

1 **F_O-F₁ coupling and symmetry mismatch in ATP synthase**
2 **resolved in every F_O rotation step**

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17

18 **Abstract**

19 The F_0F_1 ATP synthase, essential for cellular energy production, is composed of the F_0 and F_1 rotary
20 motors. While both F_0 and F_1 have pseudo-symmetric structures, their symmetries do not match. How
21 the symmetry mismatch is solved remains elusive due to missing intermediate structures of rotational
22 steps. Here, for ATP synthases with 3- and 10-fold symmetries in F_1 and F_0 , respectively, we
23 uncovered the mechanical couplings between F_0 and F_1 at every 36° rotation step via molecular
24 dynamics simulations and comparison of cryo-electron microscopy structures from three species. We
25 found that the frustration is shared by several elements. The F_1 stator partially rotates relative to the
26 F_0 stator via elastic distortion of the b-subunits. The rotor can be distorted. The c-ring rotary angles
27 can be deviated from symmetric ones. **Additionally, the F_1 motor may take non-canonical structures**
28 **relieving stronger frustration.** Together, we provide comprehensive understanding to solve the
29 symmetry mismatch.

30

31 **Key words:** F_0F_1 ATP synthase, Molecular motor, Molecular dynamics simulation

32

33 Introduction

34 Adenosine triphosphate (ATP) is mainly synthesized by the enzyme F_0F_1 ATP synthase¹⁻³. The
35 enzyme is composed of two rotary motors, the F_0 and F_1 motor, and these motors are connected by
36 two stalks, a central rotor and a peripheral stalk (Fig. 1a). The membrane-embedded F_0 motor rotation
37 driven by the proton motive force causes the central rotor rotation. The latter induces a series of
38 conformational changes in the F_1 motor, where ATP is synthesized from adenosine diphosphate
39 (ADP) and inorganic phosphate (Pi). The F_0F_1 ATP synthase is also known as a reversible machine,
40 which can hydrolyze ATP to pump protons against the proton motive force.

41 The F_1 motor contains the central rotor (γ/ϵ -subunit in bacterial F_1) and an $\alpha_3\beta_3$ hexamer made
42 of three $\alpha\beta$ dimers (Fig. 1a). Each $\alpha\beta$ dimer has catalytic sites for the ATP synthesis reaction and take
43 three distinct chemical states and conformations⁴, which are conventionally denoted as the ATP-
44 bound state (TP for brevity), the ADP-bound state (DP), and the empty state (E). Especially, the β -
45 subunit takes markedly different conformations among the three states; for the case of the *Bacillus*
46 PS3 ATP synthase, which will be our target in this study, its C-terminal segment takes a “closed”
47 conformation in the TP state, while it takes an “open” conformation in the DP and the E states⁵. Since
48 a single-molecule measurement showed that the F_1 motor in the ATP hydrolysis mode uncovered
49 120°-stepwise rotation of the central rotor, accompanied by progress of the chemical states in $\alpha_3\beta_3$ ⁶.
50 Thus, coupled with the nucleotide-dependent conformational change in the α/β -subunits, one round of
51 the F_1 motor results in the hydrolysis or synthesis of three ATP molecules, depending on the central
52 rotor direction. The F_0 motor is made of an a-subunit and a c-ring, which is a ring-shaped c-subunit
53 oligomer working as a rotor (Fig. 1a). The number of c-subunits in the c-ring varies between 8-17
54 depending on the species⁷⁻¹³; 10 subunits in the *Bacillus* PS3 ATP synthase. Each c-subunit has one
55 key proton-relaying residue. Single-molecule experiments for *Escherichia coli* ATP synthase (10
56 subunits) identified 36° stepwise c-ring rotations¹⁴. Thus, one round of the F_0 motor was driven by the
57 transfer of the same number of protons as that of c-subunits.

58 In contrast to the mechanisms of each motor, the mechanism of the coupling between the F_0
59 and F_1 motors is less clear. Because the number of c-subunits in the c-ring is not a multiple of three in

60 most cases, the mean number of protons necessary for the synthesis of one ATP is not an integer
61 value, which is sometimes called a symmetry mismatch. There has been much debate regarding the
62 mechanism by which F_0F_1 ATP synthase resolves this mismatch¹⁵. For *Bacillus* PS3 ATP synthases,
63 the two motors are connected via a central rotor made of $c_{10}\gamma\epsilon$ and a peripheral stalk made of $b_2\delta$ (Fig.
64 1a). Some early studies suggested the elastic distortion of the central rotor, γ -subunit¹⁶⁻¹⁸. Other
65 studies have anticipated the role of δ ¹⁹. Recent cryo-EM structures of mitochondria and *Bacillus* PS3
66 ATP synthases point to distortion in b-subunits^{5,20}. From recent cryo-EM studies, the symmetric
67 mismatch and the relatively rigid rotor together imply that the $\alpha_3\beta_3$ hexamer rotate relative to the a-
68 subunit^{19,21}. Sobti *et al.* resolved three different rotational states and one sub-state in *E. coli* ATP
69 synthase. The sub-state structure essentially had the same F_1 configuration as one of the three state
70 structures, but the c_{10} -ring together with the F_1 stator, $c_{10}\alpha_3\beta_3\gamma\epsilon$, rotated relative to the F_0 stator.
71 Comparing individual conformations, we see that the b-subunits have significantly different
72 conformations. Therefore, the existence of the sub-state and the flexibility of the b-subunit may be
73 necessary for resolving the symmetry mismatch. Notably, however, the cryo-EM models in Sobti *et*
74 *al.* include structural models for four c_{10} -ring rotation states out of ten possible rotational states.
75 Perhaps other states are more fragile and/or more short-lived, so that high-resolution models could not
76 be built. Moreover, cryo-EM models provide static snapshots, as usual; for example, how the sub-
77 state arises, how the b-subunit distortion resolves the mismatch, and when and how the F_1 -motor
78 undergoes structural changes in response to the c-ring rotation remain unclear.

79 In this study, to address the F_0 - F_1 coupling and the molecular mechanisms to solve the
80 symmetry mismatch, we performed molecular dynamics (MD) simulations that mimic the ATP
81 synthesis dynamics for one round of rotation in the holo-complex of the *Bacillus* F_0F_1 ATP synthesis.
82 While many molecular simulations have been reported so far for each of the F_0 and F_1 motors^{18,22-28},
83 to the best of our knowledge, this study provides MD simulations for the first time for one round of
84 ATP synthase holo-complex. Our dynamic simulations showed the order and timing of the structural
85 changes in the three $\alpha\beta$ -pairs in the F_1 motor during ten 36° -rotation steps of the F_0 motor.
86 Furthermore, relaxation simulations with a fixed c-ring rotation angle in every 36° -step exhibited the
87 c-ring-dependent partial rotation of the F_1 stator relative to the F_0 stator and some twists in the rotor.

88 Then, motivated by the simulation results, we conducted a comparative analysis of cryo-EM
89 structures from three species, accounting for the structural changes in the ATP synthases. Together,
90 we reveal how to solve the symmetry mismatch in F₀F₁ ATP synthesis in unprecedented detail.

