TOBACCO MOSAIC VIRUS PROTEIN: KINETIC AND EQUILIBRIUM STUDIES ON THE pH-DEPENDENT TRANSITION A-PROTEIN ⟶ DOUBLE DISC.

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SUMMARY. Concentration dependent and temperature dependent stopped-flow experiments on the transition A-protein ⟶ double disc show a triphasic reaction consisting of a nucleation phase, a propagation phase and a slow redistribution of polymer size which involves the dissociation of "overshoot" aggregates into double discs and smaller aggregates. No first order rate process is observed under the present experimental conditions. Equilibrium circular dichroism data and preliminary kinetic data at various temperatures indicate a change at about 21°C which might be correlated to a partial transition double disc ⟶ helix parallelled by a further shift in the equilibrium of double disc formation; from both data the thermodynamic and activation parameters for the A-protein ⟶ double disc transition are estimated.

INTRODUCTION. Under quasi-physiological conditions (pH 7, 0.1 ionic strength, 20°C) tobacco mosaic virus protein (TMVP) exists predominantly in the double disc state (1,2) which plays an important role in the self assembly of the virus from its protein and RNA (2). Starting from A-protein (M_n=52, 500, $s_{20,w} \sim$ 4.5 S), double discs are formed by increasing temperature, decreasing pH, or increasing ionic strength (1,3-5). Durham and Klug (6), by means of sedimentation velocity technique, proved the kinetics of double disc formation from A-protein (pH 7, 0.1 ionic strength, 5⟶20°C) to be a slow process. However, in a later paper, Durham (4) cited results of Scheele and Schuster in which discs were observed to form in minutes at pH values below 7.0.

When the A-protein ⟶ double disc transition is brought about by a pH drop (pH 8⟶7), proton uptake and a decrease in dichroic absorption at 265-290 nm were observed (5). The time course of both the spectral changes and aggregation, as followed by circular dichroism (CD) and ultracentrifugation techniques, suggested that the conformational changes precede the aggregation (5). In the present study, an attempt is made to follow the two processes by stopped-flow experiments. No first order could be detected under the given experimental

Abbreviations: TMV, tobacco mosaic virus; TMVP, TMV-protein; CD, circular dichroism

conditions (pH 8→pH 7, 0.1 ionic strength, 20°C, 1-7 mg/ml); instead, above a critical protein concentration, the reaction is characterized by three phases: a rapid biphasic reaction, consisting of a nucleation and a propagation step, and a slow third process which leads to disintegration of "overshoot" aggregates (> 25 S) into double discs (~25 S).

MATERIALS AND METHODS. TMV grown in Nicotiana tabacum (var. Samsun) was isolated by differential centrifugation with 2-3 depigmentation steps using EDTA (7, 8). The protein was prepared using the acetic acid method (9). For each series of experiments, freshly prepared solutions were used. In order to clarify the solutions, high speed centrifugation (2 hrs at 100, 000 g) was applied. Equilibration with the standard buffer (K-phosphate, pH 8, 0.1 ionic strength) was provided by dialysis over a period of about 24 hours at 2°C.
UV-spectroscopy, using $A^{1\%}_{260}$ = 27 and $A^{1\%}_{280}$ = 13 for TMV and TMVP respectively (10, 11), with a Zeiss PMQ II spectrophotometer, was employed to determine the concentration. Scattering was corrected according to Englander and Epstein (12). The purity of the preparations was determined from the A_{max}/A_{min} ratio which was never less than 1.2 and 2.4 for TMV and TMVP respectively.
A Durrum-Gibson stopped-flow spectrophotometer (dead time = 1.6 msec) with 2 mm pathlength was used together with a Hewlett-Packard oscilloscope to record transmittance changes. Care was taken to deaerate the flow system and the solutions. The oscilloscope was calibrated before each experiment; instability of the deuterium lamp did not exceed 10 % of the total screen at maximum sensitivity of the oscilloscope over a period of 5 minutes. Changes in absorbance (ΔOD) were calculated following the Operation Manual of the apparatus. Comparing the results with ΔOD values obtained from a standard reaction in the Zeiss PMQ II spectrophotometer showed agreement within ± 10 %. For each measurement, 3 to 4 traces were obtained to check the reproducibility; the calculated ΔOD-values did not differ from each other by more than 13 %.
UV-difference spectra of protein solutions were obtained using a Zeiss DMR 10 spectrophotometer and matched quartz cuvettes with 1 mm pathlength.
CD spectra were obtained using a Roussel-Jouan Dichrographe II with scale expansion ($\Delta E = 2 \times 10^{-6}$/mm). Cuvettes with 1 mm pathlength were used throughout; the measured absorbance never exceeded 1.
A Beckman analytical ultracentrifuge (Spinco E) was used for particle size determination. Sedimentation coefficients, obtained from log r vs. t plots, were corrected for 20°C and water viscosity.

