Structure of Aichi Virus 1 and Its Empty Particle: Clues to Kobuvirus Genome Release Mechanism

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ABSTRACT
Aichi virus 1 (AiV-1) is a human pathogen from the Kobuvirus genus of the Picornaviridae family. Worldwide, 80 to 95% of adults have antibodies against the virus. AiV-1 infections are associated with nausea, gastroenteritis, and fever. Unlike most picornaviruses, kobuvirus capsids are composed of only three types of subunits: VP0, VP1, and VP3. We present here the structure of the AiV-1 virion determined to a resolution of 2.1 Å using X-ray crystallography. The surface loop puff of VP0 and knob of VP3 in AiV-1 are shorter than those in other picornaviruses. Instead, the 42-residue BC loop of VP0 forms the most prominent surface feature of the AiV-1 virion. We determined the structure of AiV-1 empty particle to a resolution of 4.2 Å using cryo-electron microscopy. The empty capsids are expanded relative to the native virus. The N-terminal arms of capsid proteins VP0, which mediate contacts between the pentamers of capsid protein protomers in the native AiV-1 virion, are disordered in the empty capsid. Nevertheless, the empty particles are stable, at least in vitro, and do not contain pores that might serve as channels for genome release. Therefore, extensive and probably reversible local reorganization of AiV-1 capsid is required for its genome release.

IMPORTANCE
Aichi virus 1 (AiV-1) is a human pathogen that can cause diarrhea, abdominal pain, nausea, vomiting, and fever. AiV-1 is identified in environmental screening studies with higher frequency and greater abundance than other human enteric viruses. Accordingly, 80 to 95% of adults worldwide have suffered from AiV-1 infections. We determined the structure of the AiV-1 virion. Based on the structure, we show that antiviral compounds that were developed against related enteroviruses are unlikely to be effective against AiV-1. The surface of the AiV-1 virion has a unique topology distinct from other related viruses from the Picornaviridae family. We also determined that AiV-1 capsids form compact shells even after genome release. Therefore, AiV-1 genome release requires large localized and probably reversible reorganization of the capsid.
Details of the mechanism of kobuvirus genome release are not known. However, enteroviruses are also studied as models for genome release and delivery (17–21). The surface of the enterovirus capsid contains circular depressions around icosahedral 5-fold symmetry axes called “canyons.” The canyons of many enteroviruses, including major group HRVs, are the binding sites of receptors from the immunoglobulin superfamily (22–25). Nevertheless, other enteroviruses use different regions of their capsids to recognize their receptors (26). Before genome release, enterovirus virions convert into “altered” (A) particles characterized by radial expansion of the capsid and the formation of pores at the icosahedral 2-fold symmetry axes (18–21, 27–31). This conversion into A particles may be induced by binding to receptors or by exposure to the low pH of late endosomes (17, 28, 32–34). In poliovirus and differences from the previously studied picornaviruses.

