Tobacco Mosaic Virus Protein Aggregates in Solution: Structural Comparison of 20S Aggregates with Those near Conditions for Disk Crystallization†

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ABSTRACT: Previous X-ray studies (2.8-Å resolution) on the crystals of tobacco mosaic virus protein (TMVP) grown from solutions containing high salt have characterized the structure of the protein aggregate as a bilayered cylindrical disk formed by 34 identical subunits [Bloomer, A. C., Champness, J. N., Bricogne, G., Staden, R., & Klug, A. (1978) *Nature (London) 276*, 362–368]. Under low-salt conditions, 20S aggregates are in equilibrium with 4S species and involved in the efficient nucleation of TMV assembly in vitro [Butler, P. J. G. (1984) *J. Gen. Virol. 65*, 253–279]. We have investigated by sedimentation velocity and near-UV circular dichroism (CD) measurements the structure of 20S aggregates in low salt (I = 0.1 potassium phosphate at pH 7.0 and 20 °C) and the aggregates in high salt [0.2 M $(NH_4)_2SO_4$ in I = 0.1 tris(hydroxymethyl)aminomethane hydrochloride at pH 8.0 and 20 °C, close to the conditions under which TMVP crystallizes as disk aggregates]. At high salt, we observe structures (presumably stacks of disks) having $s_{20,w}$ values around 40, 45, and 50 S, but *not* the 20S species present in low-salt buffers. The near-UV CD spectrum of 20S aggregates has been obtained for the first time, using computer techniques, from the spectra of the 4S–20S equilibrium mixture and the 4S species. This spectrum of 20S aggregates differs dramatically from that of the stacks of disks examined at both high and low salt (into which the stacks can be returned by dialysis), indicating that the difference is not a solvent effect. We suggest an open polymerization structure such as a helix for the 20S species in solution instead of a closed disk structure; this is consistent with the results of recent sedimentation equilibrium studies [Correia, J. J., Shire, S., Yphantis, D. A., & Schuster, T. M. (1985) *Biochemistry* (preceding paper in this issue)].

The capsid protein of tobacco mosaic virus (TMVP)[1] self-associates to form viruslike rods and various other equilibrium and metastable aggregates depending upon solution conditions and sample history [reviewed in Butler (1984), Hirth & Richards (1981), Butler & Durham (1977), Lauffer & Stevens (1968), and Caspar (1963)]. Among the various polymeric forms of the protein that have been reported to exist in solution are A protein [a rapidly interacting system of monomers, dimers, trimers, etc. (Lauffer & Stevens, 1968; Caspar, 1963; Ackers & Katzel, 1979)], disk protein [reported to be two-layered cylindrical closed rings each containing 17 subunits (Caspar, 1963; Butler, 1972; Durham et al., 1971)], three-turn aggregates [presumably helical protorods containing about 49 subunits (Schuster et al., 1980)], stacks of disks (Durham, 1972a; Durham & Finch, 1972), short helical rods (Shire et al., 1979), and long helical rods (Durham et al., 1971; Schuster et al., 1979; Mandelkow et al., 1981). The structural polymorphism of TMVP aggregates is further expressed in the form of two packing arrays that are found in long helical rods prepared for fiber diffraction (Mandelkow et al., 1976). Protein helical rods with either $16^1/_3$ or $17^1/_3$ subunits per turn were observed in the oriented gels, but there was only one packing arrangement per specimen. However, the factors influencing this helical packing polymorphism are not understood. Since the coat protein in the virus packs in a virtually identical $16^1/_3$ subunits per turn helix as that seen in the RNA-free long helical TMVP rods, it must be assumed that the protein–protein specific interactions in these helical rods are the same as those in the native virus. A qualitative "phase diagram" of these structures has been postulated (Durham et al., 1971) and modified (Schuster et al., 1979) on the basis of the relevance of these aggregates to the mechanism of TMVP and TMV self-assembly. Structural studies of the coat protein in these various aggregation states may lead to an understanding of the self-assembly mechanism of TMVP and TMV in terms of the protein–protein and protein–RNA interactions responsible for the nucleation and subsequent growth of the aggregating species. Also, since the long protein helices are similar in structure and dimensions to those of TMV (Franklin, 1955; Scheele & Schuster, 1975; Schuster et al., 1979; Stubbs et al., 1979; Mandelkow et al., 1981), information about the self-assembly mechanism of the protein aggregates is important in determining the function of the various coat protein aggregates in virus assembly.

The TMVP aggregate having a sedimentation coefficient of about 20 S has been shown to be involved in the formation of large helical protein aggregates (Durham et al., 1971; Schuster et al., 1979) as well as in the nucleation of virus assembly in vitro (Butler & Klug, 1971). These 20S aggregates are present in low ionic strength solutions either in equilibrium with 4S aggregates at pH 7.0 and 20 °C (Durham et al., 1971) or as metastable aggregates at pH 6.5 and 6.5 °C (Shire et al., 1979). On the basis of gross shape and size determined by electron microscopy and sedimentation velocity measurements (Durham et al., 1971; Durham & Finch, 1972; Crowther & Amos, 1971), it was concluded that the 20S species is a two-layered cylindrical disk aggregate with 17-fold

