A FLUORESCENCE STUDY OF *"IN VITRO"* TMV RECONSTITUTION

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1. Introduction

Tobacco mosaic virus can be reconstituted *"in vitro"* from its RNA and protein components [1]. It was recently shown that TMV reconstitution is a sequential process: initiation occurs at the 5′-OH end of the RNA molecule by addition of a 20 S protein double-disk [2–4] and elongation of the TMV particle then proceeds. Under the conditions used for reconstitution [5] i.e. in a medium of high ionic strength, TMV RNA possesses some 66 to 70% of its bases in double-stranded helices [6] and it is unclear how this secondary structure is involved in the reconstitution process. In the initial or reconstituted virus, the RNA is in a single-stranded helical form [7, 8]. Reconstitution is thus accompanied by a drastic change in the RNA conformation

Together with Bollen and others [9] we have developed a method for following the state of the RNA during RNA–protein interaction. Such an approach was successfully applied to the reconstitution of *E. coli* 30 S ribosomal particles. In this case we chose as an experimental principle to introduce in the reconstitution system the fluorescent marker: ethidium bromide (EB). This dye intercalates in the double-stranded regions of the RNA; therefore it was possible to follow the evolution of EB fluorescence during the reconstitution process. The same approach was applied to the TMV reconstitution system. We first showed that EB readily intercalates in TMV RNA. We have then examined the fluorescence change of EB during an assembly experiment and finally characterized the particles formed in the presence of EB. A more complete account of these results will be published elsewhere.

2. Materials and methods

Absorption spectra were performed with a Cary Model 17 spectrophotometer. Fluorescence experiments were done with a Jobin Yvon (Bearn) apparatus equipped with a thermostated cell holder. Measurements were performed in a microcell of 0.6 ml.

TMV RNA was prepared by the phenol method [10]. TMV protein was extracted from virus by the acetic acid procedure [11]; it was dissolved in bidistilled water and adjusted at pH 8 with NaOH. In these conditions, the value of its sedimentation coefficient was 4 S. In some experiments, the 4 S protein was incubated at 25° during more than 10 min in the reconstitution buffer (0.1 M Na pyrophosphate pH 7.25). The protein obtained by this procedure is called "preincubated protein" and its ultracentrifugation pattern showed that the 4 S protein had been converted into 25 S protein (double-disk).

Reconstitution experiments were performed as described by Stussi et al. [5].

RNA's from turnip yellow mosaic virus (TYMV), brome grass mosaic virus (BMV) and alfalfa mosaic virus (AMV) were extracted by standard procedures.

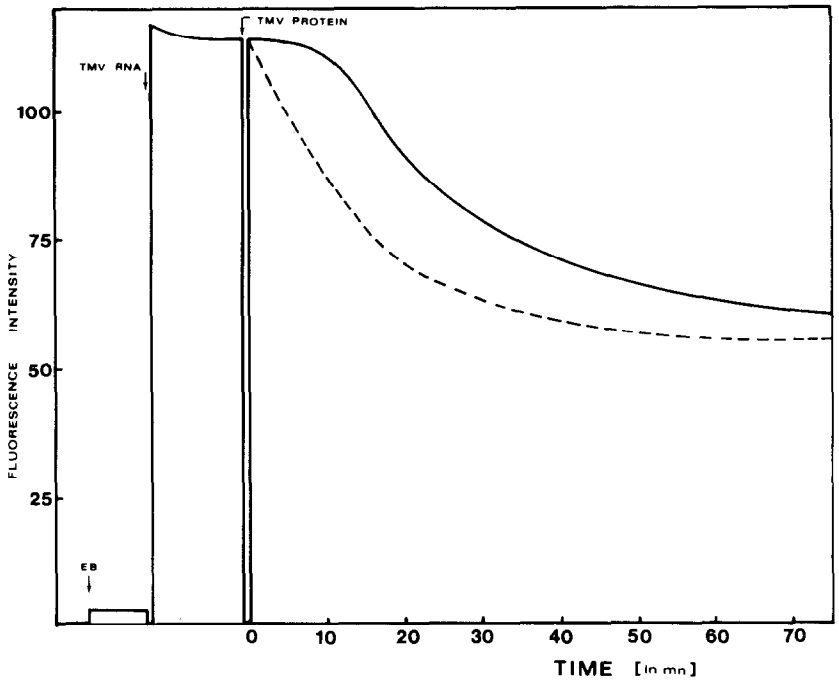

Fig. 1. TMV reconstitution in the presence of EB: the fluorescence effect. TMV RNA at a final concentration of 1.3×10^{-4}M (expressed in nucleotides) was added to a solution of EB (2.5×10^{-6}M) in the reconstitution buffer (0.6 ml). 5 mg of native TMV protein (———) or preincubated TMV protein (- - - -) was added at time 0 and the fluorescence signal recorded. The RNA to protein ratio was 1 to 20. Preincubated protein was obtained as described in the text. Reconstitution was performed at 25°. The conditions of fluorescence measurements were: λex = 540 nm and λem = 600 nm.

