Torque Generation and Utilization in Motor Enzyme $F_0F_1$-ATP Synthase

**HALF-TORQUE $F_1$, WITH SHORT-SIZED PUSHROD HELIX AND REDUCED ATP SYNTHESIS BY HALF-TORQUE $F_0F_1$**

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**Background:** ATP synthase ($F_0F_1$) is a rotary motor enzyme.

**Results:** $F_1$ with a short-sized helix-1 in $\beta$ subunit rotates with half of the normal torque and supports reduced ATP synthesis activity.

**Conclusion:** Helix-1 acts as a “pushrod” to generate torque, and torque-reduced $F_0F_1$ retains the catalytic ability of ATP synthesis.

**Significance:** Generation and utilization of the torque are crucial for motor enzymes.

ATP synthase ($F_0F_1$) is made of two motors, a proton-driven motor ($F_0$) and an ATP-driven motor ($F_1$), connected by a common rotary shaft, and catalyzes proton flow-driven ATP synthesis and ATP-driven proton pumping. In $F_1$, the central $\gamma$ subunit rotates inside the $\alpha_3\beta_3$ ring. Here we report structural features of $F_1$ responsible for torque generation and the catalytic ability of the low-torque $F_0F_1$. (i) Deletion of one or two turns in the $\alpha$-helix in the C-terminal domain of catalytic $\beta$ subunit at the rotor/stator contact region generates mutant $F_1$s, termed $F_1(1/2)$, that rotate with about half of the normal torque. This helix would support the helix-loop-helix structure acting as a solid “pushrod” to push the rotor $\gamma$ subunit, but the short helix in $F_1(1/2)$ would fail to accomplish this task. (ii) Three different half-torque $F_0F_1(1/2)$s were purified and reconstituted into proteoliposomes. They carry out ATP-driven proton pumping and build up the same transmembrane $\Delta pH$, indicating that the final $\Delta pH$ is directly related to the amount of torque. (iii) The half-torque $F_0F_1(1/2)$s can catalyze ATP synthesis, although slowly. The rate of synthesis varies widely among the three $F_0F_1(1/2)$s, which suggests that the rate reflects subtle conformational variations of individual mutants.

$F_0F_1$-ATP synthase ($F_0F_1$) catalyzes synthesis of ATP in mitochondria, chloroplasts, and bacteria in oxidative and photophosphorylation, using the energy of proton translocation caused by the proton motive force (pmf)$^2$ across membranes. Proton translocation through membrane-embedded $F_0$ portion drives ATP synthesis in membrane-protruding $F_1$ portion and, in the reverse reaction, ATP hydrolysis in $F_1$ can drive pumping back protons through $F_0$. These two reactions, the inward/outward proton translocation in $F_0$ and synthesis/hydrolysis in $F_1$, are coupled by mechanical rotation (1–10). Both $F_0$ and $F_1$ are rotary motors; $F_0$ motor is driven by downhill proton translocation, $F_1$ motor is driven by ATP hydrolysis, and the two motors share a common rotary shaft. When pmf is high, $F_0$ drives rotation of the shaft that results in ATP synthesis in $F_1$. When the chemical potential of ATP hydrolysis exceeds pmf, $F_1$ rotates the shaft and $F_0$ is forced to pump protons. Indeed, isolated $F_1$ rotates when it hydrolyzes ATP (11), and the reverse rotation by an external force results in ATP synthesis (12, 13).

ATP-driven rotation of the isolated $F_1$ has been extensively studied by using a minimum motor complex consisting of $\alpha_3\beta_3\gamma$ subunits from thermophilic Bacillus PS3 (14–16), which we also refer to as $F_1$ in this study unless otherwise indicated. The $\gamma$ subunit is a rotor subunit. Its N- and C-terminal helices form a coiled-coil and are deeply inserted into the cavity of the stator $\alpha_3\beta_3$ ring. The globular domain of $\gamma$ subunit resides outside of the cavity of the $\alpha_3\beta_3$ ring (see Fig. 1, left panel). To visualize rotation, the $\alpha_3\beta_3\gamma$ ring is immobilized on the glass surface, and a bead (or bead duplex) is attached to the globular domain of the $\gamma$ subunit. A single cycle rotates the $\gamma$ subunit $120^\circ$, consuming one ATP, meaning that $F_1$ carries out three reaction cycles per revolution. The $120^\circ$ cycle begins with a pause at $0^\circ$, then an $\sim80^\circ$-step rotation is followed by another

