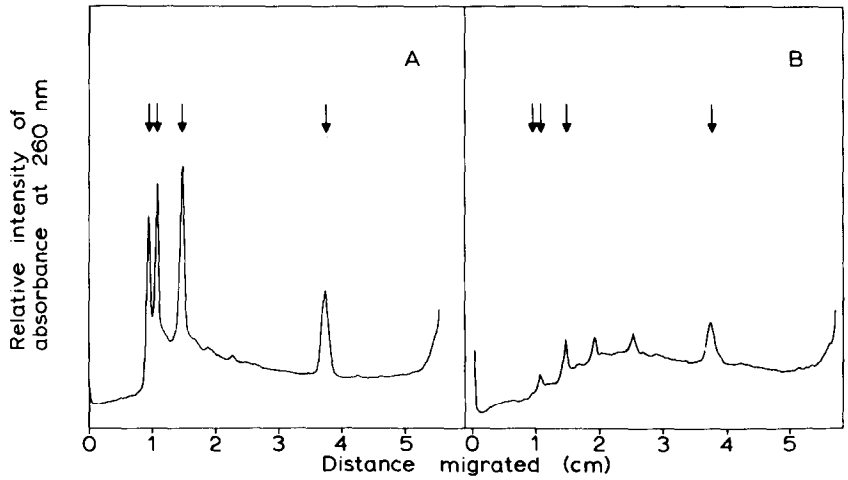

Fig. 1. Electrophoresis pattern of CCMV-RNA in 2.6% (w/v) polyacrylamide gels at 60°C. Virus was dissociated in 10% (v/v) glycerol and 2% (w/v) SDS and analyzed directly on gels. (A) Virus with intact RNA; (B) Virus with degraded RNA. The arrows indicate the positions of the RNA components isolated from biologically active virus. The anode is at the right.

itself did not associate. The formation of an icosahedral particle under these conditions was already accomplished by a small polynucleotide e.g. poly U, $n = 23$ [6].

Different methods to prepare viral protein were therefore compared to their degree of contaimination with nucleic acid. The results show that the formation of PT-D was not due to nucleic acid contaminating the viral protein preparations. The formation of PT-D can be prevented in the presence of divalent metal ions, particularly Ca^{2+}.

2. Materials and methods

CCMV, obtained from Dr. J. B. Bancroft, was grown in cowpea plants, *Vigna unguiculata* (L) Walp. var. Blackeye Early Ramshorn, and purified by the polyethylene glycol method as described by Bancroft et al. [7]. The only modification was that the leaf homogenate was kept at 4°C and at pH 4.7 for 1 hr. The purified virus was stored at 4°C in 0.1 M sodium acetate pH 5.0 containing 0.001 M sodium azide. Virus concentrations were determined with $A^{1\%}_{1\ cm}$ at 260 nm = 58,5 in 0.1 M sodium acetate buffer, pH 5.0 (Bancroft, personal communication).

The viral coat protein was prepared by the following methods:

Method A

A virus suspension (4 mg/ml) was dialyzed overnight at 4°C against 1 M NaCl, 0.001 M dithiothireitol (DTT) buffered with 0.02 M Tris–HCl pH 7.4. The virus dissociated under these conditions and the RNA was sedimented by centrifugation for 16 hr at 73 000 *g*. The top three-fourths of the supernatant was used as a viral protein solution [1].

Method B

This follows method A except that the separation of the RNA was done by zonal centrifugation. Sixty milligrams of virus (1 mg/ml) were dialyzed overnight against the buffer mentioned above. The dialyzed solution was centrifuged in a 10–50% (w/v) sucrose density gradient linear with the radius in dissociation buffer for 24 hr at 30 000 rpm in a MSE B XIV zonal rotor. After centrifugation, the contents of the rotor were fractionated and the pooled protein fractions concentrated by ammonium sulphate precipitation (0.6 saturation). After dialyzing the protein against dissociation buffer, it was used for association studies.

Method C

Virus, 10 mg/ml, was dialyzed against 0.5 M $CaCl_2$, 0.001 M DTT buffered with 0.05 M Tris–HCl, pH 7.5. After dialyzing for 6–20 hr at 4°C a white precipitate of RNA was collected by low speed centrifugation. The supernatant was then centrifuged for 2 hr at 115 000 *g* to remove residual undissociated virus. The protein solution obtained was either stored at 4°C and dialyzed against 1 M NaCl, pH 7.5 before being used for association studies, or dialyzed and used immediately.

