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Crystallization and Preliminary X-ray Analysis of Three Serotypes of Foot-and-Mouth Disease Virus

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Foot-and-mouth disease viruses from serotypes O, A and C have been crystallized. The particular strains studied include O_1K, $A10_{61}$, A22 Iraq 24/64, A24 Cruzeiro and C-S8c1. In addition, crystals have been grown of G67, a monoclonal antibody neutralization escape mutant derived from O_1K, and of virus R100, recovered after the establishment of a persistent infection in baby hamster kidney cells with C-S8c1. Empty particles, capsids which lack the RNA genome, have also been crystallized for subtypes A22 Iraq 24/64 and $A10_{61}$. In almost all cases, crystals suitable for high resolution structure determination were obtained from $(NH_4)_2SO_4$ or mixtures of polyethylene glycol and NH_4Cl.

Keywords: foot-and-mouth disease virus; empty particles; virus crystallography; crystallization; X-ray diffraction

Structural studies of viruses hold the promise of insights into a number of distinct and fascinating questions. These include the mechanisms of antigenic variation, the interactions critical to the construction and stabilization of large macromolecular assemblies, the structural bases of phenotypic changes and the mechanisms of attachment to cellular receptors. We propose to investigate these topics in a study of foot-and-mouth disease virus (FMDV§). This virus is the causative agent of foot-and-mouth disease, an economically important disease of cloven hoofed animals, especially cattle. FMDV, which constitutes the genus *Aphthovirus* in the Picornaviridae family, is a small icosahedral virus of diameter 295 Å (1 Å = 0·1 nm) composed of a single-strand positive sense RNA molecule (M_r = $2{\cdot}6 \times 10^6$) and 60 copies of each of four polypeptides (VP1 to VP3, $M_r = 24{,}000$; VP4, $M_r = 9000$); a small number of VP2 and VP4 molecules are present as the uncleaved precursor VP0.

The crystal structure of one subtype of FMDV, O_1BFS1860, has already been determined (Acharya *et al.*, 1989). Here, we report the crystallization and preliminary X-ray analysis of viruses spanning three of the seven serotypes of FMDV. The viruses studied include field isolates and laboratory generated mutants; we have also produced crystals of naturally occurring empty particles (capsids lacking the RNA genome). The field strains crystallized are O_1K (O serotype), C-S8c1 (C serotype) and three subtypes of the A serotype: $A10_{61}$, A22 Iraq 24/64 and A24 Cruzeiro. Virus O_1K is very closely related to O_1BFS1860; there are only six amino acid residue substitutions in the capsid region out of a possible total of 736 (Kitson *et al.*, 1990). About 10% of the amino acid residues in the capsid differ between the three viruses of serotype A; between the three serotypes O, A and C capsid sequence differences are around 30% (Palmenberg, 1989). Experiments to generate and sequence mutants which escape neutralization by selected monoclonal antibodies (MAbs) have mapped the location in the primary structure of antigenic sites on most of the viruses in this study (O_1K: McCahon *et al.*, 1989; Kitson *et al.*,

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§ Abbreviations used: FMDV, foot-and-mouth disease virus; MAb, monoclonal antibody; BHK, baby hamster kidney; PEG, polyethylene glycol; DTT, dithiothreitol.

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1990; $A10_{61}$: Thomas *et al.*, 1988; A22 Iraq 24/64: Bolwell *et al.*, 1989; C-S8c1: Mateu *et al.*, 1990). Three-dimensional structural information should reveal the conformational differences underlying antigenic variation within and between serotypes. Studies of the structural differences between human rhinovirus serotypes 14 and 1A (Kim *et al.*, 1989) and between poliovirus P1/Mahoney and P3/Sabin (Filman *et al.*, 1989) have already illustrated some of the strategies adopted by these viruses in order to evade immune detection. Significantly, the latter investigation also led to the identification of features in poliovirus which could not be interpreted confidently in the first structure. It seems probable, therefore, that comparison of FMDV structures will extend our understanding not only of the structural basis of serotype specificity but also of previously unseen features of the virion.

