Cryo-EM structures provide insight into how E. coli F₁F₀ ATP synthase accommodates symmetry mismatch

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F₁F₀ ATP synthase functions as a biological rotary generator that makes a major contribution to cellular energy production. It comprises two molecular motors coupled together by a central and a peripheral stalk. Proton flow through the F₀ motor generates rotation of the central stalk, inducing conformational changes in the F₁ motor that catalyzes ATP production. Here we present nine cryo-EM structures of E. coli ATP synthase to 3.1–3.4 Å resolution, in four discrete rotational sub-states, which provide a comprehensive structural model for this widely studied bacterial molecular machine. We observe torsional flexing of the entire complex and a rotational sub-step of F₀ associated with long-range conformational changes that indicates how this flexibility accommodates the mismatch between the 3- and 10-fold symmetries of the F₁ and F₀ motors. We also identify density likely corresponding to lipid molecules that may contribute to the rotor/stator interaction within the F₀ motor.
key component in the generation of cellular metabolic energy is the F₁Fₒ ATP synthase, a biological rotary motor that converts the proton motive force (pmf) to adenosine tri-phosphate (ATP) in both oxidative phosphorylation and photophosphorylation. The enzyme is comprised of two rotary motors, termed F₁ and Fₒ, that are coupled together by two stalks: a central "rotor" stalk and a peripheral "stator" stalk. The Fₒ motor spans the membrane and converts the potential energy from the pmf into mechanical rotation of the central rotor that, in turn, drives conformational changes in the catalytic F₁ motor subunits to generate ATP from ADP and inorganic phosphate (Pi). However, in most ATP synthases there is a symmetry mismatch between the rotational steps made by the F₁ and Fₒ motors—120° (corresponding to 3-fold symmetry of αβ₃ in F₁) and 36° (10-fold symmetry of the c-ring in Fₒ) in E. coli—that results in a non-integral H+/ATP ratio. To overcome this mismatch, the coupling between the two motors must be dynamic to enable the enzyme to function with high efficiency, with elastic energy stored within the complex facilitating different sized stepping between the F₁ and Fₒ motors. Hypotheses to account for the dynamic coupling have proposed flexibility within either the central or peripheral stalks, and more recently the subunit that attaches the F₁ motor to the peripheral stalk (termed δ in bacteria and OSCP in mitochondria), although the contribution made by each component has been controversial.

Although the F₁Fₒ ATP synthase is found across most forms of life, the simplest form, that contains only eight different subunits, is present in E. coli and has been used extensively as a model system for ATP synthases. Here we have used cryo-Electron Microscopy (cryo-EM) on detergent-solubilized cysteine free E. coli ATP synthase to probe its structure and understand its rotational dynamics and coupling. We describe an ensemble of 3.1–3.4 Å resolution structures of the enzyme in a series of conformational sub-states that enable key functional features to be identified. The higher resolution information obtained in the Fₒ region identifies lipids that may contribute to the rotor/stator interface and increase the interacting surface between the Fₒ stator and rotor ring. In addition to generating a comprehensive structural model of E. coli F₁Fₒ ATP synthase that provides a framework to interpret mutagenesis studies, we also describe torsional flexing of the complex and a rotational sub-step of the Fₒ motor c-ring associated with long-range conformational changes. These data indicate a model of how elastic coupling between the F₁ and Fₒ motors is mediated by a dynamic, flexible peripheral stalk.

**Results**

**Rotational sub-states of E. coli F₁Fₒ ATP synthase.** Cryo-EM maps of cysteine free E. coli F₁Fₒ ATP synthase in the presence of 10 mM MgADP were obtained at 300 kV using methods similar to those in previous studies. [Fig. 1 and Supplementary Figs. 1 and 2.] MgADP was used in an attempt to lock the rotor of the F₁-ATPase in a single rotational position, to investigate the flexible coupling between the F₁ and Fₒ motors, as well as the contribution made by nucleotides on the regulation of the F₁ motor. Hence, the F₁Fₒ ATP synthase imaged here should not be undergoing ATP synthesis or hydrolysis, and therefore should not be rotating under the conditions imaged in this study. These maps generated far superior structural information than observed previously for this complex, with the overall resolution improving from ~5 Å in previous studies to ~3 Å in this study, allowing most of the sidechains to be assigned in the model. Furthermore, masked sub-classifications focused on the Fₒ stator (subunit a and the N-termini of the b subunit) identified a series of intermediates that provided detailed information on conformational sub-states accessible to the complex (Supplementary Figs. 2–4).

