# Thermal Denaturation Equilibria of TMV Coat Proteins with Chemically Defined Differences in their Primary Structure

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Thermal denaturation of RNA free coat proteins of tobacco mosaic virus (TMV) was studied for wildtype TMV *(vulgare)* and the temperature-sensitive mutant, Ni 118. The ability to form soluble aggregates as well as the optical properties (ORD, UV-difference spectra), and the sedimentation behavior were used as criteria for the native state.

At pH 7.5, $I = 0.02$ denaturation is reversible for both proteins. The ORD data indicate that the denatured proteins contain residual secondary structure. The "melting temperatures", as defined by ORD measurements ($c_P = 0.02$ mM), are $39.5 \pm 1$ °C for *vulgare* and $27 \pm 1$ °C for Ni 118 at pH 7.5, $I = 0.02$. By means of the aggregation test ($c_P = 0.05$ mM) somewhat lower melting temperatures were found under the same solvent conditions. The difference between the primary structures of *vulgare* and Ni 118 proteins being a proline → leucine (pos. 20) replacement, the results suggest the cyclic structure of proline (20) to have a specific stabilizing function in the three dimensional protein structure. This conclusion is supported by preliminary experiments on a temperature-sensitive mutant with a threonine residue in pos. 20.

It has been shown for a number of proteins that their three dimensional structure represents the thermodynamically most favourable conformation of their polypeptide chain under physiological conditions of solvent and temperature [1]. Similarly, the formation of ordered aggregates by native protein molecules in most cases is a consequence of the specific properties of the subunits and the external conditions [2]. This has been shown in particular for tobacco mosaic virus coat protein (TMV-P) [3], which in the native state is able to form quaternary structures with [4] or without [5] ribonucleic acid. A large number of TMV mutants has been isolated and analyzed with respect to specific amino acid replacements in their coat proteins [6,7]. These closely related protein species represent a favourable material in the attempt to define correlations between amino acid sequence, folding, and aggregation on one hand and external factors on the other hand. The temperature sensitivity of several TMV mutants *in vivo* and of their coat proteins *in vitro* [8,9] has focussed our attention to temperature as the biologically most important parameter.

The following experiments give evidence for the reversibility of the thermal denaturation [10] of RNA free TMV coat protein in an aqueous medium of low ionic strength. The temperature sensitivity of the mutant Ni 118 is caused by a shift to a lower temperature of reversible denaturation. The results are discussed on the basis of the known amino acid replacement.

Abbreviations: TMV = tobacco mosaic virus; TMV-P = coat protein of TMV; analogously, *vulgare*-P and Ni 118-P. ts = temperature-sensitive; $c_P$ = concentration of protein (molarity of subunits) ; $I$ = ionic strength ($\Gamma/2$).

## Materials and Methods

TMV wild type *vulgare* and the mutant Ni 118 (replacement proline → leucine in position 20 of its coat protein [6]) were used. Purified virus was prepared from infected tobacco leaves by the polyethylene glycol method [11], native TMV-P by alkaline splitting of the virus and electrophoretic separation of protein and RNA [12].

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For further technical details see l. c. [9]. Concentrated protein solutions were prepared by isoelectric precipitation with 10% acetic acid. All the solutions used were centrifuged for 20 min at 10,000 $g$ to remove dust and precipitated protein. Sterilized vials were used for longer incubations. Protein concentration ($c_P$) was determined from the absorption at 280 m$\mu$ in 50% acetic acid using a molar extinction coefficient of 22,000 based on a subunit mol. wt. of 17,500.

