

# The Mechanism of Aggregation of the Coat Protein of Tobacco Mosaic Virus. A Comparative Study on Vulgare and Mutant Proteins

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The aggregation behaviour of tobacco mosaic virus (TMV) protein vulgare was compared to that of two mutants, A14 and Ni 725, with amino acid exchanges localized in the coat protein at positions 107 (Thr → Met, in Ni 725) and 129 (Ile → Thr, in both mutants). This behaviour, as measured by sedimentation, hydrogen ion titration, light-scattering, and near-UV absorption difference and circular dichroism (CD) spectroscopy, differs characteristically both in the range of the A-protein (pH 8) and near neutrality, whereas nuclear magnetic resonance (NMR) and far-UV CD point at only subtle, or no structural differences between the three strains.

Near pH 8, the A-proteins of both mutants sediment nearly exclusively as 8S aggregates, under conditions where vulgare protein forms a 4S/8S mixture (two-layer and three-layer aggregates, Vogel *et al.*, Z. Naturforsch., 1979); in conditions where vulgare 4S aggregates dominate, both mutants sediment as a 4S/8S mixture. The average molecular weights of the 8S proteins correspond to 12 (vulgare) to 15 (mutants) subunits. — Near neutrality both mutants titrate and polymerize more cooperatively than vulgare protein; additionally, the  $pK_{(app.)}$  of Ni 725 is shifted upwards, due to the higher  $\alpha$ -helix forming potential of Met against Thr (pos. 107). Both mutants form large aggregates ( $\geq 200S$ ) of obviously helical conformation, by the uptake of one proton per subunit, whereas 20 S-disks constituting, under the same conditions, the stable entities in vulgare protein, are made only in minor amounts. These large mutant aggregates are remarkably more stable than the vulgare “overshoot” aggregates which transiently, too, may approach s-values and turbidities similar to the mutant aggregates; conformational changes, observed prior or in parallel to the formation of vulgare overshoot and disk aggregates, are significantly retarded in the large mutant aggregates. — Raising the ionic strength seems the only way to form mutant disks and stacks of disks (20–30 S) comparable to vulgare, pointing to the different pathways of disk formation, either at neutral pH or high ionic strength. — Evidence is given that the 8S aggregates of both mutant and vulgare proteins may behave similar in aggregation, the differences mainly being inserted by the 4S (two-layer) aggregates present in vulgare protein, which near neutrality seem responsible for the direct formation of (two-layer) disks. — The non-conservative exchange in position 129, altering the environment of Trp residues (52+17?), should weaken the “extended salt-bridge system” (“pairing”) observed between the two layers of the disk (Bloomer *et al.*, Nature, 1978). A competition is suggested between the strength of this pairing, and the binding of a third layer, regulating the mode of aggregation to two-layer, to three-layer, and to higher aggregates; this is corroborated by comparison with published results on temperature-sensitive (ts I) mutants and chemically modified proteins. — To explain the effects of residue 129 on the titration of the protein we suggest a mechanical analogy, made up of a balance between the charge and state of the “carboxyle cage” (Stubbs *et al.*, Nature, 1977), as regulatory site, and the strength of the salt-bridge system, about 25 Å apart, as the site active in the process of polymerization; a signal exerted on one end of the system may be transmitted to the other end by the rigid  $\alpha$ -helical system in between. — This mechanism remains open to discussion and further investigations.

## Introduction

The A-protein of tobacco mosaic virus (TMV) is the first oligomeric state in the aggregation from isolated monomers of the RNA-free coat protein [1–4]. Depending on the conditions of the solution it sediments as a broad, often unsymmetrical peak with sedimentation constants between 4 and 8S [4];

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in neutral and mildly acidic solution, it acts as the starting material for higher molecular weight aggregates, like disks and helices [2–6].

In a study on the so-called “overshoot” aggregates [7, 8] as intermediates in the formation of disks, Vogel *et al.* [9] observed critical conditions for the formation of the “22S” and “26S”, disk-like aggregates, and the disappearance of the “4S” and “8S” A-protein aggregates\*. This let us ask the question whether the components of the A-protein may be inhomogeneous not only in molecular weights, but also in their thermodynamic state and kinetic behaviour.

Recently, Vogel *et al.* showed [10] that the “8S” species of the A-protein abruptly comes into play above distinct critical temperatures and protein concentrations, which were beyond those used by Durham and Klug [5, 6] to establish their “nucleated two-layer linear condensation model”. Light-scattering measurements, however, resulted in mean molecular weights inconsistent with Caspar’s assumption [11] that an isolated heptamer could represent the “8S” species, but could be explained by the assumption that the “8S” peak envelops a whole series of three-layer aggregates, when the number of subunits,  $n$ , increases ( $n \geq 7$ ) with increasing temperature and protein concentration, similar to the “4S” peak which envelops a whole series of two-layer aggregates, with  $n \geq 3$  [6].

During an NMR-study on the flexibility of the inner part of the disk [12] Jardetzky *et al.* [13] used methionine coat protein point mutants for identification of the flexible peptide chain segment. The ultimate mutant chosen was Ni 725, mutated from Thr (vulgare) to Met (Ni 725), in position 107, and from Ile (vulgare) to Thr (Ni 725), in position 129. This latter exchange is the only one in the spontaneous mutant A14, from which Ni 725 formerly had been derived [14]. During the NMR-investigations significant deviations were observed in the aggregation behaviour of Ni 725, compared to vulgare protein in neutral and mildly alkaline solution. Surprisingly the behaviour of A14 was found to be much more similar to Ni 725 than to vulgare pro-

tein, thus giving evidence that position 129 in the sequence is important for regulating the mode of aggregation. Moreover, the differences in the aggregation behaviour between vulgare, A14, and Ni 725 protein gave us new insights how “4S” and “8S” aggregates can react to disks and helices. The results of this comparative study are presented here. – Part of the measurements were done in D<sub>2</sub>O instead of H<sub>2</sub>O solutions, because of the comparability to the NMR measurements.

## Materials and Methods

A14 and Ni 725 proteins were prepared from stocks of viruses grown and isolated in the Heidelberg laboratories. All experimental procedures were as reported earlier [9, 10]. The concentrations of the mutant proteins were determined using an absorption coefficient at 281 nm of  $1.28 \text{ cm}^2 \cdot \text{mg}^{-1}$ , taken from the absorption difference against vulgare protein. Molar absorbance difference,  $\Delta a = a_{\text{mutant}} - a_{\text{vulgare}}$ , and molar dichroic absorption,  $\Delta \epsilon = \epsilon_L - \epsilon_R$ , were based on the subunit molecular weight of  $M_r = 17500$ . Titration experiments were performed as described in [15], the curves being recorded by use of a Radiometer REC 61 Servograph. – Some special experimental details are given in the figure legends.

Deuterium oxide solutions were made as follows: protein samples in aqueous sodium acetate buffer, pH 4.6, in the helical state, were centrifuged for 2 h at 30000 revs/min. The supernatant was carefully removed and the pellet redissolved overnight in 99.75% D<sub>2</sub>O (Merck, Darmstadt, W. Germany), brought to pD  $\approx 9$  by addition of Na<sub>2</sub>DPO<sub>4</sub> (Merck) to about 7 mM (ionic strength  $I \approx 0.02 \text{ M}$ ). The solution was again acidified by small amounts of CD<sub>3</sub>COOD (Merck), and the pelleting and redissolution procedure repeated twice. The final pD was established by addition of CD<sub>3</sub>COOD, joined by a cleaning spin and/or ultrafiltration. The time intervals at pD  $\approx 9$  were sufficient to ensure that most of the exchangeable H was replaced by D [16]. Residual H<sub>2</sub>O and HDO as checked by NMR came to a few percent. For pD the pH-meter reading was taken, using  $\text{pD} = \text{pH}(\text{meter}) + 0.4$  [17].

