RELATIONS BETWEEN TEMPERATURE SENSITIVITY, AMINO ACID REPLACEMENTS, AND QUATERNARY STRUCTURE OF MUTANT PROTEINS

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In tobacco mosaic virus (TMV) the rod like morphology of the virus particle ultimately is determined by the amino acid sequence of the coat protein subunits. During the last years mutants with single known amino acid replacements in the coat protein polypeptide chain became available (Wittmann 1962,1964, Funatsu and Fraenkel-Conrat 1964). It has been found that many of these mutants, which can be multiplied readily at green-house temperatures, produce very low amounts of infective virus particles at 30 to 35°C in the host plant, whereas the wild type, vulgare, and some of its mutants produce high amounts at 30 to 35°C as well as at 20 to 25°C (Jockusch 1964). The first class will be designated as temperature sensitive (ts) the second as temperature resistant (tr). For the majority of the ts mutants it has been shown that their defect is a consequence of the amino acid replacement(s) in coat protein (Jockusch 1964). For at least two ts mutants the presence of nonfunctional coat protein in the plant could be demonstrated (Jockusch, in preparation). In the present paper the denaturation rates of purified, RNA-free proteins of several tr and ts mutants will be given. The stabilizing effect of aggregation on ts proteins is shown by measuring the "half life times" as a function of pH.

TMV Mutants Used

The amino acid replacements of a number of mutants could be localized in the polypeptide chain by Wittmann and coworkers (Wittmann et al. 1965, Wittmann-Liebold et al. 1965). Part of the results of the temperature behavior in tobacco tissue and the chemical analysis of the coat protein may be summarized as shown in fig.1. The origin of the mutants is given in Wittmann et al. 1965. For the experiments described below mutants were

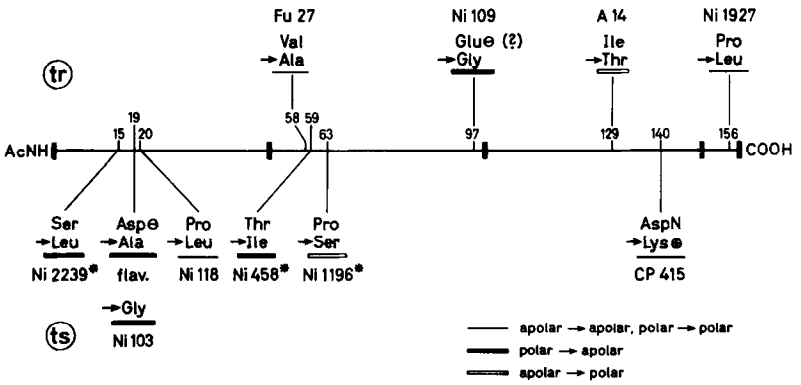

Fig.1: Amino acid replacements in coat protein and in vivo behavior of 11 mutants of TMV.
The line represents the polypeptide chain of the wild type, vulgare, consisting of 158 amino acid residues. Numbers above the line refer to the positions where the replacements have occurred. *Amino acid replacements which lead to the observed behavior in the presence of the Al4 replacement, which itself does not influence the stability at 3o°C.

selected, which are derived directly from vulgare and have only one amino acid replaced in their coat protein. These are the four ts mutants Ni 1o3, Ni 118, flavum and CP 415, the tr mutants Ni 1927 and Al4, and the wild type, vulgare. The ts mutants represent different types of amino acid replacements in different regions of the polypeptide chain.

Preparation of The Virus Proteins

Virus preparations were split at pH 1o.4, ionic strength $\Gamma/2$ = I = o.1 according to Schramm et al.(1955). "A-protein" and RNA were separated by free electrophoresis at pH 9.5, I = o.o2 in the presence of $2 \cdot 1o^{-4}$M EDTA and $1o^{-3}$M cysteine or thiodiglycol using the "Elphor VaP" apparatus (Sarkar and Wittmann, in preparation). The ratios of the extinctions at 26o mμ and at 28o mμ (corrected for light scattering) were o.55 to o.58 indicating that practically all RNA had been removed. The proteins were native as tested by their ability at 4°C to form water clear solutions at pH 1o.5, 8.o, 7.o, whereas opalescent solutions were formed reversibly at pH 5.o. The proteins were stored at pH 5.o, I = o.1, -6o°C. The Al4 protein used had been prepared by the acetic acid method (Fraenkel-Conrat 1957).