*Aggregation test:* To determine the proportion of native and denatured protein the different solubility at pH 5.0 and $I = 0.1$ was used. The test was performed as described earlier [9] with the following modifications: The protein solutions (at the temperature to be tested) were incubated in phosphate buffer pH 7.5, $I = 0.02$ (according to GREEN [13]) containing 1 mM $\beta$-mercapto ethanol. Under these conditions the denatured protein is not precipitated. After different time intervals 0.5 ml samples of the protein solution were removed from the water bath, 1.5 ml of ice cold acetate buffer pH 5.0, $I = 0.13$ were added, and the mixture (pH $\cong$ 5, $I \cong 0.1$) was kept for 20 minutes in an ice bath. The denatured protein, which precipitated under these conditions, was removed by centrifugation at 1,300 × $g$ for 10 minutes. The concentration of the native fraction in the supernatant was determined spectrophotometrically at 280 m$\mu$ (Zeiss PMQ II); to reduce light scattering the probes were diluted with an equal amount of glacial acetic acid.

*Optical rotatory dispersion (ORD) and UV spectra:* ORD spectra were measured in a Cary 60 spectropolarimeter using temperature jacketed cells (1 cm) and 0.02 mM protein solutions which were equilibrated at the desired temperature for at least 5 hours. The accuracy of the optical measurements was limited by a small amount of irreversible precipitation, which sometimes occurred after long incubation periods. UV spectra and UV-temperature difference spectra (20° vs 25, 27, 30, 35, 41 °C) were performed in a Beckman DK 2A spectrophotometer using temperature jacketed cells (1 cm).

*Sedimentation analysis:* Sedimentation velocity experiments at different temperatures (20.0 – 40.0 ± 0.2 °C) were performed in an analytical ultracentrifuge (Beckman Spinco E) using normal 12 mm cells and schlieren optics. Sedimentation coefficients were calculated from log $r$ vs $t$ plots and corrected for water viscosity at 20 °C.

## Results

### Characterization of the native and the denatured states

*Aggregation test.* Denaturated proteins were obtained by 30 min heating at 42 to 45° in the case of TMV-P *vulgare* and at 30 to 35° in the case of

Ni 118. The denatured proteins remain in solution at pH 7.5, $I = 0.02$, whereas they precipitate at pH 7.0, $I = 0.1$ (phosphate-NaCl buffer [9]) or after changing the pH to 5.0 by dialysis at high or at low temperature.

The ORD spectra of *vulgare* and Ni 118 proteins show no difference in the range of error. Therefore, only the differences between the native and the denatured states have to be discussed. As reported by SIMMONS and BLOUT [14] the ORD spectrum of native TMV-P shows a strong Cotton effect with the trough at ~230 m$\mu$ and a weak Cotton effect in the region between 285 and 300 m$\mu$. The present experiments verify these findings for both strains under investigation. Mild denaturation by heat in phosphate buffer pH 7.5, $I = 0.02$ leads to a flattening of the anomalous inflection at 290 m$\mu$ and to a decrease of the depth of the trough. The differences in the case of Ni 118-P are shown in Fig. 1. Addi-
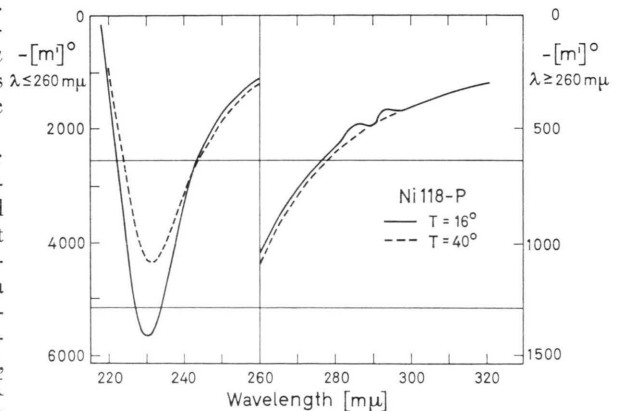


