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Title: Temperature-sensitive Mutants of The Tobacco Mosaic Virus. II. In vitro behavior. (Temperatursensitive Mutanten des Tabakmosaicvirus. II. In Vitro-Verhalten).


September 1968
A. INTRODUCTION

The biological condition, which led to the investigations on the thermal stability of the core protein of TMV mutants in vitro, is summarized in Part I. The primary structure of the protein, as determined by the protein-chemical investigations of Wittmann and coworkers (1962, 1964, 1965) and electrophoretic studies, are shown in Figure 1 along with data on the in vivo behavior (Part I).

B. MATERIALS AND METHODS

I. The isolation, propagation, and recovery of the virus mutants have been described in Part I.

II. Purification of Core Proteins

In the case of the previous in vitro studies (Jocksch, 1964), alkali-split virus was employed whose RNA was uniformly degraded by incubation with RNase. It was not, however, feasible to separate the RNA fragments completely from the protein by dialysis. For more exact studies, the preparation of RNA-free, native protein was necessary. The acetic acid method (Fraenkel-Conrat, 1957) yielded a very small amount of product since the protein must be renatured from the acetic acid solution. In the case of the alkaline cleavage of the virus, the sensitive mutant protein remained completely native, but the separation of RNA and protein by ammonium sulfate (Schramm, 1947) gave unsatisfactory results. The electrophoretic separation of cleavage products (Schramm, 1947) offered the only practical means of obtaining high yields of labile protein.

By means of carrier-free (?) electrophoresis ("Elphor VeP, Bender and Hobein, Munich) using the method of Manning, a large amount of mutant protein can be

Abbreviations used: TMV-Tobacco Mosaic Virus; NP - core protein; RNA - ribonucleic acid; RNase - ribonuclease; tr - temperature-resistant; ts - temperature-sensitive; def - defective; AR - amino acid residue; Tris = 2-amino-2-hydroxymethyl-propionol -(1,3).
purified in a relatively short time (Sarkar, 1960). Moreover, the previously specified conditions (Jockusch, 1966) were adhered to. Alih and its derivatives (except Ni5U) split so poorly in alkali that the yield of protein and the purity achieved were similar to that with *vulgare* and its derivatives. For this reason, an "acetate acid protein" was employed in the case of Alih and Ni696. No differences could be detected between the "alkali-protein" and the "acetate acid-protein" of *Alih*. Purity criterion: the degree of contamination by RNA was determined by means of the ultraviolet extinction coefficient $E_{260}/E_{280}$ (corrected for light scattering). In the case of the complete virus (5% RNA), a value of 1.2 was obtained. In the case of the "A-protein" of *vulgare* and its mutants, it was 0.55 to 0.58 indicating that the preparation was practically RNA-free. In the case of the Alih mutants, a value of 0.60 to 0.65 was obtained. Native state activity: proteins were considered to be native if they produced clear solutions at 4°C and at pH 7, and if reversible opalescence was produced at pH 5 (except Ni220H which reversibly precipitated at pH 5). Moreover, the proteins were preserved at pH 5, I (ionic strength) = 0.1 at -60°C.

### III. Buffers Employed

In general, the buffer described by Miller and Golder (1950), which has an ionic strength of $\Gamma/2 = I = 0.1$, was employed for the denaturation studies. However, at pH 8, Tris was employed for buffering instead of veronal. The buffer has the following advantages: (a) the ionic strength is definite and is not affected by the pH, (b) 80% of the ionic strength is the result of the NaCl present, (c) the ionic strength is equivalent to that of physiological saline, (d) the temperature coefficients of Miller-Golder buffers containing acetate (pH 5) and phosphate (pH 6 and 7) are small enough to be disregarded, (e) the buffers employed interfere with the determination of protein concentration.
by the measurement of UV absorption or by the ninhydrin test. In this case, 
pure phosphate buffer (I=0.1) of Green is used. For testing solubility, 
because of its high buffering capacity as compared to Miller-Golder buffer, 
the acetate buffer, pH 5.0, of Boyd was employed (directions are found in 

IV. Gel Electrophoresis of Proteins

Proteins prepared according to Part B.II. were suspended in Miller-Golder 
buffer, pH 5, precipitated with methanol-ether as in Part I, section C.V., and 
then dissolved in 8 M urea, 20 % sucrose in Aronsson-Grövall buffer. Since 
H22242 protein cannot be separated in this manner, it was precipitated by the 
addition of 2 M ammonium acetate, pH 4.8. In other cases, the acetic acid protein 
is obtained from the virus and, after removal of the RNA by centrifugation, the 
protein is precipitated isoelectrically by the addition of same water and 2 M 
ammonium sulfate. The precipitate is washed with methanol-ether and dissolved 
in urea buffer. The protein concentration is about 1 to 5 mg per ml. Electro-
phoresis was carried out as described in Part I, Section B.IV.5.

V. Tests for Native State of Proteins

Next to the macroscopic examination of protein solutions (clear, opalescent, 
precipitate), the following criteria were used for determining the native 
state: solubility at pH 5, development of soluble particles with sedimentation 
coefficients of greater than 100S, which appear as aggregates when observed under 
the electron microscope with a diameter of about 150 Å and a central canal. 