Of particular interest in FMDV is the structure of the so-called "FMDV loop", a stretch of amino acids from residues 133 to 158 of VP1 exposed on the surface of the virion. This loop, which is disordered in the structure of O_1BFS1860 (Acharya *et al.*, 1989), is a major antigenic site (see Kitson *et al.*, 1990) and is thought likely to contain the receptor attachment site (Fox *et al.*, 1989). A disulphide bond which modulates the conformation of the FMDV loop (Parry *et al.*, 1990; D. Logan *et al.*, unpublished results) is only found on O_1 subtypes. Inevitably, therefore, the question arises as to the conformation of this important feature in A and C serotype viruses. A further point of interest in the $A10_{61}$ subtype is that in this virus the RGD sequence, found within the FMDV loop in almost all sequenced FMDVs and implicated in cell attachment, is replaced by RSGD (Carroll *et al.*, 1984).

We have also crystallized a multiple site mutant derived from O_1K, G67, which contains substitutions at each of the four antigenic sites on the virus identified by Kitson *et al.* (1990) and escapes MAb neutralization at these sites. We hope to characterize the structural consequences of these substitutions and compare the effect of natural and *in vitro* generated antigenic variation.

An important problem associated with FMDV is the persistence of viral infection in the upper respiratory tract of animals which have apparently recovered from the symptoms of the disease (Van Bekkum *et al.*, 1959). Such carrier animals represent a reservoir of virus which can give rise to antigenic variants (Hedger, 1968). In a laboratory study of this phenomenon, a virus (R100), isolated after 100 passages of C-S8c1 in a persistently infected baby hamster kidney (BHK) cell line, was found to contain multiple mutations throughout the genome and to be antigenically distinct from the parent, even though it had not been subjected to the selection pressure of an immune system (Díez *et al.*, 1990). Several mutations were identified in the capsid region which had never been seen previously in FMDV (Díez *et al.*, 1990). We have crystallized R100 and hope to illuminate the rôle of these novel substitutions.

Assembled capsids lacking the RNA genome, known as empty particles, can be isolated from BHK cells infected with FMDV, especially when the infection is due to an A serotype virus. (This latter observation may be due to the apparently greater thermal stability of A serotype viruses over other serotypes (Doel & Baccarini, 1981). A comparison of the structures of A serotypes with O and C viruses may, therefore, uncover the interactions which are important for stabilization.) Empty particles normally lack not only the genomic RNA but also a maturation cleavage of the precursor protein VP0 into VP2 and VP4. For picornaviruses, the mechanism of this cleavage is thought to be autocatalytic, but remains to be properly elucidated (Acharya *et al.*, 1989; Harber *et al.*, 1991). The cleavage appears to enhance the stability of the virion. Recent structural results with poliovirus show that it releases the N terminus of VP2 to form part of an extended β-sheet across the interface between two pentameric subunits (Flore *et al.*, 1990). We have crystallized empty particles of FMDV in order to investigate the function of VP0 cleavage in virion assembly and stability, and the more general rôle of RNA in modulating the internal structure of the capsid.

Crystallization trials were performed using virus material which was the fourth, fifth or sixth passage (in bovine thyroid or BHK monolayers) of plaque-purified clones. Parental O_1Kaufbeuren (Xie *et al.*, 1987) and the monoclonal antibody escape mutant derived from it, G67 (Kitson *et al.*, 1990), were supplied by Dr G. Belsham. Monolayer cell-adapted A22 Iraq 24/64 (clone 162-154: Bolwell *et al.*, 1989) was supplied by Dr E. Ouldridge (Pittman-Moore). A24 Cruzeiro (Brazil 55), a gift from Pfizer, and $A10_{61}$ were plaque-purified at Pirbright before use in crystallization experiments. The C serotype virus C-S8c1 and R100, the variant isolated after the establishment of a persistent infection in BHK cells (Díez *et al.*, 1990), were the gift of Dr E. Domingo.