The maps obtained identified a series of states that correspond to different rotational steps of E. coli F₁Fₒ ATP synthase. The three major rotational states identified previously (termed States: "State 1", "State 2", and "State 3", assigned to describe the enzyme operating in an ATP hydrolysis direction), in which the central stalk is rotated by ~120° relative to peripheral stalk, were also observed in this study with resolutions of 2.9, 3.1, and 3.0 Å, respectively (Supplementary Fig. 2). Although these reconstructions gave the highest numerical resolution, the Fourier Shell Correlation (FSC) appeared to be dominated by the F₁-ATPase, with information in the Fₒ region blurred significantly (Supplementary Figs. 3 and 4). Hence, masked classification focused on the Fₒ motor was used on each of the main states to reveal sub-states describing movements of the complex along with increased detail in the Fₒ region (Supplementary Figs. 2–5). By examining the position of subunit y in each of the sub-states, we were able to unambiguously assign the c subunits, so that their relative position could be compared between each sub-state (Supplementary Fig. 6). Focused classification of rotational State 1 highlighted five sub-classes, here termed Sub-states 1A, 1B, 1C, 1D, and 1E; with resolutions of 3.1, 3.3, 3.1, 3.2, and 3.3 Å, respectively (Supplementary Fig. 2). The defining theme of these sub-states was flexing of the central and peripheral stalks, with the majority of the flexibility seen in the peripheral stalk which bends and twists, together with minor movements within the central stalk (Fig. 2, Supplementary Figs. 7 and 8 and Supplementary Movie 1). Independently, the movements seen between Sub-states 1B-E do not facilitate rotation of the c₁₀ ring and instead illustrate torsional flexing of the entire complex. However, comparing Sub-state 1A with 1B-E shows a ~36° rotation of subunits αβ₃yεc₁₀ (corresponding to the F₁ motor plus the c-ring) relative to the Fₒ stator (Fig. 2 and Supplementary Movie 2). Importantly, this rotation describes a ~36° movement of the c-ring in the Fₒ motor without any corresponding rotation within the F₁ motor (Supplementary Fig. 9): the αβ₃yεc₁₀ subunits are rotating as

![Fig. 1 Cryo-EM map and atomic model of E. coli F₁Fₒ ATP synthase.](image)
rotational movement of the c-ring in F₀ remained essentially the same, preserving a similar interacting surface between the rotational states (Supplementary Fig. 6). Furthermore, the position of the ψ subunit relative to the α and β subunits was in the same rotational position in all sub-states (Supplementary Fig. 9), although rotated relative to the peripheral stalk, with the F₁ enzyme locked in the same rotational state across all structures observed in this study.

The Fₐ motor stator/rotor interaction. The maps generated after classification into sub-states provided substantially improved detail in the Fₐ region, compared to that seen without masked classification or that seen previously⁴,⁵, with sidechain density observed for the majority of residues (Supplementary Figs. 4 and 5). However, the reconstructions were still anisotropic due to preferential alignment to the Fₐ-ATPase, so focused refinement using a mask of the Fₐ region was performed on Sub-state 3A resulting in a detailed map to 3.3 Å resolution of the Fₐ motor (Fig. 4, Supplementary Figs. 2 and 10). This map enabled a detailed model of the membrane-embedded α, β and c subunits to be built (Fig. 4b), which was fitted and refined to all other sub-states. The model generated complements those that have been determined for other species, particularly for the related Bacillus sp. PS3⁷ and chloroplast enzymes⁸. Importantly, because E. coli F₁Fₐ ATP synthase has been a model system for studying ATP synthase for decades⁹, the model described here provides a detailed structural framework to interpret the wealth of mutagenesis studies that have been performed on this enzyme. A particularly interesting feature seen in this study are the positions of aArg210 and cAsp61, residues that are known to be essential for proton translocation⁹. Density for these residues was resolved particularly well and suggests them to be in close proximity to each other (Fig. 4b). Furthermore residues aAsn214, aHis245, aAsn119, and aGlu219, that are also known to be important from mutagenesis studies¹⁰, form a chain of residues between the aqueous channel and rotation path of cAsp61 (Supplementary Fig. 11).

As a coordinated metal ion has been proposed to mediate protonation of the c subunit in related ATP synthases¹¹,¹²,¹³, we closely inspected our maps for ion-like density in this region. We failed to find any ion-like density adjacent to residues aGlu219 and aLeu223, residues equivalent to those shown to coordinate an ion in Polytomella mt. ATP synthase (Supplementary Fig. 12). However, we did observe a weak spherical density in the rotation path of cAsp61, adjacent to aAsn214 and aHis245, which are essential for function in E. coli F₁Fₐ ATP synthase (Fig. 4 and Supplementary Fig. 11). Given the location and shape of this density, it could represent an ion or water molecule at the protonation site of c-ring. As the distances to neighboring polar atoms are too large to hydrogen bond and the cavity in which this density resides has a van der Waals radius ~ 3 Å (Supplementary Fig. 12), the identity of this density is unlikely to be a single water
molecule surrounded by vacuum. Instead this region could correspond to a cluster of water molecules or a hydrated ion such as Magnesium, Sodium, or Phosphate. However, assigning such a density at ~3 Å resolution is challenging and clearly further work would be required to evaluate the identity and importance of this region. Hence the deposited co-ordinates do not contain any atoms in this space.

