Structure of a bacterial ATP synthase

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Abstract

ATP synthases produce ATP from ADP and inorganic phosphate with energy from a transmembrane proton motive force. Bacterial ATP synthases have been studied extensively because they are the simplest form of the enzyme and because of the relative ease of genetic manipulation of these complexes. We expressed the Bacillus PS3 ATP synthase in Eschericia coli, purified it, and imaged it by cryo-EM, allowing us to build atomic models of the complex in three rotational states. The position of subunit ε shows how it is able to inhibit ATP hydrolysis while allowing ATP synthesis. The architecture of the membrane region shows how the simple bacterial ATP synthase is able to perform the same core functions as the equivalent, but more complicated, mitochondrial complex. The structures reveal the path of transmembrane proton translocation and provide a model for understanding decades of biochemical analysis interrogating the roles of specific residues in the enzyme.

Introduction

Adenosine triphosphate (ATP) synthases are multi-subunit protein complexes that use an electrochemical proton motive force across a membrane to make the cell’s supply of ATP from adenosine diphosphate (ADP) and inorganic phosphate (Pi). These enzymes are found in bacteria and chloroplasts as monomers, and in mitochondria as rows of dimers that bend the inner membrane to facilitate formation of the mitochondrial cristae1,2. Proton translocation across the membrane-embedded F0 region of the complex occurs via two offset half-channels3,4. Studies with Bacillus PS3 ATP synthase in liposomes showed that proton translocation may be driven by ΔpH or ΔΨ alone5. The passage of protons causes rotation of a rotor subcomplex, inducing conformational change in the catalytic F1 region to produce ATP6 while a peripheral stalk subcomplex holds the F1 region stationary relative to the spinning rotor during catalysis. For the mitochondrial enzyme, X-ray crystallography has been used to determine structures of the soluble F1 region7, partial structures of the peripheral stalk subcomplex alone8 and with the F1 region9, and structures of the F1 region with the membrane-embedded ring of ε-subunits attached10,11. Recent breakthroughs in electron cryomicroscopy (cryo-EM) allowed the
structures of the membrane-embedded \( F_0 \) regions from mitochondrial and chloroplast ATP synthases to be determined to near-atomic resolutions \(^{12-15}\). Compared to their mitochondrial counterparts, bacterial ATP synthases have a simpler subunit composition. The \( F_1 \) region consists of subunits \( \alpha_2\beta_3\gamma\delta\epsilon \), while the \( F_0 \) region is usually formed by three subunits with the stoichiometry \( ab_2c_{9,15} \). Chloroplasts and a few bacteria, such as \textit{Paracoccus denitrificans}, possess two different but homologous copies of subunit \( b \), named \( b_1 \) and \( b_2 \). Each copy of subunit \( \alpha \) and \( \beta \) contains a nucleotide binding site. The non-catalytic \( \alpha \) subunits each bind to a magnesium ion (\( \text{Mg}^{2+} \)) and a nucleotide, while the catalytic \( \beta \) subunits can adopt different conformations and bind to \( \text{Mg-ADP (}\beta_{DP}\text{)} \), \( \text{Mg-ATP (}\beta_{TP}\text{)} \), or remain empty (\( \beta_E \)). Crystal structures of bacterial \( F_1-\text{ATPases} \) and \( c \)-rings from the \( F_0 \) regions of several species have been determined \(^{16-24} \). Structures of intact ATP synthases from \textit{E. coli} have been determined to overall resolutions of 6 to 7 Å by cryo-EM, with the \( F_0 \) region showing lower quality than the rest of the maps, presumably due to conformational flexibility \(^{25} \). In structures of both intact ATP synthase \(^{25} \) and dissociated \( F_1-\text{ATPase} \) \(^{17,19} \) from bacteria, subunit \( \epsilon \) adopts an “up” conformation that inhibits the ATP hydrolysis by the enzyme. In the thermophilic bacterium \textit{Bacillus} PS3, this subunit \( \epsilon \) mediated inhibition is dependent on the concentration of free ATP \(^{26-28} \). Low ATP concentrations (\( \text{eg. } < 0.7 \text{ mM} \)) promote the inhibitory \textit{up} conformation while a permissive “down” conformation can be induced by a high concentration of ATP (\( \text{eg. } > 1 \text{ mM} \)). This mechanism would allow the \textit{Bacillus} PS3 ATP synthase to run in reverse, establishing a proton motive force by ATP hydrolysis, when the ATP concentration is sufficient to do so without depleting the cell’s supply of ATP. In \textit{E. coli}, however, in the absence of a sufficient proton motive force to drive ATP synthesis, inhibition of ATP hydrolysis by subunit \( \epsilon \) persists even when the concentration of free ATP is high \(^{29,30} \).

