Cryo-EM and MD infer water-mediated proton transport and autoinhibition mechanisms of \( V_o \) complex

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Rotary vacuolar adenosine triphosphates (V-ATPases) drive transmembrane proton transport through a \( V_o \) proton channel subcomplex. Despite recent high-resolution structures of several rotary ATPases, the dynamic mechanism of proton pumping remains elusive. Here, we determined a 2.7-Å cryo–electron microscopy (cryo-EM) structure of \( V_o \) proton channel in nanodisc that reveals the location of ordered water molecules along the proton path, details of specific protein-lipid interactions, and the architecture of the membrane scaffold protein. Moreover, we uncover a state of \( V_o \) that shows the c-ring rotated by \( \sim 14^\circ \). Molecular dynamics simulations demonstrate that the two rotary states are in thermal equilibrium and depict how the protonation state of essential glutamic acid residues couples water-mediated proton transfer with c-ring rotation. Our cryo-EM models and simulations also rationalize a mechanism for inhibition of passive proton transport as observed for free \( V_o \) that is generated as a result of V-ATPase regulation by reversible disassembly in vivo.

INTRODUCTION

Active proton transport is central to controlling the balance between cellular pH and efficiency of energy metabolism. Vacular adenosine triphosphates (V-ATPases) are adenosine 5′-triphosphate (ATP) hydrolysis–driven rotary motor proton pumps that acidify the lumen of subcellular organelles in virtually every eukaryotic cell (1–3). V-ATPase is organized in cytosolic \( V_1 \)-ATPase and membrane-bound \( V_o \) proton channel subcomplexes that are coupled by a rotating central stalk and three peripheral stators (fig. S1A) (4). \( V_o \) proton channel operates by a conserved mechanism that involves clockwise rotation (as seen from the cytosol) of a ring of 10 “proteolipid” or c subunits (proteolipid- or c-ring; \( c\text{c'}/c' \) in yeast) past the C-terminal transmembrane domain of subunit a (5-7). Each proteolipid subunit has one lipid-exposed glutamic acid residue that carries protons from a cytosolic to a luminal aqueous half-channel located within a\textsubscript{CT}. Direct proton flow between the two half-channels is blocked by two arginine residues in the middle of the bilayer that transiently engage the c subunits’ glutamates in a salt bridge during c-ring rotation. Eukaryotic V-ATPase is regulated by a mechanism referred to as “reversible disassembly,” wherein \( V_1 \)-ATPase detaches from membrane-bound \( V_o \) in a nutrient-dependent manner (5). Upon disassembly of the holoenzyme, both \( V_o \) and \( V_1 \) subcomplexes become autoinhibited, with the membrane-detached \( V_1 \) losing the ability to hydrolyze MgATP and the free \( V_o \) becoming impermeable to protons (fig. S1B) (6).

Cryo–electron microscopy (EM) (cryo-EM) has been used to characterize structural details of some rotary ATPases including F- and A/V-type ATP synthases (7–10). However, the dynamic mechanism of proton pumping and the role of water in the process remain elusive. Here, we integrate a 2.7-Å cryo-EM structure of yeast \( V_o \) in lipid nanodisc (VoND) with extensive molecular dynamics (MD) simulations. We identify the locations of ordered water molecules and the presence of another structural state, which differs by a \( \sim 14^\circ \) rotation of the c-ring, findings that agree well between experiments and simulations. Moreover, the simulations show that proton transfer from the c-ring glutamates to the luminal half-channel in a\textsubscript{CT} involves transient water wires, reminiscent of the classic alternating-access mechanism of membrane transport (11). Together, our studies rationalize the energetics of c-ring rotation-driven proton transport and the molecular basis for \( V_o \) autoinhibition.

