Evidence for the role of His-142 of protein 1C in the acid-induced disassembly of foot-and-mouth disease virus capsids

Fiona M. Ellard,† Jeff Drew,‡ Wendy E. Blakemore,1 David I. Stuart2,3 and Andrew M. Q. King1

1 Pirbright Laboratory, Institute for Animal Health, Pirbright, Surrey GU24 0NF, UK
2 Laboratory of Molecular Biophysics, Oxford University, Oxford OX1 3QU, UK
3 New Chemistry Laboratory, Oxford Centre for Molecular Sciences, Oxford OX1 3QT, UK

Foot-and-mouth disease virus (FMDV) capsids are inherently labile under mildly acidic conditions, dissociating to pentamers at pH values in the region of 6.5, with the release of protein 1A and the viral RNA. This acid-induced disassembly is thought to be required for the entry of the virus genome into the host cell. Previous work has highlighted a histidine–α-helix charge-dipole interaction at the twofold axes of symmetry between pentamers and has suggested that this interaction plays a role in acid-induced disassembly. The validity of this theory has now been tested by converting the implicated residue, His-142 of protein 1C, to Arg, Phe and Asp. The effects of such changes were studied by using a previously described vaccinia virus expression system, in which synthesis and processing of FMDV capsid proteins results in the self-assembly of capsids. In agreement with the histidine–α-helix charge-dipole theory, assembly in the arginine mutant was found to be greatly reduced, while capsids of the aspartic acid mutant were considerably more stable under acidic conditions than the wild-type. Aberrant but acid-stable complexes were obtained in the phenylalanine mutant.

Introduction

Infection of cells by foot-and-mouth disease virus (FMDV) is inhibited by weak bases and ionophores that block acidification of endosomes, implying that FMDV, like many other viruses, uses endosomal acidification to trigger uncoating (Carrillo et al., 1984, 1985; Baxt, 1987). For enveloped viruses such as influenza virus, it is well known that the lowering of the pH causes alterations in the haemagglutinin protein leading to the fusion of the viral envelope with the endosomal membrane, thereby liberating the infectious core into the cytosol. However, much less is understood about how non-enveloped viruses uncoat and transfer their genomes from endosomal vesicles into the cytosol.

FMDV, a highly infectious pathogen of cloven-hoofed animals, is the most acid-labile member of the picornavirus family and provides a simple model for acid-induced endocytic entry by an non-enveloped virus. Other picornaviruses are either acid-stable, like enteroviruses and cardioviruses, or, like rhinoviruses, can acquire significant acid resistance by mutation (Skern et al., 1991; Giranda et al., 1992). In order to uncoat, rhino- and enteroviruses have first to undergo a profound structural modification (Almela et al., 1991; Prchla et al., 1994; Mosser et al., 1994) that, in the case of poliovirus (an enterovirus) and rhinoviruses of the major receptor group, is known to be promoted by contact with the appropriate cellular receptor (Fricks & Hogle, 1990; Rossmann, 1994). This specialized function of the receptor limits the range of cell surface molecules that such viruses can use to infect cells. By contrast, FMDV can use any endosomally targetted ligand on the cell surface as a receptor (Mason et al., 1993) and this property is presumably related to its pH lability.

While FMDV needs to be acid-labile to permit efficient uncoating, its capsid must be robust enough to shield the genome from the extracellular environment. How is this balance achieved? The picornavirus capsid consists of 60 copies...
of each of three surface proteins, 1B, 1C and 1D (or VP2, VP3 and VP1, respectively), and a small, internal protein, 1A (VP4). These are arranged in an icosahedral lattice of 12 pentameric units. These pentamers are the main structural intermediates in virus assembly, and it is likewise into pentamers that FMDV dissociates on mild acidification, releasing 1A and RNA. Thus, the effect of reducing the pH is to disrupt contacts between neighbouring pentamers. To explain how this occurs, Acharya et al. (1989) drew attention to the high density of histidine residues on the 1B and 1C domains lining the pentamer interface. Since the pHₐ of histidine (6.8 in solution) approximates to the pH at which FMDV dissociates, these workers proposed that dissociation is triggered by electrostatic repulsion between the protonated imidazole side-chains of these histidines.