Proton magnetic resonance spectra were taken at 360 MHz on a Bruker HX-360 of the Max-Planck-Institut für Medizinische Forschung, Heidelberg, operating in the FT mode. Protein concentrations of

\* As formerly [10] the terms “4S” and “8S” are used for simplicity, to denote two- and three-layer aggregates, bearing in mind that, in actual cases, the sedimentation constants may deviate significantly from these numerical values, and that they do not mean isolated species, but series of mutually and reversibly interacting particles.

about 10 mg/ml were used at or below room temperature. Usually a few thousand scans were accumulated to give a single Fourier transformed spectrum. Presaturation pulse (applied at the position of the HDO resonance) was obtained by using the built-in gated decoupling circuit. The power level of the decoupling channel was such that the effective  $H_1$  field covers the chemical shift range of less than 1 ppm at the 48 db attenuation of the output of the  $f_2$  channel.

## Results

In the state of the A-protein, at 20 °C and protein concentrations above about 3 mg/ml [10], at pH 7.5–9 and moderate ionic strength ( $I$ ), vulgare protein sedimented as the well known unsymmetric peak, with a leading peak at 7–8S, and a trailing boundary enveloping 4S aggregates [5, 10], shown in Fig. 1a ( $D_2O$ ,  $I = 0.02$  M, central curve) and Fig. 1b ( $H_2O$ ,  $I = 0.10$  M, lower curve). In the same conditions, however, both mutants sedimented as nearly symmetric peaks,  $s = 7$ –9S, with nearly no trailing material (Fig. 1a, upper and lower curve, and Fig. 1b, upper and central curve, for Ni 725 and A 14 protein); though in some stocks a very small amount of trailing material was found, because of its unreproducibility we suppose that this was due to a minor contamination by vulgare virus during growth. As exemplified in Fig. 1c these patterns were fully reversible, for instance by changes in  $I$ : a decrease in  $I$  to 0.01 M ( $H_2O$ ) made vulgare protein sediment nearly symmetrically as 4S protein (lower curve), whereas both mutants now showed the 4S/8S mixture (Ni 725, upper curve, and A 14, central curve), which is characteristic for vulgare protein only at higher  $I$  (Fig. 1b), or in  $D_2O$  (Fig. 1a): as shown earlier [18–20], due to its entropy driven character

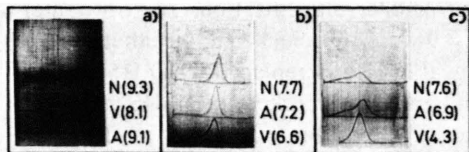


Fig. 1. Schlieren patterns of TMV-A-proteins at 20 °C, in sodium phosphate buffer, pH 8.0–8.5, protein concentrations 4–5 mg/ml. Rotor speed 47660 revs/min. V, A, N designate vulgare, A 14, Ni 725 strains, respectively. Numbers in brackets are  $s_{20,w}$ . a)  $D_2O$ ,  $I = 0.02$  M; b)  $H_2O$ ,  $I = 0.10$  M; c)  $H_2O$ ,  $I = 0.01$  M.

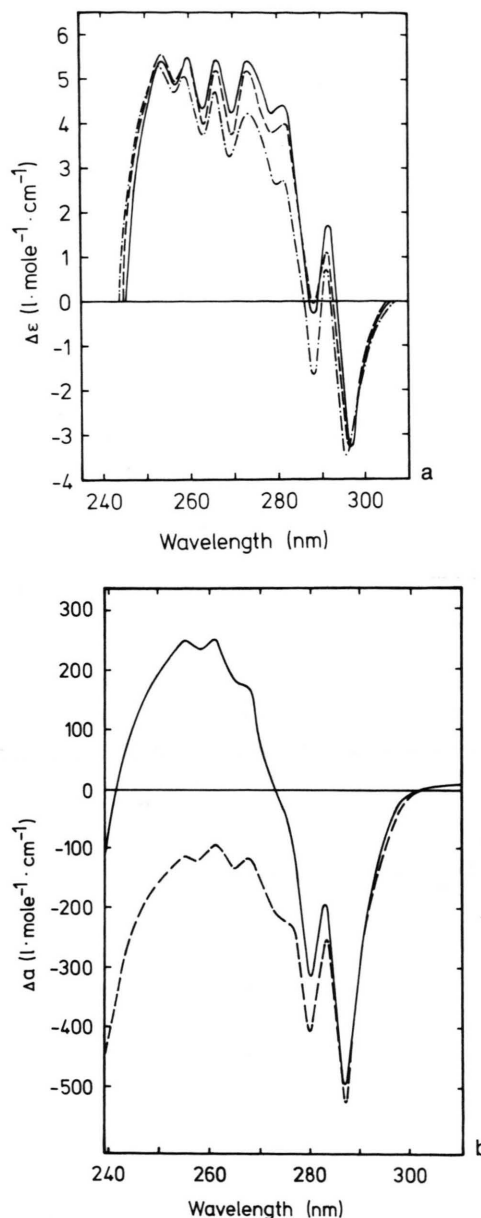


Fig. 2. Near-UV CD (a) and absorption difference (b) spectra of vulgare (---), A 14 (-----), and Ni 725 (—) protein, at 20 °C, in  $D_2O$ -sodium phosphate buffer solution, pH 8.4,  $I = 0.02$  M, protein concentration 10 mg/ml, pathlength 1 mm. For b), vulgare was in the reference cell.

the polymerization of TMV protein is favoured by increase of  $I$ , or replacement of  $H_2O$  by  $D_2O$ .

In Fig. 2a we compare the near-UV CD spectra of Ni 725 and A 14 protein to that of vulgare (in  $D_2O$ ,  $I = 0.02$  M). The more positive CD, between 255 and 296 nm, of the mutants compared to vulgare was

consistent with their higher states of aggregation (*cf.* [10], Fig. 5a + c); in the far-UV, no significant differences were found for the three samples (not shown), giving evidence that only local conformations and not the secondary structure of the mutants differed from that of vulgare protein.

In Fig. 2b we present absorption difference spectra (in D<sub>2</sub>O,  $I = 0.02$  M), with the mutant proteins in the sample and vulgare protein in the reference beam of the spectrometer. The broad positive difference band near 260 nm of Ni 725 minus vulgare protein may be due to contamination by some residual nucleotide or undegraded virus. The sharp negative difference bands at 280 and 287 nm which were similar for both mutants must be compared to the temperature-induced positive difference bands of vulgare protein, at 298, 287, and 278 nm (*cf.* [10], Fig. 5b), when the degree of polymerization rose. These positive bands were interpreted as due to the increase in amount and mean molecular weight of the two-layer (4S) aggregates; the entrance of the three-layer (8S) aggregates was supposed to be reflected by the steeper increase of the 298 over the 287 nm-band [10]. The negative difference spectra presented here (Fig. 2b) thus reflect at least two facts: some positive absorption, on one hand, due to stronger aggregation in the sample (mutants) com-

pared to the reference (vulgare) must have been overcompensated by stronger negative absorption, on the other hand; at 298 nm, both effects nearly cancel, demonstrated by the weak shoulder above 292 nm (Fig. 2b, *cf.* [10], Fig. 5b). Negative differential absorption is commonly due to a blue-shift of sample against reference absorption bands, in this case presumably due to tryptophan residues in more polar environment, in the mutants as compared to vulgare ([21], see discussion).

