EVIDENCE FOR A RAPID STRUCTURAL CHANGE IN TMV - A - PROTEIN NEAR NEUTRALITY.

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Summary: Titration of TMV-A-protein from pH 8 to 7 (20°C) or raising the temperature from 4° to 20°C (pH 7) produces, within a few minutes, a reversible change in the aromatic region of the CD-spectrum, before any extensive aggregation has taken place. This spectral change is solely a matter of the conditions of the solution and not of the history of the protein. There is no further CD-change during the slow aggregation process. Thus there must be some proton-uptake within the A-protein. The results are discussed with regard to the different interpretations of the role of A-protein or double-disc in the elongation-step of TMV-"in vitro" -self-assembly.

Introduction: There is a number of well-defined species in the self-aggregation-process of TMV-protein (1,2). One of it is the A-protein ($S_{20,w}$= 4 - 4.6 S), a mixture of low aggregates (M_w close to 5 subunits (3)). Another is the double-disc (20 S), a two-layer ring-aggregate with 2 x 17 subunits. At an ionic strength of 0.1 the A-protein is stable at 7.2<pH<10, whereas at 6.5<pH<7.2 the double-disc is the stable aggregate (1,3). Disc-formation from A-protein is a slow process of at least 6 hours and up to 2 days (1,4). Both the A-protein and the double-disc are claimed to be important species in the elongation-step of "in vitro"-self-assembly of TMV from its RNA and protein (5, 6, 7).

Abbreviations: TMV: Tobacco Mosaic Virus; CD: Circular Dichroism

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To explain the conflicting results, it was assumed "that TMV-protein has a " memory "for its previous state of aggregation" (8), i.e. there are different forms of "A-protein" according to their storing and preparing conditions. Hence the intention for this work was to investigate if there is any detectable structural change within "different A-proteins" or in the transition from A-protein to double-disc.

Experimental: TMV was grown in Nicotiana tabacum (var. Samsun) and isolated by the method of Boedtker and Simmons (9); protein was prepared by the alkaline-degradation-method of Durham (1). TMV-protein near pH 8.0 had an OD (320 nm) = 0.003 and a maximum : minimum-ratio of 2.5. Protein-concentrations (4-7mg/ml) were determined in a Zeiss-PMQ-II-Spectrophotometer using ε_{281} = 1.30 $cm^2.mg^{-1}$ (10) (pH 8) or by the biuret-method (pH 7). Phosphate-buffer-solutions (ionic strength 0.1) were used throughout. CD-spectra were measured 2-4-fold in a Roussel-Jouan-Dichrographe-II, with scale expansion ($\Delta E=2.10^{-6}$/mm), in a thermostated cuvette-holder (pathlength 0.01-1.0 mm), E (281 nm) never exceeding 1.0. CD-values are given as molar $\Delta\varepsilon = \varepsilon_L - \varepsilon_R$ (M= 17,530; 310-240nm) respectively as mean residue ellipticity $[\theta]$ (M= 111; 250-185nm). Sedimentation experiments were performed in a Beckman Spinco E ultracentrifuge with Schlieren optics; s-values were corrected for water viscosity and 20°C. Titration was performed by adding 1n HCL in a μl-syringe under stirring, heating or cooling of the solutions by dipping the cuvettes in a water-bath, and measuring immediately after pH- or T- change in the pre-thermostated cuvette-holder. Dialysis was performed for at least 36 hours. Further details are given in Table 1.

Table 1: Selected CD-values of TMV-protein under various solution and preparing conditions.

solution conditions	history of protein	$S_{20,w}$	$\Delta\varepsilon$(290nm) ($\pm$ 0.1$_5$)	$\Delta\varepsilon$(280nm)- $\Delta\varepsilon$(295nm) ($\pm$ 0.3)	$[\Theta]_{222nm}$ ($\pm$ 700)	$[\Theta]_{208nm}$ ($\pm$ 900)	$[\Theta]_{193nm}$ ($\pm$ 1,500)
pH 8.0; 20°C	directly from prep.	4.2 S	+ 1.2	+ 6.8	- 8,100	- 10,900	+ 16,000
pH 7.0; 4°C	pH 8→7 (dial. 4°)	4.0 S	+ 1.1	+ 6.6	-	-	-
	pH 4.6→7 (dial. 4°)	-	+ 0.8	+ 6.5	-	-	-
	pH 8→7 (dial. 20°, then rapid 20°→4°)	-	+ 0.8	+ 6.6	-	-	-
pH 7.0; 20°C	pH 8→7 (titration)	5.6 S (84 %) 19.5 S (16 %)	- 0.1	+ 4.3	- 8,700	- 11,400	+ 16,800
pH 7.2; 20°C	pH 8→7.2 (dial. 20°)	6.3 S (49%) 18.8 S (51%)	- 0.1	+ 4.5	- 9,500	- 12,500	+ 17,900
pH 7.0; 20°C	pH 8→7 (dial. 24 h 4° 36 h 20°)	5.2 S (25%) 18.0 S (75%)	0.0	+ 4.2	-8,600	- 10,400	+ 16,300
	pH 4.6→7 (dial. 4° then rapid 4°→20°)	-	- 0.1	+ 4.7	-	-	-

