Accelerated Publications

Structure and Function of Disk Aggregates of the Coat Protein of Tobacco Mosaic Virus†

K. Raghavendra, Judith A. Kelly, Lamia Khairallah, and Todd M. Schuster*

Department of Molecular and Cell Biology, U-125, and Institute of Materials Science, University of Connecticut, Storrs, Connecticut 06268

Received June 16, 1988; Revised Manuscript Received August 2, 1988

ABSTRACT: Experiments have been carried out on the coat protein of tobacco mosaic virus (TMVP) to test for the occurrence of the previously postulated RNA-induced direct switching, during in vitro assembly of tobacco mosaic virus (TMV), of the subunit packing from the cylindrical bilayer disk to the virus helical arrangement. No evidence was found for such RNA-induced switching and no evidence for the direct participation of the bilayer disk in either the nucleation or elongation phases of the in vitro virus assembly. Instead, virus assembly proceeds by an initiation step involving the binding of the RNA to the previously characterized two-plus turn helical aggregate that is formed from small oligomers of subunits. However, a bilayer disk, which has been characterized in high ionic strength crystals, has been observed in low ionic strength virus assembly solutions *only* as a transient species upon depolymerization of dimers of bilayer disks formed in solution at high ionic strength, and not as an equilibrium species of TMVP.

The general problem of the relationships of the secondary and tertiary structure of a macromolecule in a crystal to that in solution has been studied by a variety of methods in the case of proteins. Many enzymes retain activity in the crystalline state (Hajdu et al., 1988; Makinen & Fink, 1977), and hemoglobin and myoglobin can bind oxygen when crystallized. Considerably less is known about the influence of the salt solutions used for crystallization on the quaternary structure of proteins that form extended assemblies. One of the first such protein assembly systems to be crystallized and solved at high resolution was the 34-subunit aggregate of tobacco mosaic virus coat protein (TMVP),[1] which crystallizes as a dimer of bilayer disks[2] having 17 subunits per layer (Bloomer et al., 1978). There has been considerable interest in this structure since it was proposed that the bilayer disk plays a key role in the self-assembly process of TMV through an initial RNA recognition reaction that triggers assembly [reviewed in Butler and Durham (1977), Hirth and Richards (1981), Stubbs (1984), Bloomer and Butler (1986), and Okada (1986)]. In this model, the bilayer disk initiates virus self-assembly by binding to a specific region on the RNA, which then induces a structural switching from the cylindrical disk to helical subunit packing array. In addition, a major role for the bilayer disk in the growth phase of TMV assembly has also been proposed [reviewed by Bloomer and Butler (1986)], but this point has been controversial for some time [reviewed by Schuster et al. (1980), Hirth and Richards (1981), and Okada (1986)]. Central to this controversy has been the assumption that the bilayer disk structure, which is seen in crystals as a dimer, also exists in solution and can undergo a direct structural change resulting in helical packing of subunits.

We have undertaken a series of studies to characterize the solution properties of bilayer disks (Raghavendra et al., 1985, 1986, 1988; Correia et al., 1985) and report here on the postulated switch in subunit packing arrangement from the cylindrical to helical structure. Since extended subunit assemblies such as tubulin, other cytoskeletal proteins, actin, tropomyosin, flagellin, and hemoglobin S can each exist in different subunit packing arrangements, the subject of conformational switching at the quaternary structure level is of general interest for an understanding of the functional properties of these protein assemblies.

The self-assembly of TMV from its isolated RNA and capsid protein requires that some of the protein be in the form of a 19–20S aggregate for the nucleation of the process at 0.1 M ionic strength (Butler & Klug, 1971). The sedimentation coefficient of a structure of the size of the bilayer disk was estimated to be 17–19 S (Caspar, 1963), and when the bilayer disk was identified in the crystal (Finch et al., 1966), it was presumed to correspond to the 20S nucleating species (Butler & Klug, 1971). The assignment of the bilayer disk structure, which has been demonstrated only in protein crystals grown in high ionic strength buffers, as being the 20S nucleating aggregate seen in solutions of low ionic strength has been questioned on the basis of physical–chemical studies in solution (Schuster et al., 1980; Raghavendra et al., 1985, 1986, 1988; Correia et al., 1985) and fiber diffraction data (Namba & Stubbs, 1986). On the basis of hydrodynamic, spectroscopic, and structural data, we concluded that the 20S nucleating species is not a bilayer disk but rather a short helical rod slightly longer than two helical turns with $16^1/_3$ subunits per

† This research has been supported by a grant from the National Institutes of Health to T.M.S. (AI 11573).

[1] Abbreviations: TMV, tobacco mosaic virus; TMVP, tobacco mosaic virus coat protein; RNA, ribonucleic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; *I*, ionic strength expressed in molar concentration units.

[2] We use the term TMVP "bilayer disks" to refer to the two-layer, 17 subunit per layer, cylindrical structure identified and solved in crystals of TMVP grown from high ionic strength buffers (Bloomer et al., 1978). We, and others, have previously referred to this structure simply as a "disk" or "double disk" (Lauffer, 1975), but this nomenclature becomes ambiguous when discussing two-layer structures and their aggregates.