Viruses were grown on BHK-21 cell monolayers. Upon attaining >90% cytopathic effect, the monolayer was detached by shaking; the cells and medium were pooled and then cooled to 4 °C. For preparations of O and A serotype viruses, the cells were lysed by addition of Triton X-100 to a final concentration of 0·1% (w/v). Purification of the virus and empty particles followed methods very similar to established protocols (Brown & Cartwright, 1963; Rowlands *et al.*, 1975). The most significant difference was that the sucrose gradient centrifugation of R100 was done in the presence of 1·5 M-NaCl to prevent aggregation (Díez *et al.*, 1990). Virus recovered from sucrose gradients was concentrated by centrifugation as described by Fox *et al.* (1987). The high salt concentrations used for R100 were maintained in the process. Sucrose gradient fractions containing serotype A empty particles were pooled and an equal volume of 100% saturated $(NH_4)_2SO_4$ at pH 7·6 added. The mixture was left for 48 hours at 4 °C and precipitate collected by centrifugation at 2600 *g* for 60 minutes. The empty capsids were resuspended at a concentration

of 10 mg/ml in 0·75% $(NH_4)_2SO_4$, 20 mM-Tris, 50 mM-sodium phosphate (pH 7·6) and stored at 4 °C (Fox *et al.*, 1987).

Analysis by electrophoresis on discontinuous polyacrylamide gels (Laemmli, 1970) of FMDV empty capsids of all three A serotype viruses included in this study revealed, surprisingly, that a significant degree of cleavage of VO0 into VP2 and VP4 had occurred. Both VP2 and VP4 remained associated with the empty capsid. This result contrasts with some published reports on FMDV (Rowlands *et al.*, 1975; Doel & Chong, 1982) and poliovirus (Jacobson & Baltimore, 1968) which found that only a negligible amount of VP0 cleavage occurs in empty capsids. However, other published evidence suggests the possibility of appreciable VP0 cleavage in empty capsids of both these picornaviruses (Rweyemamu *et al.*, 1984; Maizel *et al.*, 1967). Typically, our experiments indicated that at most, only 20% of the VP0 molecules initially present remained uncleaved. The reason for this observation remains unclear but is under investigation.

Trypsin-treated O_1BFS1860 was prepared as described (Fox *et al.*, 1987).

All stock solutions used in crystallization trials were filtered through a 0·2 μm filter and contained 3 mM-NaN_3. A 100 mM-sodium phosphate buffer (pH 7·6) was used throughout and should be assumed to be present in all the solutions described below (except where indicated otherwise). Virus and empty particle concentrations of 5 to 20 mg/ml were used in the trials. Particulate matter was removed from virus samples by microcentrifugation immediately before use. Crystallization trials were carried out by vapour diffusion (sitting drop) and microdialysis methods. In sitting drop experiments, performed using microbridges supplied by Crystal Microsystems (Harlos, 1992), typically 5 to 10 μl of virus was mixed with an equal or smaller volume of the precipitating solution from the well. The method of microdialysis has been described (Fox *et al.*, 1987).

Diffraction experiments were carried out at the Science and Engineering Research Council's Synchrotron Radiation Source (SRS) facility at Daresbury, Cheshire, England. Experiments were performed at a constant temperature, usually 21 °C, using radiation from the 5T wiggler magnet on stations 9.5 and 9.6, with the machine operating at 2 GeV and 100 to 300 mA. The wavelength used was 0·89(±0·01) Å. The crystals were mounted in quartz capillary tubes (in accordance with agreed disease security protocols). All data collected on station 9.6 were recorded photographically with an Arndt-Wonacott rotation camera using CEA X-ray film. These data were collected using the American method (Rossmann & Erickson, 1983), since most crystals survived only one or two exposures in the beam. On station 9.5, data were collected on a Marresearch Hendrix-Lentfer imaging device. The increased sensitivity of the detector permitted multiple exposures (in the range 5 to 35) from each crystal. Typically, data were collected over a 0·5° oscillation at 1° intervals.