The increasing tendency for aggregation in the mutants as compared to vulgare is also seen in the proton magnetic resonance spectra of Fig. 3 taken under almost exactly the same condition as that of Fig. 2. We note that the spectrum becomes slightly, but progressively broader as we go from vulgare (a) to A14 (b) and to Ni 725 (c) with exception of the two sharp peaks ( $\delta = 1.17$  and  $\delta = 1.41$  ppm) reported earlier [13]. This broadening would arise from the increase in the effective rotational correlation time of the protein molecule due to increased aggregation in the mutants as compared to vulgare. One notices that this phenomenon bears similarity to that observed with vulgare when one goes from 4S particles to disks (*cf.* [13], Fig. 1a + b). Particularly interesting is the pronounced decrease of the peak intensity at  $\delta = 3.2$  ppm probably attributable to the

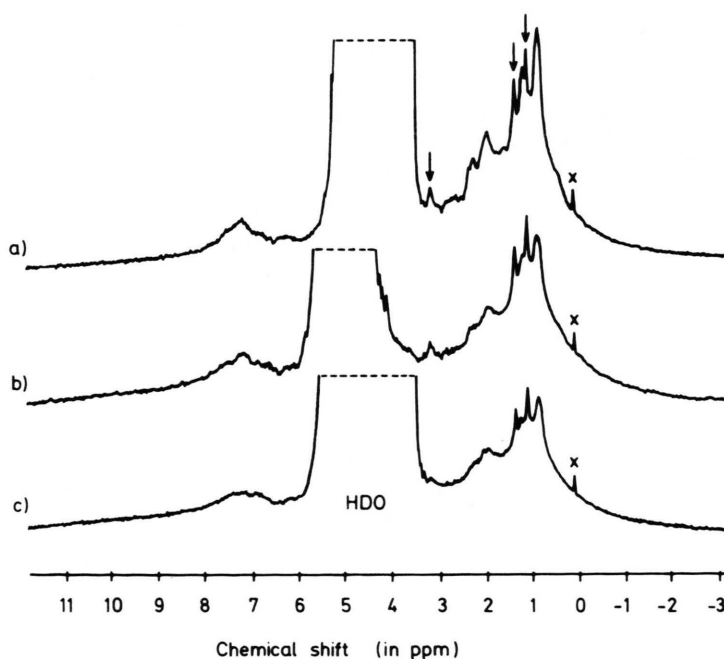


Fig. 3. Proton magnetic resonance spectra of vulgare (a), A14 (b), and Ni 725 (c) proteins, at 24 °C in D<sub>2</sub>O-sodium phosphate buffer, pD 8.5 ± 0.2,  $I = 0.02$  M, protein concentration 9.8 mg/ml, the three spectra drawn with the same gain. Chemical shifts are given from the sharp signals designated by x, from internal 3(trimethylsilyl) tetradeutero-sodium propionate (TSP). Arrows point at the signals at  $\delta = 1.17$ ; 1.41; 3.20 ppm referenced in the text.



$\varepsilon$  protons from the only two Lys residues (53 + 68) to exist in TMV protein (see below).

When a presaturation pulse  $f_2$  was applied at the position of HDO resonance, prior to the application of the sampling pulse, the resonance intensity of vulgare (pD 8.5) was dramatically decreased to almost complete disappearance (experiments not shown) except for some of the methyl and methylene resonances which presumably arise from mobile regions [13], as was observed earlier in myosin [22]. In the case of TMV proteins the resonance from, for example, the aromatic protons was completely diminished, while this was not the case when the presaturation pulse was applied at the similar power level (48 db attenuation of  $f_2$  output) on samples of higher alkaline conditions (pD 10.8). The loss of resonance intensity observed at pD 8.5 is a result of saturation transfer from the  $C_\alpha$ -protons (resonating under the HDO peak) to most other protons in the rigid region *via* spin diffusion [22]. The mechanism is operable only when the rotational correlation time ( $\tau_r$ ) of the protein is so large that  $\omega_0 \tau_r \gg 1$  [22], which is entirely consistent with a large  $\tau_r$  ( $\approx 8 \times 10^{-8}$  s) expected from the Stokes-Einstein relation for a molecular weight corresponding to the 8S protein (254 000 daltons, see below) and the resonance angular frequency  $\omega_0$  ( $= 2\pi \cdot 360 \times 10^6$  Hz). The fact that the effect of presaturation pulse was small at pD 10.8 indicates that in this pD range the proteins dissociate into smaller particles, most likely into monomers (17 500 daltons). Nevertheless, the aromatic resonances remained broad and no single Phe, Tyr, or Trp residue was traceable, showing the lack of internal mobility in any of the aromatic residues, even at pD 10.8.

The apparent homogeneity of the A-protein of A14 (Fig. 1a, b) prompted us to try to measure by light-scattering [10] the molecular weight of the 8S component (Fig. 4). The graph of the reciprocal of the molecular weight observed ( $1/M$ ) against the protein concentration ( $c$ ), according to (see [23])

$$1/M = (1/M)_0 + 2Bc$$

yields a straight line, at  $c = 5$ –20 mg/ml. From the slope, the second virial coefficient,  $B$ , is obtained as  $4.7 \times 10^{-5}$  mol ml g $^{-2}$ , in good agreement with the theoretical value [23],  $B = 3.4 \times 10^{-5}$  mol ml g $^{-2}$ , for an uncharged globular protein, with the dimensions of a cyclic heptamer [11] which should have a diameter of about 75 Å. An increase in volume

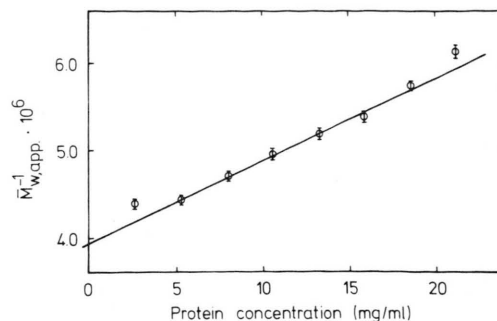


Fig. 4. Plot of the apparent reciprocal molecular weight, obtained by light-scattering, of A14-protein, as function of its concentration, at 20 °C, in H<sub>2</sub>O potassium phosphate buffer solution, pH 8.0,  $I = 0.1$  M. Experimental procedure and evaluation of the data was as described [10, 23].

(molecular weight) [23] or in charge [23, 24] increases the numerical value for  $B$  (see discussion).