The peripheral stalk of E. coli ATP synthase is constructed by a homodimer containing two b subunits. Each subunit consists of a long alpha helix with three sections (Supplementary Fig. 14). The N-terminal section (b1-b45) resides in the membrane and bracess against the a subunit, the middle section (b46-b135) forms a ~130 Å long right-handed coiled coil and the C-terminal section (b136–b154) loops back to cap the attachment to the F1-ATPase. The helices within the parallel right-handed coiled coil are offset by 5½ residues with respect to one another. This offset shows a striking correlation to the arrangement that was predicted previously using crosslinking studies,22 showing a staggered homodimeric right-handed coiled coil (Supplementary Fig. 15). Although the first four residues of subunit a were not resolved in our maps, residues 8 to 16 form a helix that sits on the periplasmic side of the membrane and which interacts with the N-terminus of the adjacent b subunit and the C-terminus of one of the c subunits (Supplementary Fig. 16a). Residues 42–88, 100–107 and 147–156 of subunit a also appear to reinforce the interactions with the other b subunit (Supplementary Fig. 16b).

When all the density corresponding to protein had been assigned, tube-like non-protein density likely corresponding to lipid molecules were observed in multiple locations within the membrane bound F0-motor (Supplementary Fig. 17). The strongest of these densities were observed between residues a1225–aQ234 and a single c subunit, where three of these tubes of density were present on the periplasmic side and one on the cytoplasmic side of the enzyme (Fig. 5 and Supplementary Movie 3). This additional density was observed in most of the maps but was clearest in the highest resolution maps, Sub-states 1C and 3A, and the focused F0 map of Sub-state 3A. Without the lipid(s), the interaction surface between subunit a and the c-ring would be limited to three of the ten c subunits, whereas with the lipid-mediated interaction this is increased by an additional c subunit to four (Fig. 5). Although the detail of the maps was insufficient to establish the identity of the lipid(s) in this bridge region, the arrangement of these lipid(s) suggests an intriguing mechanism whereby, in addition to direct protein-protein contacts, the strength of the stator/rotor interaction is increased by using lipid(s) to bridge between the interacting surfaces. As well as the strong density observed for the lipid bridge, weaker density corresponding to the lipid bilayer was also observed around the F0 motor, with 61 lipid chain-like densities (Supplementary Fig. 17), as well as the lipid plug in the center of the c-ring (Fig. 5b). These lipids encompassed the entire c-ring and are reminiscent to that seen around the Vc motor from Saccharomyces cerevisiae.23 To investigate whether the densities observed could be attributed to lipids, LC-MS-based lipidomics24 was performed on the same detergent-solubilized E. coli F1F0 ATP synthase imaged in this study. Phosphatidylethanolamines, phosphatidylylycerols and cardiolipins were all observed, with an increase in the relative abundance of cardiolipins compared to E. coli membrane (Supplementary Fig. 18), showing that E. coli lipids were co-purified with the protein.

MgADP induces a conformational change in the F1 motor. The maps presented here were generated from material imaged in the presence of 10 mM MgADP and all showed the enzyme in the “autoinhibited” state, with the C-terminal domain of the ε subunit (εCTD) in the “up” inhibited position interacting with the F1 catalytic subunits (Fig. 6a). The conformation of the F1 motor is different to that seen in previous cryo-EM studies, in that the catalytic “β1” subunit (as defined in reference 25) has bound ADP, Mg and P1, and is seen in a “half-closed” state, which is prevented from closing fully by the εCTD (Fig. 6a, Supplementary Figs. 19 and 20). This conformation is remarkably similar to that seen in the crystal structure of the isolated F1 motor25.
Fig. 4 The F<sub>0</sub> stator of E. coli F<sub>0</f> F<sub>1</sub> ATP synthase. a Sub-state 3A F<sub>0</sub> focused cryo-EM map of the E. coli F<sub>0</sub> stator, masked to remove c-ring for clarity (subunit a in orange, subunit b in pink and lipid-like densities in wheat). Highlighted orange region in the intact enzyme on right shows area depicted, with viewpoint indicated with an eye. b Molecular model of the F<sub>0</sub> stator, as viewed from the c-ring, showing discussed residues at the stator/rotor interface. Colors as in Fig. 1 (subunit a in orange and subunit b in pink) with the proton half-channels shown as light blue inverted surfaces (made with the program HOLLOW<sup>46</sup>) and potential proton path shown with black arrow. Essential aArg210<sup>27</sup> (dark blue) is located in the center of the membrane, adjacent to cAsp61<sup>28</sup> (shown in c). aGln252 is situated proximally to aArg210 and function can be retained when these residues are interchanged with one another<sup>37</sup>. aGlu219<sup>32</sup>, aAsp119, aHis245<sup>33</sup>, and aAsn214, which are required for proton translocation, are situated between aAsp61 and aArg210, whereas aSer199 is situated at the end of the periplasmic half-channel and cAsp61, adjacent to density that could be a ion/water molecule (magenta sphere). aSer199 is situated at the beginning of the cytoplasmic proton channel. N-termini and C-termini of subunits a and b labeled.