91

92 Results and Discussion

93 Simulating the rotation dynamics in the ATP synthase holo-complex

94 The *Bacillus* PS3 ATP synthase cryo-EM study provides holo-complex structure models in the three
95 rotational states: A (PDB ID: 6N2Y, Fig. 1a left), B (PDB ID: 6N2Z), and C (PDB ID: 6N30)⁵. In all
96 of these holo-complex models, F₁ was in the catalytic dwell. We introduce the numbering of the three
97 αβ pairs, αβ1, αβ2, and αβ3, counterclockwise starting from the α/β-subunits furthest from the b-
98 subunit (Fig. 1a, middle). In the A state, αβ1, αβ2, and αβ3 are in the E, TP, and DP states,
99 respectively. The structural change from A to B (the AB process) involves the counterclockwise
100 rotation of c₁₀-ring by 3×36°, together with the progress of the chemical steps in the three αβ pairs;
101 αβ1, αβ2, and αβ3 change to the DP, E, and TP states, respectively. Similarly, the transitions from B
102 to C (the BC process) and from C to A (the CA process) involve rotations by four and three 36° c-ring
103 rotation steps, respectively, coupled with the chemical state changes in αβ pairs. We call this whole
104 process the 3-4-3 pathway, assuming that the entire process starts and ends in the A state.

105 Given the 3-4-3 pathway in the three cryo-EM rotational states, we envisioned to simulate the
106 process that the F₁ motor in the A state would reach the B state when the F₀ c₁₀-ring rotates
107 counterclockwise by 3×36°. To simulate the conformational change in the AB process, we need to
108 model in a way that each αβ pair can accommodate both A and B state conformations. For this
109 purpose, we employed a double-basin model that encodes A and B state conformations separately for
110 each αβ system (see Methods for details). The double-basin model concisely builds two distinct
111 conformational basins controlling both the energy barrier in between and the relative stabilities of the
112 two basins²⁹⁻³¹ (Fig. 1b). Also, for the BC and CA processes, introducing the double-basin models that

113 connect the B and C states and that connect the C and A states, we expected to observe the respective
114 state transitions in the F_1 motor while the c_{10} -ring rotates $4 \times 36^\circ$ and $3 \times 36^\circ$ degrees, respectively (Fig.
115 1c). To ensure the energetics of the ATP synthesis process, we set the summation of the relative
116 stabilities of the three $\alpha\beta$ double-basin models in the AB, BC, and CA processes to $\sim +36$ kcal, which
117 approximates the free energy increase for the synthesis of 3 ATP molecules from ADP and Pi.

118 In the simulations, we rotated the c_{10} -ring by 36° with a constant angular velocity over 10^6
119 MD steps, followed by a c_{10} -ring pause for 9×10^6 MD steps. Note that 10^4 MD steps were denoted as
120 one frame. For each case, we repeated the same simulations ten times with different stochastic forces.
121 The structural transitions in each $\alpha\beta$ are monitored by its reaction coordinate χ , which takes negative
122 and positive values when $\alpha\beta$ is in the pre- and post- states in the double-basin system, respectively.
123 For the AB process, we repeated this rotation step four times (the upper c-ring panel of Fig. 2ab). For
124 the BC and CA processes, we began with 10×10^6 MD equilibration steps in the initial c_{10} -ring angle
125 and then repeated the rotation step four times (the upper c-ring panel of Fig. 2cd and Fig. 2e,
126 respectively).

127 How many F_0 c-ring rotation steps are necessary to induce the F_1
128 nucleotide state transition?

129 We monitored the structural changes in each $\alpha\beta$ (Fig. 2, χ pre \rightarrow post) as well as other structures.
130 When all three χ 's in F_1 changed from negative to positive values, we regarded F_1 to have completed
131 its transition. Fig. 2a shows a representative trajectory of the AB process: (1) The $\alpha\beta 1$ started
132 transitions from the E state to the DP state at the ~ 100 th frame immediately after the c-ring rotated
133 36° , followed by rapid transitions back and forth. (2) The $\alpha\beta 3$ made a transition from the DP to the TP
134 state at the ~ 2200 th frame when the c-ring completed the third 36° rotation. (3) Finally, $\alpha\beta 2$ transitioned
135 from the TP to the E state at the ~ 2500 th frame, corresponding to the dissociation of a synthesized
136 ATP molecule. At this stage, all three $\alpha\beta$ pairs completed their transitions (blue triangle in the figure).
137 This was within the third rotation step. Thus, in this trajectory, three c-ring rotation steps induced a
138 complete structural transition in F_1 during the AB process. Of the ten repeated trajectories, six showed

139 similar results; three c-ring rotation steps induced complete F_1 transitions. In the two trajectories, four
140 c-ring rotation steps were necessary to induce F_1 transitions (one case depicted in Fig. 2b). The
141 remaining two did not complete the F_1 transition at the end of the simulations (Supplementary Fig.
142 1a). Thus, as expected from the cryo-EM structures, we observed that in the majority of cases, the F_1
143 structural transitions in the AB process were completed with the three 36° -c-ring-rotation steps.

144 Next, we describe the BC process. For this process, we first took the 3000th frame snapshot in
145 the representative trajectory of the AB process (shown in Fig. 2a) as the initial structure. Fig. 2c
146 shows the typical trajectory of the BC process. In this trajectory, the $\alpha\beta 1$ and $\alpha\beta 2$ pairs exhibited
147 unstable fluctuations until $\alpha\beta 3$ transitioned from the TP to the E state at the ~ 4200 th frame. Once $\alpha\beta 3$
148 is in the E state, both $\alpha\beta 1$ and $\alpha\beta 2$ are stabilized in the TP and DP states. The overall structural change
149 in F_1 was completed after the fourth c_{10} -ring rotation step. All trajectories which complete
150 conformational changes (four out of ten) were completed with four c-ring 36° -rotation steps
151 (Supplementary Fig. 1b). Therefore, we conclude that four 36° -rotation steps are necessary to induce
152 complete structural changes in F_1 in the BC process with this initial structure. We mentioned about the
153 BC process on the 4-3-3 pathway in Supplementary Text 1.

154 Finally, the CA process is simulated. First, we started the simulation with the 5000th frame
155 snapshot of the representative BC process trajectory in the 3-4-3 pathway. Fig. 2e shows a typical
156 trajectory (Supplementary Fig 1d). Initially, $\alpha\beta 3$ gradually changed from the E to the DP state
157 between the 200th and 900th frames. Then, $\alpha\beta 1$ made the transitions from the TP to the E state and
158 settled down in the E state at the ~ 3100 th frame right when the third c-ring rotation step in the CA
159 process was over. This is quickly followed by the transition in $\alpha\beta 2$ from the DP to the TP state around
160 the 3300th frame when the overall conformational change in this process was over. The F_1 transition
161 was completed within the third 36° -c-ring-rotation step in six of the ten trajectories tested. The F_1
162 transition was completed within the second and fourth 36° -rotation steps, each in one trajectory. The
163 remaining two trajectories did not exhibit complete structural changes. Therefore, the dominant
164 pathway in the CA process completes the F_1 state transitions by the three c-ring rotation steps. We
165 mentioned about the CA process on the 4-3-3 pathway in Supplementary Text 1 and Fig. 2bd.