Fig. 1. ORD of native and heat denatured coat protein of TMV Ni 118. Conditions: pH 7.5, $I = 0.02$, 1 mM $\beta$-mercaptoethanol, $c_P = 0.02$ mM. Solutions measured at the temperatures indicated. Path length 1 cm. For [$m'$]: mean residue weight $m = 115$. The horizontal grid gives the optical rotation $-\alpha$, one unit being 0.1° ($\lambda \leq 260$ m$\mu$) and 0.025° ($\lambda \geq 260$ m$\mu$) respectively.

tional UV-temperature difference spectra with Ni 118-P in the range between 230 and 360 m$\mu$ show $\Delta\varepsilon$-minima at 284 and 293 m$\mu$ using one sample at 20° as reference and another one at $T \geq 25°$ in the sample beam. The result which resembles the difference spectrum of 67% formamide denatured TMV-P vs native TMV-P [15] supports the suggestion [14] that tryptophane might be involved in the second Cotton effect.

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Sedimentation analysis revealed a difference between the native proteins of the two strains, Ni 118-P having a lower sedimentation coefficient than *vulgare*-P in the whole range of $c_P$. In both cases the concentration dependence shows the typical behavior of reversibly aggregating systems (Fig. 2). Un-
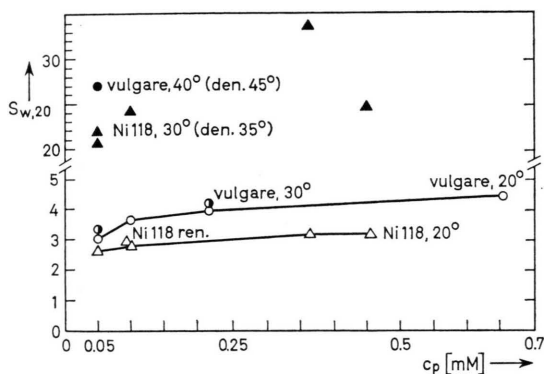
Fig. 2. Sedimentation coefficients of TMV-P *vulgare* and Ni 118 at different temperatures. Values have been corrected for the temperature dependence of water viscosity. Conditions: pH 7.5, $I = 0{,}02$, 1 mм $\beta$-mercapto-ethanol.

der the conditions of the aggregation test one can assume a preponderance of the trimer (mol. wt 52,000), whereas at the lower $c_P$ of the ORD experiments considerable amounts of the monomer are to be expected [16]. Upon denaturation the sedimentation coefficients increase from values close to 3 S to values between 20 and 40 S depending on the conditions of the experiment. These aggregates appear to be highly polydisperse (cf. Fig. 5).

### Equilibrium and reversion experiments

Thermal denaturation and renaturation after heat treatment at temperatures near the equilibrium of denaturation were measured with the aggregation test (Fig. 3). The results prove the denaturation to be reversible under constant solvent conditions. This conclusion is based on the following observations:

a) Denaturation at an appropriate temperature, about 35° for *vulgare*-P and about 25° for Ni 118-P, reaches an equilibrium after several hours.

b) A $\geqq 90\%$ denatured sample of either protein can be renatured partially by incubating for several hours at 30 to 35° *(vulgare)* or 20 to 25° (Ni 118).

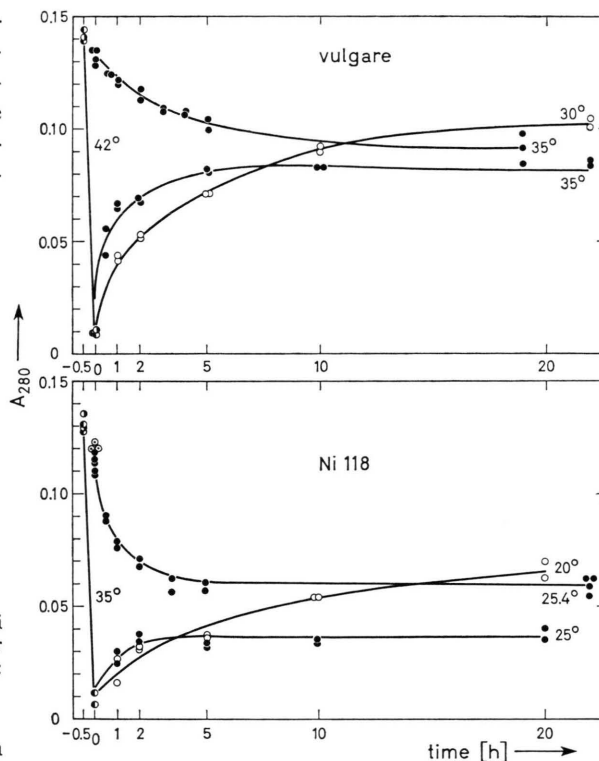[16] M. LAUFFER and C. L. STEVENS, Advances Virus Res. **13**, 1, 31 [1968].

