Engineering Viable Foot-and-Mouth Disease Viruses with Increased Thermostability as a Step in the Development of Improved Vaccines

Roberto Mateo,† Eva Luna, Verónica Rincón, and Mauricio G. Mateu*
Centro de Biología Molecular “Severo Ochoa” (CSIC-UAM), Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain

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We have rationally engineered foot-and-mouth disease virus to increase its stability against thermal dissociation into subunits without disrupting the many biological functions needed for its infectivity. Amino acid side chains located near the capsid intersubunit interfaces and either predicted or found to be dispensable for infectivity were replaced by others that could establish new disulfide bonds or electrostatic interactions between subunits. Two engineered viruses were normally infectious, genetically stable, and antigenically indistinguishable from the natural virus but showed substantially increased stability against irreversible dissociation. Electrostatic interactions mediated this stabilizing effect. For foot-and-mouth disease virus and other viruses, some evidence had suggested that an increase in virion stability could be linked to an impairment of infectivity. The results of the present study show, in fact, that virion thermostability against dissociation into subunits may not be selectively constrained by functional requirements for infectivity. The thermostable viruses obtained, and others similarly engineered, could be used for the production, using current procedures, of foot-and-mouth disease vaccines that are less dependent on a faultless cold chain. In addition, introduction of those stabilizing mutations in empty (nucleic acid-free) capsids could facilitate the production of infection-risk-free vaccines against the disease, one of the economically most important animal diseases worldwide.

Foot-and-mouth disease (FMD) (45) affects cloven-hoofed animals, including cattle, pigs, sheep, and goats, and is one of the economically most important animal diseases worldwide. For example, the series of outbreaks in the United Kingdom during 2001 led to economic losses of over £6 billion in that country alone (48) and provided a harsh reminder to the European Union of the devastating consequences of the disease. FMD is enzootic in many countries on four continents, where a critical preventive method is vaccination. The causative agent is a picornavirus, FMD virus (FMDV) (29, 45), and the current commercial vaccines are based on chemically inactivated FMD virions (5). In one typical recent year, 2000, FMD vaccines generated $284 million in sales, representing over a quarter of the entire livestock biological business (20). Unfortunately, a number of problems severely limit the efficacy of the vaccine. One of these problems is that FMD virions are remarkably thermolabile, and moderate heat induces their dissociation into pentameric subunits in the vaccine, leading to dramatic losses of immunogenicity (8, 10, 36). Thus, a faultless, expensive cold chain from the production plant is required. Climate and socioeconomic factors lead with some frequency to ruptures of the cold chain and failure of the vaccine to protect the inoculated animals.

FMD vaccines based on genetically modified virions with normal infectivity (for virus growth during vaccine production) but higher thermostability would be desirable to minimize the requirement for a faultless cold chain, without a need to modify the production procedures in current use. However, virions present stringent biological restrictions that could impose an impassable limit on their conformational stability. These restrictions arise from the exquisite structural optimization viral particles have evolved as a consequence of the many complex functions they must perform during the virus life cycle (31, 34, 37). In particular, some evidence has suggested that for FMDV, as for other viruses, an increase in virion stability could be linked to a reduction in infectivity (32), perhaps by impairing structural rearrangements or dissociation of the capsid.

To date, no or very few protein-engineering attempts have been aimed at the stabilization of pathogenic viruses or their capsids or of any other large supramolecular assembly. However, current knowledge of the three-dimensional structure of FMDV (2, 12, 19, 25) and the relatively simple organization of its capsid (Fig. 1a) facilitates to some extent its rational modification. The capsid is formed by only three different proteins (VP1, VP2, and VP3) and a small internal polypeptide (VP4). During assembly of FMDV and other picornaviruses, one copy of each capsid protein folds to yield a protomer subunit, five protomers form a pentameric subunit, and 12 pentamers assemble to form the capsid (40). In this study, we rationally engineered the FMD virion to increase its stability against thermal dissociation into pentameric subunits without disrupting the many biological functions needed for its infectivity. Our two-step approach involved (i) the identification of amino acid residues at interpentamer interfaces that are of little importance for viral function and (ii) the structure-guided replacement of some of those residues by others that could establish further interpentamer interactions. The results show that, at
least for this virus, the stability of the virion against its disso-
ciation into subunits may not be selectively constrained be-
cause of any functional requirement for infectivity. The mod-
ified viruses obtained, and others similarly engineered, could
provide the basis for thermostable FMD vaccines that could be
prepared without the need to alter the proven, economical
procedures in current use. In addition, in the form of empty
capsids, the higher intrinsic stability conferred by the stabi-
lizing mutations could more than compensate for the destabi-
lization caused by the lack of RNA (13), improving the
suitability of empty capsids for future development of infec-
tion-risk-free, fully immunogenic FMD vaccines.