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[1] Abbreviations: TMV, tobacco mosaic virus; TMVP, tobacco mosaic virus protein; CD, circular dichroism; A protein, TMVP in low states of aggregation, sedimenting at 3–8 S, depending upon the total A-protein concentration; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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symmetry. The only direct detailed evidence for the disk structure so far has been provided by X-ray diffraction studies at 2.8-Å resolution on the protein aggregate in the crystalline state (Bloomer et al., 1978), which clearly characterized a disk structure having a pair of cylindrical rings with 17 subunits in each ring. However, unlike the 20S species in low ionic strength solution, the aggregates of the protein that were shown to be disks were crystallized from high ionic strength solutions containing 0.3 M ammonium sulfate in Tris-HCl (I = 0.1) at pH 8.0 and room temperature (Leberman et al., 1974), wherein the aggregates in the supernatant solution are presumably limited stacks of disks (Durham & Finch, 1972). The question one may then ask is the following: Are the 20S aggregates present in low ionic strength solutions, conditions used for the in vitro virus assembly, and the disk aggregates crystallized from high ionic strength solutions identical structurally? There is no well-characterized reference state in solution with which to compare the structure of the 20S aggregates except the crystalline disk aggregate available in the solid state. Spectroscopic studies performed on various aggregates of the protein in solution are under various solution conditions and sample histories (Vogel, 1973, 1982; Vogel & Jaenicke, 1974). In addition, Schuster et al. (1980) have shown that the 20S boundary exhibiting s values ranging from ~18 to 25 S, depending on pH, is in fact an interacting system consisting of aggregates up to about 3 helical turns with about 49 subunits. Also, recently Correia et al. (1981, 1985) have determined by equilibrium sedimentation measurements the number of subunits in 20S aggregates as 39 ± 2 in contrast to 34 subunits present in the crystalline disk aggregate. These observations indicate a structure for the 20S solution aggregate which is different from the two-layered disk aggregate identified in TMVP crystals.

We report here results obtained using sedimentation velocity and near-UV CD spectroscopy techniques on 20S aggregates in low ionic strength buffer used for the in vitro virus assembly. The aggregates in high ionic strength, conditions close to those used for crystallization of disk aggregates, are used as reference for structural comparison with the 20S aggregates. The near-UV CD spectrum of these high-salt species exhibits significant differences when compared to that of 20S aggregates. We infer that the structure of 20S aggregates, the nucleating species in virus assembly, hitherto unequivocally referred to as disks, differs from that of the aggregating species near conditions for crystallization of disks. We propose a helical structure instead of a closed cylindrical disk structure for 20S aggregates in low ionic strength solution on the basis of our results presented here and the supporting evidence in the preceding paper (Correia et al., 1985).

Materials and Methods

Sample Preparation. TMV (common strain) was isolated from infected tobacco leaves (kindly supplied by Dr. C. A. Knight) and purified by the method of Paglini & Lauffer (1968) as modified by Shire et al. (1979). TMVP was prepared by the modified acetic acid degradation method of Scheele & Lauffer (1967), dialyzed against the appropriate buffer for 48 h, and stored at 0 °C. All ionic strengths are reported in molar concentrations. High ionic strength buffers (collectively referred to as "high salt") were prepared as follows: 0.3 M $(NH_4)_2SO_4$ in I = 0.1 Tris-HCl, pH 8.0, total I = 1.0 (HS-3); 0.2 M $(NH_4)_2SO_4$ in I = 0.1 Tris-HCl, pH 8.0, total I = 0.7 (HS-2); 0.1 M $(NH_4)_2SO_4$ in I = 0.1 Tris-HCl, pH 8.0, total I = 0.4 (HS-1). Low ionic strength buffers (collectively referred to as "low salt") used were the following: I = 0.1 potassium phosphate, pH 7.0 (LS-1); I = 0.1 Tris-HCl, pH 8.0 (LS-2). All buffers were prepared with double-distilled water and reagent-grade chemicals. pH measurements were made at 21–24 °C with a Radiometer Copenhagen PHM 64 digital pH meter connected to a Radiometer Copenhagen combination glass electrode (GK 2321C); 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) but prevents bacterial contamination and minimizes proteolysis which could lead to the formation of irreversible aggregates (Durham, 1972b). The precipitate formed during high-salt dialysis was removed by centrifugation at 6600g for 15 min at 4 °C. The supernatant was diluted to 3.0–3.5 mg of protein/mL (unless otherwise specified), and aliquots were either kept at 0 °C or equilibrated for 48 h at 20 °C. Samples of TMVP in LS-1 at 5 °C or LS-2 at 5 and 20 °C were used to obtain reference spectra of 4S or of 4S and 6.5S aggregates, respectively. Protein samples at 0 °C were warmed to 5 °C for approximately 30 min prior to measurements at 5 °C. Protein concentrations were determined from absorption measurements made with a Cary-Varian 118C spectrophotometer and corrected for light scattering (Englander & Epstein, 1957). The extinction coefficient for TMVP was taken to be ϵ_{281} = 1.30 $(mg/mL)^{-1}$ cm^{-1} in I = 0.1 potassium phosphate, pH 8.0, 4–5 °C (Fraenkel-Conrat & Williams, 1955).