On the other hand, the “true” molecular weight, obtained by extrapolation of  $1/M$  to zero concentration, yielded  $M_0 = 254$  000 daltons, more than twice the value expected for a heptamer ( $M_0 = 122$  500). However, the experimental value is consistent with the molecular weight estimated by Schramm and Zillig [1], by means of ultracentrifugation, for the 8S component of vulgare protein ( $M_0 = 260$  000), and is comparable to the upper limit of the molecular weight of vulgare 8S-protein measured by light-scattering [10], on the average corresponding to about 12 subunits per three-layer aggregate ( $M_0 = 210$  000). In this regard we must take into account that in general the sedimentation constants of the 8S-component of A14-protein are slightly higher (8–9.5S) than those of vulgare protein (7–8.5S) (see above), which, in A14, may be due to the non-existence of smaller aggregates and the lack of interaction between 4S and 8S.

The deviations from linearity, below 5 mg/ml and above 20 mg/ml (Fig. 4), are easily explained by an increase of dissociation, below 5 mg/ml, and the need for using higher virial coefficients, above 20 mg/ml, respectively. On the other hand, the linearity amongst 5 and 20 mg/ml by no means prove the existence of a single “8S species” built of 14–15 subunits. A balance seems more reasonable, between increase of aggregation and increase of  $B$ , with increasing concentration. If we assume that like in vulgare protein (*cf.* [10], Fig. 4b), between 5 and 20 mg/ml the mean molecular weight increases by 40 000 daltons, an increase of  $B$  from  $4.7 \times 10^{-5}$  to

$6.1 \times 10^{-5}$  mol ml  $g^{-2}$  would be sufficient for balancing; this seems acceptable taking into account changes in volume and shape of the particles.

Titration experiments have revealed useful details on the state of TMV protein aggregation [15, 25, 26]. Titration curves obtained in  $H_2O$ , at 22 °C and 4 mg/ml protein concentration, are shown in Fig. 5, at two different ionic strengths, for vulgare, A14, and Ni 725 protein (from above). Each titration started near pH 5, with protein in the helical state, and was done to about pH 8 (lower curves), then the titration was reversed, down to the starting point (upper curves). Upward and downward titration revealed the hystereses in proton binding and release already described [15, 26], most pronounced in vulgare protein, markedly less in both mutants, at ionic strength 0.1 M (Fig. 5a), and intermediate at ionic strength 0.01 M, with only minute differences between the three strains (Fig. 5b). The shape of the titration curves of both mutants are mutually more similar compared to vulgare, at  $I = 0.1$  M; at  $I = 0.01$  M, however, any difference nearly vanishes. Below pH 8, at  $I = 0.1$  M, vulgare protein binds 80% of the first proton in a relatively broad step ( $pK_{app.} = 7.0$ ;  $\Delta pH_{80\%} = 0.8$ ); in A14 protein, the corresponding step is markedly more cooperative, at the same position ( $pK_{app.} = 7.05$ ;  $\Delta pH_{80\%} = 0.3$ ); in Ni 725 protein, the cooperativity is identical to A14 protein, the step being shifted to higher pH values ( $pK_{app.} = 7.3$ ;  $\Delta pH_{80\%} = 0.3$ ) (Fig. 5a). At  $I = 0.01$  M the cooperativity of the first step is large and similar for the three strains, the number of protons bound per subunit in this step being significantly higher than 1 (least in Ni 725), with  $pK_{app.}$ 's at 6.6 (vulgare), 6.7 (A14), and 7.3 (Ni 725) (Fig. 5b). The pronounced steps observed for vulgare protein at  $I = 0.1$  M (Fig. 5a), with  $pK_{app.} \approx 6.0$  for the downward curve, and  $pK_{app.} \approx 6.8$  for the upward curve, matching each about 0.5 protons per subunit, correlating to the steep increase and decrease, respectively, in turbidity [9, 15], are markedly reduced or cancelled in the mutants, and at the low ionic strength. Neglecting small shifts on the abscissa of less than 0.1 pH, all curves coincide if compared at equal ionic strength, below any low pH step on each curve.

Fig. 6 summarizes some typical results upon the sedimentation of the vulgare and mutant proteins near the step for the first proton uptake during titration (Fig. 5). As shown earlier [9], vulgare protein slowly forms disk aggregates (20–22S) from A-

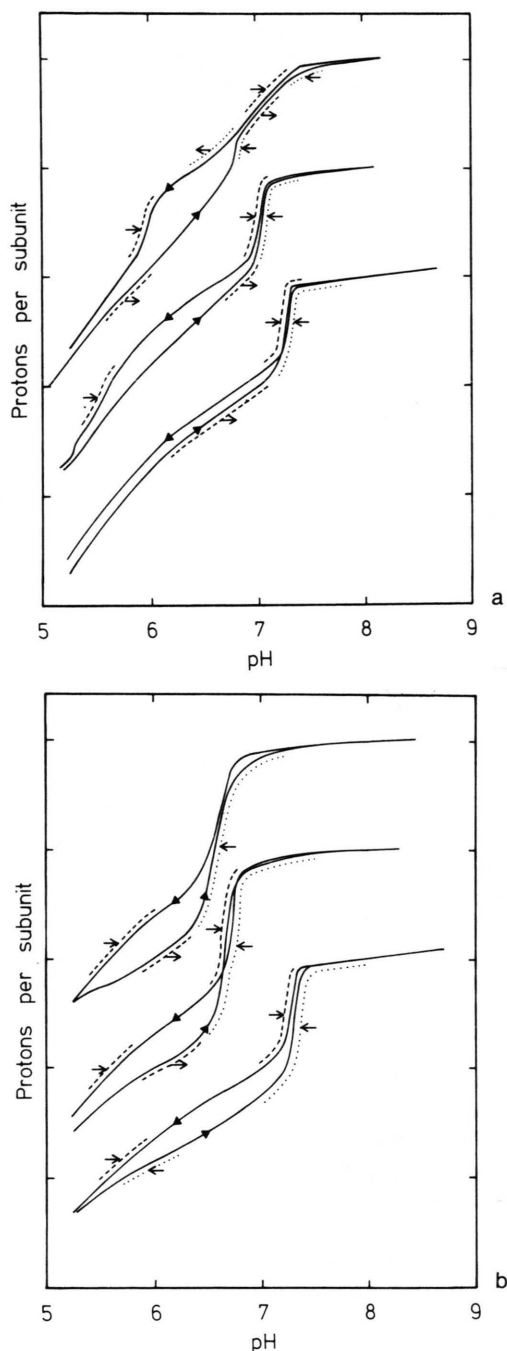


Fig. 5. Titration curves of (from above) vulgare, A14, and Ni 725 proteins, in 0.1 M (a) and 0.01 M (b) KCl solutions, at 22 °C; protein concentrations 4 mg/ml. Experimental procedure was as described in the text, and in [15]. Ranges of significant drifts of pH with time, due to slow depolymerization/polymerization processes of the sample, are indicated by arrows and dotted (downward drifts) and broken (upward drifts) lines along the curves, their meaning having been discussed in [15].