MATERIALS AND METHODS

Site-directed mutagenesis and subcloning. Substitution of amino acid residues
on the capsid of FMDV C-S8c1 (46) was carried out by site-directed mutagenesis
of plasmid p3242/C-S8c1 (4) using the QuikChange system (Stratagene).
The mutations were confirmed by automated DNA sequencing. The mutagenized
segments were subcloned in the infectious plasmid pO1K/C-S8c1 essentially as
previously described (31, 33). The presence of the engineered mutations and
absence of any other mutation in the subcloned segment were confirmed by
sequencing.

Transcription of viral RNA and electroporation of eukaryotic cells. FMDV
RNA was transcribed from linearized nonmutated and mutant pO1K/C-S8c1
plasmids by using the Riboprobe in vitro transcription system (Promega) and
was used to transfect BHK-21 cells by electroporation, following previously described
procedures (31). The same amount of every mutant RNA was used for trans-
fection. In each experiment, the same amount of nonmutated RNA was used as
a positive control. A negative control (no RNA) was also included.

Titration and amplification of viruses and extraction of viral RNA. Virus titers
were determined at least in duplicate or triplicate in standard plaque assays.
When needed, the progeny viruses were amplified by a minimum number of
passages in BHK-21 cell monolayers at the highest possible multiplicity of infec-
tion and titrated again. RNA derived from viral populations obtained by trans-
fec tion or infection was extracted using Trizol (Invitrogen) and precipitated with
ethanol. The RNA was reverse transcribed to DNA and amplified by PCR as
previously described (31). The reverse transcription-PCR tubes were incubated at a constant temperature (generally 42°C) for dif-
f erent amounts of time, and the titers of the remaining virus in each case was
determined in plaque assays. To confirm borderline results with some mutants,
a second, more sensitive method had to be used. Essentially, virus suspensions
were used without dilution at the highest possible titer (up to about 10⁷ PFU/ml).
Aliquots (1 ml) of the virus suspension were incubated at a constant temperature
(usually at 42°C or 4°C, as indicated) for different amounts of time, and the titer
of remaining virus was determined as indicated above. Nonmutated C-S8c1 virus
obtained in parallel transfection or infection experiments, along with the differ-
ent engineered mutants, was used in each heat inactivation experiment as a
positive control to normalize the results. The experimental data were fitted to
first-order exponential decays by using the program Kaleidagraph (Abelbeck
Software), which allowed the determination of the inactivation rate constant
(31).

Thermal-dissociation assays. Aliquots of ³⁵S-labeled purified virions were incu-
bated at a constant temperature (generally 42°C or 4°C, as indicated) for different
amounts of time, transferred to ice, loaded in 7.5% to 45% sucrose
density gradients, and centrifuged at 4°C in an SW40 rotor (Beckman instru-
ments) at 18,000 rpm for 18 h. The fractions were fractionated in 0.5-ml aliquots,
and the radioactivity was determined using a liquid scintillation counter.
In general, the nonmutated virus and the nonmutated control in the same experiment
were purified and processed in parallel to exclude possible differences in the
dissociation rate due to slightly differing final conditions between preparations
and experiments. The experimental data were fitted to first-order exponential
decays by using the program Kaleidagraph (Abelbeck Software), which allowed
the determination of the dissociation rate constant (31).