Although bacterial ATP synthases have been subjected to extensive biochemical analysis, high-resolution structural information is lacking for the intact enzyme or the membrane-embedded proton-conducting subunit \( a \) and the associated subunit \( b \). We determined structures of intact ATP synthase from \textit{Bacillus} PS3 in three rotational states by cryo-EM. The structures reached overall resolutions of 3.0 to 3.2 Å (Fig. 1), allowing construction of nearly complete atomic models for the entire complex. The structures reveal how loops in subunit \( a \) of the bacterial enzyme fill the role of additional subunits in the \( F_0 \) region of the mitochondrial enzyme. Most significantly, the structures provide a framework for understanding decades of mutagenesis experiments designed to probe the mechanism of ATP synthases.

Results and discussions

\textit{Structure determination and overall architecture}

Subunits of \textit{Bacillus} PS3 ATP synthase, including subunit \( \beta \) bearing an N-terminal 10×His tag, were expressed from a plasmid in \textit{E. coli} strain DK8, which lacks endogenous ATP synthase \(^{31,32} \). The complex was extracted from membranes with detergent, purified by metal-affinity chromatography, and subjected to cryo-EM analysis (Fig. 1 - figure supplement 1). Three conformations corresponding to different rotational states of the enzyme were identified by ab-initio 3D classification and refined to high resolution. The 3D classes contain 45, 35, and 20% of particle images and the overall resolutions of the corresponding cryo-EM maps were 3.0, 3.0, and 3.2 Å, respectively (Fig. 1 - figure supplements 2 and 3). Estimation of local resolution
suggests that the F₁ regions of the maps, which are larger than the F₀ regions and appear to dominate the image alignment process, are mostly at between 2.5 and 3.5 Å resolution, whereas the F₀ regions were limited to lower resolution (Fig. 1 - figure supplement 3). Focused refinement of the F₀ region and peripheral stalk subunits ab₂c₈₁₀ and δ (corresponding to the subunit OSCP in mitochondrial ATP synthase) improved the resolution of the F₀ regions considerably for all three classes but not enough to resolve density for most of the amino acid side chains. An improved map of the F₀ region was obtained by focused refinement of the membrane-embedded region only, excluding the soluble portion of subunit b with particle images from all three classes (Fig. 1 - figure supplement 2). Overall, amino acid side chain detail can be seen for subunits α, β, γ, δ, ε, a, c₁₀-ring, and the transmembrane α-helices of b₂ (Fig. 1 - figure supplement 4). The soluble region of the two b-subunits was modeled as poly-alanine (Table. 1).

The general architecture of the enzyme resembles *E. coli* ATP synthase and the more distantly related spinach chloroplast enzyme but with striking differences. As observed previously in a *Bacillus* PS3 F₁-ATPase crystal structure (PDB 4XD7), the three catalytic β subunits adopt “open”, “closed”, and “open” conformations, different from the “half-closed”, “closed”, and “open” conformations seen in the auto-inhibited *E. coli* F₁-ATPase, and the “closed”, “closed”, and “open” conformations seen in chloroplast ATP synthase and most mitochondrial ATP synthases. This difference, with the half-closed βD₃ of the *E. coli* enzyme appearing as open in the *Bacillus* PS3 enzyme, suggests species-specific differences in inhibition by subunit ε (Fig. 1B, pink density), which inserts into the α/β interface and forces βD₃ into the open conformation.

Thermophilic proteins achieve stability at high temperature through mechanisms that include an increased number ionic interactions, shorter loops between secondary structure elements, and tighter packing of hydrophobic regions. Comparison of individual subunit structures from the F₁ regions of ATP synthases from thermophiles (*Bacillus* PS3 and *Caldalkalibacillus thermarum* [PDB 5HKK]) and mesophiles (*E. coli* [PDB 3OAA], *Paracoccus denitrificans* [5DN6], and *Spinacia oleracea* chloroplast [PDB 6FKF]) did not show clear evidence of tighter packing or shorter loops in the thermophilic complexes. However, there are more ionic interactions, including those with distances < 4 Å and > 8 Å, in the thermophilic F₁-ATPase structures than in the mesophiles, suggesting that these interactions may play a role in stabilizing the thermophilic complexes.

In the F₀ region, one copy of subunit b is positioned at a location equivalent to that of the mitochondrial subunit b, while the second copy occupies the position of yeast subunit δ (mammalian A6L) on the other side of subunit a (Fig. 1B). Despite the different c-ring sizes (10 c-subunits in *Bacillus* PS3 versus 14 in spinach chloroplasts), the backbone positions of subunits ab₂ from *Bacillus* PS3 overlap with subunits abb’ from spinach chloroplast ATP synthase (Fig. 1 - figure supplement 5A). Comparison of the atomic model of the F₀ region from *Bacillus* PS3 and the backbone model of the *E. coli* complex from cryo-EM at ~7 Å resolution (PDB 5T4O) showed significant structural differences in transmembrane α-helices of subunit b relative to subunit a (Fig. 1 - figure supplement 5B). Rather than reflecting true differences between *E. coli* and *Bacillus* PS3 ATP synthase structures, these deviations likely suggest that the 6 to 8 Å
resolution *E. coli* maps were not at sufficient resolution to allow accurate backbone tracing of F\(_O\) subunits.