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2 The abbreviations used are: pmf, proton motive force; fps, frames per second; ACMA, 9-amino-6-chloro-2-methoxyacridine; $F_1(1/2)$, mutant $F_1$ that rotates with half-torque of the wild-type $F_1$; pN·nm, piconewtons nanometers.
pause, and lastly, an ~40°-step rotation occurs. F1 waits for a substrate ATP during the pause at 0° (“ATP-waiting dwell”) and rotates ~80° upon ATP binding. A pause at ~80° (“catalytic dwell”) is ~2 ms, independent of ATP concentration, representing the period required for hydrolytic cleavage of ATP and release of P0 from the catalytic site.

The torque of ATP-driven F1 motor estimated from rotation speed, bead radius, rotation radius, and viscosity of aqueous medium amounts to ~40 pN·nm (17). The energy required for a 120° rotation (~40 pN·nm × 2π/3 radians) is roughly equal to the free energy liberated from hydrolysis of an ATP molecule (~90 pN·nm) under physiological conditions and, therefore, energy conversion by F1 motor is very efficient. The torque of F1 is generated from sequential interactions between a rotor γ subunit and three catalytic β subunits in the stator αβ3 ring (18). According to the crystal structures (19, 20), β subunits can interact directly with the γ subunit at the two positions, the “sleeve” and the “orifice” regions (see Fig. 1, left panel). A mutant F1, whose γ subunit is truncated at the C terminus and loses interactions with the αβ3 ring at the sleeve region, rotates with half of the normal torque (21). Inversely, F1 devoid of the entire N-terminal helix, which preserves the sleeve interactions but loses many interactions at the orifice, also rotates with half of the normal torque (22). Therefore, the orifice interactions alone can produce half of the total torque, and the orifice interactions, probably with the assistance of some of the orifice interactions, produce another half.

At the orifice region, the helix-1-loop-helix-2 structure in the C-terminal domain of the β subunit makes contact with the γ subunit (19). The loop in the structure contains a well-conserved DELSDED acidic cluster sequence, but its acidic nature is not essential for torque generation (23). Recently, it was shown that deletion of three or four residues in the loop does not impair ATP synthesis activity of F0F1 (24). If neither acidic residues in the loop nor the full-length loop itself are essential for rotation, what structural features of the orifice region are responsible for torque generation? Here, we report isolation of mutant F1s that exert half of the normal torque, indicating that the full-length helix-1 acts as a “pushrod” for the γ subunit to generate half of the total torque. Then, we addressed the next question. If the F1 portion of F0F1 wastes a large fraction of the energy transferred from the F0 motor, can it synthesize ATP?

The results show that it can, although slowly.

**EXPERIMENTAL PROCEDURES**

**Strains, Plasmids, and Proteins—Escherichia coli** strain JM109 was used for the genetic manipulation, and JM103 Δ(uncB-uncD), which does not express *E. coli* F0F1, was used for the expression of F1 and F0F1 from thermophilic *Bacillus* PS3. Mutations of *Bacillus* PS3 F1 were introduced into the plasmid pKAGB1/HC95, which expresses the α(C193S)ββ(His10 tag at N terminus)γ(γ107C/1210C) complex of F1. Wild-type and mutant F1 were expressed and purified as described (25) except for two procedures. Heat treatment to precipitate heat-labile *E. coli* proteins was carried out at 64 °C for 20 min for wild-type F1, but at 60 °C for 10 min for the mutants. The procedure to remove endogenously bound nucleotides was omitted for the mutants because mutants with-
and $\Gamma$ and $x_i (i = 1, 2)$ are the radius and rotation radius of each bead, respectively (30). The beads rotating with about the same rotation radius were selected and analyzed. Torque was also estimated from fluctuation analysis (31) and defined as

$$\ln[P(\Delta \theta)/P(-\Delta \theta)] = N\Delta \theta/k_BT$$

(Eq. 3)

where $N$ is torque, $k_B$ is the Boltzmann constant, $T$ is temperature ($T = 298$ K), $\Delta \theta = \theta(t + \Delta t) - \theta(t)$, $\Delta t$ is 4 ms, and $P(\Delta \theta)$ is the probability distribution of $\Delta \theta$. One can estimate $N$ from this equation without the value of $\xi$.