Structural analyses and molecular modeling. The Protein Data Bank atomic
coordinates for the crystal structure of FMDV virions C (isolate C-S8c1) (25),
are available in thin-walled

Partially purified virion preparations were ob-
tained by centrifugation through sucrose cushions as indicated above, and
the amount of viral protein was estimated by sodium dodecyl sulfate-urea-poly-
acrylamide gel electrophoresis. Equal numbers of virions were applied to nitro-
cellulose sheets and subjected to enzyme immunodot assays as previously
reported (25), using a representative panel of monoclonal antibodies (SD6, 4G3,
4C4, 5A2, 7D1, 7A1, 1G5, 2E5, and 5C4) against different epitopes at each of
the identified antigenic sites in FMDV C-S8c1 (25, 34, 35).

Structural analyses and molecular modeling. The Protein Data Bank atomic
coordinates for the crystal structure of FMDV virions C (isolate C-S8c1) (25),
O (2, 19), and A (12) were inspected using the programs InsightII (Biosym
Technologies), RasMol (42), and/or Pymol (DeLano Scientific, Inc.). Contact
and solvent accessibility analysis and modeling of mutations were done with the
program Whatif (49) using the coordinates of all possible pairs of contacting
subunits with different symmetry within the capsid (the pentamer interfaces
involve basically VP2-VP2 and VP2-VP3 pairs). Prediction of energetically fa-
vorable disulfide bonds was done using the programs MODIP (14) and Disulphide
by Design (16).

RESULTS

Rational design of FMDV variants aimed at the introduc-
tion of further interactions between capsid subunits. Knowl-
eedge of the three-dimensional structure of FMDV (2, 12, 19,
25), including variant C-S8c1 (a clone from vaccine strain
C, Santa Pau-Spain/70 that we use as a model) (46), allowed a
study by our group that involved a complete alanine scanning
of the interpentamer interfaces in FMDV through the individ-
ual mutation of each of the 42 residues in each capsid pro-
tomer whose side chains were involved in interpentamer inter-
actions (31). Nearly all of those side chains were found to be
important for viral infectivity. The FMDV structure, and those

FIG. 1. (A) Schematic quaternary structure of the FMDV capsid. Numbers 1, 2, and 3 denote proteins VP1, VP2, and VP3, respectively, in one protomer subunit. A pentameric subunit that includes the labeled protomer is delimited by thick lines. A black pentagon, triangle, or ellipse indicates the position of a capsid fivefold, threefold, or twofold symmetry axis, respectively. (B) A cartoon view that includes parts of three neighboring VP2 subunits (colored red, blue, or green) belonging to different pentamers in the FMDV C-S8c1 capsid. Their N-terminal segments (Nt) (colored magenta, cyan, or yellow) form a β
annulus around a threefold axis (white line).
A2065K, and A2065H, the values in italics represent the inactivation rate constants obtained using a different, more sensitive type of assay (see the text). For the disrupting viral function and infectivity.

amino acid replacements aimed at the introduction of inter-

H11003 at 45 h posttransfection (p.t.) (from 39 transfections), 6.4

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nulus backbone (25) and the above-mentioned programs were

computation of disulfides. However, the tracing of the

subunits (25) (Fig. 1b). These segments are not well ordered,

annulus formed by the N-terminal segments of three VP2

axis, where three pentamers are joined together by a short

ising spot was the region at each capsid threefold-symmetry

were constructed (Table 1 and Fig. 2a). An additional prom-

bility. Considering these restrictions, three Cys double mutants

vealed that the mutations were still present in the progeny

normal infectivity (T2023C/A3145C and T2007C/T2008C) re-

further mutations were present in the entire capsid. Finally, the

mutations were still present in the progeny populations, and no

normal infectivity (T2023C/A3145C and T2007C/T2008C) re-

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<table>
<thead>
<tr>
<th>Virus</th>
<th>Relative infectivity (normalized titer)</th>
<th>Inactivation rate (min⁻¹)</th>
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<tr>
<td></td>
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<td>55 h p.t.</td>
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<tr>
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**TABLE 1. Relative infectivities and thermal-inactivation rates of engineered FMDVs**

a Titors are normalized relative to that of the nonmutated control virus. For the latter, the absolute average titers ± standard deviation were 3.5 × 10⁶ ± 5.4 × 10⁵ at 45 h posttransfection (p.t.) (from 39 transfections), 6.4 × 10⁶ ± 8.6 × 10⁵ at 55 h p.t. (from 29 transfections), and 2.8 × 10⁶ ± 4.5 × 10⁵ at 95 h p.t. (from 10 transfections).

