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Characterization of Crystals of Satellite Panicum Mosaic Virus

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Satellite panicum mosaic virus (SPMV) has been purified from pearl millet and obtained in a variety of different crystal forms, at least two of which appear suitable for high resolution X-ray diffraction analysis. The first is of cubic space group $P4_232$ with $a = b = c = 183.1$ Å and two virus particles in the unit cell. The second is of a primitive orthorhombic space group with $a = 166.1$ Å, $b = 266.7$ Å and $c = 269.1$ Å, with four virus particles in the unit cell. While the cubic crystal has as its asymmetric unit one twelfth of the icosahedron, or five capsid protein subunits, the asymmetric unit of the orthorhombic crystals is an entire particle. The cubic crystals diffract to at least 2.8 Å resolution. We have also succeeded in crystallizing, but not yet characterizing the master virus, PMV.

Keywords: satellite virus; X-ray diffraction; crystallization; panicum

There are three well characterized, protein encapsidated, satellite viruses of plants, each of $T = 1$ icosahedral symmetry (reviewed by Francki, 1985). These are satellite tobacco necrosis virus (STNV†; Kassanis, 1962) satellite tobacco mosaic virus (STMV; Valverde & Dodds, 1986; Dodds, 1991), and satellite panicum mosaic virus (SPMV; Masuta *et al.*, 1987). These have as their master viruses tobacco necrosis virus (TNV), tobacco mosaic virus (TMV), and panicum mosaic virus (PMV; Niblett & Paulsen, 1975), respectively. The host plants for the first two master-satellite pairs are chiefly tobacco, while that for the last pair is millet. In every case, the satellite virus can replicate in the host cell only when it is co-infected with its master virus upon which it relies for enzymatic assistance (Francki, 1985).

The structures of both STNV (Liljas *et al.*, 1982), which has a genome size of 1239 nucleotides and a capsid protein molecular mass of 21,000 Da, and STMV (Larson *et al.*, 1993a), of genome size 1059 bases and capsid protein molecular mass of 17,500 Da, have been determined by X-ray crystallography to high resolution (Jones & Liljas, 1984; Larson *et al.*, 1993b). In the latter case, segments of double helical genomic RNA were observed associated on the interior of the capsid with each pair of dyad related proteins. Each RNA segment contained seven base-pairs plus unpaired 3'

terminal nucleotides at either end (Larson *et al.*, 1993a).

SPMV (Niblett & Paulsen, 1975) is the smallest of all of the encapsidated satellite viruses (Buzen *et al.*, 1984; Masuta *et al.*, 1987). It has a genome length of 850 nucleotides and a capsid protein of 157 amino acid residues, two less than for STMV. It shows no amino acid or nucleotide sequence homology with either STMV, STNV, or its own master virus, PMV. Furthermore, it shows no immunological cross reactivity with any of those viruses. Nonetheless, experience would suggest that it may be structurally similar to the other satellite viruses and that the organization of its RNA within the capsid might be similar as well.

Pearl millet was grown from seed in a temperature controlled greenhouse and infected mechanically with a mixed inoculum of PMV and SPMV approximately three weeks after planting. After three additional weeks, the leaves exhibited strong symptoms of infection and were harvested. From about one kilogram of millet stems and leaves, nearly one and a half grams of the crystalline satellite virus could be obtained using a modified procedure of Masuta *et al.* (1987). The major alteration to the purification procedure was the replacement of the sucrose gradient centrifugation step by PEG-NaCl fractionation. Precipitate was redissolved in 0.1 M phosphate buffer (pH 7.0; 1 ml per µg of tissue). At this point the preparation contained a significant excess of SPMV over PMV as shown by SDS-PAGE. The concentration of the solution, that contained both viruses as well as 0.1 M phosphate buffer (pH 7.2), was slowly raised to 6% (w/v) PEG 8000 by adding 0.25 volume of 30% PEG 8000 and

† Abbreviations used: STNV, satellite tobacco necrosis virus; SPMV, satellite panicum mosaic virus; STMV, satellite tobacco mosaic virus; PMV, panicum mosaic virus; TNV, tobacco necrosis virus; TMV, tobacco mosaic virus; PEG, polyethylene glycol.

allowing it to stand at 4°C for 24 hours. This resulted in the precipitation, in amorphous form, of virtually all of the PMV. Following centrifugation of the PMV, the supernatant concentration of PEG 8000 was increased to 10% (w/v) and NaCl was added to a final concentration of 0.2 M. After 4 hours at 4°C the microcrystalline precipitate of SPMV was recovered by centrifugation. Both the PMV and the SPMV fractions were judged to be better than 95% pure, based on silver stained SDS-PAGE gels.

Crystals were mounted in quartz capillaries by conventional techniques and diffraction photographs recorded on Buerger precession cameras of crystal to film distance 90 mm. The X-ray source was an Enraf-Nonius Diffractis generator with conventional sealed tube operated at 40 kV and 32 mA. Crystals were subsequently examined on a San Diego Multiwire Systems multiwire area detector system with a Rigaku RU-200 generator fitted with a Supper graphite crystal monochromator as X-ray source. Using the multiwire detector system the unit cell dimensions were confirmed and refined.