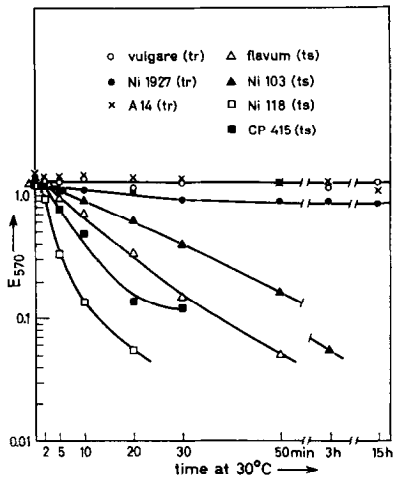

Fig.2: Loss of solubility at pH 5 during incubation at 30°C, pH 7.0, I = 0.1 in the presence of 10^{-3}M ethyl mercaptane. Ordinate: protein content of supernatant given as OD_{570} of the ninhydrin test. 1 OD_{570} ≙ 10^{-5}M protein in the test solution (4-fold diluted incubation mixture).

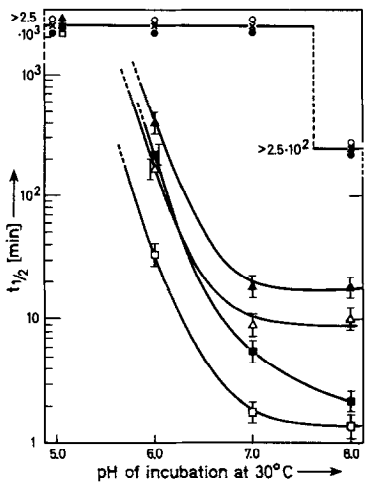

Fig.3: Dependence of half life times, measured by the solubility test, on pH at 30°C, I = 0.1 in the presence of 10^{-3}M ethyl mercaptane.
A possible error of ± 20% is indicated at each point.

Buffers Used for Denaturation Experiments

Buffers used for dialysis were prepared according to Miller and Golder (1950). However, Tris was used instead of barbiturate

for pH 8. The ionic strength in all cases was I = o.1. Buffers were 10^{-3}M either with respect to cysteine or to ethyl mercaptan. To test for solubility at pH 5 acetate buffer of pH 5.o, I = o.1 according to Boyd (o.1 M sodium acetate, o.o33 N acetic acid) was used because of its high capacity compared to the Miller-Golder buffers.

Kinetics of Denaturation

Since native TMV protein is soluble (in the form of rod like aggregates) at pH 5, the loss of solubility at pH 5 has been followed in order to obtain a relative measure for the stability of the mutant proteins (cf.Kunitz 1947). The protein solutions were adjusted to an OD_{28o} of 1.1o which approximately corresponds to $5 \cdot 10^{-5}$ M with respect to the polypeptide chain (Fraenkel-Conrat 1957). The aggregates were dissolved by dialysis against pH 1o.5 in the cold and the desired pH was achieved by lowering the pH of the dialysis buffer stepwise (pH 8.o, 7.o, 6.o, 5.o). Buffers of the final pH contained ethyl mercaptan. The protein solutions were equilibrated at 2o°C (time "o") and then placed in a water bath of 3o.o°C. At different times o.5 ml were taken and diluted immediately into 1.5 ml of ice cold Boyd buffer. The resulting pH in all cases was $5.o \leqq pH \leqq 5.1$. The samples were then centrifuged for 4 min at 13oo g. The protein content of the supernatant was determined with the ninhydrin reagent after alkaline hydrolysis. The resulting OD_{57o} values are proportional to the protein concentration at least up to an OD_{57o} of 1.7. Fig.2 shows the kinetics of the loss of solubility at pH 5 when the proteins are kept in phosphate-NaCl buffer of pH 7.o, I = o.1, at 3o°C. Under these mild conditions denaturation becomes evident within minutes in the case of ts mutants, while the tr proteins remain soluble or at the most are slightly affected within 15 hrs. Ni 118, the most sensitive mutant protein, does not lose its solubility measurably within 3o min at 2o°C under otherwise identical conditions. The loss of solubility at 3o°C and I = o.1 has been measured with parallel samples at pH 8.o, 6.o, and 5.o. At pH 5.o all proteins are practically stable at 3o°C. Although no clear first order kinetics are observed in the loss of solubility, a "half life time" $t_{\frac{1}{2}}$ may be defined for the conditions employed at which the concentration of soluble protein has decreased to