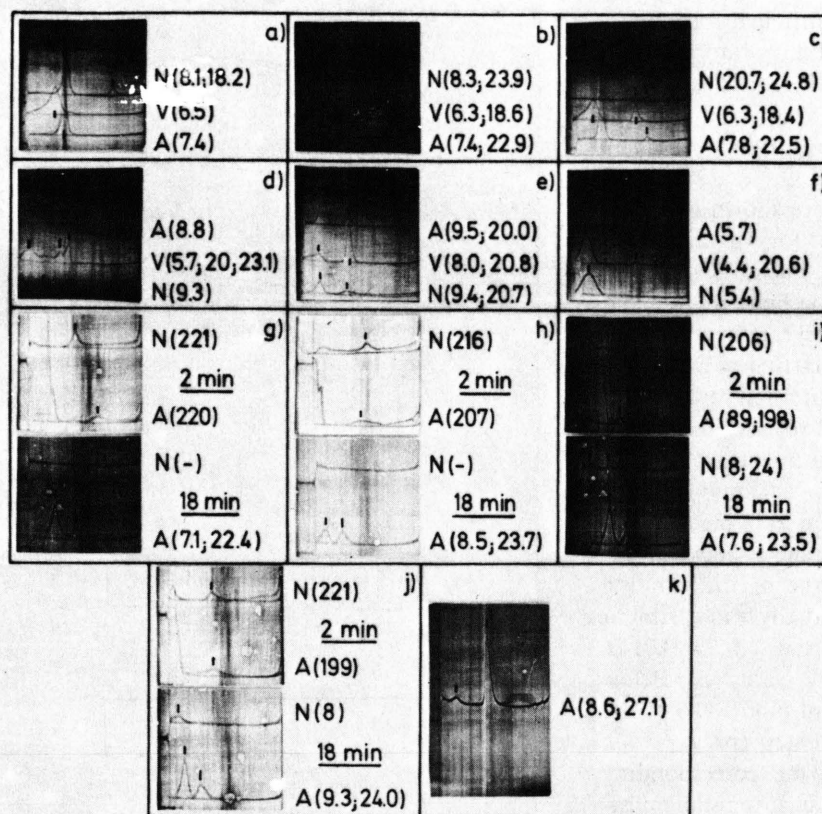


Fig. 6. Schlieren patterns of TMV-proteins (V, A, N, see Fig. 1) in potassium phosphate buffer, near the titration steps around neutral pH shown in Fig. 5. Numbers ( $s_{20,w}$ ) refer to the peaks indicated by vertical bars (sedimentation from left to right). Solutions were heated from 0 °C to 20 °C (a–c) or 22 °C (d–k) within about 2 min, except in j) (heating time about 10 h). Protein concentrations 10 mg/ml (a–c, k) and 5 mg/ml (d–j). Ionic strengths  $I = 0.1$  M (a–e, g–j), 0.01 M (f), and 0.5 M (k). a) pH 7.3, 14 h at 20 °C; b) pH 7.15, immediately after heating; c) the same, pH 7.0; d) to e) near the  $pK_{app}$ 's for each V, A, N given in the text and in Fig. 5, immediately (d) and 24 h (e, f) after heating, at  $I = 0.1$  M (d, e) and 0.01 M (f); g) to j) pH 6.85, 2 min (upper 2 curves) and 18 min (lower 2 curves) after reaching maximal rotor speed (35 600 revs/min), immediately (g), 1 (h) and 3 (i) days after rapid heating, and about 1 day after slow heating (j); k)  $I = 0.5$  M, pH 8.5, about 3 h after heating.

protein either in direct reaction, below critical temperatures and above critical pH values (e. g., 22 °C, pH 7.1), or via rapidly formed overshoot aggregates ( $\approx 26S$ ), beyond these critical conditions; this is corroborated by Fig. 6b–e. In equilibrium about 85% (w/w) disk aggregates can coexist with residual A-protein [27].

Such behaviour was not observed with the mutants: at the outset of the titration step, in these and many similar experiments, the amount of 20S material never exceeded about 15% of the total, even after one or two days at room temperature (Fig. 6a–e). Regarding the possibility of slight contamination by vulgare protein this amount of disks should represent an upper limit made, near neutral pH, by the mutants per se. Lowering slightly the pH rapidly gave way to a lot of heavy material (Fig. 6b–d, g) sedimenting at 200S and above, in coexistence with smaller amounts of 7 to 24S material. Within 3 days only minor redistributions had taken place (Fig. 6e, h, i). Also, at pH 6.85 slow heating was nearly as effective as rapid heating (Fig. 6j, compare g). Thus, the tendency towards formation of disk-like aggregates

( $\approx 20S$ ) is small in A14 and Ni725 proteins: the binding of 1 proton per subunit (Fig. 5a) makes large aggregates, considerably more stable than those observed for vulgare protein at pH 6.5 [28, 29], and all the more than those near pH 7 [9].

Lowering the ionic strength shifted the oligomer distribution both in A14 and Ni725 A-protein from exclusively 8S to 4S/8S equilibrium (Fig. 1c): from this we supposed to make easier 20S disk aggregates at low ionic strength, at the outset of the titration steps (Fig. 5b). This assumption proved to be wrong: no 20S material was made (Fig. 6f). On the other hand, raising the ionic strength to 0.5 M, at pH 8 or higher, easily formed 20S aggregates from vulgare protein [4, 5]: for A14 protein this procedure was unique forming 20–30S aggregates (disks, and stacks of disks) (Fig. 6k) in rate and extent comparable to vulgare protein. Obviously, a minimum ionic strength is necessary for the formation of disks, irrespective of the aggregates present as starting material; and, as was suggested earlier [9, 27, 30], at elevated pH and ionic strength the structure of the disks and their mechanism of formation are some-

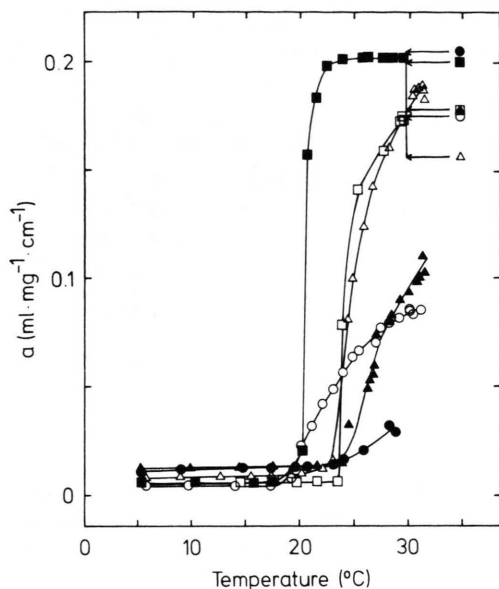


Fig. 7. Change in specific turbidities (reduced to 1 mg/ml, 1 cm pathlength, at 320 nm) of TMV-proteins, caused by stepwise increase in temperature, near the steps in the titration curves (Fig. 5), in potassium phosphate buffer, protein concentration 5 mg/ml. Filled symbols:  $I = 0.10$  M; open symbols:  $I = 0.01$  M. (●, ○) vulgare, pH 7.0 and 6.6; (▲, △) A14, pH 7.1 and 6.7; (■, □) Ni 725, pH 7.3 and 7.3. The vertical line at 30 °C, and the arrowheads indicate the turbidities attained by a sudden raise (within 1.5 min) in temperature, from 4 °C to 30 °C, for the samples indicated at the right, by the respective symbols.

what different from those near neutrality, at  $I = 0.1$  M (see also below).