Analytical Ultracentrifugation. Sedimentation velocity experiments were performed by using a Spinco Model E ultracentrifuge with an AN-D rotor and 12-mm double-sector cells having either Kel-F or charcoal-filled Epon centerpieces and plane or 1° positive quartz windows. Rotor preequilibration and cell loading were done at the run temperature either in a constant-temperature air chamber or in a cold room, and the rotor temperature was controlled during the run within 0.5 °C of the set temperature. Schlieren patterns were photographed on either Kodak metallographic plates or Tri-X ortho film, and peak distances were measured on 8–10 photos by using a microcomparator with 10-fold magnification. Measured sedimentation values were corrected for the density and viscosity of the buffers and are reported as $s_{20,w}$ values. The density and the viscosity of each of the buffers were experimentally determined and used in obtaining the correction factors at 5 and 20 °C. The correction factors, in the standard sedimentation velocity equation, for the buffers at the respective temperatures are 1.777 and 1.166 for HS-3, 1.708 and 1.21 for HS-2, 1.637 and 1.075 for HS-1, and 1.573 and 1.033 for LS-1 (or LS-2). Weight fractions of the various species were determined from comparator tracings of the photographs by measuring peak areas with a planimeter and correcting for radial dilution and bar angle.

CD Spectroscopy. CD spectra were recorded by using a Cary 60 spectropolarimeter with a CD-6002 attachment. Calibration of the instrument was performed with d-10-camphorsulfonic acid following the procedure of Adler et al. (1973). The optimal signal to noise ratio (S/N = 0.005°) was achieved with the following settings: wavelength expansion, 5 nm/division; time constant, 3 or 10 s; scan speed, 10 nm/min; full-scale range, 0.1°. Sample cell temperature was controlled to within ±0.02 °C by using a Lauda K-2/R circulating water bath. A water-jacketed quartz cylindrical cell of 5-mm path length was used. The temperature in the cell was measured with a calibrated thermistor probe. A series of four to eight spectra in the near-UV (330–240-nm) region were taken for each sample, and the data were visually averaged. The mean residue ellipticity, $[\theta]$, is calculated by using a mean residue weight of 111.0 for TMVP and is expressed as degrees cen-

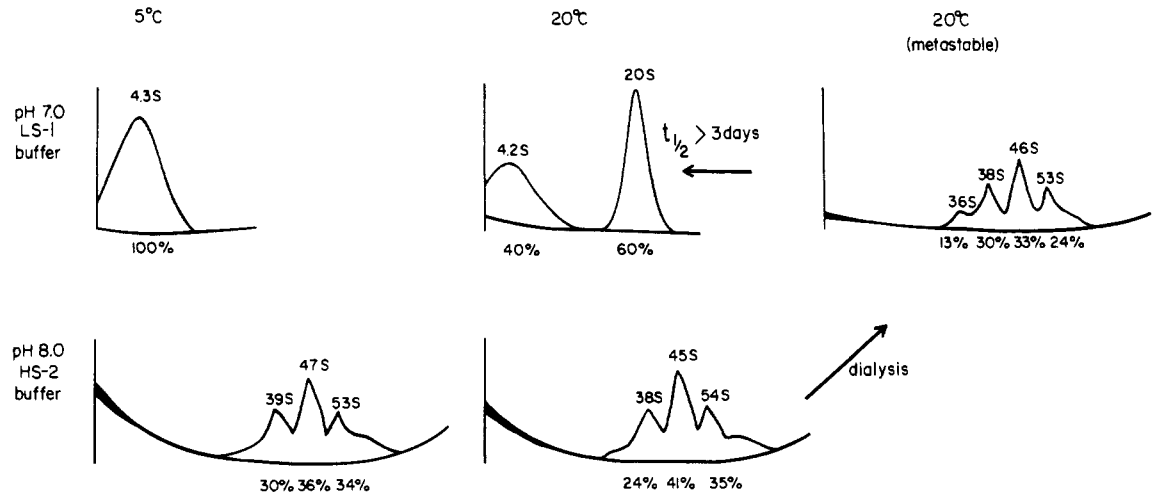

FIGURE 1: Schematic representation of sedimentation patterns of TMVP aggregates in low-salt LS-1 (I = 0.1 potassium phosphate, pH 7.0) and high-salt HS-2 [0.2 M $(NH_4)_2SO_4$ in I = 0.1 Tris-HCl at pH 8.0, total I = 0.7] buffers at 5 and 20 °C. Note the metastable behavior of the high-salt stacked disks returned to low salt and the absence of 4S and 20S aggregates in HS-2 buffer. Protein concentrations were between 3.0 and 3.5 mg/mL.

timeter squared per decimole. The spectra of 4S aggregates and the equilibrium mixture of 4S and 20S species were confirmed by Peter Previlige on samples provided by us with a Jobin Yvon Mark V dichrograph making use of the facilities of Dr. G. D. Fasman of Brandeis University.

The averaged CD spectra were digitized at 1-nm intervals by using a manually operated 10-bit analog to digital converter interfaced to a minicomputer. The spectra were corrected by subtracting the base line (cuvette + buffer). In some cases, the spectra showed a parallel shift from the solvent base line (due to random drift and not because of scattering) in the region of 310 and 330 nm where the dichroic absorption is zero (Vogel & Jaenicke, 1974), and hence, $[\theta]$ between 310 and 330 nm was adjusted to zero by adding or subtracting an additional constant term in this region. This correction was never more than about 6% of the maximum at 272 nm. The reason for choosing the wavelength region of 310–330 nm for TMVP, an extensively aggregating system, is to see whether scattering causes CD signals in the form of long tails in this region (Tinoco et al., 1980). While we do observe such a scattering artifact for the CD spectra of long helical aggreagates of TMVP at pH 6.5 and 5.5 (unpublished results), the aggregates reported here do not exhibit such effects. When the CD spectra of TMVP stacked disks in HS-2 buffer (see Figure 1) were measured in 1.0- and 5.0-mm path-length cells at 3.5 and 2.0 mg/mL, respectively, no differences could be detected. Furthermore, the spectra of all the aggregates shown in Figures 2–5 were reproduced with two different optical detection systems employing side- and end-window types of photomultiplier tubes at two different distances from the sample cell. These results rule out any significant distortions of the CD spectra by light scattering.

