

A comparative differential scanning calorimetric study of tobacco mosaic virus and of its coat protein ts mutant

V.N. Orlov, S.V. Kust, P.V. Kalmykov, V.P. Krivosheev, E.N. Dobrov*, V.A. Drachev

A.N. Belozersky Institute of Physical and Chemical Biology, Moscow State University, Moscow 119899, Russia

Received 13 July 1998

Abstract The differential scanning calorimetry (DSC) 'melting curves' for virions and coat proteins (CP) of wild-type tobacco mosaic virus (strain U1) and for its CP ts mutant ts21-66 were measured. Strain U1 and ts21-66 mutant (two amino acid substitutions in CP: I21 → T and D66 → G) differ in the type of symptoms they induce on some host plants. It was observed that CP subunits of both U1 and ts21-66 at pH 8.0, in the form of small (3–4S) aggregates, possess much lower thermal stability than in the virions. Assembly into the virus particles resulted in a DSC melting temperature increase from 41 to 72°C for U1 and from 38 to 72°C for ts21-66 CP. In the RNA-free helical virus-like protein assemblies U1 and ts21-66 CP subunits had a thermal stability intermediate between those in 3–4S aggregates and in the virions. ts21-66 helical protein displayed a somewhat lower thermal stability than U1.

© 1998 Federation of European Biochemical Societies.

Key words: Ordered aggregation; ts mutation; Differential scanning calorimetry; Thermal stability; Tobacco mosaic virus; Coat protein

1. Introduction

The coat protein (CP) of tobacco mosaic virus (TMV) has for many years served as a model in the study of regulation of ordered protein aggregation. Depending on the conditions (such as concentration, pH, temperature, ionic strength and composition of the medium) this protein can in solution form a large number of different specific ordered aggregates (for reviews see Butler [1] and Bloomer and Butler [2]). In particular, at pH near 8.0 and low ionic strength TMV CP exists in the form of an equilibrium dynamic mixture of monomers, two-layer trimers and pentamers, which is called A-protein, and at pH ≤ 6.0 and moderate ionic strength the protein forms virus-like helical aggregates called repolymerized protein (RP).

Recently interest in TMV CP has again strongly increased as it was found that the ability of different TMV isolates to produce systemic infection or necrotic reaction on *Nicotiana sylvestris* plants carrying gene N' is determined by the CP amino acid sequence [3–6]. The formation of necrosis is considered to be a defense reaction of a host plant, preventing the

spread of an infection. Culver and co-workers analyzed a large number of TMV mutants with defined substitutions in the CP molecule and came to the conclusion that the capacity of wild-type (U1) strain of TMV to escape an induction of necrotic reaction in N' hosts is determined by a higher (as compared to necrosis-inducing strains) stability of the quaternary structure of helical RP [7,8].

We have analyzed a new TMV ts mutant, ts21-66, carrying two amino acid substitutions (I21 → T and D66 → G) in the CP, and found that it induces formation of small necroses on leaves of *N. sylvestris* N' [9]. In the present work we have compared a stability of the wild-type (U1) and ts21-66 TMV virions and their CPs employing the method of differential scanning calorimetry (DSC). As far as we know, up to now only one DSC study of TMV virions and CP thermal denaturation has been published [10].

2. Materials and methods

2.1. Virus growth and purification and preparation of virus coat proteins

The wild-type (U1) TMV and ts21-66 mutant were grown at 24°C in a greenhouse on *Nicotiana tabacum* var. Samsun plants. The viruses were purified by chloroform-butanol treatment, polyethylene glycol precipitation and several cycles of differential centrifugation [11].

U1 and ts21-66 CPs were prepared by the acetic acid method [12] and stored in 5 mM phosphate buffer (PB) pH 8.0. To obtain helical RP, 1/9 volume of 0.5 M PB pH 5.0 was added to a coat protein preparation (3–5 mg/ml) in 5 mM PB pH 8.0 at 0°C, the mixture was kept for 2 h at 20°C and RP was pelleted by 90 min centrifugation at 105 000 × g. RP was resuspended for a night at 4°C in 50 mM PB pH 5.6 and clarified by centrifugation at 15 000 × g. Before DSC experiments the samples were dialyzed against the appropriate buffer.