Fig. 3. Denaturation kinetics of TMV-P *vulgare* and Ni 118 as measured by the aggregation test. From $-0.5$ to 0 hours $\sim 90\%$ denaturation at temperatures indicated. 0 hours zero time for kinetic measurements. Upper curves, denaturation at temperatures near equilibrium. Lower curves, renaturation $\sim 90\%$ denaturation at temperatures indicated. 0 hours=zero for each protein. Incubation at pH 7.5, $I = 0.02$, 1 mм $\beta$-mercapto-ethanol, $c_P = 0.05$ mм; aggregation at pH $\cong 5$, $I \cong 0.1$. Ni 118, $\odot$, samples measured without prior centrifugation.

With increasing renaturation temperature the initial rate of renaturation increases whereas the final yield in renatured protein decreases.

The lesser degree of reversibility in the case of Ni 118 (Fig. 3) may be due either to the higher temperature used for denaturation (35°) as compared to the specific „melting temperature" of this protein (see below) or to a certain amount of irreversible precipitation.

Taking the depth of the trough as a measure for the fraction of the native protein, the temperature dependence of the denaturation equilibrium has been studied (Fig. 4). To preclude effects of denaturation *kinetics* as far as possible, the solutions were incubated at the respective temperatures for several hours, since time dependent measurements of optical rotation at 232 m$\mu$ had shown that it takes about 50 min to reach the final state of de-
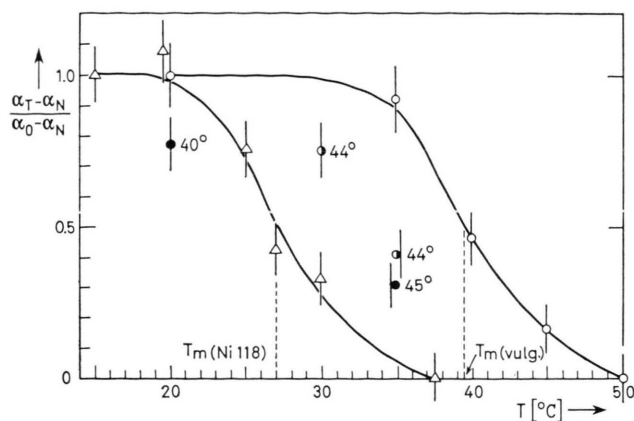
Fig. 4. Denaturation equilibria as measured by ORD. The $\alpha$-values at 230 m$\mu$ (trough) were used for the calculation. N = native protein, D = fully denatured protein, T = protein at temperature $T$. $(\alpha_T - \alpha_N)/(\alpha_D - \alpha_N)$ gives the fraction of native protein at temperature $T$. Denaturation, ○ *vulgare*, △ Ni 118. Renaturation, ◑, ● *vulgare* denatured at temperature indicated at symbol, renatured at temperature given on abscissa. Conditions, pH 7.5, $I = 0.02$, ● 1 m$M$ $\beta$-mercapto-ethanol, $c_P = 0.02$ m$M$, ◑ without mercapto-ethanol, $c_P = 0.013$ m$M$. An error of $\pm 3\%$ in the measurement of $\alpha_T$ is indicated.

naturation equilibrium at 40 °C (TMV-P *vulgare*). When solutions of *vulgare*-P were denatured at different temperatures (30 min) and afterwards kept at lower temperatures for several hours, the ORD spectrum of the native protein was partially restored. Thus the sigmoidal shape of the temperature dependence of the depth of the trough again indicates the reversibility of the reaction. The same experiment with Ni 118 was difficult to perform the optical measurements being disturbed by small amounts of precipitated protein.