Fig. 5 Lipid bridges contribute to the stator/rotor interaction. Cryo-EM density of Sub-state 3A, centered on the F<sub>0</sub> region. Density corresponding to lipids (colored in wheat) “bridge” the interaction between subunits a (orange) and c (gray). a When viewed from within the membrane, density can be seen for lipids around the F<sub>0</sub> motor. Three lipid-like densities on the periplasmic side (labeled with black arrows) are the strongest of these densities, suggesting that they are well ordered. b When viewed from the periplasmic side (view rotation axis shown), the interaction between residues ile225-Gln234 of the stator subunit a and c ring is facilitated by three lipid densities (highlighted with blue background), increasing the interaction to four rather than three c subunits (numbered one white circles).

(Fig. 4, Supplementary Fig. 19). However, inspection of the nucleotide binding pockets in the cryo-EM structures showed density for nucleotide in all six of α and β subunits, whereas only four were observed to be occupied in the isolated F<sub>1</sub> motor crystal structure<sup>25</sup>. In the cryo-EM maps of the intact F<sub>1</sub>F<sub>0</sub> enzyme in the presence of 10 mM MgADP, all α subunits contained ATP and Mg<sup>2+</sup>, whereas the contents of the β subunits varied: β1 bound ADP, Mg<sup>2+</sup> and P<sub>i</sub>, β2 bound only ADP, and β3 bound ADP and Mg<sup>2+</sup> (Fig. 6b and Supplementary Fig. 20). This composition contrasted with that observed in the crystal structure of E. coli F<sub>1</sub>-ATPase soaked in 1 mM AMP-PNP, where β1 bound ADP, Mg<sup>2+</sup> and SO<sub>4</sub><sup>2-</sup>, and β2 and β3 bound only SO<sub>4</sub><sup>2-</sup><sup>25</sup>. The presence of nucleotide in all α and β subunits in our cryo-EM maps, likely reflects the relatively high concentration of MgADP used during sample preparation.

**Discussion**

The high-resolution information obtained by cryo-EM analysis of E. coli ATP synthase presented here, provides information on how the complex adapts its conformation during rotation to accommodate symmetry mismatch between the F<sub>0</sub> and F<sub>1</sub> components, the potential role of lipids to increase the rotor/stator interface, and the conformational changes that are induced by binding of MgADP.

Strikingly, of the three F<sub>1</sub> rotational states, defined by the angle of the γ-subunit relative to the peripheral stalk, only one showed two F<sub>0</sub> rotational sub-states, as defined by the rotation angle of the c<sub>10</sub>-ring relative to the stator. This can be explained as a consequence of the potential elastic flexibility in the peripheral stalk (“the spring”) seen in this study and the symmetry mismatch between the F<sub>1</sub> and F<sub>0</sub> motors. In State 1 the un-stretched spring corresponds to an F<sub>0</sub> rotation angle in-between two of its 10 preferred rotational sub-states (Fig. 3b). These two correspond to the observed Sub-states 1 and 1”, which have similar quantities of stretch (close to 1/20 of a revolutions), and therefore also energy stored in the spring. By contrast, in States 2 and 3 the spring is close to its relaxed energy state in the observed single F<sub>0</sub> rotary states, and the adjacent F<sub>0</sub> rotary states are inaccessible.
that the two mechanisms; twisting and flexing in the peripheral and central stalks (seen in this study on E. coli), and a hinge in subunit $\delta$/OSCP (seen in Polytomella$^{15-16}$), could combine to allow a fluid motion across a ~60–80° sub-step of the enzyme. The data presented in this manuscript shows the enzyme in its autoinhibited conformation, with the F$_1$-ATPase in a single rotational state. Hence, further work will need to be performed on this enzyme to investigate whether the peripheral and central stalks retain the same properties when the enzyme is under load either synthesizing or hydrolyzing ATP.

