Cryo-EM structure of the mature dengue virus at 3.5-Å resolution

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Regulated by pH, membrane-anchored proteins E and M function during dengue virus maturation and membrane fusion. Our atomic model of the whole virion from cryo–electron microscopy at 3.5-Å resolution reveals that in the mature virus at neutral extracellular pH, the N-terminal 20-amino-acid segment of M (involving three pH-sensing histidines) latches and thereby prevents spring-loaded E fusion protein from prematurely exposing its fusion peptide. This M latch is fastened at an earlier stage, during maturation at acidic pH in the trans-Golgi network. At a later stage, to initiate infection in response to acidic pH in the late endosome, M releases the latch and exposes the fusion peptide. Thus, M serves as a multistep chaperone of E to control the conformational changes accompanying maturation and infection. These pH-sensitive interactions could serve as targets for drug discovery.

Dengue virus is a prevalent mosquito-borne flavivirus that is endemic across tropical and subtropical regions, causing diseases ranging from self-limiting fever to lethal hemorrhagic fever and shock. Each year, more than 50 million people are infected1. Dengue virus is also a potential biothreat agent2. Currently, there are neither licensed vaccines nor specific antiviral therapies against dengue infection. Indeed, the spread of dengue virus is recognized as a major urban public health concern by the World Health Organization1.

Viral membrane proteins have critical roles during the life cycle of enveloped viruses such as the dengue virus, particularly during entry into a host cell. The two dengue virus membrane proteins, E and M, undergo dramatic structural changes from the immature, fusogenic form of the virion3 and then again at the time of infection. These two proteins are expressed in a polyprotein that is cleaved to yield the precursor of M (prM, consisting of M and a leading segment, pr) and E4,5. Then, upon exposure to the neutral pH of the endoplasmic reticulum, prM binds to E to form the ‘spiky immature’ form of the virus3, the spikes consisting of trimers of the outward pointing domain II of E. E and prM then undergo further maturation that includes three steps: (i) triggered by low pH in the trans-Golgi network (TGN), formation of pr-stabilized dimers of E lying on the surface, producing the ‘smooth immature’ virus3; (ii) cleavage of prM by the furin protease in the TGN into the pr and M portions4; and (iii) triggered by neutral pH in the extracellular space, shedding of pr upon release from the cell to yield the fusion-competent, ‘smooth mature’ virion3. Later, during infection, dengue virus enters the cell through receptor-mediated endocytosis, and fusion of virus membrane with endosomal membrane is triggered by low pH in the late endosome5,6. Although cryo-EM has provided low-resolution in situ structures of immature viruses and of mature virions7,8, and X-ray crystallography has provided high-resolution ex situ structures of some domains of E and prM9–11, what is not known is how changes in pH bring about these structural and transitional transformations.

We set out to explain the interplay between E and M during viral maturation and infection by solving the cryo-EM structure of a native, mature dengue virus. Here we report the 3.5-Å structure of the mature dengue virion in its native form as determined by cryo-EM single-particle reconstruction. We discovered a latch-type interaction between E and M, mediated by pH-sensing histidine residues, that holds E in place and prevents premature exposure of its fusion peptide. This structure also provides insight into histidine-based, pH-sensitive maturation processes that spring-load E for later exposure of its fusion peptide at the right time, move M to the latch site and engage the M latch on E. Thus, the structure reveals that, in response to shifts in pH, M chaperones E through the dramatic conformational changes required for several stages of dengue virus maturation and infection.

RESULTS

Structural validation

Cryo-EM micrographs of purified dengue virions recorded in a Titan Krios microscope revealed spherical (mature) particles among...
partially mature, irregular or incomplete ones (Fig. 1a), as previously observed. We eliminated the partially mature virions as much as possible through visual inspection and obtained 32,596 spherical particles from 1,103 films. Subsequently, we computationally selected 9,288 ‘good’ particles (Supplementary Fig. 1a) that strictly conformed to icosahedral symmetry by use of a global orientation-center search method based on a multipath simulated annealing algorithm. The final reconstruction (Fig. 1b–d and Supplementary Movie 1) had an effective resolution of 3.5 Å (ref. 14; Supplementary Fig. 1b), and we validated it by identifying amino acids in the density map (Supplementary Figs. 1c,2–4).

