POLYMERIZATION OF TOBACCO MOSAIC VIRUS PROTEIN IN Na PYROPHOSPHATE

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1. Introduction

The role of Na pyrophosphate in the "*in vitro*" reconstitution of TMV is not yet understood. Fraenkel-Conrat and Singer [1] maintain that the highest yield of reconstituted nucleoprotein particles is obtained in Na pyrophosphate buffer. On the other hand Leberman [2] thinks that the attribution to this salt of some property which facilitates the reconstitution process must be treated with a certain amount of caution.

We describe in this paper the polymerization of TMV protein subunits in Na pyrophosphate as a function of pH and ionic strength at 20° and will show that other polyphosphates (Na triphosphate and a long chain Na polyphosphate) influence the polymerization process in a similar way to that of pyrophosphate. Discrepancy between the present results and those published by other authors will be discussed.

2. Materials and methods

2.1. *Tobacco Mosaic Virus (TMV)*

The strain of TMV used was the Strasbourg common strain. The virus was multiplied in *N. tabacum* var. Judy Pride for 15 days and purified by polyethyleneglycol precipitation [3].

2.2. *TMV-protein isolation*

TMV-protein was isolated according to the acetic acid method [4]. It was dissolved in bidistilled water maintained at pH 8.0, and had a $S_{20,w}$ of 4. The A_{260}/A_{280} ratio varied from 0.58 to 0.63 depending on the preparation.

2.3. *Ionic strength (I.S.)*

The ionic strength of the pyrophosphate buffer was calculated taking into account the four acidities of pyrophosphate: the value of the coefficient relating the molarity to the I.S. of Na pyrophosphate was established for each pH value (see table 1).

2.4. *Experimental conditions*

The required I.S. was obtained by adding various quantities of 1.5 I.S. Na pyrophosphate buffer to a sample of 4 S protein at 10 mg/ml. The mixture was incubated for 10 min at 20°, submitted to a low speed centrifugation and the supernatant was analyzed by analytical ultracentrifugation. The $S_{20,w}$ values were calculated taking into account the viscosity and the density of the pyrophosphate buffer. After 5 hr of fixation with formaldehyde (1% final concentration) the supernatant was examined in the electron microscope. Other experiments were performed with Na triphosphate (crystallized) and a long chain Na polyphosphate (Graham's salt), with an average chain length of 10 ("Na decaphosphate") (kind gifts of Professor J.P. Ebel [5]). In these cases it was not possible to estimate the I.S., so that the polyphosphate buffers were used at the same molarity as the Na pyrophosphate buffer.

3. Results

3.1. *TMV protein polymerization in the presence of Na pyrophosphate*

The polymerization of dissociated TMV protein in Na pyrophosphate buffer was studied as a function

Table 1
Coefficient relating the molarity to the ionic strength at different pH values in Na pyrophosphate buffer.

pH	6.0	6.25	6.50	6.75	7.0	7.25	7.50	8.0
I.S. (M) =	5.34	5.80	6.06	6.36	6.60	6.75	6.88	6.90