1. Ultracentrifugation.

This was carried out using a Spinco Model B analytical ultracentrifuge 
(Beckman Instruments) with an An-D rotor. In order to avoid strong shearing 
forces upon injection of the sample, a covered cell was used into which the 
Sample could be pipetted. It was not, therefore, layered with buffer (with the
exception of the urea protein), but was filled with 0.7 ml of protein solution in order to create an adequate sedimentation path. All runs were carried out at 20°C. The sedimentation coefficient S was calculated as follows:

\[ \log S = 13.1818 + \frac{\log 4}{4} \ln r = (2 \log f + \log \Delta t) \]

where \( S \) (s)

\( r \) (cm) the radial distance of the gradient from the axis.

\[ \Delta \ln r = \ln r(t_2) - \ln r(t_1) \]

\( \Delta t \) (min) = \( t_2 - t_1 \), the time period between photographs and \( f \) (min\(^{-1}\)) the speed of rotation of the rotor.

The viscosity was measured at 20°C using an Ostwald viscosimeter.

2. Electron Microscopy.

Protein aggregates were analyzed by the negative staining method of Brenner and Horner (1959). A drop of the sample was applied with a capillary to the grid, placed under a vacuum and dried. The same procedure was repeated with 2% phosphotungstic acid at pH 5. The electron micrographs were kindly prepared by Dr. H. Frank and Mr. Berger (Max Planck Institute for Virus Research) using a Siemens Electron Microscope I.

C. RESULTS

I. Electrophoretic Behavior of Proteins in 8 M Urea

Sengbusch (1965) found that intact virus particles of some mutants, where protein analyses (Wittmann, 1962) showed no differences between them and the parent strain, possessed a slight mobility in the electrical field in weakly alkaline solutions at intermediate ionic strength. This is explained by amino acid substitutions of the type: aminodicarboxylic acid → aminodicarboxylic acid amide. These are not normally detected by protein analysis. RNA-free proteins in 8 M urea were analyzed by gel electrophoresis in order to exclude the
possibility that substitutions, in addition to those indicated by Wittmann, may occur, and to characterise several substitutions where it is not known whether the aminodicarboxylic acid or its amide is present (Wittmann, 1962). Figure 2 shows the bands that were obtained. Two classes of bands are seen: some, which migrate like *vulgare*, and some which migrate like *flavum*. This difference is based upon a single charge difference (Wittmann et al., 1965).

By some derangement during aggregation (secondary bands) or carbamylation of amino groups by cyanate (Cole and Mecham, 1965; in alkaline medium), the results were in agreement with the analyses of Wittmann as well as the electrophoretic studies of Sengbusch. Earlier, it was shown that in the case of N1103 and N1696, an aspartic acid residue is substituted in position 19 of the polypeptide chain and that the single aspartic acid in *peptide I*. N1109 does not migrate at a slower rate than *vulgare* as one would expect from the protein-chemical results (Wittmann, 1962) and the findings of Sengbusch (1965). There are two possible explanations for these results: (a) the protein contains an additional substitution of aminocarboxylic acid — aminodicarboxylic acid which is not detectable by protein analytical methods. This additional substitution neutralises the charge loss caused by the glutamic acid — glycine substitution in the case of urea-extracted protein. (b) We are dealing with nothing more than the original parent strain. In any case, it is out of the question that the protein contains no substitution different from *vulgare*.

If one begins with only the results obtained with electrophoresis, the following principle is formulated: All proteins, which migrate more slowly than the *vulgare* protein in alkaline solvents, are to (temperature-sensitive). However, not all te-proteins migrate more slowly than *vulgare* protein. The mutants, M116 and M1196, which have both lost a proline by mutation, form only weak and diffuse bands. In alkaline 8 M urea solutions, they exhibit more aggregations
than residual protein. In this regard, previous investigations with the analytcal centrifuge have shown that Mill8 protein in this solvent sediments about twice as fast as does vulgar protein.

II. Pattern Tests with Mill8 and Vulgar Proteins

Mill8 was selected for these tests since it is a sensitive mutant and its protein exhibits no charge substitution as compared to the vulgar protein. This was done so that the effects that were observed would not be dependent on different isoelectric points.

1. Speed of Denaturation.

For the following studies, the protein, which was at pH 5, I=0.1, was dialyzed for at least 48 hours at 4°C against repeated changes of buffer. The protein solution was then incubated in a water bath under the actual experimental conditions. At specified times, 0.5 ml of protein solution was withdrawn and pipetted into 1.5 ml of ice cold Boyd buffer, pH 5. After thorough mixing, the sample was allowed to set for several minutes in an ice bath and then centrifuged for 4 minutes at 1,300 x g (solubility test). Denatured protein under these conditions will be found in the sediment after centrifugation. The protein content of the supernatant fractions was determined after alkaline hydrolysis using the ninhydrin reagent according to Moore and Stein (1954). The protein concentration for this experiment was calculated from the following relationship: 1.10 O.D. 280 cm/µl = 5 x 10⁻⁵ M (Fraenkel-Conrat, 1957).