**RESULTS**

**Torque Mutants of $F_1$—$F_1$** with decreased torque is expected to rotate beads slowly when compared with the wild-type $F_1$. For the search for structural features responsible for the torque generation, various mutants of *Bacillus PS3* $F_1$ were expressed in *E. coli*. They are, in most cases, expressed in smaller amounts and are less heat-stable than the wild-type but purified without difficulty. The purified $F_1$ was immobilized onto the nickel-nitritolactiic acid-coated glass surface through His-tagged $\beta$ subunits and, 291-nm polystyrene beads were attached on the $\gamma$ subunit as a rotation probe. We observed the rotation of duplex beads in 2 mM ATP under the microscopic field with a camera (250 fps). Under these experimental conditions, ATP-waiting dwell and catalytic dwell are too short to be observed, and continuous rotations with occasional pauses by ADP-Mg inhibition or for other reasons were observed. Among beads rotating at various speeds, the rotations at the fastest speed were taken to be the unobstructed rotation of native molecules.

We first focused on amino acid residues that are located at the rotor/stator interface narrower than 3.5 Å at the orifice region. Except for the residues in the 390DELSDSED396 sequence of the $\beta$ subunit that were already demonstrated not to be critical for torque generation, we replaced these residues with alanine and made $F_1$ variants containing mutations $\gamma$G398A/S399A/D400A, $\delta$D83A/R84A/G85A, $\epsilon$G85A/L86A, $\zeta$G88A, $\eta$Y90A/N91A, or $\zeta$N91A/S92A/N93A. We found that these mutants rotated beads at speeds similar to the wild-type $F_1$, ~20 revolutions/s (data not shown), indicating that specific interactions between side chains of these residues do not significantly contribute to torque generation. Next, we made two mutant $F_1$s, $\beta\Delta^{388}$GMDE$^{391}$ and $\beta\Delta^{392}$LSD$^{394}$, that have a deletion in the 388GMDELSSED396 sequence of the $\beta$ subunit (Fig. 1, right panel), but again, they showed rotation at a normal speed (Fig. 2, upper panels).
Then, we deleted one or two turns of the helix-1 that precedes the 390DELSDED396 sequence, and βΔΔLQDI383, βΔ376RYKE379, and βΔ377YKELQDI383 were made (Fig. 1, right panel) (superscripts are abbreviated hereafter). These three mutant F1s rotated more slowly than the wild-type F1 (Fig. 2, lower panels), and we focused on these mutants.

