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Scanning calorimetric studies of the stability of tobacco mosaic virus and aggregates of its coat protein

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Abstract. The thermal denaturation of the common strain of a rod-shaped plant virus, tobacco mosaic virus, has been investigated by differential scanning calorimetry, and compared to that of various aggregation states of its coat protein and to that of three other TMV strains. The state of the virions was monitored by electron microscopy and analytical ultracentrifugation. The observed endotherms could be analysed in terms of a stepwise dissociation of the virions. The transition temperatures of the three successive structural changes increased with decreasing pH, from pH = 8.0 to pH = 5.0, although the corresponding enthalpy changes did not vary appreciably with pH. TMV-HR showed a stronger pH dependence of the transition temperatures than the other strains, probably reflecting the importance of the changes in affecting the charged amino acids of its coat protein. The first step of the dissociation, which correlates with the breaking up of the virions into three or four shorter rods, implies a conformational change of the particle that may be related to the first step of the in situ decapsidation of TMV.

Key words: Microcalorimetry – Thermodenaturation – Rod shaped viruses – Tobacco mosaic virus – Decapsidation

Introduction

Ultrasonic absorption measurements on protein aggregates and virions have provided evidence that structural fluctuations exist in isometric viruses and protein shells (Cerf et al. 1979) as well as in rod-shaped viruses such as tobacco mosaic virus and its protein aggregates (Michels et al. 1985). These results induced us to look for the possible presence of enthalpy fluctuations in such systems, by differential scanning microcalorimetry. The presence of irreversible or quasi-irreversible thermally induced transitions prevented us from making accurate measurements of heat capacities, proportional to the magnitude of enthalpy fluctuations (Hill 1960). However, controlling the samples by electron microscopy and analytical ultracentrifugation allowed us to follow the fate of the particles upon heating.

We describe here results obtained with four TMV strains and several aggregates of their coat protein, at pH values ranging from pH = 5.0 and to pH = 8.0. They provide evidence for the importance of nucleic acid-protein interactions in the stability of the virions and for a stepwise dissociation of the virions. The first step implies a conformational change that may be related to the very first step of the in situ decapsidation of TMV.

Materials and methods

TMV-S (Vulgare strain, maintained in Strasbourg: Lebeurier and Hirth 1966) was multiplied in *Nicotiana tabacum* (var. Samsun), and purified from the plant sap by chloroform extraction and polyethyleneglycol precipitation (Van Wechmar and Van Regenmortel 1970), followed by high and low speed centrifugations in 10 mM sodium phosphate, 1 mM EDTA buffer pH 7.2.

Three other TMV strains were also used in this study: Dahlemense (TMV-D: Holling and Huttinga 1976), U2 (TMV-U2: Altschuh et al. 1981) and Holmes Ribgrass (TMV-HR: Oshima and Harrison 1975). Inoculums were gifts from Dr. Van Regenmortel. Multiplication and purification were as for TMV-S.

Protein was prepared by acetic acid dissociation of the virus (Fraenkel-Conrat 1957) and used immediately or stored at pH 4.6 (Durham et al. 1977).

Concentrations were estimated by U.V. absorbancy, using the following specific absorbancies: A (260 nm) = 3.28 cm^2/mg for TMV after correction for light scattering

Abbreviations: TMV, tobacco mosaic virus; TMV-S, Strasbourg (or Vulgare) strain; TMV-D, Dahlemense strain; TMV-HR, Holmes Ribgrass strain; TMV-U2, strain U2 of TMV; DSC, differential scanning microcalorimetry

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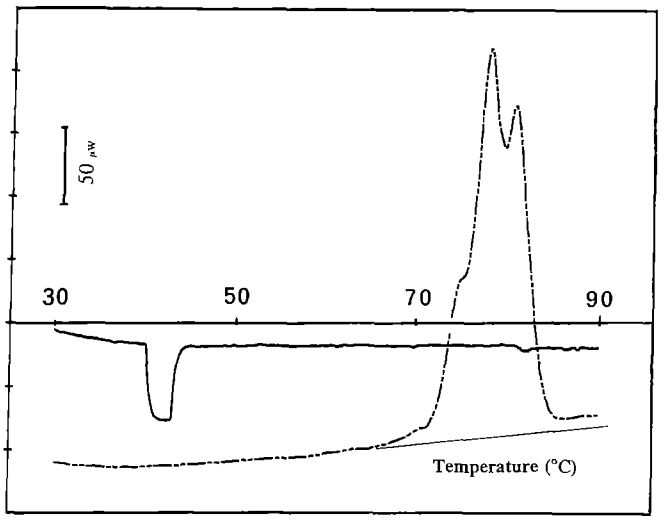

