Disk Aggregates of Tobacco Mosaic Virus Protein in Solution: Electron Microscopy Observations†

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ABSTRACT: Previous studies of the coat protein of tobacco mosaic virus (TMVP) have shown that TMVP presumably exists as linear stacks of two-ring cylindrical disks in the 0.7 M ionic strength buffer used for crystallizing the disks for X-ray diffraction studies [Raghavendra, K., Adams, M. L., & Schuster, T. M. (1985) *Biochemistry 24*, 3298-3304]. The spectroscopic and sedimentation studies of solutions of TMVP under these crystallizing conditions have demonstrated a long-term metastability of these disk aggregates when they are placed in 0.1 M ionic strength buffers, as are used for reconstituting tobacco mosaic virus from TMVP and viral RNA. The present work describes an electron microscopic study of TMVP disk aggregates under the same solution conditions employed in the previous spectroscopic and sedimentation studies. The results show that in the pH 8.0 0.7 M ionic strength crystallization buffer TMVP exists as stacks of disks which range in size from about 6 to 24 layers, corresponding to 3-12 2-layer disk aggregates having 17 subunits per layer. These TMVP aggregates persist in a metastable form in 0.1 M ionic strength virus reconstitution buffer with no apparent changes in structure of the stacked disks. The results are consistent with the conclusions of the solution physical-chemical studies which suggest that the disk structure may not be related to the 20S TMVP aggregate that is the nucleation species in virus reconstitution.

Self-assembly of tobacco mosaic virus (TMV), from its isolated RNA and capsid protein [tobacco mosaic virus protein (TMVP)], requires a 19-20S aggregate of TMVP for nucleation of the reaction in low ionic strength buffers (Butler & Klug, 1971). In addition to exhibiting self-assembly with homologous RNA, TMVP alone can undergo stereospecific self-association, resulting in helical rods of variable length but otherwise having the same dimensions and subunit packing arrangement as the virus (Franklin, 1955; Durham et al., 1971; Stubbs et al., 1977; Schuster et al., 1979; Mandelkow et al., 1981). It has been shown that the 19-20S TMVP aggregate also nucleates this RNA-free proton or temperature-induced self-association of TMVP, which results in the formation of helical virus-like rods (Durham et al., 1971; Schuster et al., 1979).

The structure of the capsid protein (M_r = 17 530) has been determined previously in crystals, grown from solutions of high ionic strength buffers, in which the protein forms dimers of 2-layered cylindrical disk aggregates each of which has 17 subunits per layer (Bloomer et al., 1978). The sedimentation coefficient of the monomer two-layer disk aggregate had been estimated previously to be 18-20 S (Caspar, 1963). It was also presumed that the disk aggregate was built of subunits packed in a similar way as in the helical virus (Caspar, 1963). When the two-layered disk was identified in the crystal (Finch et al., 1966), it was then presumed to correspond to the 20S nucleating species in solution. Although electron microscopy (Durham et al., 1971; Durham & Finch, 1972) of 19-20S TMVP from low ionic strength buffers (assembly conditions) revealed images of flat disklike structures with a central hole, the method cannot distinguish between a 2-layered disk of 17 subunits per layer and a 2-turn helix (of $16^1/_3$ subunits per turn) when the aggregates are viewed end-on, even by employing rotational averaging methods (Crowther & Amos, 1971; Finch et al., 1966).

The assignment of the disk as the 20S nucleating species in virus self-assembly has been questioned on the basis of recent physical-chemical studies in solution (Raghavendra et al., 1985; Correia et al., 1985) and on the basis of a comparative structure analysis of fiber (Namba & Stubbs, 1986) and crystal diffraction data (Bloomer et al., 1978). The results of Raghavendra et al. suggest that the structure of the nucleating aggregate may not be related to the two-layer disk aggregate in the crystal. Furthermore, those studies indicated that the TMVP aggregates which form in solutions of crystallization buffers have unusual stability properties and are most likely stacks of disk aggregates. On the basis of circular dichroism spectra of the stacked disk aggregates in high ionic strength buffers at pH 8.0 as a reference, and spectra of the same material transferred to a low ionic strength buffer at pH 7.0, it was concluded that the structure of this form of the protein is the same under both salt conditions and that the disks formed at pH 8.0 in high ionic strength buffers are metastable for many days at pH 7.0 (Raghavendra et al., 1985). However, in low ionic strength buffer identical with that used for virus assembly, the CD spectrum of the stacked disks is different from that of the nucleating 20S species. Also, molecular weight measurements by sedimentation equilibrium have shown that the 20S boundary consists of a narrow size distribution of protein aggregates, 39 ± 2 subunits (Correia et al., 1985), not 34 subunits as expected for the cylindrical disk structure characterized in crystals. Furthermore, the sedimentation coefficient and area of the 20S boundary have been shown to exhibit a continuous pH dependence, going from 19.5 S (20%) at pH 7.2 to 23.4 S (95%) at pH 6.5, which represents a continuous polymerizing system with aggregates up to 3 helical turns of 49 subunits at pH 6.5 (Schuster et al., 1980;