166 Altogether, our simulation predominantly showed the 3-4-3 pathway, which is in harmony
167 with the cryo-EM studies. Additionally, we noticed a common feature as suggested in a classic
168 work^{1,32}: Of the three F_1 $\alpha\beta$ structure transitions in each process, the $\alpha\beta$ that changes from the TP to
169 the E state is a bottleneck that tends to decide the number of necessary 36° -rotation steps. ATP
170 synthesis in this enzyme has the rate-limiting process of the product dissociation.

171

172 Partial rotation of the F_1 stator accompanied by the b-subunits 173 bending

174 Recent cryo-EM studies by Murphy *et al.*¹⁹ and Sobti *et al.*²¹ reported more than three rotational states
175 for ATP synthases from *Polytomella* sp. and *E. coli*, respectively, both of which contain a c_{10} -ring. In
176 both cases, they found varying degrees of rotation of the F_1 stator relative to the F_0 stator, raising the
177 possibility of a partial rotation of the F_1 stator as a mean to solve the symmetry mismatch. However,
178 these structural models contain only a limited c-ring rotation step. How was the symmetry mismatch
179 solved in every 36° -rotation step? To address this point, we calculated the rotary angle θ of the F_1
180 stator relative to the F_0 stator during the trajectories (Fig. 3a-e); here, the F_1 stator and the F_0 stator
181 mean F_1 $\alpha_3\beta_3$ and F_0 a-subunit, respectively. For this purpose, we fixed the rotation axis of the F_0 c_{10} -
182 ring to the z-axis and F_0 a-subunit to the positive x-axis and monitored the rotary angle θ of F_1 $\alpha_3\beta_3$
183 around the z-axis (further details in the Methods). Fig. 3a-e plot the time courses of θ for the same
184 five trajectories as in Fig. 2a-e.

185 First, we examined the F_1 stator angle θ of the AB process (Fig. 3ab). The F_1 stator started to
186 rotate $\sim 20^\circ$ counterclockwise dragged by the $c_{10}\gamma\varepsilon$ rotation. In addition, the γ -subunit is distorted \sim
187 10° (the fourth panel of Fig. 3a). When the c-ring rotates counterclockwise, the rotation up to 10°
188 tends to be absorbed by the distortion of the γ -subunit. In the subsequence process within the AB
189 process in the 3-4-3 pathway (Fig. 3a), the F_1 stator tends to return to the initial angle. Importantly,
190 the return of the F_1 stator angle occurred together with the structural transition in F_1 . Once F_1 adopts

191 conformations compatible with the cryo-EM structure of the B state, the F_1 stator can accommodate
192 the angle $\theta \sim 0$ (Fig. 3f, left).

193 Next, we analyzed the simulation trajectories of the BC process in the 3-4-3 pathway (Fig.
194 3c). When the c_{10} -ring started to rotate at the 1000th frame (the fourth cumulative c-ring rotation step
195 is denoted as $n = 4$), the F_1 stator is dragged by the rotor rotation, similarly to the above case ($n = 1$).
196 At the 1000th frame, while the c_{10} -ring rotated by 36° , the F_1 stator rotated by $\sim 20^\circ$. However, in
197 sharp contrast to the above AB process, the F_1 stator angle never returned to the initial angle ($\sim 0^\circ$)
198 until the simulation ended ($n = 5 - 7$) (Fig. 3f, right). At the end (the 5000th frame), the F_1 stator
199 was rotated by $\sim 20^\circ$. At this stage, the accumulated rotation in the c_{10} -ring was $7 \times 36^\circ$, counting from
200 the A state, whereas the F_1 motor made two progressive transitions rotating the chemical states by
201 $2 \times 120^\circ$. The difference in the rotation angle, $7 \times 36^\circ - 2 \times 120^\circ = 12^\circ$, may be adsorbed by the
202 counterclockwise rotation of the F_1 stator.

203 Notably, the rotation of the F_1 stator in the BC process is accompanied by a large distortion of
204 the b-subunit (the right-end extrusion in Fig. 3f right), in which a part that is close to F_1 is rotated
205 counterclockwise, whereas a part of the b-subunit bound to the F_0 a-subunit remains in the original
206 position. The distortion of the b-subunit is a passive conformational change. Owing to this distortion,
207 elastic energy is charged into the b-subunit. We propose that the presence of the b-subunit, in addition
208 to the balance of rotation angles discussed later, is the reason why the BC process requires more c-
209 subunits rotation than other processes (Supplementary Text 2). To quantify the motion of the b-
210 subunit, we performed principal component analysis (PCA) on the b-subunit; the first principal
211 component (PC1) in the AB process represents a counterclockwise tilt along the F_1 stator (Fig. 3g,
212 left). The time course of the PC1 value (the third panel in Fig. 3a) indicates that it changes closely in
213 parallel with the F_1 stator angle θ . Note that a positive PC1 indicates a counterclockwise distortion of
214 the b-subunit (the direction of the arrow in Fig. 3g).

215 Third, we examined the CA process of the 3-4-3 pathway. As shown in Fig. 3e panel, we
216 observed a clockwise rotation of the F_1 stator back to the initial angle. The b-subunit also followed
217 clockwise tilting back to the relaxed structure in the A state (third panel in Fig. 3e). Therefore, it can

218 be said that the CA process resolves the distortion caused by the counterclockwise rotation
219 accumulated in the AB and BC processes. Lastly, we also mentioned the rotation of F₁ stator for the
220 4-3-3 pathway in [Supplementary Text 1](#).

221

222 Relaxation simulations with the fixed c₁₀-ring rotary angles

223 While these simulations provided dynamic view to resolve the symmetry mismatch, the obtained
224 structures are inherently transient due to a relatively fast rotation of c₁₀-ring. To obtain well-
225 equilibrated structures at every 36°-c₁₀-ring-rotation steps, which were not seen by cryo-EM studies,
226 we conducted further MD simulations of relaxation. We fixed the c₁₀-ring rotation step at $n \times 36^\circ$ -
227 rotation steps for $n = 1 \sim 10$ and conducted further 2000-frame simulations ten times with the initial
228 structure taken from the $n \times 1000$ th frame snapshots in the previous trajectories.

229 We found a clear trend in the AB process. For $n = 1$, we started the simulations from the
230 1000th frame snapshot of the representative trajectory ([the red curve in Fig. 4a](#)), as well as from the
231 trajectory in which the F₁ rotated the least amount at the 1000th frame among ([the blue curve in Fig.](#)
232 [4a](#)). Both sets of simulations steadily showed counterclockwise rotations of the F₁ stator with varying
233 degrees of rotation angles up to $\sim 30^\circ$ ([Fig. 4b, panel 1](#)). Thus, our simulation models gave consistent
234 results with recent cryo-EM studies¹⁹. We also monitored the distortion of rotor c₁₀γε ([Supplementary](#)
235 [Fig. 3](#)). In the $n = 2$ state, the F₁ stator was rotated $\sim 20^\circ$, and then rotated clockwise to $\sim -10^\circ$ in the
236 c-ring state $n = 3$. Although the angle of the initial structure in $n = 3$ had a much more
237 counterclockwise rotated, we found its clockwise rotation back to $\sim 0^\circ$ during the relaxation simulation
238 ([the left-side green curve in Fig. 4a](#)). Thus, the clockwise return of the F₁ stator at $n = 3$ is a robust
239 process.