The concentrations of intact viruses, A-proteins and RPs were determined by UV spectroscopy using the following coefficients: $E_{260}^{0.1\%} = 2.30$ for intact viruses and $E_{280}^{0.1\%} = 1.30$ for A-proteins and RPs of both viruses [11]. For light-scattering suspensions of intact viruses and RPs true values of absorption determined by the extrapolation method [11] were used in calculations.

2.2. Differential scanning calorimetry

Calorimetric measurements were carried out in a differential adiabatic scanning microcalorimeter DASM-4 ('Biopribor', Russian Academy of Science, Pushchino, Russia) with a 0.47 ml capillary platinum cell, interfaced with an IBM-compatible computer. In all experiments the heating rate was 1°C/min. The reversibility of the thermal transition was checked by reheating the samples after cooling from the first scan. The calorimetric traces were corrected for the instrumental baseline by subtracting scans for the reheatings of the samples. A constant pressure of 2 atm was always maintained to prevent possible degassing of the samples on heating.

The TMV CP molecular weight was taken to be 17.5 kDa. In the case of virus preparations the concentration unit included three nucleotides of the virus RNA interacting with each protein subunit.

2.3. Analytical centrifugation

Analytical centrifugation of the virus and protein samples (1–2 mg/ml) was performed in a Beckman E centrifuge equipped with a scanner at $\lambda = 280$ or 290 nm, 20°C and 20 000–60 000 rpm.

*Corresponding author. Fax: (7) (095) 939-3181.
E-mail: dobrov@pcman.genebee.msu.su

Abbreviations: TMV, tobacco mosaic virus; CP, coat protein; RP, repolymerized protein; DSC, differential scanning calorimetry; PB, phosphate buffer

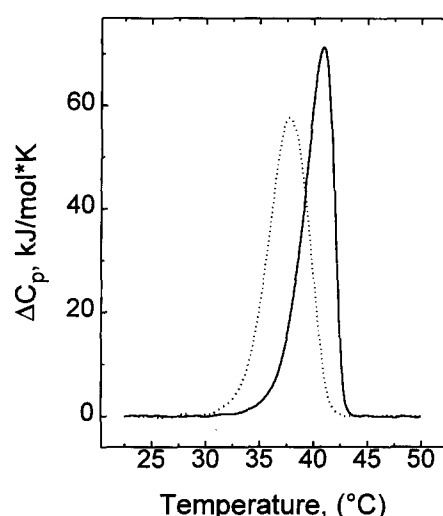


Fig. 1. DSC scans for U1 strain (solid line) and ts21-66 mutant (dotted line) 3-4S A-proteins preparations in 30 mM PB pH 8.0. Protein concentration is 2.0 mg/ml.

3. Results

3.1. DSC melting of coat protein aggregates and TMV virions at mildly alkaline and neutral pH

As was mentioned in Section 1, at pH 8.0 and low ionic strength TMV CP exists in the form of small (3 or 4S) A-protein aggregates (see also Table 1). Previously we reported that ts21-66 CP in the form of the A-protein by several criteria displays a somewhat lower thermostability than wild-type (U1) CP [9] and behaves in this respect like CPs of the other mutants of this type [13,14]. This conclusion was confirmed by the results of DSC experiments. In the case of U1 CP in 30 mM PB pH 8.0 a single rather large and narrow heat absorption peak was observed at 40.9°C, while in the case of ts21-66 CP a similar peak was found at 37.8°C (Fig. 1). For both proteins this transition was irreversible and no additional peaks were observed on further heating up to 100°C. For U1 CP our results agree with those of Mutombo et al. [10].