Overall structure

Inside the envelope, the capsid of the virus was disordered, as described previously. On the surface of the virion, Asn67 and Asn153 of every E subunit were glycosylated (Fig. 1c), as previously observed. We built an atomic model for the asymmetric unit (Fig. 2a,b and Supplementary Figs. 2,5), which contains three E subunits and three M subunits (Fig. 1c). The three quasiequivalent copies of E are very similar to each other, as are those of M, as illustrated by their superposition in Supplementary Figure 5a. The averaged r.m.s. deviation of the locations of the Ca atoms among the three quasiequivalent copies of E and M was only 1.2 Å, the largest difference being in the loop that connects domains II and III. Therefore, we averaged these three copies (Supplementary Movie 1).

Mature dengue virion has a smooth icosahedral outer surface covered by E protein (Fig. 1b and Supplementary Movie 1) with M protein underneath. Five E monomers surround each fivefold axis in the shape of a starfish, three surround each threefold axis and two surround each twofold axis (Fig. 1b and Supplementary Movie 1), as established in previous studies at resolutions of 9 Å (ref. 8) and 24 Å (ref. 7). Each of the large triangles in Figure 1b,c outlines an asymmetric unit. Two adjacent triangles contain three E-M-M-E heterotetramers of membrane proteins E and M, making one ‘rhombic raft’ (Fig. 1c and Supplementary Movie 1). E-M:M-E heterotetramers bind neighboring E-M-M-E heterotetramers (Fig. 1b) through E to E interactions, mainly hydrophilic ones (Supplementary Fig. 1c and Supplementary Table 1) at interfaces along the lateral edges of E. These E and M proteins anchor to an underlying lipid bilayer envelope through their transmembrane helices E-T1, E-T2, M-T1 and M-T2 (Figs. 1 and 2b and Supplementary Figs. 3,4,5b,6a). Apart from the last three residues of M at its C terminus, all residues of E and M are ordered in the structure, thus permitting atomic modeling for both full-length proteins, leaving out the last three amino acids of M (Fig. 2a,b, Supplementary Figs. 2,3 and Supplementary Movie 1).

In situ structure of E

The in situ structure of the full-length dengue E protein contains four domains, the transmembrane domain and the domains I, II and III that comprise the ectodomain (Fig. 2a,b and Supplementary Fig. 2). Our atomic model of the transmembrane domain of E, which anchors domain III to the membrane, consists of three perimembrane helices E-H1, E-H2 and E-H3 at the N terminus and two transmembrane helices E-T1 and E-T2, all interlinked by loops (Fig. 2b,c and Supplementary Figs. 3,5a,d,e).

We also identified structural elements crucial to the movement of E’s domains during viral maturation and membrane fusion by comparing our in situ structure of E at atomic resolution to other available structures (including pseudoatomic models) of the ectodomain of E (ref. 9,11). Superposition of all of these structures by matching Ca atoms of their domain I revealed a relative rotation of domain II (Fig. 2d), with domain I held in place by interaction with the domain III, anchored to the transmembrane domain, with the latter anchored to the membrane. We propose that the β hairpin (Val197–Val208; β strands f and g) is the axle of this rotation, as illustrated by the arc that connects the tips of all but one of the domain II structures (Fig. 2d and Supplementary Fig. 5c). (The exception is the 9 Å pseudo-atomic model.) The location of this hairpin (as measured from Val197) is ~12 Å away from the previously suggested hinge of Gly190 (ref. 11).

We propose that rotation about this axle would enable the conformational change of E required for the virus to begin fusion with endosomal membrane and release its core into the cytoplasm. The richness of hydrophobic residues on and around this hairpin (Fig. 2e) is conducive of such a rotation. Indeed, small molecules that bind to the cavity next to this hairpin have been shown to block viral entry, presumably by hindering the relative rotation about the β hairpin and preventing conformational change required for fusion.