RESULTS

Cryo-EM structure of \( V_o \) at 2.7-Å resolution

Using a dataset of >700,000 \( V_o \) ND particle images, we were able to reconstruct a 3.1-Å map of the complex. Furthermore, three-dimensional (3D) classification resulted in five distinct reconstructions solved at resolutions between 2.7 and 3.6 Å (fig. S2). The 2.7-Å map of \( V_o \) ND (Fig. 1A and movie S1) resembles a conformation of the complex as seen in previous lower-resolution structures (12–14) but reveals finer structural details for residues involved in proton transport, including those of a\textsubscript{CT}’s transmembrane helices 7 and 8 (Fig. 1A, bottom) and the c-ring (Fig. 1B). The cryo-EM densities for glutamic acid residues exposed to the lipid bilayer indicate a “proton-locked” conformation (as seen in crystal structures of c-rings of related rotary ATPases (15)). For instance, the carboxyl groups of c2\textsubscript{E137} and c4\textsubscript{E145} are in hydrogen bonding distance to the backbone carbonyl oxygens of c2\textsubscript{M59} and c4\textsubscript{M67}, respectively (Fig. 1B, bottom left and right insets). In contrast, there is little EM density beyond c1\textsubscript{E137}’s γ carbon (Fig. 1B, top left inset), suggesting that its carboxyl group is deprotonated. c4\textsubscript{E108}, on the other hand, appears well resolved (Fig. 1B, top right inset), consistent with its carboxyl group forming a salt bridge with a\textsubscript{CT}’s R735.

The 2.7-Å map also resolved several ordered lipid molecules, some of which form a bilayer within the central cavity of the c-ring, presumably to prevent transmembrane leakage of protons and other ions (Fig. 1C and fig. S3A). Several individual lipid molecules seen
at the periphery of $\alpha_{\text{CT}}$ and subunits e and f likely contribute to the stability of the complex (fig. S3B). Next to the cytosolic and luminal half-channels that provide water access to the c-ring glutamates (Fig. 1B, gray density), $\alpha_{\text{CT}}$ has two additional cavities, one open to the cytosol and another toward the lumen (Fig. 1D, middle). The cavity that opens to the cytosol is occupied by a tightly bound lipid molecule that prevents direct access for water molecules from the cytoplasm to the c-ring (Fig. 1D, left). A cavity that opens to the lumen side of $\alpha_{\text{CT}}$ is occupied by density that we modeled as the glycosylation precursor dolichol-P-P-(GlcNAc)$_2$Man$_x$ (right). The pyrophosphate moiety is stabilized by the side chains of $\alpha$K538, $\alpha$K593, $\alpha$S534, and the backbone amide of $\alpha$L608.

**Membrane scaffold proteins stabilize $\nu_o$**

Our cryo-EM maps of $\nu_o$ND displayed several belt-like densities wrapping around the membrane-exposed parts of the c-ring and $\alpha_{\text{CT}}$. Similar features have been described for other nanodisc-embedded membrane proteins and were ascribed to the Apolipoprotein (Apo) A1–derived membrane scaffold protein (MSP) used for nanodisc
reconstruction (17). Unexpectedly, focused classification revealed three distinct tubular densities wrapped around the bilayer exposed region of the c-ring (Fig. 2A), with the density at the periphery of the cryo-EM map of V_o ND, provides a rationale for the intermediate belt leaves sufficient space (~15 Å) to accommodate bulk lipid-ring (Fig. 2B, left and right, and Fig. S4), the middle belt leaves sufficient space (~15 Å) to accommodate the transmembrane helices of, for example, transient receptor potential vanilloid 5 (TRPV5) (30 Å; 6o1u) (20). While the top and bottom belts appear to be in direct contact with residues of the c-ring (Fig. 2B, left and right, and Fig. S4), the middle belt leaves sufficient space (~15 Å) to accommodate bulk lipid (Fig. 2B, middle). Together, this arrangement of the MSPs, as seen in our cryo-EM map of V_oND, provides a rationale for the improved stability of the nanodisc-reconstituted complex.