240 In the BC process, we observed similar but not identical trends. In the c₁₀-ring state $n = 4$, the
241 F₁ stator rotated counterclockwise by $\sim 20^\circ$, which is very similar to the c₁₀-ring state $n = 1$. This
242 angle was maintained in the c-ring states $n = 5$ and 6. Particularly, in the $n = 6$ state, we also
243 observed marked, albeit incomplete, structural transitions in the F₁ motor. [Supplementary Fig. 4](#)

244 shows that $\alpha\beta 3$ exhibited irreversible and bottleneck transitions from the TP to E states in four of the
245 ten trajectories. In the c-ring state $n = 7$, which is the final state in the BC process, the F_1 stator is
246 slightly, but not completely, turned to reach $\sim +10^\circ$. A simple estimation may help our understanding:
247 the c-ring rotated by $7 \times 36^\circ$ from the A state, while the F_1 motor rotated by $2 \times 120^\circ$. The difference
248 was 12° .

249 Finally, in the CA process, we found the result to be somewhat similar to that of the AB
250 process (Fig. 4d 8-10). In the c_{10} -ring state $n = 8$, the F_1 stator rotated counterclockwise by $\sim 30^\circ$,
251 similar to the c_{10} -ring state $n = 1$. We found that the rotor was significantly distorted at $n = 8$, which
252 is different from the $n = 1$ case. The F_1 stator slightly rotated back but remained rotated at $\sim 10^\circ$ in the
253 c-ring state $n = 9$. Finally, after the full round, the F_1 stator settled down at the original 0° -angle. To
254 check if the settled down was robustness, we simulated from another initial state in which the F_1 stator
255 rotated 20° (the right-side green curve in Fig. 4a). As we expected, we found the same settled down,
256 therefore, we conclude that the F_1 stator settles down to $\sim 0^\circ$ when the system is fully relaxed after one
257 round of the c_{10} -ring.

258 In summary, we see that the F_1 stator constantly rotates back and forth, dragged by the c_{10} -
259 ring rotation and by the chemical state change in F_1 , but eventually returns to its initial position after
260 the full round. We have discussed the angle change from a typical trajectory, and in the next section,
261 we will discuss it using more statistical values.

262

263 On the symmetry mismatch between F_O and F_1

264 The stepwise rotations of the F_O c_{10} -ring and F_1 motor shown in Fig. 5a (upper panel) illustrate the
265 symmetry mismatch. By symmetry, the ideal elementary rotation steps of the c_{10} -ring and F_1 motor
266 were 36° and 120° , respectively. We assume that the c_{10} -ring and the F_1 motor rotation steps coincided
267 at the ground state angle, the A state, chosen as the angle 0° (this is an approximation, but it turned out
268 reasonable). Then, the 120° -step of the F_1 motor was flanked by $3 \times 36^\circ = 108^\circ$ and $4 \times 36^\circ = 144^\circ$ of
269 the c_{10} -ring steps with 108° -step closer. It is reasonable to assume that a smaller deviation in the angle
270 corresponds to a lower energy of frustration. Thus, the $3 \times 36^\circ = 108^\circ$ step of the c_{10} -ring may be

271 realized to accommodate the F_1 motor 120° -state. In the same way, the 240° -step of the F_1 motor was
272 flanked by $6 \times 36^\circ = 216^\circ$ and $7 \times 36^\circ = 252^\circ$ of the c_{10} -ring steps with 252° -step closer. Obviously, the
273 360° -step was realized both by the c-ring and the F_1 motor without frustration, which led to the 3-4-3
274 pathway, consistent with all three cryo-EM studies. This minimal-deviation-rule directly provides the
275 simplest reasoning for the 3-4-3 pathway. The same rule may be applicable to c-rings composed of
276 subunits other than ten (Fig. 5b). The c_8 -ring, the c_9 -ring, and the c_{11} -ring may exhibit the 3-2-3, 3-3-3,
277 and 4-3-4 pathways starting from the ground state.

278 The next question is how to resolve the mismatch between the F_O c_{10} -ring and the F_1 motor in
279 the primary rotation states B ($n = 3$) and C ($n = 7$) in the 3-4-3 pathway, namely the difference
280 between 120° and 108° in the B state and the difference between 240° and 252° in the C state.
281 Structures provided by Guo *et al.* suggested that the mismatch of the range $\sim 12^\circ$ can mostly be
282 compensated by the rotation of the F_1 stator via the distortion of the b-subunit (Table 1, Fig. 5c, and
283 Supplementary Text 3).

284 The mismatch between the F_O c_{10} -ring and the F_1 motor increases after the 36° c-ring rotation
285 from the primary states A, B, and C, namely the rotation states $n = 1, 4$, and 8. This range of
286 mismatches cannot be absorbed by the F_1 stator rotation. Instead, it is realized by the combinations of
287 the three types of elastic structural changes in the structures of Sobti *et al.* and Murphy *et al.* (Fig. 5c
288 and Supplementary Text 3): a) the b-subunits accommodate $\pm(7 - 14)^\circ$, which appears as the
289 rotation of the F_1 stator, b) the c_{10} -ring rotation deviates $\pm(11 - 13)^\circ$ from the ideal angles, and c) the
290 rotor distortion is in the range of $\pm(4 - 11)^\circ$. In contrast, the F_1 motor showed a fluctuation of only
291 $\sim \pm 2^\circ$. It is a key feature that the F_1 motor maintains the canonical angle with slight fluctuations in
292 all cryo-EM structures. The corresponding angles monitored in our MD simulations are consistent
293 with these experiments (Fig. 5a, the second and third panels), except that the MD simulations
294 assumed uniform 36° -rotation steps of the c_{10} -ring, which is not rigorously the case in cryo-EM
295 structures.