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Correia et al., 1985). These results led to the suggestion that the nucleating protein species of TMV self-assembly is not the 2-layer (17 subunits per layer) cylindrical disk but a 2-plus-turn short helical rod (Raghavendra et al., 1985; Namba & Stubbs, 1986).

We report here direct visualization by electron microscopy of the stacked disk aggregates in solution conditions from which disk aggregates crystallize, the identical conditions used in the CD and sedimentation measurements. Further evidence is presented to show that these aggregates exhibit sufficient stability to allow them to be dialyzed into self-assembly buffer solutions for spectroscopic comparison with the other association states of TMVP. Of particular interest are the forms of the capsid protein which participate with RNA in the self-assembly of the virus, a subject of considerable study [for reviews, see Hirth & Richards (1981), Butler (1984), Stubbs (1984), and Lomonossoff & Wilson (1985)].

Materials and Methods

Sample Preparation. TMV (common strain) and TMVP preparations were described previously as were sample handling, buffer preparation, dialysis techniques, and concentration determinations (Raghavendra et al., 1985). All samples were checked by analytical velocity sedimentation and CD measurements to confirm reproducibility with previous studies. All ionic strengths are reported in molar concentrations. High ionic strength buffer (referred to as "high-salt", HS, buffer) was 0.2 M $(NH_4)_2SO_4$ in I = 0.1 M tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl), pH 8.0, total I = 0.7 M, and the low ionic strength buffers (referred to as "low-salt", LS, buffer) were I = 0.1 M potassium phosphate, pH 7.0, and I = 0.1 M Tris-HCl, pH 8.0. All buffers were prepared with double-distilled water and reagent-grade chemicals. pH measurements were made at 21–24 °C by using a Radiometer (Copenhagen) PHM 64 digital pH meter connected to a Radiometer (Copenhagen) combination glass electrode (GK 2321C). Special precautions, developed previously, were employed to eliminate plant proteases (Shire et al., 1979a; Steckert, 1982) and avoid bacterial contamination. These precautions were shown to be effective in yielding highly purified, intact, and protease- and nuclease-free solutions of TMVP as judged by oligonucleotide assays and electrophoresis (Steckert & Schuster, 1982; Potschka, 1983); 1 mM NaN_3 was routinely used in buffers since it does not affect the protein aggregation (Schuster et al., 1979) or CD spectra (Vogel, 1982) or virus assembly (Shire et al., 1979a) but prevents bacterial contamination and proteolysis which could lead to the formation of irreversible aggregates (Durham, 1972).

Electron Microscopy. The protein samples, originally at 3.0–3.5 mg/mL concentration, were diluted to about 1.4 mg/mL just before grid preparation. In each case, a 5-μL drop of the sample was allowed to rest on the carbon–formvar-coated copper grid for about 4 min, and then the grid was washed with 10–15 drops of a 1% aqueous solution of uranyl acetate (pH <4.0). The grids were prepared either in the cold room (4 °C) or at room temperature (20 °C) depending upon the original sample temperature. TMV (concentration 0.1 mg/mL) was used as an internal standard in some grid preparations. The diameter of the virus is 180 Å. Grids were viewed by using a Philips 301 electron microscope operating at 80 kV, and the micrographs were recorded with Kodak SO163 film.