In situ structure of M protein

In our cryo-EM structure, 72 of the 75 residues of the full-length M protein are resolved (including two domains: an extended domain of its first 20 amino acids and a transmembrane domain; Fig. 2a–c and Supplementary Fig. 2). M has three portions (Fig. 2f and Supplementary Fig. 3): an extended N-terminal loop (amino acids 1–20, named M1–20), an amphipathic perimembrane helix (amino acids 21–40, named M-H) and a pair of transmembrane
helices (amino acids 41–75, named M-T1 and M-T2) (Fig. 3 and Supplementary Fig. 6a). The perimembrane and transmembrane helices serve to anchor the M protein to the membrane.

Val2 of M_{1-20} (Fig. 2f) is bound in a pocket of E (pocket 1; Fig. 4a,b). M_{1-20} also contains Thr16 (Fig. 2f), where the loop bends and turns to the other side of helix α8 of E (Supplementary Fig. 5b,c). This bend is very close to the ‘hole’ formed between two E subunits (Supplementary Fig. 7), a feature described previously6 and discussed below.

The perimembrane helix of M (M-H) starts at Ser21 and strikes the outer leaflet of the viral membrane at an angle, with a kink at Lys27 (Fig. 2f and Supplementary Fig. 3a). The first part (before the kink), which is mainly hydrophilic but with one tryptophan (Trp26) inserted into the membrane, is slightly outside the head-group region (Supplementary Fig. 6a). The second part (after the kink), which is buried in the head-group region, is amphipathic, like the perimembrane helices of the E protein.

The two transmembrane helices of M, M-T1 and M-T2, are shorter than the transmembrane helices of E (Fig. 2b and Supplementary Fig. 5a,b). Unlike those in E, these two transmembrane helices in M contain mostly hydrophobic residues, with the exception of several hydrophilic residues in the head-group region of the lipid (Fig. 3b and Supplementary Fig. 6a). The last resolved residue of M, Pro72, is located at the edge of the head-group region of the outer leaflet.

Membrane–protein interactions

The discernible features in the membrane density of the reconstruction suggest the existence of some lateral order among the phospholipids of the lipid bilayer (Supplementary Fig. 6b,c), as is the case for bacteriophage PRD1 (ref. 17). The membrane is bent to an angular shape at the distal ends of the transmembrane helices of E and M, where the membrane thickness is reduced from 42 Å to 30 Å (Fig. 1d and Supplementary Fig. 6d,e) because of the short lengths of these transmembrane helices. Indeed, for each transmembrane helix of M, there are just four helical turns, and for each transmembrane helix of E, there are just five. This angular membrane shape is in sharp contrast to the spherical membrane shape observed in alphaviruses18, where the transmembrane helices are noticeably longer (seven helical turns), cross a fully relaxed envelope and reach underlying capsid proteins.

The two transmembrane helices of E, E-T1 and E-T2, are oriented vertically and span the hydrophobic region of the membrane, with their interconnecting loops buried within the head-group region of the inner leaflet (Fig. 2b,3a and Supplementary Fig. 6a). They form a coiled coil (Fig. 3a), with hydrophobic surfaces facing outward and

Figure 2  Atomic model of the E-M-M-E heterotetramer. (a) Side view of the averaged heterotetramer. (b) Side view of the atomic model of the tetramer shown in ribbon with glycans at Asn63 and Asn157 of E shown as sticks. The M_{1-20} loop binds to a groove in E (Fig. 4a,b). (c) The color scheme of the domains of E-M follows previous work8–11: E domain I (DI), red; E domain II (DII) yellow; E domain III (DIII), blue; E transmembrane (TM) domain, cyan; M_{1-20}, magenta (ectodomain); and M TM domain, orange. (d) Hinge in DII of E. The blue arc, centered on a β-hairpin of DII (asterisk), connects the tips of DII in the various conformations: sE (H), solubilized E Harvard crystal structure9; sE (P), solubilized E Purdue crystal structure11; immature, E ectodomain crystal structure1; mature 9 Å, our in situ atomic structure of full-length E in the cryo-EM structure; mature 3.5 Å, pseudo-atomic model obtained by fitting to a 9-Å mature virion cryo-EM structure11. TM, DI and DIII are from our cryo-EM structure. (e) Stereo view of the hydrophobic environment of the β-hairpin (solid golden ribbon with sticks) in DII of E. This domain (atoms colored golden for C, red for O, blue for N, yellow for S) is shown together with surrounding environment (semitransparent ribbons with sticks, atoms colored white for C, red for O, blue for N, yellow for S) to illustrate hydrophobic interactions. Except for residues at the top and bottom surfaces of the protein, almost all the residues of the hairpin and its surrounding residues are hydrophobic, as indicated by the atom types. (f) Ribbon model of M color-coded from blue at the N terminus through red at the C terminus, with key residues mentioned in the text shown as sticks.