Crystals of SPMV were grown under a wide variety of conditions ranging from pH 4.5 to 8.5 and using both ammonium sulfate and PEG as precipitating agents. Two crystal forms, however, demonstrated particular promise as subjects for a high resolution structure analysis based on their size, quality and diffraction properties. These crystals were grown by vapor diffusion methods using glass depression plates in plastic sandwich boxes (see McPherson, 1982) at 4°C over a period of about one month. The reservoir solution was 37% saturated ammonium sulfate in water. The sample droplets were composed of 10 µl of a 10 mg/ml virus solution (buffered with 20 mM potassium phosphate) plus 10 µl of the reservoir. These crystals can also be grown in Crychem sitting-drop plates (Charles Supper Co., Natick, MA) from 30% saturated ammonium sulfate in water. The habit was that of a cube and the maximum size of the crystals was 0.7 mm on an edge.

The observed diffraction pattern from the crystals of cubic habit could be indexed on a lattice consistent with a primitive unit cell of dimensions $a = b = c = 183.1$ Å. From photographs and from three-dimensional multiwire area detector data the reciprocal lattice was shown to possess 432 symmetry with $00l \neq 2n$ systematically absent. This is consistent only with cubic space group $P4_232$. The volume of the unit cell is 6.18×10^6 Å³ which can accommodate two icosahedral virus particles. This cell, having a volume to mass ratio of $V_m = 2.5$ contains an amount of solvent somewhat less than the orthorhombic crystals of STMV studied earlier (Larson *et al.*, 1993b). This further implies that the virus particles must be centered at 23 symmetry points within the unit cell. Thus the asymmetric unit of the crystals is one twelfth of the virus particle, and this includes five capsid protein subunits plus whatever associated nucleic acid might

satisfy icosahedral constraints. These crystals diffract strongly and highly redundant data to 3 Å resolution has been collected. We believe that when the crystals are grown larger they will diffract at least as well as the STMV crystals used in our earlier analysis (Day & McPherson, 1992).

The second crystal form had the morphology of thick plates with beveled edges, sometimes reaching maximum dimensions of greater than 0.7 mm with thickness of 0.2 mm. These crystals were grown by vapor diffusion methods using glass depression plates in plastic sandwich boxes (see McPherson, 1982) at 23°C over a period of about ten days. The reservoir solution was 30% saturated ammonium sulfate in water. The sample droplets were composed of 8 µl of an 8 mg/ml virus solution buffered with 0.1 M cacodylate (pH 6.8) plus 8 µl of the reservoir.

The diffraction pattern from these crystals could be indexed on a primitive orthorhombic lattice consistent with real space dimensions of $a = 166.1$ Å, $b = 266.7$ Å and $c = 269.1$ Å. At this point we could not assign a space group unequivocally due to the large cell dimensions. If it is assumed that there are four virus particles in each unit cell of volume 12.58×10^6 Å³, then the volume per particle would be almost exactly the same as for the cubic crystals above. This means that the four virus particles occupy general positions within the unit cell and that the asymmetric unit of the crystals must be an entire particle or 60 capsid protein subunits plus associated nucleic acid. The diameter of the virus, as judged from electron micrographs (Francki, 1985; Masuta *et al.*, 1987), is 170 Å; hence, the unit cell can be only one virus particle thick along the a direction. These orthorhombic crystals do not appear to exhibit the diffraction quality of the cubic crystals.

Crystals of PMV were obtained after resuspending the virus pellet in water and centrifuging at low speed to clarify. Vapor diffusion yielded small octahedral crystals of about 0.1 mm maximum dimension in both Crychem plates and glass depression plates contained in plastic boxes (McPherson, 1982). The microdrops were formed by combining 8 µl of PMV (15 mg/ml) and 2 µl of 0.1 M phosphate buffer (pH 7.4) with 10 µl of the precipitant solution. The precipitant solutions producing the best results to this point are 3.5% (w/v) PEG 8000, 4% (w/v) PEG 20,000, or 30% saturated ammonium sulfate. All crystals were grown at room temperature over 2 to 10 days.

Presently, the PMV crystals have not been characterized because of their small size. We are optimistic, however, that this problem will be overcome and that we can proceed with the three-dimensional structure analysis of the master virus as well as its satellite.

We are in the process of undertaking a full three-dimensional structure analysis of both forms of the SPMV crystals we have described here using molecular replacement approaches combined with phase averaging and extension. Given the likelihood

that SPMV and STMV will bear a reasonable resemblance to one another, we anticipate that these methods will prove successful. We are further hopeful that the structure of SPMV, combined with the results obtained in the STMV analysis, will allow us to define in more detail the conformation and distribution of the genomic RNA within the virus particle.

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