Recently, these ideas were refined by Curry et al. (1995), who noted that residue 142 of 1C is located near to an α-helix formed by residues 89–98 of 1B in the neighbouring twofold-related pentamer. The interface between each pair of pentamers contains two copies of this residue on either side of the twofold axis of symmetry, each with its side-chain located at the positive end of the dipole that is associated with the orientation of the peptide bonds in an α-helix. Protonation of these histidines would give rise to repulsive electrostatic interactions across the interfaces, which could lead to pentamer dissociation. Twomey et al. (1995) identified two histidine residues as potential capsid destabilizers at low pH, based on the net positive charge of residues in their respective neighbourhoods, one being His-1C-142, as predicted by Curry et al. (1995), and the other His-1C-145. Both are conserved among all seven FMDV serotypes (Table 1). However, only in the case of His-1C-142 is this conservation unique to FMDV, His-1C-145 being structurally conserved throughout all picornaviruses, including the acid-resistant enteroviruses, and therefore a less likely candidate.

In view of the various lines of evidence pointing to His-1C-142 as a key trigger of uncoating, we set out to investigate the role of this amino acid in the acid-induced uncoating of FMDV.

Table 1. Histidine residues in the pentamer interface of FMDV serotypes

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<th>Serotype</th>
<th>1B</th>
<th>1C</th>
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<tr>
<td></td>
<td>21 56 65 87 88 93 157 209</td>
<td>142 145 192</td>
</tr>
<tr>
<td>A₁₀₀₁ (OAO)</td>
<td>H V F H H H H H H H H H H H H H</td>
<td>H H H H H H H H H H H H H H H H H</td>
</tr>
<tr>
<td>O₁ K</td>
<td>H V H K S H H H H H H H H H H H H H</td>
<td>H H H H H H H H H H H H H H H H H</td>
</tr>
<tr>
<td>C-S8C1</td>
<td>H H A P K G H H H H H H H H H H H H H</td>
<td>H H H H H H H H H H H H H H H H H</td>
</tr>
<tr>
<td>Asia1 PAK/1/54</td>
<td>T H H K S H H H H H H H H H H H H H</td>
<td>H H H H Q H H D</td>
</tr>
<tr>
<td>SAT1 BOT/1/68</td>
<td>T E K H K G H H H H H H H H H H H H H</td>
<td>H H H D</td>
</tr>
<tr>
<td>SAT2 RHO/1/48</td>
<td>T E K Q K S H H H H H H H H H H H H H</td>
<td>H H H D</td>
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<tr>
<td>SAT3 BEC/1/65</td>
<td>T E K H K A H Y H H H H H H H H H H H H</td>
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We have previously described the synthesis and self-assembly of FMDV capsids using a vaccinia virus expression system (Abrams et al., 1995). Such ‘artificial’ particles resemble antigenically ‘natural’ empty capsids purified from cells infected with the same strain of serotype A FMDV and undergo an identical pH-dependent disassembly (Curry et al., 1995). We report here on the altered assembly/disassembly properties of cDNA clone-derived FMDV capsids in which His-1C-142 has been replaced by alternative amino acids.

Methods

Plasmid construction. Manipulation of DNA constructs was performed by standard methods (Sambrook et al., 1989). DNA used for transfection into eukaryotic cells was purified by centrifugation on caesium chloride gradients. Plasmid pCA2 containing the P1-2A and 3C⁺⁰⁺ regions of FMDV strain A₁₀₀₁ has been described previously (Abrams et al., 1995). In order to introduce a bacteriophage T7 RNA promoter upstream of the viral protein coding sequences, a HindIII–DraI fragment from pCA2 containing the FMDV sequences was cloned into pBluescript II KS (+) with HindIII and HindIII to give pKSCA2. Mutagenesis of the nucleotides encoding amino acid 142 of 1C was achieved by overlap PCR (Higuchi et al., 1988) using Pco (Boehringer Mannheim), a proof-reading thermostable DNA polymerase, and sets of primers as follows: for the His-1C-142 has been replaced by alternative amino acids.