Results and Discussion

Stacked Disks. Figure 1a shows the short stacks of disks that exist in the supernatant of the HS crystallizing buffer at pH 8.0. Under these conditions, analytical ultracentrifuge analysis shows three main sedimenting boundaries, 38 S, 45 S, and 54 S, with broad trailing and leading shoulders on the slowest and fastest boundaries, respectively (Raghavendra et al., 1985), but no 18–20S aggregates are seen. In Figure 1a, almost half of all the particles are seen on end. Those on their sides range from 6 to 24 layers of subunits in length. However, the majority of the images correspond to stacks of 6, 8, and 10 layers of subunits. Figure 1b (inset) shows enlargements of some short stacks where the pairing between the two layers within a disk can be seen. This kind of detail was shown previously in electron micrographs for very long "irreversible" TMVP stacked disk rods (Finch & Klug, 1971; Unwin & Klug, 1974) and short stacks of disks (Nonomura & Ohno, 1974). In most of the stacks, the central 40-Å hole is visible as is the case in the TMV particles included for reference in Figure 1a. In all of our specimens, the single layers of subunits are more discernible in stacked disks than in the whole virus where the helical array of subunits has a pitch of 23 Å (Franklin, 1956). This difference in staining is seen consistently and has been noted by others previously. The greater ease with which the uranyl acetate stain penetrates between subunit layers in the disk structure compared with the helical subunit array demonstrates the qualitative difference in the two packing arrangments.

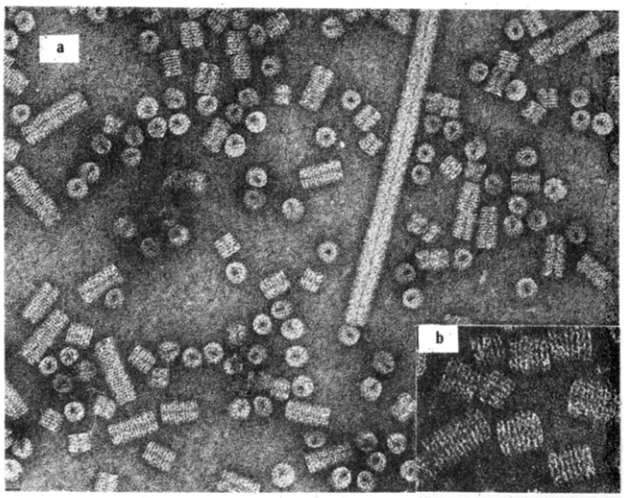

FIGURE 1: (a) Electron micrograph of disk aggregates of TMVP present in HS crystallization buffer [0.2 M $(NH_4)_2SO_4$ in I = 0.1 M Tris-HCl, pH 8.0, total I = 0.7 M], 20 °C. Disk aggregates were obtained by dialyzing at 4 °C the 4S protein, from LS buffer (I = 0.1 M Tris-HCl, pH 8.0), and then equilibrating at 20 °C for 48 h. Note the presence of a portion of a helical TMV particle, diameter 180 Å, which was added for calibration purposes. Under these conditions in solution, TMVP exhibits three sedimentation boundaries having $s_{20,w}$ values of 38, 45, and 54 S. (b) Electron micrograph of disk aggregates of TMVP: an enlarged region of (a) showing the pairing between the two layers within each disk in the stacks.

Stability of Stacks of Disks. Stacked disks can be formed in the HS crystallizing solutions either by dialyzing the dissociated 4S TMVP at 4 °C from pH 8.0 LS buffer and then equilibrating at 20 °C for 48 h (Figure 1a) or by first equilibrating the protein in LS buffer at pH 7.0 and 20 °C for 48 h to the 40:60 mixture of the 4S/20S species and then dialyzing into the HS crystallizing buffer for 48 h [see Figure 1 of Raghavendra et al. (1985)]. Figure 2 shows a field of stacked disks formed by the latter method and then cooled to 0 °C for 4 days before the grid was prepared in the cold room. Similar distributions of stacked disks are observed when grids are prepared a few hours after the TMVP in the HS crystallizing buffer is cooled to 0 °C. Also seen in Figure 2 is evidence that there is a tendency for the stacks to form on the