Preliminary turbidity measurements (Fig. 7) supported the ultracentrifuge findings, near the titration steps (Figs 5 + 6, g–j). For the moment, the reproducibility is insufficient for quantitative evaluation of the measurements, because of their high sensitivity to small changes in pH or temperature. Qualitatively they gave evidence that, with stepwise heating, both mutants aggregate much stronger and more cooperatively than vulgare, at  $I = 0.1$  M; these differences got smaller at  $I = 0.01$  M, or nearly vanished by rapid heating, when the turbidity of vulgare protein approached that of the mutants. After some time the turbidity of vulgare protein began to decrease (“overshoot”, [9, 28, 29]), those of the mutants remained remarkably stable (not shown), in parallel to the sedimentation behaviour. This gave evidence that, provided the slow formation of vulgare disks is overcome by rapid heating [7, 9, 28, 29], vulgare protein can behave like the mutants which *per se* do not form disks.

A clear-cut correlation was shown to exist between the CD spectra of vulgare protein, and its mode of aggregation: going from A-protein via disks, or related compounds, to helices, the near-UV CD intensity first decreased, in parallel to the formation

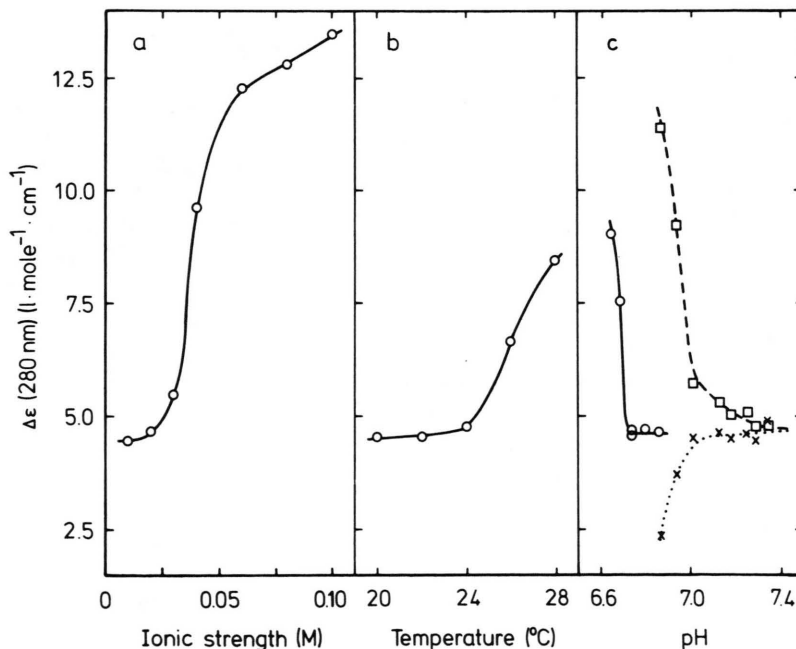


Fig. 8. Change of CD (280 nm) of A14-protein, under conditions favouring the polymerization, in potassium phosphate buffer solution, protein concentration 4.7 mg/ml. a) as function of the ionic strength,  $I$ , at pH 6.80,  $T = 22$  °C; b) as function of the temperature, at pH 6.80,  $I = 0.02$  M; c) as function of the pH, at  $T = 22$  °C: (—○—)  $I = 0.02$  M; 10 min after  $\Delta T$  from 0 °C; (---□---)  $I = 0.10$  M; 10 min after  $\Delta T$  from 0 °C; (···×···)  $I = 0.10$  M; 24 h at 22 °C.



of disks, then it increased, when helices came into play [27, 30]. The lack of considerable amounts of A14–20S aggregates observed in the ultracentrifuge (Fig. 6), near the first step in the titration curves (Fig. 5), was corroborated by the lack of a decrease in CD intensity, at 280 nm, when observed soon after raising the polymerizing conditions (Fig. 8): neither at low (Fig. 8a–c), nor at ionic strength 0.1 M (Fig. 8a + c), such a decrease was found. The steep increase agrees together with the strongly co-operative proton uptake (Fig. 5). Only when re-investigated one day later (Fig. 8c) a decrease was observed, giving evidence that a slow conformational change had taken place within the large aggregates ( $\geq 200S$ ), without significant changes in the state of aggregation (Fig. 6g–i). In special conditions such a phenomenon had been observed for vulgare protein, too [9], though normally the decrease of CD was finished prior to the start of the measurements [27]. – Comparable results were obtained in  $D_2O$  solutions, at  $I = 0.02$  M (not shown).

CD spectra representative for the different states of A14 protein aggregation, at 22 °C, are shown in Fig. 9: at pH 8, as A-protein (compare Fig. 1b), at pH 6.85, 10 min and one day after heating from 0 °C

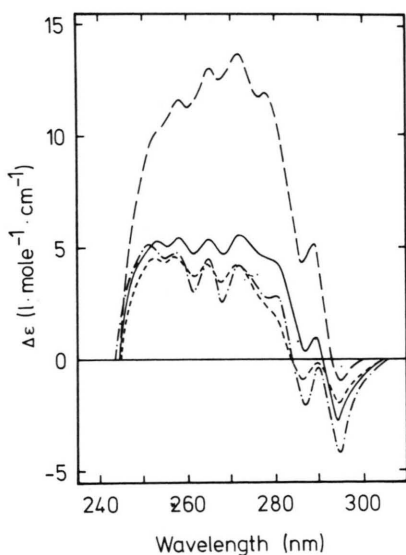


Fig. 9. Representative CD spectra of A14-protein, in different states of aggregation (compare Fig. 8), at 22 °C, in potassium phosphate buffer solution. (—)  $I = 0.1$  M, pH 8.0, protein concentration  $c_p = 4.7$  mg/ml; (---)  $I = 0.1$  M, pH 6.85,  $c_p = 4.7$  mg/ml, 10 min after  $\Delta T$  from 0 °C; (- · -) the same conditions, after 24 h at 22 °C; (· · ·)  $I = 0.5$  M, pH 8.5,  $c_p = 9.5$  mg/ml, 2 h after  $\Delta T$  from 0 °C and  $\Delta I$  from 0.1 M.

(compare Fig. 6g + h), and at pH 8.5, at ionic strength 0.5 M (compare Fig. 6k). As far as comes out from CD, by comparison with the states of vulgare protein, the large aggregates are first in a helix-like conformation, then they change to a disk-like conformation. The differences between the disk-like spectra below pH 7 ( $I = 0.1$  M) and at pH 8.5 ( $I = 0.5$  M), most obvious at the negative bands near 295 nm, are in full agreement with the corresponding differences observed with vulgare protein ([27, 30], see above).