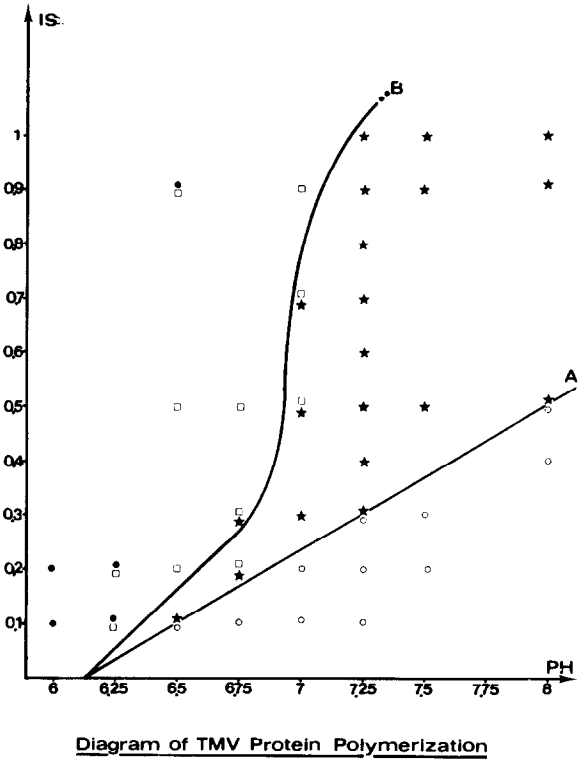

Diagram of TMV Protein Polymerization

Fig. 1. Diagram of the different types of aggregates formed by TMV protein in Na pyrophosphate. 10 mg/ml of native TMV protein in solution in bidistilled water maintained at pH 8.0 was added to Na pyrophosphate buffer of various ionic strengths and pH's. The mixture was maintained during 10 min at 20°. After a short low speed centrifugation the supernatant was analysed by analytical ultracentrifugation. The $S_{20,w}$ values were corrected for the viscosity and density of Na pyrophosphate buffer. The $S_{20,w}$ values plotted on the diagram indicate the state of aggregation of TMV protein for each I.S. value of the pyrophosphate buffer at various pHs. O: $S_{20,w}$ = 8.5 S; ★: $S_{20,w}$ ranging from 21 to 28 S; □: $S_{20,w}$ ranging from 29 to 34 S; •: $S_{20,w}$ larger aggregates, about 100 S. A Line: equilibrium between 8.5 S and 25 S; B Line: equilibrium between 25 S and 32 S.

of I.S. between pH 6.0 and 8.0. Under these conditions the different aggregates formed had sedimentation constants of 8–9.5 S; 21–28 S; 29–34 S and greater. We shall use in the text the terms 8 S, 25 S and 32 S, respectively, to characterize these aggregates. The range of existence of the different aggregation states of TMV protein is indicated on the diagram of fig. 1.

At pH values below 7.0, the I.S. has little effect: single protein helices form with a higher sedimentation constant. Above pH 7.0 the protein subunits seem to cluster into 8 S aggregates at low ionic strength with very little protein remaining in the 4 S state (fig. 2a)

At variance with the observation of Durham et al. [6] this 8 S aggregate is systematically found at pH 6.5–8.0 at I.S. values where no 25 S is formed. Moreover, increasing the I.S. between pH 6.5 and 8.0 leads to the formation of both 8 S and 25 S aggregates (fig. 2b). These two kinds of aggregates are found in equivalent amounts along the lower line A (fig. 1). Above 0.3 I.S. and between pH 7.0 and 8.0 the amount of 25 S increased while the amount at 8 S decreased (area between lines A and B, fig. 1). For example, at pH 7.25 (pH value at which TMV reconstitution is routinely performed) only 25 S aggregates are formed between 0.4 and 0.8 I.S. (fig. 2c, d). Electron microscopic observations of aggregates obtained under these conditions at pH values between 7.0 and 8.0 showed the presence of 2 rings, 3 rings and 4 rings disks in the same preparation.

In order to determine whether the 8 S aggregate represents an intermediate state in the formation of the 25 S aggregate, the following experiments were performed. Dissociated protein was dialysed against Na pyrophosphate buffer at pH 7.25, 0.1 I.S.; an aliquot fraction analyzed by analytical ultracentrifugation showed a material migrating with a $S_{20,w}$ = 8 S. When the material was dialyzed against Na pyrophosphate buffer (0.5 I.S.) at the same pH, a new com-