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an SP6 RNA polymerase promoter ten bases upstream of the authentic virus 5’ end. These non-virus-derived bases were reduced to GG, and the SP6 RNA polymerase promoter was replaced by a T7 RNA polymerase promoter, by using PCR. The resulting construct, pT7S3, proved to be slightly more infectious than its parent. An infectious cDNA clone containing the A161 capsid genes was produced by replacing the O1K capsid-encoding regions of pT7S3 with those of A161 (Carroll et al., 1984) to produce pT7S3::A10X161. The FMDV-encoding sequences from pMR15 (Ryan et al., 1989) were transferred into pBluescript II SK (+). O1K sequences from L to the 2A/2B junction (NrdI–Apal) were replaced with the corresponding A161 sequences from pMR53 (Ryan et al., 1991). A BssEI–Xhol fragment from this construct was inserted in pT7S3. Virus can be recovered from this construct when RNA transcripts produced in vitro with T7 RNA polymerase are electroporated into BHK21 cells: this virus has been referred to as OAO.

### Processing and assembly studies

Monolayers of BHK21 cells (95 % confluent) were trypsinized and washed in PBS before being resuspended at a concentration of approximately 10⁷ cells/ml in HEPES-buffered saline (Chu et al., 1987) containing vTF7-3, a recombinant vaccinia virus expressing bacteriophage T7 RNA polymerase. Cells were incubated at 37 °C with occasional shaking for 1 h and cooled on ice and an equal volume of cold HEPES-buffered saline was then added. Plasmid DNA was added to the cells at a concentration of 125 µg/ml DEAE–dextran (5 µg/ml) (Gauss & Lieber, 1992) was added to increase electroporation efficiency. The cells were transfected by electroporation using the Bio-Rad Gene Pulser and Gene Pulser disposable cuvettes with an electrode gap of 0.4 cm. Cells were electroporated by using a capacitance of 250 mF and two pulses of 280 V. Cells were kept on ice for 15 min after electroporation before being added to flasks containing Dulbecco’s minimum essential medium supplemented with 5 % foetal calf serum and 10 mM HEPES, pH 7.3. The cells were cultured for 14–15 h before being labelled for 2 h with 35S EXPRE3S0S protein-labelling mix (DuPont NEN) at a concentration of 4 MBq/ml in methionine- and cysteine-free Eagle’s medium. The medium was removed and any unattached cells were recovered. The attached cells were rinsed in calcium- and magnesium-free PBS and were then detached from the flask by using cell-dissociation solution (Sigma C-5914). The cells were pelleted and resuspended in calcium- and magnesium-free PBS. This suspension was divided into the number of samples required and then the cells were pelleted and the medium was removed. The cells were resuspended in 265 µl of a phosphate–citrate–KCl McIlvaine-type buffer (Dawson et al., 1969) of the stated pH and ionic strength of 0.1 M containing 0.5 % NP-40, which resulted in their lysis. In all cases, the actual pH was tested by using pH paper and was found to be only a few tenths of a pH unit above the predicted values. The resultant suspensions were incubated at room temperature for 15 min, after which the pH of the suspensions was adjusted to 7.6 by the addition of 735 µl of the appropriate neutralization solution; this resulted in an ionic strength of 0.15 M from the buffer component of the mixture. Since the ionic strength of the mixtures would also be affected by the amount of cell constituents present, the different pH treatments were performed on aliquots of the same infected cell suspension. After the pH of the samples was readjusted to 7.6, they were clarified by centrifugation at 12,000 g in a Sorvall Microspin for 1 min. The supernatants were loaded onto 15–45 % sucrose gradients in phosphate–citrate–KCl buffer pH 7.6 (ionic strength of the buffer without sucrose, 0.15 M) and centrifuged at 202,000 g at 15 °C for 2.25 h. Labelled virus proteins were immunoprecipitated from 200 µl samples of each gradient fraction by using a polyconal guinea pig antiserum to purified A161 virus by the method of Firestone & Winguth (1990). The proteins were then analysed by 10 % SDS–PAGE followed by autoradiography and densitometry using a Bio-Rad model 620 video densitometer.