Figure 3  Hydrophobic interactions. (a) Close-up view of the transmembrane helices (E-T1 and E-T2) and perimembrane helices (E-H1 and E-H2) of E protein (cyan ribbons). The boundaries of the outer and inner leaflets of the phospholipid bilayer are marked. (b) Close-up view of the transmembrane helices (M-T1 and M-T2) and the perimembrane helix (M-H) of M protein (orange ribbons). The sticks represent atomic models of selected side chains.
multiple hydrophilic residues (threonine and serine) facing inward, reminiscent of the configuration of a leucine zipper, although with hydrophobic residues (threonine and serine) for interaction between the coiled α-helices instead of hydrophobic residues (leucine). These interactions explain why a functional mosaic cannot be constructed from E-T1 and E-T2 of two different viruses19.

The three perimembrane helices of E (E-H1, E-H2 and E-H3) and one perimembrane helix of M (M-H) lie horizontally among the head groups of the outer leaflet of the envelope (Fig. 3 and Supplementary Fig. 6a). Indeed, our structure revealed that helices E-H1 and E-H3 are amphipathic, with top halves containing mainly hydrophobic residues facing outside and bottom halves containing mainly hydrophobic residues and interacting with the alkyl groups of the membrane. The smallest of the perimembrane helices, E-H2, is a two-turn 3_10 helix that is not resolved in the 9-Å structure. It extends slightly outside the head-group region and contains mainly hydrophilic residues.

**Interactions between E and M**

M and E proteins interact primarily through hydrophobic contacts. M_{1–20} lines a groove in the side of E that faces the membrane on the viral envelope (Fig. 4a,b, Supplementary Fig. 8a and Supplementary Movie 2). The area of the buried surface between M and the ectodomain of E is 1,138 Å² (Fig. 4b). To our surprise, we found no hydrogen bonds between M and the ectodomain of E despite the extensive interactions between the proteins. Their binding affinity results from three hydrophobic interactions. First, Val2 of M (Fig. 4c and Supplementary Fig. 3) inserts into a pocket in E (pocket 1; Fig. 4a,c, Supplementary Fig. 8b,c and Supplementary Movie 2) formed by Leu216, Leu218 and Met260 on one side of helix αB of E. Second, His7, Met10 and Leu12 of M (Fig. 4e and Supplementary Fig. 3) form another hydrophobic core with residues of E that include His209 and Trp212 (pocket 2; Fig. 4d, Supplementary Fig. 8d,e and Supplementary Movie 2). Third, Trp19 of M (Fig. 4e and Supplementary Fig. 3) is encompassed by a deep recess (pocket 3; Fig. 4a,e, Supplementary Fig. 8f,g and Supplementary Movie 2) that includes Trp206 and His261 (on the other side of helix αB of the same E monomer noted in pocket 1). These hydrophobic interactions result in the stability of mature dengue virus.

Indeed, many of the aforementioned residues are highly conserved among all flaviviruses or among just all dengue viruses (Supplementary Fig. 9). In M, His7, Leu12 and Trp19 are strictly conserved among all flaviviruses (with a single exception of a methionine in Powassan tick-borne encephalitis in the position corresponding to Leu12), and Val2 can be replaced only with residues containing branched hydrophobic side chains (that is, leucine and isoleucine). For pocket 1, Leu216 and Leu218 of E are strictly conserved among flaviviruses, and Met260 is conserved among different strains of dengue virus. In pocket 2, Trp212 of E is strictly conserved, and His209 is strictly conserved, except for yellow fever and St. Louis encephalitis. For pocket 3, Trp206 of E can only be replaced by an aromatic phenylalanine, and His261 is conserved among dengue and Japanese encephalitis subtypes. The conservation of these residues is consistent with critical roles of these interactions.

