TOBACCO MOSAIC VIRUS RECONSTITUTION AT LOW IONIC STRENGTH

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1. Introduction

According to Butler and Klug [1], tobacco mosaic virus (TMV) reconstitution proceeds from a protein double disk (20 S). This protein aggregate recognizes specifically the 5'-OH end of the TMV-RNA [2] and the continued growth of the particles occurs from the same aggregate. However, Okada and Ohno [3] and Richards and Williams [4] have recently questioned the role of the 20 S protein aggregate in the elongation process and have claimed that the 4 S aggregate exclusively is a protein source for the growth of TMV. But neither the Cambridge group, nor the Japanese and the American group have studied the quality of the reconstituted material obtained in the different conditions used.

The present paper investigates under well defined conditions the role of the 4 S and 20 S aggregates and the quality of the reconstituted material.

2. Material and methods

2.1. *Source of the TMV protein*

The quality of the native protein and its ability to give aggregates more or less rapidly depend on how it is prepared and stored, and the purification of the virus is especially important. The TMV was purified by the PEG* precipitation procedure [6] and the protein was isolated from a fresh virus preparation by the acetic acid method [7]. The RNA contamination was extremely low (the A_{260}/A_{280} ratio varied from 0.57 to 0.60). The protein was maintained in solution in double distilled water adjusted to pH 8.0. Protein concentrations were determined with a Zeiss spectrophotometer, assuming an extinction coefficient $E^{0.1\%}_{280} = 1.27$ [7] for protein solution at pH 8.0.

In these conditions of preparation, analytical ultracentrifugation analysis showed only one peak, the mean s_{20w} value of which is 4 S (fig. 1 a). No other aggregate was present. Generally, protein preparations were used immediately. Further chromatography on DEAE–cellulose column was found unnecessary in our conditions of preparation. This protein had no "memory" as suggested in some cases [8].

2.2. *Mixing of RNA and protein*

RNA was prepared by the phenol procedure and dissolved in 0.01 M phosphate buffer pH 7.0. The RNA was diluted to 100 μg/ml with sodium pyrophosphate (NaPP) buffer pH 7.25 of final ionic strength (IS) 0.1 or 0.5. Then the 4 S or 20–25 S protein solution (1.2 ml to 1.8 mg/ml) in NaPP buffer at the same IS was added to start the reconstitution process. The protein had a final concn. of 0.1%. The temperature used was 24°C.

The mean value of the s_{20w} of the protein aggregates was 20 with 0.1 IS and 25 with 0.5 IS. We consider the 20 S protein aggregate to be the same of that obtained by Durham and Klug [9] in their phosphate buffer and to consist of double disks. It is not yet clear if the 25 S aggregates observed at 0.5 IS in NaPP buffer were also double disks (with an increased value of s_{20w} due to the IS of the buffer) or to aggregates of more than two disks (3 or 4 disks for example). In any case this point is not very important because apparently whatever the nature of the aggregate

* PEG = polyethyleneglycol.

Table 1
Reconstitution yield at various ionic strengths.

		IS of reconstitution buffer	Yield (% of RNA coated)
Expt. A		0.5 (control)	60
		0.1	3
Expt. B		0.5 (control)	74
	a)	0.3	88
	b)	0.1	84

a and b) Native protein was preincubated for 2 hr at 24°C in the same reconstituted buffer: (NaPP buffer pH 7.25).
b) This protein preparation was the one illustrated in fig. 1b.

it was very efficient in the reconstitution process [14].

In some experiments the protein was preincubated for 2 hr in NaPP buffer at pH 7.25 but at various IS: 0.5, 0.3, 0.15 and 0.1 and was added to RNA solutions at the corresponding IS.

2.3. *Time of reconstitution*

Fluorescence studies [5] have shown that a good reconstitution was obtained in about 20 min. Our preliminary experiments have shown that TMV particles reconstituted in this way were unstable and that their infectivity was nonproducible. After 2 hr of reconstitution, the maximal yield was obtained and we observed reproducible levels of infectivity. Consequently a 2 hr reconstitution period was used in the experiments.