African green monkey kidney cells (ATCC CCL-81) grown in 50 150-mm-diameter plates in minimal essential medium (MEM) with 10% fetal bovine serum (FBS) at 37°C in a 5% CO₂ incubator to 80% confluence were used for AiV-1 infection. The medium was aspirated from the plates, and the cells were washed with 5 ml of serum-free MEM. The cells were infected with 2 ml of the virus diluted to obtain a multiplicity of infection (MOI) of 0.2 in serum-free MEM and incubated for 3 h at 37°C in a 5% CO₂ incubator with gentle shaking every 30 min. Subsequently, 18 ml of MEM, supplemented with 10% FBS and 1 mM L-glutamine, was added to each plate, followed by incubation at 37°C. After 72 h, a cytopathic effect was observed, and the cells were pelleted by centrifugation at 9,000 rpm for 15 min. The virus was precipitated by adding PEG 8000 and NaCl to final concentrations of 10% and 0.5 M, respectively. The solution was centrifuged at 9,000 rpm for 10 min, and the supernatant was discarded. The resulting pellet was resuspended in 10 ml of 20 mM HEPES (pH 7.5) and 150 mM NaCl at 4°C and then homogenized with a Dounce tissue grinder. DNase and RNase were added to final concentrations of 10 μg/ml, and the solution was incubated at 37°C for 30 min. Subsequently, trypsin was added to a final concentration of 80 μg/ml, and the solution was incubated at 37°C for an additional 30 min, followed by centrifugation at 4,500 rpm at 10°C for 10 min. The clarified supernatant was layered over a 25% (wt/vol) sucrose cushion and centrifuged in a Beckman Ti 50.2 rotor at 48,000 rpm at 10°C for 2 h. After centrifugation, the supernatant was discarded, the pellet was resuspended in 2 ml of 20 mM HEPES (pH 7.5) and 150 mM NaCl at 4°C, and the virus suspension was layered onto a 10 to 40% potassium tartrate gradient and centrifuged in an SW40 rotor at 36,000 rpm at 10°C for 90 min. The gradient layer containing the virus was collected by piercing the wall of the tube with a syringe and needle. The virus-containing fraction was transferred to 20 mM HEPES (pH 7.5) and 150 mM NaCl buffer using sequential centrifugations, and buffer additions were made using Vivaspin (Sigma-Aldrich) columns at 4°C.

**Preparation of empty AiV-1 particles by heating.** The stability of AiV-1 was determined as the temperature at which 50% of its RNA genome was accessible to fluorescent RNA-binding dye Sybr green II. Virions at a concentration of 0.02 mg/ml were incubated with Sybr green II (3,000X diluted from the stock solution according to the manufacturer’s instructions), and the mixture was heated from 25 to 95°C in 1°C increments with a 2-min incubation time at each temperature in a real-time PCR instrument (Roche LightCycler 480). The fluorescence signal increases as the dye interacts with RNA that is released from the thermally destabilized particles, or the dye might be able to enter the particles. The thermal stability of the virus was estimated as the temperature corresponding to an increase in the fluorescence to 50% of the maximal value obtained when all virions were thermally denatured. The measurements were carried out in triplicates.

**Single particle data acquisition and image processing.** A solution of freshly purified AiV-1 (50 μl at concentration 2 mg/ml) was heated to 53°C for 10 min, and 3.5 μl of this sample was then immediately applied onto holey carbon grids (Quantifoil R2/1, mesh 300; Quantifoil Micro Tools) and vitrified by being plunged into liquid ethane using an FEI Vitrobot Mark IV. Grids with the vitrified sample were transferred to an FEI Titan Krios electron microscope operated at 300 kV aligned for parallel illumination in nanoprobe mode. The column of the microscope was kept at −196°C. Images were recorded with an FEI Falcon II direct electron detection camera under low-dose conditions (20 e⁻ /Å²) with under-focus values ranging from 1.0 to 3.0 μm at a nominal magnification of 47,000, resulting in a pixel size of 1.73 Å/pixel. Each image was recorded in movie mode with 1-s total acquisition time and saved as seven separate movie frames. In total, 3,893 micrographs were acquired. The frames from each exposure were aligned to compensate for drift and beam-induced motion during image acquisition using the program SPIDER (38).

**Icosahedral reconstruction of the AiV-1 empty particles.** Regions comprised of the AiV-1 empty particles (324×324 pixels) were extracted from the micrographs using the program e2boxer.py from the package EMAN2 (39), resulting in 11,606 particles. Contrast transfer function (CTF) parameters of incoherently averaged particles from each micrograph were automatically estimated using the program ctffind4 (40). Subsequently, the particles were separated into two half-data sets for all of the subsequent reconstruction steps to follow the gold standard procedure for resolution determination (41). The structure of AiV-1 determined by X-ray crystallography was used to initiate the reconstruction. The phases of the initial model were randomized beyond a resolution of 40 Å. The images were processed using the package RELION (42). The data set of 11,606 particles was subjected to multiple rounds of two-dimensional (2D) classification and 3D classification, resulting in a near-homogeneous set of empty AiV-1 particles. Subsequent refinement was performed using the 3dautoarfin program, with the starting model from the previous reconstruction low-pass filtered to a resolution of 60 Å. The reconstruction was followed by another round of 3D classification, where the alignment step was omitted and the estimated orientations and particle center positions from the previous refinement step were used. The resulting map was masked with a threshold mask and B-factor sharpened (43). The resulting resolution was determined at the 0.143 Fourier shell correlation of the two independent reconstructions.