Fig. 1. Original calorimetric recording of heat absorptions of a 5.6 mg/ml TMV-S solution at pH 7.1 (- - - -) and the corresponding buffer (—). The endotherm on the baseline corresponds to a 50 μW calibration mark; scanning rate: 1°C/min

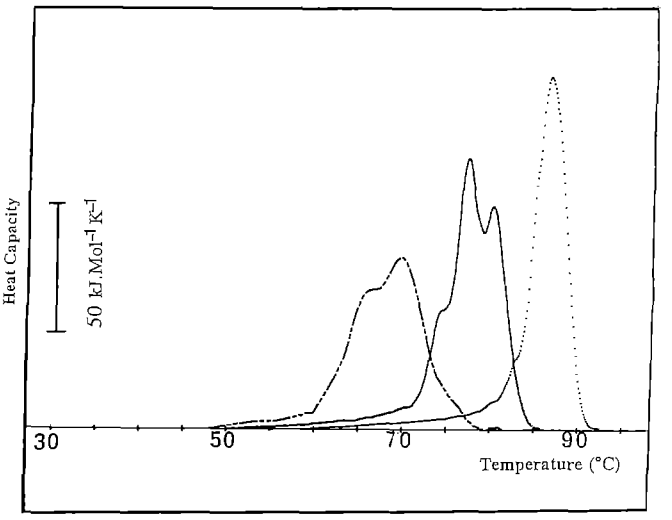

Fig. 2. DSC scans for TMV-S at pH: 5.6 (····); 7.0 (—) and 8.0 (- - - -). The baseline has been subtracted as described in Materials and methods; scanning rate: 1°C/min

(Winter and Gent 1971) and A (275 nm) = 1.29 cm²/mg for pTMV, after twenty fold dilution in 0.02 M sodium borate buffer pH 9.0 to dissociate all protein aggregates that may have formed at neutral or low pH (Michels et al. 1985). All samples used in this work were dialyzed for 24 h against the appropriate buffer at an approximate concentration of 3–5 mg/ml, unless otherwise specified.

Since the stability of TMV and pTMV varies considerably with pH and not only with temperature, we used buffers that exhibit a small pH variation with temperature (Dawson et al. 1969):

0.02 M sodium acetate buffer, for $4.5 \leq pH \leq 5.5$
0.02 M sodium phosphate buffer, for $5.0 \leq pH \leq 7.5$
0.02 M sodium borate buffer, for $8.0 \leq pH \leq 9.0$.

The calorimetric experiments were performed in a high-sensitivity differential scanning calorimeter DASM-4 (Privalov et al. 1975) equipped with matched 0.46 ml platinum cells. Unless otherwise specified the heating rate was set at 1°C per min.

Figure 1 shows a typical DSC scan of a solution of TMV-S at pH 7.1, together with that of the corresponding solvent. Heat capacity increments corresponding to simple transitions may be obtained from the thermogram by subtraction of a baseline given by the heat capacity values of native and totally denaturated protein obtained by linear extrapolation of the heat capacity before and after the process, to the transition temperature. In this case the baseline is approximated by a step curve. Here we used, instead, a sigmoidal curve as a baseline. The apparent specific excess heat capacity $\Delta C\,p$ shown in Figs. 2, 4, 5 and 6 is the difference between the apparent specific heat capacity curve and this baseline. The total experimental enthalpy, $\Delta H_{cal,t}$ was calculated from the area under the peak. Most of our DSC scans show three peaks or shoulders at temperatures T_1, T_2 and T_3 (see for instance the profile obtained at pH 7 in Fig. 2). We therefore interpreted all endotherms using a cooperative four state sequential model (see next section).

Enthalpy changes are expressed in kJ/mole of coat protein subunit. For virions this concentration unit includes the three nucleotides of the viral RNA associated with each subunit (Michels et al. 1985). Heats Δh_{cal} and Δh are given in J/g of subunit.