Since 20S TMVP aggregates generally coexist with the A protein in solution, it was of interest to us to generate by computer the near-UV CD spectrum of pure 20S species. The CD spectrum of 20S species thus obtained could then be compared to that of high-salt aggregates present in solution conditions close to those used for disk crystallization (Leberman et al., 1974). The method used to obtain the computer-generated CD spectrum consists of measuring near-UV CD spectra of 4S aggregates and the equilibrium mixture of 20S–4S aggregates in LS-1 buffer at 5 and 20 °C, respectively. Weight fractions of 4S and 20S species in the equilibrium mixture were determined from peak areas obtained during sedimentation velocity measurements. The weighted contribution of 4S aggregates thus computed from the sedimentation data and the reference CD spectrum of 100% 4S species was subtracted from the CD spectrum of the 4S–20S mixture. The resulting spectrum, when scaled appropriately, will be the near-UV CD spectrum of 20S aggregates. The complete CD analysis, viz., digitizing, manipulating, computer simulating, and plotting, was done with the help of programs written by Dr. W. T. Windsor.

Results and Discussion

Aggregates of TMVP near Conditions for the Crystallization of Disk Aggregates. Our initial experiments were carried out in HS-3, HS-2, and HS-1 buffers. In general, the extent of association increases with increasing ionic strength. In HS-3 and HS-2 buffers, there are no boundaries sedimenting slower than 38 S whereas in HS-1 there are association states smaller than the single two-turn disk, i.e., 4 or 8 S depending upon temperature. When the CD spectra of HS-1 solutions of TMV protein were corrected for the presence of these small aggregates, the resulting spectra were the same as those obtained in HS-2 buffers although the sizes of the stacked-disk aggregates in HS-2 buffer are larger (38–54 S) than those found in HS-1 buffer (27–49 S). These results indicate that the self-association of disks which leads ultimately to crystal formation does not induce detectable conformation changes in the protein. It should be noted that we did not observe any 20S boundaries in any of the high-salt solutions investigated. Apparently, the single disk is not favored under these conditions because of its strong tendency to self-associate. We proceed from these results to use HS-2 CD spectra as reference spectra of the crystallographically characterized disk structure of TMV coat protein (Bloomer et al., 1978). Our argument therefore assumes that the TMVP subunits present in the supernatant disk aggregates of crystallizing buffers have the same structure as is found in the crystals of disks.

The results illustrated in Figure 1 summarize the states of aggregation in low ionic strength buffer at pH 7.0 and in high ionic strength buffer at pH 8.0 at 5 and 20 °C; whereas the association equilibrium in LS-1 (the usual virus reconstitution buffer) is temperature dependent, that in HS-2 buffer is not. It is the protein in the 20S boundary in LS-1 at 20 °C that was shown previously to be necessary for the initial nucleation of virus assembly using TMVP and TMV–RNA (Butler &

Table I: Sedimentation and Circular Dichroic Measurements of TMVP Aggregates[a]

buffer condn[b]	T (°C)	$s_{20,w}$ in S (app wt fraction in %)						$[\theta]_{252}/[\theta]_{272}$	$[\theta]_{280} - [\theta]_{295}$ (deg·cm²·dmol⁻¹)
					High Salt				
HS-2 (I = 0.7)	5				39 (30)	47 (36)	53 (34)	0.9	147
	20				38 (24)	45 (41)	54 (35)	0.8	149
HS-1 (I = 0.4)	5	4.6 (69)	30 (9)		39 (14)	48 (8)		1.3	140
	20	8.5 (48)	27 (9)	34 (13)	41 (18)	49 (12)		1.2	134
					Low Salt				
LS-1 (I = 0.1)	20[c]			36 (13)	38 (30)	46 (33)	53 (24)	0.9	148
	5	4.3 (100)						1.4	138
	20	4.2 (40)	20 (60)					1.5	110
	20		20 (100)[d]					1.5	90

[a] All protein concentrations were between 3.0 and 3.5 mg/mL. [b] HS and LS are described under Materials and Methods and in the figure legends. [c] HS-2 aggregates at 20 °C dialyzed to LS-1 buffer conditions (see text). [d] Calculated for 100% 20 S (see text).

Klug, 1971). Two new findings are reported in Figure 1. First, the dialysis of stacked disks at 20 °C (38–54 S) into low ionic strength buffer, where the equilibrium state of TMVP association is 4 and 20 S, results in a negligible shift in the distribution of stacked-disk sizes. Second, under these conditions, the rate of depolymerization of the metastable stacked disks is very slow, with a half-time greater than 3 days. These results made it possible for us to compare, under identical solution conditions, the structures of disks and the 20S virus-assembly nucleating species.