0006-2960/88/0427-7583$01.50/0 © 1988 American Chemical Society

turn. There is a significant structural difference between the helical and cylindrical packing arrangements of subunits. It has been shown by Champness et al. (1976) that axial intersubunit contacts in the bilayer disk are totally different from those in the helix. These conclusions then lead to the question of the role in TMV assembly, if any, of the bilayer disk structure that is observed in the crystals. In order to pursue this question, it is necessary to obtain the isolated bilayer disk in solution. Previous attempts have always resulted in multimers of bilayer disks ranging in size from trimers to dodecamers, i.e., 6–24-layer stacks of subunits having sedimentation coefficients of 36–53 S, depending upon the method of dialysis into high ionic strength crystallizing buffers. None of these structures act as nuclei in initiating virus reconstitution with RNA (Raghavendra et al., 1985, 1986).

We report here a protocol, adapting the solution conditions originally used to crystallize TMVP, developed for preparing dimers of bilayer disks having a sedimentation coefficient of 28 S and show that this is the species that forms crystals with the same space group and unit cell dimensions as those obtained previously (Leberman et al., 1974). We also show by electron microscopy that the 28S aggregates are coaxial dimers of bilayer disks and demonstrate that the 28S dimer dissociates to 4S subunits and a transient 17S species, which is probably an isolated bilayer disk but which does not nucleate or otherwise participate in virus assembly. From these results we conclude that there is no direct switching in quaternary subunit packing from the cylindrical bilayer disk or its dimer to the helical array and that the formation of the helical assembly nucleus from disks requires depolymerization of the bilayer disk. Although we have studied the composition of TMVP solutions at and near conventionally used virus assembly conditions, we have failed to find evidence for the existence of a stable isolated bilayer disk structure in solution.

Materials and Methods

Virus, Protein, and RNA Preparation, Analytical Ultracentrifugation, and Electron Microscopy. These methods have all been described in detail previously (Raghavendra et al., 1985, 1986; Shire et al., 1979a; Steckert, 1982).

Solution Conditions. High-salt (protein crystallization) buffers used were 0.2 and 0.3 M $(NH_4)_2SO_4$ in I = 0.1 Tris-HCl, to give total ionic strengths of 0.7 and 1.0, respectively, at pH 8.0 and 20 °C. Low-salt (virus assembly) buffer was I = 0.1 potassium phosphate at pH 7.0 and 15, 17, or 20 °C, as described below. The pH values of buffer solutions were measured as described previously (Raghavendra et al., 1985).

Virus Reconstitution. TMV RNA stored in virus assembly buffer at about −70 °C was thawed to cold room temperature (2–4 °C) for 2 h. It was then transferred to an air box maintained at the temperature of the virus assembly experiments and equilibrated for 30 min. TMVP solutions at the reconstitution temperature of 17 or 20 °C were added to RNA, rapidly mixed, and incubated for 10 min before sedimentation velocity measurements were carried out in an analytical ultracentrifuge. The final weight ratio of TMVP to RNA used in the virus assembly experiments was ≥20:1.

Sample Handling and Crystallization. It has been shown by Klug and co-workers that TMV protein solutions of 8.0 mg/mL crystallize as dimers of bilayer disk aggregates from pH 8.0, total ionic strength = 1.0 [0.3 M $(NH_4)_2SO_4$ plus I = 0.1 Tris-HCl] buffer at room temperature (Leberman et al., 1974; Finch et al., 1974). As we are interested in relating the quaternary structure of the aggregates in solution to that in the single crystal, we have developed a protocol and chosen two representative $(NH_4)_2SO_4$ concentrations of 0.2 and 0.3 M in order to observe intermediates, if any, and the final states of aggregation in each solution before crystallization. All the samples were prepared by dialyzing a pH 8.0, 4S aggregate (*monomer* ⇄ *trimer*) at a protein concentration of 3.5 or 8.0 mg/mL, pH 8.0 from I = 0.1 Tris-HCl and 0 °C to I = 0.7 or 1.0 (total ionic strength) buffers at pH 8.0 and 20 °C for 6 h. After dialysis, the solutions were subjected to a cleaning spin (10000 rpm for 15 min at 20 °C) and were cooled to 15 °C [which produces better crystals according to Leberman et al. (1974)] for 12–14 h before transferring to 20 °C. Electron microscopy was carried out on 3.5 mg/mL samples whereas ultracentrifuge and dissociation experiments were performed on both 3.5 and 8.0 mg/mL solutions. Solutions of 8.0 mg/mL were transferred to room temperature, which varied between 15 and 27 °C during the 2–3-week period of crystallization experiments. In one case, protein crystals appeared in 2–3 days in solutions of about 50 mg/mL TMVP maintained at 20 °C. We were also able to grow protein crystals by the hanging drop method from I = 0.4 solutions after adding 2% (w/v) poly(ethylene glycol) (M_r 8000) to the protein solution and setting up 20-μL drops against 1.0 mL of I = 0.4 buffers containing 10–15% poly(ethylene glycol). Crystals were photographed with a standard phase contrast Nikon optical microscope.