Protein concentrations were determined spectrophotometrically, assuming $A^{1\%}_{1\ cm}$ at 280 nm to be 12.7 in 1.0 M NaCl, pH 7.4 (Bancroft, personal communication). RNA was determined by the orcinol method.

Viral RNA was analyzed by electrophoresis in 2.6% (w/v) polyacrylamide gels according to Loening at 60°C [8,9]. Virus was dissociated at room temperature in a solution of 10% (v/v) glycerol and 2% (w/v) sodium dodecyl sulphate (SDS) in water. The mixture was subjected directly to electrophoresis with a current of 3.5 mA per gel for 3.5 hr. The gels were scanned at 260 nm or stained with 0.01% (w/v) Toluidine Blue in 40% (v/v) methoxy-ethanol in water. Gels were destained in water.

Electron micrographs of virus particles and protein aggregates were obtained with a Siemens Elmiskop 101. The material was negatively stained over holes in a carbon film with 1% (w/v) uranyl acetate in double-distilled water.

Sedimentation analysis was performed in an MSE analytical ultracentrifuge equipped with Schlieren optics and an ultraviolet absorption scanner.

All buffer solutions were made at room temperature and contained 0.001 M DTT. Solutions of pH 5.0 to 6.3 were buffered with 0.05 M sodium acetate and above pH 6.3 the solution was buffered with 0.05 M Tris–HCl, unless otherwise stated.

3. Results and discussion

The results of the three methods used to prepare

Table 1
Yield and spectral properties of virus coat protein preparations

Method*	Yield**	A_{280}/A_{260}***
A	50%	1.40 ± 0.10
B	60–80%	1.55 ± 0.05
C	90%	1.70 ± 0.05

* A, B and C refer to the respective methods described under Materials and methods.
** Percentage of theoretical yield based on a protein content of the virus of 76% (w/w) [10].
*** The absorbance ratio of 280 and 260 nm indicates the presence of residual nucleic acid. A_{280}/A_{260} = 1.75 indicates less than 0.25% (w/w) residual RNA.

coat protein are summarized in table 1. Compared are percentages of theoretical yield and absorbance ratios. The percentage of theoretical yield was calculated by assuming that the protein content of the virus is 76% by weight [10]. The yield increased from about 50% in method A to more than 90% in method C. The absorbance ratio at 280 and 260 nm indicates the amount of residual RNA. This ratio varied from 1.3 for method A to 1.75 for method C, indicating a RNA contamination of about 2.3% and less than 0.25% (w/w) respectively.

The contaminating RNA in protein prepared by method A or B is probably low molecular weight RNA, which is not precipitated by 1.0 M NaCl and subsequent centrifugation during 16 hr at 73 000 *g*. Ammonium sulphate precipitation of the protein used in method B improved the absorbance ratio but still did not eliminate all of the RNA. A_{280}/A_{260} increased from 1.35 to 1.50 in a typical experiment.

It was not possible to use ionic exchange chromatography to purify the protein because it is insoluble under the conditions of low ionic strength and neutral pH, necessary to bind the residual RNA to the column. We therefore used $CaCl_2$ (method C), which precipitates the RNA quantitatively as has been previously described for brome mosaic virus [11]. The contaminating low molecular weight RNA in methods A and B is due to degradation of the RNA in the virus particles prior to dissociation in 1 M NaCl. Details of the RNA degradation process inside the virus will be published elsewhere. Fig. 1A shows the electrophoresis pattern of intact CCMV-RNA and Fig. 1B the pattern of strongly degraded CCMV-RNA. Both were run at 60°C after application of about 12 μg of RNA. It is clear from fig. 1 that large amounts of degraded RNA can be present in purified virus and these may contaminate protein prepared by method A or B.

In contrast to what was observed by applying method A or B, almost no variation in spectral properties was found for protein prepared according to method C, irregardless of the degree of RNA degradation in the original virus preparation. This has been verified repeatedly by preparing protein from different batches of virus, each of which contained different amounts of low molecular weight RNA, as determined by polyacrylamide gel electrophoresis.