Ethidium bromide (EB) was a gift of Dr. Cobb of the Boots and Pure Drug Co. (Nottingham, England).

3. Results

3.1. *EB–TMV RNA interaction*

It has been established that EB intercalates between the stacked base levels of DNA with high affinity and, as a result, a considerable enhancement of fluorescence. EB also binds weakly to the phosphates of the polynucleotide chain mainly by electrostatic interaction without fluorescence stimulation. From a study of the EB optical properties in the presence of RNA or polynucleotides, it was concluded that the dye only intercalates in the bihelicoidal regions [12]. The available intercalation sites present in an RNA molecule can be titrated by EB. In the reconstitution conditions (0.1 M sodium pyrophosphate pH 7.25), the fluorimetric titration curve is very similar in shape to that obtained with 16 S ribosomal RNA from *E. coli* [9]. From a Scatchard plot of these data, one can deduce the total number of intercalation sites i.e. 6.5 per 100 bases. If one assumes that the intercalation sites are independent of each other, then these sites can be split in two classes: 3 strong sites with an affinity constant $K = 5 \times 10^6$ M^{-1} and 3.5 weaker sites with $K = 5 \times 10^5$ M^{-1}. Very similar results were obtained with AMV RNA. A smaller number of intercalation sites (five) were found in TYMV and BMV RNA's. Under the same conditions, EB absorption or fluorescence are not altered by the presence of TMV protein or initial virus at concentrations close to those found in the reconstitution mixture. Moreover, no effect of EB on the state of aggregation of the TMV protein was observed by ultracentrifugation analysis. Thus, in the reconstitution experiments, no artefact due to some interaction of dye with a component distinct from free RNA was expected.

Table 1
Fluorescence of EB in the supernatants.

	Direct measurement	Measurement after RNAase digestion
TMV RNA without protein	7.2	3.7
TMV RNA with protein	22.5	15.2

Supernatants were obtained as described in the text. The fluorescence of EB was measured directly and after pancreatic RNAase digestion (0.6 ml of supernatant was incubated for 4 hr at 25° with 10 μg of enzyme).

3.2. *TMV reconstitution in the presence of EB*

A typical assay was performed as follows: to a solution of EB (2.5×10^{-6} M) in the reconstitution buffer, we added first TMV RNA at a molarity of 1.3×10^{-4} M (expressed in nucleotides) and left the temperature to equilibrate at 25°. A stoichiometric amount of TMV protein was then added (the RNA to protein ratio in weight in the mixture is 1:20) and the fluorescence recorded as a function of time. When the native protein (4 S) was first preincubated at 25° for 1 hr in the reconstitution buffer at a concentration around 10 mg/ml and then added to the RNA–dye mixture, an immediate decay of fluorescence was observed (the half-time was 10 min). After 1 hr, the fluorescence signal stabilized to half its initial value (fig. 1). When native protein (10 mg/ml) prepared in bidistilled water adjusted to pH 8 with NaOH was added to the RNA–dye mixture, the same phenomenon occurred but with an initial lag of 10 min (fig. 1). The difference in behaviour between the two protein preparations can be related to the finding by Butler et al., that reconstitution is initiated by a 20 S protein double-disk [4]. This view has been confirmed in a more detailed kinetic study (Guilley et al., to be published). The 10 min lag appears to be a measure of the conversion time of 4 S protein into the 25 S disk form under our reconstitution conditions [13].

No decrease in fluorescence was observed when TYMV, BMV or AMV RNA's were used, in the reconstitution experiments, instead of TMV RNA. This indicates a high affinity of TMV protein for TMV RNA. The magnitude of the effect in the homologous system depends on the RNA to protein ratio. A 50% decrease in fluorescence intensity is only observed when the RNA to protein ratio reaches or exceeds the stoichiometric one. The decay of EB fluorescence which occurs during the TMV reconstitution (fig. 1), could be due either to a decrease in the quantum yield of the intercalated dye, or to expulsion of the dye. We have therefore tried to measure the amount of free dye before and after reconstitution. Two solutions of EB (10^{-5} M) and TMV RNA (1.4×10^{-4} M) in the reconstitution buffer were incubated for 2 hr at 25°, either in the absence or in the presence of viral protein in stoichiometric proportions. The two solutions (final volume: 5.5 ml) were centrifuged at 65,000 rpm in a SW 65 rotor for 12 hr at 4°. The fluorescence of the supernatants was then measured directly and after RNAase digestion (table 1). The supernatants always contained some traces of RNA since their fluorescence was sensitive to the pancreatic RNAase digestion. Nevertheless we observed that the amount of free EB was higher when reconstitution had taken place. This is direct evidence for EB chasing during TMV reconstitution. A more quantitative interpretation will be given elsewhere (Favre et al., to be published).

3.3. *Isolation and characterization of TMV particles reconstituted in the presence of EB*

We have examined the formation, the morphology and the infectivity of TMV particles reconstituted in the presence of EB. For this purpose, various EB concentrations (10^{-7} M to 10^{-4} M) were added to solutions of TMV RNA (300 μg in 6 ml of pyrophosphate buffer). Then, incubation was performed at 25° for 4 hr in the presence of 6 mg of TMV protein. The reconstituted material was purified by two successive ultracentrifugators (2 hr at 105,000 *g*), analyzed by spectrophotometry, examined by electron microscopy and tested for infectivity.