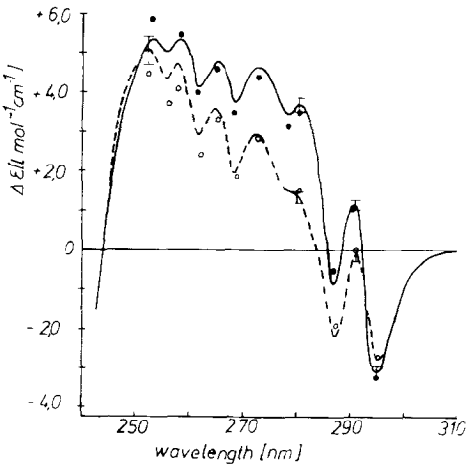

Fig. 1: Near-UV-CD-spectrum of TMV-protein under different solution conditions. Cp = 4-7 mg/ml.; pathlength 1mm; potassium-phosphate-buffer 0.035-0.047 m.
a) ——pH 8.0; 20°C; 4.2 S
b) ••• pH 7.0; 4°C; 4.0 S
c) --- pH 7.0; 20°C; up to 1 hour after titration from pH 8.0; 16% disc (19.5 S) maximal (Fig. 2a)
d) ooo pH 7.0; 20°C; dialyzed for 36 h at 20°C; 75% disc (18.0 S) in equilibrium with 25% A-protein (5.2 S) (Fig. 2b)

Results: Fig. 1a shows the near-UV-CD-spectrum of TMV-protein at pH 8; 20°C, identical to that at pH 7; 4°C (Fig. 1b). After rapid titration from pH 8 to 7 (20°C) the CD-spectrum is changed to Fig. 1c; this spectrum was scanned 3-fold within 1 hour after titration, without any detectable change in shape. During this time sedimentation was started; the respective pattern is shown in Fig. 2a, proving the formation of 16% double-disc within 1 hour. Further aggregation (Fig. 2b and Table 1) does not produce any further change in the CD-spectrum (Fig. 1d). Between 260-290 nm spectra 1a and 1c differ mostly, but only less or no change is observed at 295nm and, within the greater limit of error, near 250nm or in the far-UV-CD-spectrum (Fig. 1 and Table 1).

The CD-changes were tested immediately after the rapid pH-or

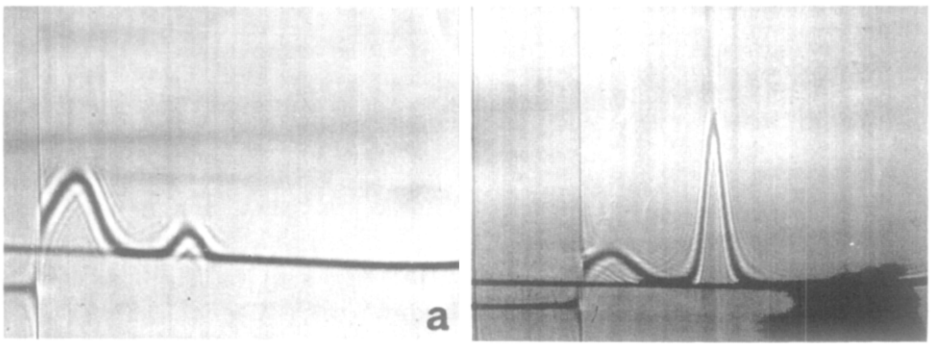

Fig. 2: Schlieren pattern of TMV-protein in different aggregation states. Cp = 4-7 mg/ml; potassium-phosphate-buffer 0.035-0.047 m; pH 7.0; 20°C
a) 16% disc (19.5 S); photograph was taken 1 hour after titration from pH 8.0. Corresponds to CD-spectrum Fig. 1c).
b) 75% disc (18.0 S) in equilibrium with 25% A-protein (5.2 S), after 36 h dialysis at 20°C. Corresponds to CD-spectrum Fig. 1d).

T-change with the 290nm-band (which characteristically decreases to zero) and the difference between the positive 280nm- and the negative 295nm-band (which is independent of zero-line position). The results are given in Table 1, proving that a preparation which had been in the disc-form (Fig. 2b), then being chilled to 0°C for some minutes (6,8) and measured at 4°C, has a CD-spectrum not characteristic for the disc (Fig. 1d), but for the "real" A-protein (pH 8;20°C, Fig. 1a+b). In contrast, a preparation which had been in the helix-form (pH 4.6), brought to pH 7 at 4°C, and after raising the temperature to 20°C, in fact has a spectrum characteristic for the disc, but without aggregation.

Discussion: Concerning the CD-measurements, the presented results may be summarized as follows:

1) TMV-A-protein at pH 8; 20°C, is identical to A-protein at pH 7; 4°C.

2) A-protein at pH 7; 20°C, is not identical to A-protein at pH 8, but preformed in structure to double-disc.
As there is a proton-uptake of about 0.4 H^+/subunit in going from pH 8 to 7 at 20°C, but none at 4°C (11), 1) and 2) gives evidence that this happens within the A-protein, not necessarily in the disc (3).

3) There is no evidence for " a "memory" of TMV-protein for its previous state of aggregation" (8). In contrast, the structure of the protein is solely given by conditions of the solution, not by its history.

4) There is no change in CD-pattern between pH 7.2 and 6.5 (12), but there is a steady increase in CD-intensity below pH 6.5 (12, 13) proportional to the increase in aggregates > 30 S (4, 12). Because of its structure and its range of existence, A-protein, preformed to disc, would be a good reagent for the growth of TMV-rods in the sense of Butler and Klug (8), i.e. " the growing end of the nucleoprotein-rod may be acting as a sensor for the disc-like state of the A*-protein", but there is no explanation for the different rates of reconstitution according to the preparation conditions of A-protein.

5) The structural change must be a very local one, because it is observed only in the spectral range of tyrosine and tryptophane, not in the phenylalanine and peptide range. Thus, the gross conformation of the 2 forms of A-protein and of the disc is the same. As tryptophane is the only species absorbing above 290 nm (14), and there is only a slight change in intensity of the 295 nm-band, this long-wavelength-absorbing tryptophane residue should not be engaged in the structural change.

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