As can be seen in Fig. 1, TMV A-protein heat transition traces had practically no denaturational increment of heat capacity. This may mean that the degree of TMV CP molecule surface hydrophobicity does not change significantly on thermal denaturation.

After heating to 50°C, about 70% of TMV CP coagulated and were pelleted by low-speed centrifugation (15 min, 14 000 rpm, Eppendorf centrifuge). The rest of the protein had a

sedimentation coefficient of about 3S as judged by analytical centrifugation (Table 1). After heating to 60°C all protein was sedimented at 14 000 rpm. In contrast to the majority of other proteins, TMV CP coagulation in this conditions was not accompanied by additional exothermal or endothermal DSC signals. Recently, quantitative models describing DSC 'melting curves' for the case of irreversible protein denaturation followed (or not followed) by coagulation have been put forward by the groups of Sanchez-Ruiz [15] and Kurganov [16].

A large size of the heat absorption peak at 38 or 41°C and an absence of additional peaks at higher temperatures testify against our previous suggestion [9], that only partial denaturation of U1 and ts21-66 CPs takes place at these low temperatures and prove that complete denaturation of TMV CP has occurred. Thus, at pH 8.0 TMV CP displays surprisingly low thermostability.

On heating of intact U1 TMV suspensions in the same conditions (Fig. 2A) no heat absorption peaks were observed up to 55°C, and only at higher temperatures a broad peak with a maximum at 72°C and a major shoulder at 70°C was observed. For intact ts21-66 the shape and the peak positions of DSC scans in 30 mM PB pH 8.0 did not differ from those of U1 to the limit of accuracy of the method (Fig. 2A). Further heating resulted in protein coagulation. If after heating to 85°C the samples were spun at 14 000 rpm, all the protein was pelleted, but 80–100% of virus RNA remained in the supernatant. This means that TMV virions disruption precedes coagulation and in all probability, TMV CP molecules undergo denaturation just as they are released from the virus particles.

Thus, incorporation into virions strongly (by about 30°C) increases thermostability of TMV CP molecules, and in the case of the virus suspensions the heat absorption peaks observed correspond in fact to the temperatures of disruption of quaternary (intersubunit) protein-protein interactions and RNA-protein interactions.

As follows from Fig. 2A, incorporation into the virions also abolishes a difference in the thermostability between ts21-66

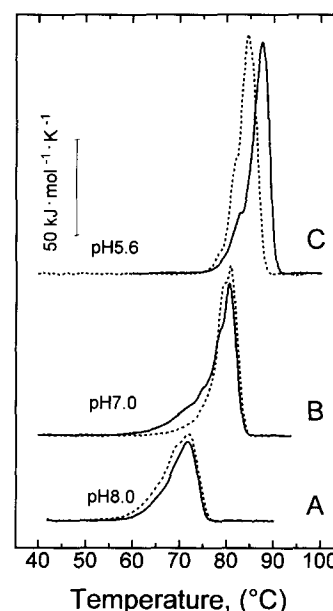


Fig. 2. DSC scans for preparations of intact U1 (solid line) and ts21-66 (dotted line) viruses. Virus concentration is 2 mg/ml. A: 30 mM PB pH 8.0; B: 30 mM PB pH 7.0; C: 50 mM PB pH 5.6.

Table 1
Sedimentation coefficients of U1 and ts21-66 virions and their proteins^a

Sample	S_{20}°
U1 virions in 30 mM PB pH 8.0	175 ± 5
ts21-66 virions in 50 mM PB pH 5.6	170 ± 8
U1 helical RP in 50 mM PB pH 5.6	160 ± 15
ts21-66 helical RP in 50 mM PB pH 5.6	135 ± 15
U1 A-protein in 30 mM PB pH 8.0	3.7 ± 0.4
ts21-66 A-protein in 30 mM PB pH 8.0	2.6 ± 0.3
U1 A-protein in 30 mM PB pH 8.0 after DSC melting up to 50°C and low speed (14 000 rpm) centrifugation (30% of initial material)	2.9 ± 0.4

^aMean values of 3–5 experiments are presented.