**DISCUSSION**

A model for maturation and infection

Our atomic structure of the mature virion, together with previous models of the immature particle and the post-fusion form of E,3,4,9–11,23,24, clarifies the picture of dengue virus maturation, through multiple pH-sensitive stages (Fig. 5).

At neutral pH in the endoplasmic reticulum, in the immature spiky virus (stage 1; Fig. 5a) the three domains II of each E trimmer point outward15,23. Low pH in the TGN triggers rotation of each of the
Viral membrane fusion proteins face two challenges to productively fuse with host cells. First, every fusion protein has to arrest in a high-energy, pre-fusion form during folding, before reaching its low-energy, post-fusion form. Second, those fusion proteins that are pH-sensitive must distinguish the low pH in the TGN during egress from the low pH in the late endosome during infection. Fusion should occur in the latter stage but not the former. The three classes of viral fusion protein overcome these challenges with different strategies.

Class I fusion proteins, such as those found in influenza viruses and HIV, use a ‘hidden knife’ strategy. They overcome the first challenge by expressing their fusogenic peptides in the middle of an inactive precursor, whose cleavage, either in the TGN (HIV) or later (influenza) leaves this peptide at a new N terminus to prime fusion. Those that are pH-sensitive, like influenza hemagglutinin, overcome the second challenge by passing through the low pH in the TGN in an uncleaved form. Later, the low pH in the late endosome during infection exposes the fusion peptide from a primed (cleaved) virus. Exposure of the fusion peptide by pH-insensitive fusion proteins, as found in HIV, is triggered during infection by binding to membrane receptors.

Class III fusion proteins, as found in rhabdoviruses, baculoviruses and some herpesviruses, use a ‘reversible form’ strategy, specifically a reversible pH-driven conformational change. The fusion protein takes its fusion incompetent ‘pre-fusion’ form through the TGN but adopts its fusion competent ‘pre-fusion’ form at higher pH outside the cell, as reviewed in ref. 25.

Our structure revealed how the class II viral fusion protein in dengue virus uses a ‘multistep chaperone’ strategy, involving a second membrane protein (M) that is used to aid in the folding, trafficking and function of the fusion protein (E). One review suggested that the action of a protein such as E2 in alphavirus might be conceptualized as a chaperone. Because M in dengue virus performs many roles in a
complicated, multistep process widely dispersed over space and time, because it cannot be recycled as a result of cleavage and irreversible conformational changes, because its anchoring in the membrane is critical to its function, and because it responds to pH in the TGN, the extracellular environment and the endosome, we describe M as a membrane-anchored, pH-sensing, multistep chaperone.

For these fusion strategies, countermeasures can be devised against viral infection. The special dependency of class II fusion proteins on chaperone proteins may be their Achilles heel. Indeed, in addition to providing insight into the mechanisms of viral maturation and fusion, our structure identifies specific interactions between the dengue virus fusion protein and its chaperone protein in atomic detail that are critical for maturation and infection. These specific interactions are potential targets for future therapeutic intervention. Indeed, the small peptide enfuvirtide blocks pro-fusion folding of the gp41 fusion protein in HIV with itself by competing for a binding site on gp41 (ref. 26). By contrast, small molecule analogs of dengue M protein that block its access to any or all of the three pockets in E might disrupt the function of the chaperone protein M and thereby abolish dengue virus maturation and/or trigger premature exposure of the fusogenic peptide of E from the mature virus. Such small molecules could serve as leads for drug discovery.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Atomic coordinates of the mature dengue virion have been deposited in the Protein Data Bank: 3J27. Cryo-EM density maps of the virion and the averaged tetramer have been deposited with the Electron Microscopy Data Bank: EMD-5499 (averaged subunit) and EMD-5520 (full virion).