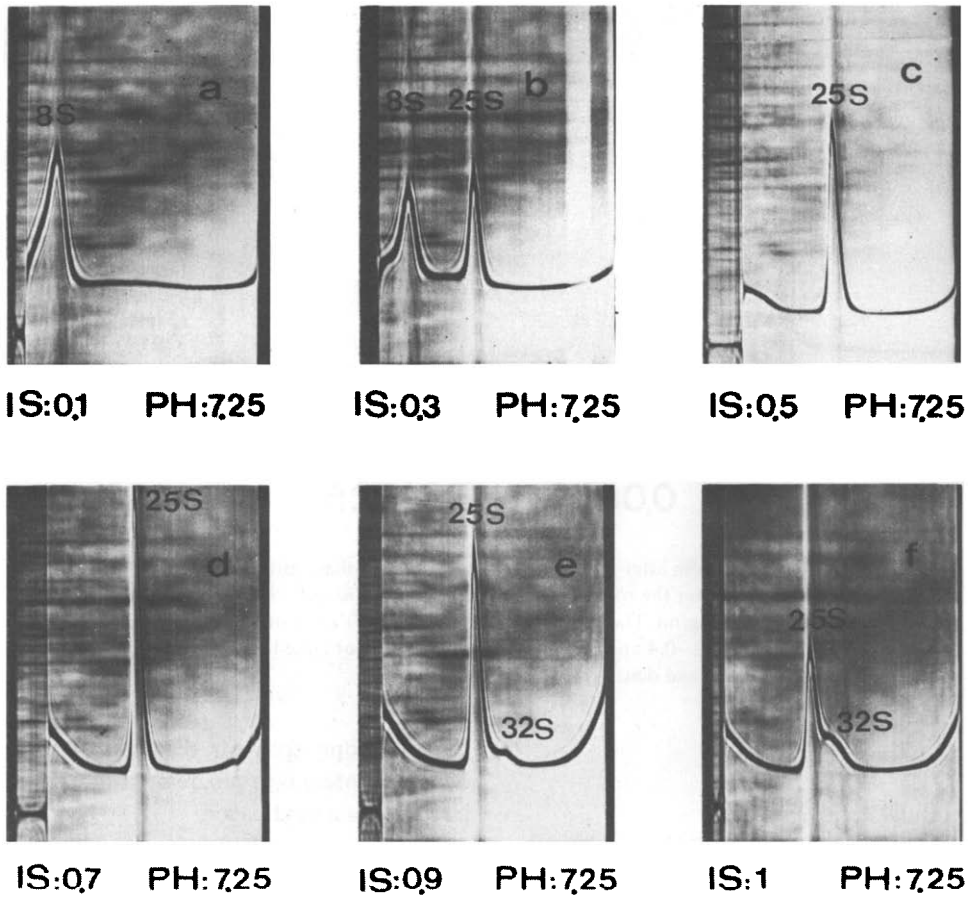

Fig. 2. Sedimentation patterns of TMV protein after 10 min at 20° in Na pyrophosphate buffer at various ionic strengths at pH 7.25. The patterns indicate the relative amounts of various types of aggregates. All photographs were taken 15 min after the rotor reached a speed of 47,600 rpm. Sedimentation is from left to right.

ponent was obtained with a $S_{20,w}$ of 25. When this preparation was back-dialyzed against the same buffer but at 0.1 I.S., the 8 S component was formed again. Dissociated protein ($S_{20,w}$ = 4 S) could be re-obtained by back-dialysis against the same buffer at 0.067 I.S. It seems therefore that the 8 S component represents an intermediate state between the 4 S and 25 S aggregates. It should be noted that when a 25 S preparation in 0.7 I.S. pyrophosphate pH 7.25 was back-dialyzed against 0.1 I.S. Na phosphate pH 7.0 (instead of pyrophosphate) no 8 S component was formed but only a single component of 20 S was observed.

We were also interested to know whether the polyphosphate buffers would permit the polymerization of the TMV protein. The same experiments were performed in Na triphosphate buffer (0.03–0.06 and 0.15 M) or "Na decaphosphate" buffer (0.015 and 0.05 M) at pH 7.25 under the same conditions used with Na pyrophosphate. The results showed that at molarities corresponding to those used with Na pyrophosphate similar discrete aggregates were obtained after 10 min incubation at 20° (fig. 3).