**Cryo-EM structure determination and refinement.** The initial model, derived from the native AiV-1 structure obtained by X-ray crystallography (44), was fitted into the B-factor-sharpened cryo-EM map and subjected to manual rebuilding using the programs Coot and O, and coordinate and B-factor refinement using the programs CNS and phenix (45–48).

**Data analysis.** The volumes of the particles were calculated using the programs Mamba and Voidoo of the Uppsala Software Factory (49). The volume of the RNA genome was accessible to fluorescent RNA-binding dye Sybr green II. Virions at a concentration of 0.02 mg/ml were incubated with Sybr green II (3,000X diluted from the stock solution according to the manufacturer’s instructions), and the mixture was heated from 25 to 95°C in 1°C increments with a 2-min incubation time at each temperature in a real-time PCR instrument (Roche LightCycler 480). The fluorescence signal increases as the dye interacts with RNA that is released from the thermally destabilized particles, or the dye might be able to enter the particles. The thermal stability of the virus was estimated as the temperature corresponding to an increase in the fluorescence to 50% of the maximal value obtained when all virions were thermally denatured. The measurements were carried out in triplicates.
TABLE 1 AiV-1 virion and empty particle structure quality indicators

| Parameter                        | AiV-1 empty particle cryo-EM structure | AiV-1 virion crystal structure |
|----------------------------------|----------------------------------------|-------------------------------|
| Space group                      | NA ‡                           | 123                           |
| Unit cell dimensions             | a, b, c (Å)                    | 350.80, 350.80, 350.80         |
|                                  | α, β, γ (°)                    | 90, 90, 90                    |
| Resolution (Å)                   | 4.2                           | 72.0–2.3 (2.34–2.30)          |
| Rmerge ‡                        | NA                           | 0.15 (0.63)                   |
| ⟨h⟩/⟨h⟩                          | NA                           | 6.0 (1.8)                     |
| Completeness (%)                 | NA                           | 91.0 (94.0)                   |
| Observation multiplicity         | NA                           | 3.2 (3.0)                     |
| No. of observations              | NA                           | 902,384 (43,198)              |
| No. of unique observations       | NA                           | 282,853 (14,471)              |
| Rwork                            | 0.34                         | 0.33                          |

No. of:

- Protein atoms*                  | 5,455                        | 5,791                         |
- Water atoms*                    | NA                           | 147                           |

RMSD

- Bond lengths (Å)                | 0.016                        | 0.013                         |
- Bond angles (°)                 | 1.57                         | 1.43                          |

Ramachandran statistics (%)

- Preferred regions               | 89.1                         | 95.7                          |
- Allowed regions                 | 8.4                          | 4.0                           |
- Disallowed regions              | 2.5                          | 0.3                           |

Avg atomic B factor (Å²)          | 127.8                        | 22.9                          |

‡ Rmerge = Σ|I−|Σ|I| − (⟨h⟩/Σ⟨h⟩)^2. * Statistics are given for one icosahedral asymmetric unit. ‡ Statistics for the highest-resolution shell are indicated in parentheses. † Determined according to the criterion of Molprobity (78). ‡ NA, not applicable.