## Discussion

The results presented above can be summarized as follows:

a) The first proton uptake below pH 8 is shifted by about 0.3 pH to the alkaline, if, in position 107 of the amino acid sequence, threonine in vulgare is replaced by methionine in Ni 725 protein.

b) In position 129, the replacement of isoleucine by threonine, in A14 and Ni 725 as against vulgare protein, reinforces the tendency for 8S instead of 4S aggregates, in mildly alkaline solution and moderate ionic strength, and for helices instead of disks, near neutrality. This clear relation between the existence of 4S and the formation of two-layer disk aggregates, as well as the existence of 8S and the formation of helical aggregates, is strongest support to earlier suggestions that subunits in the 4S oligomeric series are arranged in two layers [6, 11], and that material sedimenting in the leading 8S peak of the A-protein is qualitatively different from the material sedimenting in the trailing 4S boundary [9, 10].

c) These amino acid exchanges do only alter local structures as proved by the similarity of the NMR and the far-UV CD spectra for the three strains, and also of the near-UV CD spectra, if one compares similar states of aggregation. The exchange in position 129 affects the environment of a tryptophan residue. There are hints (see below) that also the mechanism of the aggregation reactions are similar if one compares similar states of aggregation in the three strains.

d) “8S” aggregates are significantly larger than heptamers, with numbers of subunits between 12 and 15, if sedimenting between 8 and 9.5S (compare also [10]).

e) “Disks” are different, and are made by different pathways, whether they are made by lowering the pH ( $I = 0.1$  M), or raising the ionic strength

(pH 8.5). These differences are most obvious, too, around a tryptophan residue (compare also [9, 27, 30]).

f) The lack of two-layer aggregates (4S and disks) strongly reinforces the cooperativity of the polymerization processes, and weakens the hystereses observed for the proton uptake plus association, and the proton release plus dissociation of the aggregates. This gives evidence that not only the metastability of large helices [26, 28, 29] but also the existence of stable or metastable intermediate sized aggregates contributes to the hystereses observed.

a) is easily explained by the higher  $\alpha$ -helical conformational parameter [31, 32] of methionine compared to threonine: in going from the disk to the virus-helix, the inner part of the protein, which is flexible in the disk [12, 13, 33], is transformed to an  $\alpha$ -helix (V-helix, [34]), between residues 102–112. The tendency for this transformation will be higher with increasing average  $\alpha$ -helical conformational parameter of the amino acids participating. The shift in  $pK_{app}$  proves that  $\alpha$ ) the proton uptake in question actually occurs close to the V-helix, in the neighbourhood of which negative charges may cluster to form a "carboxyle cage" [34] capable of binding protons with abnormal high  $pK$ , and that  $\beta$ ) the protein aggregates thereby formed are really helices, similar to the virus-helix enwrapping the RNA.

For explanation of b) and c) we consider the structure of the vulgare disk as revealed by the X-ray resolution to 2.8 Å [33]. This analysis revealed an "extended salt-bridge system", at 60 to 80 Å radial distance from the centre of the particle, linking the two layers of the disk. This salt-bridge system contributes to the "pairing distortion" observed at 65 to 80 Å radial distance [12, 35], supposed to stabilize the two-layer aggregates (4S and disks, [6, 36]) as compared to helices. Members of the salt-bridge system are Asn 127, Glu 131, and Arg 134 from the "LR"- $\alpha$ -helix in the upper ring of the disk, and Glu 50 plus Lys 53, Glu 22 plus Asp 19, Lys 68 plus Asp 66, in the lower ring. Considering the sequence, Ile (vulg.)/Thr (A 14) 129 and Trp 52 are midway this system, with Trp 17, Trp 70 plus 72 neighbouring. Replacing the non-polar Ile 129 by the more polar Thr obviously causes the blue-shift observed in the aromatic spectra of A 14 and Ni 725 against vulgare protein (Fig. 2b), as matters stand by Trp 52 and, perhaps to a lesser extent, Trp 17. Of course, positioned in an  $\alpha$ -helix the side chain of amino acid

129 points in opposite direction from the salt-linkages made by residues 127, 131, and 134; however, the field of the groups surrounding the salt-bridges is no short-range quantity, and besides of this, A-protein differs slightly in conformation from disks, as revealed by the different CD spectra, attributed to changes near Tyr residues 70 and 72 [30]. Furthermore, we notice the particular effect on the Lys residues, as members of the salt-bridge system, in going from vulgare to A 14 and Ni 725, as revealed by the NMR signals at  $\delta = 3.2$  ppm (Fig. 3).

Salt-bridges are most stable in hydrophobic environment: thus, the salt-bridge interaction between the upper and lower subunits in the two-layer aggregates should be weakened by the exchange from Ile to Thr. In the three-layer aggregates, the pairing between two layers should be disturbed by competition with the third layer. *Vice versa*, weakening the interaction between two layers should favour three-layer, or even larger aggregates. In going from disks to helices the salt-bridge system is completely disrupted [33], in accordance with earlier conclusions [30] that, during this transition, the neighbouring Trp residues 17 and 52 are influenced. This easily explains why A 14 and Ni 725 proteins tend to form 8S instead of 4S aggregates, and why 8S aggregates tend to helix instead of disk formation.

There are several more observations all pointing to the importance of the axial inter-subunit interactions ("pairing", meaning, in addition to the salt-bridge system, one hydrophobic contact between positions 54 and 74/75, and hydrogen bonds between positions 59 and 147/148, [33]) for the specific and correct aggregation in TMV protein strains and mutants:

amino acid exchanges within or nearby this pairing frequently lead to temperature-sensitivity of the coat protein (*tsI*-behaviour) [37, 38]: Ni 2239: Ser (15)  $\rightarrow$  Leu; flavum, refluvescens, Ni 103, Ni 696: Asp (19)  $\rightarrow$  Ala, Val, Gly, Ala; Ni 118: Pro (20)  $\rightarrow$  Leu; Ni 458: Thr (59)  $\rightarrow$  Ile; Ni 1196: Pro (63)  $\rightarrow$  Ser; Ni 116: Asp (66)  $\rightarrow$  Gly; Ni 2068: Tyr (139)  $\rightarrow$  Cys; CP 415: Asn (140)  $\rightarrow$  Lys. It was clearly demonstrated [38–41] that *tsI*-behaviour is due to "non-specific" aggregation of the coat protein, as was found, too, by chemical modification of the two Lys groups 53 and 68 [42]. A 14 does not belong to the *ts* mutants [38, 39]: obviously its non-conservative exchange against vulgare is less active than those in the mutants itemized. However, the raise in the charge

and polarity of Tyr 139 of vulgare protein, by oxidation with N-Br-succinimide, induces the formation of 8S and helical, instead of 4S and disk aggregates [43, 44], similar as found in A14 and Ni 725: Tyr 139 was shown to reside very close to Asp 66 and Lys 68 [33, 45].

Taken together this shows that there must be a delicately balanced competition between the axial pairing between two layers, and the specific or non-specific bonding to other subunits. From this point of view, three-layer aggregates may be regarded in between two-layer aggregates, and helices. Provided the three-layer (8S) aggregates from vulgare, A14 and Ni 725 protein are comparable, this finds support by their CD spectra (Fig. 2a, compare [27], Fig. 2, and [30], Fig. 1), their free enthalpies of formation [10], and their strong tendency to form large aggregates, obviously of helical conformation, instead of short, disk-like structures (Figs 6–9). This in turn supports earlier suggestions [9], that in direct reaction the two-layer aggregates ("4S") from vulgare form disks ("22S"), and three-layer aggregates ("8S") form short overshoot-aggregates ("26S").

