TOBACCO MOSAIC VIRUS RECONSTITUTION IN THE PRESENCE OF 8 S TMV-PROTEIN COMPONENT

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1. Introduction

Until now the only aggregates considered as playing a role in tobacco mosaic virus (TMV) reconstitution were the double-disks (20 or 25 S) for the initiation of rod formation and, depending upon the authors, either the native protein (4 S) [1, 2] or the double-disks [3, 4] for elongation of the rods. TMV protein, however, can polymerize into a number of other organised structures under different conditions of pH, temperature and ionic strength (IS) [5–7]. Among the various aggregates obtained in sodium pyrophosphate (Napp) buffer we have described [7] the formation of an 8 S component at low IS. This aggregate, observed on several conditions by Durham and Klug [8], was systematically found by us [7] when the protein was prepared as described below and by Rodionova [9] and also by Ohno et al. [10] under other conditions. According to Caspar [11] the 8 S aggregate may correspond to the association of 7 monomeres stacked on three levels. In this paper we shall describe the role of 8 S component in the initiation and elongation process and the quality of the reconstituted material obtained.

2. Material and methods

2.1. *Tobacco mosaic virus and source of TMV protein*

The strain of TMV and the technique used to isolate TMV protein have been published elsewhere [4–7].

2.2. *Polymerization of the protein*

a) After 10 min of incubation in the presence of Napp buffer 0.1 IS at pH 7.25, about 50–70% of the 4 S protein polymerize in such a way as to give rise to an 8 S binding in the ultracentrifuge. Protein prepared in this manner will be referred to as '8 S protein'. Occasionaly, however, the 8 S protein contained, in addition to the 4 S–8 S material, a small amount of 25 S aggregate. Such material was not used in the work described below.

b) When 4 S protein was incubated for 10 min in Napp buffer 0.5 IS at pH 7.25, we obtain only 25 S aggregate [4]. The preincubated material was centrifuged for 10 min at low speed, and the supernatant, containing the 25 S aggregate, was stored at room temperature for not more than two days. Protein treated in this manner will be referred to below as '25 S protein'.

2.3. *Reconstitution procedure*

0.5 ml Of TMV-RNA (100 μg/ml) in Napp buffer 0.1 IS, pH 7.25 was added to 0.5 ml (2 mg/ml) of 8 S TMV-protein in the same buffer. The 8 S protein was always added in stoichiometric proportion to the RNA, but depending upon the experiment it was either used alone or in combination with 25 S protein in the proportion indicated below. As a control, we added 25 S protein to the RNA in the proportions indicated in the text. The RNA was always added to the reaction mixture first and all the reactions were done at 0.1 final IS.

2.4. *Study of the reconstitution process*

The reconstitution was studied by following the increase in turbidity (310 nm) of the reaction mixture as described previously [12].

2.5. *Bioassay and RNase treatment*

One aliquot of the reconstituted material was treated with RNase as described elsewhere [4]. To determine the specific infectivity the untreated and treated samples were inoculated at known concentrations upon *Nicotiana tabaccum* var. *Xanthi necroticum* [4].

2.6. *Electron microscopy*

Electron micrographs of reconstituted material were obtained after negative staining with 1% uranyl formiate.

3. Results

3.1. *Kinetics of reconstitution in the presence of 8 S protein*

Several different types of experiment were done involving various combinations of 8 S and 25 S protein and TMV RNA. The results of the study of reconstitution kinetics are shown in fig. 1.

Curve A is for a control experiment in which the ratio RNA/25 S protein was 1:4. The amount of 25 S protein is too small to permit the reconstitution process to proceed to completion and the O.D. increase is hence very small.

Curve B: in this experiment, RNA and 8 S protein were mixed in stoichiometric proportion and then the 25S protein was added in a ratio RNA/25 S protein of 1:4. Under these conditions the yield was only slightly higher than that observed in curve A.

Curve C: in this case 8 S protein was added to the RNA to give an RNA: protein ratio of 1:4. An equal amount of 25 S protein was then added followed immediatly by addition of more 8 S protein to yield a final 1:20 ratio between RNA and 8 S protein. Under these conditions the reconstitution process went faster than for the experiment shown in curve B and the yield was considerably increased.