Protonation states of essential glutamates control water channel access

In our 2.7-Å map, we placed water molecules into density peaks using the “Find Waters” routine available in Coot (see Materials and Methods) (Fig. 3, A and B). To evaluate the authenticity of the putative water molecules, we computed the Q-score, which measures the resolvability of the atoms (Fig. 3B, left) (21), and the distance between water and the adjacent oxygen and nitrogen atoms (Fig. S5A). Both these measurements are within the range of expected values for either crystal or cryo-EM structures (21, 22). Further support for the authenticity of the modeled water in our 2.7-Å map comes from the presence of densities resembling water molecules between Q80’s side chain and V7’s carbonyl oxygen in all eight copies of the c subunits [c(1)-(8)] (Fig. S5B). Using this protocol, we modeled a total of ~180 water molecules, ~40 of which are within the putative proton path (Fig. 3, A and B, and Fig. S5C). Most notable are water molecules connecting the side-chain nitrogens of R799 to the transmembrane entry channel and between c(1)E137’s carboxyl group and the side chain of dH796 (Fig. 3B, see arrowheads in left panel).

To further validate the water assignments, we carried out all-atom MD simulations starting with the model derived from our 2.7-Å map embedded in bulk water (i.e., water standard density in randomized positions). Compared to protein motion, which takes up milliseconds, water molecules move on the nanosecond time scale. By overlaying a large number of individual MD coordinate frames, a residence probability map of stably bound water molecules can be obtained (Fig. 3C, blue dots). The positional overlap of the statistically probable water clusters in the simulations and the putative water molecules detected in the cryo-EM structure is notable (compare red beads and blue dots in Fig. 3C, and see fig. S6). This overlap not only supports the validity of the cryo-EM map–derived water assignment but also demonstrates the usefulness of near-atomic resolution cryo-EM structure–based MD simulations in the context of biochemical activity. The fact that some MD-derived water clusters are not clearly resolved in the cryo-EM map (and vice versa) could be due to (i) the extent of positional accuracy in the 2.7-Å cryo-EM structure, (ii) the assumptions imposed by the classical force field used in the MD simulations, and (iii) the difference in the temporal scales of the cryo-specimen plunge freeze and the simulations. Whereas the cryo-EM density represents an average of multiple chemical states present during the millisecond time frame of plunge freezing, classical MD can capture only one state at a time on a nano- to millisecond time scale (23). Nevertheless, the simulations identify charged and polar residues in the luminal and cytosolic half-channels that form hydrogen bonds to the ordered water, keeping the water columns stable in both channels (Fig. S7).

Next, we performed two separate MD simulations to explore the role of water dynamics in the transfer of the proton from the c-ring glutamates to the luminal half-channel in aCT (Fig. S8, A and B). In eukaryotic V-ATPase, a critical residue involved in this process is a strictly conserved glutamic acid (aE789 in yeast) that is located at the entry of the luminal half-channel across from c(1)E137 (Fig. 3B). In the first simulation, the donating c(1)E137 was protonated, and the receiving aE789 was deprotonated (Fig. 3D, left). In the second simulation, the protonation states of the two residues were reversed (Fig. 3D, right). The simulated dynamics of the water molecules suggests that proton transfer from c-ring glutamic acid residues (cE137, c′E145, or c′′E108) to aE789 occurs via a cluster of water molecules, whose lifetime depends on the protonation states of the two residues. At the onset of the transfer, when a proton resides on, e.g., c(1)E137, a network of hydrogen-bonded water molecules (hereafter referred to as “water wire”) connects the carboxyl groups of c(1)E137 and aE789 for >50% of the structures derived from three independent 500-ns simulations (Fig. 3E, blue vertical lines). These water wires enable transfer of the proton from c-ring essential glutamates to aE789 (Fig. 3F). After the transfer, when the c-ring glutamate is ionized, and aE789 is protonated, no water wires are observed in the simulation (Fig. 3E, red line). This disruption of the water wire after proton transfer ensures that protons cannot leak back from the luminal half-channel into the aCT-c-ring interface once proton transfer has occurred. Note that some water molecules are retained near c(1)E137 after deprotonation (Fig. 3G), likely to stabilize the newly ionized glutamic acid. The negatively charged c(1)E137 is also stabilized by transient hydrogen bond formation with cY66 as observed in the 2.7-Å map (Fig. 1B, top left inset, and Fig. S9). Together, dynamics of the water wires is coupled to the charge and conformational states of key acidic residues for controlling proton transport during c-ring rotation.