RESULTS AND DISCUSSION

Structure of AiV-1 virion and capsid proteins. The crystal structure of the AiV-1 virion was determined to a resolution of 2.1 Å using X-ray crystallography. The diffraction data were affected by perfect hemihedral twinning. The structure determination process has been described elsewhere (37), but for completeness of the discussion the basic crystallographic and structure quality indicators are reprinted here (Table 1). The maximum outer diameter of the AiV-1 capsid is 318 Å. The capsid is built from major capsid proteins VP0, VP1, and VP3 arranged with pseudo-T3 icosahedral symmetry (Fig. 1A). VP1 subunits form pentamers around 5-fold axes, while VP0 and VP3 alternate around the icosahedral 3-fold axes. The three capsid proteins have jellyroll β-sandwich folds with β-strands named according to the picornavirus convention B to I (53). Two antiparallel β-sheets forming the cores of the subunits contain strands BIDG and CHEF, respectively (Fig. 1A). Loops of the capsid proteins are named according to the β-strands they connect. N termini of the major capsid proteins are located on the inside of the capsid, whereas C termini are exposed at the virion surface. A complete model of the AiV-1 icosahedral asymmetric unit could be built apart from residues 1 to 12, 56 to 63, and 76 to 111 of VP0, residues 84 to 87 and 234 to 253 of VP1, and residues 221 to 223 of VP3.

Topology of AiV-1 virion surface is distinct from the previously structurally characterized picornaviruses. While structures of numerous viruses from the Picornaviridae and Dicistroviridae families have been determined previously, AiV-1 is the first characterized representative of the Kobuvirus genus. AiV-1 shares less than 26% sequence identity with poliovirus 1, HRV14, EV71, human parechovirus 1 (HPeV-1), and hepatitis virus A (Table 2) (54–57). Structure-based phylogenetic analysis indicates that AiV-1 is quite distinct from all previously determined structures of picornaviruses and dicistroviruses (Fig. 1D). Most of the differences are located at the capsid surface. Our high-resolution analysis did not confirm the presence of the previously proposed astrovirus-like protrusions in the AiV-1 capsid (1, 16). Instead, the AiV-1 virion is rather spherical in shape with plateaus around 3-fold axes, slight depressions at 2-fold axes, and low protrusions around icosahedral 5-fold axes that are encircled by circular depressions that we, according to the enterovirus convention, call canyons (Fig. 2A and B). However, the AiV-1 canyon is shallower and has a different shape to that of enteroviruses (Fig. 2C). The differences in the capsid topology between AiV-1 and other enteroviruses are due to different lengths of loops of the capsid proteins that form the capsid surface. The central wall of the enterovirus canyon is formed by CD loops of VP1, whereas the outer wall is formed by the EF loop of VP2 called puffed and a loop before β-strand B of VP3 called knob, the CD loop of VP3, and the GH loop of VP1 (Fig. 3C, D, G, and H). However, capsid protein VP3 of AiV-1 lacks the knob loop and its VP0 (a homologue of enterovirus VP2) contains only a very small puff, including a single seven-residue α-helix (Fig. 3A, B, E, and F). Interestingly, the puff and knob loops of AiV-1 are shorter that those in HPeV-1, which lacks the canyon altogether (Fig. 3I and J). However, the BC loop of VP0 of AiV-1 is relatively elongated and forms a protrusion on the capsid surface located in the volume occupied by the puff and knob of enteroviruses (Fig. 3E to J). Therefore, the BC loop of AiV-1 VP1 substitutes for the missing knob and small puff of AiV-1 and forms the outer wall of the canyon (Fig. 3A, B, and F). Moreover, the GH loop of AiV-1 VP1 is relatively short and fills the canyon instead of reinforcing its outer wall, as is the case in poliovirus 1 (Fig. 3A and B). The long C-terminal arm of poliovirus 1 VP1, which wraps around a side of the puff, is not structured in AiV-1, resulting in a relative broadening of the canyon (Fig. 3A to D). Furthermore, the depth of the AiV-1 canyon is further diminished by the α3 helix in the CD loop of AiV-1 VP1 that partially fills the volume of the canyon (Fig. 3A and B). In summary, the differences in the surface-exposed loops make...
AiV-1 unique among picornaviruses and dicistroviruses characterized to date. They are reflected in the isolated position of AiV-1 in the structure-based evolutionary tree (Fig. 1D). The differences in surface topology indicate the possibly of a different type of receptor binding in AiV-1 than is found in enteroviruses.