5o% of the concentration at the reference time. These values were calculated taking 2 min as the reference time and are shown as a function of pH in fig.3.

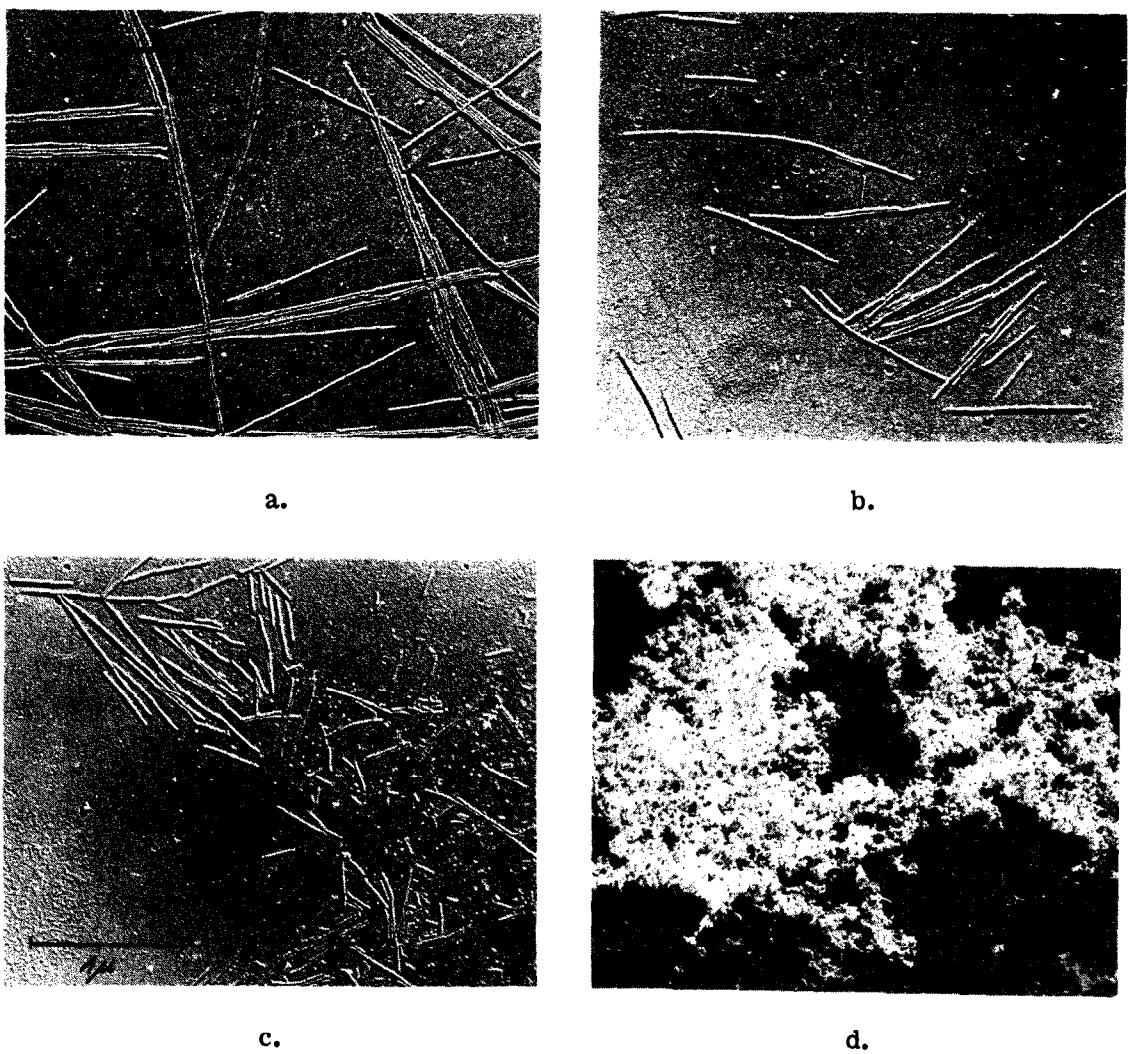

Fig.4: Aggregates formed by wild type protein (a, b) and the most sensitive protein (c, d) upon 8-fold dilution with acetate buffer pH 5.o, I=o.1. Samples were taken from the experiment shown in Fig.2. a) vulgare time "o" b) vulgare 15 min at 3o°C c) Ni 118 time "o" d) Ni 118 15 min at 3o°C. In the case of Ni 118 time "o", numerous disk like aggregates can be seen in addition to rods. No rods or disks were observed in the 3o°C treated sample of Ni 118.

Electron Microscopy

Samples of the experiment shown in fig.2 were taken at times "o min" and "15 min" of the 30°C treatment and diluted 8-fold into Boyd buffer pH 5. Electron microscope specimens were prepared by agar filtration and shadowed with platinum-palladium at an angle of 20°. Fig.4 shows that the native protein forms rods under these conditions and that the insolubility of denatured samples is due to the formation of large disordered aggregates.

Conclusions

There are two classes of defective missense mutations with respect to the state of the protein involved: 1) the regularity of the molecular conformation is retained but function is lost, as in the case of Siegel's (1962) mutant PM2 of TMV, 2) the unique ordered conformation and hence function is lost because of thermal instability. The ts mutants of TMV mentioned here apparently are models of the second type: all the mutant proteins listed in fig.1 below the line would be lethal in homoiothermic animals such as man. The second type may be "leaky" in the genetic sense and such a leakiness