Another rotary state of autoinhibited V_o

Aside from the 2.7-Å map, which is representative of the dominant population of autoinhibited V_oND (~95%; Fig. 4A), 3D classification
revealed a minor, hitherto unseen autoinhibited rotary state solved at 3.6 Å (~5%, class 4) (Fig. 4B). This minor substate population showed the d-ring/subunit d subcomplex rotated by ~14° relative to aCT in the direction of proton pumping. This clockwise rotation of the c-ring brings c(1)E137 close to aCT’s two arginine residues R735/799 and exposes c″E108 to bulk water entering from the cytosolic half-channel (see arrow in Fig. 4B). The 14° rotation of the c-ring/d subcomplex did not lead to disruption of the binding interface between the distal end of aNT and subunit d, resulting in a compression/ outward bending of aNT’s coiled-coil segment (Fig. 4, C and D; fig. S10; and movie S2). Therefore, autoinhibited Vo in lipid nanodisc can exist in at least two distinct states: the previously described, predominant state 3 (12–14), and a less abundant state, hereafter referred to as state 3’.

**Free energy landscape of c-ring rotation suggests molecular origins of autoinhibition**

Unlike the isolated membrane sector of F-ATP synthase, Fo, which acts as a passive proton pore (24), free Vo does not conduct protons along a pH gradient under physiological conditions (25). The molecular origin of this difference in activity between the two related proton channel subcomplexes is not known. To explore the energetics of side-chain interactions in the autoinhibited state and during c-ring rotation, we applied MD simulations to the 2.7-Å state 3 model.
of the V_o. However, even in fully active holo V-ATPase, ATP hydrolysis–driven clockwise rotation of one c-ring glutamic acid past a_CT occurs on the millisecond time scale [~18 μmol/(min × mg) => ~300 ATP/s => ~100 rps => ~1000 H^+/s => ~1 H^+/ms (26)], with a substantially slower dynamics expected for autoinhibited V_o. This time scale remains, however, beyond the reach of standard, all-atom MD simulations for membrane protein complexes the size of V_o. To capture such millisecond—or even slower rotation events, we combined string optimization with adaptive biasing force simulations (see Materials and Methods for details) (27–29). Our calculations reveal the most favorable reaction path on the free energy landscape of c-ring rotation, showing five distinct minima referred to as substates A, B,
C, D, and E (Fig 4E, left, and movies S3 and S4). Substate A has the lowest free energy as it results from the initial MD equilibration of the 2.7-Å state 3 model (compare Fig. 4, A and F, top left). Subsequent rotation of the c-ring follows an uphill free energy path via three local minima, B, C, and D, before reaching substate E, which has a free energy similar to substate A. While all substates show interaction of the two c-ring glutamates and aceCT’s two arginines (Fig 4F), the higher free energy (lower stability) of substates B, C, and D suggests that some of the inter- and intra-subunit interactions at the aceCT-c-ring interface are lost, or strained, in these intermediates. Such a destabilizing effect is most prominent in the transition between substates C and D via the “transition state” (TS), which involves the disruption of the interaction of c′E108 with the two arginines (Fig 4F, bottom left). The transition between C and D is the rate-limiting step of the ~14° c-ring rotation connecting substates A and E and requires an activation energy of ~6.5 kcal/mol, corresponding to a time constant of ~110 ms (see Materials and Methods for kinetics calculations). Together, the free energy profile underlying the rotation pathway reveals that the aceCT-c-ring interface is dominated by the electrostatic attraction between the c-ring essential glutamates (cE113 and c′E108) and the aceCT essential arginines (aR735 and aR799) and that disruption of one of these interactions represents the slowest step of the 14° rotation from A to E. It is noteworthy that the simulated substate E resembles the experimentally determined cryo-EM structure of state 3′ (compare Fig. 4, B and F, bottom right), a finding that lends support to our simulation protocol.