The high-resolution information obtained for the intact E. coli complex provides a framework to further understand the proton path in F$_0$, along with the multitude of mutagenesis studies that have been performed in this region (Fig. 4b). For example, density for aArg210$^{17,27}$, which is essential for F$_0$ rotation, is positioned adjacent to the proton translocating residue cAsp61$^{17}$ on the rotor ring (Fig. 4b). Radiation damage in cryo-EM causes the preferential loss of carboxylates, making the confident assignment and precise building of glutamates and aspartics a challenging task.$^{19}$ Other studies of ATP synthases have focused on organisms that utilize a glutamic acid to translocate protons, whereas E. coli F$_0$ ATP synthase utilizes aspartic acid, cAsp61, to translocate protons. As aspartic acid contains a shorter sidechain than the glutamic acid, the possible rotamer positions after electron irradiation are fewer giving an advantage over other systems when attempting to assign the conformation of this sidechain. Although we cannot unequivocally assign the precise nature of this interaction, the cAsp61 adjacent to aArg210 appears to be in an outward facing conformation compared to cAsp61 residues exposed to the membrane (Fig. 4b), which corroborates crystallographic studies on the isolated c-ring$^{10}$. The position of aArg210 likely prevents short circuiting of the protons half-channels, as suggested by other studies$^{19}$. Unlike other structural studies on related ATP synthases$^{11,18,31}$, the interface between the a and c subunits remained largely similar across the classified maps. This could be a hallmark of the cCTD/MgADP inhibited state, a specific feature of E. coli F$_1$F$_0$ ATP synthase or an artifact of the methods used to classify the particles. Further studies on uninhibited enzymes and ATP synthases from other organisms will be needed to delineate this structural feature.

A deep funnel-like evagination can be observed on the cytoplasmic side of the membrane that likely facilitates deprotonation of the c-ring near aSer199, which is adjacent to cAsp61. The proton path on the periplasmic side of the membrane less direct, with a smaller channel leading to aGlu219 (Fig. 4b). Residues aGlu219$^{32}$, aAsp192$^{29}$, aHis248$^{29}$, aAsn241$^{34}$, which have been found to be required for proton pumping$^{20,33}$, form a chain of residues between the periplasmic channel and the rotation path of cAsp61 (Supplementary Fig. 11). Moreover, mutagenesis studies on E. coli F$_1$F$_0$ ATP synthase have shown that residues aGlu219 and aHis245 can be substituted for one another and still retain function$^{36}$, suggesting that their function is coupled. Although a coordinated metal ion has been proposed to mediate protonation of the c subunit$^{11}$ in related ATP synthases via this route$^{11,18,21}$, close inspection of our maps (Supplementary Fig. 13) failed to show any non-peptide density in this region. This could be explained by the fact that the residues which coordinate the ion in Polytomella mt ATP synthase, aHis248 and aHis252, correspond to aGlu219 and allele223 in the E. coli enzyme and so the local geometry needed for metal binding would be lost in the bacterium. Instead we observed weak ion/water-like density at the rotor/stator interface adjacent to the functionally essential residues aAsn214 and aHis245, which would directly interact with the proton binding cAsp61 residue as the c-ring rotates potentially facilitating protonation (Fig. 4b and Supplementary

Fig. 6 The F$_1$ motor changes conformation to the autoinhibited state in 10 mM MgADP. a Masked maps of the $\alpha$, $\beta$, $\gamma$, and $\epsilon$ subunits showing the $\beta_1$ subunit (labeled) “half-closed” (black arrow describes direction of movement in $\beta_1$ subunit) onto the cCTD (labeled). The C-terminal domain of the $\epsilon$ subunit is in an “up” conformation. b Nucleotide and Mg$^{2+}$ occupancy of the $\alpha$ and $\beta$ subunits, with difference density (blue mesh) of nucleotides shown (with equivalent Mitochondrial F$_1$ nomenclature$^5$; $\beta_1 = \beta_\text{M1}, \beta_2 = \beta_\text{E1}, \beta_3 = \beta_\text{E2}$).

because thermal excitation is insufficient to stretch the spring through the required 1/10 of a revolution (Fig. 3b). Hence, the flexibility of the peripheral stalk is sufficient to provide the conformational freedom to accommodate the symmetry mismatch. However, as ~40% of the particles processed in this study were not classified into the sub-states observed, it is certainly speculative to infer the elastic energy stored in this system. Hence further studies on the exact nature of this coupling will be needed to understand the interaction fully. Still, the simple observation of four discrete rotational sub-steps in the F$_0$ motor highlights how a symmetry mismatch between the 3-fold F$_1$-ATPase and 10-fold F$_0$ motor can be accommodated (Fig. 3a).