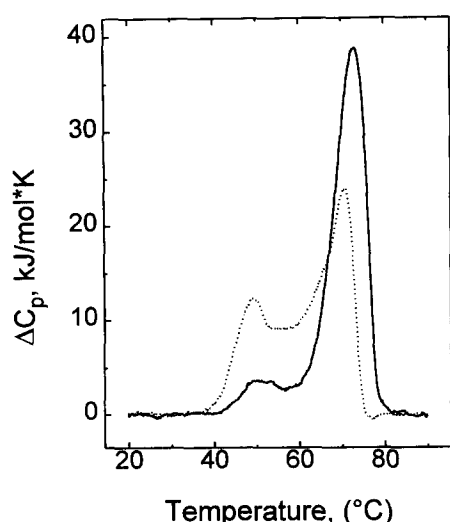


Fig. 3. DSC scans for U1 (solid line) and ts21-66 (dotted line) helical RP preparations in 50 mM PB pH 5.6. Protein concentration is 2 mg/ml.

and U1 CPs. This testifies to a similarity of protein-protein and RNA-protein interactions in particles of wild-type TMV and ts21-66 mutant, and suggests that assembly into virions forces ts21-66 subunits into a conformation rather close to that of U1 CP.

We also measured DSC melting curves for the intact virus suspensions at pH 7.0 (this pH is not very suitable for measurements of TMV CP melting curves due to the presence of a mixture of different types of aggregates). In 30 mM PB pH 7.0 U1 and ts21-66 virions preparations also had similar DSC melting curves with a major peak located at about 80.6°C (Fig. 2B). The heterogeneity of the peaks, although evident, was lower than at pH 8.0.

3.2. DSC melting of coat protein aggregates and TMV virions at mildly acidic pH

In 50 mM PB pH 5.6 U1 and ts21-66 TMV CP exists in the form of helical virus-like RPs [1,2,9]. The DSC melting curve of U1 RP had one major peak at 73.2°C and a much smaller one between 50 and 55°C (Fig. 3). The size of the small peak varied in different experiments and in all probability this peak corresponded to an admixture of either aberrant aggregates or of so-called stacked disks, while the major peak should correspond to normal RP. Similar DSC curves for U1 RP were obtained by Mutombo et al. [10].

DSC curves for ts21-66 RP differed from those of U1 RP (Fig. 3). The main peak position was shifted to a somewhat lower temperature (70.9°C) and the area under the lower-tem-

perature peak usually was somewhat larger. This means that in the case of ts21-66 mutant amino acid substitutions I21→T and D66→G (resulting in necrosis induction on *N. sylvestris* plants) induce some decrease in the thermostability of the helical aggregates. These results support the hypothesis of Culver and colleges [7,8] that strain U1's ability to escape induction of the plant defense system (necrotic reaction) is determined by an increased RP stability.

At pH 5.6 some increase in thermostability as compared to pH 7.0 was also observed for the intact virus preparations (Fig. 2C). At this pH, in contrast to pH 7.0 or 8.0, U1 and ts21-66 virions displayed different stability, U1 having a T_m (87.6°C) about 3°C higher than that of ts21-66 (84.6°C). The difference between T_m values of intact viruses at this pH was similar to that between their respective RPs.

The major peak of U1 RP melting had a maximum at 73.2°C (Fig. 3) and that of intact U1 at 87.6°C. The only difference between RP and the virions is the presence in the latter of an RNA molecule. Thus, intravirus RNA makes a significant contribution to TMV virion stability, increasing the disruption temperature of both U1 and ts21-66 particles by 14°C.

On analytical centrifugation U1 and ts21-66 virion preparations used for DSC melting gave one rather homogeneous peak with a sedimentation coefficient of about 175S characteristic of full-length TMV particles (Table 1). On centrifugation unheated U1 and ts21-66 RPs samples in 50 mM PB pH 5.6 also gave one band with a sedimentation coefficient of 120–165S but this band was significantly less homogeneous than in the case of the virions.