The simulation described above started from the autoinhibited state, which is a unique rotary state due to the presence of two carboxylic acids in the aceCT-c-ring interface (cE113 and c′E108; Fig. 4A). During regular function of this motor, however, only a single glutamate is at the interface for most of the time, with the two neighboring carboxyls of cE113 and c′E108 moving past aceCT only once every 360° rotation of the c-ring. We, therefore, performed a second simulation with the same starting model, albeit wherein one of the aceCT-c-ring interactions can accommodate the ~14° c-ring rotation between two states (Fig. 4, C and D; fig. S10; and movie S2), it is obvious that clockwise or anticlockwise rotation by either state would require the disruption of at least one of these interactions. Because the interaction of aceCT and d, which is only seen in free V0, links the stator (subunit a) to the rotor (c-ring-d subcomplex) of the V0 motor, it has been proposed that this interaction is responsible for autoinhibition (30). However, we and others have previously reported that removal of either aceNT or d from V0 does not result in measurable proton transport (31, 32). This suggests that V0 autoinhibition must be ascribed to additional thermodynamic or kinetic factors that distinguish between the V0 and F0 proton channels.

The most notable difference between the two systems lies in the structure of the c-ring, featuring identical transitions in both V0 and V1, in contrast with the three distinct transitions in V1, arising from the unique structure of cE108 (Fig. 1B and fig. S11). From the three c-ring transitions in V1 autoinhibition, the 2-glu transition is both structurally and kinetically similar to the c-ring rotation in free F0 because it features neighboring glutamic acids at the interface and occurs at a rate similar to that of passive proton transport in F0 (fig. S11B) (24). This means that while the “F1-like” 2-glu transition in V1 contributes substantially to autoinhibition, it is unlikely to be the sole cause, as the corresponding transitions in F0 allow for some passive transport (~3% of F1F0 activity) (24, 33). The 1-glu transition occurs within ~0.6 or ~20 ms, depending on the direction of c-ring rotation, which is notably faster than the 2-glu transition. This suggests that the unique 0-glu transition, estimated to take at least ~180 ms, represents a rate-limiting component of c-ring rotation. The dominant role of the 0-glu transition is also supported by mutagenesis experiments. Mutating either cE108 or c′E145 results in complete inhibition of proton pumping by the holoenzyme (34), indicating that when the length of the 0-glu transition is increased to a three or four helix gap, even the free energy of ATP hydrolysis is insufficient to drive c-ring rotation.

In summary, our cryo-EM structures of states 3 and 3′, together with the MD-derived free energy profiles and kinetic constants, point to two separate causes for V0 autoinhibition: (i) the interaction of the total path of the 0-glu transition is ~3 times longer than that of the simulated A′-to-E′ transition (36 Å versus 12 Å, respectively), and despite the similar end-point free energies, an even larger time constant of ~180 ms can be extrapolated for this transition (see legend to fig. S11 for calculation). Thus, we consider a number of distinct transitions that characterize the overall c-ring rotary dynamics in an isolated V0 (fig. S11A) and find that the 0-glu transition represents the slowest step, followed by the 2-glu and 1-glu transitions.
Concluding remarks and outlook
Here, we present the 2.7-Å cryo-EM structure of yeast Vo, in lipid nanodisc, revealing previously unseen details of specific protein-lipid interactions, membrane scaffolding proteins, and bound water molecules in the proton path. From all-atom MD simulations, we conclude that proton transfer from c-ring essential glutamates is facilitated by alternating access of bulk water that is gated by the protonation state of the proton accepting residue (αE789 in this case) at the opening of the luminal half-channel. A second cryo-EM structure, together with MD simulation of c-ring rotation, confirms that this rotary dynamics is energetically favorable in the direction of active proton transport. The data also suggest that there are at least two origins for Vo autoinhibition: the interaction of αNT with d and the asymmetry in the spacing of the essential c-ring glutamic acid residues. While autoinhibition, c-ring asymmetry, and the αNT-d interaction are unique to V-ATPase, alternating access of water molecules into the ace-ring interface is likely an aspect of rotary ATPase mechanism that is conserved in the related F- and A-ATP synthases. Our studies demonstrate the power of integrating cryo-EM and MD simulations not only to cross-validate the structural models and functional implications but also to be able to explore the dynamic mechanism of this membrane integral nanomotor with atomic detail.