2.4. *RNAase treatment*

The reconstituted material was ultracentrifuged. After the first ultracentrifugation [10] the pellets were resuspended in double distilled water and treated with RNAase for 10 min at 37°C (0.14 μg RNAase/A unit at 260 nm). After RNAase treatment, the reconstituted material was ultracentrifuged for 2 hr at 105 000 g as was the untreated material. The pellets were resuspended in 0.01 M phosphate buffer pH 7.0.

2.5. *Reconstitution yield*

The reconstitution yield was estimated by the method of Stussi et al. [10] or in some cases by following the increase in light scattering (at A of 310 nm) which is approximately proportional to the rod length and concentration of the particles over the range of the reaction [11]. The absorption was recorded with a Beckman Acta III recording spectrophotometer.

2.6. *Electron microscopy*

Electron microscopy was done according to the method previously described [9] and the histograms were obtained according to the procedure described in detail elsewhere [12].

2.7. *Bioassay*

Native and reconstituted TMV in 0.01 M phosphate buffer pH 7.0 were diluted in the same buffer at chosen concentrations to obtain between 10 and 100 local lesions per half leaf. Before inoculation, 25 μl of aqueous bentonite (10 mg/ml) were added to 2 ml of each solution. The TMV suspensions were assayed on the local lesion host *N. tabacum* var. *Xanthi necroticum* using latin square inoculation of at least 8 half leaves for each suspension tested.

3. Results

3.1. *Reconstitution at low ionic strength*

Experiments reported in table 1 (experiment A) showed that when 4 S protein (fig. 1 a) was added to TMV-RNA in NaPP buffer pH 7.25 at 0.5 and 0.1 IS the yield of reconstitution was 60% and 3%, respectively. When the 4 S protein was preincubated at 0.1 IS, a small peak of 20 S appeared (fig. 1 b) which represented about 10% of the main 4 S peak estimated by the area under the peaks. In this case, the yield of reconstitution was 84% and was slightly higher than that at 0.5 IS (the control). At 0.3 IS a large 25 S peak was observed and the yield was of the same order as at 0.1 IS (table 1).

In experiments made with a different protein preparation, the 4 S protein preincubated in NaPP buffer at 0.1 IS formed only 8 S aggregates (fig. 1 c) and in this case the reconstitution yield was low: 7%. These