**Absence of hydrophobic pocket in VP1.** The genome release of some enteroviruses can be initiated by the interaction of the virus with receptors with an immunoglobulin fold that bind to the canyon (25, 53, 58). At the bottom of the canyons of some enteroviruses is a small aperture leading to a hydrophobic pocket within the β-barrel core of the VP1 subunit (54, 55). The pocket is filled with a lipid moiety called a "pocket factor," which was proposed to function in the regulation of virus stability and uncoating (25). Importantly, compounds binding to the VP1 pocket with high affinity have been demonstrated to increase enterovirus stability, block its genome release or receptor binding, and prevent virus infection (59–64). However, in comparison to enteroviruses the β-sandwich core of AiV-1 VP1 is filled with hydrophobic side chains of residues and does not form the pocket (Fig. 3A and C). In this respect, AiV-1 is similar to other picornaviruses such as cardioviruses (65–67), parechoviruses (56, 68), and aphthoviruses.
(32, 69). Therefore, capsid-binding inhibitors are unlikely to be effective against AiV-1 and other kobuviruses.

**Structural changes of AiV-1 capsid associated with genome release.** It has been shown previously that virions of enteroviruses convert to A particles before the genome release (17, 28, 33, 34). The A particles are characterized by an expanded virion diameter and channels in the capsid that were speculated to serve in the release of the RNA genomes. The empty capsid shells produced after the genome release were named B particles, which are structurally similar to the A particles. It was demonstrated that the formation of A particles and the genome release of many picornaviruses might be induced nonphysiologically by heating the virions to 42 to 56°C (21). In order to study the genome release of AiV-1, its virions were gradually heated, and the genome release was monitored (Fig. 2E). AiV-1 virions released their RNA rather abruptly at 53°C. Electron microscopy of AiV-1 virions heated to 53°C for 10 min identified 95% of empty capsids, which were used to reconstruct the empty particle to a resolution of 4.2 Å (Fig. 2G and Table 1). The AiV-1 empty capsid is expanded by 7.6 Å in diameter relative to the native virus (Fig. 2A and B). The volume of the particle increases from 4.8 × 10^6 Å^3 to 5.5 × 10^6 Å^3. The structure of the empty particle differs from that of the native virion, mostly in the contacts between the pentamers of capsid protein protomers. In the native AiV-1 virion, the interpentamer contacts are mediated by strand F of VP3 (Fig. 2C and D). In the empty AiV-1 particle, the N-terminal arm of AiV-1 VP1 becomes structurally reorganized upon the formation of the empty particle. Residues 1 to 30 become disordered, and residues 31 to 35 refold into a new structure with the last resolved residue pointing toward the particle center (Fig. 4A and B). This is in contrast to the previous observation of the A particle of CVA16, in which the N terminus of VP1 was exposed at the capsid surface (Fig. 4C and D). The differences in the positioning of the N-terminal arm of VP0 might influence the stability of the empty capsid, since enteroviruses, which produce empty particles after genome release, have the same type of interpentamer interaction mediated by the N terminus of VP0/VP2 as that of AiV-1 (Fig. 1C).

The N-terminal arm of AiV-1 VP1 undergoes structural reorganization upon the formation of the empty particle. Residues 1 to 30 become disordered, and residues 31 to 35 refold into a new structure with the last resolved residue pointing toward the particle center (Fig. 4A and B). As a consequence, the interpentamer interface is reduced from 2,750 to 1,400 Å^2 (see Fig. 5). Furthermore, residues 139 to 144 of VP0 form new interactions with the core of subunit VP3 (Fig. 4B). At the same time, residues 55 to 60 of VP0 became structured in the empty AiV-1 particle and residues 67 to 75 retain the same structure as in the native virions (Fig. 2C and D and Fig. 4A and B). Therefore, the N-terminal arm of AiV-1 VP0 does not appear to be externalized from the empty particle. This is in contrast to the presumption that the N-terminal part of AiV-1 VP0 is functionally homologous to the VP4 of other picornaviruses and might therefore play a role in the transport of the virus genome across the endosomal membrane into the host cytoplasm. However, it is possible that in vivo, because of interactions with an as-yet-unknown receptor, the empty AiV-1 particles dissociate into pentamers and the VP0 N termini could interact with the membranes.