**Half-torque F1**—Torque of the three mutants relative to that of wild-type F1 was estimated from three different analyses. First, continuous rotation of 209-, 291-, and 350-nm duplex beads in 2 mM ATP was analyzed. The rotation speed of each mutant F1 was obtained from the consecutive, smooth 10 revolutions taken from the five fastest-rotating molecules. As the size of bead increases, viscous load increases and rotation slows down (Fig. 3A). With beads of the same size, viscous load is constant, and torque is obtained from the equation (torque = viscous load x rotation speed) (Fig. 3B). The averaged torque with three different sizes of beads is 46 pN-nm (wild-type), 29 pN-nm (βΔRYKE), 26 pN-nm (βΔLQDI), and 22 pN-nm (βΔYKELQDI). Second, rotation of 291-nm duplex beads in 200 nM ATP was observed. At this low ATP concentration, F1 of wild-type F1 was estimated from three different analyses. First, continuous rotation of 209-, 291-, and 350-nm duplex beads in 2 mM ATP with a high speed camera (4,000 fps). Under these conditions, viscous load of bead rotation is negligible, ATP-waiting dwell is not observed, and only catalytic dwell is observed at each 120° interval. The histogram of durations of the catalytic dwells is fitted by a double exponential function defined by two time constants (Fig. 4B). Previous studies assigned two time constants to the two sequential catalytic events on the enzyme, ATP hydrolysis and P1 release (16). Two of the F1(1/2)s have time constants (βΔRYKE, 0.5 and 1.2 ms; βΔYKELQDI, 0.5 and 1.1 ms) that are very similar to those of the wild-type F1 (0.4 and 1.4 ms). The βΔLQDI mutant has slightly longer time constants (0.8 and 1.3 ms). Enzyme turnover rates calculated from the above values are 560 s⁻¹ (wild-type), 590 s⁻¹ (βΔRYKE), 480 s⁻¹ (βΔLQDI), and 630 s⁻¹ (βΔYKELQDI). ATP hydrolysis activities measured in the bulk solution containing 2 mM ATP are 330 s⁻¹ (wild-type), 270 s⁻¹ (βΔRYKE), 110 s⁻¹ (βΔLQDI), and 330 s⁻¹ (βΔYKELQDI) in the presence of lauridimethylamine-N-oxide, which is thought to relieve ADP-Mg inhibition. Taking into account that purified F1s may contain some fraction of inactive molecules, these values are compatible with values obtained from rotation. To summarize the above results, enzyme kinetics of F1(1/2) are not changed much from those of wild-type F1.

**Proton Pumping and ATP Synthesis**—Bacillus PS3 F1F0, containing F1(1/2) was expressed in _E. coli_ and purified. The purified mutant F0F1s are as stable as the wild type. To avoid complexity caused by regulatory function of the e subunit, the C-terminal domain of the e subunit (eCΔ) was deleted and will be termed wild-type F0F1 hereafter (26, 33, 34). The purified F0F1 was reconstituted into proteoliposomes, and ATP-driven proton pumping was monitored with the quenching of ACMA fluorescence that reflects pH gradient across membranes (ΔpH). Upon the addition of ATP, quenching started with a short lag, progressed at a maximum rate, and leveled off at the stationary phase (Fig. 5A). The established ΔpH was completely
abolished by the addition of nigericin, an H⁺-K⁺ antiporter that can dissipate ΔpH. In general, quenching time courses of the three F₀F₁(1/2)s are very similar to each other. The fastest quenching progress rates of F₀F₁(1/2)s are about 40% of that of wild-type F₀F₁, and the maximum quenching level at the stationary phase with F₀F₁(1/2)s was ~60% of that of wild-type F₀F₁. Thus, F₀F₁(1/2)s can pump protons, but the final ΔpH built up is much smaller than that of wild-type F₀F₁.

Using the same proteoliposomes, we observed ATP synthesis driven by acid-base transition and valinomycin-induced K⁺...
**DISCUSSION**

**Short-sized Helix-1 Impairs Energy Conversion but Not Chemical Catalysis**—Properties of the half-torque mutants are summarized in Table 1. As seen, when the mutant enzymes hydrolyze ATP without the burden of the viscous load, their enzymatic kinetics are not very different from those of the wild-type enzyme. However, once they have to do work such as rotating large beads and pumping protons, their defects become obvious. Thus, shortening of helix-1 does not impair chemical catalysis but does impair mecanochemical energy conversion.

**Torque Generation in F1(1/2)**—The half-torque mutants we reported here retain the sleeve interactions. In addition, they do not lose all orifice interactions but retain the interactions at the conical entrance region of the subunit, which apparently has only the interactions with the sleeve interactions. In addition, they do not lose all orifice interactions but retain the interactions at the conical entrance of the orifice of αβ3 ring that touches the globular portion of the γ subunit (Fig. 1). F1 with the truncated γ subunit, which apparently has only the interactions with the conical entrance region of the αβ3 ring, can generate a small torque that barely supports unidirectional slow rotation of a load-negligible gold bead (35). It appears, therefore, that the...