Table I summarizes the sedimentation data for high-salt aggregates in HS-2 (I = 0.7) buffer, conditions close to those used for the crystallization of disk aggregates. Also included are the results in HS-1 buffer (I = 0.4) to show the effect of ionic strength and temperature on the formation of aggregates in high salt. In HS-2 buffer at 5 °C, the protein exists as a mixture of 39S, 47S, and 53S aggregates. The 4S and 20S boundaries are conspicuously absent. Vogel (1982) has also reported the absence of 20S aggregates in his studies on the effect of salts on TMVP. The weight fractions of the boundaries (see Table I) indicate that the amount of each aggregate is about one-third of the total. At 20 °C, the aggregates continue to be present as 38S, 45S, and 54S species with a small redistribution in their relative weight fractions. Hence, temperature does not strongly influence the state of aggregation of the protein which is mainly controlled by the ionic strength under these conditions, in contrast to the situation at low ionic strength and pH 7.0. On the other hand, the aggregation behavior of the protein in HS-1 buffer is more temperature dependent than in HS-2 buffer as revealed by the sedimentation boundaries (Table I). The 4S (amounting to two-thirds of total) and 8S (representing half the total) boundaries are the major ones at 5 and 20 °C, respectively. Higher aggregates such as 27 and 30 S and others at about 35, 40 and 50 S are also present, but in low concentration. From these observations, it is clear that in HS-1 buffer (I = 0.4) the aggregation leading to large aggregates is incomplete and 4S and 8S species predominate at 5 and 20 °C, respectively. Under these conditions, temperature thus has a pronounced influence on the conversion of lower aggregates from 4 to 8 S but only a marginal effect in the formation of large aggregates. Previously, TMVP aggregates of sizes, viz., 28, 37, and 45 S were obtained (Durham, 1972a) in high ionic strength (I = 1.0) solutions at pH 8.0 and 5 °C and interpreted as "stacks of disks" on the basis of electron microscopic results (Durham & Finch, 1972). The 4S boundary was observed as a trailing peak, and the 28S boundary was the major component observed in the earlier work. Aggregates sedimenting at 27 S were also observed under high-salt conditions by Vogel (1982). It is reasonable to assume that the >20S aggregates

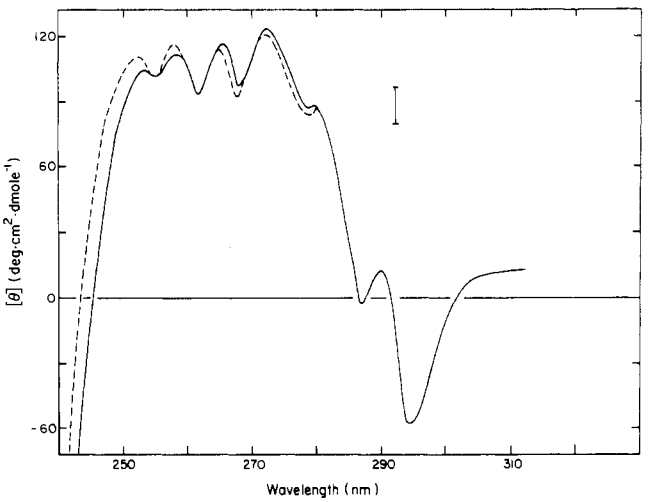

FIGURE 2: Near-UV CD spectra of TMVP aggregates in high-salt HS-2 buffer [0.2 M $(NH_4)_2SO_4$ in I = 0.1 Tris-HCl at pH 8.0, total I = 0.7] at 5 (---) and 20 °C (—). See Table I and Figure 1 for a description of aggregation states. The error bar indicates the uncertainty in the $[\theta]$ value which is ±8 units. See Materials and Methods for the parallel shift in the spectra from the base line beyond 300 nm.

observed by us in HS-1 and HS-2 buffers are probably stacks of disks of different sizes, as indicated by their s values. At the higher ionic strengths, the equilibrium tends toward the formation of stacks of disks which may be conducive to the crystallization of disk aggregates. Thus, from our sedimentation data, one can broadly classify the species in high-salt conditions into two categories: A protein consisting of 4S and 8S aggregates which dominate at I = 0.4 solutions and stacks of disks at I = 0.7.

CD measurements and sedimentation runs were simultaneously performed on aliquots of the same samples in order to correlate the results of the two techniques. Near-UV CD data on high-salt aggregates in HS-2 (I = 0.7) and HS-1 (I = 0.4) buffers are shown in Figures 2 and 3, respectively. The CD spectra in the wavelength region 330–240 nm exhibit fine structure in the aromatic Cotton effects with the positive peaks centered arount 252, 257, 264, and 272 nm along with a shoulder at about 280 nm and negative bands appearing at about 287 and 295 nm. The band at 295 nm is predominantly due to Trp chromophores (Schuster et al., 1980) but also contains a small contribution from Tyr residues. Between 290 and 270 nm, there are overlapping bands contributed by both Tyr and Trp residues. The fine structure in the bands observed below 270 nm is entirely due to Phe chromophores. A similar assignment of dichroic bands in the near-UV CD spectra of TMVP to Phe, Tyr, and Trp chromophores was reported by

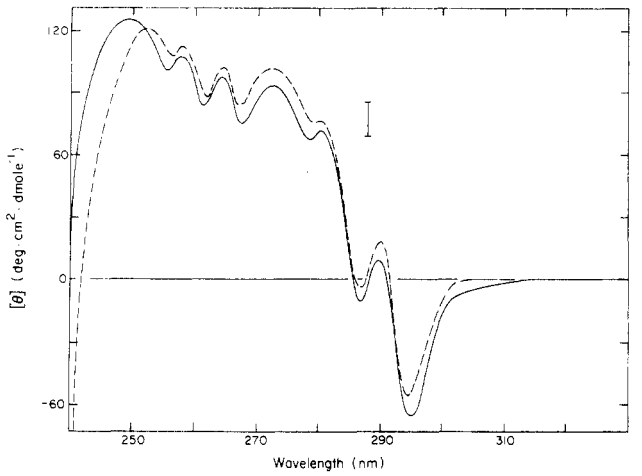

FIGURE 3: Near-UV CD spectra of TMVP aggregates in high-salt HS-1 buffer [0.1 M $(NH_4)_2SO_4$ in $I = 0.1$ Tris-HCl at pH 8.0, total $I = 0.4$] at 5 (—) and 20 °C (- - -). See Table I for a description of aggregation states. The error bar indicates the uncertainty in the $[\theta]$ value which is ±8 units.