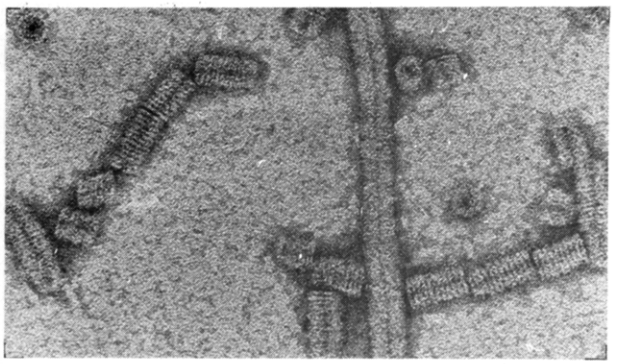

FIGURE 2: Electron micrograph of disk aggregates of TMVP present in HS crystallization buffer (as in Figure 1a) which were obtained by dialyzing a 40:60 equilibrium mixture of 4S and 20S aggregates formed originally in LS buffer (I = 0.1 M potassium phosphate, pH 7.0) at 20 °C and cooled to 0 °C. Note the end-to-end aggregation of disk aggregates. Under these conditions in solution, TMVP exhibits three sedimentation boundaries having $s_{20,w}$ values of 39, 47, and 53 S.

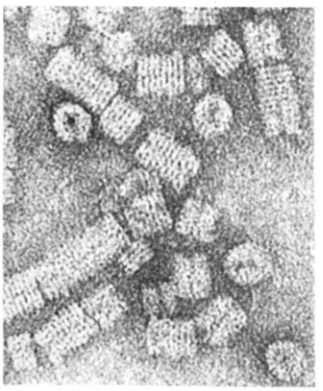

FIGURE 3: Electron micrograph of disk aggregates of TMVP prepared as in Figure 1 and then dialyzed to virus assembly conditions, pH 7.0 LS buffer (I = 0.1 M potassium phosphate) and 20 °C. Note the similarity between the disk aggregates in this figure and in Figure 1a. The sedimentation pattern of this sample is the same as that seen in Figure 1 solutions. The true equilibrium state of TMVP under these conditions is a 40:60 mixture of 4S and 20S aggregates, respectively.

grid end-to-end aggregates with themselves [and with whole TMV (not shown here) when it is added for calibration purposes]. However, this extent of self-association is not seen by sedimentation measurements under these conditions. For this reason, the population distribution of the sizes of stacked disks cannot be quantitatively determined by electron microscopy. Sedimentation analysis of the TMVP solution from which samples are taken for Figure 2 contained three boundaries of about equal area with $s_{20,w}$ values of 39 S, 47 S, and 53 S. Most of the unaggregated stacks are in the range of 6–10 layers of subunits under these conditions.

As was shown previously by sedimentation analysis (Raghavendra et al., 1985), these stacks of disks exhibit unusual stability properties. An example of the unusual metastability is seen in Figure 3 which shows a specimen, prepared as in Figure 1a, that has been dialyzed at 20 °C from pH 8.0 HS buffer into the pH 7.0 LS buffer. Previous sedimentation analysis showed that the half-time for depolymerization to the true equilibrium state of a 40:60 mixture of 4S and 20S boundaries, respectively, is greater than 3 days. It is this metastability which made it possible previously to carry out spectroscopic and hydrodynamic measurements on the disk aggregates under the exact conditions used for TMV self-assembly. The present results confirm that the stacked disks remain structurally intact when changing from crystallizing

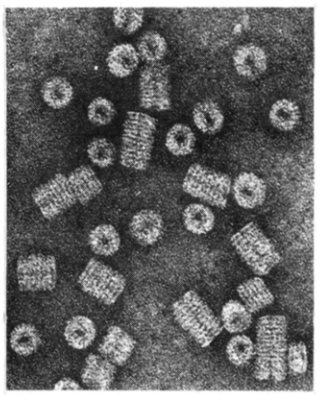

FIGURE 4: Electron micrograph of disk aggregates of TMVP prepared as in Figure 1 dialyzed to pH 6.5 LS buffer (I = 0.1 M potassium phosphate) and 4 °C. Note the similarity between the disk aggregates in this figure and in Figures 1 and 3. The sedimentation pattern of this sample is the same as that seen in Figure 1 solutions. The true equilibrium state of TMVP under these conditions is all 4 S.

to assembly conditions. A further test of the stability of the stacked disks was carried out by cooling to 4 °C a pH 8.0 HS sample, dialyzing it for 8 h at 4 °C into a pH 6.5 LS buffer, and storing it at 0 °C for 12 days. Again, the stacked disks remained intact, as is seen in Figure 4, and they did not dissociate within those 12 days to their true equilibrium state, which in this case is 100% 4S protein (Shire et al., 1979b).