a) Effect of Temperature. Figure 3 shows the denaturation of Mill8 protein at 20°C and 30°C. The samples contained 5 x 10⁻⁵ M protein in Miller-Golder buffer, pH 7.0, I=0.1, 10⁻³ M ethylmercaptan. The capacity to form soluble aggregates at pH 5 disappears at 30°C with a half time of t½=1.6. At 20°C, t½ > 10⁵ minutes. The "melting point" of the tertiary structure of Mill8 protein (Kausemann, 1954) as a consequence of an amino acid substitution lies...
between 20°C and 30°C, while it is greater than 30°C in the case of the *vulgare* protein. However, at 20°C, appreciable denaturation occurs after 5 hours (Table 1).

b) Effect of Mercaptan. Duplicate samples with $5 \times 10^{-5}$ M *Mii18* protein in Miller-Golder buffer, pH 7.0, I = 0.1, were incubated at 30°C with and without $5 \times 10^{-3}$ M ethylmercaptan. In order to assimilate the test conditions, the Boyd buffer contained $10^{-2}$ M ethylmercaptan. Even with a 100-fold increase in SH-groups over those in the protein, no differences in the speed of denaturation during incubation could be found (Figure 4). Nevertheless, the studies were carried out in the presence of mercaptan in order to avoid any troublesome interferences by heavy metal contamination.

2. Aggregation during Dialysis.

If, during the investigations on the progress of denaturation, the pH is lowered to 5, optimal aggregation to rods is achieved in this case as well as by dialysis. In the following studies, the behavior of *Mii18* protein and *vulgare* protein was studied at 20°C and 30°C at pH 7.0 or by dialysis at pH 5. These were carried out using $10^{-4}$ solutions of *Mii18* and *vulgare* protein.

All of the incubation buffers contained $10^{-2}$ M ethylmercaptan. The dialysis was against Miller-Golder buffer at pH 5.0. The incubation and dialysis times for all samples were 5 hours, after which the samples were centrifuged for 10 minutes at 1300 g. The relative concentrations of the sediments and supernatants were determined by measurements of the $E_{280}$ after evaporation and dissolution in 10 M urea, pH 7. The supernatants were centrifuged in the analytical ultracentrifuge and the soluble fractions were studied under the electron microscope.

Table 1 gives the fractions of the supernatant and precipitate and the sedimentation coefficients of the proteins in the supernatants. Figure 5 shows the sedimentation profile, while Figure 6 shows the electron micrographs of aggregates found at pH 5. The results are (a) by dialysis pH 7 → 5 at 20°C and 30°C,
vulgare protein forms aggregates of greater than 100 S in which, as the
electron microscope controls show, the subunits are arranged in a hollow
cylinder form. The fraction of protein that precipitates in this case is
less than 1%. At pH 7 and I = 0.1, the protein remains in a clear solution
in the form of smaller aggregates of ca. 30 S. This\footnote{The sedimentation coefficient values are not corrected to a constant viscosity.}
corresponds to a
double ring of about 2 x 17 protein subunits.\cite{Casper, 1963}. At I = 0.02,
and after storage at 4°C, a component with a value of 4 S was found. This
corresponds to the actual "A protein" with three subunits per particle
\cite{Casper, 1963}. (b) Like vulgare, \textit{Mii18} protein after dialysis pH 7 \to 5 at
20°C, forms soluble aggregates of more than 100 S which appear as rods (hollow
cylinders) under the electron microscope. About 99% of the protein in this
case forms into aggregates. However, at 30°C and I = 0.1, this value is 75%:
and at I = 0.02, 98% of the protein forms an insoluble precipitate. The
somewhat higher stability at higher ionic strengths can be shown to be repro-
ducible. At pH 7 \to 5, I = 0.1, the vast majority of the protein in solution is
in the form of rods. Hence, it has been concluded that the 30°C-denaturation of
\textit{Mii18} protein is an all-or-none occurrence, that is, either the protein precipi-
tates or rods are formed at pH 5. At pH 7, I = 0.1, after 5 hours at 20°C, a
slight precipitate is formed. At 30°C, 91% of the protein precipitates. At
I = 0.02, however, the solution remains clear and particles are found with a
sedimentation coefficient of 36 S.

\footnote{Viscosity measurements were carried out on A proteins at pH 7, aggregates at
pH 5, and virus particles at pH 7. With a protein concentration of 10^{-4} M, the
viscosity of the aggregates was only 6% higher than that of the pure solution,
It is concluded that it would not be important to correct for such a small change.}
(c) The question now was if the 36 S component obtained by incubating
Mlll8 protein at 30°C, pH 7, I = 0.02, still possessed the capacity to form
aggregates in an orderly fashion when dialyzed against pH 5 buffer in the
cold. It was found that after incubation for 30 minutes at 30°C followed
by dialysis at 4°C against Miller-Golder buffer, pH 5.0, I = 0.1, 95 % of
the protein precipitated. Contrary to an earlier assumption (Jochamk, 1964),
the protein remaining in solution at 30°C at low ionic strength is no longer
native. On the other hand, 'vulgar' protein can be kept under similar conditions
for at least 24 hours at 30°C without losing its capacity to form soluble
aggregates at pH 5 in the cold.