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

Z.H.Z., X.Z., P.G. and X.Y. designed experiments. J.M.B., X.Z. and X.Y. cultured cells and purified virus samples. X.Z., X.Y., P.G., J.M.B. and Z.H.Z. obtained cryo-EM images. Z.H.Z., J.M.B. and X.Z. participated in the image processing and three-dimensional reconstruction from the Polara data. X.Z. obtained a 7 Å structure from the Polara data. P.G. refined the structure to 3.5 Å resolution with the Titan Krios data and built the atomic models. P.G., X.Z. and Z.H.Z. interpreted the structure and drafted the manuscript. P.G., X.Z., Z.H.Z. and S.S. finalized the manuscript. G.B. and Q.Z. participated in discussion and interpretation of the results. All authors reviewed the final manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Isolation of dengue virus. C6/36 cells were cultured at 33 °C in the presence of 5% CO₂. During cell passaging, we detached cells from flasks by vigorously shaking each flask a few times, avoiding exposure of cells to trypsin. Twenty-seven Corning T125 flasks, each containing C6/36 cells in 50 ml of medium, were infected by dengue virus type 2 New Guinean strain. Five days after infection, cell culture medium was collected and centrifuged in a Beckman centrifuge (11,000 g) for 50 min to pellet and discard large debris. PEG-8000 was added to the supernatant to a final concentration of 7% (w/v). The sample was left at 4 °C for 8 h and subsequently centrifuged in a Beckman centrifuge (11,000 g) for 45 min to collect the virus-containing pellet. The virus was resuspended in TNE buffer (50 mM Tris, 140 mM NaCl and 5 mM EDTA, pH 7.4) by soaking the pellet in the buffer for 20 min. The resuspended sample was then loaded at the top of a glycerol–potassium tartrate gradient (10% to 40% potassium tartrate, 30% to 7.5% glycerol, from top to bottom) and centrifuged for 12 h at 120,000 g (Beckman Coulter SW41) at 4 °C. A band was located at about three-fourths distance from the top of the gradient. The gradient material above the band was removed with a pipette; then, the virus-containing band was carefully collected with another pipette. The collected viral sample (1 ml) was diluted to ~12 ml by TNE buffer and pelleted for 2 h at 120,000 g (Beckman Coulter SW41) at 4 °C to remove gradient material and to concentrate the sample. The pelleted viral sample was resuspended in 100 µl of TNE buffer for cryo-EM.

Cryo–electron microscopy imaging and initial structure determination. Each aliquot (~2.5 µl) of freshly prepared dengue virus sample was placed onto a Quatrofoil 2/1 grid (Quatrofoil), blotted with filter paper and plunged into liquid nitrogen-cooled liquid ethane to make cryo-EM grids. CryoEM images were first recorded as focal pairs (targeted defocus values of −1 µm for close-to-focus images and −2.5 µm for far-from-focus images) on a 16-megapixel charge-coupled device (CCD) camera (TVIPS) in a Polara G2 cryo-EM instrument (FEI Company) operated at 300 kV. These images had a magnification of ×93,000 and a pixel size of 0.97 Å/pixel. The measured defocus values of these images ranged from −0.3 µm to −2.5 µm, as determined manually using ctfit of EMAN27. The imaging electron dosage was 17 e⁻/Å². Approximately 40,000 particles were selected from 5,000 focal pairs using winboxer of IMIRS package28,29. This data set was used to obtain a 7-Å resolution reconstruction with IMIRS package28,29 from a final data set of ~3,300 particles.

To improve the resolution of our reconstruction, we subsequently took images of the frozen grids from the same batch of samples in an FEI Titan Krios cryo–electron microscope operated at 300 kV. These images were recorded at a calibrated magnification of ×57,500 (±5,000 nominal magnification) on Kodak SO-163 Electron Image Films, with a dosage of 25 e⁻/Å². The defocus values of these cryo-EM images ranged from ~0.7 µm to ~2.5 µm, as determined automatically with CTFFIND3 (ref. 30).

Micrographs were digitized in Nikon CoolScan scanners at a pixel size of 6.35 µm on the film. Considering the calibrated magnification of ×57,500, the pixel size of the digitized images was 1.104 Å/pixel. Approximately 32,569 particles were selected from the 1,103 films by the boxer program in EMAN package27 with the assistance of ETHAN31 automatic boxing.

