POLYMERIZATION OF TOBACCO RATTLE VIRUS PROTEIN

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1. Introduction

Tobacco rattle virus (TRV) was first reassembled *in vitro* from its RNA and protein components by Semancik and Reynolds [1] and Abou Haidar [2] but the conditions they used were different. Experience with the *in vitro* reassembly of tobacco mosaic virus has shown that the state of aggregation of the protein is important both for initiation and elongation of the rod [3–6] and this is likely to be true also of the similarly constructed TRV. We have therefore investigated the aggregation of TRV protein as a function of pH, ionic strength and temperature.

2. Material and methods

2.1. Virus

The TRV strain CAM was a gift from Dr. B.D. Harrison, and was multiplied in *Nicotiana Clevelandii* and purified as described by Sänger [7]. Further purification was obtained by centrifugation in a CsCl gradient.

2.2. Protein extraction

Protein was extracted from the virus by the acetic acid method of Semancik [8], but mercaptoethanol (0.5 ml/l) was added to the water during dialysis. The protein was then precipitated by an equal volume of saturated $(NH_4)_2\ SO_4$ and resuspended in distilled water. The A_{260}/A_{280} ratio varied from 0.55 to 0.6 depending on the preparation, indicating almost no contamination by residual RNA.

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2.3. Experimental conditions

Aliquots of the suspension of TRV protein in water were precipitated again by $(NH_4)_2\ SO_4$ and resuspended in the required buffer, dialyzed at 2° overnight against that buffer, and allowed to stand for 2 hr at the chosen temperature before study. We used buffers of ionic strengths from 0.01 to 0.8: sodium acetate for pH 4.5, 4.7, 5.0, 5.2 and 5.5; sodium phosphate for pH 6.0 and 7.0; glycine-NaOH for pH 8.0 and 10.0.

For each buffer and each pH the value of the coefficient k relating the molarity (M) to the ionic strength (μ) has been calculated, using the pK's tabulated in [9].

Protein samples at a concentration of about 4 mg/ml were examined in a Spinco Model E analytical ultracentrifuge. The viscosities and densities of the various buffers were measured at 20° using an Oswald viscosimeter and a Mohr balance, respectively. The same viscosity and density correction (the factor can be as high as 1.46, for acetate buffer pH = 4.5, μ = 0.6) was used at all temperatures (2°, 20° and 30°) to obtain the $s_{20,w}$ values of the sedimentation coefficients.

For electron microscopy the protein samples (at about 0.1 mg/ml) were fixed for at least 3 hr with formaldehyde diluted in the corresponding buffer (1% final conc.). The specimen grids were examined in a Siemens Elmiskop 1 A electron microscope, at a nominal magnification of 40,000, after negative staining with 1% uranyl formate.

3. Results

3.1. Aggregation states of TRV protein at 2°

The analysis of the protein in the various buffers shows that at 2°, essentially two types of components