For the control, samples (about 0.5–1 ml) at the same concentration and ionic environment (nature of the buffer, pH ...) were heated in test tubes in a Haake thermostat at a rate of 0.95°C/min. Samples were removed at temperatures predetermined by analysis of the corresponding thermograms, chilled in ice, diluted two- to five-fold in 0.1 M sodium phosphate buffer, pH 5.8, and examined in a Spinco Model E analytical ultracentrifuge equipped with Schlieren optics. Aliquots were also diluted twenty-fold with 0.02 M sodium acetate buffer pH 5.8, negatively stained with 2% uranylacetate, and examined in a Hitachi H 600 electron microscope, at a nominal magnification of 40 000.

Results

1. Thermal denaturation of the common strain virus: TMV-S

We first checked (not shown) that the profiles of the DSC scans did not depend upon the virus concentration in the range 1.7 to 4.9 mg/ml, providing evidence that the thermograms corresponded to intraparticle transitions, and not to changes in interparticular interactions. Differential scanning calorimetry scans of the TMV-S virions at three pH values are given in Fig. 2, after subtraction of the baselines as described in Materials and methods. Figure 2 shows that the thermal stability increased with decreasing pH. The DSC profiles of TMV-S display a succession of three major endotherms. This is most clearly visible at neutral pH.

To test for the reversibility of the observed transitions, DSC scans of control samples were recorded up to a

predetermined temperature (usually a transition temperature T_i estimated by the least square fit on a complete DSC scan). The samples were cooled down to 25 °C at a rate of ca 1°/min in the microcalorimeter cell, and a full DSC scan recorded again. All successive transitions were irreversible under our experimental conditions. After heating up to T_1, for instance, the first transition was no longer observable in the subsequent DSC scan, whereas the peaks at $T > T_1$ were still present in the thermogram, although with a reduced amplitude. A similar observation was made for the second transition (DSC scans not shown). All samples behaved alike.

The profiles changed only very little when the scanning rate was varied from 0.5 K min^{-1} to 2.0 K min^{-1}. Structural transitions, although irreversible, are therefore rapid compared to the scanning rate, and not kinetically limited. Using results of recent simulations of calorimetric scans for irreversible systems (Freire et al. 1990), this observation allowed us to treat the system as if the three steps were reversible. Among several reversible three steps systems that were compared to the data, only one turned out to be successful. It assumes that each of the three steps involves the same cooperative unit, of m protein subunits. Details of the model, its comparison with the data and further implications for the denaturation mechanism will be described in another paper (Cerf et al., to be published). Using this model, the number of cooperative units was found to be close to $m = 5$.

All three transition temperatures were found to decrease with increasing pH, from $T = 83$ °C at pH = 5.6 to $T = 62$ °C at pH = 8.0 for the first transition, for example (see also Fig. 7). This change of the stability shows up in the variation with pH of the positions of the peaks of the thermograms of Fig. 2. Transitions enthalpies, on the other hand, showed little variation with pH, and therefore with temperature; the total enthalpy ΔH_{cal} of all transitions was about 750 kJ/mole. The following values were found for the transition enthalpies ΔH_i, $i = 1, 2, 3$: $\Delta H_1 \simeq 190$ kJ/mole; $\Delta H_2 \simeq \Delta H_3 \simeq 290$ kJ/mole. It is to be noted that ΔH_1, ΔH_2 and ΔH_3 were largely unaffected by reasonable manipulations of the baseline. Altogether we estimate that the values indicated above are correct to within 10%.

We monitored the dissociation state of the virions by electron microscopy and analytical ultracentrifugation. Figure 3 shows the electron micrographs and ultracentrifugation pattern of TMV-S heated up to T_3 at pH 7. At $T < T_1$ virions were present as 3000 Å rods sedimenting at 176 S. At T_1, the sedimentation pattern contained a second peak sedimenting at 140 S, corresponding to particles of about one-third of the initial length, in good agreement with the shorter particles seen together with the long ones in electron micrographs. We also checked that a sample of TMV-S heated up to T_1 and then chilled in ice contained less than 15% material that would precipitate during low speed sedimentation. Deposited on a 10–40% sucrose gradient, the low speed supernatant gave rise to only two bands after 2.5 h ultracentrifugation in a SW 28 rotor at 25 000 rpm, even if the gradient was heavily overloaded. No other fast or slowly sedimenting material could be detected on a sucrose gradient or during the onset of the analytical ultracentrifugation run. Denaturation was therefore limited at this stage. At T_2, the shorter rods broke apart into smaller, polydisperse, aggregates. After T_3 the material was totally denatured, and an important precipitate could be seen in the test tubes, that could be eliminated by low speed centrifugation. As precipitation of protein is usually an exothermic process (Privalov and Khechinashvili 1974), the enthalpy of this third transition is very likely underestimated.