b Inactivation rate constants at 42°C of virions present in the supernatant from transfected cell cultures. For the nonmutated control and mutants D3069E/T2188A, A2065K, and A2065H, the values in italics represent the inactivation rate constants obtained using a different, more sensitive type of assay (see the text). For the nonmutated control virus or most mutants, the averages ± standard deviations corresponding to several independent experiments are indicated.

c NA, not applicable (not enough virus could be recovered).

d ND, not determined.

e NA, not applicable (virus either reverted to wild type or acquired a compensating mutation).

f Obtained by introducing both mutations into the infectious clone.

This mutant showed a delayed infection; viral progeny approached the yield obtained with the nonmutated virus, but at longer times postinfection.

severe selective constraints found at the subunit interfaces, has been used as a guide for the design of potentially tolerated amino acid replacements aimed at the introduction of interpentamer disulfide bonds or electrostatic interactions without disrupting viral function and infectivity.

**Disulfide bridges.** Two programs that predict mutations leading to energetically favorable disulfide bonds in proteins were used. Only a few potentially good target residues were found, and most of those residues were needed for virus viability. Considering these restrictions, three Cys double mutants were constructed (Table 1 and Fig. 2a). An additional promising spot was the region at each capsid threefold-symmetry axis, where three pentamers are joined together by a short β annulus formed by the N-terminal segments of three VP2 subunits (25) (Fig. 1b). These segments are not well ordered, and their atomic coordinates could not be reliably used for the computation of disulfides. However, the tracing of the β-annulus backbone (25) and the above-mentioned programs were used as rough guides to select five further Cys mutants (Table 1). The structural data suggested the β annulus could be flexible enough to relax and accommodate the introduced disulfide bridges with good stereochemistry.

**Electrostatic interactions.** Ion pairs are much less stereochemically demanding than disulfide bonds. However, again, very few residues could be mutated to form interpentamer ion pairs without removing any of the side chains involved in infectivity. Considering the restrictions, two double mutants that could form new interpentamer salt bridges or short-range ionic interactions were chosen (Table 1 and Fig. 2b). A third mutant was designed by targeting Asp3069 in VP3 (Table 1 and Fig. 2c). We knew that this interfacial residue could not be replaced by Ala without a lethal effect on infectivity. However, a stereochemically more conservative replacement by Glu could be tolerated while bringing the carboxylate near enough to Lys2198 in VP2 to form a semiburied salt bridge. Finally, we also considered for mutation capsid residues exposed to solvent, not involved in contacts with other residues, and located very close to the interpentamer interfaces but far away from regions known to be functionally important. We expected residues that fulfilled all these conditions to be dispensable for viability and targeted Ala2065 in VP2 (Table 1 and Fig. 2d). Virtual mutagenesis suggested that mutation of Ala2065 to Lys and/or His could lead to an interpentamer salt bridge or short-range electrostatic interaction with Glu3137 in VP3.

**Infectivity of engineered FMDV mutants.** The chosen mutations were introduced in an FMDV infectious clone, susceptible cells were transfected with the same amount of viral RNA from any of the 13 mutant clones, and the infectivity of each mutant was determined (Table 1). Six Cys mutants showed a drastic reduction in infectivity relative to the nonmutated control. Sequencing of the two Cys mutants with approximately normal infectivity (T2023C/A3145C and T2007C/T2008C) revealed that the mutations were still present in the progeny population.