The results of crystallization experiments are summarized in Table 1. All of the O and C type viruses in this study crystallized as rhombic dodecahedra from $(NH_4)_2SO_4$. Crystals of O_1K were grown by microdialysis against 11 to 12% saturated $(NH_4)_2SO_4$. These diffract to 2·6 Å and belong to space group *I*23 (Table 2). Thus, they are essentially identical to crystals of O_1BFS1860 (Fox *et al.*, 1987), which is not surprising given the very high sequence identity between these two viruses. To crystallize G67, 21 to 23% saturated $(NH_4)_2SO_4$ was required. Typically, crystals of G67 (0·4 mm × 0·4 mm × 0·2 mm) are about twice the dimensions of O_1K crystals and were found to be about twice as resistant to radiation damage. Although the two viruses crystallized with identical morphologies, processing of the X-ray diffraction data revealed a

Table 1

Summary of crystallization results

Crystal	Precipitant	Additives	Method[a]	Typical dimensions (mm³)
O_1K	11–12% $(NH_4)_2SO_4$[b]	—	MD	0·2 × 0·2 × 0·1
G67	21–23% $(NH_4)_2SO_4$	—	MD	0·4 × 0·4 × 0·2
C-S8c1	9–12% $(NH_4)_2SO_4$	10 mM-DTT	VD	0·4 × 0·4 × 0·2
R100	13% $(NH_4)_2SO_4$	10 mM-DTT	VD	0·35 × 0·35 × 0·15
Trypsin-treated O_1BFS1860	2·25–2·75% PEG 4000[c]	2 M-NH_4Cl	VD	0·15 × 0·15 × 0·05
$A10_{61}$ virus (type I)	2·5–3·0% PEG 20,000	2 M-NH_4Cl	VD	0·4 × 0·4 × 0·2
$A10_{61}$ virus (type II)	3·0% PEG 4000	2 M-NH_4Cl	VD	0·15 × 0·15 × 0·08
$A10_{61}$ empty	2·5–4·0 M-NH_4COOH	—	MD	0·25 × 0·25 × 0·13
A22 Iraq 24/64 virus	3·0–5·0% PEG 4000	4 M-NH_4Cl	VD	0·3 × 0·3 × 0·15
A22 Iraq 24/64 empty	2·0–3·0% PEG 20,000	4 M-NH_4Cl	VD	0·3 × 0·3 × 0·15
A24 Cruzeiro	4·0–5·0% PEG 4000	4 M-NH_4Cl	VD	0·3 × 0·3 × 0·15

100 mM-sodium phosphate buffer (pH 7·6) used throughout, except for R100 (100 mM-Tris (pH 8·0), or 100 mM-Hepes (pH 7·6)).

[a] MD, microdialysis; VD, vapour diffusion.

[b] Concentrations of $(NH_4)_2SO_4$ are given as the percentage of saturation.

[c] Concentrations of PEG are given as percentage (w/v).

Table 2
Summary of X-ray diffraction analysis

Crystal	Space group	Resolution limit[a] (Å)	Unit cell parameters (Å)	Packing density[b] (Å^3/Da)
O_1K	*I*23	2·6	$a = 345$	2·7
G67	Pseudo *I*432[c]	2·9	$a = 345$	2·7
C-S8c1	*I*23	3·5	$a = 348$	2·8
Trypsin-treated O_1BFS1860	Trigonal/ hexagonal	3·0	$a = 635$ $c = 320$	2·5[d]
$A10_{61}$ virus (type I)	*R*3	3·0	$a = 296$ $\alpha = 62·3°$	2·6
$A10_{61}$ virus (type II)	*I*23	3·4	$a = 347$	2·8
$A10_{61}$ empty	$C222/C222_1$	3·0	$a = 590$[e] $b = 560$[e] $c = 490$[e]	2·7[f]
A22 Iraq 24/64 virus	*I*222	3·0	$a = 328$ $b = 342$ $c = 364$	2·7
A22 Iraq 24/64 empty	*I*222	3·0	$a = 328$ $b = 342$ $c = 364$	2·7
A24 Cruzeiro	$C222/C222_1$	3·5	$a = 950$[e] $b = 700$[e] $c = 500$[e]	2·8[g]