296 In all the AB, BC, and CA processes, the first 36° -c-ring-rotation step from the primary
297 rotation states induces the counterclockwise rotation of the F_1 stator ($n = 1, 4, 8$). This is in agreement

298 with the findings of Murphy *et al.*¹⁹. The second 36°-c-ring-rotation step tends to keep the F₁ stator
299 rotated, which is often accompanied by significant distortion of the rotor and incomplete transitions in
300 the F₁ motor ($n = 2, 5, 9$). The F₁ motor may take structures different from canonical three-fold rotary
301 states. These are strongly frustrated states and are likely too dynamic to be modeled at high resolution
302 in cryo-EM studies. The simulations showed relatively diverse configurations. Here, we suggest an
303 interesting scenario: In some of these states, the F₁ motor may utilize the 80° sub-step to the so-called
304 binding-dwell. Namely, the 80° sub-step of the F₁ motor is close to $2 \times 36^\circ = 72^\circ$ step of c₁₀-ring, the
305 200° sub-step is close to both $5 \times 36^\circ = 180^\circ$ and $6 \times 36^\circ = 216^\circ$ of c₁₀-ring, and the 320° sub-step of the F₁
306 motor is close to $9 \times 36^\circ = 324^\circ$ step of c₁₀-ring, which may serve to relieve the highly frustrated
307 energies. In the last 36°-c-ring-rotation step of the AB, BC, and CA processes, the F₁ stator finally
308 rotates clockwise back to near the original angle ($n = 3, 7, 10$); and the final angles differ in the three
309 processes: $\sim -10^\circ$, $\sim +10^\circ$, and $\sim 0^\circ$ for the AB, BC, and CA processes, respectively.

310

311 Conclusions

312 Using the recently obtained cryo-EM structures of the *Bacillus* PS3 F₀F₁ ATP synthases, we carried
313 out MD simulations of the holo-complex that mimicked three cycles of ATP synthesis, the AB, BC,
314 and CA processes, and one round of rotor rotation. We found that the AB, BC, and CA processes
315 completed the respective structural changes in F₁ with the highest probabilities when the c₁₀-ring
316 made three, four, and three 36°-rotation steps, which is consistent with the experimental results. At all
317 ten 36°-step of c₁₀-ring rotations, we investigated the holo-complex structural changes that resolve the
318 symmetry mismatch between F₀ and F₁. The symmetry mismatch was resolved by the distortion of a
319 few parts. First, the b-subunit distortion led to the rotation of the F₁ stator back and forth relative to
320 the F₀ stator. Second, the rotor itself is distorted to a lesser extent. Third, the comparative analysis of
321 cryo-EM structures from the three species showed that the c-ring rotary angles can be deviated from
322 symmetric ones. Since the movement of the β-subunit in αβ₂ is suppressed by the b-subunit and ε-
323 subunit, a stronger torque is required to overcome this barrier during the BC process. Simulation

324 results, together with the comparative analysis of recent cryo-EM structure models, reveal molecular
325 reasoning to resolve the symmetry mismatch.

326 Since we adopted a simple approach to simulates rotary motions of the ATP synthase holo-
327 complex, it has some limitations. First, since we used a classical MD with a coarse-grained molecular
328 representation, the chemical reaction itself was only dealt with conformational changes correlated
329 with chemical reactions. Second, since we adopted a predefined time course of c_{10} -ring rotation, we
330 have not been able to reproduce the effects of stochastic proton transport. Third, since some parts of
331 the b-subunit around δ -subunit was missing in cryo-EM models, we cannot deny the possibility that
332 this missing region contribute to the conformational change. Despite these limitations, our MD
333 simulations revealed the mechanical coupling between F_1 and F_O with reasonable accuracy in detail,
334 which is sufficient to fully explain the solution of the symmetry mismatch in ATP synthases.

335

336 Materials & Methods

337 Model building

338 We used the *Bacillus* PS3 ATP synthase structure model for the A state (Protein Data Bank (PDB)
339 ID, 6N2Y) obtained by cryo-EM as the reference structure for the corresponding A state in our MD
340 simulations⁵. The model consists of eight proteins and 22 subunits, $\alpha_2\beta_3\gamma\delta\epsilon$. Based on the PDB
341 structure, we modeled loops for the missing regions in the α -subunit using MODELLER³³. For the
342 sake of structural symmetry, the range of residues used for three α - and β -subunits was made the
343 same: I8 to S501 for α -subunits and T2 to M469 for β -subunits. In addition, one of the β -subunits
344 (called β_2 in the original PDB data) contains a C-terminal region that is neither modeled nor has a
345 sequence assigned. This region was excluded from the simulation system. We established the
346 coordinate system such that the center of masses of the F_0 c_{10} -ring was set at its original point, the
347 rotation axis of the c_{10} -ring coincided with the z -axis, and R169 of the F_0 α -subunit was on the x -axis.

348 We also prepared reference structure information for B and C states. Notably, we did not use
349 the structural models given in the PDB for these states. Instead, we mostly used the structure
350 information for the A state because we assumed that the A state was the ground-state form. In the
351 structure-based simulation, the reference structure for each subunit was used to define the lowest
352 energy state of the subunit, except for the structures of $\alpha_3\beta_3$. Assuming a change in the chemical state
353 and the corresponding change in the stable conformation, we repositioned the reference structures in a
354 rotary manner. For example, the reference structure information of $\alpha\beta_1$ in the B state, which is in the
355 DP state, was copied from the reference structure of $\alpha\beta_3$ in the A state, having the same DP state.
356 Similarly, all reference structure information could be obtained from those in the A state.

357

358

359 MD simulation setting

360 We performed coarse-grained MD simulations using the reference structure described above. In the
361 coarse-grained representation, one amino acid was treated as one particle located at the $C\alpha$ position.
362 We primarily used the energy function AICG2+^{34,35}, which has been intensively used in simulations of
363 large molecular complexes^{25,36,37}. In this function, the reference structure was assumed to be the most
364 stable conformation, and many parameters inside were determined from the atomic interactions in the
365 all-atom reference structures (detail in [Supplementary Text 4](#)).

366 In this study, to investigate the mechanical coupling between F_O and F_1 parts, we designed a
367 minimal setup that mimicked ATP synthesis reactions induced by the c-ring rotation driven by the
368 proton-motive-force in a simple design. Assuming the c-ring rotation driven by the proton-motive
369 force, we made the c-ring rotate with a predefined time course. In the F_1 part, for each $\alpha\beta$ pair that
370 sandwiches the ATP catalytic site, we set multiple basins that encode ATP-bound (TP), ADP-bound
371 (DP), and empty (E) state conformations. When the state transition settled to the new state, we
372 considered that the corresponding chemical event occurred (detail in [Supplementary Text 5](#)).

373 In our minimal design, the F_O a-subunit was a rigid part of the F_O stator and was fixed to the
374 initial structure and position. The F_O c_{10} -ring was treated as a rigid body and rotated by design around
375 the z-axis with a predefined schedule.

376 With these setups, we ran simulations on several processes: AB, BC, and CA. First, in the AB
377 process, the simulation started from the reference structure built from PDB ID: 6N2Y. In the
378 simulation, the c_{10} -ring rotated stepwise 36° by 36° as designed. The coupling between F_O and F_1 led
379 the three double-basin systems of the F_1 -motor to exhibit their conformations and reach the post-state
380 (state B). Then, we selected a representative snapshot from the trajectory and treated it as the initial
381 model for the subsequent simulation of the BC process. The simulation of the BC process began with
382 this model. After three or four c-ring 36° -rotation steps, we also picked a representative snapshot of
383 the initial structure of the CA process. Finally, we performed a CA simulation. In each simulation, we
384 repeated 10 MD runs with different stochastic forces using CafeMol version 2.1³⁸. Unless otherwise
385 noted, we took 4×10^7 MD steps, 5×10^7 MD steps, and 5×10^7 MD steps for the AB, BC, and CA

386 processes, respectively. We used underdamped Langevin dynamics at 323 K temperature and set the
387 friction coefficient to 2.0 (CafeMol unit); default values were used for the others.