Of the five mutants aimed at the introduction of electrostatic interactions, mutants T2053D/Q2057K and Y2200H/I3189D showed severely reduced infectivity. The single mutants Q2057K and I3189D were also essentially noninfectious (not shown), which indicated that the effects of the double mutations were not due to the introduction of pairwise interactions. In contrast, mutations A2065H and A2065K were tolerated without a significant loss of infectivity. Again, the introduced mutations were still present in the progeny populations, and no further mutations were present in the entire capsid. Finally, the
D3069E mutation was in itself lethal. In four replicate experiments viruses could be recovered, but sequencing showed that, in three out of the four populations recovered, the virus had reverted to the parental genotype. Remarkably, in one experiment, the virus responded to the introduction of the D3069E mutation not by reversion, but by acquiring during its replication a second-site, compensatory mutation that restored nearly normal infectivity. This involved a Thr-to-Ala replacement at position 2188 in VP2, spatially close (but not adjacent) to the original mutation (Fig. 2e); no further mutations were present in the entire mutant genome. We then introduced, in the parent infectious clone, mutations D3069E and T2188A together and T2188A alone. The titer of the double mutant was not severely reduced relative to the nonmutated control. The titer of the T2188A single mutant was severely reduced at shorter times posttransfection but largely recovered at longer times posttransfection (Table 1). Further, the progeny viruses yielded essentially normal titers (about $10^7$ PFU/ml) when used for infecting cells. The entire genome of the engineered and amplified D3069E/T2188A double mutant was sequenced, again showing that the introduced mutations were preserved and that no other mutations had occurred. These results confirmed a compensating, nonadditive effect of mutation T2188A on D3069E. In all, about half of the mutants were either normally or residually infectious and could be tested for thermostability.

Thermal inactivation of the infectivity of engineered FMDV mutants. We then analyzed the effects of mutations on the rate of inactivation of virion infectivity at 42°C by using a simple assay, as described previously (31) and in Materials and Methods (Table 1). Mutations T2188A and A2065K did not significantly affect the rate constant. The double mutations T2023C/A3145C, N2114C/G3192C, K2002C/E2006C, T2007C/T2008C, Y2200H/I3189D, and D3069E/T2188A led to a moderate increase in the inactivation rate, while mutant A2065H yielded a slightly reduced inactivation rate (close to the significance limit), relative to the nonmutated control. To confirm that the differences in inactivation rates between some of these mutants and the parental virus were real, a second, more sensitive assay to analyze thermal inactivation had to be used, as described in Materials and Methods. The results confirmed that D3069E/T2188A is inactivated faster, and A2065H somewhat more slowly, than the unmodified virus (Table 1 and Fig. 3). Similar differences were observed with highly purified virions (not shown).
Thermal dissociation of engineered FMDV mutants. Based on indirect evidence, we had assumed that, under our experimental conditions, thermal inactivation of FMDV infectivity was caused essentially by virion dissociation into pentamers. To prove or disprove this assumption, nonmutated FMD virions were radioactively labeled with [35S]Met and purified, and their rates of dissociation into subunits were directly determined by measuring the percent intact virion particles (isolated by ultracentrifugation in density gradients) as a function of time. As expected, and as shown in Fig. 4 for one experiment using the nonmutated virus, the reduction in virion (140S)-associated radioactivity was paralleled by an increase in pentamer (12S)-associated radioactivity. At 50°C, the dissociation rate approached the inactivation rate (not shown). However, at 42°C (Fig. 5, left) or 4°C (Fig. 5, right), purified virions were inactivated much faster than they were dissociated into subunits; thus, two processes with different rates appear to be involved. This result implied that we also had to directly determine the rate of dissociation of the mutant virions into pentamers. For example, the loss of immunogenicity of FMD vaccines is not caused by virus inactivation (the virions in them are already chemically inactivated) but by their heat-induced dissociation of virosomes into pentamers.

Electrophoresis under reducing and nonreducing conditions suggested that in T2023C/A3145C, but not T2007C/T2008C, the disulfide bonds could actually be formed (data not shown). Thus, we analyzed the dissociation kinetics of three infectious mutants: T2023C/A3145C, D3069E/T2188A, and A2065H. These viruses were radiolabeled, purified, and incubated at a moderate temperature (42°C), likely reached in cases of cold-chain failure, and also at low temperature (4°C), to mimic a situation in which the vaccine could be kept refrigerated.