In the structures we obtained for the E. coli enzyme we did not observe any significant hinge movement in the $\delta$ subunit (Supplementary Fig. 21), that corresponds to OSCP in mitochondrial ATP synthases, and which has been proposed to provide flexible coupling in other systems$^{11}$. In contrast to Polytomella mt. ATP synthase, where the peripheral stalk remains essentially unchanged during rotation$^{11}$, the E. coli peripheral stalk bends and twists substantially. This difference could derive from Polytomella mt. ATP synthase having a large bridged peripheral stalk, the structure of which appears likely to inhibit substantial movement within this component$^{11}$ so that, in this species, flexibility is generated primarily by the junction between it and the F$_0$ motor that is formed by the OSCP subunit. However, it has been proposed$^{11}$ that this hinge is also found in bovine mitochondrial ATP synthase$^{26}$, raising the possibility that the mechanism by which the stator flexes might be different between eukaryotes and prokaryotes. Although further work will be needed to investigate this proposal, it raises the possibility that the hinges present in the E. coli stator could represent a novel drug target, especially since a number of agents are known to target the flexing OSCP subunit in mitochondrial ATP synthases$^{11}$. An alternative hypothesis is
Fig. 11). However, the observation of such a ion/water density is highly speculative and future more detailed maps along with other experiments will be needed to confirm the presence and significance of such an entity.

Our model also places aGln252 adjacent to aArg210 (Fig. 4b), and multiple studies have shown that if these residues are substituted for one another, function can be retained17,38 with the arginine residue still being able to contact cAsp61 and facilitate rotation in our model. The maps also show density for residues 10–20 of subunit a, which is located adjacent to the N-terminus of subunit b (Supplementary Fig. 16a). Truncation of these amino acids has been shown to interfere with the interaction between the a and b subunits, resulting in a non-functional enzyme35. This, together with multiple contacts between both b subunits and transmembrane helices of subunit a (Supplementary Fig. 16b), point to this being a load-bearing element that clamps subunits b together and prevents them from deforming during rotation. Mutations in this area have a detrimental effect on the enzyme and result in changes in crosslinking analyses39,40.

The lipid bridge we observe at the rotor/stator interface (Fig. 5) suggests a mechanism whereby lipids can form a functional component of the complex to facilitate transient interactions between the stator and rotor. Although lipids have been identified elsewhere in previous studies1,4, the lipids we observe in E. coli F1Fo ATP synthase appear to mediate an interaction between the rotor and stator. This lipid bridge increases the interacting surface between subunit a and the c-ring so that it encompasses four of the ten c subunits, with three interacting directly through protein-protein contacts and one through the lipid bridge (Fig. 5). Although it is unclear why the E. coli enzyme employs such a mechanism, an attractive hypothesis would be that the lipids facilitate a transient interaction that would decrease the energy required for rotation while still contributing to the interaction surface that is important to maintain complex integrity. F1Fo ATP synthases from other organisms with larger c-rings, such as chloroplast18, do not appear to have the specific local geometry that would facilitate the lipid bridge we observe in E. coli, as the horizontal42 or inclined membrane helices can bend to the curvature of the c-ring, suggesting that this feature could be restricted primarily to bacteria or ATP synthases with smaller c-ring sizes. For example, in chloroplast F1Fo, ATP synthase18 the a subunit is able to interact with four of the fourteen c subunits in the rotor ring and the long transmembrane helices in subunit a can adapt to the curvature of the c-ring. Inspection of the maps generated from porcine43 and Bacillus sp. PS317 F1Fo ATP synthase in light of our finding, shows similar densities in this region, though this was not highlighted by the authors of these studies (Supplementary Fig. 22), suggesting that this lipid mediated interaction may be conserved across some species containing smaller c-rings. During synthesis the F1 motor would rotate in a clockwise direction when viewed from the periplasm (Fig. 5). This would result in c-ring rotation away from the lipid bridge, hence if the lipid were to remain in place under synthesis the interaction to the a subunit must be stronger than that to the c subunit. The role lipids play in the function of membrane proteins has been an area of considerable interest and controversy, with the lipid environment often appearing to influence function. Mitochondrial ATP synthase requires cardiolipin to function, which has been shown to bind to conserved lysine residues in mitoatp1 ATP synthases44. Lipids functioning as bridges between protein subunits within and between complexes may facilitate transient dynamic interactions within membranes and several studies have identified lipids that are essential for the oligomerization of a considerable range of membrane proteins45.