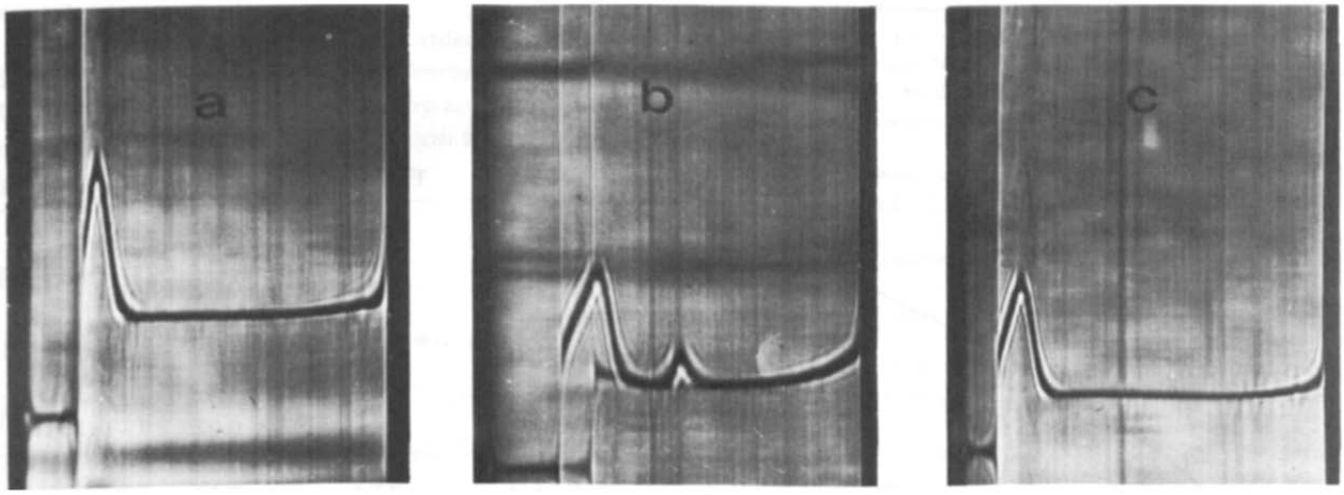

Fig. 1. Sedimentation patterns of TMV protein at 24°C in Na pyrophosphate buffer at 0.1 IS and pH 7.25. All photographs were taken 16 min after the rotor reached a speed of 47 600 rpm. Sedimentation was from left to right. a) Native protein 3 mg/ml, s_{20w} = 4. b and c) Two different protein preparations preincubated for 2 hr in Na pyrophosphate buffer 0.1 IS 3 mg/ml: b) s_{20w} = 4 and 20. The proportion 20 S/4 S aggregates was from 1/10 estimated by the area under the peaks. c) s_{20w} = 8 The S values were corrected for the effects of temperature and solution density to water at 20°C but were not extrapolated to infinite dilution.

experiments showed that 4 S protein can aggregate more or less easily at 0.1 IS, according to the quality of the preparation. When the 4 S protein was incubated at 0.15 IS and the reconstitution performed in the same buffer, very similar results as those reported in table 1 experiment B(b) were obtained but more regularly. In many other experiments of the same type we obtained a 70 to 85% yield when the ratio 20 S/4 S indicated a mean value of ten 20 S aggregates per RNA molecule. When the average number of 20 S aggregates per RNA molecule was less, depending on the protein preparation, the yield decreased considerably.

3.2. *Kinetics of reconstitution at low ionic strength*

3.2.1 Stability of 25 S aggregates

25 S aggregates were preformed by incubation of 4 S protein for 10 min in NaPP buffer 0.5 IS [13]. When this material was brought to 0.1 IS, it rapidly dissociated into 4 S protein. But when it was initially mixed with 4 S protein, it dissociated much more slowly.

3.2.2. Reconstitution

Four experiments were done involving various combinations of 25 S aggregates, 4 S protein and RNA. The yield was studied by following the increase in light scattering.

i) Preformed 25 S aggregates were mixed with RNA in stoichiometric proportion in 0.1 IS. We obtained rapidly a high reconstitution yield (curve A, fig. 2).

ii) A stoichiometric mixture of 4 S protein and RNA in 0.1 IS was mixed with 25 S aggregates in the ratio 1/20. The reconstitution yield was similar (curve B) but was achieved more slowly.

iii) When the ratio of 4 S-RNA/25 S was changed to 1/10 or 1/1, both the yield and rate of reaction were decreased (curve C).

iv) When reconstitution was attempted using only a stoichiometric mixture of 4 S protein and RNA, the yield was very small (curve D).

3.3. *RNAase stability and infectivity of the reconstituted material*

Comparison of the histograms of the reconstituted material obtained at low ionic strength before and after RNAase treatment showed that RNAase treatment significantly decreased the mean length of the particles, proving that the structure of the particles was unstable and the protection of the RNA incomplete. However, material reconstituted in 0.5 IS was not affected by RNAase treatment.