When protein in 1.0 M NaCl pH 7.5 was dialyzed against 0.2 M NaCl of pH 5.0 or pH 5.7 the protein associated into PT and into a mixture of PT and PT-D respectively. PT sedimented with S20.W of about 52 S and PT-D with S20.W of about 110 S. The PT-D preparation was more inhomogeneous than PT as shown in the electron micrographs in fig. 2A and 2B.

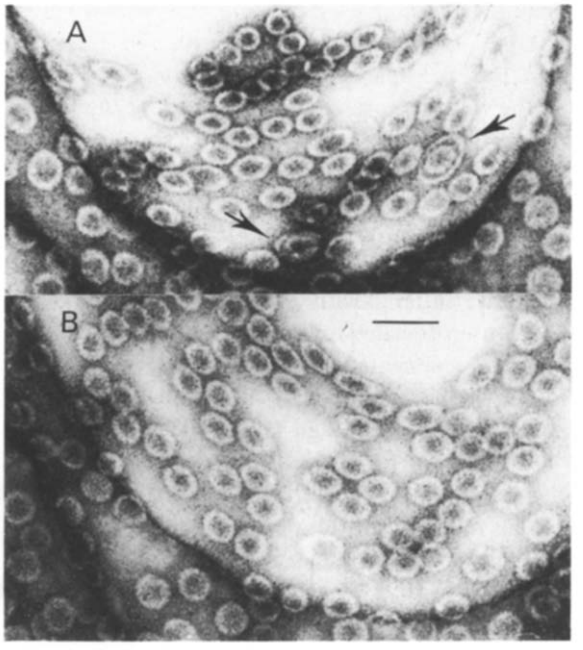

Fig. 2. Electron micrographs of protein agggregates obtained if CCMV-protein was dialyzed from 1 M NaCl, pH 7.5 to 0.2 M NaCl pH 5.7 (A) or pH 5.0 (B). The aggregates were negatively stained with 1% (w/v) urany acetate in water. The bar represents 50 nm.

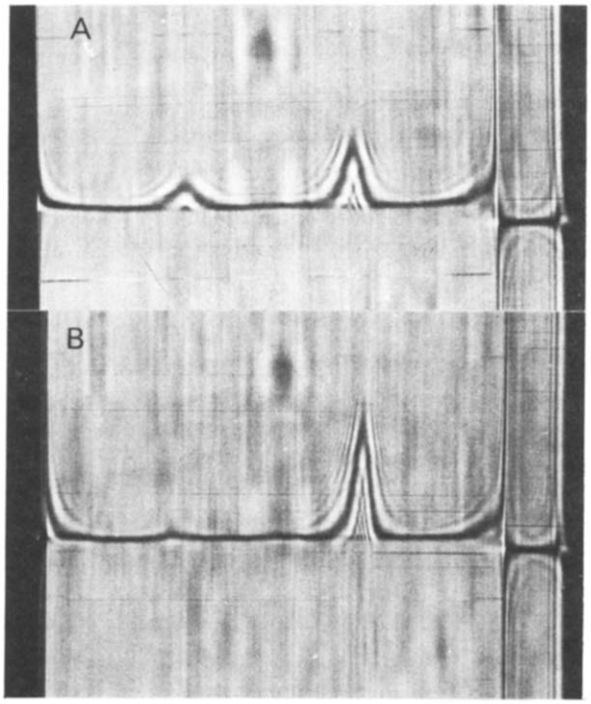

Fig. 3. Schlieren sedimentation patterns of CCMV-protein after dialyzing from 1.0 M NaCl, pH 7.5 to 0.2 M NaCl, pH 5.7 in the presence of either 0.01 M EDTA (A) or 0.01 M $CaCl_2$ (B). Protein concentrations: 3.35 mg/ml. The picture was taken 14 min after the rotor reached a speed of 30 000 rpm at 20.0°C. The sedimentation is from right to left.