**Flexibility in the peripheral and central stalks**

As expected, the most striking difference between the three rotational states of the *Bacillus* PS3 structure is the angular position of the rotor (subunits \(\gamma\alpha_0\)) (Fig. 2A, Video 1). The structure of the ATP synthase, with three \(\alpha\beta\) pairs in the F\(_I\) region and ten \(c\)-subunits in the F\(_O\) region, results in symmetry mismatch between the 120\(^\circ\) steps of the F\(_I\) motor and 36\(^\circ\) steps of the F\(_O\) motor.

The 120\(^\circ\) steps of the F\(_I\) motor gives an average rotational step of 3.3 \(c\)-subunits, with the closest integer steps being 3, 4 and 3 \(c\)-subunits. By comparing the positions of equivalent \(c\)-subunits in different rotational states, the observed rotational step sizes in the three rotational states of the ATP synthase appear to be almost exactly 3, 4 and 3 \(c\)-subunits (Fig. 2B). At the present resolution, the structures of subunit \(a\) and the \(c\)-ring do not appear to differ between rotary states. Similar integer step sizes were found in yeast ATP synthase \(^{37}\) and V-ATPase \(^{38}\), which also contain 10 \(c\)-subunits. However, non-integer steps were seen in the chloroplast (14 \(c\)-subunits) \(^{15}\) and bovine (8 \(c\)-subunits) \(^{39}\) ATP synthases, indicating that the \(c\)-subunit steps between the rotational states of rotary ATPases likely depends on the number of \(c\)-subunits.

The unequal number of \(c\)-subunit steps between rotational states or the different interactions made by the three \(\alpha\beta\) pairs with the \(b_2\delta\) peripheral stalk could lead to a variable rotation speed for the \(c\)-ring in the active enzyme, analogous to kinetic limping in kinesin motors \(^{40}\). Alternatively, flexibility in the enzyme could maintain a constant rotational velocity. Indeed, flexibility is thought to be important for the smooth transmission of power between the F\(_I\) and F\(_O\) regions, which often have mismatched symmetries \(^{41-43}\). Earlier studies suggested that the central stalk (subunits \(\gamma\) and \(\epsilon\) in bacteria) is the main region responsible for the transient storage of torsional energy in rotary ATPases \(^{44-47}\). Comparison of the three rotational states of the *Bacillus* PS3 enzyme also shows that C-terminal water-soluble part of subunit \(b\) displays the most significant conformational variability between states, while the subunits in the F\(_I\) region show little flexibility beyond the catalytic states of the \(\alpha\beta\) pairs (Fig. 2C; Video 1). The structure of the yeast ATP synthase F\(_O\) dimer \(^{12}\), which lacked the the F\(_I\) region and an intact peripheral stalk, showed that the \(c\)-ring and subunit \(a\) are held together by hydrophobic interactions rather than by the peripheral stalk. In *Bacillus* PS3 ATP synthase, the peripheral stalk is structurally simpler and more flexible than in yeast mitochondria \(^{14}\), suggesting that the bacterial subunits \(a\) and the \(c\)-ring are also held together by hydrophobic interactions and not the peripheral stalk. Given that these structures represent resting states of the bacterial ATP synthase, additional subunits, such as those in the central stalk, may show flexibility while under strain during rotation.