As yet there was no structural explanation for the extreme pH dependence of the kinetic properties [28, 29], and the critical conditions [9, 15] observed for vulgare protein during polymerization. This extreme pH dependence does not reflect in its titration curve: vulgare protein takes up its first proton, below pH 8, in a relatively broad step (Fig. 5a, upper curve). However, A14 and Ni 725 protein titrate much more cooperatively (Fig. 5a, central and lower curve), under the same conditions. On the same premises of similarity supposed above the cooperative effects observed in vulgare protein may be contributed by the proton uptake and polymerization of its three-layer aggregates, and smeared by the two-layer aggregates. Several observations shown above provide indirect evidence for this; also, Schuster *et al.* observed the sudden onset of overshoot formation in vulgare protein, at pH 6.5, near 15 °C (*cf.* Fig. 2 in [28]), where, at pH 8 and otherwise comparable conditions, the sudden increase of 8S aggregates was reported [10]; however, most strikingly the cooperative effects in the polymerization of vulgare protein coincide exactly with the cooperative titration of A14 protein: at 22 °C,  $I = 0.1$  M, protein concentration 4–5 mg/ml, above pH 7.1 we observe only proton uptake by vulgare, none by A14 protein; this is the range of the very slow, probably direct

formation of disks ("22S") from vulgare two-layer aggregates [9]. Near pH 7.1, both starts the proton uptake by A14 three-layer aggregates, and the rapid formation of small vulgare overshoot aggregates ("26S"), which slowly decompose to disks, too [9]. Hence, the sudden appearance of overshoot aggregates follows from the higher cooperativity of the transition, not, as suggested [9], their lower  $pK_{app}$ . (In fact, the  $pK_{app}$  of A14 protein seems slightly higher, not lower, than that of vulgare protein.) Finally, the increase, near pH 6.75, in the vulgare protein CD [27] occurs at the same pH below which the hysteresis loop opens (Fig. 5a, upper curve, and [26]), and where the three-layer aggregates of A14 protein start to take up the second proton. – Such a mechanism, assuming reactions with different cooperativity proceeding simultaneously, implies that aggregates can be formed with differently charged subunits built into (for instance, below pH 6.75, in vulgare protein). A redistribution of protons should take place, which may contribute to the metastability of the overshoot aggregates: this deserves further investigation. At pH 6.5, a change of CD with time was observed, from a more "helix-like" to a "disk-like" spectrum [27], pointing to a conformational change in these overshoot aggregates, which was not observed for those above pH 6.8 (Vogel, unpublished).

As yet any search for groups failed [4] that could be responsible for the anomalous titration, outside the "carboxyle cage" [34]. Thus, how can a change in the stability of the pairing so efficiently be transferred to the carboxyle cage, and *vice versa*, about 25 Å apart, to alter mode and extent of proton uptake and aggregation so dramatically? We feel that the analogy suggested by Fig. 10 provides a useful working hypothesis to answer this question: the core of the TMV-protein subunit is made up by 4 more or less radially oriented  $\alpha$ -helices [12, 33, 34] which give some rigidity to the whole system. That is to say, a structural change at one end of the subunit can exert some stress, pull or torsion, which may be transferred, without to much dissipation, over some distance across the subunit. Especially, at the N-terminal end (which in  $\alpha$ -helices is less stable than the C-terminal side, [46]) of the LR-helix (pos. 114–134, in the disk) significant structural alterations have been shown to occur in going from disks to helices [33, 34], or by binding of oligonucleotides [47]. Thus, forces may be exerted, too, by "loading"

the carboxyle cage with one or two protons, and the extent of the overall changes resulting in the subunit structure will be determined by opposite forces, exerted by the pairing defined above. If the strength of the "spring" ( $\equiv$  free enthalpy of intersubunit bonding) (Fig. 10) is high, as in vulgare two-layer aggregates, one proton makes disks, and two protons are needed for helices. If the strength of the "spring" is weakened, either reversibly by addition of a third layer, or irreversibly by mutation, one proton is sufficient to make helices. This implies that the net-charges of vulgare, A14, and Ni725 A-proteins are identical: this follows from the similar points of isoelectric precipitation, in vulgare and A14 protein [48], together with the similar shapes of the titration curves, below the steps (see above).

The analogy presented here may be called a "thermodynamic" one. However, there are hints that the conformation of the helices formed by A14-protein by the uptake of one proton per subunit are

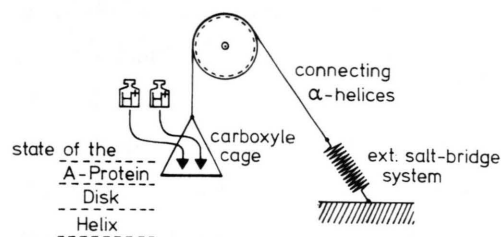


Fig. 10. Suggested analogy for the balance between the strength of the "extended salt-bridge system" [33] and the charge and state of the "carboxyle cage" [34], leading to different states of aggregation in TMV-proteins. For further explanation see text.

metastable (*cf.* Fig. 8c). Being aware the risk to overdo the analogy we were amused to see how easily this simple picture can be adapted to different problems: regarding the different "damping" effects by exchange of the "springs" ( $\equiv$  activation enthalpies) we can make a "kinetic" analogy; adding the "weights" as a whole we can make overshoot aggregates; dividing them into pieces and adding them part by part, to individual subunits, we can gradually approach the equilibrium.— Following well known models on enzyme mechanisms we can regard the "carboxyle cage" as a regulatory site, the protons (and the RNA) as effectors, and the site of the "pairing" as one of the active sites, active in forming different states of polymerization, which is the biological function of TMV-protein.

There remains to explain e), the differences between disks made by protons, or by salt. It was pointed out [33] that, because of the stability of disks in very high ionic strength, a simple view of the instability of isolated salt-bridges in high ionic strength does not apply to a salt-bridge system with hindered access to solvent. Under these conditions, in the crystal, two water molecules are included into the "extended salt-bridge system" [33]. However, we do not know how many water molecules may be included in solution, at moderate ionic strength, in the A-protein and in the disk near neutrality. There is clear evidence for structural differences between disks at moderate and at high ionic strength, manifested in CD by the negative bands near 295 nm [27], attributed to Trp residues 17 + 52 [30], close to the salt-bridge system. We may ask the question whether in vulgare A-protein, though both temperature and ionic strength favour aggregation, the equilibrium between two-layer and three-layer aggregates is shifted to three-layer aggregates by a raise in temperature, but to two-layer aggregates by a raise in ionic strength, by extracting water from the interface between the upper and lower layer of the two-layer aggregates, leaving a minimum of two water molecules per subunit. Indeed, temperature and ionic strength have different effects on the structure of the A-protein, *prior* to the formation of disks: increasing temperature makes all CD bands above 260 nm more positive ([10], Fig. 5a, and [30], Fig. 6), which is the direction of making higher-than-two-layer aggregates, and increasing the ionic strength makes them more negative ([27], Fig. 6, and unpublished results), the direction for making two-layer aggregates. This view deserves further investigation, but it would explain how the action of salt can overcome the intrinsic tendency of A14-protein for helices instead of disks, and finds some support by the very peculiar aggregation behaviour of Dahlemense protein [49], which, at low ionic strength, forms 7S aggregates, but 4S aggregates at moderately elevated ionic strength. This strain, too, has non-conservative exchanges against vulgare, besides others, within the salt-bridge system.

In this paper we presented a lot of observations which all combine together to give more profound insight than previously available into the complex mechanism of aggregation of TMV-protein. We absolutely realize that there are some speculations included, but we hope that they will help to stimu-



late useful discussions, and new experimental investigations, for prove or disprove, in many laboratories in the world, in order to obtain a complete understanding of this system, in the near future.

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#### Note added in proof:

After submitting this paper for publication, an article [50] appeared presenting a model similar to Fig. 10, using terms from allosteric enzymes: the balance between the network of hydrogen bonds in the disk (R-form) and the V-column in the helix (T-form) is supposed to determine the cooperative binding of the RNA which generates the R → T transformation.

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