The cryo-EM structure of the F1 motor described here also provided information on the structural changes and nucleotide occupancy introduced by the binding of MgADP. Currently, only two studies have provided cryo-EM maps of an ATP synthase in which conditions have been manipulated to image the sample with added nucleotide13,46. One of these studies, which exposed the E. coli enzyme to 10 mM MgATP for 45 s45, described the molecular events that occur upon ATP binding and showed that the C-terminal domain of the inhibitory subunit ε47 was removed from the central cavity when ATP was bound. Our present maps show that a short incubation of 10 mM MgADP induces the catalytic subunits to bind this ADP (Fig. 6) which, in turn, induces a partial “closure” of the β subunit that is blocked in a “half-closed” position by the inhibitory ε subunit in a manner reminiscent of that seen in the crystal structure of isolated E. coli F1-βATPase25 (Supplementary Fig. 19). Interestingly, the nucleotide occupancy differed to that in the isolated F1 enzyme, with ADP seen in all catalytic sites and P i bound along with MgADP in the “half-closed” β subunit (Supplementary Fig. 20). As no P i was intentionally introduced into the system, it is likely that this component was either introduced with the purchased ADP as a contaminant or is already present in the enzyme preparation. Trypsin treatment of E. coli F1-β ATP synthase after incubation with 5 mM MgADP+P i has previously suggested that the cCTD would adopt a down conformation in the presence of MgADP+P i, and single molecule studies49,50 have also suggested that the conformation of the cCTD would differ in the presence of MgADP+P i. However, our cryo-EM work did not detect any structures in which the ε subunit was in a down conformation, which could be due to the concentration of P i being below the threshold to induce changes in the ε subunit or due to the short incubation time used. Nevertheless, the observation that MgADP induces a single β subunit to close but is blocked half-way by the cCTD in the up position, strongly suggests that a key function of the ε subunit is to increase the efficiency of the enzyme by preventing E. coli F1Fo ATP synthase from entering a low energy MgADP inhibited state, which is known to inactivate the enzyme16,51 (Supplementary Movie 4).

In summary, the cryo-EM study presented here has generated a comprehensive structural model of E. coli F1Fo ATP synthase, providing a framework to understand mutagenesis studies together with yielding insight into the flexibility of the peripheral and central stalks. The range of rotary sub-states together with the lipid bridge observed describe an attractive mechanism by which the F1 and F0 motors can be coupled with minimal energy loss. In addition, the structural rearrangement observed upon binding of MgADP suggests that the ε subunit can function as a brace to prevent the complex falling into the MgADP inhibited state.

**Methods**

**Protein purification.** The E. coli F1Fo ATP synthase protein was prepared as described in Sobti et al. 202012. Cysteine free E. coli ATP synthase (plasmid generated in Ishmukhametov et al. 200513, which has had all cysteines residues substituted with alanine and a His-tag introduced on the β subunit) was expressed in E. coli DK8 strain31. Cells were grown at 37 °C in LB medium supplemented with 100 μg mL−1 ampicillin for 5 h. The cells were harvested by centrifugation at 5000 × g, providing ~1.25 g cells per litre of culture. Cells were resuspended in lysis buffer containing 50 mM Tris/Cl pH 8.0, 100 mM NaCl, 5 mM MgCl2, 0.1 mM EDTA, 2.5% glycerol and 1 μg mL−1 DNase I, and processed with three freeze thaw cycles followed by one pass through a continuous flow cell disruptor at 20 kPSI. Cellular debris was removed by centrifuging at 7200 × g for 15 mins, after which the membranes were collected by ultracentrifugation at 100,000 × g for 1 h. The ATP synthase complex was extracted from membranes at 4 °C for 1 h by resuspending the pellet in extraction buffer consisting of 20 mM Tris/Cl pH 8.0, 300 mM NaCl, 2 mM MgCl2, 100 mM sucrose, 20 mM imidazole, 1% glycerol, 4 mM dithiothreitol and EDTA-free protease inhibitor tablets (Roche). Insoluble material was removed by ultracentrifugation at 100,000 × g for 30 min. The complex was then purified by binding on Talon resin (Clontech) and eluted in 150 mM imidazole, and further
purified with size exclusion chromatography on a 16/60 Superose 6 column equilibrated in a buffer containing 20 mM Tris/Cl pH 8.0, 100 mM NaCl, 1 mM digitonin, 1 mM MgCl2. The purified protein was then concentrated to 11 μl (6 mg ml−1), and snap frozen and stored for grid preparation. The protein used in this study came from the same preparation as used in Sobti et al. 2019, which contains activity assays for this enzyme, showing ~80 s−1 turnover per enzyme (~9 μm2 s−1) even after 8 h at room temperature, as well as sensitivity to DCCD, showing coupled F0F1 complexes. SDS PAGE was performed to assess protein purity (Supplementary Fig. 23), with bands identified using mass spectrometry.