Curve D: 25 S aggregate was mixed first with the RNA at a ratio of 1:4 followed by addition of 8 S protein at a ratio of 1:20. The rate of reconstitution

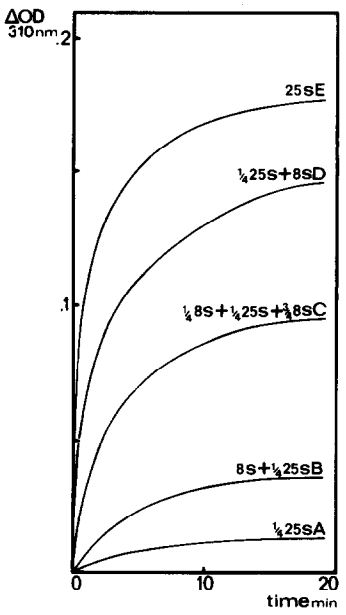

Fig. 1. Kinetics of reconstitution followed by light scattering at 310 nm. 4 S Protein was incubated for 10 min in 0.1 or 0.5 IS Napp buffer at pH 7.25 to obtain respectively 8 S or 25 S proteins and were then centrifuged at low speed to remove denaturated material. For each sample 50 μg RNA (100 μg/ml) were added to 1 mg of 8 S protein (2 mg/ml) and 250 μg of 25 S component (10 mg/ml) according to the conditions indicated in the text. For the control sample (*Curve E*) RNA was added to 1 mg of 25 S component. All experiments were done at 0.1 final IS in 1 ml of Napp buffer.

and the yield were similar to those obtained when the 25 S protein was mixed with RNA in stoichiometric proportion (*Curve E*).

3.2. *Competition between 8 S protein and 25 S aggregate*

The results of the experiment described above seem to indicate that the affinity of the 25 S component for the initiation site is very high. In fact when this aggregate is the first in contact with TMV-RNA, the reconstitution process can take place in spite of the presence of large quantities of 8 S protein. To test the ability of 8 S protein to interact with the RNA, we have performed experiments in which the 8 S material was preincubated with the RNA before addition of 25 S protein. The results are summarized in fig. 2.

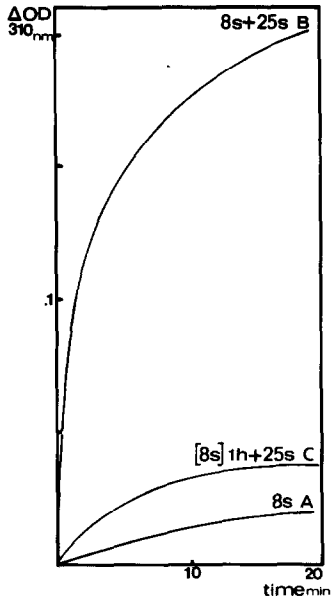

Fig. 2. Competition between 8 S and 25 S protein. The reconstitution process was followed by light scattering at 310 nm. All experiments were done in Napp buffer pH 7.25 0.1 IS. The ratio RNA 8 S and RNA 25 S is always stoechiometric for the *curve C* the mixture RNA + 8 S protein was maintained for 1 hr at room temperature before addition of 25 S protein.

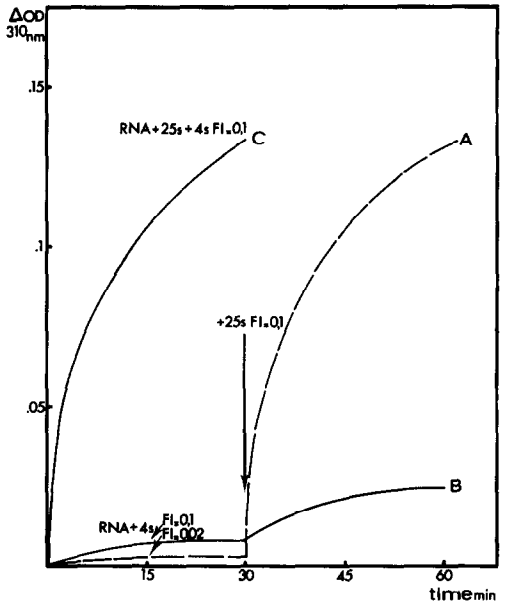

Fig. 3. Kinetics of reconstitution, in the presence of 4 S protein, followed by light scattering at 310 nm. All experiments were performed in Napp buffer pH 7.25 0.1 IS. *Curve A.* RNA + 4 S protein (R = 1:20) were preincubated in Na phosphate buffer 0.02 IS pH 7.25 for 30 min followed by addition of 25 S protein (R = 1:4). *Curve B.* RNA + 4 S protein (R = 1:20) were preincubated in Napp buffer 0.1 IS for 30 min, then 25 S protein was added (R = 1.4). *Curve C.* control: RNA + 4 S protein (R = 1:20) + 25 S protein (R = 1:4). R = ratio between RNA and protein.

Curve A: the RNA was added to 8 S component alone in a ratio RNA/8 S protein of 1:20. The yield of reconstitution is very low.

Curve B: in this experiment RNA and 8 S protein were mixed in stoichiometric proportion and an equal amount of 25 S protein was immediately added to the reaction mixture. The rate and the yield of reconstitution are similar to those observed when 25 S protein was mixed to RNA (*curve E*, fig. 1).