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**TABLE 1**

| Mutants      | Torque$^a$ | $k_{cat}$(ATP) | Catalytic events$^b$ | $k_{cat}$(Pi) | ATP synthesis |
|--------------|------------|----------------|----------------------|---------------|---------------|
| Wild-type    | 46, 50, 47 | 2.0            | 0.37, 1.1            | 64            | 8.8 ± 0.8     |
| βARYKE       | 29, 34, 22 | 1.6            | 0.48, 1.2            | 38            | 2.5 ± 0.4     |
| βΔLQDI       | 26, 29, 23 | 0.7            | 0.81, 1.4            | 40            | 1.8 ± 0.1     |
| βΔKELQDI     | 22, 29, 22 | 1.3            | 0.46, 1.1            | 43            | 1.0 ± 0.2     |

$^a$ Torque values (from left) from Figs. 3, B, D, and F.

$^b$ Life times of two catalytic events (ATP cleavage and P$_i$ release) that occur on the enzyme at the catalytic dwell (from Fig. 4).

$^c$ Typical values of ACMA fluorescence quenching (Fig. 5).

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**FIGURE 4.** Kinetic constants of F1(1/2)s. A, histogram of ATP-waiting dwell of rotation of 291-nm duplex beads in 200 nM ATP recorded by 250 fps. The ATP binding rate constant ($k_{on}$) is obtained from a fitting curve (solid line), constant $\times \exp(-k_{on} \times [ATP] \times t)$; B, histogram of the catalytic dwell of rotation of a 40-nm gold bead in 2 mM ATP recorded by 4,000 fps. The data are fitted by a double exponential function (solid line), constant $\times \exp(-1/\tau_1) \times t) - \exp(-1/\tau_2) \times t$.

**FIGURE 5.** Activities of F0F1(1/2) s. A, ATP-driven proton pump. Acidification of the inside lumen of F0F1(1/2)-containing proteoliposomes by proton pumping was monitored with fluorescence quenching of ACMA. The reaction was started by the addition of 1 mM ATP. B, ATP synthesis activity. The F0F1(1/2)-containing proteoliposomes were pre-equilibrated with the low K$^+$, acidic mixture and then injected into a 10-fold volume of the high K$^+$ basic mixture. The acidic mixture contained ADP, P$_i$, and valinomycin. The basic mixture contained ADP, P$_i$, and luciferin/luciferase. Final concentrations of F0F1, ADP, and P$_i$ were 11.4 nM, 0.5 mM, and 10 mM, respectively. ATP produced was monitored with emission of luciferin, and the initial slope of exponential fitting curve between 0 and 6 s was taken to be ATP synthesis activity as shown in the right side of the figure. Experimental details are described under “Experimental Procedures.”
sleeve interactions coordinated with the interactions at the conical entrance would be responsible for the generation of torque in F1(1/2)s.

**Helix-1 with Sufficient Length Is Necessary for Torque Generation, Acting as Pushrod**—The orifice interactions by themselves produce at least half of the torque of the ATP-driven F1 motor (21, 35). Given that the interactions at the conical entrance region contribute only a small fraction of the torque, the majority of the torque produced at the orifice is attributed to the interactions of the helix-1-loop-helix-2 with the convex coiled-coil region of the γ subunit. Concerning the central loop, the conserved DELSDED sequence is not necessary for the torque generation (23). Even the mutants with the shortened loop can synthesize ATP (24) and produce normal torque (Fig. 2). Instead, this work reveals a critical role of full-length helix-1 in the generation of torque. When helix-1 is shortened by one helical turn (5.4 Å) by deletion of either the RYKE or the LQDI segments, torque is reduced by half. When the segment RYKELQDI, which forms two turns (10.8 Å) in helix-1, is truncated, torque is again reduced by no more than half. It is clear from these results that the physical length, rather than residue-specific interactions, of helix-1 is important for torque generation. In addition, the fact that deletion of one turn and of two turns gives the same result implies the presence of a critical length for helix-1 function. Crystal structure (36), single molecule study (37), and rapid scan atomic force microscopy (38) show that C-terminal domains of three β subunits in F1 undergo bending motion sequentially and alternately during the catalytic cycle. The helix-1-loop-helix-2 is a protruding body of the C-terminal domain toward the γ subunit (Fig. 1), and it can protrude more toward the γ subunit by a bending motion (36). It appears that a protruding solid body of the helix-1-loop-helix-2 physically pushes the convex part of the coiled-coil of the γ subunit and assists the γ subunit to rotate (39). To transmit the force, helix-1, and probably helix-2 as well, must play the role of pushrod with sufficient length and stiffness.