388 Data Availability

389 The cryo-EM structures used in this paper are available download from the Protein Data Bank under
390 PDB IDs 6N2Y, 6N2Z, and 6N30 for the *Bacillus* PS3 ATP synthase; 6WNQ, 6OQV, 6OQR, 6OQS,
391 6OQT, 6OQU, 6PQV, 6OQW, and 6WNR for the *E. coli* ATP synthase; 6RDH, 6RDW, 6RDZ,
392 6RE8, 6REB, and 6RES for the *Polytomella* sp. ATP synthase.

393

394 Code Availability

395 All MD simulations in this paper was performed by CafeMol software. It can be downloaded from
396 <https://www.cafemol.org>.

397

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493

494 Author Contributions

495 S.K. and S.T. conceived and designed the project; T.N. developed the simulation code; S.K.
496 performed the simulations; S.K. and T.N. analyzed the data; S.K. assembled figures, and all authors
497 discussed the results and were involved in the manuscript writing process.

498

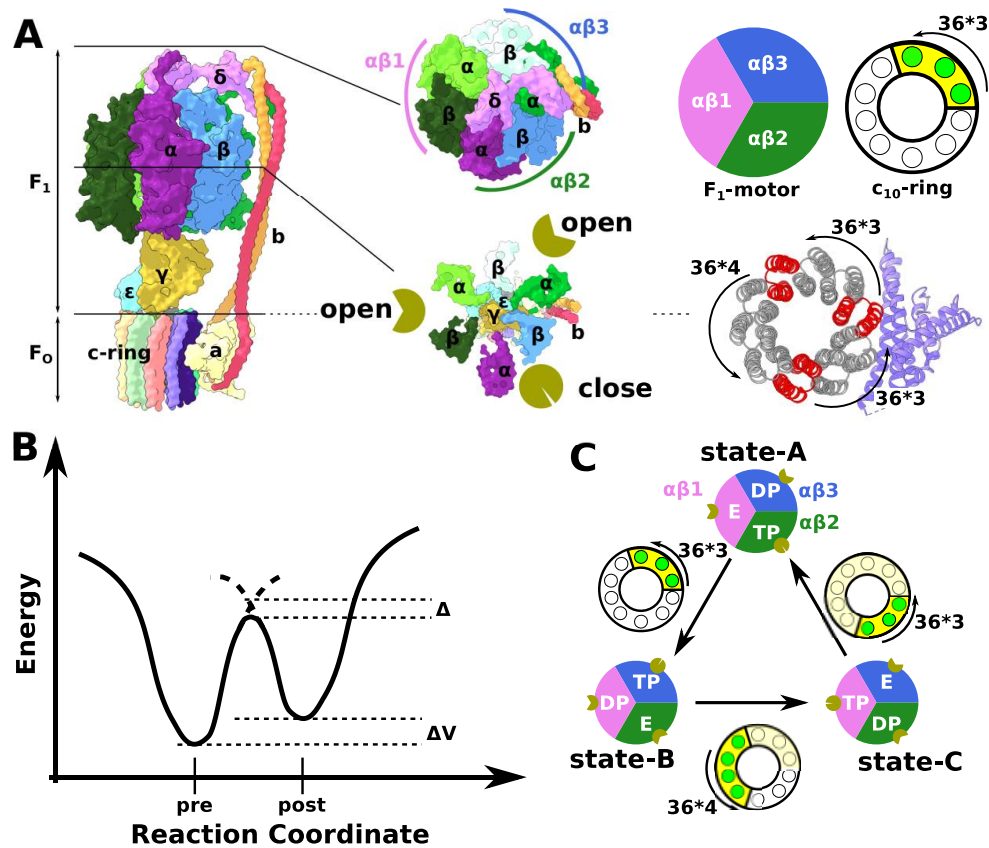
499 Competing Interests statement

500 The authors declare no competing interests.

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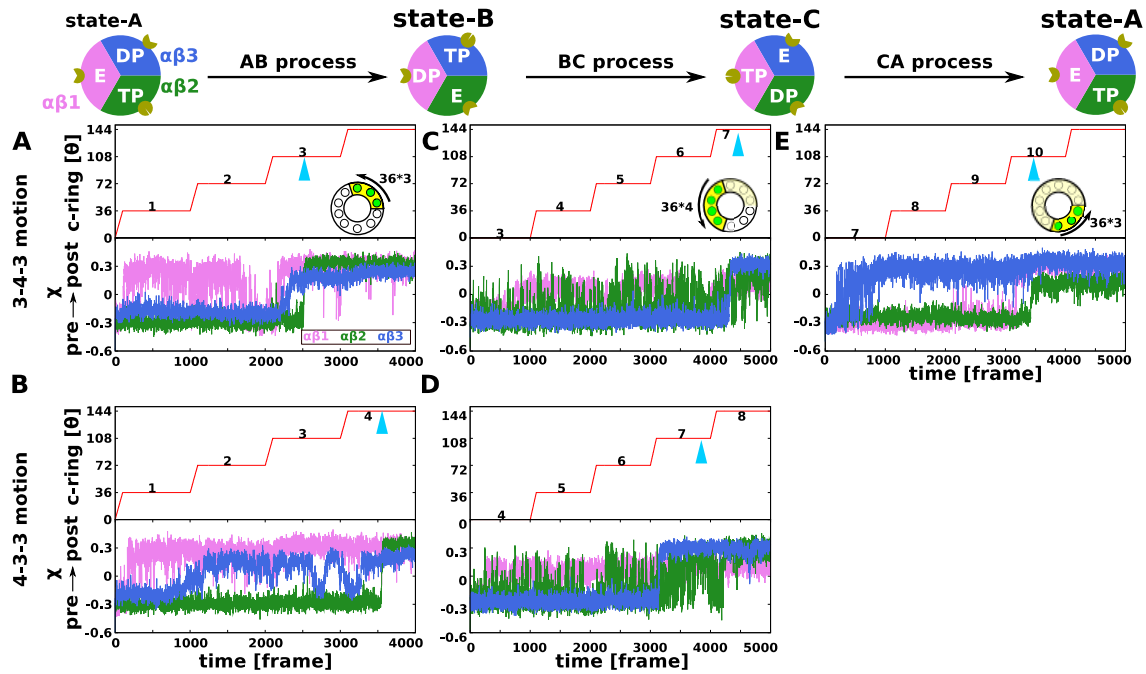