[a] This represents the limit of the data we have collected. In many cases, useful diffraction extends to higher resolution.
[b] Calculated assuming a relative mass of the virion of $7·5 \times 10^6$ Da.
[c] See the discussion in the text.
[d] Assuming 6 virions/unit cell.
[e] Estimated visually from oscillation photographs.
[f] Assuming 8 virions/unit cell.
[g] Assuming 16 virions/unit cell.

peculiar form of disorder in the G67 crystals. In space group *I*23, the reciprocal lattice possesses higher symmetry than the molecular transform which it samples. There are two possible (and distinct) orientations, separated by 90°, for particles possessing icosahedral symmetry in the *I*23 unit cell; for G67 both of these orientations are present within the same crystal, which appears to be constructed of mosaic blocks of *I*23 cells orientated randomly in either of the two ways. The net effect of this disorder is to introduce 4-fold symmetry into the diffraction data which may therefore be processed as space group *I*432. The problem of handling such data will be discussed elsewhere.

A new crystal form of trypsin-treated O_1BFS1860 was obtained in 2·25 to 2·75% (w/v) polyethylene glycol (PEG) 4000, 2 M-NH_4Cl (see Fox *et al.*, 1987). The crystals are hexagonal plates and diffract to around 3·0 Å. The diffraction data indicate a large primitive unit cell with trigonal or hexagonal symmetry (Table 2).

Relatively large crystals of C-S8c1 grew by vapour diffusion from 9·5 to 11·5% saturated $(NH_4)_2SO_4$ only in the presence of 10 mM-dithiothreitol (DTT). However, after around ten days, these crystals turned opaque and lost the ability to diffract X-rays. Data to 3·5 Å were collected and the space group found to be *I*23. Under the conditions used to crystallize C-S8c1 (but with the addition of 1·5 M-NaCl to prevent amorphous aggregation) only small crystals (maximum dimension 0·1 mm) of R100 were produced. These failed to diffract X-rays. More recently, much larger crystals (maximum dimension 0·35 mm) of R100 have been grown simply by using 100 mM-Tris (pH 8·0) or Hepes (pH 7·6), instead of 100 mM-sodium phosphate buffer at pH 7·6. These crystals have yet to be tested for X-ray diffraction. Interestingly, they do not suffer from the rapid degradation observed for C-S8c1 crystals.

Initial crystallization trials on A serotype viruses with $(NH_4)_2SO_4$ produced either amorphous precipitate (A24, A22) or crystals which diffracted only poorly (A10). Amorphous precipitate was also obtained using PEG, either on its own or with NaCl. However, these viruses were found to remain soluble in concentrations of NH_4Cl up to 5 M and mixtures of PEG and NH_4Cl yielded good crystals of all three subtypes and of A22 empty particles. Although the viruses crystallized spontaneously in these solutions, improved yields were obtained in some cases by various seeding techniques.