X-ray Diffraction. To determine the space group and unit cell dimensions of the crystals, a 0.51 × 0.33 × 0.30 mm crystal was mounted in a glass capillary for X-ray diffraction measurements. Precession photographs using nickel-filtered copper radiation from a Rigaku rotating anode (70 mA, 36 kV) with a crystal to film distance of 10 cm, a precession angle of 7.5°, and an exposure time of 19 h were taken. Data were also collected with a Nicolet multiwire proportional chamber detector and a Rigaku rotating anode (240 mA, 40 kV). With a crystal to detector distance of 30 cm, measurable diffraction data were observed at a resolution of 3 Å, indicating high-quality, well-ordered crystals.

Results and Discussion

Relationship between 28S TMVP in Solution and Single Crystals of Dimers of Bilayer Disks. At pH 8.0, I = 0.1, and 5 °C, solutions of TMVP subunits (M_r 17530) exhibit a single schlieren boundary, in velocity sedimentation analysis, having a sedimentation coefficient of about 4 S, depending upon protein concentration. This boundary represents a reversible self-associating reaction mixture with a weight-average molecular weight corresponding to that of a trimer (Ackers & Katzel, 1979). Figure 1 shows the result of increasing the ionic strength, temperature, and protein concentration of such solutions in order to achieve crystallization conditions. At ionic strengths of 0.7 and 1.0, at 20 °C, and at a protein concentration up to 10.0 mg/mL, a single symmetrical 28S sedimenting boundary is formed with no evidence of intermediate species between 4S and 28S and no higher association states. Solutions of 8.0 mg/mL form well-defined orthorhombic crystals ($P22_12_1$) within 2–3 weeks (Leberman et al., 1974; Raghavendra et al., 1988) or, as we have found, within 2–3 days at 50 mg/mL. The crystals have unit cell dimensions of a = 228.1 Å, b = 223.4 Å, and c = 174.5 Å, which are within 0.2% of those reported by Klug and co-workers (Leberman et al., 1974). Further, they have shown that the unit cell consists of dimers of bilayer disks that are related by a dyad axis located between the disks (Finch et al., 1974). Such crystals were used to obtain the structure of the TMVP subunit and the bilayer disk (Bloomer et al., 1978).

Electron microscopic investigations of solutions containing 28S aggregates reveal large numbers of coaxial four-layer

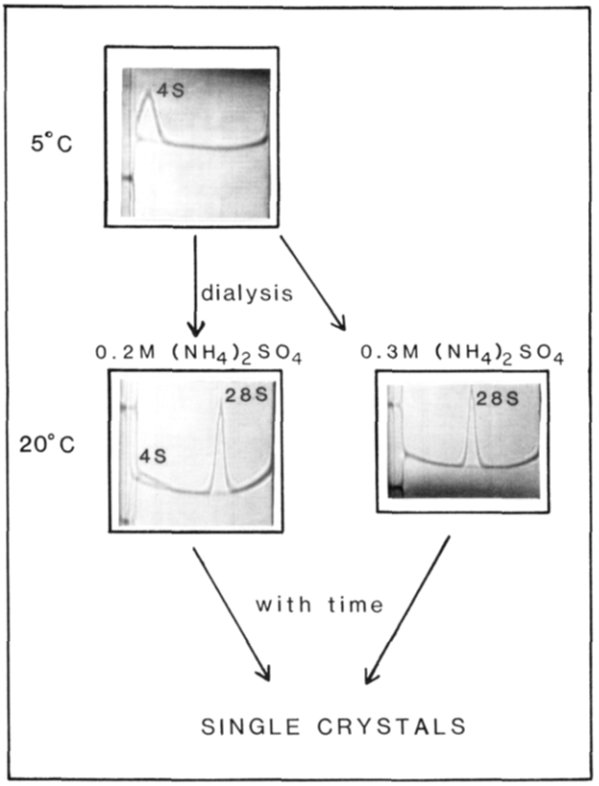

FIGURE 1: Formation and crystallization of 28S dimers of bilayer disks from 4S subunits of TMVP by increase in temperature, ionic strength, and protein concentration. The 5 °C buffer was pH 8.0, I = 0.1 Tris-HCl, and the 20 °C buffers contained in addition 0.2 and 0.3 M $(NH_4)_2SO_4$ to give total ionic strength values of 0.7 and 1.0, respectively, with 85% and 100% 28S TMVP. The protein concentration was 3.5 mg/mL. Sedimentation proceeds from left to right in 12-mm path-length cells photographed at 40° and 50° schlieren bar angles. Note that both 28S boundaries are symmetrical. No faster sedimenting boundaries were observed. Increase of the protein concentration to 8.0 mg/mL results in 28S crystallization in 2–3 weeks.

