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Metal-free Southern Bean Mosaic Virus Crystals*

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Native southern bean mosaic virus contains a significant number of Mg^{2+} and Ca^{2+} ions. These can be removed by treatment with EDTA causing the virus to swell by 7% in radius at alkaline pH values. The swollen virions are susceptible to protease and nuclease digestion. They are likely to be an intermediate during assembly and disassembly.

Crystals of the metal-free virus have been grown and were found to be approximately isomorphous with the orthorhombic type III southern bean mosaic virus crystals (Akimoto, T., Wagner, M. A., Johnson, J. E., and Rossmann, M. G. (1975) *J. Ultrastruct. Res.* 53, 306-318), although the cell dimensions are longer by 2%. Native rhombohedral type II crystals disintegrate on changing the pH or increasing the ionic strength of the mother liquor. Damage can be prevented by addition of ethylene glycol. At alkaline pH values, these crystals also show a 2% increase in their cell dimensions as well as a significant alteration in their diffraction patterns.

In the type II and III crystals, the viruses pack with only their 5-fold axes in contact. Thus, the difference of the apparent swelling in solution and in the crystals may be one of differential swelling over the virus surface.

Atomic absorption spectroscopy has shown that there are approximately 120 magnesium and 80 calcium ions firmly bound to each native southern bean mosaic virus particle (Hsu *et al.*, 1976), although Hull (1978) found 1 well-bound Ca ion per subunit or 180 per virion. It is difficult to disassemble the virions without first removing these divalent cations (Hsu *et al.*, 1976). The resistance of the virions to disassembly by sodium dodecyl sulfate (Sehgal, 1973) suggests that a major stabilizing effect is generated by protein-protein interactions (Kaper, 1973), apparently mediated by cations which probably form bridges between carboxylate ions of adjoining protein subunits (Bancroft, 1970). Durham and co-workers (Durham, 1977; 1978; Durham *et al.*, 1977) have suggested that the calcium ion concentration in cytoplasm ($\simeq$1 μM) as compared to that in the extracellular fluid ($\simeq$1 mM) is likely to be a contributing factor to the disassembly of the virus on entering the cell.

SBMV[1] is an isometric virus of average diameter 284 Å (Johnson *et al.*, 1976) with a particle mass of 6.6×10^6 daltons (Yphantis, 1964). The RNA, which is encapsulated by the protein coat, comprises 21% of the total particle weight (Ghabrial *et al.*, 1967). The protein coat contains 180 subunits each of approximately 28,500 daltons (Tremaine, 1966; Ghabrial *et al.*, 1967; Sehgal and Sinha, 1974) implying a $T = 3$ icosahedral surface lattice (Caspar and Klug, 1962). The native virus was first crystallized by Magdoff (1960). Three additional crystalline forms of the cowpea strain have been reported (Akimoto *et al.*, 1975). The rhombohedral type II crystals have been the subject of an extensive study resulting in a 5-Å resolution electron density map (Suck *et al.*, 1978).

On treatment of SBMV with EDTA (Wells and Sisler, 1969; Brown and Hull, 1973), the sedimentation coefficient decreases from 115 S to 100 S, a phenomenon which has been interpreted as a swelling of the virions (Hsu *et al.*, 1977; Sehgal and Sinha, 1974). The swollen virus loses essentially all trace of metal ions (Hsu *et al.*, 1976) and becomes sensitive to protease and nuclease attack. The swelling process is reversible on addition of both calcium and magnesium, with full integrity and infectivity restored to the particle (Hsu *et al.*, 1976). Furthermore, in the presence of 0.4 M Cl^- ions at pH 7.5, the virus disassembles into two subviral entities, one of which contains protein particles with a diameter of approximately 130 Å,[2] while the other is composed of protein-RNA aggregates (Hsu *et al.*, 1977; Sehgal and Hsu, 1976). Sehgal and co-workers (Hsu *et al.*, 1977; Sehgal and Hsu, 1976) have suggested that the RNA is associated only with the pentamers at the 5-fold vertices of the virus particle. Hull (1977) has also studied the swelling of the SBMV-like viruses and concluded that the protein coat is stabilized by divalent cations, a pH-dependent protein-protein interaction, and salt links between protein and RNA.