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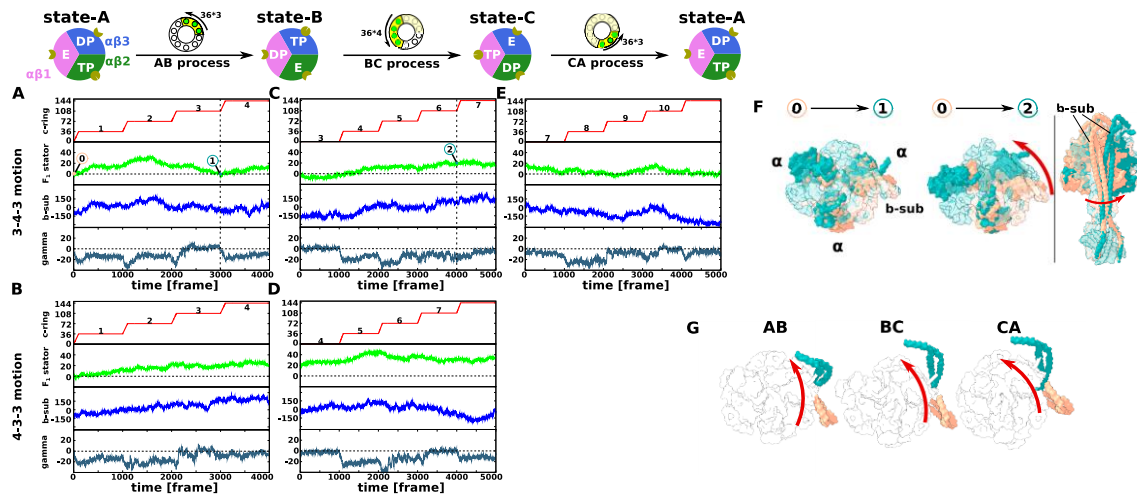
505 **Fig. 1 Structure of F₀F₁ ATP synthase and the simulation system.**

506 a. (Leftmost) The structure of *Bacillus* PS3 F₀F₁ ATP synthase holo-complex (the A state, PDB ID:
 507 6N2Y). The F₁ motor (top half) contains δ : pink; $\alpha_3\beta_3$ hexamer made of three $\alpha\beta$ pairs, $\alpha\beta_1$: lime
 508 green and green; $\alpha\beta_2$: violet and sky blue; $\alpha\beta_3$: yellow green and white; γ : dark yellow; ϵ : cyan. The
 509 F₀ motor contains c₁₀-ring: pastel-colored barrel-shaped objects; a-subunit: pale yellow; b-subunits:
 510 pale red and orange. In the 6N2Y structure, $\alpha\beta_1$, $\alpha\beta_2$, and $\alpha\beta_3$ take the E, TP, and DP states,
 511 respectively, and these take the open, closed, and open structures, respectively indicated by golden pie
 512 chart pictures. (Right) Guo *et al.* built three different ATP synthase conformations: A (PDB ID:
 513 6N2Y), B (6N2Z), and C (6N30). The structure differences from A to B (AB), B to C (BC), and C to
 514 A (CA) contain three, four, and three 36°-c₁₀-ring-positional-rotation in a counterclockwise direction,
 515 respectively. The red c-subunits indicate the ones closest to the a-subunit in the A, B, and C states. **b.**
 516 The schematic view of the double-basin model. "pre" and "post" indicate the minimum energy
 517 structures for the pre- and post-structures, respectively. Δ and ΔV are parameters to control the barrier

518 height and relative stability between two minima. **c.** The conformational change cycle in the ATP
 519 synthesis mode.
 520



521
 522 **Fig. 2 Structure change in the F₁ motor during MD simulations of one round c₁₀-ring rotations.**
 523 **a-e.** Representative MD simulation trajectories for the AB (panel A), BC (panel C), and CA (panel E)
 524 processes in the 3-4-3 pathway, and for the AB (panel B) and BC (panel D) processes in the 4-3-3
 525 pathway. Results of all the 10 trajectories are given in **Supplementary Fig. 1**. Each panel shows the
 526 c₁₀-ring rotation time schedule (upper) and the reaction coordinate for the structure changes: χ of the
 527 αβ1, αβ2, and αβ3 (red, green, and blue, respectively) (bottom). The triangle mark colored cyan shows
 528 the timing when all the three αβ's completed their structural changes. The CA process trajectories in
 529 the 4-3-3 motion is shown in **Supplementary Fig. 2**. One frame of time corresponds to 10⁴ MD steps.
 530



531

532 **Fig. 3 F_0 - F_1 coupling during MD simulations of one round c_{10} -ring rotations.**

533 **a-e.** Each panel plots, from the top to bottom, the rotation angle of the c_{10} -ring, the rotation angle of

534 the F_1 stator, the first principal component about the b-subunit motions, and the rotation angle of the

535 rotor defined by the angle of the upper part of γ -subunit relative to the rotation angle of c_{10} -ring (the

536 moving average over 10 frames) for the same trajectories as those shown in Fig. 2a-e. **f.**

537 Superimposed snapshots shown in Fig. 3a-c trajectories 0, 1, and 2, respectively. The snapshot at time

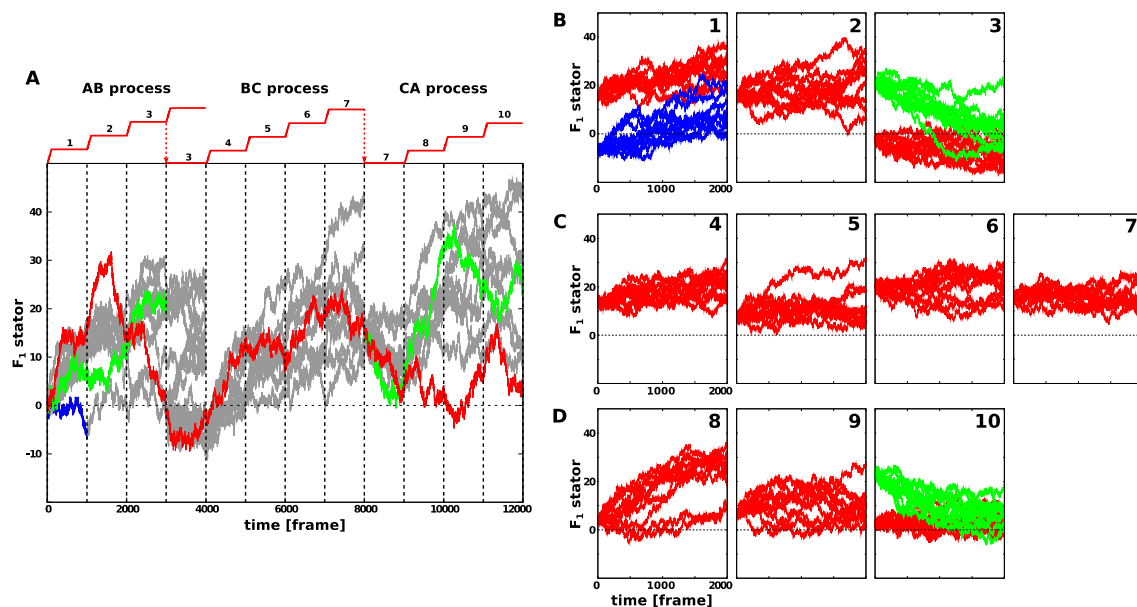
538 point 0 is colored dark salmon, and those at time point 1 and 2 are colored dark cyan. The three α -

539 subunits are opaque to make it easier to see, and the others are translucent. **g.** The direction of

540 structural change of the first principal component in the principal component analysis for the b-

541 subunit. The first principal component value increases from dark salmon to dark cyan.