Mutant T2023C/A3145C could be purified, but it readily dissociated into pentamers, even at 4°C (not shown). In contrast, mutant D3069E/T2188A dissociated into pentameric subunits much more slowly than the nonmutated virus under the same experimental conditions, both at 42°C (Fig. 6a) and at 4°C (Fig. 6b). Mutant A2065H also dissociated into subunits much more slowly than the nonmutated virus, both at 42°C (Fig. 6c) and at 4°C (Fig. 6d). All these results have been reproduced in independent experiments and using different purified preparations of both the mutant and the nonmutated control. The difference in stability against thermal dissociation between mutant A2065H or D3069E/T2188A and the nonmutated virus was significantly reduced (almost abolished) in the presence of 1 M NaCl (V. Rincón and M. G. Mateu, unpublished results), as expected if electrostatic interactions, which would be screened in the presence of high salt concentrations, were indeed involved.

Genetic stability of thermostable FMDV mutants. To evaluate the possibility of an inverse relationship between virus stability and biological functioning, and for vaccine development based on thermostable variants, it was important to ascertain whether the stabilizing mutations could be genetically preserved. Although the stable mutants were as infectious as
the nonmutated virus, a rapid loss of the mutations during virus replication would suggest some biological disadvantage due to a somewhat impaired viral function. We subjected mutants D3069E/T2188A and A2065H to serial amplifications in duplicate and sequenced the progeny populations. In the case of A2065H, after three passages the mutation was fully preserved in both series; after seven passages, a fraction of the progeny virus genomes had acquired the forward mutation H2065P and an accompanying mutation, V2107I, at a spatially very close residue; however, even after nine passages, the original A2065H mutation was still present in a large fraction of the progeny virions. In the case of D3069E/T2188A, the original mutations were fully preserved in both series, at least during eight passages. Thus, both mutants were genetically fairly stable, allowing a large amplification of the engineered viruses.

Antigenicity of thermostable FMDV mutants. The antigenic structure of FMDV C-S8c1 and those of FMDVs belonging to other serotypes have been extensively studied by us and other groups (25, 34, 35, 39). The interpentamer interfaces and the residues we targeted for stabilization are located far away from the known antigenic regions, and various approaches suggest that no further immunodominant antigenic sites may generally exist on FMDV (34, 35). However, for its potential use in vaccine development, it was important to experimentally confirm that introduction of the stabilizing mutations did not alter the antigenic specificity of the virus. We compared the antigenicity, using enzyme immunodot assays, of the thermostable A2065H and D3069E/T2188A mutants with that of the parental C-S8c1 virus using a large representative panel of virus-neutralizing monoclonal antibodies directed against nine different epitopes located at the five different antigenic sites and