EXPERIMENTAL PROCEDURES

The Swollen, Metal-free Virus in Solution—The SBMV (cowpea strain) was isolated from leaves of the cowpea plant (*Vigna unquiculata* L.) by a modification (Johnson *et al.*, 1974) of the procedure of Ghabrial *et al.* (1967). Virus at a concentration of 10 to 20 mg/ml was dialyzed against distilled water for 12 h at room temperature. Dialysis was then performed at room temperature for 24 h against 0.05 M phosphate buffer at pH 7.5, containing 0.04 M EDTA. High molecular weight impurities were removed by a low speed centrifugation (27,000 $\times$ *g*) for 15 min. Finally, the swollen virus was sedimented by ultracentrifugation (39,000 rpm in a Beckman type 40 rotor for 2 h). The virus was resuspended in 0.02 M phosphate buffer containing 0.01 M EDTA at pH 7.5 and stored at 4°C. Care had to be taken to avoid touching the dialysis bags and glassware, thus avoiding contamination with bacteria and ribonuclease which degrade the swollen virus. Addition of 0.001 M sodium azide was also useful to inhibit bacterial growth.

The swelling of the virus was confirmed by use of the analytical ultracentrifuge and found to be consistent with Sehgal's (Hsu *et al.*, 1977; Sehgal and Sinha, 1974) results. Atomic absorption spectros-

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[1] The abbreviations used are: SBMV, southern bean mosaic virus; PEG, polyethylene glycol.

[2] I. Rayment, unpublished results based on solution x-ray scattering.

copy[3] showed the absence of calcium and magnesium to better than 1 part in 10^6. In addition, small angle x-ray scattering was used to check on the viral diameter.[4] At least seven orders of diffraction could be observed from solutions containing approximately 250 mg/ml of virus. The results are shown in Table I. The native, unswollen virus diameter is consistent with the value of 284 Å determined from type I single crystals (Johnson *et al.*, 1976). However, the metal-free virus is swollen only at high pH. This result compares to the swelling of cowpea chlorotic mottle virus (Bancroft *et al.*, 1967; 1968) which swells by 7% above pH 7, although, in this case, the removal of metal ions is not a prerequisite for swelling.

Crystals Grown from Metal-free Virus Solution—Crystals were grown by vapor diffusion (*cf.* McPherson, 1976). The crystallization droplets were prepared by placing 30 μl of virus solution together with a given volume (Table II) of M_r = 6000 PEG solution. These were equilibrated against a PEG solution. When PEG was added to the virus, it was of the same concentration as in the equilibrating solution.

Octahedrally shaped crystals could occasionally be grown at pH 4.5 to 7.5 using 15 mg/ml of virus with an equal proportion of PEG solution equilibrated against 8% PEG solution. More reproducible orthorhombic crystals were grown from higher virus concentration (Table II). At pH 7.5, these crystals were very thin, but at a more acidic pH, they had greater thickness. Better crystals, unfortunately at low pH, showed good birefringence with well developed {001} and {110} faces. Bragg maxima could be observed on "stills" to 4 Å resolution using an Elliott rotating anode x-ray generator and perpendicular focusing mirrors (Harrison, 1968). The crystals were very sensitive to radiation damage with a lifetime of 4 h at most. This effect was somewhat alleviated by cooling the crystals to 4°C with a cold air stream. Under these conditions, their life was extended to between 8 and 12 h. It was then possible to take precession photographs (μ = 1° 30′) for all three major zones. The space group of crystals grown at pH 6 was found to be approximately isomorphous with orthorhombic native type III SBMV crystals. The unit cell had dimensions of a = 552 ± 1 Å, b = 341 ± 1 Å, c = 551 ± 1 Å, representing less than a 2% increase over the native crystals (a = 541 ± 1 Å, b = 334 ± 1 Å, c = 541 ± 1 Å).

The Treatment of Native Rhombohedral Type II Crystals with EDTA—Due to the difficulty encountered in growing crystals of the metal-free virus at high pH, attempts were made to modify the virus within the crystal lattice. However, type II crystals can only be grown to useful size in the pH range of 5 to 6 and modification of pH causes them to dissolve. In addition, dialysis of the crystals against concentrations of EDTA greater than 0.05 M resulted in the crystals cracking and dissolving. Fortunately, it was possible to alter the pH of the crystals and to introduce EDTA after careful treatment with ethylene glycol. The treatment consisted of successive dialysis against 10, 10, 15, 20, and 25% solutions of ethylene glycol for 24 h buffered with 0.01 M phosphate and containing 0.01 M EDTA, giving solutions at pH 5.5, 6.5, 7.0, 7.5, and 8.0, respectively. These crystals diffract to at least 4.0 Å resolution as compared to 2.8 Å for the type II native form.