**Nucleotide binding in the F\(_I\) region and inhibition by subunit \(\epsilon\)**

The structure of the F\(_I\) region of the intact *Bacillus* PS3 ATP synthase and the earlier crystal structure of the dissociated F\(_I\)-ATPase (PDB 4XD7) \(^{19}\) both show that the three catalytic \(\beta\)-subunits (\(\beta_E\), \(\beta_T\), and \(\beta_D\)) adopt “open”, “closed”, and “open” conformations, respectively (Fig. 3A). In the crystal structure, which was prepared in the presence of CyDTA (trans-1,2-Diaminocyclohexane-N, N', N'-tetraacetic acid monohydrate) as a chelating agent, there was no nucleotide in the three noncatalytic sites of the three \(\alpha\)-subunits and the only nucleotide in a catalytic site was an ADP molecule without a Mg\(^{2+}\) ion in the \(\beta_T\) site. In contrast, all three non-catalytic sites in the cryo-EM map are occupied by Mg-ATP, while a Mg-ADP molecule and a
Bacillus PS3 ATP synthase is found in a conformation that has been proposed to allow ATP synthesis while ATP hydrolysis is auto-inhibited. In this state subunit $\varepsilon$ maintains an up conformation and inserts into the $\alpha_{DP}\beta_{DP}$ interface, forcing $\beta_{DP}$ to adopt an open conformation (Fig. 3A, lower, dashed box)\(^{19}\). In the crystal structure (PDB 4XD7)\(^ {19}\), the C-terminal sequence of subunit $\varepsilon$ was modeled as two $\alpha$-helical segments broken at Ser 106, while the cryo-EM structures show the C-terminal part is in fact entirely $\alpha$-helical. In comparison, subunit $\varepsilon$ from the auto-inhibited E. coli F$_1$-ATPase structure (PDB 3OAA)\(^ {17}\) maintains its two C-terminal $\alpha$-helices (Fig. 3B), with its $\beta_{DP}$ adopting a half-closed conformation that binds to Mg-ADP. The C-terminal $\alpha$-helix of the E. coli subunit $\varepsilon$ inserts slightly deeper into the $\alpha_{DP}\beta_{DP}$ interface but overall in a manner similar to that of the Bacillus PS3 subunit $\varepsilon$. However, the second $\alpha$-helix in E. coli is offset by a ten-residue loop that allows it to interact with subunit $\gamma$. This interaction (Fig. 3B, lower, dashed box) may stabilize the up conformation of subunit $\varepsilon$ in E. coli, explaining why auto-inhibition in E. coli does not depend on ATP concentration\(^ {29,30}\) while in Bacillus PS3 it does. Interestingly, during ATP synthesis, Bacillus PS3 subunit $\varepsilon$ was proposed to maintain the up conformation\(^ {27}\), suggesting that it only blocks ATP hydrolysis but not ATP synthesis. For a canonical ATP synthase, the substrates ADP and Pi bind to an open $\beta_E$. The $\beta_E$ subsequently transitions to become the closed $\beta_{DP}$ and then $\beta_{TP}$, driven by rotation of the central rotor, producing an ATP molecule that is ultimately released when the closed $\beta_{TP}$ converts back to an open $\beta_E$\(^ {7}\). For the Bacillus PS3 ATP synthase to produce ATP with subunit $\varepsilon$ in the up conformation, substrate would need to bind to the $\beta_{DP}$ site instead of the usual $\beta_E$ site, with an ATP molecule produced on transition to a closed $\beta_{TP}$. The cryo-EM maps show that a clash between subunit $\varepsilon$ and $\beta_{TP}$ blocks the central rotor turning in the direction of ATP hydrolysis while it is still free to turn in the direction of ATP synthesis (Fig. 3C), which could explain the ability of subunit $\varepsilon$ to selectively inhibit ATP hydrolysis\(^ {27}\).
mammalian mitochondrial ATP synthases contain subunit f, which has a transmembrane α-helix adjacent to the transmembrane α-helix 1 of subunit a (Fig. 4A, right), anchoring subunit b between α-helices 5 and 6 of subunit a. The location of the loop between α-helices 3 and 4 of the Bacillus PS3 subunit a suggests that it serves a similar structural role, compensating for the lack of subunit f in bacteria. The loop forms an additional interface with subunit b near the periplasmic side of the membrane region and may interact with the N terminus of subunit b in the periplasm as well. Two interfaces are also present between the second copy of subunit b and subunit a, one with the first transmembrane α-helix, and the other with the hairpin of α-helices 3 and 4 (Fig. 4A). The structure suggests that two interfaces are necessary for subunits a and b to maintain a stable interaction.

Proton translocation through the F_0 region

The Bacillus PS3 ATP synthase structure implies a path for proton translocation through the bacterial complex involving two half-channels and similar to the paths described for the mitochondrial and chloroplast enzymes. The cytoplasmic half-channel consists of an aqueous cavity at the interface of subunit a and the c-ring (Fig. 4B, left). The periplasmic half-channel is formed from a cavity between α-helices 1, 3, 4 and 5 of subunit a, and reaches the c-ring via a gap between α-helices 5 and 6 (Fig. 4B, right). In the atomic model, both channels are visible when modelling the surface with a 1.4 Å sphere that mimics a water molecule (Fig. 4B). The channels are wide and hydrophilic, suggesting that water molecules could pass freely through each of the channels before accessing the conserved Glu 56 of the c-subunits. During ATP synthesis, protons travel to the middle of the c-ring via the periplasmic half-channel and bind to the Glu 56 residue of a subunit c (Fig. 4C). Protonation of the glutamate allows rotation of the ring counter-clockwise, when viewed from F₁ towards F₀, delivering the subunit c into the hydrophobic lipid bilayer. Protonation of the remaining nine subunits in the c-ring returns the first glutamate to subunit a, now into the cytoplasmic half-channel, where it releases its proton to the cytoplasm due to interaction with the positively charged Arg 169 of subunit a. The proposed channels are consistent with a series of experiments probing water accessibility of residues in the E. coli ATP synthase subunit a by mutating them to cysteines and testing their accessibility by Ag⁺ ions. Residues that are close to the c-ring, such as S202, S206, N214, and I249 (A161, T165, N173, and G214 in Bacillus PS3) are among the most accessible by Ag⁺, suggesting that the half-channels do not exclude Ag⁺. Therefore, it is likely that Na⁺ ions, which are similar in size to Ag⁺ ions, are also not excluded by subunit a. It is also known that the c-rings from Na⁺- and H⁺-driven ATP synthases have different affinities for Na⁺ and H⁺, and that the c-ring of the Na⁺-driven ATP synthase from Ilyobacter tartaricus does not bind K⁺ or Cs⁺ ions. Together, these results suggest that ion selectivity in ATP synthases is probably determined by the c-ring, not subunit a.