RESULTS.
A. Control experiments. Doubly distilled water was mixed 1:1 with 0.5 M and 3 M NaCl ($\Delta\eta$ and Δn comparable to TMVP solutions) in order to check for viscosity and schlieren effects; no such effects were detected. When A-protein (pH 8, 0.1 ionic strength, 9.2 mg/ml) was mixed 1:1 with phosphate buffer (pH 8, 0.1 ionic strength), no transmittance changes were observed at the most sensitive oscilloscope settings indicating the absence of dilution and temperature artifacts.

B. Stopped-flow experiments: A-protein → Double disc. The reaction A-protein (pH 8, 0.1 ionic strength) + 0.1 M KCl/0.006 N HCl (final pH = 6.9) was followed at 281 nm, 24°C, at different initial protein concentrations (2.2 - 13.4 mg/ml). In terms of transmittance changes, the results at low concentration values (< 3 mg/ml) show a slow decrease in transmittance giving ΔOD values of < 0.01 after 10 minutes. This effect is too small to be analyzed quantitatively. At higher concentration values, the transmittance changes can be described by 3 phases (Figure 1): an initial "lag phase" (slow decrease in transmittance), followed by a

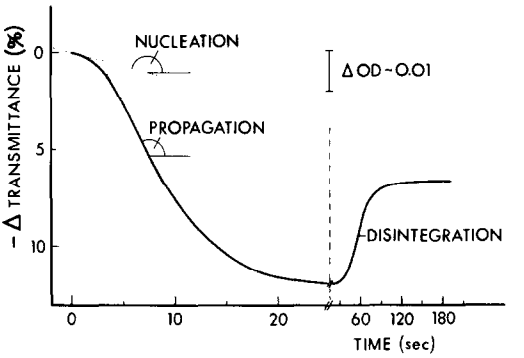

Fig. 1. Kinetic trace of a representative stopped-flow experiment of the triphasic reaction of TMVP. c = 5.0 mg/ml, T = 20.0°C, λ = 281 nm. Comparable properties were obtained at λ = 320 nm.

rapid decrease in transmittance which attains a maximum value. This rapid biphasic reaction, in the time range of seconds, is followed by a slow increase in transmittance in the minute range. Table I summarizes results at different concentration values.

The first and second phases were analyzed according to the equation:

$$v = k\, c^{n} \qquad (1)$$

where v is the reaction velocity as measured from the initial slope of the kinetic trace, k is the rate constant, c is the protein concentration, and n is the reaction order. From the least squares plot of log v vs. log c (Figure 2), the average values of n for the first and second phases were calculated to be 3.04 ± 0.24 and 2.64 ± 0.34 respectively. Different protein samples gave identical profiles of the total reaction and the same value for n, although the absolute values of the rate constants showed large scatter.

Table I. Concentration dependence of the rates of the transition A-protein → double disc , T = 24.0°C

c(mg/ml)	ΔOD_{max}	τ [a](sec)	v_N(OD/sec) [b]	v_P(OD/sec) [b]
6.69	0.075	2.9	0.0047	0.0120
5.35	0.034	3.6	0.0015	0.0050
4.01	0.023	5.2	0.0007	0.0029
2.68	~0.014	(equilibrium after 10min)	0.0002	
1.34	~0	(equilibrium after 10min)		

a τ = halftime of maximum change of transmittance

b Reaction order of nucleation (N): $n = 3.15 \pm 0.24$
Reaction order of propagation (P): $n = 2.61 \pm 0.35$

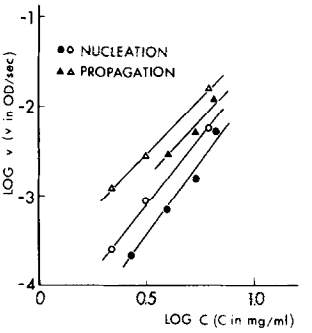

Fig. 2. Determination of the reaction order of nucleation and propagation in the pH-dependent transition A-protein → double disc, T = 24.0°C. Data of two experiments are characterized by open and filled symbols.