### Results

#### Effects of changes in amino acid 142 of 1C on assembly of empty capsids

Changes in the capsid protein sequence could potentially have three different effects. They could interfere with proteolytic processing of P1 or with one of the later stages of the assembly pathway, preventing formation of pentamers or assembly of pentamers to empty capsids. The ways in which the different mutations affected processing and assembly were studied by using a transient expression system. BHK21 cells were infected with vTF7-3 and transfected with the various test plasmids as described. After labelling with 35S EXPRE3S0S protein-labelling mix, the cells were lysed in buffer at pH 7.6. The products were treated as described. A representative sample of the results obtained is shown in Fig. 1. In preparations of OAO virus (a cDNA-derived FMDV expressing the A161 capsid), the processing products 1ABC and 1CD were obtained in approximately equal amounts. By contrast, in all of the protein products derived from the capsid expression cassettes, pKSCA2 or its mutated derivatives (pKSM3, -4, -5), 1CD was present in larger amounts than 1ABC. This preference of 3C for the 1BC junction has also been observed when the FMDV-encoding sequences from pCA2 are expressed from a recombinant vaccinia virus (Abrams et al., 1995). Similar results were previously obtained with analogous cassettes of FMDV subtypes A23 Cruzeiro and O1K. The distinctive cleavage preference in the case of the infectious cDNA (OAO) compared with the capsid-expressing cassettes may reflect the specificity of 3CD protease in the former.

Curry et al. (1995) observed that in natural empty capsids from three different subtypes of FMDV (A16, A22, and A24), most of the 1AB protein had cleaved to 1A and 1B, while empty capsids expressed from vaccinia virus encoding A161 sequences contained essentially intact 1AB. This contrasted with our results, where empty capsids produced by OAO virus were found to contain hardly any cleaved 1AB and a significant amount of 1AB could even be observed in virions. Curry et al. (1995) found that cleavage of 1AB in empty particles is time dependent, and this probably accounts for the contrasting results; in the present studies, cell lysates were loaded directly onto sucrose gradients with minimal sample preparation, whereas the method used by Curry et al. (1995) entailed extensive manipulations before sucrose gradient purification. Recent structural analysis by Curry et al. (1997) suggests that the 1AB cleavage observed in their preparations may occur at a slightly different position from normal and may therefore be due to adventitious protease action.

The percentage of the processed capsid proteins that assembled into empty capsids was examined for the various constructs. For capsid proteins derived from the wild-type
Fig. 1. Processing and assembly of capsid proteins from cells lysed at pH 7-6. Cells were either infected with OAO virus (a) or were infected with vTF7-3 and transfected with pKSCA2 (wild-type) (b), pKSM3 (His → Phe mutant) (c), pKSM4 (His → Arg mutant) (d) or with pKSM5 (His → Asp mutant) (e). Samples were analysed on 15–45% sucrose gradients. Fractions from the top of the gradient are shown on the left. FMDV proteins were immunoprecipitated with guinea pig antibodies to purified A1061 virus and analysed on 10% SDS–PAGE gels.

The pH stability of empty capsids produced from OAO virus, pKSCA2 and two of the mutants (His → Asp and His → Phe) was examined. Samples of transfected and labelled cells were lysed at a range of different pH values but in buffers of constant ionic strength and incubated at room temperature for 15 min before the pH of the samples was returned to 7-6. These were then size-fractionated by centrifugation through 15–45% sucrose gradients and the fractions were immunoprecipitated. The amount of processed capsid protein present in each sample was then quantified by densitometry. A representative sample of the results is shown in Fig. 2. As was expected, OAO virions (Fig. 2) were very sensitive to acidification, only a very small percentage remaining at pH 6-2, while empty capsids proved more resistant. This result correlates well with the observations of Curry et al. (1995), except that a lower pH was required to cause 50% loss of assembled capsids in the present study, which is almost certainly due to the presence of cellular components. These will cause an increase in the ionic strength and could contain molecules that might bind to and stabilize the virus capsids against acidification. Some variation in pH stability between replicate experiments was observed, which was probably due to variations in ionic strength between experiments. However, the pH at which empty capsids produced from pKSCA2 started to disassemble was approximately the same as for empty capsids produced from OAO virus. It is more difficult to analyse the results from the His → Phe mutant, the virus peak being flattened in comparison to the wild-type. However, assembly products of the same or higher sedimentation coefficient than normal empty capsids proved comparatively resistant to acid treatment. Empty capsids produced by the His → Asp mutant proved very resistant to acid treatment; indeed, the proportion of assembled capsid was consistently found to be slightly higher at the lowest pH studied, 5-0, than at pH 5-6, the mean increase from 10 to 14% being significant (P < 0-05).