In eukaryotes, subunit a is encoded by the mitochondrial genome, limiting genetic interrogation of the roles of different residues. In contrast, numerous mutagenesis studies have been performed on bacterial subunits a and b, with E. coli ATP synthase being the most frequently studied. A single G9D mutation in the E. coli subunit b (positionally equivalent to Y13D in Bacillus PS3), results in assembled but non-functional ATP synthase, while multiple N-terminal mutations in subunit b can either disrupt enzyme assembly or ATP hydrolysis. In Bacillus PS3, Tyr 13 is part of the transmembrane α-helix of subunit b and is adjacent to Gly 188 of subunit a (Fig. 4B, figure supplement 3, dashed box). In E. coli subunit a, Gly 188 is replaced by a leucine (Leu...
275 Therefore, the G9D mutation in *E. coli* not only introduces a charged residue into a
276 hydrophobic transmembrane α-helix, but also creates a steric clash with Leu 229 of subunit a,
277 explaining why the mutation leads to an inactive enzyme. Remarkably, the single N-terminal
278 membrane-embedded α-helix in each of the two copies of subunit b in the *Bacillus* PS3 ATP
279 synthase forms different interactions with subunit a (Fig. 4A). One surface interacts with
280 transmembrane α-helices 1, 2, 3, and 4 of subunit a while the other interacts with α-helices 5 and
281 6 and the loop between α-helices 3 and 4 of subunit a. Given that the N-terminal α-helix of
282 subunit b makes interactions with different regions of subunit a, it is not surprising that
283 mutations in this region are often detrimental to the assembly and activity of the complex. Cross-
284 linking experiments suggested that the N terminus of the two copies of subunit b are in close
285 proximity with each other 62. However, the atomic model shows that the transmembrane α-helix
286 of the b-subunits are on opposite sides of subunit a, suggesting that the cross-linking results may
287 be due to non-specific interactions of b-subunits from neighboring ATP synthases.

288 In *E. coli*, Arg 210 of subunit a (Arg 169 in *Bacillus* PS3) tolerates the fewest mutations 63–66.
289 Recent structures of rotary ATPases suggest that the importance of this residue derives from its
290 role in releasing protons bound to the Glu residues of the c-subunits as they enter the cytoplasmic
291 half-channel, as well as preventing short-circuiting of the proton path by protons flowing
292 between half-channels without rotation of the c-ring 18,38,39,67,68. Other residues in the *E. coli*
293 subunit a identified by mutation as being functionally important include Glu 196 (Glu 159 in
294 *Bacillus* PS3) 69,70, Glu 219 (Glu 178) 69–71, His 245 (Ser 210) 65,72,73, Asp 44 (Asp 19) 74, Asn
295 214 (Asn 173) 63, and Gln 252 (Gln 217) 71,75 (Fig. 4D). When mapped to the *Bacillus* PS3
296 structure, only Glu 196 (Glu 159 in *Bacillus* PS3) is close to the cytoplasmic half-channel.
297 Extensive mutations of *E. coli* Glu 196 showed that enzyme activity depends on the charge and
298 polarity of the residue with Glu > Asp > Gln = Ser = His > Asn > Ala > Lys 69. Therefore, the
299 negative surface charge from Glu 196 (Glu 159) near the cytoplasmic half-channel facilitates
300 proton transport across the lipid bilayer. The atomic model of subunit a also suggests that other
301 residues such as *Bacillus* PS3 Thr 165, Asn 162, Glu 158, Tyr 228, and His 231, which are close
302 to the cytoplasmic half-channel, may contribute to channel formation. Many functional residues
303 identified by mutagenesis are clustered around the periplasmic half-channel. In the atomic model
304 of the *Bacillus* PS3 subunit a, Asp 19 and Glu 178 are close to the periplasm, while Ser 210, Asn
305 173, and Gln 217 are deeper inside the membrane. Among these residues, Glu 178 and Ser 210
306 are considered to be more important to enzyme function than Asn 173 and Gln 217, as mutations
307 of corresponding residues in *E. coli* are more likely to abolish the proton translocation by the
308 complex 58. The Glu 219/His 245 residues in *E. coli* 73 also occur in the *S. cerevisiae* (His
309 185/Glu 223) and human (His 168/Glu 203) mitochondrial ATP synthases (Fig. 4 - figure
310 supplement 2). These residues do not appear to be close enough to form a hydrogen bond in the *S.
311 cerevisiae* F0 dimer structure 12. In *Bacillus* PS3 subunit a, the His residue is replaced by a serine
312 (Ser 210) that similarly does not appear to close enough to Glu 178 to form a hydrogen bond.
313 Interestingly, although many of these functional residues appear important, their mutation to
314 amino acids that cannot be protonated or deprotonated often does not completely abolish proton
315 translocation 58. The atomic model of *Bacillus* PS3 subunit a shows that the proton half-channels
316 are wide enough for water molecules to pass through freely. This observation suggests that the
317 function of these conserved polar and charged residues is not the direct transfer of protons during
318 translocation. Rather, their presence may help maintain a hydrophilic environment for water-
319 filled proton channels. This role allows different species to use unique sets of polar and charged
residues forming their proton half-channels. This variability suggests a remarkably flexible proton translocation mechanism for this highly efficient macromolecular machine.