FIG. 6. Thermal-dissociation kinetics of purified FMDV mutant D3069E/T2188A (a and b) or A2065H (c and d) at 42°C (a and c) or 4°C (b and d). The remaining percent of intact (nondissociated) 140S virions is represented in a logarithmic scale as a function of the incubation time. All data were fitted to single-order exponential processes. (a and b) Mutant D3069E/T2188A (triangles) and the corresponding nonmutated control (circles) were purified and assayed in parallel. The average values and error bars (standard deviations) corresponding to two independent measurements at 42°C (a) or 4°C (b) are shown. The average dissociation rate constants for D3069E/T2188A (k\textsubscript{mut}) versus the nonmutated control virus (k\textsubscript{wt}) were as follows: k\textsubscript{mut}/H11005 = 0.033 h\textsuperscript{-1} versus k\textsubscript{wt}/H11005 = 0.106 h\textsuperscript{-1} at 42°C and k\textsubscript{mut}/H11002 = 0.022 days\textsuperscript{-1} versus k\textsubscript{wt}/H11002 = 0.075 days\textsuperscript{-1} at 4°C. Further, independent preparations of D3069E/T2188A and its nonmutated control were also analyzed at 4°C and yielded similar results (not shown). (c and d) Mutant A2065H (triangles) and the corresponding nonmutated control (circles) were purified and assayed in parallel. The average values and error bars corresponding to two independent measurements at 42°C (c) or three measurements at 4°C (d) are shown. The average dissociation rate constants for A2065H versus the nonmutated control virus were as follows: k\textsubscript{mut}/H11005 = 0.041 h\textsuperscript{-1} versus k\textsubscript{wt}/H11005 = 0.094 h\textsuperscript{-1} at 42°C and k\textsubscript{mut}/H11002 = 0.004 days\textsuperscript{-1} versus k\textsubscript{wt}/H11002 = 0.055 days\textsuperscript{-1} at 4°C. The analysis of an independently engineered A2065H mutant and its nonmutated control at either temperature yielded similar results (not shown).
Virions can be stabilized without impairing biological function and infectivity. Virus variants with increased resistance to inactivation of infectivity by heat are not generally represented in FMDV populations (32) despite these being quasispecies and containing a multitude of other phenotypic variants (17). This and other observations supported the possibility of a selective pressure that opposes a substantial increase in the thermostability of FMDV and other viruses, perhaps because it would hamper intracellular nucleic acid release. On the other hand, the results presented here show that it is possible to greatly increase the thermal stability of FMDV against dissociation into subunits without noticeably disrupting any viral function. In infection experiments with the two mutant viruses which showed an increased resistance against dissociation, the virus yield was not reduced relative to that of the parental virus; in addition, preliminary experiments indicate that the multiplication cycle of these mutants proceeded at the same rate as that of the parental virus. The above observations are not contradictory, as it was found that heat inactivates the infectivity of the FMD virion before dissociation into subunits occurs. A possible scenario that could explain the results we observed with FMDV is that moderate heat could provide the energy needed to prematurely trigger a conformational rearrangement of the metastable virion, leading to its biological inactivation prior to dissociation. Under physiological conditions, such rearrangement could occur inside the cell (perhaps triggered by a pH decrease in the endosomes), facilitating the subsequent dissociation of the capsid and the release of the viral genome. Experimental evidence in different viral systems indicates that, in vitro, heat may provide the energy needed to trigger the same processes, like capsid conformational changes and dissociation, which are triggered in vivo by other conditions, like binding to the cell receptor or a decrease in pH. If that hypothesis is correct, ease of inactivation of viral infectivity may be favored by biological selection, and no variants with increased resistance to thermal inactivation of infectivity could be found, consistent with what we observed (A2065H showed only a small decrease in its inactivation rate). In contrast, because the viruses we engineered are much more resistant to dissociation into subunits but proved nearly as infectious as the nonmutated virus, leading to similar virus yields, we believe it is reasonable to propose that, within certain limits, ease of viral-particle dissociation may not be critically favored by biological selection, because it is not the rate-limiting step in the infection process. Of course, the results obtained, as is generally the case with in vitro experiments, provide no absolute proof that the same situation occurs in vivo. In addition, there is the possibility that an increase in stability against dissociation, even if it does not disrupt biological function, could somehow slightly reduce the biological fitness of the virus, placing those mutants at some competitive disadvantage against less stable variants (this is under study). However, the results show that such a slight disadvantage, if present, would not be enough to prevent normal propagation and infectivity of the mutants in the absence of competing virus, for example, during vaccine production. To summarize, the results reveal that, for FMDV at least, efficient viral function and instability against particle dissociation (and subsequent genome release) need not be inextricably linked.

Molecular basis for the increased thermostability of the engineered FMDVs. Engineered disulfide bonds would be formed in an oxidizing extracellular medium (e.g., a vaccine formulation), but not in the reducing intracellular environment; thus, we expected those bonds to stabilize the virion with no adverse effects on its infectivity, even if there was an inverse stability/infectivity relationship. Natural disulfide bonds are indeed involved in stabilizing some virus capsids (3, 27, 50). Unfortunately, our disulfide approach was not successful. Intramolecular disulfide bonds artificially introduced at positions where natural disulfides are present in homologous proteins (23), including a bacteriophage capsid (3), proved stabilizing in several cases. However, most protein oligomers, including the FMDV capsid, have no adequate disulfide-bonded homologs. Engineered nonhomologous intermolecular disulfide bridges in oligomeric proteins either stabilized or destabilized the oligomer, depending on the specific mutation and target protein (9, 21, 41). In addition, in our case, those Cys mutations predicted to be energetically more favorable targeted residues involved in virus viability and could not be used.

Perhaps because charge-charge interactions act nondirectionally and over relatively long distances, our ion pair approach did lead to thermostable FMD virions. Two out of five engineered viruses (D3069E/T2188A and A2065H) were nearly as infectious as the nonmutated virus and much more resistant against thermally induced dissociation into subunits. As for disulfide bonds, the net effect of salt bridges on protein...
stability and association has been found to depend on many factors (7, 44), but a number of engineered intermolecular ion pairs did favor reversible association of some proteins (24, 43).