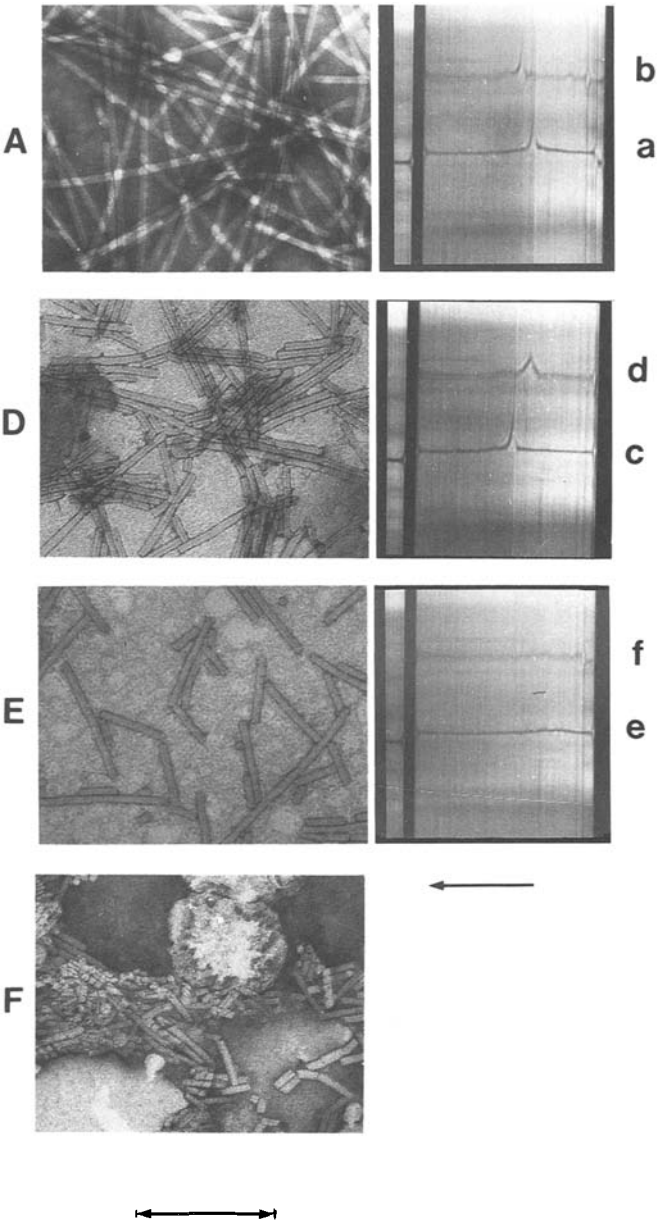

Fig. 3. Control of the thermal denaturation of TMV-S at pH 7.0 by electron microscopy (capital letters) analytical sedimentation (lower case letters). *A*, *a*: native virions at 25 °C, *b*, *c*: $T = 55$ °C, resp. 67 °C, *D*, *d*: $T = T_1 = 74$ °C (first peak of the DSC), *E*, *e*: $T = T_2 = 76$ °C (main peak of the DSC), *F*: $T = 78$ °C. The micrograph corresponds to the unique small field of short rodlike particles that could be found on two electron microscopy grids. The arrow indicates the direction of sedimentation, from right to left. Scale bar = 3000 Å (the length of a native TMV particle)

2. *Thermodenaturation of the aggregates of the coat protein of TMV-S*

Three major types of aggregates have been described for the coat protein of TMV, corresponding to various conditions of pH and ionic strength (Durham et al. 1971). Below about pH 6.5, the protein assembles into a single helix whose architecture is very similar to that of the virus, except that its length is undetermined because it does not contain RNA. At neutral pH and moderate ionic strength, TMV protein sediments at 20 S, forming a short helix made of about 39 subunits, with a pitch and a diameter very similar to those of the virus (Correia et al. 1985). At higher pH much smaller aggregates are present, sedimenting at 4 S. The 4 S → 20 S transition can be induced by increasing the temperature from 4 °C to 25 °C under suitable pH and salt conditions (Durham et al. 1971; Schuster et al. 1979).