At a constant initial protein concentration of 10.0 mg/ml, A-protein was mixed 1:1 with 0.1 M KCl/0.006 N HCl at various temperatures (15-25°C, Table II). At 15.0 and 17.5°C, there is a slow decrease in transmittance which attains a ΔOD value of about 0.01 and 0.02 after 10 minutes. At 20.0 - 25.0°C, a triphasic reaction, similar to Figure 1, was observed. The plot of ΔOD vs. T, shown in Figure 3, indicate a sigmoidal behavior similar to that observed earlier (5, 13). From the plot of log v vs. 1/T for the nucleation phase (cf. TableII), an activation energy of 38.9 ± 2.9 kcal/mole of trimer is calculated. The

Table II. Temperature dependence of the rates of the transition A-protein → double disc , c = 5.0 mg/ml

T(°C)	ΔOD_{max}	τ (sec)	v_N(OD/sec)	v_P(OD/sec)
25.0	0.179	2.33	0.0044	0.0334
22.5	0.152	4.40	0.0021	0.0161
20.0	0.058	7.87	0.0014	0.0036
17.5	~ 0.021	(equilibrium after 10min)	0.0008	0.0003
15.0	~ 0.013	(equilibrium after 10min)		

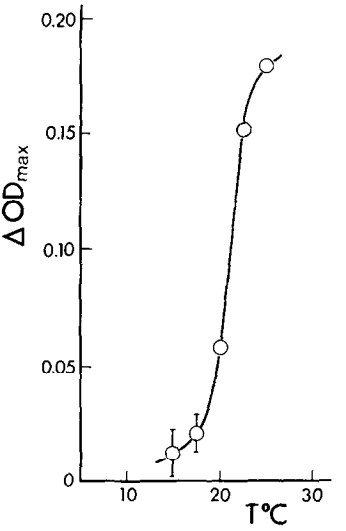

Fig. 3. Temperature dependence of the pH-dependent transition A-protein → double disc. c = 5.0 mg/ml.

corresponding activation parameters are: ΔH^* = 37.3 kcal/mole of trimer, ΔS^* = 143 ± 8 e.u.

C. Sedimentation Velocity Experiments. Earlier equilibrium ultracentrifugation studies (5) indicate that the initial state (pH 8, 0.1 ionic strength) and the final state (pH 7, 0.1 ionic strength) correspond to the A-protein and double disc respectively. In the present study, the time course of the sedimentation patterns was followed after mixing A-protein at an initial concentration of

6 mg/ml with 0.1 M KCl/0.006 N HCl in the stopped-flow apparatus (24.0°C). At a time 16 minutes after mixing, the sedimentation pattern at 56,000 rpm showed 3 components with S-values of 4.8, 27 and 30. After incubating the same sample for 43 hours at ~20°C, the sedimentation pattern showed only 2 components having S-values of 6.3 and 22.

D. Circular Dichroism and UV-Difference Spectra. CD measurements of the A-protein ⟶ double disc transition were done at various temperatures (14.3 - 25.0°C). The plot of the logarithm of the equilibrium constant derived from proton uptake and CD data (5) vs. 1/T shows 2 lines which intersect at about 21°C (Figure 4). From the slopes and intercepts of the straight lines obtained