Cryo-EM grid preparation. One microlitre of 100 mM ADP/100 mM MgCl2 (pH 8.0) was added to an aliquot of 9 μl of purified cytochrome f. E. coli F0F1 ATP synthase at 11 μl (6 mg ml−1) and the sample incubated at 22 °C for 30 s, before 3.5 μl was placed on glow-discharged holey gold grid (Ultrafalcon R1.2/1.3, 200 Mesh). Gridded grids were blotted for 3 s at 22 °C, 100% humidity and flash-frozen in liquid ethane using a FEI Vitrobot Mark IV (total time for sample application, blotting and freezing was 45 s).

Data collection. Grids were transferred to a Thermo Fisher Talos Arctica transmission electron microscope (TEM) operating at 200 kV and screened for ice thickness and particle density. Grids were subsequently transferred to a Thermo Fisher Titan Krios TEM operating at 300 kV equipped with a Gatan BioQuantum energy filter and K3 Camera at the Pacific Northwest Centre for Cryo-EM at OHSU. Images were recorded automatically using serial EM at 881,000 magnification yielding a physical pixel size of 1.08 Å (K3 operating in super resolution mode: 0.54 Å per pixel micrographs). A total dose of 48 electrons per Å2 was used spread over 77 frames, with a total exposure time of 3.5 s. 9342 movie micrographs were binned by a factor of 2, and two for State 3). 3D classiﬁnement in Relion 3.0, yielding nine deconvolution yielding a physical pixel size of 1.08 Å (K3 operating in super resolution classification, using a mask to generate a model of the membrane region (Supplementary Fig. 10). See Supplementary Fig. 2 for detailed flowchart describing this classification and Supplementary Fig. 24 for FSC curves.

Model building. Models were built and reﬁned in Coot56, PHENIX57 and ISO-LDE58 using pdb IDs 3oa252 (E. coli F1F0-ATPase), 1awb59 (N-terminal domain of E. coli subunit 6) and 6ny260 (Bacillus sp. PS3 ATP synthase) as guides. Auto-sharpening implemented in PHENIX20 was performed on the focused model of Sub-state 3A, and this higher detailed information was used to build the F0 region before progressing to the other sub-states for local reﬁnement. See Supplementary Fig. 25 for model to map FSC curves and Supplementary Table 1 for all data collection and reﬁnement statistics.

Mass spectrometry. Protein complexes were digested with trypsin and dried using a vacuum concentrator (SpeedVac, ThermoFisher Scientiﬁc). The co-puriﬁed lipids were resuspended in 60% acetonitrile by sonication for 10 min and analyzed by LC-MS based lipidomics.24 Briefly, the lipid samples were separated on a C18 column (Acclaim PepMap 100, C18, 75 μm × 15 cm; Thermo Scientiﬁc) by Dionex UltiMate 3000 RSLC nano System, and then analyzed by a hybrid LTQ-Orbitrap XL mass spectrometer (Thermo Scientiﬁc). A binary buffer system was used with buffer A [ACN: H2O (60:40), 10 mM ammonium formate, 0.1% formic acid] and buffer B [IPA: ACN (90:10), 10 mM ammonium formate, 0.1% formic acid]. The phospholipids were separated with a gradient of 22 to 99% buffer B at a ﬂow rate of 300 nL min−1 over 30 min. The LTQ-Orbitrap XL was operated in negative ion mode and in data-dependent acquisition with one MS scan followed by three MS/MS scans.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Maps and models have been deposited in the EMDB and PDB with the following accession codes: Sub-state 1A; EM-20167 and PDB-6OQX [https://doi.org/10.2210/pdb6OQX/pdb]. Sub-state 1B; EM-20168 & PDB-6OQS [https://doi.org/10.2210/pdb6OQS/pdb]. Sub-state 1C; EM-20169 and PDB-6OQT [https://doi.org/10.2210/pdb6OQT/pdb]. Sub-state 1D; EM-20170 and PDB-6OQU [https://doi.org/10.2210/pdb6OQU/pdb]. Sub-state 1E; EM-20454 and PDB-6PQV [https://doi.org/10.2210/pdb6PQV/pdb]. Sub-state 2A; EM-21854 and PDB-6WNQ [https://doi.org/10.2210/pdb6WNQ/pdb]. Sub-state 2B; EM-20171 and PDB-6OQX [https://doi.org/10.2210/pdb6OQX/pdb]. Sub-state 3A; EM-20172 and PDB-6OQY [https://doi.org/10.2210/pdb6OQY/pdb]. Sub-state 3B; EM-21855 and PDB-6WNR [https://doi.org/10.2210/pdb6WNR/pdb]. Sub-state 3A Fv focused: EM-21419 and PDB-6VWK [https://doi.org/10.2210/pdb6VWK/pdb].

The source data underlying Supplementary Fig 18 are provided as a Source Data file. Other data are available from the corresponding authors upon reasonable request.

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