The cell dimensions of the EDTA-treated crystals at pH 8.0, measured on μ = 1° 30′ precession photographs, were a = 339 ± 1 Å and c = 780 ± 1 Å. Native crystal cell dimensions were found to be a = 334 ± 1 Å and c = 765 ± 1 Å on films taken with the same camera to avoid uncertainties of crystal-to-film distances. Again, this is only a 2% increase in size. However, the small angle scattering of the mother liquor, surrounding the ethylene glycol and EDTA-treated crystal at pH 8.0, showed a 7% increase in diameter over native virions (Table I).

Powder diagrams of crushed crystals were studied to investigate the difference between these two results. Such a photograph shows a radial average of intensities in reciprocal space without resolving individual diffraction maxima. The pattern obtained is thus similar to that obtained from small angle solution scattering and can be used to measure the mean viral diameter in the crystal lattice. There was a 4% increase in viral diameter for ethylene glycol/EDTA-treated crystals at pH 8.0. Furthermore, the diffraction of the ethylene glycol/EDTA-treated crystals showed significant differences from that of the native crystals.

TABLE I

Virus diameter determined from small angle x-ray scattering

pH	EDTA-treated	Approximate concentration	Diameter
		mg/ml	*Å*
7.5	no	250	287.9 ± 1.8
7.5	yes	250	306.5 ± 2.1
4.0	yes	250	290.2 ± 2.3
8.0	yes[a]	70	309.0 ± 1.7

[a] Solution scattering from the mother liquor surrounding the type II crystals treated with a 25% solution of ethylene glycol and 0.01 M EDTA.

TABLE II

Conditions for crystallizing swollen SBMV

pH	Maximum virus solubility	Virus concentration	Buffer	Virus:PEG	PEG[a] concentration	Approximate crystal shape
	mg/ml				%	*mm*
7.5	>160	32	0.02 M phosphate	3:1	8	1 × 1 × 0.2
6.5		40	0.02 M phosphate	3:1	6	1 × 1 × 0.2
5.0		55	0.02 M phosphate	1:0[b]	3	0.8 × 0.8 × 0.3
4.0	≃50	50	0.02 M citrate	1:0[b]	2.5	1 × 1 × 0.6

[a] Concentration of PEG was the same in the equilibrating solution and the solution added to the virus sample.

[b] No PEG in virus solution. Use of PEG here is entirely for the purpose of concentrating the latter through vapor diffusion.

RESULTS AND DISCUSSION

Akimoto *et al.* (1975) showed that the virus particles are in contact at their 5-fold vertices and are separated by 318 Å along this axis for both the type II and III crystal forms. Thus, the particles are not spherical but protrude at their 5-fold vertices and are flattened at their quasi-3-fold axes (Suck *et al.*, 1978). In measuring radii with small angle x-ray scattering or from powder photographs a mean radius is obtained. In contrast, cell dimensional changes observed in the type II and III crystals measure the maximum diameter of the virions. Since the latter is changed by only 2%, it must be concluded that the virus becomes almost spherical on swelling. Such a large conformational change is supported by the intensity changes of the single crystal diffraction pattern. Nevertheless, it is possible that such changes might be caused in part by alteration in the electron density of the solvent corresponding to the "salt effect" observed by Perutz (1954). Although the type III crystals do show a small cell dimension change at pH 4.0, the low angle x-ray-scattering results would suggest that the virus is not swollen at acidic pH values.

The 80 and 120 Ca^{2+} and Mg^{2+} sites per virion, respectively (Hsu *et al.*, 1976), together with the crystallographic structure determination (Suck *et al.*, 1978), permits speculation as to their position and role within the particle. It was found that uranyl acetate substitutes well at a position on the quasi-3-fold axis possibly corresponding to 60 calcium sites. These observations are supported by noting that the protein recovered after disassembling the swollen virus with KCl exists as trimers in the presence of Ca^{2+}, whereas a higher aggregation state predominates in the presence of magnesium.[2] Thus, removal of calcium might permit the separation of the closely associated trimers around the quasi-3-fold axes (Suck *et al.*, 1978) and hence cause the virus to become more spherical.

Further studies will be directed at collecting 3.5-Å resolution data for the pH 8.0 ethylene glycol/EDTA-treated crystals. A difference electron density map with respect to the native

[3] S. S. Abdel-Meguid, personal communication.

[4] K. Fricks and D. L. D. Caspar, personal communication.

data may throw light on the conformational changes incurred on removing metal ions from the virus.

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