CONCLUSIONS

It has been found that stacked disks at pH 7.0 and 20 °C of TMVP do not participate in either the nucleation or the elongation phase of TMV assembly (M. L. Adams and T. M. Schuster, unpublished results). These results along with spectroscopic and hydrodynamic measurements have suggested that the 20S TMVP species which initiates TMV assembly is not the two-layer disk structure which has been characterized by X-ray crystallography. The present results have confirmed the existence of stacked disk aggregates under the conditions of our previous solution studies (Raghavendra et al., 1985) and have further suggested that the rate of switching in solution from the cylindrical disk structure seen in crystals to the helical subunit packing structures of TMVP may be too slow to be relevant to TMV self-assembly. A similar conclusion was drawn recently by Namba and Stubbs (1986) on the basis of structural considerations.

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Calcium–Proton and Calcium–Magnesium Antagonisms in Calmodulin: Microcalorimetric and Potentiometric Analyses†

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ABSTRACT: Microcalorimetry, pH potentiometry, and direct binding studies by equilibrium dialysis or gel filtration were performed to determine the thermodynamic functions $\Delta H°$, $\Delta G°$, and $\Delta S°$ guiding the interactions of Ca^{2+}, Mg^{2+}, and H^+ with bovine brain calmodulin. At pH 7.5, Ca^{2+} and Mg^{2+} binding are both endothermic with enthalpy changes of 19.5 and 72.8 kJ·(mol of calmodulin)$^{-1}$, respectively. These enthalpy changes are identical for each of the four ion-binding domains. The affinity constants also are identical with intrinsic values of 10^5 M^{-1} for Ca^{2+} and 140 M^{-1} for Mg^{2+}. Ca^{2+} and Mg^{2+} do not compete for the same binding sites: at high concentrations of both ions, a calmodulin–Ca_4–Mg_4 species is formed with an enthalpy value of 24.4 kJ·mol^{-1} with respect to calmodulin–Ca_4 and −28.8 kJ·mol^{-1} with respect to calmodulin–Mg_4. Moreover, in the presence of high concentrations of Ca^{2+}, the affinity of each of the four ion-binding domains in calmodulin for Mg^{2+} is decreased by a factor of 4 and vice versa, indicative of negative free-energy coupling between Ca^{2+} and Mg^{2+} binding. Protons antagonize Ca^{2+} and Mg^{2+} binding in a different manner. Ca^{2+}–H^+ antagonism is identical in each of the four Ca^{2+}-binding domains in the pH range 7.5–5.2. Our analyses suggest that three chemical geometries, probably carboxyl–carboxylate interactions, are responsible for this antagonism with ionization constants of $10^{6.2}$ M^{-1} in the metal-free protein. Mg^{2+}–H^+ antagonism also is identical for each of the Mg^{2+}-binding sites but is qualitatively different from Ca^{2+}–H^+ antagonism. The localization of the putative Mg^{2+}-binding sites and the structural basis of the Ca^{2+}–H^+ antagonism have been discussed.

In eukaryotic cells, calmodulin (CaM)[1] is a vector in the stimulus-response coupling since it is sensitive to raised [Ca^{2+}] and subsequently activates a number of enzymes. Since activation is critically dependent on the number of Ca^{2+} ions bound to the vector [for review, see Cox (1984)], it is of crucial importance to know in detail the characteristics of Ca^{2+} binding to the latter and the influence, even small, of other ions present in the cell. Unfortunately, controversy exists regarding the affinities of the four Ca^{2+}-binding domains, the cooperativity in binding, and the influence of Mg^{2+} and monovalent cations on Ca^{2+} binding. Seven different groups [mentioned by Burger et al. (1984); also Ogawa and Tanokura (1984)], who performed direct binding studies in buffers of sufficient ionic strength to eliminate electrostatic effects (Debye & Hückel, 1923), experienced that the shape of the binding isotherm is close to the ideal Langmuir isotherm, which led us to assume a simple model of four independent Ca^{2+}-binding sites of equal affinity (Burger et al., 1984). However, from studies using ^{43}Ca NMR (Andersson et al., 1982) and fast kinetics of Ca^{2+} off-rates (Bayley et al., 1984), it appears that CaM contains two low- and two high-affinity pairs of sites

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[1] Abbreviations: CaM, calmodulin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; PIPES, 1,4-piperazinediethanesulfonic acid.

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