(d) Stabilisation by phosphate ions: The precipitation of Mlll8 protein
at 30°C in Miller-Golder buffer, pH 7, I = 0.1, is not purely an ionic strength
effect. If one incubates the protein under similar conditions but in pure
phosphate buffer, pH 7, I = 0.1, the solution will be only opalescent and
not removed by centrifugation at 1,300 x g. After dialysis at 4°C against
buffer, pH 5, I = 0.1, only a fraction of the protein precipitates. In
contrast to chloride ions, the phosphate ion has a stabilising effect at least
in the case of the solubility behavior of the Mlll8 protein.

3. Nature of the Precipitates.

From the spectrum of the solvents which are capable of dissolving the protein
precipitates, one can draw some conclusion as to the nature of the bonds involved
in holding the aggregate together (Summary: Jaenicke, 1965). Using Mlll8
protein, the following studies were carried out: A 10⁻⁴ M solution of native
protein in buffer, pH 7, I = 0.1 with 10⁻³ M cysteine, was held at 30°C for
40 minutes and then mixed with an equal volume of Boyd buffer, pH 5, I = 0.1
(the resulting pH was at 5). The suspension was divided up and the fractions were
centrifuged at 1,300 x g for 10 minutes. 21 % of the protein was recovered in the
supernatant fractions. The sediments were suspended in equal volumes of various
solvents as shown in Table 2. The protein concentration in the supernatants was determined on the basis of the $I_{250}$ (Table 2) after centrifugation for 10 minutes at $1,300 \times g$. It was found that (a) the coherence of the aggregates is not the result of covalent bonding. Mutual exchanges between aliphatic side chains appear to be the major contribution (solubility in sodium dodecyl-sulfate). (b) The binding forces differ qualitatively from those which hold the native protein aggregate together (higher alkali resistance). (c) At pH 5, at $4^\circ C$ as well as at $30^\circ C$, a noteworthy renaturation occurs.

### III. Comparative Denaturation Kinetics of Vulgare Mutants

#### 1. Denaturation at pH 7.

The four tv strains, vulgare, Alk, Kl109, Kl1927, and the five ts strains, flavus, reflexus, Cl15, Kl103, and Kl115, were compared at pH 7, I = 0.1, $30^\circ C$, with reference to the velocity of protein denaturation (Figure 7). These were tested as described in Section C.III.1. With the exception of reflexus, which is derived from flavus by way of the vacans strain, all of the mutants differ from vulgare by only a single amino acid substitution (with regards to Kl109, however, see Section C.I.). With regards to their ability to form soluble aggregates at pH 5, in the case of the ts-mutants, half-lives of 1.8 minutes (Kl118) to 80 minutes (reflexus) were observed. Kl1927 was more strongly denatured than vulgare whose half life was $>2.5 \times 10^3$ minutes which was greater than that of the most stable ts-mutant. The protein from Alk, however, is even more stable. After 15 hours, practically no denaturation can be detected using the solubility test in the case of the vulgare protein.

#### 2. Effect of pH

Multiple samples were suspended in buffers of pH 8.0, 6.0, and 5.0 as described in Section C.III.1, incubated, and tested. Figure 8 shows the corresponding curves for pH 6.0; at pH 5.0, after 15 hours, practically no
decrease in solubility was detected. At pH 6, the proteins behaved the same as at pH 7.

The half-lives obtained in this manner are summarized in Table 3. As the incubation pH approached the isoelectric point, the denaturation velocities were decreased by a factor of \( >100 \) to \( >1000 \) (M115).

IV. Comparison of Several All Mutants

In Figure 9 is shown the kinetics of the loss of solubility of several A11 mutant proteins incubated at pH 7, I=5.1, as described in Section C.II.1. The half-lives found were: M1511 = 20 minutes; M1696 = 30 minutes; M1196 = 180 minutes; M12519 = \( >2.5 \times 10^2 \) minutes.

One finds that (a) the mutant M1696, which is differentiated from flavum by two very distinct substitutions in the polypeptide chain, is somewhat more stable than flavum: M1696 \( t_\frac{1}{2} = 30 \) minutes; flavum \( t_\frac{1}{2} = 20 \) minutes. (b) Also, an amide substitution can cause the behavior (see C.I.). The protein of M1511 has a similar half-life to that of flavum. (c) The protein of the ts mutant M12519 is as stable as that of A11 at 30°C. Also, no precipitation was observed during dialysis of alkaline-split virus against pH 5 buffer at 35°C. In agreement with the protein analyses of Stettmann-Liebold et al. (1965), in both mutants, no substitution could be found and from these and the in vivo studies (Part I), one must conclude that in the case of M12519, it is not the envelope protein that is altered but rather there is the mutation of a second gene for temperature sensitivity. (d) M1220 precipitates independently from the 30°C influence almost completely at pH 5, I = 0.1.

V. Wild Strains

In the case of the in vivo studies (Part I), it was shown that the wild strains, dehlsense, U2, and Holmes' Rib Grass, are ts. In the case of the
in vitro studies, the test conditions for U2 and HRG in contrast to those for 
vulgaris must be somewhat altered since isoelectric precipitation occurs at a 
higher pH. It was diluted with Boyd buffer, pH 5.5, I = 0.1, instead of pH 5 
(see Rentchler, in preparation). Incubation at pH 9.0, I = 0.1, 10^{-3} M ethyl-
mercaptan, 30°C for 4 hours, gave the following results: U2 and HRG are 
completely stable; dahlmanse protein denatures very slowly; t_{1/2} 300 minutes.