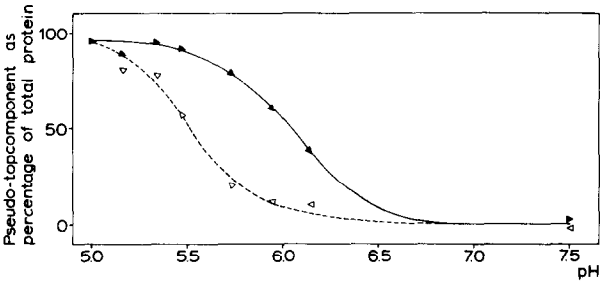

Fig. 4. The dissociation and association of CCMV-protein as a function of the pH in 0.2 M NaCl and 0.01 M $CaCl_2$, expressed at PT component percentage of total protein. The percentage of PT was determined from the areas of PT and dissociated protein in the ultraviolet absorption scan. (——▶——▶——) Dissociation behaviour of PT if the pH was increased from pH 5 to 7.5 by dialysis to a higher pH. (- - - ◁ - - ◁ - - -) Association of CCMV-protein if the pH was lowered from pH 7.5 to 5 by dialysis.

The two preparations differed in their stability. The PT-D preparation often precipitated during storage while PT at pH 5.0 remained soluble for months at 4°C. The association of the protein into PT and PT-D at pH 5.7 in 0.2 M NaCl was not appreciably influenced by the protein preparation method used as judged from sedimentation analysis. Contaminating RNA did not interfere with the formation of PT-D.

The formation of PT-D at pH 5.7 could be suppressed by the addition of Ca^{2+} ions in the dialysis procedure. The sedimentation patterns of the association products in the presence of 0.2 M NaCl and 0.001 M DTT at pH 5.7, with either 0.01 M EDTA or 0.01 M $CaCl_2$ are shown in fig. 3A and 3B respectively. The addition of Ca^{2+} ions probably induced a compact conformation of the protein at pH 7.5, necessary for the formation of an intact icosahedral particle like PT at pH 5.0 in 0.2 M NaCl.

The partition of protein among PT and dissociated protein in 0.2 M NaCl, 0.01 M $CaCl_2$ from pH 7.5 to 5.0 depended on the pH and ionic strength of the protein solution from which the dialysis was started.

Fig. 4 shows how the partition of protein differed in the association and dissociation reaction. The pH at which 50% of the protein was present as PT was about 5.5 in the association reaction and about 6.0 in the dissociation reaction. PT as the percentage of total protein was determined from the areas in the ultraviolet absorption scans of dissociated protein and total protein. A similar hysteresis effect has been found in acid–base titration studies with CCMV [12]. This effect, as well as that of divalent ions, is in agreement with the model for the association and dissociation of CCMV as proposed by Bancroft et al. [13].

The control of the self-association and the dissociation of CCMV-protein offers good prospects for the study of the kinetic and thermodynamic aspects of these processes without any interference of byproducts like PT-D.

Acknowledgements

The investigations were subsidized by the Netherlands Founation for Chemical Research (SON) with financial aid from the Netherlands Organization for

the Advancement of Pure Research (ZWO). The regular discussions with Drs. J. Lyklema, J. P. H. van der Want, A. van Kammen, C. Veeger and J. Visser during the course of this work are very much appreciated.

References

[1] Bancroft, J. B. and Hiebert, E. (1967) Virology 32, 354–356.
[2] Bancroft, J. B. (1970) Advan. Virus Res. 16, 99–134.
[3] Bancroft, J. B. and Flack, J. H. (1972) J. Gen. Virol. 15, 247–251.
[4] Bancroft, J. B., Wagner, G. W. and Bracker, C. E. (1968) Virology 36, 146–149.
[5] Bancroft, J. B., Bracker, C. E. and Wagner, G. W. (1969) Virology 39, 324–335.
[6] Bancroft, J. B., Hiebert, E. and Bracker, C. E. (1969) Virology 39, 924–930.
[7] Bancroft, J. B., Rees, M. W., Dawson, J. R. C., McLean, G. D. and Short, M. N. (1972) J. Gen. Virol. 16, 69–81.
[8] Loening, U. E. (1967) Biochem. J. 102, 251–257.
[9] Reijnders, L., Sloof, P., Sival, J. and Borst, P. (1973) Biochim. Biophys. Acta 324, 320–333.
[10] Bancroft, J. B., Hiebert, E. Rees, M., and Markham, R. (1968) Virology 34, 224–239.
[11] Yamazaki, H. and Kaesberg, P. (1963) J. Mol. Biol. 7, 760–762.
[12] Johnson, M. W., Wagner, G. W. and Bancroft, J. B. (1973) J. Gen. Virology 19, 263–273.
[13] Bancroft, J. B., Rees, M. W., Johnson, M. W. and Dawson, J. R. O. (1973) J. Gen. Virology 21, 507–513.