Discussion

Adjacent pentamers in the FMDV capsid are held together by ionic interactions between residues 6, 7 and 8 of protein 1B from adjacent pentamers and a Ca$^{2+}$ ion present at the threefold axes and by several weak interactions, notably the
A key histidine residue in uncoating of FMDV

Fig. 2. Assembly and stability of capsid proteins from cells lysed into buffers of differing pH. Cells were either infected with OAU virus or were infected with vTF7-3 and transfected with pKSCA2 (His wt), pKSM3 (Phe) or pKSM5 (Asp). Samples were subjected to the stated pH and analysed on 15–45% sucrose gradients as in Fig. 1. FMDV proteins were immunoprecipitated with guinea pig antibodies to purified A1061 virus and analysed on 10% SDS–PAGE gels. The amount of processed capsid protein present in each lane was quantified by using a Bio-Rad densitometer and the proportion of the total processed capsid protein (i.e. 1AB + 1C + 1D) present in each lane was calculated.

A series of hydrogen bonds that knit the non-β-barrel strands β-A1 and β-A2 of 1B to strand β-F of the 1C β-barrel (Acharya et al., 1989). Another force favouring assembly is the hydrophobic free energy gained when amino acid residues are transferred from a bulk solvent phase to the substantially less polar environment of the interior of a protein. These interactions must be of sufficient strength to hold pentamers together at neutral pH but not so strong that the protonation of a few histidine residues cannot cause disassembly.

The charge characteristics of the amino acid residues in the pentamer interface are quite conserved in FMDV serotypes A, O, C and Asia1. The overall degree of conservation is less if the South African Territories (SAT) serotypes are included in the comparison, but these possess amino acid sequences that are more divergent from the other serotypes and crystal structures have not yet been solved for them. Although this makes sequence alignment uncertain, three histidines in this area appear to be conserved across all seven serotypes (Table 1). These correspond to positions 1B-157, 1C-142 and 1C-145 in A1061. While the first is located on the inner surface of the capsid, the other two are located on the pentamer–pentamer interface and are more likely candidates to be involved in uncoating (Fig. 3). As noted in the Introduction, Curry et al. (1995) drew particular attention to His-1C-142 as a likely trigger for uncoating. Since undertaking these studies, detailed electrostatic calculations by van Vlijmen et al. (1998) have confirmed His-1C-142 as the residue predicted to have the greatest destabilizing effect on pentamer–pentamer contacts at low pH. The difference in $pK_a$ of an imidazole side-chain in its buried (i.e. assembled) and solvated (i.e. free pentamer) states provides a direct measure of the free energy contributed by the protonation of that side-chain to the acid-induced dissociation of the virion. In the case of His-1C-142, the predicted perturbation in $pK_a$, from 6.8, typical for a solvated histidine, to just 2.1 in the assembled type A capsid, is particularly dramatic.

These predictions appear to be borne out by our mutagenesis studies, which demonstrate that substitution of this one residue is sufficient either to stabilize or to destabilize the capsid, depending on the choice of substituent. That an
aspartate should have rendered the capsid so acid-stable is, however, very interesting. Van Vlijmen et al. (1998) calculated that approximately 20% of the free energy of dissociation contributed by His-1C-142 is attributable to the polarizing effect of the helix dipole. If this positive pole exacerbates the destabilizing effect of a protonated (positively charged) histidine, it would be expected to have a stabilizing effect in the presence of a negatively charged residue at 1C-142. This appears to be the case, since, although the switch to a negatively charged aspartate somewhat reduced the efficiency of assembly, those capsids that were assembled remained in relatively constant amounts as the pH was reduced from 7.6 to 5.6. The reduced efficiency of assembly at neutral pH may be explained by the disruptive effect of negative charges, two per pentamer interface, incompletely neutralized by the helix dipole. By the same token, the reproducible increase in assembled capsid as the pH was further reduced from 5.6 to 5.0 was probably due to partial protonation of those aspartates and a consequent reduction in net negative charge.

Replacing the histidine with an uncharged phenylalanine similarly resulted in the proportion of processed proteins present in the empty capsid region of the gradient being relatively unaffected by the pH of the lysis solution. However, the formation in this mutant of products that sedimented both faster and slower than normal empty capsids could indicate that they were structurally aberrant and/or far less stable at pH 7.6 than the normal capsids. There are unlikely to be extensive changes in the structure of the protein, however, since proteolytic processing, which is sensitive to incorrect folding (Ypma-Wong & Semler, 1987), occurred in the normal way in this mutant.