MATERIALS AND METHODS
Cryo-EM structure determination
Purification and lipid nanodisc reconstitution of yeast Vo
The Vo proton channel subcomplex of the yeast H+–V-ATPase was purified and reconstituted into lipid nanodiscs using MSP MSP1E3D1 as described (13, 35).

Cryo-EM grid preparation and data acquisition
Three-microliter aliquots of Vc,ND peak fractions from the Superose size exclusion chromatography run (0.3 mg/ml) were applied to glow-discharged gold grids (UltrAuFoil 1.2/1.3), blotted for 2 to 3 s, and then plunge-frozen in liquid ethane using a Leica EM GP (Leica). The grids were loaded into a Thermo Fisher Titan Krios (300 keV) equipped with a BioQuantum energy filter and a Gatan K2 Summit camera. Movies were acquired in super-resolution electron counting mode with a physical pixel size of 1.08 Å (0.54 Å/pixel in super-resolution mode), an exposure rate of 5 electrons/pixel per second, and a total exposure time of 10 s divided into 25 frames. In-fly frame alignment and dose weighting were performed with MotionCor2 (36), and contrast transfer function (CTF) parameters were estimated with Gctf (37). A total of 4083 movies were then selected based on defocus ranges and CTF quality parameters.

Image processing
Each super-resolution movie stack was saved as a new aligned movie stack with 1.08 Å/pixel by MotionCor2 (36) and then imported into RELION3 (38) and CTF corrected using Gctf (37) for further processing (fig. S2, A and B). Aligned micrographs were then generated using RELION3’s own implementation for motion correction. The averages of ~200 manually picked particles were used as templates for autopicking. A total of ~1.8 M particles were automatically selected and then, after 2D/3D classification, low-convergence classes were discarded. The remaining ~0.7 M particles were subjected to 3D autopicking and the map was subsequently improved by particle-based motion correction, B-factor weighting, and CTF refinement in RELION3 (particle polishing and CTF refinement) (39). At this stage, the resulting map had a resolution of ~3.1 Å as determined according to the gold-standard Fourier shell correlation (FSC) at 0.143 correlation (40). To distinguish potential high-correlating structural heterogeneity, we performed another round of 3D classification (K = 8) with a high weighting factor (T = 20) and skipping the orientation search (41). Five of eight classes were selected based on visibility of side-chain densities and connectivity. Particles in each class were then subjected to 3D autorefinement to yield final maps with resolutions ranging from 2.7 to 3.6 Å (fig. S2, B and C), with rotational heterogeneity of the c-ring representing the main distinguishing feature between classes (fig. S2, D and E). Each final map was processed using nonisotropic B-factor sharpening as implemented in Phenix (42).

Focused classification on MSPs of the lipid nanodisc
The density representing the lipid nanodisc was segmented out by removing the protein-derived density using the UCSF (University of California, San Francisco) Chimera (43) and low-pass filtered to generate a mask for the nanodisc. Using the mask, ~700,000 particles that yielded the 3.1-Å map were subjected to 3D classification without orientation search. The 3D classification revealed substantial heterogeneity of MSP binding to the Vo complex with the predominant population of MSP bound to Vo, displayed in Fig. 2 (see also fig. S3).