Vogel & Jaenicke (1976) and Budzynsky (1972).

The differences in aggregation behavior of TMVP in HS-2 and HS-1 buffers seen by sedimentation data (Table I) can be correlated with the corresponding CD spectral changes, as shown in Figures 2 and 3. For this purpose, $[\theta]$ values at 252, 272, 280, and 295 nm were chosen as markers. Figure 2 represents the CD spectra of stacks of disks present in HS-2 buffer at 5 and 20 °C which are almost indistinguishable from each other, consistent with their sedimentation data. In HS-1 buffer ($I = 0.4$), where 4 and 8 S are the major boundaries at 5 and 20 °C, respectively (Table I), the spectra in Figure 3 exhibit differences when compared with the CD spectra of stacks of disks (see Figure 2). The following differences are observed: (a) $[\theta]$ has a maximum value at 250 nm for the 4S-dominated and at 252 nm for the 8S-dominated aggregates of TMV protein in HS-1 buffer (Figure 3) whereas this band is reduced for the stacks of disks in HS-2 buffer (Figure 2) at 20 °C; (b) $[\theta]$ at 272 nm, on the other hand, has a lower value for the former than for the latter; (c) changes in $[\theta]$ at 280 and 295 nm are not significant. Furthermore, the ratio of $[\theta]_{252}$ to $[\theta]_{272}$ and $[\theta]_{280} - [\theta]_{295}$ tabulated in Table I can be used to characterize the aggregating species. It can be readily seen that the ratio has a value of 0.8–0.9 for the stacks of disks and 1.3 or 1.2 for the protein sample with a 4S or an 8S boundary, respectively, as the major component. Hence, it is clear from these observations that $[\theta]_{252}/[\theta]_{272}$ distinguishes the mixture of large aggregates with $s_{20,w} > 30$ S present in HS-2 buffer ($I = 0.7$) from the 4S or 8S dominated aggregating species in solutions of HS-1 ($I = 0.4$). On the other hand, $[\theta]_{280} - [\theta]_{295}$ varies only slightly, within 15 deg·cm²·dmol⁻¹, and does not follow a regular pattern for the varying salt conditions and temperatures of the high-salt solutions. Thus, the reduction in the intensity of the 252-nm band and a corresponding increase in that of the 272-nm band in going from 4S or 8S aggregates to stacks of disks reflect an increase in the flexibility of Phe chromophores and ordering of Tyr and Trp residues (Strickland, 1976; Vogel & Jaenicke, 1974, 1976). Finally, we have found that the CD spectra of the stacks of disks in HS-1 resemble those in HS-2 qualitatively when the contributions due to either 4S or 8S (as measured in LS-2 buffer at 5 and 20 °C, respectively) were subtracted from the spectra of the former. This implies that the CD spectra of stacks of disks do not depend upon the sizes of the aggregates under these conditions.

Stability and Reversibility of High-Salt TMVP Aggregates. The stability of stacked disks of TMVP formed in HS-2 buffer was studied following changes in pH, ionic strength, and temperature. An aliquot of protein containing these large aggregates in HS-2 buffer was dialyzed against LS-1 buffer ($I = 0.1$) for 6 h at 20 °C and then equilibrated at that temperature for about 70 h. Results of sedimentation velocity measurements on a typical sample are summarized in Table I and Figure 1. It appears that the large aggregates are quite stable for long periods of time and partially depolymerize slowly under these conditions to form small amounts of 36S material. [The same behavior was observed when the HS-2 aggregates were dialyzed against pH 6.5 potassium phosphate ($I = 0.1$) buffer.] This is in contrast to the expected equilibrium distribution of 20S and 4S aggregates in LS-1 buffer at 20 °C. However, 4S aggregates do appear when the sample is allowed to stand for about 12 days. Cooling the protein solutions to 5 °C resulted in further depolymerization with the formation of a 4S boundary corresponding to 20% of the total aggregates, but the larger aggregates were still present. Interconversion among large aggregates and the formation of 36S material after dialysis to LS-1 buffer and the formation of trace amounts of 4S aggregates upon cooling to 5 °C resulted in negligible changes in the CD spectrum (see Comparison of CD Spectra of Stacks of Disks and 20S Aggregates). This result is in accordance with the previous observation that the CD spectrum of TMVP at pH 8.0 in high ionic strength buffers does not change when the size of the disk stacks changes.