VI. In Vitro Stability of Virus Particles

From the fact that during virus recovery, heat precipitation of plant proteins 
at 60°C for 10 minutes is utilized and that this method allows the recovery of 
both tr and ts mutants, it has been tentatively assumed that complete ts virus 
particles have the same high thermal stability as the vulgaris virus (Jockusch, 
1964). In making this assumption, however, it is necessary to consider the 
following: (a) The conditions in the crude plant extracts are not defined, parti-
cularly with regards to the actual initial virus concentration, which is not 
known. (b) The treatment time is relatively short as opposed to the time of 
in vivo growth (several days). (c) Particularly in the case of the ts mutants, 
one often obtains unpredictable and often low virus yields which could well be 
the consequence of the heat treatment. In the following experiment, it was 
determined if incubation of ts virus particles for long periods of time at high 
temperatures rendered them RNase sensitive. Solutions of purified virus containing 
1 mg/ml of strains vulgaris (tr), All (tr), MI18 (ts) flavum (ts), CPh15 (ts) 
were incubated in Sorenesn phosphate buffer, pH 7.0, I=0.17, at 50°C. at times 
0, 5, 15, 25, and 40 hours, samples were withdrawn, mixed with an equal volume 
of cold solution containing 10 mg/ml RNase in phosphate buffer, pH 7, and then 
incubated for 1 hour at 37°C. After that, the samples were diluted in ice cold 
phosphate buffer (1:1000 for vulgaris, All, and MI18; 1:200 for flavum and CPh15) 

2 Worthington Biochemicals, Freehold, N.J. U.S.A.
and held in an ice bath until the test. Infectivity was tested on the Xanthi tobacco strain. Controls: untreated vulgare solutions containing 1.0, 0.2 and 0.02 µg/ml virus. Vulgare solutions were incubated for 40 hours at 50°C without subsequent RNase treatment. Vulgare RNA, 20 µg/ml, untreated as well as virus solution treated with RNase were diluted to 5 µg/ml for the test. The results are shown in Figure 10.

None of the strains showed a significant decrease in infectivity. As the dilution series for the vulgare virus shows, it doesn't matter if the test was carried out in the saturation range of the standard curve. After treatment at 50°C for 40 hours, vulgare virus not treated with RNase showed no significant difference in infectivity as compared to virus treated with RNase. Under the same conditions, treatment of free RNA with RNase was 100% effective in inactivation.

D. CONCLUSIONS

I. Relationship of In Vivo and In Vitro Behavior

The known functions of the TMV envelope protein are (a) in the cytoplasm of the host plant, they aggregate specifically with virus RNA in forming virus rods in the presence of other proteins and nucleic acids. (b) During the extracellular phase of the infection cycle, they provide the RNA encased in the viable virus rod with adequate protection against external factors, particularly the action of RNase. (c) In spite of this stability, they permit the release of RNA during the primary process of infection. The physicochemical basis is known for only one of the functions. Function (b) is not, according to Section C.VI., temperature sensitive in the case of the to-mutants. The following results show that the aggregation of RNA-free envelope proteins at low pH is an appropriate model for the coaggregation of the proteins with RNA: (a) The defective mutant, RM2, does not form stable virus particles in the plant under greenhouse conditions.
In vitro, at low temperatures, its proteins aggregate in such a way so as to form open helices which do not have the conformation necessary to protect RNA from RNase (Zaitlin and Ferris, 1964). (b) The mutant H1220h, at high as well as at low temperatures, constructs very small amounts of stable virus - its protein precipitates also in the cold upon dialysis against buffer, pH 5.0, I = 0.1, whereas the remaining mutants form rod-shaped aggregates. (c) Ts mutants of Class I, in contrast to the wild type vulgare and the tr mutants, can form only very reduced amounts of virus at 32°C in vitro - Their proteins lose up to 50% of the capability to form orderly aggregates in pH 5.0 buffer after incubation at 30°C, pH 7, I = 0.1, in 2 to 180 minutes. In contrast, the capabilities of the tr proteins in this respect remains undiminished for many hours. Obviously, in this case, low pH can alter the phosphate residues of RNA during the aggregation process if the fine structure of the rod-shaped aggregates ('stacked disk' form) is not in close enough harmony with that of the virus, (Macrohelix) (Franklin and Commerer, 1955).

Considered collectively, the in vivo and in vitro investigations hshow the unequivocal conclusion that temperature sensitivity of mutants of Class I is dependent on the temperature sensitivity of the envelope protein subunits.

II. The Aggregation Defect as A Consequence of The Irreversible Alteration of Conformation

In the case of the first in vitro studies on ts mutants of TMV (Jockusch, 1964), aggregation and heat treatment were carried out simultaneously (dialysis at high to low pH at 30°C). It was presumed consequently that high temperature during the aggregation process produced denaturation. The newer in vitro studies (this investigation and Jockusch, 1960) showed, however, that the denaturation of envelope protein subunits occurs also at constant high pH and
that the disordered aggregation during the subsequent pH reduction is a consequence of this denaturation.