Virus $A10_{61}$ crystallized as parallelepipeds in solutions containing 2·5 to 3·0% PEG 20,000 and 2 M-NH_4Cl. For seeding experiments, sitting drops containing between 1·8 and 2·6% PEG 20,000 and 2 M-NH_4Cl were left to equilibrate for at least five days before being seeded with a single crystal or fragment. The seed was washed in a solution containing 1% PEG 20,000, 2 M-NH_4Cl until its

edges became very slightly rounded and then stabilized in 3% PEG 20,000 2 M-NH_4Cl before transfer to the pre-equilibrated drop. By this technique, crystals of dimensions around 0·35 mm × 0·35 mm × 0·18 mm were frequently obtained. The maximum observed dimension was 1·1 mm. The crystals diffract to at least 3 Å and belong to space group *R*3. From the unit cell dimensions (Table 2) we expect one virion, centred on the crystallographic 3-fold axis, to be present in each unit cell. Thus, there are 20 protomers in the asymmetric unit yielding a high degree of redundancy for non-crystallographic averaging.

$A10_{61}$ virus also crystallized with a morphology identical to that of O_1BFS1860 crystals in a single drop containing 3% PEG 4000, 4 M-NH_4Cl, reaching dimensions of 0·15 mm × 0·15 mm × 0·08 mm. These crystals diffract to 3 Å and, like the crystals of O and C serotype viruses described above, belong to space group *I*23 (Table 2).

Crystals of $A10_{61}$ empty particles were obtained by microdialysis against 2·5 to 4 M-NH_4COOH. These crystals, which have a completely different morphology to the crystals of whole virions, diffract to about 3·0 Å and were found to belong to a face-centred orthorhombic space group (*C*222 or $C222_1$) with a large unit cell (Table 2).

Crystals of the virus A22 Iraq 24/64 were grown using 3 to 5% PEG 4000, 4 M-NH_4Cl mixtures, but only after accidental displacement of the cover slip had permitted a degree of evaporation from the sitting drop, a result which proved to be easily reproducible. Such crystals were poorly formed, presumably because of over-rapid growth. However, they were a useful source of seeds for microseeding and streak seeding (Stura & Wilson, 1990). Crystals grown from these seeds were used to macroseed further drops when they reached a size of 0·05 to 0·1 mm. Successful macroseeding required careful washing of the seed in 2% PEG 4000, 2·5 M-NH_4Cl until slightly rounded corners appeared and stabilization in 5% PEG 4000, 4 M-NH_4Cl. Ultimately, crystals of maximum dimension of about 0·25 mm were obtained. These diffract to high resolution (3 Å) and are in the space group *I*222 (Table 2). There are two virions in the unit cell (centred at 0,0,0 and $\frac{1}{2},\frac{1}{2},\frac{1}{2}$) yielding 15-fold non-crystallographic symmetry.

Identical crystals of A22 empty particles grew spontaneously in 2 to 3% PEG 20,000, 4 M-NH_4Cl. These conditions were then shown to promote growth of seeds of A22 virus particles prepared as described above. The crystals of empty particles diffract almost as well as those of the virus; they also belong to space group *I*222 and are essentially isomorphous (Table 2).

In only one well, containing virus A24 Cruzeiro mixed with 4% PEG 4000, 4 M-NH_4Cl, was spontaneous growth of single crystals observed. These had a rhombic dodecahedral morphology identical to the O and C serotype crystals described above. Further crystals, of typical dimensions 0·25 mm × 0·25 mm × 0·12 mm, were obtained by streak seeding with a cat whisker (Stura & Wilson, 1990) in wells containing virus pre-equilibrated with 4·5 to 5% PEG 4000, 4 M-NH_4Cl. X-ray diffraction was observed to a resolution limit of around 3·5 Å. Initial analysis indicates a face-centred orthorhombic space group with large cell edges (*C*222 or $C222_1$). The space group and cell parameters of the rhombic dodecahedral crystals appear to be identical to composite crystals of a prismatic habit which were first observed for this virus under the same crystallization conditions. The superior appearance of the single crystal does not, unfortunately, arise from improved internal order.

Structure determination has focused on the most tractable crystal forms and is now complete or well advanced for O_1K, G67, C-S8c1, $A10_{61}$ and for the virus and empty particles of A22 Iraq 24/64. These results will be presented in due course.

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Note added in proof: The R100 crystals described in the paper diffract to a resolution limit of 3·5 Å.