At EB concentrations lower than 10^{-5} M, the reconstitution yield, the morphological aspect of the particles and their specific infectivity were not affected. At higher EB concentrations, we observed a parallel decrease in reconstitution yield and specific infectivity. This can be understood on the basis of the electron microscopy observations. In the absence of EB or with low EB concentrations, we observed the two typical 700 Å and 3000 Å populations of particles (fig. 2A) [5, 14]. At higher EB concentrations, a striking reduction of the number of the 3000 Å particles

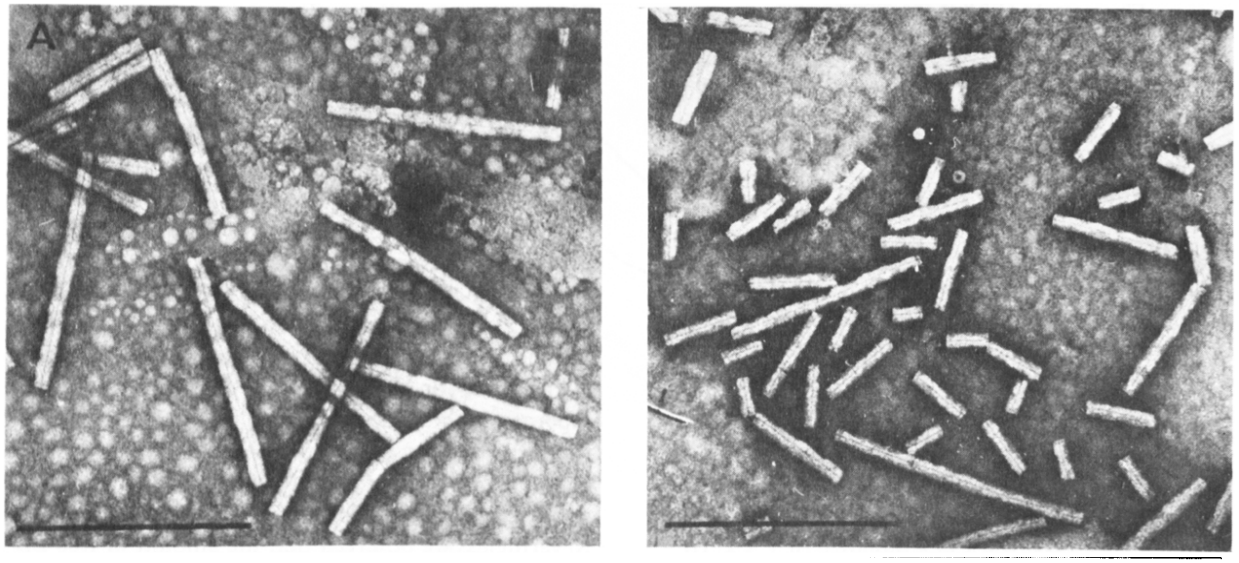

Fig. 2. Electron micrographs of the negatively stained particles obtained during reconstitution experiments performed without EB (A) and with EB at a final concentration of 2.5×10^{-5} M (B). Magnification: × 96000; scale marker: 3000 A.

was observed (fig. 2B). Apart from this effect on the length of the particles, their morphological aspect remained unaffected.

4. Discussion

It is known that TMV RNA possesses, in solution of moderate ionic strength, a large proportion of its bases in bihelicoidal regions [6]. We have verified that this is really the case under our reconstitution conditions since a large stimulation of EB fluorescence occurred in the presence of TMV RNA. This RNA possesses 6.5 sites of intercalation of EB per 100 bases, which is characteristic of a high secondary structure.

During the TMV assembly process, a decay of EB fluorescence is observed. This effect is specific for TMV RNA and its magnitude is maximum for a stoichiometric protein to RNA ratio. This finding strongly suggests that the effect is directly related to the TMV assembly process. Direct evidence for chasing of the dye was obtained, since the concentration of free EB increased after reconstitution. This is supported by the fact that particles obtained in the presence of EB had an unchanged morphological aspect. At EB concentrations higher than 10^{-5} M, the reconstitution yield and concomitantly the number of 3000 Å particles decreased. Indeed, EB stabilizes the bihelicoidal regions of TMV RNA and inhibits the formation of long rods. When this was taken into account, it was found, that the specific infectivity of the 3000 Å particles remained unchanged. Therefore, the particles formed in the presence of dye are identical in every respect to those formed in its absence. The dye has a small effect at low concentration but appears to block the elongation process at high concentration.

We have also shown, that in the reconstitution buffer the double disks (25 S) necessary for the initiation of reconstitution are formed from native protein in about 10 min.

Our method provides a direct method of following TMV RNA uncoiling during the virus reconstitution. This method should prove of interest for the study of nucleic acid–protein interactions.

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