Cryo–electron microscopy structure refinement. To improve the resolution of the three-dimensional (3D) reconstruction, we processed the Titan Krios particle images with a recently developed procedure, global orientation–center search by multipath simulated annealing (MPSA)33, as implemented in EMAN27. In this procedure, we subjected all of the selected particle images to an iterative process. The 7-Å resolution structure from the Polara data set was used as the starting model. In each iteration, the orientation and center of each particle were determined by MPSA global search through seven trials, each trial starting with a different initial guess. If for four of the seven trials the determined orientation and center converged, we considered this particle ‘good’. The tolerances for orientation and center convergence were 3° and 3 pixels, respectively. In this way, we determined the orientation and center parameters for each particle and ruled out ‘bad’ particles. ‘Good’ particles were used for 3D reconstruction with the m3d3 module of EMAN without class averaging. The reconstruction was subjected to Fourier amplitude scaling similar to that in ref. 32, temperature (B) factor sharpening (we used a B factor of 40 Å²), low-pass filtering (to the current best resolution) and solvent flattening, and then the resulting density map was used as the reference of the next iteration of refinement. The overall result of these manipulations is comparable to a simple B-factor sharpening with a B factor of 240 Å²; using Fourier amplitude scaling allowed us to generate a map with more natural structure factors (Fourier amplitudes). To avoid possible model bias, no atomic model was referred to or used throughout this iterative refinement process of the density map, and only the cryo–EM reconstruction obtained from the experimental cryo-EM images was used as the model for refinement. Upon reaching convergence of the refinement and no further improvement in the resolution of the 3D reconstruction on the basis of Fourier shell correlation and on evaluation of side chain densities map, a final map was reconstructed from 9,288 ‘good’ particle images from the Titan Krios data set. A total of 16 iterations of MPSA refinement were performed to reach convergence. Twelve iterations were done with data from the first imaging session to refine the structure to 4.2-Å resolution. Four more iterations were done with all data to refine the structure to the final resolution. Astigmatic CTF correction was performed.

The final map was used to derive an intermediate atomic model (see below). This intermediate model was Fourier-transformed, and the resultant Fourier amplitudes were radially averaged to produce a one-dimensional ‘structure factor’ profile as a function of spatial frequency. Fourier amplitude scaling was done by using this ‘structure factor’ profile to suppress noise. After the amplitude scaling, a temperature factor of 40 Å² was deconvoluted from the map to sharpen high-resolution features, and the resulting map was low-passed to 3.3 Å with cosine apodization at the edge of Fourier truncation.

Building the atomic model for the virion and the averaged heterotetramer. We first averaged the density of the three heterotetramers within a rhombic region (Fig. 1c). We used Coot33 and REMO34 to build the atomic models for E and M proteins on the basis of this averaged density map. The protein backbone was first traced with the ‘baton’ tool in Coot. The resulting Ctn model was converted into a full-atom model with REMO. We used the CNS package35 to refine the E-M-M-E heterotetramer structure by pseudo–crystallographic methods as previously described36 with its twofold symmetry as a noncrystallographic symmetry restraint. Then, one half of the tetramer, containing one copy of E and one of M, was fitted into the density of each of the three copies of E and M in an asymmetric unit. The resulting atomic model was refined by the CNS package35 against the map of the entire virion, withicosahedral symmetry as a noncrystallographic symmetry constraint.

We then built atomic models for the glycans at Asn67 and Asn153. Atomic models for both a single sugar of N-acetyl-glucosamine (NAG) and a disaccharide with two NAGs were built for Asn67 and Asn153, respectively. Densities for additional sugars on these two glycosylation sites exist but are poorly ordered and were therefore not modeled. These additional sugars are more apparent in lower-resolution density maps, suggesting their flexibility.

The full model was refined again in CNS as described above (R-factor: 29.3%, see R-factors of individual resolution bins in Supplemental Table 2). We also added sugars to the atomic model for the averaged tetramer and refined that model. The final R-factor for the averaged tetramer is 28.8% at 3.5 Å.

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