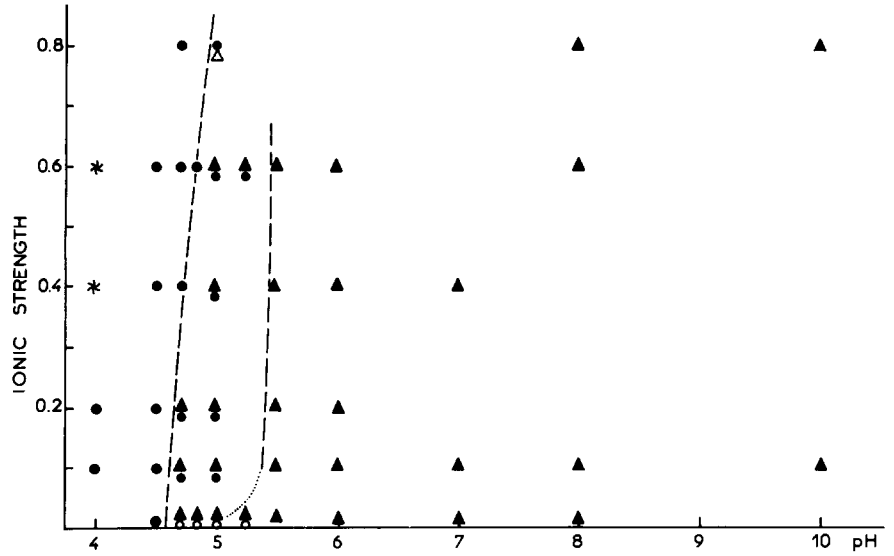

Fig. 1. Diagram showing the range of existence of the different types of TRV protein aggregates as a function of pH and ionic strength (μ) at 2°. Symbols: ●, ○ = 4 S protein; ▲, △ = 36 S aggregate. The relative amounts of 4 S and 36 S components are indicated according to the following convention: ▲ More than 90% of 36 S. ● More than 90% of 4 S. (◭) Approx. equal amounts of 4 S and 36 S (from 30 to 70% of 4 S or 36 S). (◌◭) 10 to 30% of 4 S. (◬) 10 to 30% of 36 S. The symbol * indicates that the protein is denatured and precipitates.

were obtained, sedimenting at $s_{20,w}$ = 2 to 4 S and 28 to 36 S, respectively.

Calculations similar to those made by Caspar [10] for TMV protein aggregates showed that monomeric TRV protein sediments at about 2 S, its dimer at 4 S. A two-layer disk consisting of two stacked rings of 26 subunits of molecular weight 24,000 daltons (or 32 subunits of 19,000; see discussion in Tollin and Wilson [11] sediments at 33–36 S. Thus the slowly sedimenting species probably corresponded to monomers and/or dimers and the faster one to disks. In the text they will be referred to as 4 S and as 36 S components, respectively.

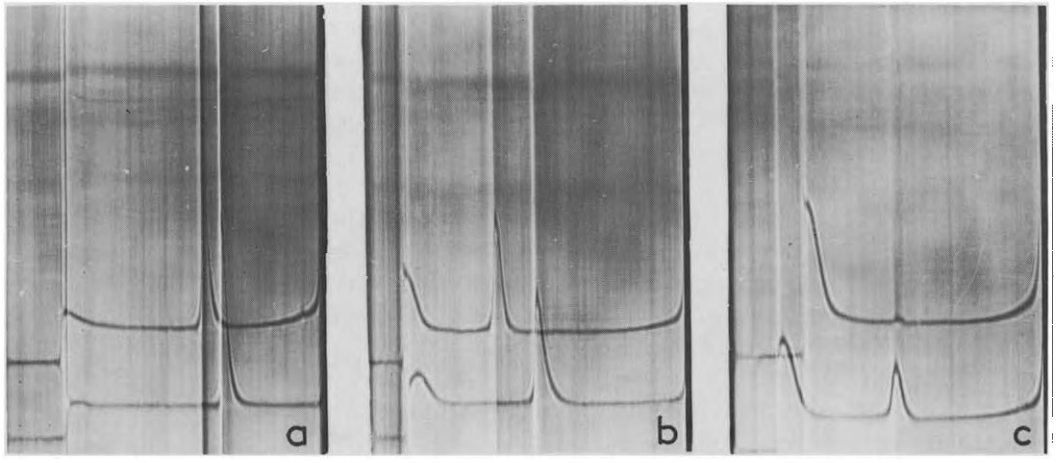

Fig. 2. Sedimentation patterns of TRV protein at 2°. a) Glycine-NaOH buffer, pH 8, μ = 0.01 (bottom) and 0.8 (top). b) Na acetate buffer, pH 5, μ = 0.01 (bottom) and 0.6 (top). c) Na phosphate buffer, pH 4.7, μ = 0.2 (bottom) and 0.4 (top). Protein conc.: about 4 mg/ml. Photographs were taken about 30 min after reaching 47,660 rpm.