In order to learn if the observed protein aggregation was reversible and not the result of proteolysis, the following experiments were performed. Protein in HS-2 buffer containing stacks of disks was dialyzed against very low ionic strength (0.005 M) potassium phosphate buffer, pH 7.0 and 5 °C. Only approximately 25% of the total protein depolymerized to 4S aggregates instead of the expected complete depolymerization to 4S species. The sample was then dialyzed against 67% acetic acid, followed by dialysis against 0.1% acetic acid and then LS-1 buffer ($I = 0.1$), all at 5 °C. This procedure allows the large protein aggregates that were formed in high salt to go through the last few steps of the usual TMV protein preparation (Scheele & Lauffer, 1967). This reversible denaturation procedure results in complete depolymerization to monomers and extensive chain unfolding followed by refolding and 4S formation. A further equilibration of the sample (with a protein concentration of 2.3 mg/mL) at 20 °C for 48 h resulted in the equilibrium distribution of 4S (57%) and 20S (43%) aggregates. Since this is close to the normal behavior of the protein under these conditions (Durham, 1972a; S. Shire and T. M. Schuster, unpublished results), we can clearly rule out the possibility of proteolysis (Durham, 1972b) as the cause of metastability of the stacks of disks in our TMVP samples. Thus, it appears that the stacks of disks formed in HS-2 buffer, close to the crystallization conditions of the disk aggregates, are very stable with respect to changes in solution conditions and depolymerize very slowly but do not result from proteolysis or other irreversible changes.

Comparison of CD Spectra of Stacks of Disks and 20S Aggregates. After characterizing the sedimentation and CD properties of TMVP aggregates, presumably stacks of disks, we were interested in comparing the structure of these species with that of the 20S aggregates used in TMV reconstitution and believed to be in the form of disks (Durham & Finch, 1972). In order to compare the near-UV CD spectra of 20S aggregates and stacks of disks under the same solvent con-

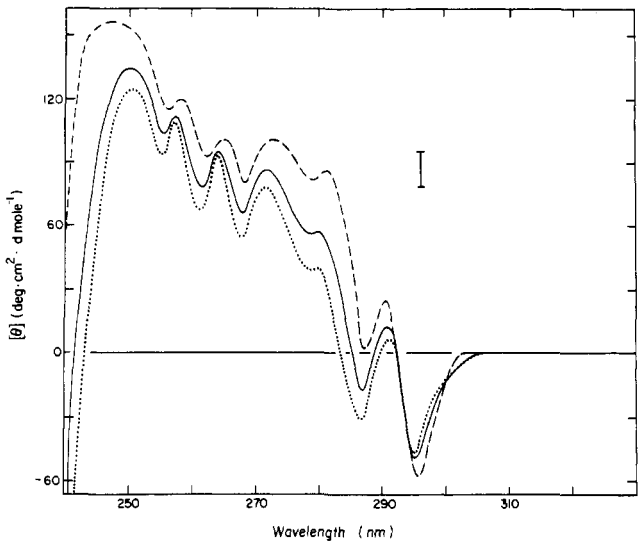

FIGURE 4: Near-UV CD spectra of TMVP aggregates in low-salt LS-1 buffer (I = 0.1 potassium phosphate, pH 7.0) at 5 (---) and 20 °C (—) and computed spectrum of 20S aggregates (···). See Table I and Figure 1 for a description of aggregation states. The error bar indicates the uncertainty in the $[\theta]$ value which is ±8 units.

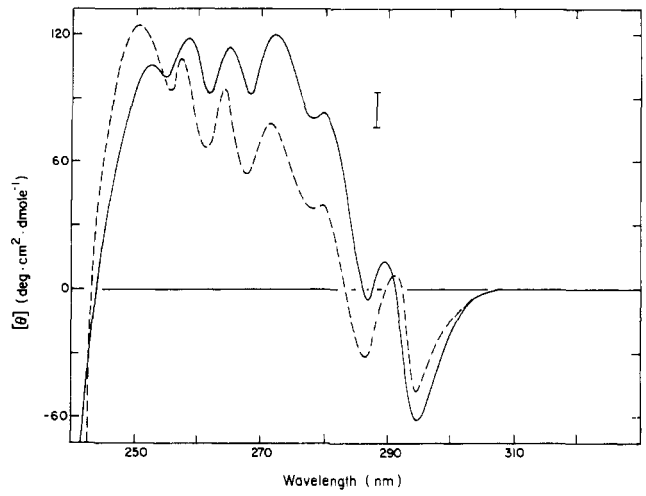

FIGURE 5: Near-UV CD spectra of TMVP aggregates in low-salt LS-1 buffer (I = 0.1 potassium phosphate, pH 7.0) at 20 °C: HS-2 aggregates returned to LS-1 (—) and computed spectrum of 20S aggregates (---). See Table I and Figure 1 for a description of aggregation states. The error bar indicates the uncertainty in the $[\theta]$ value which is ±8 units.

ditions, viz., LS-1 buffer (I = 0.1), we first obtained the computer-generated CD spectrum of 100% 20S aggregates by the method outlined under Materials and Methods. Figure 4 shows the CD spectra of 4S aggregates at 5 °C, a 20S–4S equilibrium mixture at 20 °C, and 100% 20S aggregates. The contribution of 4S aggregates to the CD spectrum of the 20S–4S mixture depends upon its composition, which is about 40% (see Table I). The intensities of the 280- and 287-nm bands in the CD spectrum of 20S aggregates (bereft of 4S species) are appreciably reduced when compared with those of the 20S–4S equilibrium mixture (Figure 4), also reflected in $[\theta]_{280} - [\theta]_{295}$ values given in Table I. The difference between $[\theta]_{280}$ and $[\theta]_{295}$ is 90 deg·cm²·dmol⁻¹ for 20S aggregates as compared to 110 deg·cm²·dmol⁻¹ for 20S–4S mixtures and 138 deg·cm²·dmol⁻¹ for 4S aggregates. On the other hand, $[\theta]_{252}/[\theta]_{272}$ has a value of 1.4–1.5 (see Table I) for 4S and 20S aggregates. This does not reflect relatively less intense bands in the wavelength region of 240 and 290 nm (Figure 4) in the CD spectrum of 20S aggregates as compared to that of 4S aggregates which might result from an overall flexibility in the aromatic groups of the protein (Vogel & Jaenicke, 1974).