structures with a common central hole appearing in various states of loose packing and stacking (Figure 2). The four-layer structures correspond to coaxial dimers of the bilayer disk characterized in crystals by Finch et al. (1974), who suggested that the crystal packing produced antiparallel dimers of the bilayer disk in the unit cell. These layered structures stain very differently from helical arrays of TMVP subunits as observed in native virus or in RNA-free TMVP rods formed at pH values lower than 7.0. Helical packing arrays of TMVP do not exhibit dark banding patterns in electron micrographs because of presumed tight subunit packing that prevents stain penetration (Williams & Fisher, 1970; Durham et al., 1971; Durham & Finch, 1972; Finch et al., 1974; Nonomura & Ohno, 1974; Raghavendra et al., 1986). However, under the crystallization conditions used here, we have not observed by sedimentation analysis extended aggregates of the kind seen in the electron micrographs of the same solutions. This implies that the large loose aggregates (Figure 2) are the result of surface or drying effects. On the basis of our previous molecular weight and sedimentation velocity measurements of the two- and three-turn helical aggregates of TMVP containing $16^1/_3$ subunits per turn (Shire et al., 1979b; Schuster et al., 1980; Correia et al., 1985) and recent sedimentation velocity and electron microscopy studies on disk aggregates (Raghavendra et al., 1985, 1986, 1988; K. Raghavendra and T. M. Schuster, unpublished results), we estimate that a dimer of bilayer disk aggregates (i.e., four layers of subunits) containing 17 subunits per layer should have a sedimentation coefficient in the range of 27–30 S.

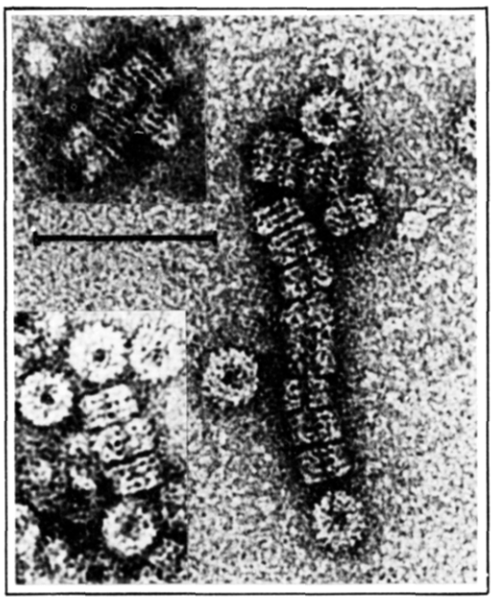

FIGURE 2: Electron micrograph of 28S dimers of bilayer disk aggregates (i.e., four layers of subunits) of TMVP present in I = 0.7 solutions as prepared in Figure 1. The bar represents about 600 Å. The four-layer structures are coaxial, have a common central hole, and exhibit clear striations between layers. Also, in-register extended stacking seen here is not observed as >28S aggregates in the sedimentation patterns of the same solutions used in Figure 1.

Our results on the 28S boundary indicate that this is the same structure observed in the crystal, and since we observe no other species in crystallizing buffer solutions, it is likely that the 28S four-layer structure is the actual crystallizing species as well. A similar conclusion was drawn earlier about the structure of TMVP aggregates in crystallizing buffers but was based on sedimentation velocity and electron microscopy results on TMVP in noncrystallizing buffers (Durham, 1972; Durham & Finch, 1972). It was previously shown that, upon changing TMVP solution conditions to go from 4S to 28S, a transient intermediate 8S species is formed on the pathway to the final 28S state of aggregation (Vogel, 1982) and therefore is probably not involved in crystal formation. Further confirmation of this conclusion is provided by our sedimentation analysis of I = 0.4 solutions in which predominantly the 8S aggregate forms and persists for long times without leading to crystallization (K. Raghavendra and T. M. Schuster, unpublished results). The conclusion that the structure seen in TMVP crystals is the same as that seen in solution, under otherwise identical conditions, is consistent with the notion that the packing forces in the crystal are not sufficiently strong to alter the quaternary subunit arrangement and state of aggregation of TMVP dimers of bilayer disks at high ionic strength.

Properties of 28S and 17S Aggregates under Virus Assembly Conditions. The 28S dimers of disks do not participate in the TMV assembly reactions under I = 0.7 crystallization conditions (data not shown). We were interested in studying the properties of these aggregates when they were taken to the commonly used low-salt virus assembly conditions. Figure 3A summarizes the results of dialyzing high ionic strength (I = 0.7) solutions of the 28S dimer of the bilayer disk into low ionic strength (I = 0.1) virus reconstitution buffers. These experiments were performed at 17 °C to slow the rate of formation of 20S from 4S subunits. Within a few minutes after the change of solution conditions, we observe that the 28S structure depolymerizes to 4S subunits and a transient 17S species. Figure 3B shows the time course of 28S depo-