The structure of HPeV-1, which also contains VP0 in mature virions, was recently determined (56). However, in contrast to AiV-1, empty particles of HPeV-1 rapidly dissociate into pentamers after the genome release (56, 70). Major differences between AiV-1 and HPeV-1 are in the interpentamer interactions mediated by the N-terminal arm of VP0 (Fig. 1A and B). While in AiV-1 the β1 and β2 strands extend the β-sheet CHEF of VP3 from the icosahedral asymmetric unit from a neighboring pentamer, in HPeV-1 the N-terminal arm forms a loop that stretches around the icosahedral 2-fold axis and the β-strands extend the β-sheet of the VP3 subunit from the same pentamer (Fig. 1A and B). The differences in the positioning of the N-terminal arm of VP0 might influence the stability of the empty capsid, since enteroviruses, which produce empty particles after genome release, have the same type of interpentamer interaction mediated by the N terminus of VP0/VP2 as that of AiV-1 (Fig. 1C).
The reduction in the interface area cannot be directly converted to binding energies, but the reduced interpentamer contacts indicate that the expanded AiV-1 particles might be prone to the formation of capsid pores for genome release or to disassembly into pentamers.

**Differences between kobuvirus and enterovirus genome release.** The structure of the empty particle of AiV-1 is different from that of empty virus particles. "A" particles of CVA7, EV71, poliovirus 1, HRV-2, and CVA16 contain two types of pores located at icosahedral 2-fold axes and between icosahedral 2-fold and 5-fold symmetry axes (Fig. 4C and D) (18–21, 27–31). In the CVA16 A particle, the pores at 2-fold axes were proposed to allow externalization of VP1 N termini that then translocate to the channels between 2-fold and 5-fold axes (Fig. 4C and D) (29). In addition, the pores at 2-fold axes were speculated to serve as channels for the release of VP4 subunits and the RNA genome. The borders of the channels located at 2-fold axes in enterovirus particles are formed by helix α3 from the CD loop of VP2 and EF loop of VP3 (29, 72). In contrast to the enterovirus A and empty particles, the empty particle of AiV-1 does not contain...
any pores (Fig. 2B). Aphthovirus equine rhinitis A virus (ERAV) also forms empty particles with compact capsids that do not contain any pores for genome release (32). Nevertheless, the empty particles of ERAV rapidly dissociate into pentamers. The most likely positions for the formation of channels in the AiV-1 capsid are the borders of the pentamers of capsid protein protomers and specifically areas around the icosahedral symmetry axes. Helices /H9251 of VP0 subunits related by a 2-fold axis move 1.8 Å away from each other when the AiV-1 virion converts to the empty particle (Fig. 4A and B). Nevertheless, the movement does not result in the formation of a channel around the 2-fold axis (Fig. 2B and 4B). The channels at the 2-fold axes observed in the A and empty particles of enteroviruses are also not of sufficient size and require expansion in order to allow the genome release (Fig. 2D) (29, 71, 72). It is therefore possible that an opening in the AiV-1 capsid at the 2-fold axis might serve as a channel for genome release; however, more extensive local reorganization of the capsid than in enteroviruses would be required. This possibility is to some extent supported by the presence of residues with neutral and negatively charged side chains in the vicinity of the 2-fold symmetry axis of AiV-1, similar to the situation in enteroviruses (Fig. 6A to C) (73). The negative charge might provide a “slippery” surface facilitating the egress of viral RNA (74).