DSC scans for the three types of aggregates 4 S, 20 S and helix of TMV-S protein are given in Fig. 4: only the helical aggreate presented two distinct peaks in its thermogram above 25 °C. The nature of the first transition has not yet been investigated in detail.

Transition temperatures and enthalpy changes were found to be: $T = 41$ °C and $\Delta H = 320$ kJ/mole for 4 S aggregates at pH = 8.54; $T = 52$ °C and $\Delta H = 440$ kJ/mole for 20 S aggregates at pH = 6.52; $T = 58$ °C and 72 °C, and $\Delta H = 95$ kJ/mole and $\Delta H = 310$ kJ/mole at pH = 5.0, respectively for the two successive peaks of the DSC scan of the helical aggregates.

We also investigated the fully reversible temperature induced 4 S → 20 S transition at pH 6.5, starting from 0 °C and heating the solution at a scan rate of 1 °C/min^{-1}. The first peak, at 14.8 °C, corresponds to the reversible formation of 20 S aggregates. The second at 50 °C was comparable to that observed in the thermogram of the 20 S specimen, and corresponds to the irreversible denaturation of the latter aggregates (data not shown). The 4 S → 20 S polymerization enthalpy calculated from this scan was about 67 kJ/mole and the temperature of formation of 20 S was 14.8 °C at pH = 6.52. These values agree well with those obtained by Sturtevant et al. (1981) in their extensive study of the polymerization of TMV coat protein.

3. *Thermodenaturation of other strains of TMV*

Figure 5 shows the thermograms corresponding to virions of three other strains of TMV: TMV-U2, TMV-D and TMV-HR, at neutral pH. The DSC scan of TMV-D was very similar to that of TMV-S (see Fig. 2), whereas the three major peaks were more separated for TMV-HR, and much closer together for TMV-U2 (where the succession of three endothermic transitions was clearly visible at pH 7.9; DSC scan not shown). Analytical centrifugation and electron microscopy controls have shown that these transitions reflect the same structural changes as described above for TMV-S. At the highest temperatures TMV-U2 underwent an exothermic transition that corresponds to the formation of long ribbons, also obtained with purified coat protein of TMV-U2 (electron micrographs not shown).

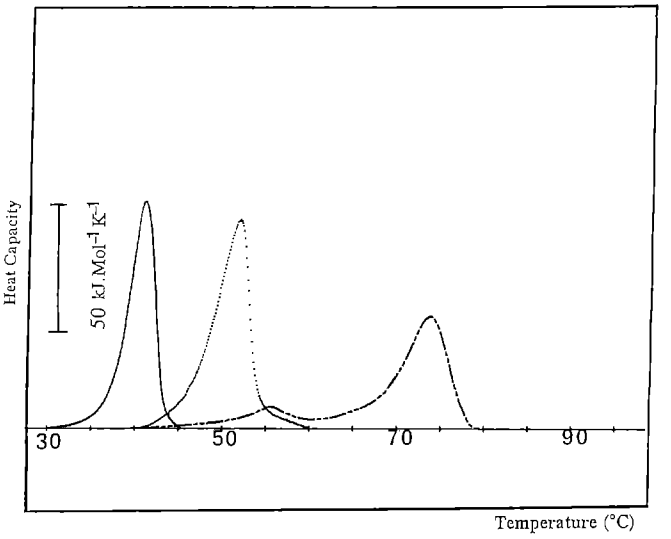

Fig. 4. DSC scans (from 25 °C to 90 °C) for various aggregates of the coat protein of TMV-S: —— 4S aggregates, in 0.02 M sodium borate buffer pH 8.54; ····· 20S aggregates, in 0.10 M potassium phosphate buffer pH 6.52; – – – – helices, in 0.02 M sodium acetate buffer pH 5.0. Baseline subtraction was as in Fig. 2; scanning rate: 1 °C/min. The 20S sample, first dialyzed in the cold against the pH 6.52 buffer, was allowed to heat slowly to 25 °C (Schuster et al. 1979). Denaturated protein was removed by low speed centrifugation before microcalorimetry