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Supplementary materials:

Table 1

Figure 1 - figure supplement 1-4

Figure 4 - figure supplement 1-3

Video 1

Material and Methods

Protein expression and purification

E. coli strain DK8, in which the genes encoding endogenous ATP synthase subunits were deleted \(^\text{31}\), was transformed with plasmid pTR-ASDS \(^\text{32}\) encoding Bacillus PS3 ATP synthase with a 10× His tag at the N terminus of subunit β. Transformed E. coli cells were grown in 2×TY medium at 37 °C for 20 hours before being harvested by centrifugation at 5,400 g. Cell pellets were resuspended in lysis buffer (50 mM Tris-HCl pH 7.4, 5 mM MgCl\(_2\), 10 % [w/v] glycerol, 5 mM 6-aminocaproic acid, 5 mM benzamidine, 1 mM PMSF) and lysed with three passes through an EmulsiFlex-C3 homogenizer (Avestin) at 15 to 20 kbar. All protein preparation steps were performed at 4 °C unless otherwise stated. Cell debris was removed at 12,250 g for 20 min and the cell membrane fraction was collected by centrifugation at 184,000 g for 1 h. Membranes were washed twice with lysis buffer before being resuspended in solubilization buffer (50 mM Tris-HCl pH 7.4, 10 % [w/v] glycerol, 250 mM sucrose, 5 mM 6-aminocaproic acid, 5 mM benzamidine, 1 mM PMSF) and solubilized by the addition of glycol-diosgenin (GDN) to 1 % (w/v) and mixing for 1 h at room temperature. Insoluble material was removed by centrifugation at 184,000 g for 45 min and solubilized membranes were loaded onto a 5 ml HisTrap HP column (GE Healthcare) equilibrated with buffer A (solubilization buffer with 20 mM imidazole, 300 mM sodium chloride, and 0.02 % [w/v] GDN). The column was washed with 5 column volumes of buffer A, and ATP synthase was eluted with 3 column volumes of buffer B (buffer A with 200 mM imidazole). Fractions containing ATP synthase were pooled and concentrated prior to being loaded onto a Superose 6 increase 10/300 column (GE Healthcare) equilibrated with gel filtration.
buffer (20 mM Tris-HCl pH 7.4, 5 mM MgCl₂, 10 % [w/v] glycerol, 150 mM sodium chloride, 5 mM 6-aminocaproic acid, 5 mM benzamidine, 0.02 % [w/v] GDN). The peak corresponding to *Bacillus* PS3 ATP synthase was pooled and concentrated to ~10 mg/ml prior to storage at -80 °C.

**Cryo-EM and image analysis**

Prior to grid freezing, glycerol was removed from samples with a Zeba spin desalting column (Thermo Fisher Scientific). Purified ATP synthase (2.5 μL) was applied to homemade nanofabricated EM grids 76 consisting of a holey layer of gold 77,78 that had been glow-discharged in air for 2 min. Grids were then blotted on both sides in a FEI Vitrobot mark III for 26 s at 4 °C and ~100 % RH before freezing in a liquid ethane/propane mixture 79. Cryo-EM data were collected with a Titan Krios G3 electron microscope (Thermo Fisher Scientific) operated at 300 kV equipped with a Falcon 3EC direct detector device camera automated with EPU software. Data were recorded as 60 s movies at 2 seconds per frame with an exposure rate of 0.8 electron/pixel/second, and a calibrated pixel size of 1.06 Å.