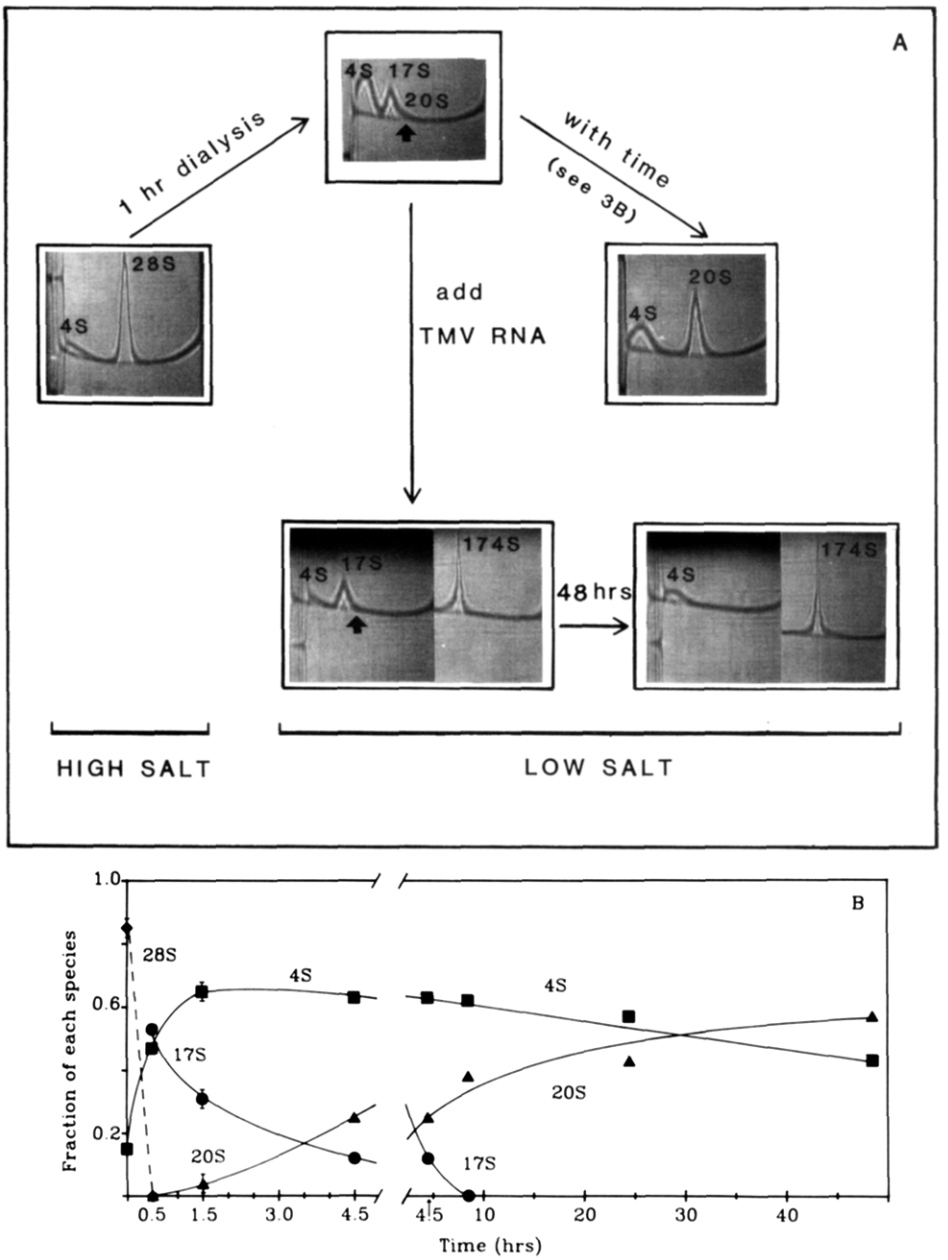

FIGURE 3: (A) Sedimentation patterns of TMVP aggregates in the absence of RNA (upper three photos) showing the dissociation of 28S dimers of bilayer disks brought from 20 °C and high ionic strength ("high-salt") protein crystallization buffer (i.e., $I = 0.7$, pH 8.0, and as in Figure 1) to 17 °C and low ionic strength ("low-salt") virus assembly conditions (pH 7.0, $I = 0.1$ potassium orthophosphate) by rapid dialysis. The protein concentration was 3.5 mg/mL, and the direction of sedimentation of the boundaries is from left to right. The 28S dimers dissociate to 4S and transient 17S aggregates. Subsequent formation of 20S aggregates is from 4S [see (B)]. The 174S schlieren boundary present after the addition of RNA (lower two photos) represents fully constituted TMV. The slight thickening of the leading edge of the 17S boundary (arrow) corresponds to less than 0.2 mg/mL 20S TMVP. However, for the concentration of RNA used in this experiment (0.16 mg/mL), a concentration of 20S of only 0.2 mg/mL would be adequate to initiate assembly by specific binding to all the RNA even if as many as four 20S molecules per RNA strand were required for stable nucleation. Note that the concentration of 17S remains unchanged at 1.0 mg/mL after virus reconstitution (lower left schlieren pattern), but without the leading edge of 20S (arrow). All of the coat protein for reconstitution was provided by the 4S boundary for the growth phase following nucleation by the trace 20S boundary. Control experiments with 4S protein alone do not yield reconstituted virus. Additional control experiments at times longer than 1 h after dialysis revealed larger amounts of 20S than shown here, which was how the boundary was measured and identified. The composition of the unreacted TMVP immediately after virus reconstitution (lower left schlieren photo), 95% 17S and 5% 4S, slowly converts to 100% 4S (lower right schlieren photo). This final concentration of unreacted TMVP is below the critical concentration for 20S formation under these conditions (Durham, 1972). (B) Time course of depolymerization of 28S dimers to 4S and transient 17S aggregates and formation of 20S aggregates after changing from high to low ionic strength as in the upper part of (A). Times for all points except those at 0.5 h correspond to the dialysis time of 1 h plus time of experiment. Details of the experimental conditions are as in (A). The data points at 0.5 h were obtained by rapid dilution rather than dialysis. TMVP at 35 mg/mL, in the high-salt buffer, as in (A), was diluted at 17 °C into the low-salt buffer at a 1:10 ratio to give a final concentration of 3.5 mg/mL, followed by immediate analytical ultracentrifuge analysis. Note that at the earliest time measurement, 0.5 h, all 28S bilayer disk dimers have depolymerized to form about 50% 17S and 50% 4S. The data points at 4.5 h are duplicated in the two parts of the time course graph.