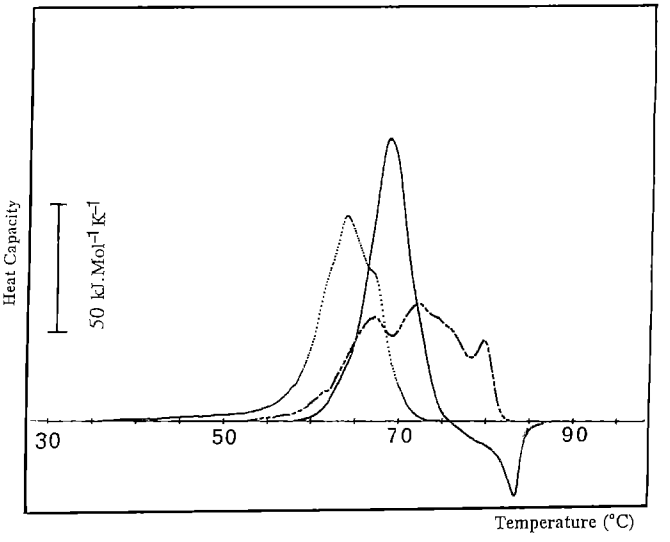

Fig. 5. DSC scans at neutral pH for strains TMV-U2 (–), TMV-D (····) and TMV-HR (– – – –). Baseline subtraction was as in Fig. 2; scanning rate: 1 °C/min

Comparison of Figs. 2 and 5 also shows that the thermostability of the common strain of TMV-S was higher than that of any other strain tested at neutral pH. This result also held at other values of the pH, ranging from pH = 5.0 to pH = 8.0 (data not shown). The variation with pH of the transition temperatures of TMV-U2 and TMV-D was similar to that observed for TMV-S (data not shown), but this variation was significantly more

important for TMV-HR. Figure 6 shows the DSC scans for TMV-HR at three values of the pH, showing a much larger dependence of peak position on pH than for TMV-S (compare with Fig. 2). The differing behaviour of T_1, the temperature of the first transition, for TMV-S and TMV-HR is illustrated in Fig. 7. Similarly, T_2 and T_3 varied more rapidly with pH for TMV-HR than for TMV-S (not shown).

For none of the four strains of TMV, did $\Delta H_{cal,t}$ vary substantially with pH. ΔH_{cal} values for TMV-D and TMV-HR were similar to those of TMV-S. For TMV-U2, $\Delta H_{cal,t}$ was smaller by about 90 kJ/mole, a value that correlated well with that of the negative (exothermic) peak of the corresponding thermogram (Fig. 5). The contribution to ΔH_{cal} of the "normal" thermal denaturation was therefore comparable for TMV-U2 and for the other three strains.

ΔH_1 was also approximately independent of pH, except for TMV-HR. Its average value for TMV-D was nearly the same as for TMV-S (ca. 190 kJ/mole), but it reached ca. 300 kJ/mole for TMV-U2. For TMV-HR it rose from ca. 120 kJ/mole at pH 5.5 to ca. 300 kJ/mole at pH 7.0. ΔH_2 and ΔH_3 were close to each other for TMV-S, TMV-D and TMV-HR and did not vary appreciably with pH. The values of ΔH_2 and ΔH_3 could not be estimated for TMV-U2, since an exothermic reaggregation took place at high temperature.