III. Stabilisation by Low pH or RNA

The reason of denaturation of proteins by heat is the thermal movement of the polypeptide chain components away from the configuration characteristic of the native molecule, which leads to the formation of stronger bonds of the polypeptide components of the same molecule in a different manner or with another molecule (We, 1971). From the structure of the protein aggregate and the complete virus particle, one is able to comprehend how the degree of freedom for thermal movement by the polypeptide chain is reduced as a result of the small distance between subunits in the A-protein. Consequently, it is plausible to assume that low pH as well as RNA produce thermal stabilisation of the subunits during aggregation. In this regard, it can be also said that the pH dependence of the denaturation speed at pH ≥ 7 is practically nonexistent.

IV. The Importance of A Single Amino Acid Residue

As a result of the protein-chemical analyses of Wittmann and co-workers, it is possible to correlate the in vivo and in vitro behavior of mutant proteins not only with each other but also with the known amino acid substitutions. In this case, however, a simple, generalised rule cannot be established (Wittmann-Liebold et al., 1965; Jockusch, 1966). For that reason, only a few specific cases can chosen where a plausible explanation can now be given.

1. The Proline Residue in Positions 20, 63, and 156

M118 (Proline → leucine in position 20) and M1927 (proline → leucine in position 156) constitute a very clear example of the effect of the position of the substitution in the polypeptide chain on the stability of mutant proteins (Jockusch, 1966; Wittmann-Liebold et al., 1965). M1927 is 1. The C-terminal
end (position 158) of the polypeptide chain lies on the surface of the virus particle (Harris and Knight, 1952) and, as a consequence, also on the surface of each subunit. N118 is exposed. The substitution at position 20 is not detectable serologically in the intact virus and appears, therefore, at least from the observations with the whole virus particle, to lie more towards the interior (Sengbusch, 1965). Obviously, the proline at position 20 stabilizes a sharp bend in the polypeptide chain which is necessary for the functioning of the subunit (orderly aggregation). The proline residue, however, is not important in the spontaneous production of these sharp bends at higher temperatures. In contrast to this, the three dimensional fixation of either of these amino acid residues by the proline residue in position 156 does not appear to be decisive in the reaggregation and the back-folding of the proteins denatured with urea; hence, it follows that the last three amino acids can be generally absent (Sengbusch and Wittmann, 1965). The temperature sensitivity of N1196 (proline → serine in position 63) can be understood as well as that of N118 but it is not possible at this time to give an explanation for the quantitative differences between these two mutants.


The group, aspartic acid - proline, which is found in *nigra*, is also found in hemoglobin and causes, in the latter case, the break in the alpha-helix region (Perutz et al., 1966; Ouzzo, 1966). The end of the helical region is quite generally an important prerequisite for formation of the structure of globular proteins. In the group 19-20 in the case of TMV envelope proteins, either proline or aspartic acid can be substituted. In both cases at 20-25°C, the functional tertiary structure will be improved but the disaggregated protein will be labile at high temperature. The aspartic acid has been
substituted by various amino acid residues in different mutants:

(other amino acid substitutions are shown in parenthesis)

All of the substituted amino acid residues result in the appearance of the protein. However, the native state half-lives measured at 30°C, pH 7, I = 0.1, differences shown above. The fact that the substitution, amino dicarboxylic acid ↔ amino dicarboxylic acid amino is aspartic acid, asparagine at position 19 in M1511 is a speculation based on physicochemical similarities between these proteins and those of flavus, M103, and M1696.

In must remain undecided, however, whether the stabilizing effect of aspartic acid is the result of a mutual exchange with the solvent (Ouss0, 1966) or the result on an intramolecular salt bond.

3. The Wild Strain Protein

The four wild strains, vulgare, dahlemense, U2 and Holmes’ Rib Grass (HRG) are in vivo and in vitro. Obviously, they have been selected under natural conditions for temperature resistance. The proteins from dahlemense (Wittmann-Liebold and Wittmann, 1963) and U2 (Wittmann, 1965; Rentschler, in press) have been analyzed for their primary structure. The protein-chemical basis of their temperature resistance is shown in Figure 1. Positions, where mutations can lead to temperature sensitivity are similar to those of vulgare in the case of the other wild strains. However, those positions which do not affect behavior vary widely (M116 and M12068 belong to the stable mutants. The proline residue in position 156 is involved with enzymatic stabilization (Frogita and Fraenkel-Conrat, 1960)).
I. The "Lipophilic" Protein of Nil204

The protein of Nil204 differs from all the other mutant proteins studied in that (a) it is reversibly precipitated at pH 5, I = 0.1, 4°C, and (b) it cannot be precipitated from an aqueous solution with methanol-ether. Its strong lipophilic behavior can be explained by the substitutions: serine → leucine (position 15) and threonine → isoleucine (position 153) if one assumes that they lie in the vicinity of the surface of the subunit. Both of the substitutions are of the type: polar → apolar (see Figure 1).

SUMMARY

The proteins of several temperature sensitive (ts) and temperature-resistant (tr) strains of TMV (Table, Part I), for which the amino acid sequence is known due to the work of Wittmann et al., have been separated from the RNA in the native state and investigated with respect to their thermal stability.

Model experiments with the wild type (vulgare) and the most sensitive mutant (Nil18) show that:

(a) Upon dialysis from pH 7 to 5 at 30°C, Nil18 protein forms a precipitate and only a small proportion of soluble aggregates, whereas Nil18 protein at 20°C and vulgare protein at both temperatures form soluble aggregates with sedimentation coefficients of several hundred S (Fig. 5, Table I).