Reversibility of the denaturation includes reversibility of particle association as proved by ultracentrifugation. The results given in Fig. 5 show partial reversion of the sedimentation behavior after slowly cooling down the denatured Ni 118-P at 25, 20, and 15 °C for 20 hours (in total) before centrifuging at 20 °C (Fig. 5 c). The yield of renatured protein did not exceed 30 per cent. Contrary to the ORD results which might be explained by a series of structures with different stabilities, the sedimentation experiment clearly shows the coexistence of native and denatured molecules in solution as *two distinct species*. The fraction of the protein which showed the low sedimentation coefficient ($\sim 3$ S) of native TMV-P, after "tempering" at pH 7.5, $I = 0.02$ was equally able to form ordered aggregates of several hundred S upon subsequent dialysis against
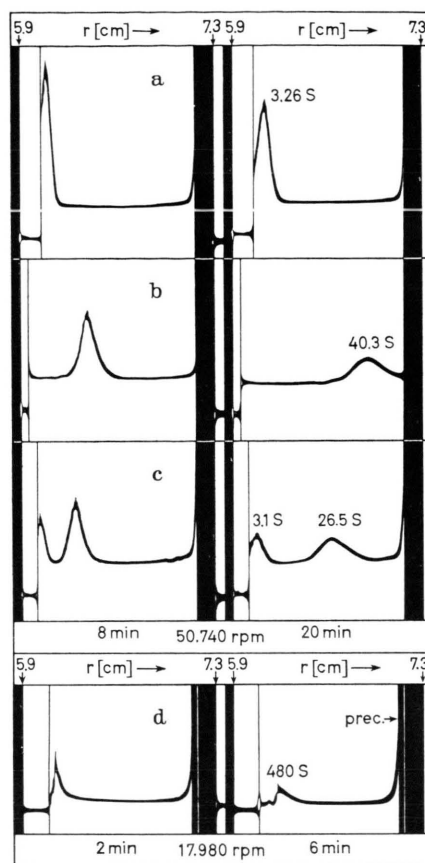


Fig. 5. Denaturation and renaturation of Ni 118 protein as shown by sedimentation analysis. 8 ml of protein solution were subjected to the various treatments, after each treatment 1 ml was removed for analytical centrifugation. a) Native protein at 20°, b) after incubation at 35° for 30 min, c) after tempering at 25, 20, and 15° for 20 hours, d) aggregated at 4° overnight, run at 20°. Conditions: $c_P = 0.37$ m$M$, 1 m$M$ $\beta$-mercapto ethanol. For a), b), c) : pH = 7.5, $I = 0.02$, for d) pH = 5.0, $I = 0.1$. Sedimentation coefficients not corrected for temperature dependence of water viscosity.

| Property | Method | $c_P$ [mM] | *vulgare*-P | Ni 118-P |
|---|---|---|---|---|
| $[m']_{230}$ native | ORD | 0.02 | − 5460 | − 5640 |
| $[m']_{230}$ denat. | ORD | 0.02 | − 3930 | − 4250 |
| Sed. coeff. [S] | UC | 0.1 | 3.67 | 2.75 |
| $T_m$ [°C] | AT | 0.05 | $\sim 36$ | $\sim 25$ |
| $T_m$ [°C] | ORD | 0.02 | 39.5 | 27 |
| $K_{N \to D}$, 35°C | ORD | 0.02 | $\sim 0.1$ | $\sim 20$ |
| $\Delta_{N \to D}$, 35 °C [Kcal/mole] | ORD | 0.02 | + 1.47 | − 1.92 |