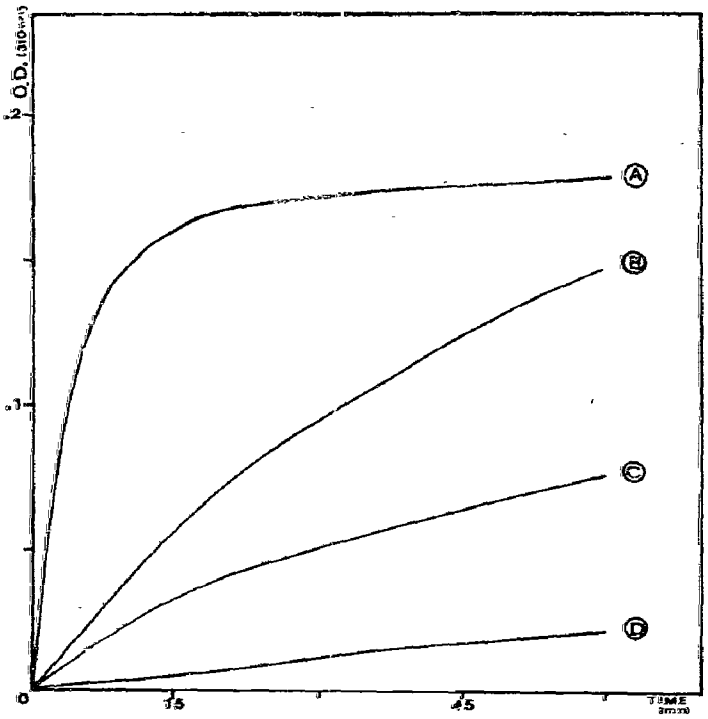

Fig. 2. Kinetics of reconstitution followed by light scattering. All experiments were done in NaPP buffer pH 7.25, 0.10 IS at 24°C. A) 50 μg RNA + 1 mg 25 S protein*. B) 50 μg RNA + 1 mg 4 S protein + 250 μg 25 S protein*. C) 50 μg RNA + 1 mg 4 S protein + 150 μg 25 S protein*. D) 50 μg RNA + 1 mg 4 S protein.

* 25 S protein was obtained by incubation for 10 min in NaPP buffer 0.50 IS pH 7.25 at 24°C.

Table 2
Quality of reconstituted material.

Material	Treatment	Concentration of inoculum in μg/ml to obtain 50 lesions per half-leaf (8 half leaves)
Original TMV		0.010
TMV reconstituted in 0.15 IS	− RNAase	0.025
	+ RNAase	0.025
Original TMV		0.025
TMV reconstituted in 0.1 IS with preincubated protein	− RNAase	0.10
	+ RNAase	1.0

Original TMV preparations and reconstituted material were in Na phosphate buffer at pH 7.0 and 0.01 M.

The specific infectivity of the virus reconstituted at low ionic strength was generally the half that of the virus reconstituted at 0.5 IS. But if the specific infectivity of the RNAase treated particles of the control (0.5 IS) was not significantly decreased, a drastic decrease (10-fold) of the specific infectivity of the RNAase treated material reconstituted at low ionic strength was observed as indicated in table 2. This proves that the quality of the material reconstituted at low ionic strength was poor. Electron micrographs showed some gaps in the protein coat of the particles.

4. Discussion

The data presented show that reconstitution occurs at low ionic strength provided that 20 or 25 S aggregates are present in relatively large amount. This confirms the role of 20 S aggregates in the initiation process as demonstrated by Butler and Klug [1] and confirmed by other groups [3, 4]. In our conditions, at low ionic strength, the yield of reconstituted material is very high (84–88%) and the transformation of 4 S protein to 20 S at this IS is extremely slow. It is therefore likely that 4 S protein participates in the reconstitution; it should be noted that the history of our 4 S protein does not imply a memory effect. These observations are in agreement with those reported by others [3, 4], however the rate of reconstitution is faster when the only material present is 20 or 25 S aggregates, and this seems to indicate that this structure is better adapted than that of the 4 S for rod elongation. This point of view, in agreement with Butler and Klug [14] is reinforced by the results obtained at high ionic strength where 20 or 25 S aggregates predominate or at low ionic strength using preformed disks. On the other hand, the particles grown from 4 S protein are unstable, sensitive to RNAase and have a relatively low specific infectivity. Thus, it seems reasonable to assume that in the natural condition, both the initiation and the elongation process occur by means of double disks.

Acknowledgements

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