If protonation of His-1C-142 is solely responsible for dissociation of the pentamers, it might be expected that when this residue was changed to a positively charged arginine residue, capsid assembly could not occur. Assembly was, indeed, very inefficient. However, small amounts, too low to quantify, of assembled products were observed at pH 7.6, which may have contained some true empty capsids. This might suggest that the protonation of 1C-142 is a necessary but not an entirely sufficient condition for the uncoating of capsids at low pH.

In addition to His-1C-142, His-1C-145 is located nearby and might have a subsidiary function in uncoating, whilst His-1B-21, also located at the pentamer–pentamer interface, is comparatively conserved in serotypes A, O, C and Asia1. Although His-1B-21 is replaced by threonine in the SAT serotypes, there is a conserved histidine two residues upstream in these viruses. In the A19 capsids used for this study, 1B-93 is a histidine residue, which is in close proximity to its rotational partner across the twofold axis and could account for some tendency for the capsids to uncoat. Van Vlijmen et al. (1998) concluded that protonation of His-1C-145 should have a significant effect on capsid destabilization, but that 1B-21 should have a stabilizing effect at low pH. Two other histidines, at 1B-87 and 1B-88, are not conserved and were not considered by van Vlijmen et al. (1998) to be relevant to acid lability, as the

Fig. 3. Structure at the pentamer interface, showing the histidine residues present in the vicinity of 1C-142 between twofold-related pentamers in A1061 FMDV. The pentamer interface passes horizontally through the centre of the figure. The side-chains of only the histidine residues are shown; the rest are represented by a trace of the protein main chain. Labels associated with the twofold-related pentamer are indicated by primes. The twofold-related helices are indicated by dotted cylinders, with ‘+’ at the positive end of the dipole. The figure was produced by using Berkeley-enhanced RASMOL 2.6-ucb (Sayle & Milner-White, 1995); the coordinates were kindly provided by E. Fry (Laboratory of Molecular Biophysics, University of Oxford). The diagram on the right indicates the approximate region of the pentamer interface represented in the figure.
pK of the former was predicted to be less than 4.0 under all conditions and that of the latter was predicted to decrease on disassembly (i.e. to exert a slight stabilizing influence). Further mutational studies will be needed to confirm these predictions.

The occurrence of structural changes within the pentamers as the pH changes must also be considered. Such alterations have been noted previously for Mongo virus, which also uncoats via pentamer dissociation (Kim et al., 1990). These structural changes involve a movement of the G–H loop of 1D, an ordering of the G–H loop of 1C between residues 176 and 182, the displacement of a bound phosphate near the G–H loop of 1D and the movement of the carboxy terminus of 1B. Kim et al. (1990) postulated that some of these structural changes could be due to protonation of His-205 in the G–H loop of 1D and His-250 of 1B. It is worth noting that His-250 of 1B in Mengo virus is in a position not that far removed from the corresponding virion for three subtypes of type A FMDV with empty capsids. Curry et al. (1995) observed that the empty capsid was more stable, by 0.5 pH unit on average, than the corresponding virion for three subtypes of type A FMDV (A2b, A1061 and A2). This difference was not found to be associated with the cleavage of 1AB [a result that has recently been confirmed by Knipe et al. (1997)], but was found to be correlated with the presence of RNA and the associated ordering of the network of sequences lining the interior surface of the capsid. In particular, the virion is more ordered than the empty capsid in the region of the threefold axes of symmetry (Curry et al., 1997). Differences are observed in the amino terminus of 1D and the carboxy terminus of 1A and to a lesser extent in residues 153 and 154 in 1C. The way in which this ordering affects acid sensitivity needs to be investigated.

This study has not investigated the role of the RNA in the increased sensitivity to acidification of virions in comparison with empty capsids. Curry et al. (1995) observed that the empty capsid was more stable, by 0.5 pH unit on average, than the corresponding virion for three subtypes of type A FMDV (A2b, A1061 and A2). This difference was not found to be associated with the cleavage of 1AB [a result that has recently been confirmed by Knipe et al. (1997)], but was found to be correlated with the presence of RNA and the associated ordering of the network of sequences lining the interior surface of the capsid. In particular, the virion is more ordered than the empty capsid in the region of the threefold axes of symmetry (Curry et al., 1997). Differences are observed in the amino terminus of 1D and the carboxy terminus of 1A and to a lesser extent in residues 153 and 154 in 1C. The way in which this ordering affects acid sensitivity needs to be investigated.

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References


poliovirus, can infect normally insusceptible cells via the Fc receptor.


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