As the 20S aggregates exist in equilibrium with 4S species in low ionic strength buffer LS-1 at 20 °C, conditions used for the efficient in vitro virus assembly (Table I; Butler & Klug, 1971), we chose these solution conditions to make a structural comparison between 20S aggregates and the stacks of disks. As mentioned in the previous section, protein aggregates in HS-2 buffer (I = 0.7) were dialyzed to LS-1 buffer (I = 0.1) for 6 h at 20 °C and allowed to remain at 20 °C for about 70 h after dialysis. CD spectra of the stacks of disks in HS-2 and LS-1 buffers did not show differences, consistent with their sedimentation data (Table I and Figure 1). However, surprisingly large spectral differences between 20S aggregates and stacks of disks were observed, as shown in Figure 5, in the near-UV CD spectra of these species in LS-1 buffer (see also Table I). From the figure, we observe that the intensities of all the bands except 252 nm of 20S aggregates are considerably reduced and the shape of the spectrum is also very different from that of stacks of disks. From the data in Table I, it may be seen that $[\theta]_{252}/[\theta]_{272}$ is 1.5 for 20S aggregates as opposed to 0.9 for the stacks of disks. In addition, $[\theta]_{280} - [\theta]_{295}$ is 90 deg·cm²·dmol⁻¹ for the former and 148 deg·cm²·dmol⁻¹ for the latter. In particular, the differences in the intensities of the bands at 272, 280, 290, and 295 nm in the CD spectra of the two aggregating species suggest that the Tyr and Trp residues show more ordering in the stacks of disks than in the 20S aggregates. These spectral differences between the stacks of disks and 20S aggregates did not result from solvent or temperature effects since these conditions are the same for the two sets of spectra.

Throughout our argument we have assumed, as have others, that the mixture of large aggregates observed in solution conditions close to those used for crystallization of disk aggregates represents stacks of disks. If the disk structure in the crystalline state is preserved in stacks of disks in the same solution conditions, then the CD spectral differences we observe between the 20S aggregates and stacks of disks suggest significant structural differences in the two forms of TMVP.

Recent sedimentation equilibrium data (Correia et al., 1981, 1985; Correia, 1981) have provided accurate molecular weight determinations for the equilibrium 20S species at pH 7.0 and 20 °C (Durham et al., 1971) and the metastable 20S species at pH 6.5 and 6.5 °C (Shire et al., 1979). Correia et al., (1985) have determined the number of subunits in 20S aggregates to be 39 ± 2. This stands in contrast to 34 subunits present in the crystalline disk aggregates (Finch et al., 1974; Bloomer et al., 1978) and indicates a nondisklike structure for the 20S aggregates in solution, conditions used for virus assembly. We suggest a helical structure just larger than two turns for the 20S aggregates, on the basis of the near-UV CD spectroscopy and sedimentation velocity data presented in this paper and the supporting data of Schuster et al. (1980) and Correia et al. (1985). Although the secondary structure and folding of the TMVP subunit are essentially unchanged in the crystalline disk and the helical rod packing arrangements (Bloomer et al., 1978; Stubbs et al., 1979; Mandelkow et al., 1981), there are differences in the axial subunit contacts. It is presumably these differences which result in the different side-chain environments reflected in the near-UV CD spectra reported here.

Summary and Conclusions

TMVP aggregates in high ionic strength buffers (I = 0.7), close to the conditions for the crystallization of disk aggregates,

exist as a mixture of large aggregates, presumably stacks of disks, with $s_{20,w} > 30$ S. Aggregates sedimenting at 20 S are absent under these conditions. Once formed, these stacks are very stable and slowly depolymerize when the pH, ionic strength, and temperature of the solution are varied but undergo complete, reversible depolymerization when treated with acetic acid. In relatively lower ionic strength solutions (I = 0.4), 4S and 8S aggregates predominate over higher aggregates at 5 and 20 °C, respectively. Near-UV CD spectra of stacks of disks show flexibility around Phe residues and ordering of Tyr and Trp chromophores. In particular, the ratio of $[\theta]_{252}$ to $[\theta]_{272}$ distinguishes stacks of disks (<1) from the other aggregates with 4–8 S as the major boundary (>1).

Near-UV CD spectra of the stacks of disks in high salt at pH 8.0 and low salt at pH 7.0 are identical with each other but exhibit striking differences when compared to the spectrum of 20S aggregates in low salt. $[\theta]_{252}/[\theta]_{272}$ for 20S species is as high as 1.5 and $[\theta]_{280} - [\theta]_{295}$ as low as 90 deg·cm^2·dmol^{-1} when compared to 0.9 and 148 deg·cm^2·dmol^{-1}, respectively, for the stacks of disks. These differences indicate structural differences between the two aggregated species. Our results suggest that the structure of disks crystallized from high-salt conditions may differ significantly from that of 20S aggregates in solution, bringing into question the structure (previously assigned to be disks) of the nucleating protein species in virus assembly. Reconstitution studies using stacked disks and TMV–RNA have shown that at 20 °C in LS-1 buffer, stacked disks (as described in Figure 1) do not participate in either the nucleation or the elongation phase of tobacco mosaic virus self-assembly (M. L. Adams and T. M. Schuster, unpublished results). The 20S nucleating species may, in fact, be a helix, not a disk.

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