**Critical Length of Helix-1**—Very recently, more research on the helix-1-loop-helix-2 structure was published by Mnatstakanyan et al. (40). They found that a mutant of *Bacillus PS3 F0F1* Δγ (Δβ(LQDI+LSD)), can catalyze ATP synthesis, although slowly, but Δ10 (Δβ(QDIIAIL+LSD)) cannot. From the difference between Δ7 and Δ10, Mnatstakanyan et al. (40) concluded that the critical length of the helix-1-loop-helix-2 structure is around ~10 Å shorter than the native length. Our results clearly show that shortening one turn (~5 Å) of helix-1 (βΔLQDI and βΔRYKE) is enough to abolish half of the torque. Therefore, we agree on the presence of a critical length but propose that the critical length of the helix as a solid body is ~5 Å shorter than the native length. Most likely, Δ10 is also a half-torque mutant and has the catalytic ability of ATP synthesis, although it might have been very weak and escaped the assay employed in Ref. 40. Actually, Δ10 retains proton-pumping activity, although weak (40), which should not happen without torque.

**Low-torque F0F1 Builds Up a Small ΔpH**—Given that F1(1/2) can use only a fraction of the energy of ATP hydrolysis for driving the motor, it is reasonable to assume that F0F1(1/2) can also use only a fraction of the energy for proton pumping. Indeed, the final ΔpH built up by the proton-pumping action of F0F1(1/2) is significantly smaller than ΔpH built up by wild-type F0F1. Final ΔpH could be determined by equilibrium between proton-pumping activity and back pressure of pmp and/or equilibrium between proton-pumping activity and passive leakage of protons through membranes. In any case, however, the fact that three kinds of F0F1(1/2)s build up the same final ΔpH indicates that the final ΔpH is directly determined by the magnitude of the torque, that is, by the energy conversion yield. This agrees with the thermodynamic prediction that ΔpH is directly related to the torque (n × ZΔpH = torque × 2π/3; where n is the number of transported protons per ATP, and Z is a constant).

**Low-torque F0F1 Can Synthesize ATP Slowly under Thermodynamically Favorable Conditions**—If the same inefficient energy conversion occurs in the ATP synthesis reaction of F0F1(1/2), the F1(1/2) portion of F0F1(1/2) can use only a fraction (~45 pN·nm per 120° rotation) of the total energy provided by the F0 portion. Despite this defect, F0F1(1/2) can synthesize ATP (Fig. 5B). This might be because of the small free energy required for ATP synthesis under actual experimental conditions. The reaction mixtures contained 0.5 mM ADP and 10 mM Pi. The ADP we used contained about 0.05% ATP as measured by luciferase assay, and the free energy required for ATP synthesis would amount to ~40 pN·nm, which the F1(1/2) portion of F0F1(1/2) can manage to exert. Therefore, low-torque F0F1 has catalytic ability and can synthesize ATP under optimum thermodynamic situations.

Because the free energy defines the direction and equilibrium but not the rate of the reaction, it is not surprising that each of the three F0F1(1/2)s catalyzes ATP synthesis at a different rate. Subtle variations in the enzyme can affect the rate, and the reason for the slow ATP synthesis rate of F0F1(1/2)s cannot be explained with certainty. During the ATP synthesis reaction, the F0 portion provides energy to the F1 portion via rotation of the γ subunit. The F1 portion uses this energy to induce the otherwise thermodynamically unfavorable conformational changes to β subunits that lead to ATP synthesis. We speculate that with insufficient ability to utilize energy, F0F1(1/2) would fail frequently and take a long time to induce such changes, or even make uncoupled rotations frequently without inducing such changes. We observed that the F0F1(1/2)s have larger uncoupled ATP hydrolysis activity than wild-type F0F1, which is probed by resistance to inactivation by dicyclohexylcarbodiimide, a reagent that prevents proton translocation and hence rotation of F1. This observation favors the scenario of the uncoupled rotation but does not exclude other interpretations.