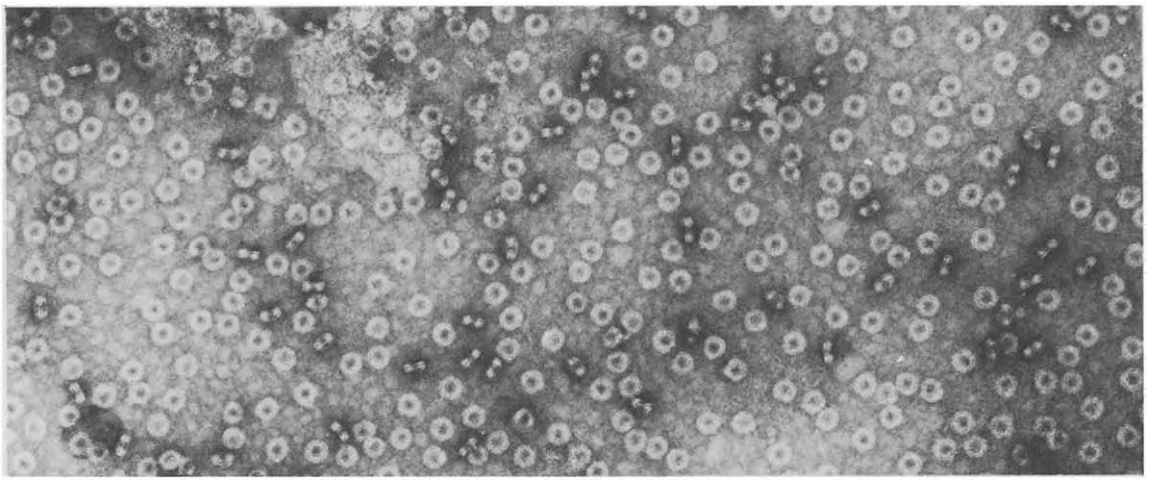

Fig. 3. Electron micrograph of a sample of TRV protein in pH 7 phosphate buffer (μ = 0.01), fixed in 1% formaldehyde prior to negative staining with 1% uranyl formate (× 108,000).

Fig. 1 illustrates their stabilities at 2°. At pH below 4 and μ above 0.2 the protein was denatured and precipitated. At pH above 5.5 the predominant component was 36 S, but some 4 S (less than 10%) was always present, even at pH 8 or 10, whatever the ionic strength (fig. 2a). Between pH 4.7 and 5.2, at very low ionic strength (μ = 0.01), the 36 S species was always predominant. At pH = 5.0 and μ above 0.1, both components were present in approximately equal amounts, but the exact relative proportions were not well reproducible, probably because slight pH differences displace the equilibrium (fig. 2b). At pH 4.7, the proportion of 36 S dropped drastically as μ increased from 0.2 to 0.4 (fig. 2c). At μ = 0.8, no 36 S was present at all. In this region of the phase diagram between pH 4.7 and 5.2, at μ greater than 0.4, we also sometimes observed a component sedimenting at 22–25 S. It was only present in minute amounts and never coexisted with a 36 S peak. Its significance is not yet known.

We have checked that the nature of the buffer did not influence the conformation of the protein: the sedimentation patterns in phosphate and acetate buffers were similar, at pH 4.7, 5.0 and 6.0, respectively.

In conditions where 36 S predominated, many disks could be seen on the electron micrographs (fig. 3). The thickness of particles seen on edge confirmed that they were probably two-layer disks.

3.2. Influence of the temperature

Offord [12] and Semancik and Reynolds [1] noticed an aggregation of TRV protein at 30°. This observation prompted us also to investigate the behaviour of the protein at 20° and 30°.

At 20° we obtained essentially the same sedimentation patterns and electron micrographs as at 2°, in the same buffers.

Some ultracentrifugations were performed at 30° and showed that the stability of 4 S is almost unaffected by temperature. However, conditions producing 36 S at 2° or 20°usually gave rise to faster sedimenting material, at 30°. For example, at pH 4.7 or 5.2, μ = 0.01, only one peak was observed, at 40–45 S. At μ above 0.5, at the same pH, the protein was dissociated as at 2° (fig. 4a, bottom). At pH 8.0 and μ=0.01 or 0.1, the major peak sedimented at about 30 S, and may correspond to the 36 S double disk; minor faster peaks were also observed at about 52 S, 70 S and 105 S. At μ = 0.5, at the same pH, the major peak sedimented at 47 S, and a minor one at 73 S; no material sedimented at 36 S or less (fig. 4a, top). Fig. 4b is an electron micrograph of TRV protein at 30° in glycine buffer pH 8, μ = 0.8, in which aggregates of double disks may be seen. For other ionic strengths, at the same pH, the pictures are very similar.