is indeed found in the ts mutants of TMV. Its degree, i.e.the production of infective particles at high temperature may be used to establish the order of the in vivo stability as Ni 118, flavum < CP415, Ni 103 < Ni 1927, A14, vulgare. The in vitro order has been found to be slightly different in preliminary experiments (Jockusch 1964) and could now be confirmed on the basis of "solubility half life times" at pH 7 or 8 as Ni 118 < CP 415 < flavum < Ni 103 ≪ Ni 1927, A14, vulgare. No simple rule concerning the relations between temperature sensitivity and the type of amino acid replacements (charge and polar or apolar character of the side chains involved) can be deducted from fig.1, and so far the material is too small for statistical analysis. Conclusions concerning the role of certain single amino acid residues will be given in a later publication (Jockusch in prep.).

The strong pH dependence of the stability of the proteins, which for the four ts mutant proteins mentioned here leads to a more than 100-fold (Ni 103, flavum, CP 415) or more than 1000-fold (Ni 118) increase of half life times when the pH of incubation is lowered from 7.0 to 5.0 apparently is a consequence of the formation of the quaternary structure in which the subunits are

forced into the correct conformation by each other. This explanation perhaps applies to many if not all cases in which the temperature sensitivity of the "synthesis" of a protein is observed (Edgar and Epstein, 1965).

A more complete report on the biological and physicochemical behavior of a number of tr and ts mutants of TMV will appear in "Zeitschrift für Vererbungslehre" (Jockusch, in preparation).

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