Finally, it should be noted that although F0F1(1/2) can synthesize ATP, the synthesis reaction reaches equilibrium when ATP concentration is 4 or 5 orders of magnitude lower than in the case of wild-type F0F1. This means that cellular ATP concentration supported by F0F1(1/2) would be very low and could hardly sustain normal cell activities. Thus, natural selection would not allow field organisms to have a low-torque version of F0F1, even if it had the catalytic ability of ATP synthesis.

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REFERENCES

1. Yoshida, M., Muneyuki, E., and Hisabori, T. (2001) ATP synthase—a marvelous rotary engine of the cell. *Nat. Rev. Mol. Cell Biol.* 2, 669–677.

2. Boyer, P. D. (2002) A research journey with ATP synthase. *J. Biol. Chem.* 277, 39045–39061.

3. Senior, A. E., Nandanaciva, S., and Weber, J. (2002) The molecular mechanism of ATP synthesis by F,ATP synthase. *Biochim. Biophys. Acta* 1553, 188–211.

4. Kinosita, K., Jr., Adachi, K., and Itoh, H. (1997) Direct observation of the rotation of F,ATPase. *Nature* 386, 364–370.

5. Weber, J. (2006) ATP synthase: subunit-subunit interactions in the stator stalk. *Biochim. Biophys. Acta* 1757, 1162–1170.

6. Nakamoto, R. K., Baylis Scallon, J. A., and Al-Shawi, M. K. (2008) The rotary mechanism of the ATP synthase. *Arch. Biochem. Biophys.* 476, 43–50.

7. Junge, W., Siewaff, H., and Engelbrecht, S. (2009) Torque generation and elastic power transmission in the rotary F,ATPase. *Nature* 459, 1117–1124.

8. Dürer, M. G., Zarrabi, N., Cipriano, D. J., Ernst, S., Glick, G. D., Dunn, S. D., and Birsch, M. (2009) 36’ step size of proton-driven c-ring rotation in F,ATP synthase. *EMBO J.* 28, 2689–2696.

9. von Ballmoos, C., Wiedenmann, A., and Dimroth, P. (2009) Essentials for ATP synthase F,ATP synthase. *Biochim. Biophys. Acta* 178, 649–672.

10. Nakano-Matsui, M., Sekiya, M., Nakamoto, R. K., and Futai, M. (2010) The mechanism of rotating proton pumping ATPases. *Biochim. Biophys. Acta* 1797, 1343–1352.

11. Noji, H., Yasuda, R., Yoshida, M., and Kinosita, K., Jr. (1997) Direct observation of the rotation of F,ATPase. *Nature* 386, 299–302.

12. Itoh, H., Takahashi, A., Adachi, K., Noji, H., Yasuda, R., Yoshida, M., and Kinosita, K. (2004) Mechanically driven ATP synthesis by F,ATPase. *Nature* 427, 465–468.

13. Rondelez, Y., Tresset, G., Nakashima, T., Kato-Yamada, Y., Fujita, H., Takeuchi, S., and Noji, H. (2005) Highly coupled ATP synthesis by F,ATPase single molecules. *Nature* 433, 773–777.

14. Senior, A. E., Nadanaciva, S., and Weber, J. (2002) The molecular mechanism of ATP synthesis. *Annu. Rev. Biophys. Biomol. Struct.* 31, 245–268.

15. Shimabukuro, K., Yasuda, R., Muneyuki, E., Hara, K. Y., Kinosita, K., Jr., and Yoshida, M. (2003) Coupling of rotation and catalysis in F,ATPase revealed by single-molecule imaging and manipulation. *Cell* 130, 309–321.

16. Yasuda, R., Noji, H., Kinosita, K., Jr., and Yoshida, M. (1998) F,ATPase is a highly efficient molecular motor that rotates with discrete 120 degree steps. *Cell* 93, 1117–1124.