Modeling
Model building was started from our earlier 3.5-Å model [6c6l.pdb; (13)]. The model was iteratively improved using reciprocal and real-space refinement as implemented in Phenix (42), as well as manual model building in Coot (44). For reciprocal space refinement, the box size of the final masked map was reduced to 140 × 124 × 128 pixels and sampled with a d_min of 2.8 Å to generate structure factors. Reciprocal-space refinement included xyz coordinates and individual atom displacement parameters using map phases and Ramachandran restraints. Hydrogens were added to improve geometry and reduce clashes. Each round of reciprocal space refinement was followed by manual model building in Coot and two macro cycles of real-space refinement using global minimization and grid search-based rotamer fitting (grid search-based rotamer fitting was omitted in final stages of the refinement). Model refinement was iterated until quality measures [R/Rfree in reciprocal-space refinement, MolProbity (45), and EMRinger (46) scores] remained stable. During the refinement process, the pixel size of the map was adjusted from 1.08 to 1.076 Å using the EMRinger score as a measure of quality of fit. Water molecules were placed into distinct density peaks as identified with the “Find Water” routine in Coot using the masked map. The search parameters were 3 root mean square deviation for density peaks and between 2.4 and 3.4 Å for distances to polar side chain or backbone atoms or other water molecules. The automatically placed waters were manually edited to remove waters placed into density belonging to, e.g., lipid molecules or MSP, and to add waters missed due to uncertainties in amino acid side-chain positions. Atomic displacement parameters (B-factors) were estimated using Phenix reciprocal and/or real-space refinement. Lipid molecules and the dolichol-PPi-GlcNAc3Man1 ligand were modeled using a map that was obtained by masking out density belonging to protein.
Graphical representations of maps and models were generated in the UCSF Chimera (43) and visual MD (47).

**Structure validation**

Proper protein geometry was validated using the validation tools implemented in Coot (44), Phenix (42), and the MolProbity webserver (48). Model-to-map FSC was estimated using phenix.validation_cryom. The density resolvability for protein and water molecules was evaluated quantitatively as Q-score (21).

**PISA analysis**

Contact areas and binding energies of the interfaces between c-ring and a subunit (αCT for V_o) were analyzed using the protein interfaces, surfaces and assemblies (PISA) web server (www.ebi.ac.uk/pdbe/pisa/) (49).

**MD simulation protocol**

**System setup**

All MD simulations were initiated using the 2.7-Å model from class 1 (state 3) using NAMD2.12 (50). Two computational assays were generated to represent the pre- and post-proton transfer states, whereby E137 was either protonated or deprotonated. Using the membrane builder of the online server CHARMM-GUI (51), the protein was embedded in a model membrane with a lipid composition of phosphatidylcholine (PC):phosphatidylethanolamine (PE):phosphatidylinositol (PI):phosphatidylinerine (PS) 48%:21%:27%:4% to closely mimic the experimental conditions. The molecular assembly was solvated by 60,196 transferable intermolecular potential with 3 points (TIP3P) water molecules, representing a unit cell of initial dimensions equal to 160 Å by 160 Å by 116 Å. Na+ and Cl− ions were then added to ensure electric neutrality and set the ionic concentration to 100 mM. The complete computational assay, including the protein, lipids, water molecules, and ions, amounted to nearly 310,000 atoms. After 5000 steps of conjugate-gradient energy minimization, it was simulated for 5 ns at 310 K, with all protein heavy atoms restrained to their initial positions, using a force constant of 5 kcal/mol per Å². Last, all geometric restraints were removed, and the computational assay was thermalized for 500 ns. Three repeats of the 500-ns MD simulations were performed each for the pre- and post-proton transfer state.

**Simulation protocol**

All MD simulations were performed in the isobaric-isothermal ensemble with the MD program NAMD 2.12 and the CHARMM36 all-atom force fields for proteins and lipids. Periodic boundary conditions were applied. The temperature was maintained at 310 K using Langevin dynamics with a damping constant of 0.5 ps⁻¹. The pressure was fixed at 1 atm using the Langevin piston method. Van der Waals and electrostatic short-range interactions were smoothly truncated with a 12-Å cutoff, and a switching function was applied at 10 Å. Long-range electrostatic forces were computed with the particle mesh Ewald algorithm. The equations of motion were integrated with the reversible reference system propagation algorithm (r-RESPA) multiple time step propagator, with an effective time step of 2, 2 and 4 fs for bonded, short- and long-range nonbonded contributions, respectively.