In the case of FMDV, further considerations are important: (i) capsid disassembly is irreversible, and thus, kinetic rather than thermodynamic effects should be taken into account (18); (ii) any single mutation introduces not one but many (60) identical interactions, and cooperativity could further potentiate the effects (51); and (iii) the introduction of additional charges, by altering the capsid electrostatic potential, could decrease any repulsive effects of equally charged groups nearby (2, 11).

A possibility that is consistent with the observed effects of the D3069E and T2188A mutations assumes that 60 strong salt bridges between Glu3069 and Lys2198 would be formed as intended, impairing capsid dissociation to the point of compromising virus viability. In the double-mutant context only, Ala2188 (close to both Glu3069 and the interpentamer interface [Fig. 2e]) could facilitate some local rearrangement, leading to disruption of a few interpentamer interactions, thus reducing the impairment of capsid dissociation caused by D3069E alone (this is under study). An electrostatic effect does appear to be involved in the stabilization by D3069E/T2188A, as the presence of a high salt concentration (which would screen those interactions) substantially reduced the difference in stability between the mutant and the nonmutated virus.

The stabilizing effect of mutation A2065H may be simply explained if 60 ion pairs between protonated His2065 and Glu3137, less strong than the putative salt bridges between Glu3069 and Lys2198, are formed as intended. Again, the presence of a high salt concentration greatly reduced the difference in stability between mutant A2065H and the nonmutated control, indicating that, as expected, an electrostatic effect is indeed involved in the stabilization by the A2065H mutation.

Thermostable viruses for improved FMD vaccines. This study also provides proof of principle that it is possible to rationally engineer virions with increased thermostability against dissociation for vaccination or other purposes. When the D3069E/T2188A and A2065H mutations were combined in a triple mutant, the individual stabilizing effects were not additive (Rincón and Mateu, unpublished). However, viral particles in which mutation A2065H or D3069E/T2188A was engineered separately are already much more resistant to thermal dissociation into subunits than the parental virus, probably enough to relax the need for a strict cold chain to prevent dramatic losses of immunogenicity during vaccine distribution and use. In addition, these two viruses are normally very infectious and genetically fairly stable, allowing their normal growth during vaccine production by current procedures. The infectivity of these mutants can be as easily abolished as that of the parental virus, either by heat or by treatment with binary ethylenimine, a chemical used for virus inactivation during vaccine production (Rincón and Mateu, unpublished). This would help to ensure, during vaccine production, that no infectious viral particles remain in the final product. The engineered mutations did not alter the antigenic specificity of the viral particle, either. In summary, these engineered viruses may fulfill the prerequisites for their use in virus-based FMD vaccines with higher thermostability against dissociation, helping to prevent losses of immunogenicity.

Empty (nucleic-acid-free) FMDV capsids are also being considered as improved FMD vaccines (1, 22, 26, 28, 30, 38). These would fully preserve antigenicity and immunogenicity but would avoid the need to grow and manipulate infectious viruses, thus eliminating the risk of virus escape or incomplete inactivation during vaccine production (6, 47). However, the need to improve empty-capsid stability will be even greater than for current virion-based vaccines, because the empty capsid appears to be even less stable than the virion, due to a role of the RNA molecule (13). The engineering of the thermostabilization mutations described in this study (or equivalent mutations) on empty capsids may increase their stability enough to more than compensate for their intrinsic instability, facilitating their production and use as safe FMD vaccines and contributing to the control of this important disease worldwide.

Conclusions. This study provides (i) evidence that no relationship necessarily exists between ease of viral-capid dissociation and viral function and infectivity, (ii) evidence that thermostabilization of a virion against dissociation can be achieved by modifying electrostatic interactions between capsid subunits, and (iii) rationally engineered viruses that, together with others similarly modified, could form the basis of FMD vaccines that could be prepared using current commercial procedures but that would be less dependent on a faultless cold chain. The results also encourage the use of the ion pair approach to increase the stability of other virions, viral capsids (including the FMDV capsid), and supramolecular complexes for biotechnological or nanotechnological applications.

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REFERENCES