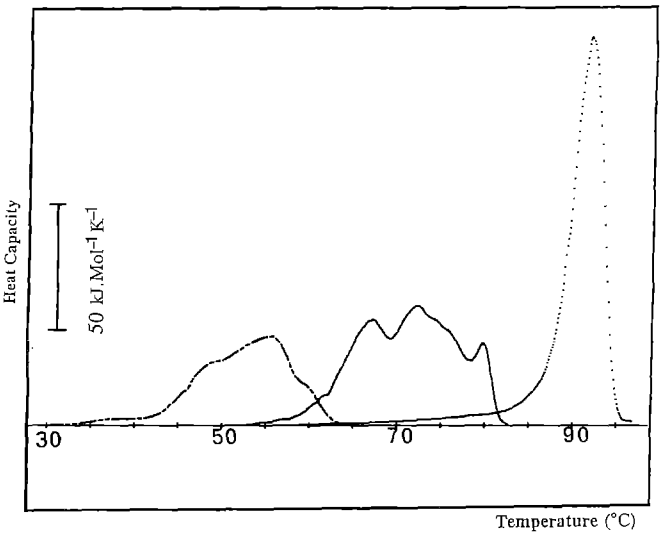

Fig. 6. DSC scans of TMV-HR at pH: 5.6 (···), 7.0 (—) and 8.0 (— - - —). Baseline subtraction was as in Fig. 2; scanning rate: 1°C/min

Discussion

We have shown that the combined use of differential scanning calorimetry, electron microscopy and ultracentrifugation provides information on the mechanism of thermal decapsidation and denaturation of tobacco mosaic virus. Microcalorimetric methods yielded the transition temperatures and enthalpies, whereas control experiments near the transition temperatures provided information about the state of assembly of the particles.

The overall similarity of the results obtained with four strains of TMV, including TMV-HR that differs considerably from the common strain in the primary structure of its coat protein, points toward similar mechanisms of stability and thermal dissociation in all cases. An exception was provided by the behaviour of the coat protein of TMV-U2 which forms specific filamentous aggregates at high temperature, after complete dissociation of the virions.

DSC provides further evidence for an essential contribution of RNA-protein interactions to the stability of the virions. At similar pH the first transition temperature of the virion was, indeed, always much higher than that for the protein, be the latter helical or not. Also, $\Delta H_{cal,t}$ was always much larger for virions than for protein aggregates, by a factor of approximately two. This result also holds for the helical protein aggregate which, apart from the absence of RNA, possesses a structure that is very similar to that of the virion (Namba et al. 1989). The values of $\Delta H_{cal,t}$ derive from the total areas under the peaks of the thermogram and are, of course, independent of any model used to analyse the details of the thermograms. They are probably underestimated because they include the endothermic irreversible denaturation of the virion, accompanied by an irreversible precipitation of denaturated material. Since TMV contains 95% protein and only 5% RNA, it is however unlikely that the important difference of the values of $\Delta H_{cal,t}$ for virions and for protein aggregates can be attributed to differences in the irreversible precipitations at high temperature. Heats of denaturation for TMV proteins ranged from $\Delta h_{cal} = 19$ J/g at pH 8.0 (4S protein) to $\Delta h_{cal} = 25$ J/g at pH 7.0 (20S aggregates) or pH 6.0 (helices), the corresponding transition temperatures being 41°C, 52°C, and 56°C, respectively. These values are close to those observed for many proteins at similar transition temperatures (Fig. 6 in Privalov and Khechinashvili 1974), although in the latter experiments transitions were reversible. For virions, $\Delta h_{cal} \simeq 40$ J/g is comparable to or higher than the highest values obtained for proteins such as ribonuclease and chymotrypsin. Furthermore, it did not vary with the transition temperature. This latter result is confirmed by close inspection of Fig. 1, that shows that the heat capacities of native and denaturated TMV are similar to each other, once extrapolated to the same temperature. In fact, as shown by Sturtevant et al. (1981), the specific heat capacity of native TMV protein is abnormally high. Its value $c_p = 1.7$ J/°/g at 10°C is much closer to those observed by Privalov and Kechinashvili (1974) for denaturated soluble proteins than to the average value of native ones ($c_p = 1.3$ J/°/g).

The stronger pH dependence of the stability of TMV-HR very likely reflects the importance of the amino acid changes affecting basic or carboxylic residues of its coat protein, as compared to the other strains tested. The polypeptide chains of the coat proteins of TMV-HR and TMV-S differ by 22 such changes. Many of these changes are located at subunit contacts, rendering impossible in TMV-HR several salt links that stabilise the quaternary structure of the common strain: the second charge cluster