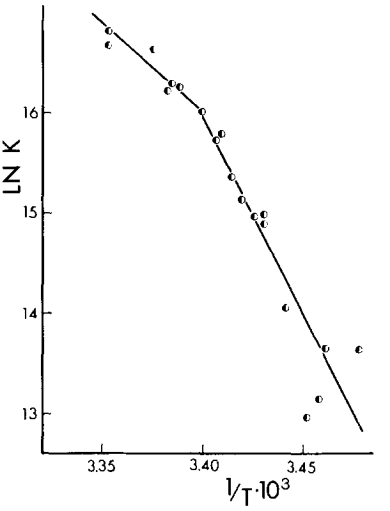

Fig. 4. Determination of the thermodynamic parameters of the pH-dependent transition A-protein ⟶ double disc from H^+-uptake and CD data.

from a linear regression analysis, ΔH and ΔS are evaluated, making use of the relation:

$$\ln K = -\frac{\Delta H}{R}\,\frac{1}{T} + \frac{\Delta S}{R} \qquad (2)$$

The results are summarized in Table III. The UV-difference spectrum (pH 8 vs. pH 7) shows significant changes at 281, 290 and 297 nm confirming earlier findings (5).

DISCUSSION. The concentration dependence (Table I) and temperature dependence (Table II) of the transition A-protein ⟶ double disc prove double

Table III. Thermodynamic parameters from CD data

T(°C)	ΔH (kcal/mol)	ΔS(e.u.)	ΔF (kcal/mol)
< 21	77.5 ± 13.2	295 ± 45	-9.3
> 21	35.5 ± 4.9	153 ± 16	-9.5

disc formation to be a complex reaction accompanied by subtle conformational changes (5). Under the present experimental conditions, no first order rate process is observed. Instead, higher orders of about 3.0 and 2.6 are obtained for the nucleation and propagation steps. The reaction order for nucleation is in quantitative agreement with the value predicted by Oosawa's theory of helical polymerization (15) and with experimental values obtained for actin (16) and insulin (17). The rapid biphasic reaction suggests cooperative interactions; this conclusion is strengthened by the existence of a critical protein concentration below which no double discs are formed (3, 4, cf. Table 1).

The slow third phase of the triphasic reaction (Figure 1) is tentatively explained as a slow redistribution of polymer size (18) which involves the dissociation of "overshoot" aggregates (> 25 S) into double discs (~25 S) and smaller aggregates (~6 S) (cf. $s_{20,w}$ after 16 min and 43 hrs).

Such "overshoot" polymerization phenomena have actually been observed for TMVP (19) and explained theoretically by Scheele and Schuster (20). The condition for "overshoot" to occur, i.e., a slow nucleation step and fast subsequent propagation steps, is satisfied by the present results (cf. v_N and v_P in Tables I, II). If one assumes the trimer to be the polymerizing unit, one may speculate that the nucleation reaction is: 3 trimers → nonamer. With such a nucleus, which might be a special helical assembly formed, propagation is easily achieved by the reaction of nonamers and/or attachment of trimers to the growing helix. Using this concept it is easy to see that "overshoot" might lead to helical aggregates or stacked discs.

The activation energy of 38.9 kcal/mole for the nucleation step is comparable to values observed for other association reactions (e.g. (21)). $\Delta S^* > 0$ may be correlated to changes in charge and/or solvation in the assembly process.

Preliminary kinetic data for the second phase show an Arrhenius plot having

two slopes with a transition temperature at about 21°C. The same behavior is exhibited by equilibrium CD data at various temperatures (Figure 4). The value $\Delta H = 77.5 \pm 13.2$ kcal/mole at $<21^{\circ}C$ is in good agreement with the value of 77 kcal/mole obtained for the "trimer-disc transformation" by Taniguchi (3). At $>21^{\circ}C$, a value of $\Delta H = 35.5 \pm 4.9$ kcal/mole is obtained which is of the order of magnitude of the value calculated for the change in electrical work associated with the polymerization of charged cylinders (Lauffer (1)). From the magnitudes of the ΔH-values above and below 21°C, the appearance of another mode of assembly (e.g. helix) is suggested at $T > 21^{\circ}C$ besides an additional shift of the equilibrium A-protein $\rightleftharpoons$ double disc.

It is interesting to note that the ΔF values (Table III) are close in magnitude; they are in fact comparable to the ΔF values of -8.8 kcal/mole and -8.95 kcal/mole obtained for the helix and disc formation respectively by Butler et al (22). This indicates a delicate equilibrium maintained between the double disc and the helix state.

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