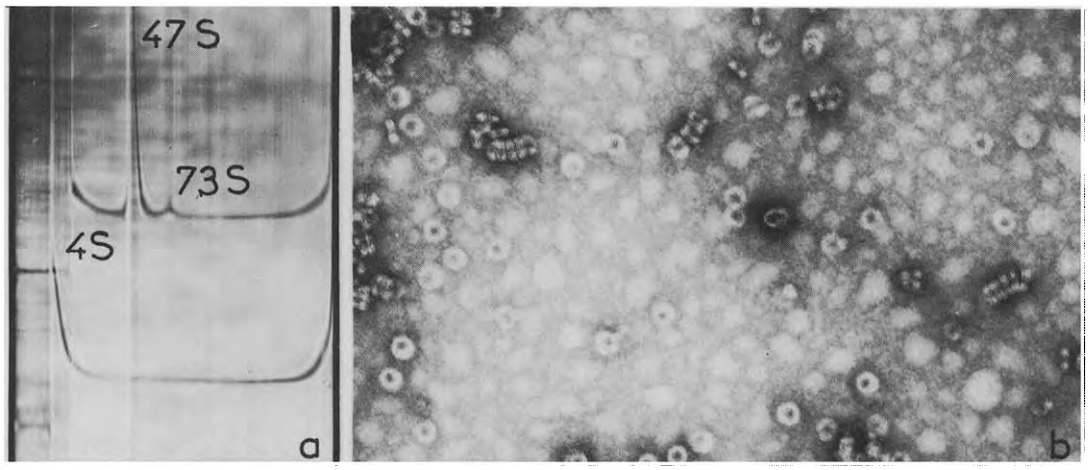

Fig. 4. TRV protein aggregation at 30°. a) Sedimentation of TRV protein (4 mg/ml) in phosphate pH 4.7 buffer (bottom) and glycine pH 8.0 (top), both at $\mu = 0.5$. The photograph was taken 8 min after reaching 47,660 rpm. b) Electron micrograph of a formaldehyde fixed, negatively stained protein sample in pH 8.0, $\mu = 0.8$ buffer (× 108,000).

4. Discussion

Ionic strength and pH have almost the reverse effects on TMV and TRV proteins. For TMV, the smallest aggregates are found at pH above 7.0 and low ionic strength [3]; for TRV they exist at pH below 5.5, and their importance increases with the ionic strength. For TMV the double disk exists in a fairly narrow range of pH and ionic strength; for TRV it is the predominant aggregate at pH above 5.5, whatever the ionic strength. On the other hand we never observed the presence of long helices or stacks of disks of TRV protein, although short aggregates of double disks form at 30°.

The state of aggregation of TRV protein changes drastically near pH 5.0. This may be attributed to the titration of the histidine group and/or carboxylic groups present. The latter however would titrate less abnormally than in TMV protein where the transition occurs at a pH close to 7.0 [4].

Semancik and Reynolds [1] succeeded in reassociating TRV RNA and protein in a pH 8, 0.25 M glycine buffer ($\mu = 0.5$) at 9°. In these conditions, according to our results, TRV protein exists predominantly in the double disk form sedimenting at about 36 S. This situation is very similar to that for TMV where optimal reconstitution conditions correspond to the aggregation of the protein into the double disk form [3–6]. Abou Haidar [2] however succeeded also in reconstructing TRV particles in a pH = 4.7 phosphate buffer of $\mu = 0.5$, at 2°, where 36 S double disks are present, but the predominant form is the 4 S protein. This raises the question of the participation of the monomers and small aggregates in the reconstitution, a point to be discussed in a further paper.

References

[1] J.S. Semancik and D.A. Reynolds, Science 164 (1969) 559.
[2] M. Abou Haidar, Thèse de 3ème cycle, Université de Strasbourg (1972).
[3] A.C.H. Durham, J.T. Finch and A. Klug, Nature New Biology 229 (1971) 37.
[4] A.C.H. Durham and A. Klug, Nature New Biology 229 (1971) 42.
[5] P.J.G. Butler and A. Klug, Nature New Biology 229 (1971) 47.
[6] C. Stussi, H. Guilley, G. Lebeurier and L. Hirth, Biochimie 54 (1972) 287.
[7] H.L. Sänger, Molec. Gen. Genetics 101 (1968) 346.
[8] J.S. Semancik, Virology 40 (1970) 618.
[9] Data for Biochemical Research, ed. R.M.C. Dawson et al. (Clarendon Press, Oxford, 1969).
[10] D.L.D. Caspar, Adv. Protein Chem. 18 (1963) 37.
[11] P. Tollin and H.R. Wilson, J. Gen. Virol. 13 (1971) 433.
[12] R.E. Offord, Ph. D. Thesis, Cambridge, 1965.