lymerization to 4S and 17S and the subsequent events. As 4S concentration increases, it begins to slowly form a 20S boundary as normally occurs when 4S TMVP at pH 7.0, 5 °C, and $I = 0.1$ is warmed to 17 or 20 °C (Durham, 1972; Shire et al., 1981). However, at 20 °C the rate of 20S formation from 4S TMVP exceeds the rate of 17S depolymeri-

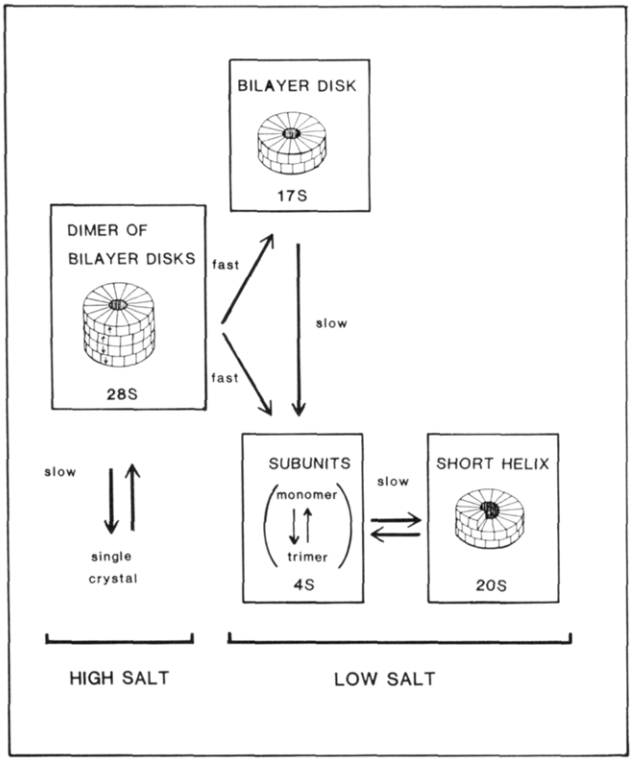

FIGURE 4: Relationship between the structure of TMVP in crystals grown from high ionic strength buffers and the structures observed in low ionic strength buffers used for virus reconstitution studies. Radial subunit alignment is schematic in these diagrams. See text for discussion of possible subunit polarity of the 17S bilayer disk. Equilibrium states are connected by double arrows. Kinetic pathways between the transient, monomeric bilayer disk at low ionic strength and other aggregation states are indicated by single arrows. The overall time course of the changes between these structures is shown in Figure 3B. Only the 20S short helix has been found to nucleate virus reconstitution by binding to homologous RNA.

zation, making it more difficult to observe the separate reactions. But at 17 °C, the rate of formation of 20S from 4S TMVP is slow enough to interpret the sequence of events. As shown in Figure 3B, the 28S dimer dissociates completely within the first 30 min after reduction of ionic strength to form about equal weight fractions of 4S and 17S protein and no 20S helical polymers. Following this rapid 28S dissociation, the newly formed 17S TMVP continues to depolymerize but more slowly and with no trace of intermediates. The 4S protein increases in concentration, initially at about the same rate as 17S depolymerizes and before any substantial formation of 20S helical polymers. After 1.5 h the rate of formation of 20S increases and the concentration of 4S begins to decrease gradually. By 8.5 h the 17S is fully depolymerized, and the rate of 20S formation slows but continues to form from 4S protein and comes to apparent equilibrium after 48 h at the 4S:20S ratio of 43:57. In the course of these reactions, the concentration of 4S is quasi-buffered when all three species are present since 4S is being produced by 17S depolymerization and consumed by 20S formation. These results suggest that 28S depolymerizes by the mechanism shown in Figure 4 and that there is no direct interconversion of 17S to 20S.