The experimental S values were slightly lower than in pyrophosphate but as no correction for viscosity was applied in this case a more precise comparison was not feasible.

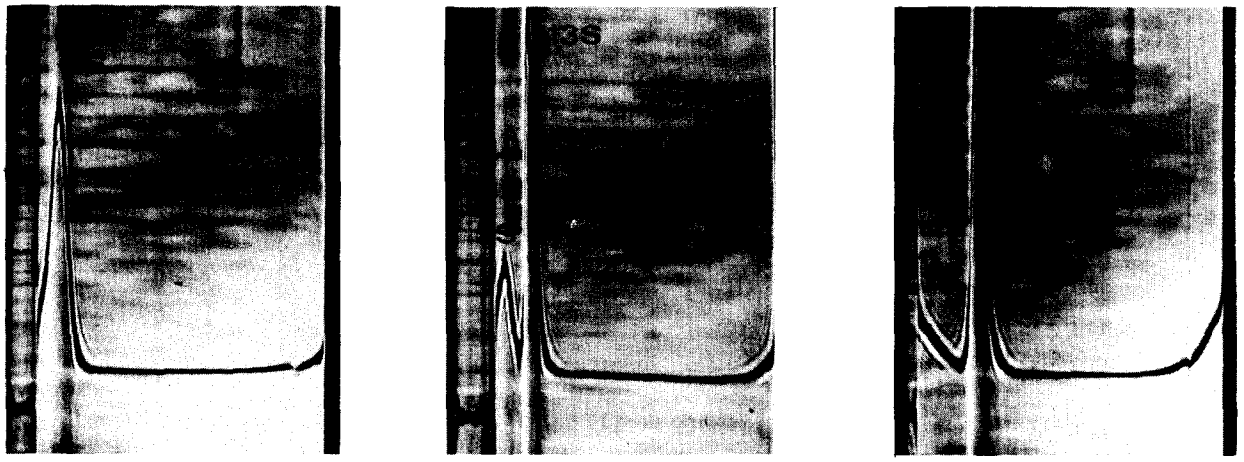

Fig. 3. Sedimentation patterns of TMV protein after 10 min at 20° in Na triphosphate buffer at 0.03, 0.06 and 0.15 M, at pH 7.25. All photographs were taken 15 min after the rotor reached a speed of 47,600 rpm. Sedimentation is from left to right. TMV protein is used at the concentration of 10 mg/ml. The molarities 0.03–0.06 and 0.15 M of triphosphate buffer are the same as those of Na pyrophosphate buffer of I.S. 0.2–0.4 and 0.9. Similar patterns were obtained with "Na decaphosphate". The $S_{20,w}$ values have not been corrected for viscosity and density.

4. Discussion

From these results it appears that:

a) The native 4 S TMV protein can polymerize very rapidly (10 min at 20°) into 25 S when it is suspended in Na pyrophosphate buffer at I.S. equal or superior to 0.3 and at pH values higher than 6.75. The rapidity of this process contrasts to the slow polymerization found in phosphate buffer [7] or carbonate buffer [8].

b) The phase diagram of the polymerization process of TMV protein in pyrophosphate (fig. 1) is markedly different from that obtained in phosphate buffer [6].

c) The S values of the polymerized protein obtained in pyrophosphate are higher than those found in phosphate buffer.

d) The process of polymerization of the 4 S component and of depolymerization of the 25 S component pass through an intermediate 8 S state.

e) The efficacy of reconstitution of the virus in pyrophosphate [1] may be linked to the rapidity of polymerization observed in this buffer.

Further work to establish the actual role played by the Na pyrophosphate in the polymerization process of TMV protein is in progress; its implications will be discussed in a next paper.

Acknowledgements

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