It was proposed previously that an “iris-like aperture” movement of the N termini of VP3 subunits might result in the opening of 10-Å diameter channels through the rhinovirus capsid, which
might enable the exit of the genomic RNA (75, 76). The narrowest constricted along the AiV-1 5-fold axis is formed by the N-terminal arms of VP3 subunits, which might perform such an “iris-like aperture” movement (Fig. 2C). However, the structures of AiV-1 VP1 and VP3 subunits, which are in contacts around the 5-fold axes, are not affected by the capsid expansion (Fig. 2D), and the pore along the 5-fold axis is obstructed in both the native and the empty particle structures (Fig. 6A, B, and E). Therefore, the 5-fold axes of the AiV-1 capsid do not seem to be likely portals for the genome release. It is possible that the iris-like aperture movements in the rhinovirus capsids may represent structural rearrangements accompanying the genome release rather than forma-

**FIG 4** Comparison of interpentamer contacts in virions and expanded particles of CVA16 and AiV-1. (A to D) Structures of AiV-1 virion (A), AiV-1 empty particle (B), CVA16 virion (C), and CVA16 empty particle (D) in the vicinity of the icosahedral 2-fold axes viewed from the particle center (left) and perpendicular to the capsid surface (right). VP1 subunits are shown in blue, VP0/VP2 are shown in green, and VP3 is shown in red. Subunits from different icosahedral asymmetric units are distinguished by color tones. N-terminal arms of VP0 in AiV-1 and VP2 in CVA16 that participate in interpentamer contacts are shown in bright green. Dashed lines indicate the putative positions of unstructured residues. N-terminal residues of VP1 are shown in bright blue. Residues of VP3-forming strands βE and βF that interact with the N termini of VP0/VP2 are shown in bright red. The positions of icosahedral 2-fold and 3-fold symmetry axes are indicated with ovals and triangles, respectively.

**FIG 5** Buried surface areas of interfaces within AiV-1 and CVA16 virions and empty particles. (A) List of buried surface areas. Individual subunits are labeled according to their relative positions shown in panel B. "Other" indicates the sum of buried surface areas of additional small interfaces. (B) Capsid surface representation of AiV-1 virion with subunits VP0, VP1, and VP3 shown in green, blue, and red, respectively. Icosahedral asymmetric units considered for buried surface calculations are labeled with letters.
tion of a pore through which the RNA exits the virion. Similarly, the 3-fold axes of the AiV-1 capsid are entirely closed by side chains located in their vicinity, and the internal face of AiV-1 capsid around the 3-fold axes is covered by negatively charged residues (Fig. 6B and D).

Comparison of the native virion and empty particle structures of AiV-1 indicates that the genome release necessitates the formation of a special pore within the capsid shell. However, the possibility of obtaining a 4.2-Å-resolution reconstruction of the empty particle indicates that the pore does not affect the overall icosahedral symmetry of the capsid or that the structural changes required for the genome release are reversible. In that case the empty capsids would not provide information on how the genome was released from the particle. The absence of pores in the AiV-1 capsid presents an obstacle for genome release. It was shown previously that the genome release of HRV2 proceeds in the 3'-to-5' direction (77). Assuming that AiV-1 genome release has the same directionality, it is not clear how the 3' end of the AiV-1 genome might induce the formation of a pore in the capsid or how it finds the special preformed pore within the capsid. The alterations in the structures of the N termini of VP2 and VP1 connected with the formation of the empty AiV-1 particle result in the removal of the negative charge that is located at the edges of the pentamers in the native virion (Fig. 6A and B). This may promote interactions of the genomic RNA with the capsid and its eventual release from the virion.

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C.S. calculated the single-particle cryo-EM reconstruction, performed crystallization, model building, refinement, and data analysis, and participated in writing the manuscript. L.P. optimized and conducted large-scale virus purifications. K.S. and T.F. participated in single-particle cryo-EM reconstruction. A.M.L. provided material for the study and participated in data analysis. P.P. designed the study, performed AiV-1 crystal structure determination, participated in data analysis, and wrote the manuscript.

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