All image processing steps were performed in cryoSPARC v2 80 unless otherwise stated. 10,940 movies were collected. Movie frames were aligned with an implementation of alignframes_lmbfgs within cryoSPARC v2 81 and CTF parameters were estimated from the average of aligned frames with CTFFIND4 82. 1,866,804 single particle images were selected from the aligned frames with Relion 2.1 83 and beam-induced motion of individual particles corrected with an improved implementation of alignparts_lmbfgs within cryoSPARC v2 81. A subset of 1,238,140 particle images were selected by 2D classification in cryoSPARC v2. After initial rounds of ab-initio 3D classification and heterogeneous refinement, three classes corresponding to three main rotational states of the enzyme were identified, containing 405,432, 314,448, and 175,694 particles images (Figure 1 - figure supplement 2). These 3D classes were refined with non-uniform refinement to overall resolutions of 3.0 Å, 3.0 Å and 3.2 Å, respectively, with the F₁ region reaching higher resolution than the F₀ region of the complex as seen from estimation of local resolution (Figure 1 - figure supplement 3). Masked refinement with signal subtraction (focused refinement) 33 around subunits ab₂c₁₀δ excluding the detergent micelle improved the map quality of the membrane-embedded region as well as the peripheral stalk for all three classes. The membrane-embedded region (subunits ac₁₀ and transmembrane α-helices of the b-subunits) was improved further by focused refinement with particle images from all three classes, yielding a map at 3.3 Å resolution. All Fourier shell correlation (FSC) curves were calculated with independently refined half-maps and resolution was assessed at the 0.143 criterion with correction for the effects of masking maps. For illustration purposes, composite maps for each of the three rotational states were generated by combining the F₁ region of the maps from non-uniform refinement, the peripheral stalk region from the maps obtained with focused refinement of subunits ab₂c₁₀δ and the map from focused refinement of the membrane-embedded region. Specifically, each map was multiplied by a mask surrounding the region of interest and the resulting maps were adjusted to similar absolute grey scale by multiplying with a constant with relion_image_handler before being merged with the maximum function volume operation in UCSF Chimera 84. These composite maps were not used for model refinement.

**Model building and refinement**

Atomic models for subunits αβ₂γδ from all three rotational states were built with Coot 85 into the maps of the intact complex from non-uniform refinement using PDB 4XD7 19 and PDB...
6FKF\textsuperscript{15} as initial models for subunits $\alpha_3\beta_3\gamma\epsilon$ and subunit $\delta$, respectively. Subunits $ac_{10}$ and the membrane-embedded regions of subunits $b_2$ were built de novo in the 3.3 Å map of the membrane-embedded region of the complex from focused refinement. Backbone models of the soluble region of subunits $b_2$ for all three conformations were built with the maps from focused refinement of the peripheral stalk. Models were refined into their respective maps with phenix.real_space_refine\textsuperscript{86} using secondary structure and geometric restraints followed by manual adjustments in Coot (Table. 1). The quality of the models was evaluated by MolProbity\textsuperscript{87} and EMRinger\textsuperscript{88}. To generate full models for all three rotational states, the model of subunits $ac_{10}$ and the membrane region of subunit $b_2$ were fit into the full maps of each conformation as three rigid bodies ($a$, $c_{10}$, and $b_2$ membrane region) with phenix.real_space_refine. For class 1 and 3, the backbone models of the soluble region of subunit $b_2$ did not fit the full maps well, and thus the fit was improved by molecular dynamics flexible fitting (MDFF)\textsuperscript{89}. The final composite model for each rotational state was generated by combining the models of subunits $\alpha_3\beta_3\gamma\delta\epsilon$, the rigid body refined subunits $ac_{10}$ and membrane region of $b_2$, and the backbone model of the soluble region of $b_2$. Figures and movie were generated with Chimera\textsuperscript{84} and ChimeraX\textsuperscript{49}.

**Figure Captions**

**Figure 1. Overall structure of Bacillus PS3 ATP synthase.** A, Cartoon of ATP synthase. B, Cryo-EM map of ATP synthase with subunits coloured the same as the cartoon. C, Example map density that allowed construction of an atomic model. Scale bar, 30 Å.

**Figure 2. Rotational states of ATP synthase.** A, Atomic models of the three rotational states of Bacillus PS3 ATP synthase with subunits coloured the same as in Figure 1. B, Top view of the c-ring and subunit $a$ of the three rotational states from the cytoplasm when the F$_1$ regions of the three states are aligned. Rotation steps of the complex between states are ~3, 4, and 3 c-subunits. C, Comparison of the atomic models of subunits $b$, $\delta$, and other F$_1$ region subunits in the different rotational states. The $b$ subunits appear to be the most flexible part of the enzyme.

**Figure 3. Inhibition of ATP hydrolysis by subunit $\epsilon$.** A, Comparison of $\beta_{DP}$ (blue) with $\beta_E$ (pink, top) and $\beta_{T_P}$ (light purple, bottom). $\beta_{DP}$ is forced to adopt an open conformation by subunit $\epsilon$ (dashed box). B, Comparison of subunits $\gamma$ (pink) and $\epsilon$ (blue) of ATP synthases from Bacillus PS3 (top) and E. coli (bottom, PDB 3OAA\textsuperscript{17}). The dashed box shows additional interaction between subunits $\epsilon$ and $\gamma$ in the E. coli complex. C, Cross section through the catalytic F$_1$ region of the Bacillus PS3 ATP synthase. Subunit $\epsilon$ (pink) in the rotor is blocked from rotating in the direction of ATP hydrolysis (clockwise) by $\beta_{T_P}$ but is free to rotate in the direction of ATP synthesis (counterclockwise).