542



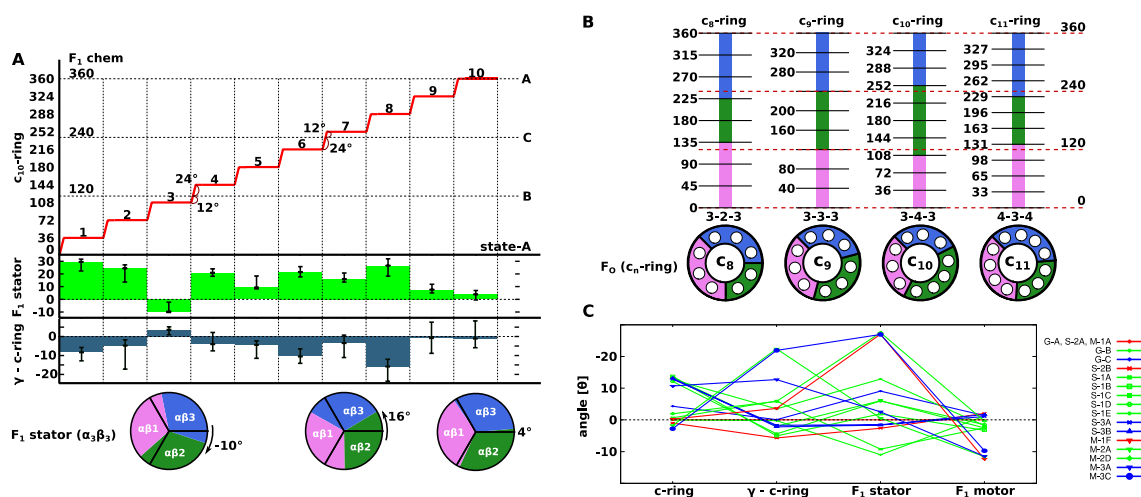
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544 **Fig. 4 Relaxation simulation with the c_{10} -ring rotation angle fixed at every $n \times 36^\circ$ step.**

545 **a.** Reference trajectories from which relaxation simulations were conducted. The F_1 stator rotation
 546 angle is depicted. Red: the representative one plotted in **Figs. 2 and 3**. Green in 0-3000th frame: one
 547 that was rotated markedly at the 3000th frame. Green in 8000-12000th frame: a case where the F_1
 548 stator was largely rotated at the end. Blue: a case where the F_1 stator was least rotated. Gray: all the
 549 other trajectories. **b-d.** Each panel plots the time course of the F_1 stator angle in the relaxation
 550 simulation with c_{10} -ring fixed to the $n \times 36^\circ$ rotation angle. Each simulation started from the snapshot
 551 of the reference trajectory in **a** at the corresponding time. **b.** the c_{10} -ring rotation state $n = 1, 2,$ and 3
 552 in the AB process. **c.** $n = 4, 5, 6,$ and 7 in the BC process. **d.** $n = 8, 9,$ and 10 in the CA process.

553

554



555

556 **Fig. 5 The F_0 - F_1 coupling and the symmetry mismatch in ATP synthesis process.**

557 **a.** Summary of the symmetry mismatch and the elastic structure changes. The stepwise c_{10} -ring
 558 rotation (the red ladder), the F_1 motor rotation (the horizontal dashed line), the F_1 stator rotation via
 559 the distortion of the b-subunit (the green bar in the second panel), and the rotor distortion (the blue bar
 560 in the third panel) are depicted for every $n \times 36^\circ$ c_{10} -ring rotation step. **b.** Predicted rotation pathways
 561 are described for systems with different number of c-subunits. **c.** Four elements of structure
 562 distortions found in cryo-EM studies. c-ring, the deviation of the c-ring rotation angle from its ideal
 563 angle. γ - c-ring, the rotary angle of the γ -subunit at its interaction site to $\alpha\beta$ minus the c-ring rotation

564 angle. F_1 stator, the F_1 stator rotation angle relative to the F_0 stator. F_1 motor, the rotation of the γ -

565 subunit relative to the F_1 stator $\alpha_3\beta_3$.

566

567 **Table 1 Rotary angles in cryo-EM structures of three ATP synthases.**

Guo	c ₁₀ -ring	n ¹⁾	$\gamma - c_{10}$ -ring ²⁾	F ₁ stator ³⁾	F ₁ motor ⁴⁾	m ⁵⁾
6N2Y (state-A)	0	0/10	0	0	0	0/3
6N2Z (state-B)	108.0(-0.0)	3	-0.2	-11.0	118.8 (1.2)	1
6N30 (state-C)	247.7(4.3)	7	0.1	9.1	238.7 (1.3)	2
Sobti						
6WNQ (2A) A ^{Guo}	0	0/10	0	0	0	0/3
6OQV (2B)	-1.0(-1.0)	0/10	-5.7	-2.6	-2.0 (2.0)	0/3
6OQR (1A) B ^{Guo}	107.3 (0.7)	3	6.0	-9.2	122.5 (-2.5)	1
6OQS (1B)	130.3 (13.7)	4	-5.1	1.9	123.3 (-3.3)	1
6OQT (1C)	130.6 (13.4)	4	-2.0	6.0	122.5 (-2.5)	1
6OQU (1D)	131.9 (12.1)	4	-4.4	6.0	121.5 (-1.5)	1
6PQV (1E)	131.2 (12.8)	4	3.5	12.9	121.8 (-1.8)	1
6OQW (3A) C ^{Guo}	239.1 (12.98)	7	-1.7	-1.5	238.9 (1.1)	2
6WNR(3B)	238.8 (13.2)	7	-2.2	-1.7	238.3 (1.7)	2
Murphy						
6RDH(1A) A ^{Guo}	0	0/10	0	0	0	0/3
6RDW(1F)	35.7 (0.3)	1	3.6	27.1	12.3 (-12.3)	0/3
6RDZ(2A) B ^{Guo}	109.8 (-1.8)	3	22.4	0.6	131.6 (-11.6)	1
6RE8(2D)	142.1 (1.9)	4	5.8	27.5	120.4 (-0.4)	1
6REB(3A) C ^{Guo}	241.3 (10.7)	7	12.7	2.6	251.5 (-11.5)	2
6RES(3C)	254.8 (-2.8)	7	21.9	26.9	249.7 (-9.7)	2

568 The angle value is in degrees. The value in parentheses is the deviation from the ideal value from its symmetry.

569 ¹⁾ The c₁₀-ring rotation step. ²⁾ The distortion of the rotor is defined by the difference between the angle of the
570 upper part of the γ -subunit and the rotation angle of the c-ring. ³⁾ The rotation angle of the F₁ stator relative to
571 the F₀ stator. ⁴⁾ The difference between the angles of the F₁ stator and F₁ rotor. ⁵⁾ F₁ motor rotation step.