17. Ariga, T., Muneyuki, E., and Yoshida, M. (2007) F,ATPase rotates by an asymmetric, sequential mechanism using all three catalytic subunits. *Nat. Struct. Mol. Biol.* 14, 841–846.

18. Abrahams, J. P., Leslie, A. G., Lutter, R., and Walker, J. E. (1994) Structure at 2.8 Å resolution of F,ATPase from bovine heart mitochondria. *Nature* 370, 621–628.

19. Bowler, M. W., Montgomery, M. G., Leslie, A. G., and Walker, J. E. (2007) Ground state structure of F,ATPase from bovine heart mitochondria at 1.9 Å resolution. *J. Biol. Chem.* 282, 14238–14242.

20. Hossain, M. D., Furuike, S., Maki, Y., Adachi, K., Suzuki, T., Kohori, A., Itoh, H., Yoshida, M., and Kinosita, K., Jr. (2008) Neither helix in the coiled coil region of the axle of F,ATPase plays a significant role in torque production. *Biophys. J.* 95, 4837–4844.

21. Kohori, A., Chiwata, R., Hossain, M. D., Furuike, S., Shiroguchi, K., Adachi, K., Yoshida, M., and Kinosita, K., Jr. (2011) Torque generation in F,ATPase devoid of the entire amino-terminal helix of the rotor that fills half of the stator orifice. *Biophys. J.* 101, 188–195.

22. Ariga, T., Muneyuki, E., and Yoshida, M. (2007) F,ATPase rotates by an asymmetric, sequential mechanism using all three catalytic subunits. *Nat. Struct. Mol. Biol.* 14, 841–846.

23. Lu, Y., Hara, K. Y., Kinosita, K., Jr., and Yoshida, M. (2003) The molecular mechanism of ATP synthesis. *Annu. Rev. Biophys. Biomol. Struct.* 31, 245–268.

24. Bowler, M. W., Montgomery, M. G., Leslie, A. G., and Walker, J. E. (2006) F,ATPase is a highly efficient molecular motor that rotates with discrete 120 degree steps. *Cell* 93, 1117–1124.

25. Ariga, T., Muneyuki, E., and Yoshida, M. (2007) F,ATPase rotates by an asymmetric, sequential mechanism using all three catalytic subunits. *Nat. Struct. Mol. Biol.* 14, 841–846.

26. Abrahams, J. P., Leslie, A. G., Lutter, R., and Walker, J. E. (1994) Structure at 2.8 Å resolution of F,ATPase from bovine heart mitochondria. *Nature* 370, 621–628.

27. Bowler, M. W., Montgomery, M. G., Leslie, A. G., and Walker, J. E. (2007) Ground state structure of F,ATPase from bovine heart mitochondria at 1.9 Å resolution. *J. Biol. Chem.* 282, 14238–14242.

28. Hossain, M. D., Furuike, S., Maki, Y., Adachi, K., Suzuki, T., Kohori, A., Itoh, H., Yoshida, M., and Kinosita, K., Jr. (2008) Neither helix in the coiled coil region of the axle of F,ATPase plays a significant role in torque production. *Biophys. J.* 95, 4837–4844.

29. Kohori, A., Chiwata, R., Hossain, M. D., Furuike, S., Shiroguchi, K., Adachi, K., Yoshida, M., and Kinosita, K., Jr. (2011) Torque generation in F,ATPase devoid of the entire amino-terminal helix of the rotor that fills half of the stator orifice. *Biophys. J.* 101, 188–195.

30. Ariga, T., Muneyuki, E., and Yoshida, M. (2007) F,ATPase rotates by an asymmetric, sequential mechanism using all three catalytic subunits. *Nat. Struct. Mol. Biol.* 14, 841–846.

31. Bowler, M. W., Montgomery, M. G., Leslie, A. G., and Walker, J. E. (2006) F,ATPase is a highly efficient molecular motor that rotates with discrete 120 degree steps. *Cell* 93, 1117–1124.

32. Ariga, T., Muneyuki, E., and Yoshida, M. (2007) F,ATPase rotates by an asymmetric, sequential mechanism using all three catalytic subunits. *Nat. Struct. Mol. Biol.* 14, 841–846.