Table I. Physicochemical properties of wildtype and temperature-sensitive TMV proteins. Abbreviations: $[m']$ = mean residue rotation; $T_m$ = equilibrium temperature of denaturation; $K$ and $\Delta G$, reaction constant and free energy of denaturation; UC = ultracentrifugation; AT = aggregation test.

acetate buffer pH 5.0, $I = 0.1$ at $4\,°C$, while the amount corresponding to the more rapidly sedimenting fraction (20 S) precipitated at pH 5.0 (Fig. 5 d).

Some of the data of this section are summarized in Table 1.

### Discussion

The foregoing results show two differences in the physicochemical behavior of *vulgare* and Ni 118 proteins:

(1) In the native state Ni 118-P either has a lesser tendency to aggregate, or a more extended conformation, or both, as shown by the lower sedimentation coefficient. Correspondigly, in "stacked disks"-aggregates observed in the electron microscope the subunits appear more loosely packed in the case of Ni 118 as compared to *vulgare* [9].

(2) Ni 118-P has a "melting temperature" $(T_m)$ more than $10°$ lower than *vulgare*-P.

The quantitative deviations in $T_m$ obtained from solubility test and ORD may be due either to the different range of $c_P$ or to the fact that different conformational alterations are measured. The ORD data prove only partial denaturation of the proteins for both strains under consideration. Contrary to the high degree of denaturation by urea [14] the C o t - t o n effect at 230 m$\mu$ does not disappear completely while the inflection in the range of tryptophane (and tyrosine) absorption is nearly completely lost. Possibly, the residual C o t t o n effect reflects a more resistant portion of the three dimensional native structure, as *vulgare*-P treated at $50\,°C$ still reacts with specific antibodies [17].

Since there occurs only partial thermal denaturation, our reversion experiments do not prove the three dimensional structure of the TMV proteins to be fully determined by the amino acid sequence (but cf. [3]). However, the experiments show the interplay of sequence *and* temperature in the process of renaturation of the partially unfolded molecule. Single amino acid replacements represent a new parameter in this type of investigation. TMV *vulgare* has pro-

line in position 20 of its coat protein. This residue is replaced by leucine in Ni 118 [6]. Leucine and proline are comparable in their solubility properties [18]. This suggests the cyclic structure of proline to be responsible for the higher stability of *vulgare*-P by reducing the rotational freedom in the polypeptide backbone in position 20 as well as in the preceding aspartyl residue, position 19 [19]. This hypothesis leads to the prediction that a mutant isolated in Berkeley, which has threonine in position 20 [7] should bevahe similar to Ni 118, although the solubility properties of leucine and threonine are rather different [18]. This prediction has been confirmed. The mutant, 430, was found to have a coat protein of similar temperature sensitivity as Ni 118 as shown by *invivo* experiments, aggregation test, ORD, and ultracentrifugation. TANFORD's equation for the conformational energy of a polypeptide chain [18], however, would predict the difference in free energy of denaturation, accompanying the replacement of a proline residue, being much lower than our observed $\Delta G$ (0.9 kcal/mole vs. 3 kcal/mole, cf. Table 1). It should also be mentioned that in addition to the proline in position 20 a negatively charged residue in position 19 seems to be necessary for the thermal stability of the TMV protein subunit [9].

*Note added in proof:* While this paper was in press a reinvestigation of the ORD of TMV-P was published [20] which leads to the conclusion that the disappearence of the extrancis Cotton effect at about 290 m$\mu$ ist also characteristic for native aggregation. Major conformational changes upon aggregation can be excluded (c. f. JAENICKE and LAUFFER, Biochemistry, in press).

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