(b) If soluble aggregates are formed, they have the typical cylindrical structure (Figure 5).

(c) The non-covalent bonds which form the precipitate differ qualitatively from those which form the ordered aggregates (Table 2).

The thermal stabilities of 18 different proteins were compared. Upon incubation at pH 7, I = 0.1, 30°C, ts-I mutants proteins lose their ability
to form soluble aggregates at pH 5 in the cold with half lives of 2 to 60 minutes (in one case 180 minutes), whereas the proteins of ts mutants retain this ability for at least several hours (Fig. 7, 9). Of the wild type proteins, daelemanense is the most sensitive. In contrast to all other mutant proteins, the protein of the defective mutant M1220h precipitates at pH 5 even in the cold. The protein of the ts-II mutant M12519 is stable at 30°C (Figure 9).

The rate of denaturation of ts proteins decreases strikingly when the pH is lowered from 7 to 5, but between pH 7 and 8, there is only a slight pH dependence (Figure 8, Table 3). Complete virus particles of ts mutants are stable at 50°C for at least 40 hours (Figure 10).

The role of certain amino acid residues in the stabilisation of the native tertiary structure is discussed. In the case of ts mutants, all the amino acid residues which are replaced are not necessary for the spontaneous formation of a functional structure at low temperature.

It was found that:

(a) All mutant proteins which have a lowered electrophoretic mobility in 2 M urea at pH 8.8 are ts, but not all ts proteins have an altered electrophoretic mobility.

(b) The proline residues in positions 20 and 63 are necessary for the thermal stabilisation of disaggregated protein, but not the proline residue in position 156.

(c) In the positions 19-20, the arrangement, Asp-Pro, most probably stabilises a bend in the polypeptide chain which is necessary for ordered aggregation. The substitution of each of the residues leads to a sensitive protein.

(d) In the case of the defective protein of M1220h, the role of polar (but not charged) and apolar side chains for the solubility properties of the protein molecule is evident.
I wish to thank Prof. Dr. G. Melchers for his generous support of this work and his critical discussion of the manuscript, and Dr. H.G. Wittmann (Max Planck Institute for Molecular Genetics) for supplying TMV mutants with known amino acid substitutions.

I would like to thank Dr. H. Frank and G. Berger (Max Planck Institute for Virus Research-Electron Microscopy), Dr. S. Sarkar, H. Tischy (unpublished methods) and Master Electrician K. Schuls for their efforts.

I wish also to thank the Farbwerken Hoechst AG (name of company) and the Max Planck Association for financial support.

LITERATURE CITED


Wittmann, H.O.: Protein studies on mutants of tobacco mosaic virus as a contribution to the problem of the genetic code (Proteinuntersuchungen an Mutanten des Tabakmosaikvirus als Beitrag Zum Problem des genetischen Codes). Z. Vererbungsl. 93, 491-520 (1962).


Fig. 1. The primary structures of the envelope proteins and the in vivo temperature behavior of $N$ mutants of TMV. Not shown are: $N1220^*$, serine $\rightarrow$ leucine in position 15; threonine $\rightarrow$ isoleucine in position 15. * In addition to the substitutions indicated, in $N1$ there is the substitution isoleucine $\rightarrow$ threonine at a position which in itself does not lead to temperature sensitivity. * In addition to this substitution, there is the substitution serine $\rightarrow$ phenylalanine at position 138, which does not lead to temperature sensitivity according to studies on another mutant. The protein-chemical data have been obtained from Wittmann, 1964; Wittmann-Liebold et al., 1965, for the mutants, and from Wittmann-Liebold and Wittmann, 1963; Wittmann, 1965, and Rentschler, in press, for the wild strains. Localization of substitutions of N103 and N1696 within peptide I by electrophoresis - separation into $tr$ and $ts$ according to Jockusch, 1964, and this work, Part I - separation of amino acid residues as polar and apolar according to Perutz et al. (1965).
Fig. 2a and 2b. Gel electrophoresis of TMV mutant proteins in 8 M urea, pH 8. (a) Vulgare derived mutants, (b) All derived mutants. Reference: Vulgare protein, 200 V, 1 h, 30 mA, 15 hours, densitometry after amido-Schwarz staining.

Fig. 3. M118 protein at pH 7.0, I = 0.1, at 30°C and 20°C. Loss of capacity at pH 5 to form soluble aggregates.
Figure 4. Denaturation kinetics of N118 protein at pH 7.0, I = 0.1, 30°C in the presence and absence of ethylmercaptan.

Figure 5. Sedimentation profiles of vulgare and N118 protein which have been dialysed for five hours at I = 0.1, from pH 7.0 to pH 5.0. Prior to analytical ultracentrifugation, the precipitated protein was

legend continued on next page
Legend for Figure 5, continued:

tumved by centrifugation for 10 minutes at 1,300 g. Pictures taken ca. 2.5 minutes after attaining running speed of $f = 17,980 \text{ min}^{-1}$ with Philpot-Svensson optics, 60° angle.
Figures 6a–d. Electron micrographs of protein aggregates of *vulgare* and *Mili8*. The results are similar to those in Figure 5 except that the solutions were not preparatively centrifuged. Undiluted aggregates were negatively stained with phosphotungstic acid. Apparatus magnification: 40,000 X; total magnification 100,000 X. *Vulgare* proteins: (a) at 20°C; (b) aggregated at 30°C. *Mili8* protein: (c) at 20°C; (d) aggregated at 30°C. In (d), in addition to cylinder-form aggregates, a number of amorphous aggregates are seen. 70–80% of the protein is found in the amorphous aggregates. Pictures taken by Dr. Frank, Max Planck Institute for Virus Research.
Figure 7. Denaturation kinetics of proteins from *vulgare* mutants and *reflavaescens*.