All the above-described DSC curves were obtained at a heating rate of 1°C/min. A decrease in the heating rate resulted in a decrease in the melting temperatures. Thus, the curves obtained cannot be considered equilibrium ones, and, strictly speaking, should be subjected to kinetic analysis (Orlov, to be published). Nevertheless, we estimated the areas under the peaks (ΔH_{cal}) and present them in Table 2. The values obtained are reproducible and agree rather closely with the analogous values reported by Mutombo et al. [10]. (taking into account that these authors used a very large absorption coefficient of 3.28 optical units for intact viruses and due to this underestimated the virus concentration and overestimated the virus enthalpy values by about 40%).

The different ΔH values for CPs and for intact viruses in Table 2 look in reasonable agreement with each other and with those for other small proteins. TMV contains only 5% of RNA and RNA contribution to the virus DSC melting curves is more or less similar to this value (data not shown). The $\Delta(\Delta H)$ between small protein aggregates and RP (about 150 kJ/mol) calculated from Table 2, which probably should

Table 2

'Melting enthalpy' changes on thermal denaturation of intact U1 and ts21-66 viruses and their protein aggregates^a

	pH 5.6		pH 7.0		pH 8.0	
	T_m (°C)	ΔH_{cal} (kJ/mol)	T_m (°C)	ΔH_{cal} (kJ/mol)	T_m (°C)	ΔH_{cal} (kJ/mol)
Intact U1	87.6	613	80.5	537	71.8	309
Intact Ts21-66	84.6	614	80.8	498	71.9	404
U1 RP	73.2	415	—	—	—	—
Ts21-66 RP	70.9	397	—	—	—	—
U1 A-protein	—	—	—	—	40.9	251
Ts21-66 A-protein	—	—	—	—	37.8	269

^a30 mM PB pH 7.0 and pH 8.0; 50 mM PB pH 5.6. Concentration of all samples 2 mg/ml. All ΔH_{cal} values have a standard deviation of $\pm 4\%$.

correspond to the enthalpy of TMV CP helical polymerization, may be compared with the value of about 70 kJ/mol determined for reversible TMV CP polymerization-depolymerization from 4S protein to the two-turn helix at 15°C and pH 6.52 in the classical Sturtevant-Lauffer work [27].

4. Discussion

An increase in a protein's thermal stability on incorporation into a supramolecular assembly is a quite common phenomenon, but in the case of TMV CP this effect is exceptionally strong. As follows from a comparison of Figs. 1 and 2, TMV virions (one of the most stable biological objects) are built from quite thermolabile blocks and the high thermal stability of the virus particles is determined almost entirely by intermolecular quaternary axial and lateral protein-protein interactions and by RNA protein interactions. Protein molecules not packed into long helical aggregates possess rather low stability.

This low stability of small (3–4S) TMV CP aggregates is probably determined by the large hydrophobic patches located on subunit surfaces [17,18]. These patches are used (and masked) in the course of formation of large aggregates (RPs, virions). The presence of surface hydrophobic patches should strongly decrease overall thermodynamic stability of monomeric and oligomeric CP subunits. In our opinion, this low stability may play a major role in the TMV life cycle. It is well known that the presence of free CP molecules strongly inhibits TMV virion disassembly [19–21]. Thus, denaturation of CP molecules released from a virion may greatly facilitate the disassembly process by lowering the concentration of assembly-active subunits. If the stability of CP molecules is low, even a small 'push' by a disassembly-triggering factor (or by some accompanying component) may be sufficient to unfold free CP subunits in a TMV-infected cell.