**Figure 4. F$_0$ region subunits and proton translocation in Bacillus PS3 ATP synthase.** A, Comparison of the F$_0$ regions from Bacillus PS3 (left) and S. cerevisiae (right). B, Cross sections through a surface representation of the F$_0$ region (simplified with rolling of a 1.4 Å sphere\textsuperscript{49}) show the cytoplasmic (left) and periplasmic (right) proton half-channels. C, Proton translocation pathway of Bacillus PS3 ATP synthase. During ATP synthesis, a proton enters the complex via the periplasmic half-channel, passing between $\alpha$-helices 5 and 6 of subunit $a$ to bind to the Glu 56 residue of a subunit $c$. The proton then rotates with the c-ring until it reaches the cytoplasmic half-channel formed between subunit $a$ and the c-ring. In the cytoplasmic half-channel, the
proton is released from the Glu residue due to its interaction with the positively charged Arg 169 of subunit a. A Glu 56 residue from each protomer of the c-ring is shown. D, Subunit a of Bacillus PS3 ATP synthase. Arg 169 is in purple, important residues for proton translocation identified by mutagenesis in E. coli ATP synthase are in pink, and other residues that appears to contribute to proton transfer in the cytosolic proton half-channel are in light purple.

Figure 1 - figure supplement 1. Bacillus PS3 ATP synthase purification. A, SDS-PAGE of GDN solubilized Bacillus PS3 ATP synthase. B, Size-exclusion chromatography of Bacillus PS3 ATP synthase.

Figure 1 - figure supplement 2. Cryo-EM image processing. A, Example micrograph of Bacillus PS3 ATP synthase. B, Representative 2D class averages of Bacillus PS3 ATP synthase. C, Image processing workflow.

Figure 1 - figure supplement 3. FSC, orientation distribution, and local resolution of the cryo-EM maps used to build atomic models. A to C, Full maps of Bacillus PS3 ATP synthase class 1, 2, and 3. D to F, Focused refinement maps of class 1, 2 and 3 including subunit δ and the F0 subunits. G, Focused refinement map of the membrane bound region only.

Figure 1 - figure supplement 4. Examples of atomic models from subunits built in the experimental cryo-EM maps.

Figure 1 - figure supplement 5. Overlay of Bacillus PS3 ATP synthase subunits ab2 with spinach chloroplast ATP synthase subunit ab′ PDB 6FKF15 (A) and E. coli ATP synthase subunits ab2 PDB 5T4Q25 (B).

Figure 4 - figure supplement 1. Comparison of subunit a structures from different organisms. A, Bacillus PS3 ATP synthase. B, S. cerevisiae mitochondrial ATP synthase (PDB 6B2Z12). C, S. oleracea chloroplast ATP synthase (PDB 6FKF15).

Figure 4 - figure supplement 2. Multiple sequence alignment of subunit a. Sequences are from bacteria (blue), mitochondria (pink), and chloroplast (black), including Bacillus PS3 (BACP3), E. coli (ECOLI), Mycobacterium tuberculosis (MYCTU), S. cerevisiae (YEAST), Homo sapiens (HUMAN), Bos taurus (BOVINE), and Spinacia oleracea (SPIOL). The functionally important Arg residues for proton translocation are in blue. Other important residues for proton translocation identified by mutagenesis in E. coli ATP synthase are in red. Strictly conserved residues are in bold. Figure was generated with ESPript 3.090.

Figure 4 - figure supplement 3. Positions of subunit b Tyr 13 and subunit a Gly 188. Side view of the F0 subunits. The residues of interest are inside the dashed box and are coloured in blue.

Video 1. Atomic models of the Bacillus PS3 ATP synthase in three rotational states.

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Figure 1
Figure 1 - figure supplement 1
Several rounds of ab-initio 3D classification and heterogeneous refinement

State 1  
405,432 particles

State 2  
314,448 particles

State 3  
175,694 particles

Missing peripheral stalk  
218,354 particles

Non-uniform refinement

Focused refinement

3.0 Å resolution  
3.0 Å resolution  
3.2 Å resolution

Masking

F1 region

Focused refinement

3.3 Å resolution  
895,574 particles

Composite map state 1

Figure 1 - figure supplement 2
Figure 1 - figure supplement 3
Figure 1 - figure supplement 4
Figure 1 - figure supplement 5
Figure 2

A  
Rotational state 1  
Rotational state 2  
Rotational state 3  

B  
Subunit $a$ and c-ring when $F_1$ aligned  

C  
State 1  
State 2  
State 3  
Subunit $b$  
Subunit $\delta$  

Figure 2
Figure 3
Figure 4
Figure 4 - figure supplement 1
Figure 4 - figure supplement 2
Figure 4 - figure supplement 3

subunit b
subunit a

bY13
aG188