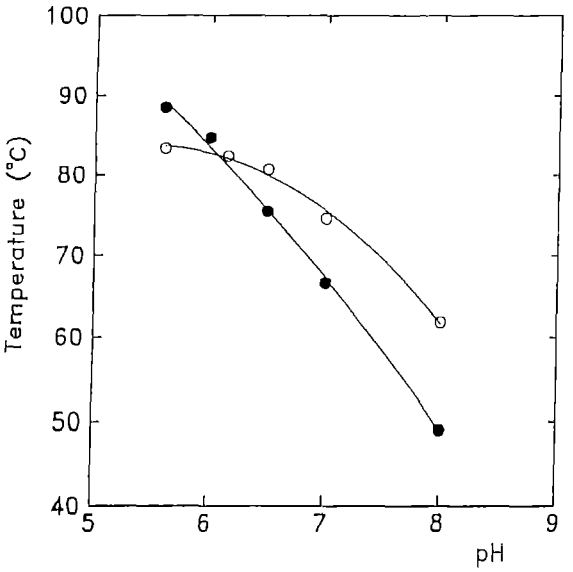

Fig. 7. Variation with pH of the first transition temperature of TMVS (o) and TMV-HR (•)

described by Namba et al. (1989) seems to be most affected. It would be of interest, from this point of view, to compare the detailed three dimensional organisation of the two strains, if that of TMV-HR were available.

Our results provide evidence that thermal denaturation of TMV proceeds in three major steps. Controls show clearly that the first step is correlated with breaking up of the virions into three or four shorter rods. This step is followed by the dissociation of the rodlets, probably accompanied by the release of their RNA. Eventually, TMV protein and RNA become totally denaturated.

The short rods obtained in the first step of the thermal denaturation most likely are broken virions, since no other major reaction products could be observed at this stage, and since it is also known that TMV protein does not reaggregate into helices in such conditions. Breaking up the virions must imply, of course, that the polynucleotide chain is broken too at the corresponding sites. RNA, however, represents only 5% of the total mass of the virions, and its hydrolysis is unlikely to show up in a DSC scan.

A striking and unexpected result is the very high ΔH_1 associated with this first transition, more than 170 kJ/mole of coat protein subunit, resulting in at least a clear shoulder in the DSC scan at neutral pH. Breaking up the virion into three or four segments results in changing the environment of only about one subunit in ten. If one assumes that this process is the sole sources of the enthalpy change, the value of ΔH_1, at the first transition, for these subunits, would then be ca. 1700 kJ/mole, a value far beyond any enthalpy change found for a protein of molecular weight 17 300. Thus, in fact, many more than 10% of the subunits must be concerned, showing that the first transition must imply a conformational change of at least an important part of the particle. Accordingly, the major structural change associated with this first transition is a conformational change of the virion. The disruption of the particles at $T = T_1$ would then be an "artefact" prior to the thermal denaturation: in this changed conformation the particle may break apart at sites where protein-RNA interactions are weakest (the strength of these interactions varies along the RNA chain, with the local nucleotide sequence).

A structural transition has already been described for the filamentous bacterial virus Pf1. It is reversible and its specific transition enthalpy is less than 25% of that described in this work (Hinz et al. 1980): $\Delta H_{cal} = 14.5$ kJ/mole of protein of M.W. = 5 000 would correspond to $\Delta H_{cal} \simeq 50$ kJ/mole for TMV protein, instead of the observed values $\Delta H = 170-300$ kJ/mole. The difference may reflect the differences in the architectures of TMV and filamentous phages.

Our experiments have been performed in vitro, in conditions that do not obviously mimick those occurring during in situ decapsidation. However, as pointed out by Wilson (1984), TMV particles pretreated at 4 °C in 0.1 M *Tris* buffer pH 8, ultracentrifuged and resuspended in a standard buffer, but not untreated virions, may be decapsidated and their RNA translated into specific proteins, once they are introduced into a wheat germ or a reticulocyte in vitro translation system. It is therefore likely that these treated particles too, have undergone an irreversible conformational change and that in this new state, virions may be uncoated by the ribosomes and translation specific factors. This possibility leads us to suggest that, at least the conformational change associated with the first step of the decapsidation mechanism observed in DSC experiments, may possess a biological significance, and induced us to investigate in more detail the structure of these "decapsidation intermediates". Preliminary results (Michels and Witz, to be published) have already shown that after dialysis against a pH 7.2 buffer, the DSC profile of pH 8 treated virions no longer exhibits the two subsidiary maxima on either side of the central peak visible in Figs. 1 and 2.

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