We have used the TMV self-assembly reaction as an assay for the reactivity of the 17S protein. As shown in Figure 3A, the protein in the 17S form does not participate in either the nucleation or elongation phase of TMV self-assembly. In the time scale of the reconstitution experiments, its concentration remains unchanged upon addition of RNA. Similar experiments performed at 20 °C showed the same results; i.e., the 17S protein does not bind to RNA or the growing nucleoprotein complex. It is known that the 20S aggregate is required for efficient TMV reconstitution since it nucleates the reaction (Butler & Klug, 1971) and at 20 °C can supply about 20% of the remaining protein during the elongation phase of virus rod growth (Schuster et al., 1980; Shire et al., 1981). The results in Figure 3A are in agreement with the previous results and demonstrate clearly that only the 4S and 20S protein aggregates participate in virus reconstitution.

Figure 4 shows schematically the sequence of depolymerization–polymerization events and the relevant structures occurring in these experiments. We assume that the 17S boundary corresponds to an isolated bilayer disk because of both electron microscopy results and hydrodynamic estimates of a structure of this size that yield a sedimentation coefficient of 17–19 S (Caspar, 1963). Electron micrographs taken on grids prepared immediately after reducing the ionic strength by dilution reveal a high concentration of bilayer disk structures lying flat (data not shown). Although such photos do not distinguish between cylindrical bilayer disks and short 20S helices, it is known that the latter do not form in the short time required for grid preparation (see Figure 3B). Furthermore, the four-layer 28S disk dimers would tend to lie on their sides on the grid (see Figure 2). Also, near-UV circular dichroism spectra show that these transient structures are neither 28S nor 20S aggregates. This leaves only a bilayer disk structure to account for both the electron microscopy and sedimentation results. If this assignment is correct, it represents the first demonstration of isolated bilayer disks in low ionic strength solutions and shows them to be quite unstable under virus assembly conditions. These results mean that the isolated bilayer disk does not participate in virus assembly and the dimers of bilayer disks are not in equilibrium with isolated bilayer disks in the low or high ionic strength buffers. The 17S bilayer disk shown schematically in Figure 4 lacks a designation of subunit polarity since the present experiments do not provide such information. The observed 17S structure may be made of either the two inner antipolar layers or the two outer polar layers of the 28S structure. However, the fact that at 15 and 20 °C as well as at 17 °C the kinetics of 28S dissociation are strongly biphasic, resulting in nearly equal 17S and 4S fractions after the initial, fast, phase, indicates not only two types of axial bonding surfaces between layers but also that disruption of one of these leads rapidly to 4S subunits. The obvious candidate for the 17S bilayer disk is the inner antipolar pair of layers in the 28S dimer as in Figure 4. Dissociation to form this structure would result in two single-layer structures that would be expected to be unstable and depolymerize to 4S protein, which initially would be present at the same concentration as the 17S protein. If dissociation of the 28S dimer occurred along the dyad axis, two identical polar bilayer disks would be produced that would not be expected to form about 50% 4S protein initially.

These direct experiments on TMVP species, which are in equilibrium with the crystalline form of the protein, have provided new conclusions about the mechanism of TMV self-assembly. First, we have been unable to find conditions in which the isolated bilayer disk, previously characterized in crystals of dimers of bilayer disks grown at high ionic strength, exists in a stable state in solution. Second, the preformed 28S dimers of the bilayer disk in solution are the crystallizing species, and the antiparallel dimers observed in the crystal are not due to crystal packing forces. Third, these dimers do not convert directly to a protein species that nucleates the virus reconstitution reaction. Instead, the 20S nucleating species forms from 4S subunits after depolymerization of bilayer disks

or their dimers. These results lead to the conclusion that there is no direct or RNA-induced switching in quaternary structure from the cylindrical bilayer disk to the helical array of subunits. Therefore, these experiments fail to demonstrate any functional role in virus assembly for the TMVP bilayer disk structure observed in solution and in crystals. Finally, the present results demonstrate that short TMVP helices and small TMVP aggregates participate in TMV assembly under the conditions used in these and past studies.

The existence of quaternary polymorphism in subunit assembly systems poses a challenge to detailed structure–function studies and provides justification for solving the structures of different subunit packing arrangements of a protein whenever possible. For example, further complexity of TMVP association is seen even at low ionic strength where two different helical forms occur below pH 6.7. Recent time-resolved low-angle X-ray scattering studies have revealed two kinetically determined polymerization paths (Potschka et al., 1988) that result in either virus-like helical rods of $16^1/_3$ subunits per turn or a long-lived metastable polymorph of $17^1/_3$ subunits per turn (Mandelkow et al., 1976). The latter form has no known biological function. The picture that is emerging for subunit assembly systems is that quaternary polymorphism can result from small thermodynamic and kinetic differences between similar packing arrangements. Even small differences in chain folding that may occur in cloned proteins may extend the range of quaternary polymorphism, as has been reported for recombinant DNA derived TMVP expressed in *Escherichia coli* (Shire et al., 1987).