According to current concepts, uncoating of a larger part of TMV virions in infected cells occurs through the action of a so-called cotranslational disassembly mechanism, when cellular ribosomes in the course of translation of the TMV 5'-proximal gene consequently 'strip off' the CP subunits from the virus RNA molecule [22,23]. As far as we know, the thermodynamic aspects of this scheme have never been considered. For the cotranslational disassembly to occur, the energy provided by the translation process should be sufficient to break RNA-protein interactions of a trinucleotide with a CP subunit and a whole system of lateral and axial protein-protein interactions (a subunit per codon). Perhaps, the capacity of the protein-synthesizing machinery to unfold the released CP molecules may help to reduce the energetic cost of the disassembly process to an acceptable level.

The low stability of the CP subunits may also explain the puzzling fact of an absence of the assembly activity of TMV CP molecules produced in *Escherichia coli* cells [24,25]. Probably, newly synthesized CP molecules can be correctly folded only with the help of some specific chaperones not present in *E. coli* cells.

It is well known that the most important change in the TMV CP ordered aggregation mode (transition from disk-type to helix-type aggregation) occurs at a pH near 7.0 [1,2]. U1 (and ts21-66) CP does not contain histidine, and 35 years ago Caspar made the brilliant suggestion [26] that this change is determined by protonation of two carboxyl-carboxylate

pairs. These pairs in TMV CP were unsuccessfully searched for for 25 years, until Namba et al. [18] on the basis of their X-ray diffraction data proposed that these carboxyl-carboxylate pairs really should be the intersubunit ones (one axial and one lateral).

Now it may be suggested that at least a part of the additional TMV virion (and RP) stability is determined by these intersubunit pairs and reflected in the pH dependence of this stability. On decrease in pH from 8.0 to 5.6 U1 virion T_m increases from 72 to 87.6°C due to gradual proton binding by these two pairs, one of which (axial pair E50-D77) is located rather close to the region mutated in ts21-66 [9]. At pH 7–8 and permissive temperatures virion formation forces ts21-66 subunits into a conformation similar to that of U1 subunits, but on acidification to pH 5.6 further tightening of mutant virion (and RP) structure probably cannot be fully accomplished due to the absence of a bulky Ile residue in position 21 and/or to the presence of a Gly residue in position 66.

Comparison of wild-type (U1) and ts21-66 mutant RP stability was one of the main aims of the present work. This question is not trivial, as for ts21-66 (and some other TMV coat protein ts mutants) it was shown that when mutant CP subunits are assembled into virions (in vivo or in vitro) the particles obtained do not differ from U1 virions in stability [9,14]. This means that in the course of the virion assembly the mutant CP subunits assume a conformation more or less close to the native one.

The results obtained in the present work (Fig. 3) demonstrate that ts21-66 RP does possess a somewhat lower thermal stability than U1 RP, and these data seem to support the Culver hypothesis that it is the decreased RP stability which causes the induction of the necrotic reaction in plants of *N. sylvestris* [7,8]. But the difference in stability is not large and possibly more careful selection of optimal conditions of mutant RP formation will produce ts21-66 RP as stable as that of U1.

One more unusual feature of TMV virion heat absorption peaks is their heterogeneity. Presumably, the DSC melting makes it possible to observe some subtle thermodynamic differences between the particles in the virus preparation, which cannot be revealed by other methods. These differences may be caused by such factors as an uneven distribution of divalent cations on different virions, 'pentenary' interparticle interactions or intraparticle heterogeneity, determined by heterogeneity of RNA-protein interactions along the length of the virion. It should be also said that in our experiments the heterogeneity of the virus DSC peaks was less pronounced than in the work of Mutombo et al. [10].

Acknowledgements: This work was supported in part by Grant 96-04-50940 of the Russian Fund for Fundamental Research.