Figure 8. Denaturation of proteins from *vulgare* mutants at pH 6.0. Scale for the time axis differs from that in Figure 7.
Figure 9. Denaturation kinetics of A-14 Mutant proteins.

Figure 10. Thermal stability of tr and ts virus particles. See text.
TABLE I

Sedimentation Characteristics of *Vulgaris* and *Nilius* Proteins

<table>
<thead>
<tr>
<th></th>
<th>1 - 0.1</th>
<th>1 - 0.2</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 7</td>
<td>pH 7+6</td>
<td>pH 7</td>
<td>pH 7+6</td>
</tr>
<tr>
<td><em>Nilius</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4°C</td>
<td>32 S</td>
<td>-</td>
<td>3-4 S</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>30 S</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20°C</td>
<td>80% 30 S</td>
<td>90.5% 500 S</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>11% Pr</td>
<td>0.5% Pr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30°C</td>
<td>9% 51 S</td>
<td>26% 270 S</td>
<td>99% 36 S</td>
<td>2% 250 S</td>
</tr>
<tr>
<td></td>
<td>01% Pr</td>
<td>74% Pr</td>
<td>1% Pr</td>
<td>98% Pr</td>
</tr>
<tr>
<td><em>vulgar</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4°C</td>
<td>-</td>
<td>-</td>
<td>4 S</td>
<td>-</td>
</tr>
<tr>
<td>20°C</td>
<td>100% 20 S</td>
<td>96.5% 500 S</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0% Pr</td>
<td>0.5% Pr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30°C</td>
<td>100% 28 S</td>
<td>96.5% 380 S</td>
<td>99% 240 S</td>
<td>98% 530 S</td>
</tr>
<tr>
<td></td>
<td>0% Pr</td>
<td>0.5% Pr</td>
<td>1% Pr</td>
<td></td>
</tr>
</tbody>
</table>

Similar studies are shown in Fig. 5. Given are the relative fractions in % of protein with the stated sedimentation characteristics (sedimentation coefficient in Svedberg units, in brackets, the next component, or "Pr" = precipitated). Determination of concentration by measurement of the $E_{280}$ in 10 M urea.
Table 2. Solubility of Ni11 Precipitates

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Temperature</th>
<th>Time</th>
<th>Fraction dissolved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miller-Golden-Buffer pH 6, I = 0.1</td>
<td>4°C</td>
<td>24 h</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Miller-Golden-Buffer pH 5, I = 0.1</td>
<td>30°C</td>
<td>24 h</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Miller-Golden-Buffer pH 10.5, I = 0.1</td>
<td>4°C</td>
<td>24 h</td>
<td>0.16</td>
</tr>
<tr>
<td>0.02 M Na-Dodecyl sulfate pH 6, I = 0.12</td>
<td>20°C</td>
<td>&lt;5 min</td>
<td>1.0</td>
</tr>
<tr>
<td>67% H$_2$SO$_4$</td>
<td>20°C</td>
<td>&lt;5 sec</td>
<td>1.0</td>
</tr>
<tr>
<td>8 M Urea pH 6, I = 0.1</td>
<td>20°C</td>
<td>&lt;5 min</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Table 3. Native state half-lives of tr and ts proteins as a function of the incubation pH.

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>pH 5.0</th>
<th>pH 6.0</th>
<th>pH 7.0</th>
<th>pH 8.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>vulgare (tr)</td>
<td>&gt;2.5·10³</td>
<td>&gt;2.5·10³</td>
<td>&gt;2.5·10³</td>
<td>&gt;2.5·10³</td>
</tr>
<tr>
<td>Ni 103 (ts)</td>
<td>&gt;2.5·10³</td>
<td>400</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Ni 118 (ts)</td>
<td>&gt;2.5·10³</td>
<td>30</td>
<td>2</td>
<td>1.5</td>
</tr>
<tr>
<td>Ni 1927 (tr)</td>
<td>&gt;2.5·10³</td>
<td>&gt;2.5·10³</td>
<td>&gt;2.5·10³</td>
<td>&gt;2.5·10³</td>
</tr>
<tr>
<td>CP415 (ts)</td>
<td>&gt;2.5·10³</td>
<td>200</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>falcum (ts)</td>
<td>&gt;2.5·10³</td>
<td>200</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>A 14 (tr)</td>
<td>&gt;2.5·10³</td>
<td>&gt;2.5·10³</td>
<td>&gt;2.5·10³</td>
<td>&gt;2.5·10³</td>
</tr>
</tbody>
</table>

Temperature 30°C, I = 0.1. Values given are the values of the $t_1/2$ (min). The native state criterion is solubility at pH 5.