**Path-finding simulations**

Following the 500-ns equilibration of the computational assay, a constant-velocity simulation was performed, wherein the c-ring was rotated by ~14°. The constant-velocity simulation was carried out over a period of 100 ns, with an angular velocity of 10⁻⁵ degrees/fs imparted to the Cα atoms of the axial acidic residues of the ring, namely GLU137, GLU108, GLU145, and GLU188. This initial pathway was further refined using the string method with swarm of trajectories in Cartesian space (27). Toward this end, 50 equally spaced structures were chosen to form the putative minimum-action pathway, which was evaluated in 15 iterations using the average dynamic drift of atomic positions. This dynamic drift was estimated from swarms of 10 trajectories consisting of 10 ps of unbiased MD prefaced by 100 ps of restrained MD at the different nodes of the rotation pathway. The overall string optimization leading to the minimum free energy pathway underlyng the 14° rotation of the c-ring corresponds to an aggregate simulation of 0.825 μs.

**Free energy calculation**

To determine the free energy change associated with the complex conformational transition between conformational states S1 and S2 along the minimum free energy pathway, a path collective variable (PCV) (52) was used in conjunction with the multiple-walker extended adaptive biasing force (MW-eABF) algorithm (29, 53). A PCV allows progress along the minimum free energy pathway to be described by means of a differentiable mathematical expression

\[
s(x) = \frac{1}{N-1} \sum_{i=1}^{N-1} e^{-\lambda(x-x_i)^2} - \frac{1}{\lambda} \ln \left( \sum_{i=1}^{N-1} e^{-\lambda(x-x_i)^2} \right)
\]

wherein \(i\) varies between 1 and \(N\), the number of intermediate structures forming the discretized minimum free energy pathway. The mean square displacements, \((x-x_i)^2\), were determined based on the positions of the Cα and Cβ atoms of the c-ring in its instantaneous conformation, \(x\), and that of the \(i\)th intermediate state, \(x_i\), of the minimum free energy pathway. \(\lambda\) is a smoothing factor comparable to the inverse of mean square displacement between successive intermediates. An ancillary variable was used to measure deviations from the minimum free energy pathway and confine sampling in a tube wrapping around the latter

\[
z(x) = -\frac{1}{\lambda} \ln \left( \sum_{i=1}^{N-1} e^{-\lambda(x-x_i)^2} \right)
\]

Loose geometric restraints were applied to ensure that the trajectory samples conformational space within a tube centered on the minimum free energy pathway and of radius equal to 2.5 Å. The 50 intermediate structures served as the initial conformations of the 50 walkers in the MW-eABF simulation. The gradients of the free energy profiles, presented in Fig. 4 and fig. S12.

**Kinetics calculation**

Kinetics calculations provide an estimate of the mean first passage time, \(\tau\), which is the inverse of the rate constant \(k\). Assuming a diffusion-dominated motion of the protein in the membrane environment, and a position-independent diffusivity over the entire reaction pathway, equal to \(D = 6 \times 10^4 \text{ Å}^2/\text{s}\) (54–56), the free energy profile obtained from the eABF simulations can be reconciled with the mean first passage time using the following expression (57)

\[
\tau = \frac{1}{k} = \frac{\sum_{j=0}^{N} e^{-\beta w(x_j)}}{\sum_{i=0}^{N} D e^{-\beta w(x_i)}}
\]
where the transition pathway is discretized over the *N* points of the curvilinear pathway, and $w(s)$ is the accompanying free energy change. The quantity appearing in the main sum represents the probability of finding the system at point *s* along the transition pathway.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/41/eabb9605/DC1

**REFERENCES AND NOTES**

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Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors. The cryo-EM maps are deposited in the Electron Microscopy Data Bank under accession numbers emd-30034 and emd-30035 for classes 1 (state 3) and 4 (state 3′), respectively. The associated models are deposited in the Protein Data Bank with accession numbers 6M0R (state 3) and 6M05 (state 3′), respectively.

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