References

- [1] Butler, P.J.G. (1984) *J. Gen. Virol.* 65, 253–279.
- [2] Bloomer, A.C. and Butler, P.J.G. (1986) in: *Plant Viruses* (Van Regenmortel, M.H.V. and Fraenkel-Konrat, H., Eds.), Vol. 2, pp. 19–57, Plenum Press, New York.
- [3] Saito, T., Meshi, T., Takamatsu, N. and Okada, Y. (1987) *Proc. Natl. Acad. Sci. USA* 84, 6074–6077.
- [4] Knorr, D.A. and Dawson, W.O. (1988) *Proc. Natl. Acad. Sci. USA* 85, 170–174.
- [5] Mundry, K.W., Schaible, W., Elwart-Tschur, M., Nitschko, H.

- and Hapke, C. (1990) in: Recognition and Response in Plant-Virus Interactions (Fraser, R.S.S., Ed.), pp. 345–359, Springer-Verlag, Berlin.
- [6] Culver, J.N. and Dawson, W.O. (1991) *Mol. Plant Microbe Interact.* 4, 458–463.
- [7] Culver, J.N., Stubbs, J. and Dawson, W.O. (1994) *J. Mol. Biol.* 242, 130–138.
- [8] Taraporewala, Z.F. and Culver, J.N. (1996) *Plant Cell* 8, 169–178.
- [9] Dobrov, E.N., Abu-Eid, M.M., Solovyev, A.G., Kust, S.V. and Novikov, V.K. (1997) *J. Protein Chem.* 16, 27–36.
- [10] Mutombo, K., Michels, B., Ott, H., Cerf, R. and Witz, J. (1992) *Eur. J. Biophys.* 21, 77–83.
- [11] Dobrov, E.N., Kust, S.V., Yakovleva, O.A. and Tikchonenko, T.I. (1977) *Biochim. Biophys. Acta* 475, 623–637.
- [12] Fraenkel-Conrat, H. (1957) *Virology* 1, 1–4.
- [13] Jockusch, H. (1966) *Z. Vererbungsl.* 98, 344–362.
- [14] Jockusch, H., Koberstein, R. and Jaenicke, R. (1969) *Z. Naturforsch.* 24b, 613–617.
- [15] Conejero-Lara, F., Sanchez-Ruiz, J.M., Mateo, P.L., Burgos, F.J., Vendrell, J. and Aviles, F.X. (1991) *Eur. J. Biochem.* 200, 663–670.
- [16] Kurganov, B.I., Lyubarev, A.E., Sanchez-Ruiz, J.M. and Shnyrov, V.L. (1997) *Biophys. Chem.* 69, 125–135.
- [17] Bloomer, A.C., Champness, J.N., Bricogne, G., Staden, R. and Klug, A. (1978) *Nature* 276, 362–368.
- [18] Namba, K., Pattanayek, R. and Stubbs, G. (1989) *J. Mol. Biol.* 208, 307–325.
- [19] Pelcher, L.E., Walmsley, S.L. and MacKenzie, S.L. (1980) *Virology* 105, 287–290.
- [20] Wilson, T.M.A. and Watkins, P.A.C. (1986) *Virology* 149, 132–135.
- [21] Wu, X., Beachy, R.N., Wilson, T.M.A. and Shaw, J.G. (1990) *Virology* 179, 893–895.
- [22] Wilson, T.M.A. (1984) *Virology* 137, 255–265.
- [23] Wilson, T.M.A. and Shaw, J.G. (1985) *Trends Biochem. Sci.* 10, 57–60.
- [24] Shire, S.J., McKay, P., Leung, D.W., Cachianes, G.J., Jackson, E., Wood, W.I., Raghvendra, K., Khairallah, L. and Schuster, T. (1990) *Biochemistry* 29, 5119–5126.
- [25] Hwang, D.-J., Roberts, I.M. and Wilson, T.M.A. (1994) *Proc. Natl. Acad. Sci. USA* 91, 9067–9071.
- [26] Caspar, D.L.D. (1963) *Adv. Protein Chem.* 18, 37–118.
- [27] Sturtevant, J.M., Velicelebi, G., Jaenicke, R. and Lauffer, M.A. (1981) *Biochemistry* 20, 3792–3800.