ACKNOWLEDGMENTS

We thank Carol Masheter for technical assistance and Dr. Philip Marcus for assistance with optical microscopy of protein crystals. Plant growth facilities were provided by the Tobacco Research Station, Windsor Locks, CT.

REFERENCES

Ackers, G. K., & Katzel, L. (1979) *J. Supramol. Struct. 10*, 216 (Abstract).

Bloomer, A. C., & Butler, P. J. G. (1986) in *The Plant Viruses* (Van Regenmortel, M. H. V., & Fraenkel-Conrat, H., Eds.) Vol. 2, pp 19–57, Plenum, New York.

Bloomer, A. C., Champness, J. N., Bricogne, G., Staden, R., & Klug, A. (1978) *Nature (London) 276*, 362–368.

Butler, P. J. G., & Klug, A. (1971) *Nature (London), New Biol. 229*, 47–50.

Butler, P. J. G., & Durham, A. C. H. (1977) *Adv. Protein Chem. 31*, 187–251.

Caspar, D. L. D. (1963) *Adv. Protein Chem. 18*, 37–121.

Champness, J. N., Bloomer, A. C., Bricogne, G., Butler, P. J. G., & Klug, A. (1976) *Nature (London) 259*, 20–24.

Correia, J. J., Shire, S., Yphantis, D. A., & Schuster, T. M. (1985) *Biochemistry 24*, 3292–3297.

Durham, A. C. H. (1972) *J. Mol. Biol. 67*, 289–305.

Durham, A. C. H., & Finch, J. T. (1972) *J. Mol. Biol. 67*, 307–314.

Durham, A. C. H., Finch, J. T., & Klug, A. (1971) *Nature (London), New Biol. 229*, 37–42.

Finch, J. T., Leberman, R., Chang, Y. S., & Klug, A. (1966) *Nature (London) 212*, 349–350.

Finch, J. T., Gilbert, P. F. C., Klug, A., & Leberman, R. (1974) *J. Mol. Biol. 86*, 183–192.

Hajdu, J., Acharya, K. R., Stuart, D. I., Barford, D., & Johnson, L. N. (1988) *Trends Biochem. Sci. (Pers. Ed.) 13*, 104–109.

Hirth, L., & Richards, K. E. (1981) *Adv. Virus Res. 26*, 145–199.

Lauffer, M. A. (1975) *Entropy Driven Processes in Biology*, Springer, New York.

Leberman, R., Finch, J. T., Gilbert, P. F. C., Witz, J., & Klug, A. (1974) *J. Mol. Biol. 86*, 179–182.

Makinen, M., & Fink, A. L. (1977) *Annu. Rev. Biophys. Bioeng. 6*, 301–342.

Mandelkow, E., Holmes, K. C., & Gallwitz, U. (1976) *J. Mol. Biol. 102*, 265–285.

Namba, K., & Stubbs, G. (1986) *Science (Washington, D.C.) 231*, 1401–1406.

Nonomura, Y., & Ohno, T. (1974) *J. Mol. Biol. 90*, 523–527.

Okada, Y. (1986) *Adv. Biophys. 22*, 95–145.

Potschka, M., Koch, M. H. L., Adams, M. L., & Schuster, T. M. (1988) *Biochemistry* (in press).

Raghavendra, K., Adams, M. L., & Schuster, T. M. (1985) *Biochemistry 24*, 3298–3304.

Raghavendra, K., Salunke, D. M., Caspar, D. L. D., & Schuster, T. M. (1986) *Biochemistry 25*, 6276–6279.

Raghavendra, K., Kelly, J. A., & Schuster, T. M. (1988) *Biophys. J. 53*, 114a (Abstract).

Schuster, T. M., Scheele, R. B., Adams, M. L., Shire, S. J., Steckert, J. J., & Potschka, M. (1980) *Biophys. J. 32*, 313–329.

Shire, S. J., Steckert, J. J., Adams, M. L., & Schuster, T. M. (1979a) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2745–2749.

Shire, S. J., Steckert, J. J., & Schuster, T. M. (1979b) *J. Mol. Biol. 127*, 487–506.

Shire, S. J., Steckert, J. J., & Schuster, T. M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 256–260.

Shire, S. J., Leung, D. W., Cochianes, G. J., Jackson, E., Wood, W. I., Raghavendra, K., Schuster, T. M., & McKay, P. (1987) *Biophys. J. 51*, 91a (Abstract).

Steckert, J. J. (1982) Ph.D. Thesis, University of Connecticut.

Stubbs, G. (1984) in *Biological Macromolecules and Assemblies* (Jurnak, F. A., & McPherson, A., Eds.) Vol. 1, pp 149–202, Wiley, New York.

Vogel, D. (1982) *Biochim. Biophys. Acta 706*, 65–79.

Williams, R. C., & Fisher, H. W. (1970) *J. Mol. Biol. 52*, 121–123.