Curve C: the experiment was the same as in curve B except that the 8 S protein was incubated with the RNA for 1 hr at room temperature before addition of the 25 S component. In this case a great inhibition of reconstitution was observed regardless at the amount of 25 S aggregate added to the mixture.

3.3. *Reconstitution with 4 S protein in Napp buffer 0.1 IS pH 7.25*

We have compared the ability of 4 S and 8 S proteins to inhibit the reconstitution reaction with 25 S protein (fig. 3). 4 S protein in 0.02 IS Na phosphate (pH 7.25), was incubated with RNA for 30 min after which 25 S protein was added and then IS was adjusted to 0.1 with sodium pyrophosphate (*curve A*). Reconstitution proceeded at a rate similar to that observed without preincubation (*curve C*). If, however, the RNA is preincubated with the 4 S protein in IS = 0.1 pyrophosphate during the same time (conditions which lead to the formation of 8 S) reconstitution after addition of 25 S protein is dramatically inhibited (*curve B*).

3.4. *Specific infectivity of material reconstituted in the presence of 8 S protein*

The material reconstituted in the presence of 8 S protein (corresponding to curve D fig. 1) and control material reconstituted with 25 S protein in 0.1 and 0.5 IS were purified to test the specific infectivity.

Table 1
Quality of reconstituted material

Experimental process for each sample		Concentration of each inoculum in μg/ml to obtain 50 lesions per half-leaf	
		− RNase	+ RNase
control RNA/25 S* = 1:20 in 0.5 IS		0.15	0.15
RNA/25 S = 1:20	in 0.1 IS	0.15	0.15
RNA/8 S** = 1:20 + 1:4 25 S		0.15	2.0

* 25 S Corresponding to the protein preincubated for 10 min in Napp buffer, pH 7,25, 0.5 IS.
** 8 S Corresponding to the protein preincubated for 10 min in Napp buffer, pH 7,25, 0.1 IS.

One aliquot fraction of each sample was treated by RNase [4]. The treated and untreated samples were inoculated upon *N. tabaccum* var. *Xanthi necroticum*. The results obtained are summarized in table 1. It can be seen that the reconstituted material at 0.1 IS is as infectious as that reconstituted at 0.5 IS when the protein used was double-disks (25 S). Moreover the reconstituted material is in both cases RNase resistant. When reconstitution was performed in the presence of 8 S protein the specific infectivity of reconstituted material is of the same order as for the control samples; however after RNase treatment the specific infectivity decreases to less than one tenth that of the control suggesting that under these conditions some mistakes occur during the coating process so that the RNA is exposed in places to the action of the enzyme. Electron microscopic observations are in agreement with this hypothesis; the material reconstituted with the 8 S protein was generally shorter and it was possible to observe gaps in places along the length of the particles.

4. Discussion

TMV assembly may be divided conceptually into two steps: (i) rod initiation, in which an unique protein aggregate, the disk, specifically recognizes and interacts with the 5′OH region of the TMV-RNA molecule, and (ii) rod elongation, in which protein material is progressively added to form the nucleoprotein helix. In the experiments described above we have shown that 8 S protein is unable to participate in TMV assembly unless a small amount of the 25 S protein initiation structure (the disk) is concurrently added to the reaction mixture. We may conclude, therefore, that 8 S protein alone does not participate effectively in rod initiation. The 8 S aggregate is, however, capable of inhibiting initiation of rod assembly by disks if it is preincubated before addition of the 25 S protein. Guilley et al. [13] have characterized and sequenced a TMV-RNA fragment which has a great affinity for the TMV protein disk. The fragment consists of a hairpin structure with single stranded regions upon each side. It seems likely that this sequence corresponds to the 5′OH region of the RNA molecule. If this is indeed the case, the observation described here may be taken to suggest a model in which 8 S protein interacts slowly with the single stranded portion of the proposed initiation sequence [13] but, unlike the disk, is unable to 'melt' the double-helix hairpin portion of the sequence. Thus, the initiation site on the RNA would be abortively blocked and elongation would not occur.

When a small amount of 25 S protein is added to the 8 S protein–RNA reaction mixture so that the growth of rods does take place in the presence of 8 S protein, the reconstituted particles are RNase sensitive and appear to contain gaps. This may indicate that the configuration of the 8 S protein is such that the helix formed from it does not assure good protection of the RNA molecule against enzymatic attack. Furthermore these results suggest that in our previous reported experiments [4] many of the reconstituted particles may have been elongated by 8 S protein instead of 4 S protein since the experiments were conducted under conditions similar to those described above, in which the protein aggregates quickly into 8 S protein. If this is the case it may explain some discrepancies between our published results and later work done in our laboratory [14] and by others [15] which seem to demonstrate that 4 S protein is able to elongate incompletely coated particles into fullength infectious rods.

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